PSYCHOLOGICAL STRESS-INDUCED DNA DAMAGE IN LYMPHOCYTES OF AEROBICALLY FIT AND UNFIT INDIVIDUALS

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Habitual aerobic exercise has a beneficial effect on many systems of the body and reduces the risk of developing certain diseases, including cardiovascular disease and some cancers. Psychological stress, which has some of the same physiological effects, has a negative influence on several systems, including immune and neuroendocrine functioning. It is possible that exercise serves to combat the detrimental effects of stress by buffering its harmful influences. The primary aim of this study was to examine one potential protective mechanism: the increased resistance of cells to stress-induced oxidative damage as a result of aerobic exercise training. In order to accomplish this, a 2 (Group assignment: stress/nonstressed) x 3 (Time: baseline/ immediately post-stressor/20 minutes post-stressor) experimental design was used. Forty participants were assigned to either a stressed experimental group or a nonstressed control group. Participants in the stress group were exposed to experimental tasks that typically cause acute stress. Oxidative damage was measured at each time point by scoring the amount of DNA damage in lymphocytes isolated from the blood. Repeated measures analysis of covariance (ANCOVA) and hierarchical multiple regression were performed to examine the differences in lymphocyte DNA damage in response to acute psychological stress in participants of varying cardiorespiratory fitness levels. It was hypothesized that group assignment would significantly influence the rise in DNA damage, indicating that the stressful tasks induced the damage. The ANCOVA supported this expectation, $F(1,38) = 4.1$, $p<0.05$, partial $eta^2 = 0.10$, however, the

regression analysis did not find that fitness levels significantly interacted with group assignment to buffer the effect of stress on DNA damage. These results should be interpreted with caution because of the small sample size and the limited variability of cardiorespiratory fitness levels in the sample. Future studies are needed to address these limitations and broaden the knowledge of how physical fitness can protect from the potentially damaging effects of psychological stress.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to sincerely thank my thesis advisor, Andrew Baum, Ph.D., and my committee members, Anthony Caggiula, Ph.D., and John Jakicic, Ph.D., for their encouragement and guidance throughout the completion of this research project. I would also like to extend my appreciation to Bret Goodpaster, Ph.D. and George Grove from the UPMC Exercise Physiology Lab for supplying their equipment and services for the cardiorespiratory exercise tests. In addition, I would like to thank Melanie Flint, Ph.D., Emily McLeod, and Bill Riehl from the Hillman Cancer Center for their support and assistance in conducting the biological assays. Lastly, I would like to thank my husband, Jared, for his support, love, and patience.

1.0 INTRODUCTION

Epidemiological evidence consistently indicates that regular physical activity and exercise reduce the risk of diseases such as cardiovascular disease, type 2 diabetes and some forms of cancer (Booth, Chakravarthy, Gordon & Spangenburg, 2002). The specific protective mechanisms that contribute to this risk reduction have not yet been fully explained, but several possibilities have been suggested. Physiological changes known to occur as a result of habitual exercise have salutary effects on health, including lowered resting heart rate and blood pressure, reduced body fat, strengthened muscles, enhanced immune function, higher maximal oxygen uptake and improved oxidative outcomes (Irwin & Friedman, 1999). People who participate in aerobic exercise over a prolonged period of time also exhibit beneficial adaptations of physiological systems that are activated during acute psychological stress, specifically the reactivity and recovery of heart rate and blood pressure in response to stress (Crews & Landers, 1987; McCubbin, Cheung, Montgomery, Bulbulian & Wilson, 1992).

Research is less clear about whether habitual exercise affects immune and endocrine responses during exposure to psychological stress or whether molecular precursors of cell dysfunction are affected. The proposed research will address this by examining psychological stress-induced oxidative damage in the nuclear DNA of immune cells in individuals ranging in degree of fitness. Examining biomarkers of oxidative stress, such as DNA damage, after an acute psychological stress task in exercise trained and sedentary individuals will broaden our

understanding of how physical fitness may protect the body from the potentially damaging effects of psychological stress.

1.1 OXIDATIVE STRESS

Acute exercise and stress increase the production of reactive oxygen species (ROS). ROS are molecules with unpaired electrons that are highly reactive and formed continuously in the body as a result of biochemical reactions and external factors. An elevated production of ROS is a precursor to harmful oxidative stress. Habitual aerobic exercise, however, is associated with a reduced production of ROS during acute bouts of exercise. This is consistent with the effects of acute and habitual exercise on hemodynamic responses and suggests that the "trained" body adapts to the challenges induced by acute exercise, rendering it more efficient at coping with subsequent stressors (Finkel & Holbrook, 2000).

Oxidative stress is defined as a balance between availability of oxidants and anti-oxidant compounds. This balance is important because of the damage that unbuffered ROS cause to multiple cellular structures including lipids, proteins, and DNA. The production of ROS is a normal activity of living cells and under ordinary conditions can be managed by bodily defenses (*i.e.* by antioxidants). Oxidative stress occurs when ROS are produced in increasingly excessive amounts and/or the buffering effects of antioxidants decrease. This compromises the body's capacity to defend against ROS, which interact with DNA, proteins, and lipids to generate oxidized bases, protein oxidation, and lipid peroxidation, respectively. These processes can cause cellular damage or degeneration and have been implicated in the onset of chronic diseases

such as cardiovascular disease, cancer, and degenerative diseases (Ames, Shigenaga & Hagen, 1993).

1.2 OXIDATIVE STRESS AND EXERCISE

It is now widely accepted that most forms of acute aerobic exercise increase oxidative stress by increasing the production of ROS (Alessio, Hagerman, Fulkerson, Ambrose, Rice & Wiley, 2000; Ashton, Rowlands, Jones, Young & Jackson *et al*., 1998; Jammes, Steinberg, Bregeon & Delliaux, 2004). It has been difficult to determine the precise location and mechanisms involved in ROS production during exercise because most human studies have relied on examinations of whole body exercise and have used indirect measures of oxidative stress. These indirect measures include by-products of oxidative damage to lipids, proteins, and DNA and require inferences about the causes of the damage. For example, several studies show that acute exercise causes elevated levels of plasma malondialdehyde (MDA), the reactive intermediate of lipid peroxidation, and carbonyls, reactive intermediates from protein oxidation (Jammes *et al*., 2004; Alessio *et al*., 2000; Tsai, Hsu, Hsu, Cheng, Liu & Hsu, 2001). These effects suggest changes in oxidative stress. Damage to nuclear DNA of leukocytes has also been shown twenty-four hours after completion of exhaustive exercise compared to resting levels, presumably as a function of increased oxidative stress (Niess, Hartmann, Grunert-Fuchs, Poch & Speit, 1996). Ashton and colleagues (1998) reported more direct measures of ROS in venous blood before and after exhaustive aerobic exercise using electron spin resonance (ESR) spectroscopy. ROS increased after the exercise, suggesting support for the general hypothesis that ROS increase during exercise.

The primary source of ROS generation during exercise is believed to be the increased respiration of mitochondria due to greater demand for energy in contracting muscles (Leeuewenburgh & Heinecke, 2001). During the enhanced oxygen consumption (VO_2) of aerobic activity the reduction of molecular oxygen in mitochondria provides the continuous generation of ATP for muscular contraction as a result of the electron transport chain. This process accounts for roughly 95-98% of the total oxygen consumption of tissues, but the small percentage of the remainder may go through one electron reduction leading to the production of superoxide radicals ($O_2^{\bullet}$), a common form of ROS in the body. Furthermore, the reduction of $O_2^{\bullet}$ produces another form of ROS, hydrogen peroxide (H_2O_2) (Jackson, 2000). The superoxide and hydrogen peroxide produced during contractile activity are then leaked from the muscle cells into the interstitial fluid where they can form hydroxyl radicals (O'Neill, Stebbins, Bonigut, Halliwell & Longhurst, 1996). Because aerobic exercise can cause a 10-20-fold increase in whole body VO_2 compared to the resting state, it is not surprising that biomarkers of oxidative stress increase in direct proportion to intensity of activity (Alessio *et al*., 2000).

Elevated oxygen consumption may not be the sole source of ROS production during exercise. Isometric exercise also induces increases in oxidative stress, although the increase is not as great as after an aerobic challenge (Alessio *et al*., 2000). Even so, this provides some evidence for alternate pathways. These other sources of ROS include phagocyte production of ROS as part of the inflammatory process, the autoxidation of catecholamines and the generation by xanthine oxidase (Jackson, 2000).

1.3 THE ROLE OF ANTIOXIDANTS

As noted, oxidative stress profiles are not entirely determined by ROS production, but can also occur from limitations in the oxidative defense system. Defense against ROS damage is comprised of antioxidant enzymes, non-enzymatic antioxidants and cellular repair mechanisms (Moller, Wallin & Knudsen, 1996). The major enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). SOD is located in the mitochondria and cytosol where it dismutates superoxide radicals into H_2O_2 and O_2. GPx and CAT serve to catalyze the decomposition of H_2O_2 in different locations. Glutathione (GSH) is an abundant non-enzymatic antioxidant that is synthesized in the liver and serves multiple functions in antioxidant defense including the removal of hydrogen and organic peroxides during GPx catalyzed reactions (Powers & Sen, 2000). Antioxidants like vitamins C and E are obtained through diet and also play a role in oxidative protection. Typically, these components of the antioxidant defense system work together, along with repair mechanisms such as DNA repair enzymes, to effectively guard against excess oxygen toxicity in the resting state and during exercise. The major forms of defense mirror more generalized immune system activity and include scavenging reactive metabolites and converting them to less reactive molecules, enhancing the resistance of biological targets to ROS attacks, facilitating the repair of damage, and triggering the expression of genes that encode antioxidant proteins and cellular repair enzymes (Sen, 1995).

1.4 HABITUAL EXERCISE AND OXIDATIVE STRESS

Repeated bouts of exercise lead to regular elevations of both ROS and defense system activity. The increase in ROS typically exceeds the capacity of the defense system, resulting in oxidative stress. Intuitively, it would seem that continual elevations of oxidative stress would be harmful; however, exercise training enhances antioxidant activity and reduces ROS damage at rest and post-exercise. For instance, after completing a 16-week treadmill walking/jogging program, participants exhibited elevated levels of GPx and decreased plasma MDA at rest and post-exercise, compared to pre-training levels (Fatouros, Jamurtas, Villiotou, Pouliopoulou & Fotinakis *et al*., 2004). Similarly, a 12-week running program resulted in greater production of erythrocyte SOD and GPx, and decreased production of TBARS after training than before training (Miyazaki, Oh-ishi, Ookawara, Kizaki, Toshinai & Ha, 2001). The results of these two studies indicate an adaptive response to exercise training characterized by smaller, more transient increases in lipid peroxidation and increases in production of antioxidants.

The majority of studies exploring the effects of habitual exercise on oxidative stress have focused on plasma measures of ROS and endogenous antioxidants. However, effects at the cellular level have also received attention, particularly investigation into DNA damage and repair. ROS have the capacity to cause a range of DNA lesions, including modified nucleotide bases and strand breaks. If modified bases are not properly repaired prior to replication the cell is in danger of potentially harmful mutations or apoptosis. Fortunately, defenses such as nucleotide excision repair (NER) exist to mend nucleotide base alterations before they damage the integrity of the genome or lead to cell death. A widely used biomarker of DNA damage is 8-hydroxydeoxyguanine (8-OH-dG), a DNA repair product found in the blood and urine (Griffiths,

Moller, Bartosz, Bast & Bertoni-Freddari *et al*., 2002). Levels of 8-OH-dG are an indication of the amount of base modification and subsequent repair that has taken place in the body.

Single cell gel electrophoresis, known as the comet assay, is another popular method used to determine the amount of oxidative DNA damage in cells. The comet assay measures single strand breaks (SSB) in DNA that occur as a result of oxidized bases. DNA from a variety of cells can be evaluated with this method; however, leukocytes, specifically lymphocytes, are the most frequently examined cells in human studies. It is thought that lymphocytes serve as sentinel cells, providing early warning signs for negative health effects from exposure to oxidative stress (Faust, Kassie, Knasmuller, Kevekordes & Mersch-Sundermann, 2004). Furthermore, investigation of oxidative damage to lymphocytes may also provide information on how common sources of oxidative stress, such as acute exercise and psychological stress, affect the immune system.

Exercise studies frequently use the comet assay to assess DNA damage because of its increased sensitivity over alternate techniques (Hartmann & Niess, 2000). Evidence indicates an association between increased amounts of DNA damage in leukocytes and exhaustive exercise and a reduction of this damage with physical conditioning (Radak, Naito, Kaneko, Tahara, Nakamoto & Takahashi, 2002; Niess *et al*., 1996; Hartmann, Plappert, Raddatz, Grunert-Fuchs & Speit, 1994; Tsai *et al*., 2001). Niess *et al*. (1996) conducted an experiment in which 5 untrained men and 6 trained men performed an incremental treadmill run. Using the comet assay, examination of leukocyte DNA SSB at baseline, 15 minutes post-run and again after 24 hours revealed an increase at the 24-hour time point, with less damage occurring for the trained participants. They also found a negative correlation between VO_2 max and amount of DNA damage after exercise. These findings are limited by a cross-sectional design, small sample size,

and the lack of attention to potential confounding variables. Despite these limitations, this study provides a valuable starting point for examining the possible protection conveyed by exercise training.

A more recent study used a rat model to reveal further evidence that habitual exercise may play a role in providing resistance to oxidative stress-induced DNA damage (Nakatani, Komatsu, Kato, Yamanaka & Takekura *et al*., 2005). Experimenters evaluated kidney and diaphragm levels of SOD activity and 8-OH-dG levels in rats that underwent a ten-week swimming program and in sedentary controls. Interestingly, no differences in the levels of 8-OH-dG were found between the groups, although SOD activity was higher in the habitually exercised rats compared to the controls. However, following the injection of an oxidative stress-producing renal carcinogen, FE-nitrilotriacetic acid (Fe-NTA), habitually exercised rats showed a suppressed induction of 8-OH-dG, while the sedentary rats had a larger increase in 8-OH-dG levels. These results indicate that habitual exercise may induce resistance to DNA damage in the presence of oxidative stress.

Adaptations in oxidative stress profiles that occur as a result of regular exercise are assumed to be in response to cellular alterations, but cellular and molecular mechanisms that are responsible for these changes have not been identified. It has been suggested that ROS may trigger the activation of specific cellular stress signaling pathways that help the cells respond to stress (Finkel & Holbrook, 2000). The common effect of activating these pathways is to modulate transcription factor activity and alter the expression patterns of genes that may play a role in cell protection. It is possible that the mere presence of elevated ROS levels serves as a signal to turn on the multitude of defenses the body has against oxidative damage (Powers & Sen, 2000). A more efficient and faster acting cellular defense system for combating ROS would

presumably be beneficial during times of elevated ROS production, whether it be during an acute bout of exercise, exposure to an oxidative stress-inducing agent, or during times of psychological stress.

1.5 PSYCHOLOGICAL STRESS AND DNA DAMAGE

Psychological stress has received considerably less attention than acute exercise as a potential producer of oxidative stress. The first evidence that psychological stress induces damage to nuclear DNA was shown in liver cells of rats exposed to a conditioned emotional stimulus (CES). The CES consisted of a communication box in which rats witnessed other rats being shocked intermittently. The rats subjected to the CES exhibited increased 8-OH-dG in nuclear DNA of the liver after the 2nd, 3rd, and 4th exposure with a return to baseline levels one hour after completion of the stressor (Adachi, Kawamura & Takemoto, 1993). Human studies identified a link between presumably stressful student exams and increased DNA damage; no associations were found during low stress periods (Forlenza, Latimer & Baum, 2000; Cohen, Marshall, Cheng, Agarwal & Wei, 2000). The observed increases in repair were used to infer an increase in DNA damage occurring during psychological stress. This assumption is supported by the observation that medical students had greater nuclear DNA damage in lymphocytes on the day of an examination (measured by the comet assay) than during the term between two examination periods (Sivonova, Zitnanova, Hlincikova, Skodacek, Trebaticka & Durackova, 2004). Furthermore, in a recent study conducted by Flint, Baum, Chambers, and Jenkins (2007) the stress hormones, epinephrine (E), norepinephrine (NE), and cortisol, induced DNA damage in murine 3T3 cells exposed in vitro. Together, these studies provide evidence that psychological

stress affects DNA damage. However, the exact mechanisms by which this occurs are not fully understood, and interactions with possible protective effects of fitness are not known.

2.0 STUDY AIMS

The aim of this study is to replicate findings that stress increases oxidative damage using a more precise measure of damage, and to determine whether habitual exercise buffers increases in nuclear DNA damage that occur during acute psychological stress. Damage to DNA in lymphocytes during an acute psychological stressor was compared among participants of varying fitness levels. The design of this study is a 2 (Group: stress/nonstressed control) x 3 (Time: baseline/immediately post-stressor/20 minutes post-stressor) experimental design with one between subjects factors (Group assignment) and one within subjects factor (Time). The primary outcome measures were DNA damage scores from the Comet Assay at each blood draw during the acute stressor period.

2.1 HYPOTHESES

It was anticipated that the group variable would significantly influence DNA damage scores across time. The stress group was expected to show increased damage at Time 2 (immediately post-stressor) compared to Time 1 (baseline), while damage in the control group is expected to remain unchanged. In addition, an interaction between group assignment and fitness level was hypothesized. This interaction should predict the change in DNA damage scores between Times 1 and 2 over and above the main effect of group assignment alone.

3.0 METHODS

3.1 PARTICIPANTS

A total of forty men and women between the ages of 18 and 25 were recruited for this study. Participants were eligible for the study if they reported being sedentary or physically fit. A sedentary adult was defined as one who has not engaged in an exercise regimen exceeding 20 minutes per day, 3 days per week over the previous six months (Jakicic, Marcus, Gallagher, Napolitano & Lang, 2003). Aerobically fit individuals were defined as those who report engaging in 30 minutes or more of aerobic exercise at least 5 days per week over the previous six months. Cardiorespiratory fitness tests were used to objectively assess levels of fitness. Participants were excluded if they currently smoked or were taking medication that would influence cardiovascular or immune measures, including excessive alcohol (>10 drinks/week) and illegal drugs. Participants with a medical condition, musculoskeletal injuries or psychiatric diagnosis that might affect activity or immune system function were also excluded. This information was collected by self-report and included incidence of cancer, asthma, arthritis, diabetes, heart disease, autoimmune disorders, major depression/anxiety, and hypertension. Participants who reported being overweight (BMI>27) were also ineligible to participate. Participants were recruited through posted flyers, and the undergraduate psychology subject pool

website at the University of Pittsburgh. Participants received either monetary compensation or credit towards their undergraduate psychology class.

3.2 MEASURES

3.2.1 Body Composition

Weight, body mass index, and percent body fat were measured using a body composition analyzer/scale (Tanita Corporation of America, Inc., Arlington Heights, Illinois).

3.2.2 Cardiorespiratory Fitness

A submaximal graded exercise treadmill test was used to assess cardiorespiratory fitness as described by Jakicic et al. (2003). The treadmill was set to a constant speed of 80.4 m/min, with the grade beginning at 0% and progressing by 2.5% at 3-minute intervals until 85% of age-predicted maximal heart rate (computed as 220 minus age) was achieved. Maximum cardiorespiratory fitness (VO_2 max) was calculated by plotting results from the submaximal test and extrapolating out to the age-predicted maximal heart rate.

3.2.3 Sleep Quality

The Pittsburgh Sleep Quality Index (PSQI) was used to examine sleep duration, sleep quality, and napping behavior over the past month (Buysse, Reynolds, Monk, Berman & Kupfer, 1989). The PSQI has high internal validity and an overall reliability coefficient of 0.83.

3.2.4 Perceived Stress

The Perceived Stress Scale (PSS) is a 14-item scale used to assess perceptions of stress by measuring the degree to which individuals appraise daily life events as unpredictable, uncontrollable, or overloading (Cohen, Kamarck & Mermelstein, 1983). The items ask about thoughts and feelings during the last month. The validity is adequate, and it has a reliability coefficient of 0.85.

3.2.5 Social Support

The Interpersonal Support Evaluation List (ISEL) was used as a measure of perceived availability of supportive social resources (Cohen, Mermelstein, Kamarck & Hoberman, 1985). The ISEL consists of 40 statements that can provide an overall functional support measure or can be separated into four separate functions of perceived support, including appraisal, belonging, tangible support and self-esteem. The ISEL has adequate validity and reliability, and has demonstrated reasonable stability across time. The internal reliability of the total scale ranges from 0.88-0.90. The internal reliability ranges for the subscales are 0.70-0.82 for appraisal, 0.62-0.73 for self-esteem, 0.73-0.78 for belonging, and 0.73-0.81 for tangible support.

3.2.6 Self-Efficacy

General self-efficacy beliefs were assessed with the ten-item Generalized Self-Efficacy Scale (GSES) (Schroder & Schwarzer, 2005). The GSES has an internal reliability coefficient of 0.85 and sufficient validity.

3.2.7 Profile of Mood States

The Profile of Mood States short form (POMS-SF) is a 37-item measure used to assess current mood states. The original 65-item form is comprised of adjectives rated on a 5-point scale from not at all to extremely and can be divided into six factor based subscales: Tension-Anxiety, Depression-Dejection, Anger-Hostility, Fatigue-Inertia, Vigor-Activity, and Confusion-Bewilderment (McNair, Lorr & Droppleman, 1971). The POMS-SF retains the reliability of the six subscales in the range of 0.78 to 0.91 and correlation with the original version has been found to be above 0.95 (Shacham, 1983).

3.2.8 Diet History

The Web-based version of the National Institutes of Health Diet History Questionnaire (NIH DHQ) was used to assess diet history over the past year. The NIH DHQ is a food frequency questionnaire (FFQ) consisting of 124 food items that includes both portion sizes and dietary supplement questions. Data indicate that this instrument provides reasonable nutrient estimates and sufficient reliability and validity (Subar, Thompson, Kipnis, Midthune & Hurwitz *et al*., 2001). DHQ data was analyzed by the National Cancer Institute Diet* Calc software.

3.2.9 Biological Outcome Measures

3.2.9.1 Baseline DNA Damage

This measure was defined as the amount of DNA damage observed in lymphocytes taken at rest 15 minutes prior to the acute psychological task.

3.2.9.2 Psychological Stress-Induced DNA Damage

This was the amount of DNA damage seen in lymphocytes taken immediately after completion of the acute psychological task.

3.2.9.3 Recovery DNA Damage

This was considered the amount of DNA damage measured in lymphocytes taken 20 minutes after the completion of the acute psychological task.

4.0 PROCEDURES

Eligibility screening occurred over the telephone prior to the first laboratory visit. During this screening the study was explained and information was collected in order to determine eligibility. If eligible, the participants were scheduled to come in to the laboratory on two separate occasions. Based on self-report of being sedentary or physically fit, an equal number of fit and unfit participants were randomly assigned to either the stressed experimental group or the nonstressed control group.

During the initial visit the study was again explained and informed consent obtained prior to participation. Information was collected on demographics, sleep quality, perceived stress, social support, self-efficacy, foods ingested in the previous 12 hours, and physical activity in the previous 12 hours. Following completion of the questionnaires, a butterfly needle was inserted into the antecubital vein of the non-dominant arm and remained in place for fifteen minutes before blood was taken. During this time participants were instructed to relax and read magazines. Additionally, an automated blood pressure cuff was placed on the dominant arm in order to record blood pressure and heart rate at 3 to 5-minute intervals throughout the study as a manipulation check of the stressors. After the first blood draw participants in the experimental condition engaged in two cognitive tasks, a speech task and the computer version of the Stroop Color and Word Test, both chosen for their known ability to elicit a stress response. For the speech task, participants were instructed to defend themselves in front of a judge for being

accused of shoplifting. They were given two minutes to prepare and three minutes to give the speech, which they were told was being recorded on a tape recorder. The speech task has been shown to effectively elicit cardiovascular and neuroendocrine responses (Stoney, Matthews, McDonald & Johnson, 1991). During the Stroop Color and Word Test participants were asked to choose the print color of a color name from color names printed in different colors. This task also elicits cardiovascular and immune responses characteristic of stress (Manuck, Cohen, Rabin, Muldoon & Bachen, 1991). Immediately following the completion of the task, a second blood draw was taken. The POMS-SF was given before and after the stress tasks to assess current mood and as an additional manipulation check of the stressors. Participants were then instructed to relax and read magazines before the third blood draw taken 20 minutes later (Figure 1). After the third blood draw the butterfly needle and blood pressure cuff were removed. Participants in the control condition underwent the same procedures as the experimental group except instead of completing the cognitive tasks they were instructed to read magazines during the stressor period. All participants also received instructions on how to access the web-based diet history questionnaire. Participants were debriefed at the conclusion of the session.

The second lab visit was scheduled for the same week or the week following the first visit. During the second lab visit the study was again explained to the participant and body composition was measured. Participants then performed the submaximal VO_2 test in order to get an estimate of their cardiorespiratory fitness.

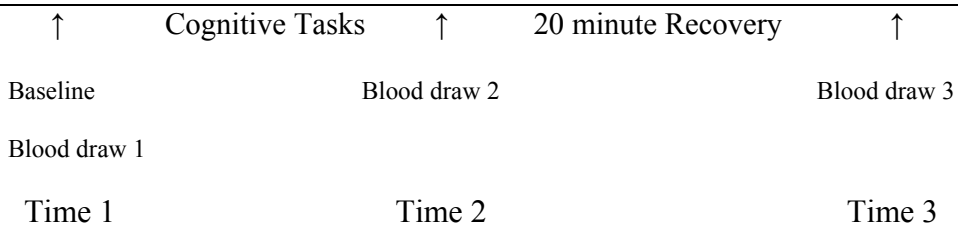

Figure 1. Timeline of blood draws.

4.1 DATA COLLECTION

A total of three 15ml blood samples were taken from each participant. Samples were placed immediately in EDTA-tubes to avoid coagulation. Mononuclear cells were isolated by Ficoll Hypaque centrifugation, as described by Kanof, Smith & Zola (1996). Cell viability was assessed by trypan blue dye exclusion and cells were counted under a microscope with a hemocytometer.

The comet assay was used to assess the amount of DNA damage in the lymphocytes. This technique is a widely accepted and reliable method, as proven in many testing circumstances (Hartmann, Agurell, Beevers, Brendler-Schwaab, Burlinson & Clay et al., 2003; Collins, Dusinska, Franklin, Somorovska, Petrovska & Duthie *et al*., 1997). As described by Collins (2004), cells were suspended in agarose gel then placed on slides and immersed in a lysis solution for one hour. Slides were then placed in an electrophoresis chamber in order to allow for the separation of damaged DNA strands. Slides were washed and stained with ethidium bromide before examination under a UV microscope. Visual scoring on each slide was based on the characterization of 100 randomly selected nucleoids, which were put into one of 5 classes (0,1,2,3,4) representing the increasing extent of DNA damage seen in the tail of the "comet".

Each comet was assigned a value according to its class and the overall score for 100 comets ranged from 0, indicating 100% of the comets were scored as being in class 0 with no damage, up to 400, in which 100% of the comets fell into class 4 with maximum damage. The same two experienced researchers using a specific pattern when moving along the slide carried out the observation and analysis. The observers had no knowledge of the identity of the slide during evaluation. Intraclass correlation (ICC) analysis was used to determine inter-rater reliability for the DNA damage scores from the two blind raters. An examination of 50% of the scores revealed that inter-rater reliability was adequately high, $r(54) = 0.96$, $p<0.01$.

In addition to measuring DNA damage, plasma catecholamines that are typically influenced by psychological stress, E and NE, were collected from the blood samples. For each sample, 7ml of blood was centrifuged and the plasma collected and stored at -20^{o} Celsius for future analysis. After all samples were collected, the plasma was unfrozen and catecholamines were measured using high performance liquid chromatography (HPLC).

5.0 DATA ANALYSIS

Data analysis was performed using SPSS version 13.0 for Windows. Descriptive statistics and frequency distributions were obtained for each variable of interest. Associations between baseline demographic data and biological outcome variables were examined with Pearson product-moment correlations in order to determine the variables to be used as covariates in further analyses. Independent t-tests were also conducted to examine if significant differences existed between the stress and control groups for demographic and psychosocial measures.

To verify that the stress manipulation was successful, 2x3 repeated measures analyses of variance (ANOVA) were conducted. Separate analyses were run using heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and POMS anxiety scores as dependent variables, with group assignment (stress/nonstressed) as the between subjects variable and time (Time 1, Time 2, Time 3) as the within subjects variable. The catecholamines, NE and E, were examined in exploratory analyses.

In order to test the response of DNA damage to the stress tasks, a 2x2 repeated measures analysis of covariance (ANCOVA) was performed using raw DNA damage scores as the dependent variable, group assignment as the between subjects variable, and time (Time 1, Time 2) as the within subjects variable. DNA damage at Time 3 (recovery period) was not included in the analyses, because a high number of participants in the sample (n=14) could not give a sufficient amount of blood at this time point. Pearson product moment correlations revealed that

age was significantly associated with DNA damage scores, $r(38)= -.33$, $p<0.05$, and was subsequently used as a covariate in the analyses.

A hierarchical multiple regression analysis was performed to test whether the strength of the effect of the stress condition on DNA damage depends on fitness level. Fitness was assessed by the scores on the VO_2 submaximal test and was treated as a continuous variable in the regression analysis. In order to carry out the regression analysis, it was necessary to calculate difference scores for DNA damage by subtracting the amount of damage at Time 2 (after the stress task) from the damage found at Time 1 (baseline). The difference score served as the dependent variable. Age was entered in the first step as a covariate, followed by fitness level and group assignment in the next steps. The interaction term, group x fitness, was entered in the final step. Relationships were determined by examining the variance in DNA damage explained by the 2-way interaction between stress condition and fitness level, over and above the main effects of each variable separately.

5.1 PARTICIPANT STATISTICS

A total of ninety-one men and women were recruited for this study. Fifty-one participants were unable to give the required amount of blood. Therefore, the final sample consisted of forty participants with twenty in the stress condition and twenty in the nonstressed control condition. The demographic information for this sample is provided in Table 1. Independent t-tests shown in Table 2 revealed that there were no significant statistical differences ($p>0.05$) in demographic variables or psychosocial measures between individuals in the stress group and those in the control group. All participants reported being non-smokers, drinking less than 10 alcoholic

drinks per week, and being in general good health. Frequency distributions for fitness scores in the overall sample, and in the control and stress groups separately are illustrated in Figures 2, 3 and 4, respectively.

Table 1. Demographic variable means and standard deviations.

	Entire Sample N=40	Stress Condition N=20	Control Condition N=20
Gender (% male)	22/40 (55%)	12/20 (60%)	10/20 (50%)
Age (SD)	21.6 (2.5)	22.1 (2.5)	21.2 (2.4)
Ethnicity (% Caucasian)	31/40 (77.5%)	15/20 (75%)	16/20 (80%)
BMI (SD)	22.4 (2.8)	22.4 (2.6)	22.6 (3.1)
Body Fat % (SD)	18.7 (6.8)	17.8 (6.9)	19.6 (6.7)
VO_2 (ml/kg/min) (SD)	45.9 (11.9)	48.9 (13.4)	42.9 (9.7)

Table 2. Independent t-tests for equality of means of demographic and psychosocial variables between the stress and no-stress control groups.

	Group Assignment	N	Mean	Std. Deviation	*t*	Sig. (2-tailed)
VO_2 (ml/kg/min)	Stress	20	42.90	9.69	-1.63	0.11
	No stress	20	48.90	13.37		
Age	Stress	20	21.15	2.43	-1.23	0.23
	No stress	20	22.10	2.47		
Gender	Stress	20	1.50	0.51	-0.62	0.54
	No stress	20	1.60	0.50		
PSS Total	Stress	20	14.90	4.88	-0.40	0.69
	No stress	20	15.55	5.31		
ISEL Total	Stress	20	18.05	6.61	-0.26	0.80
	No stress	20	19.05	15.92		
GSES Total	Stress	20	6.80	2.98	0.99	0.33
	No stress	20	5.80	3.38		
PSQI Total	Stress	20	8.45	5.20	-0.63	0.53
	No stress	20	9.50	5.35		
BMI	Stress	20	22.57	3.09	0.22	0.83
	No stress	20	22.37	2.56		
Body Fat %	Stress	20	19.64	6.74	0.84	0.41
	No stress	20	17.84	6.92		

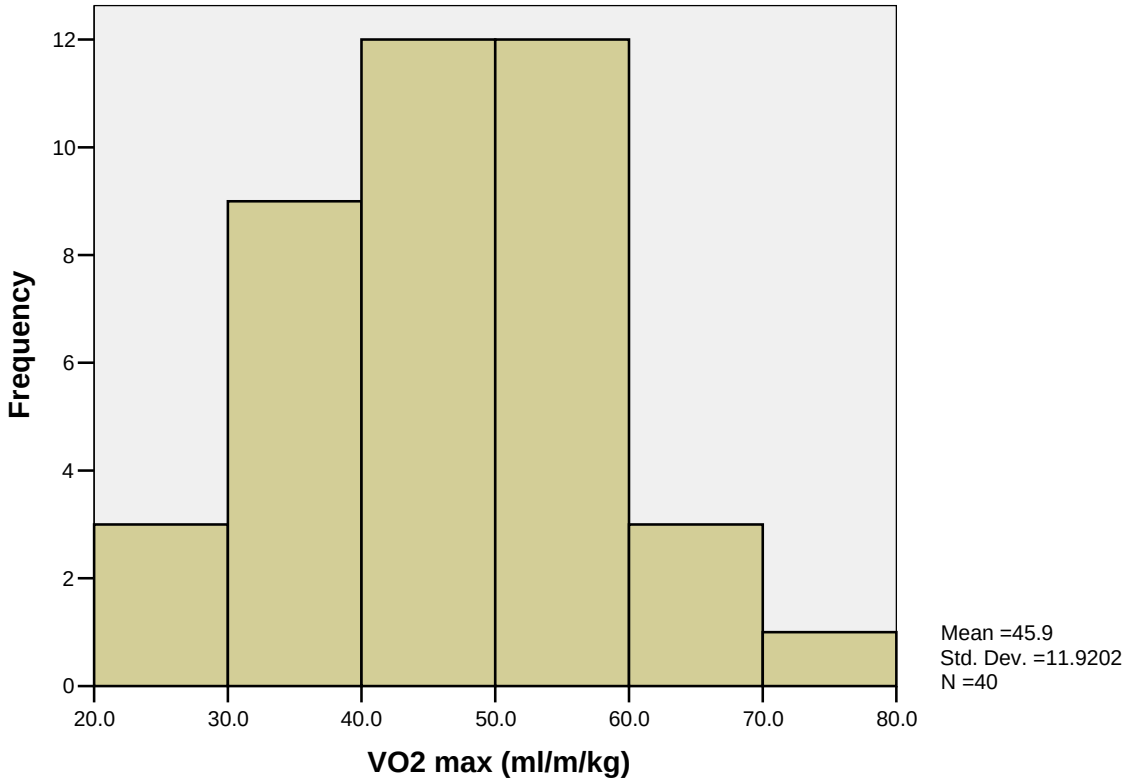

Figure 2. Frequency distribution for VO_2 max scores in the overall sample.

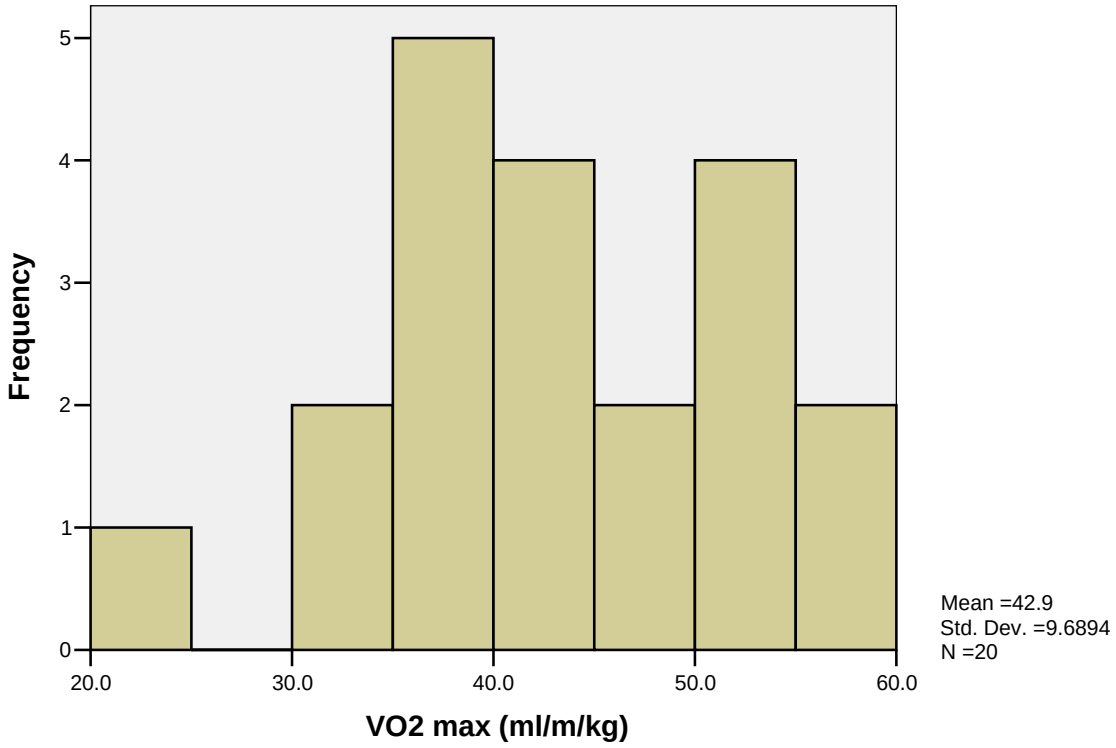

Figure 3. Frequency distribution for VO_2 max scores in the control group.

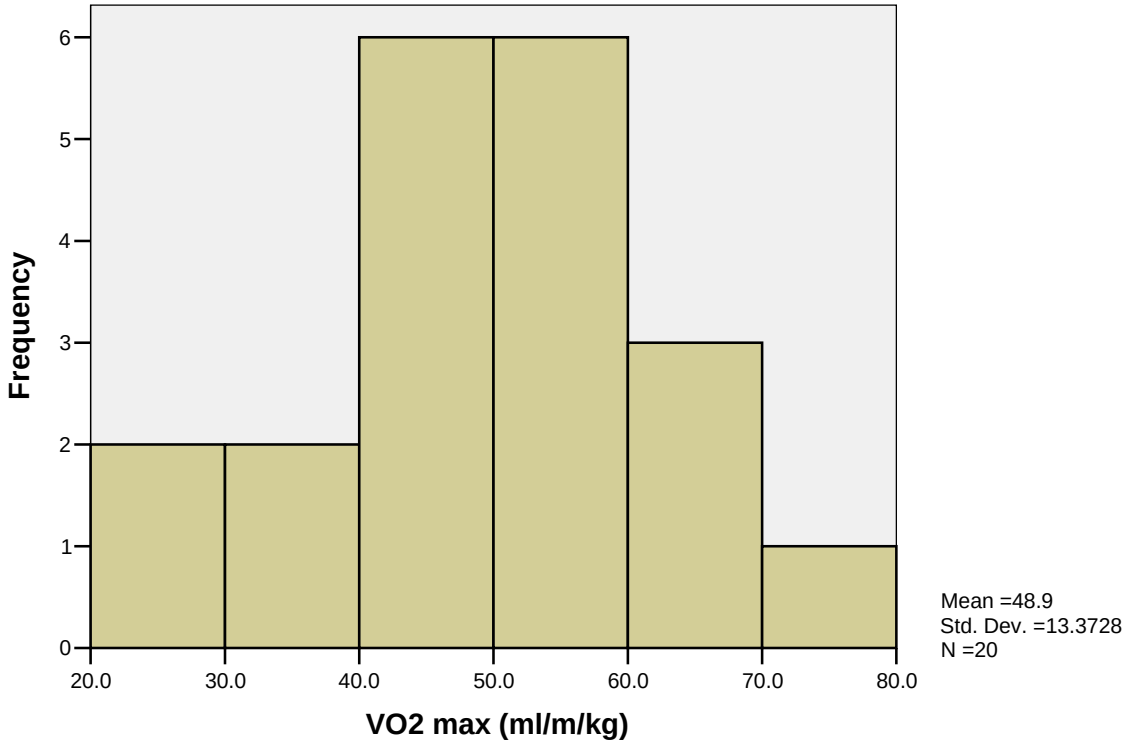

Figure 4. Frequency distribution for VO_2 max scores in the stress group.

5.2 RESULTS

5.2.1 Stress Manipulation

Results indicated that the stress manipulation was successful. The 2x3 repeated measures ANOVA yielded a significant interaction of group and time for HR, $F(1,38) = 1.44$, $p < 0.01$,

partial eta^2 = 0.28, SBP, F(1,38) = 19.45, $p < 0.01$, partial eta^2 = 0.34, and DBP, F(1,38)=28.72, $p < 0.01$, partial eta^2 = 0.43 (See Fig. 5,6,7). HR, SBP, and DBP were comparable at baseline for both stress and no-stress participants, but they diverged at Time 2 (after tasks). In the stress group, participants' HR, SBP, and DBP significantly increased at Time 2 for the stress group but had returned to near baseline levels at Time 3. Participants in the no-stress group showed little or no change over time. The POMS anxiety scores did not show an interaction effect, F (1,38) = 0.159, p>0.05, partial eta2 = 0.004.

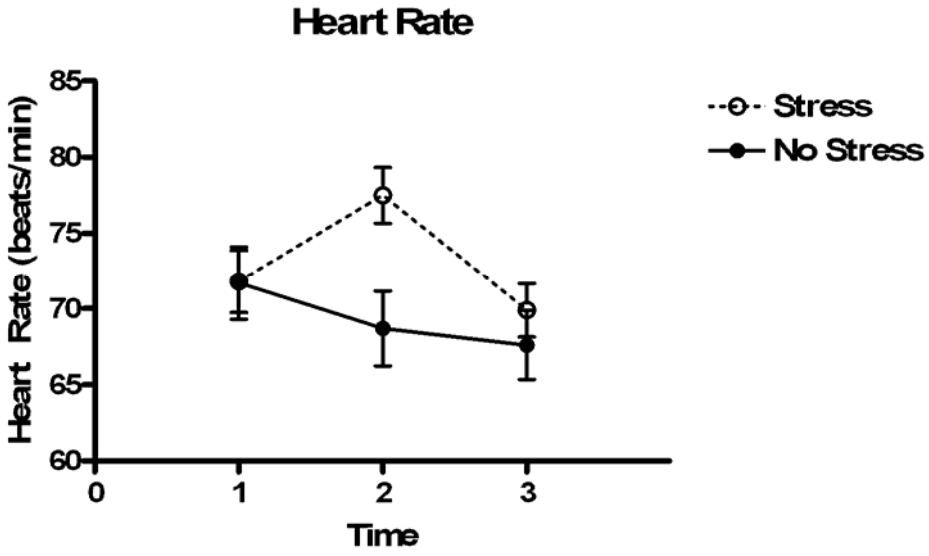

Figure 5. Effect of stress manipulation on heart rate in the stress and no-stress groups across time.

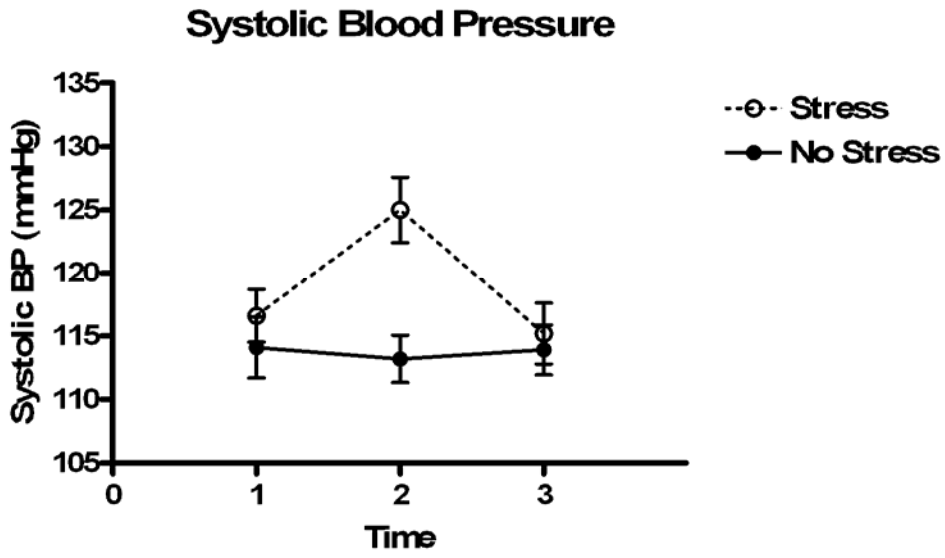

Figure 6. Effect of stress manipulation on systolic blood pressure in the stress and no-stress groups across time.

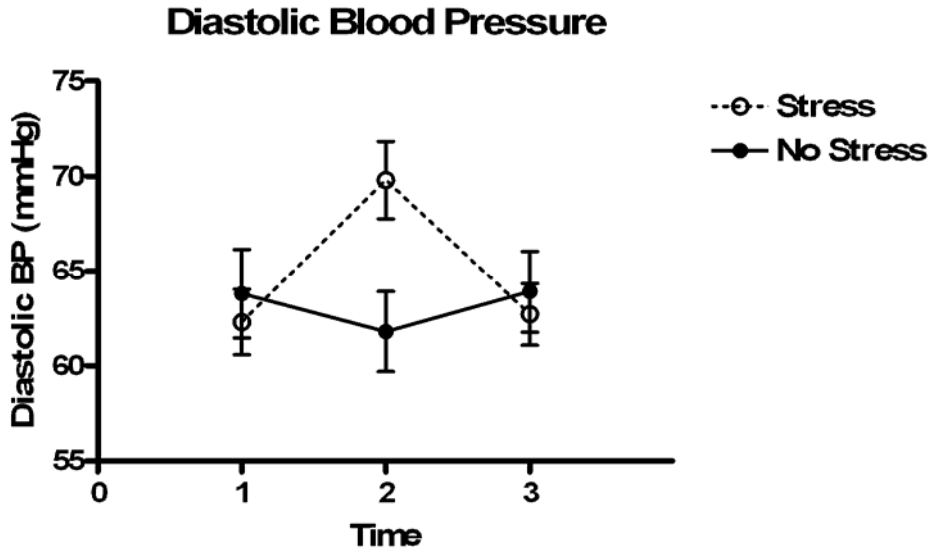

Figure 7. Effect of stress manipulation on diastolic blood pressure in the stress and no-stress groups across time.

5.2.2 Effect of Group Assignment on DNA Damage

It was expected that the stress group would have increased DNA damage after the stress tasks compared to at baseline, and that the control group would not exhibit this rise in damage. Results from a 2x2 (Group x Time) repeated measures ANCOVA supported this hypothesis. There was a significant interaction between group assignment and time, $F(1,38) = 4.1$, $p<0.05$, partial $eta^2 = 0.10$, indicating that DNA damage increased from Time 1 to Time 2 only in the stress group (see Fig. 8).

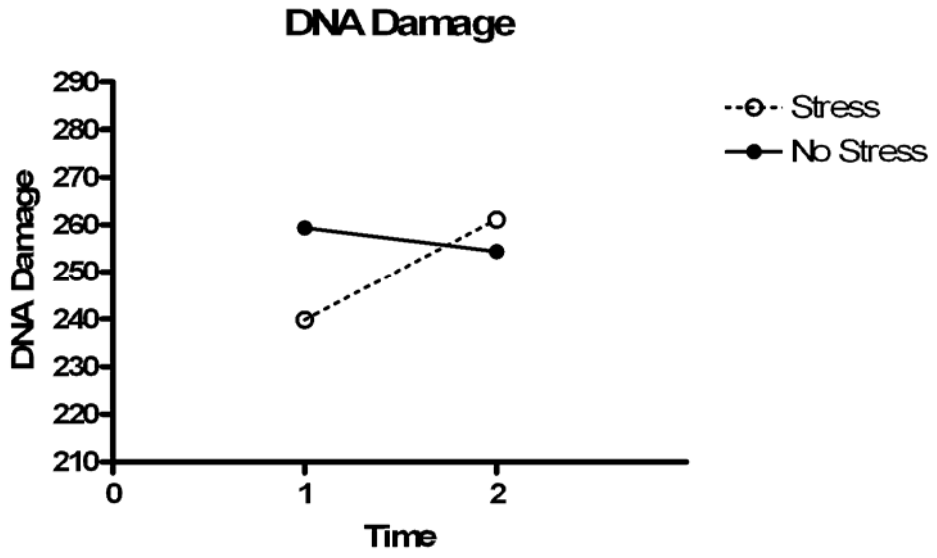

Figure 8. DNA damage across time between the stress and no-stress groups.

5.2.3 Impact of Fitness Level

It was hypothesized that the interaction between cardiorespiratory fitness and group assignment would significantly predict the change in DNA damage between Time 1 and Time 2 over and

above the main effects of each variable individually. As shown in Table 3, age and group assignment significantly predicted the variance in DNA damage difference scores. Age accounted for 11% of the variance, and group assignment accounted for 9% of the variance. No main effect for fitness occurred, and the interaction between fitness and group assignment did not have an influence over and above the independent main effects of age and group assignment. These results further support the hypothesis that the stress condition significantly contributed to the rise in DNA damage; however, the results do not support the hypothesis that fitness buffers this response.

Table 3. Regression analysis for the interaction of group assignment and fitness level on DNA damage difference scores.

Predictor	B	SE	*t*	*p*
Age	-6.27	2.72	-2.31	0.03*
Group Assignment	30.73	13.29	2.31	0.03*
VO_2 (ml/kg/min)	-1.03	0.97	-1.06	0.30
Group*VO_2 max	0.37	1.19	0.31	0.16

*$p<0.05$ (2-tailed)

5.2.4 Exploratory Analyses

Plasma catecholamine measures were examined in exploratory analyses. Based on results obtained by Flint et al. (2007), it was of interest to determine whether changes in E and NE were correlated with changes in DNA damage in response to the acute psychological stress. Pearson product moment correlations indicated that neither changes in E nor changes in NE were

significantly correlated with the changes in DNA damage in response to stress. Furthermore, repeated measures 2 (Group) x 2 (Time) ANOVA did not find a significant interaction between group and time for E, $F(1,38) = 2.89$, $p = 0.10$, partial $eta^2 = 0.03$ or NE, $F(1,38) = 2.1$, $p = 0.16$, partial $eta^2 = 0.05$, although there was a trend for E and NE to increase at Time 2 in the stress group and decrease in the control group.

6.0 DISCUSSION

Psychological stress and exercise affect overall health and cellular functioning in different ways. It is well known that habitual aerobic exercise has a beneficial effect by lowering resting heart rate and blood pressure, reducing body fat, strengthening muscles, enhancing immune function, increasing maximal oxygen uptake, and improving oxidative outcomes (Irwin & Friedman, 1999). Since psychological stress appears to negatively influence immune and neuroendocrine functioning, and to increase oxidative burden, it is possible that this negative impact is due to increases in stress hormones and ROS and the subsequent rise in DNA damage induced by psychological stress. The primary aims of this study were to expand upon previous evidence that psychological stress leads to increased DNA damage, and to determine if the benefits of exercise extend to protecting cells from this damage.

6.1 PSYCHOLOGICAL STRESS AND DNA DAMAGE

Results of this study are congruent with previous studies, and they also provide novel evidence for the impact of psychological stress on DNA damage. It has been shown that exam stress is linked to increased DNA damage (Sivonova et al., 2004), but this is the first study to find a significant effect of acute psychological stress on DNA damage in humans. This could have important implications for health if stress becomes chronic and DNA damage is not repaired by

the body's natural repair mechanisms. Although DNA damage scores at the recovery point (Time 3) were not included in the statistical analyses, of the 13 participants in the stress group that did provide Time 3 samples, trends suggest that DNA damage begins to return to baseline. This implies that damage can be repaired quickly and would probably not have a detrimental effect on cellular functioning if repair mechanisms are operating correctly or if stress is brief or readily resolved.

The mechanisms by which DNA damage occurs during acute psychological stress still remain unknown, although recent studies have begun to examine possible explanations. Flint *et al.* (2007) suggest that hormones released in the body during stress, particularly cortisol, NE and E, can cause DNA damage within 10 minutes. This was shown by the in vitro exposure of murine 3T3 cells to the stress hormones. The authors propose that the hormones induce damage by binding to cell surface receptors and triggering a series of intracellular signals, which have yet to be determined. It is important to recognize that circulating cells in the human body may respond very differently and more research is needed to bridge the gap between the response of murine cells and the response of active cells in humans. With this in mind, analyses using E and NE measures were conducted in the present study simply to explore their relationship to the increased DNA damage in lymphocytes in response to acute psychological stress. Although there was a trend for E and NE to increase at Time 2 in the stress group and decrease in the control group, the results do not indicate a significant relationship between these hormones and DNA damage. It is possible that the significance of this relationship would be strengthened by a larger sample size. It is also possible that ROS play a bigger role in the induction of damage in cells circulating in the human body and the influence of catecholamines on damage is minor in

comparison. Future studies are needed to measure plasma ROS in response to stress in order to better understand the influence of acute psychological stress on cellular processes.

6.2 THE INFLUENCE OF CARDIORESPIRATORY FITNESS

Contrary to our hypothesis, cardiorespiratory fitness levels did not buffer the impact of acute psychological stress on DNA damage. It was hypothesized that as a result of habitual aerobic exercise fit individuals would have stronger antioxidant and DNA repair responses and would be better prepared to resist DNA damage in response to psychological stress. One reason for why this effect was not seen is that the sample recruited for this study did not exhibit a wide range of cardiovascular fitness levels. As can be seen in the distribution graph of Figure 2, the fitness scores are evenly distributed, but the range is limited. With a mean VO_2 max of 45.9 ml/kg/min and most falling between 40 and 60 ml/kg/min this sample is considered above average in fitness compared to the average population of 18-25 year olds. According to the American College of Sports Medicine, men aged 18-25 need a score of at least 44.5 ml/kg/min and women need a score of at least 35.2 ml/kg/min to be in the top 50^{th} percentile. This makes it difficult to discuss these results with any degree of certainty because the analysis may have been considerably different had more unfit individuals been included in this study.

6.3 LIMITATIONS

Several limitations are present in this study. The narrow range of cardiorespiratory fitness levels represented in this sample is a major limitation. It is possible that the sample was high in fitness scores because of the exclusion criteria placed on the recruitment of participants. Participants were only allowed in the study if they were not overweight, did not smoke, did not drink excessively and were in general good health. The possibility exists that these criteria effectively excluded a large percentage of unfit individuals. Another possibility is that individuals interested in fitness were more drawn to the study because of the fitness test involved.

One strategy to avoid this would be to widen the inclusion criteria, although that would also increase the variability of the sample. Because participants have assigned themselves to their fitness levels a considerable amount of experimental control associated with randomization is not available. The purpose of keeping the criteria strict in this study was to eliminate any differences that may exist between individuals of varying fitness levels, including BMI, body fat, and alcohol consumption, that could have had an impact on the dependent variable. In order to improve on this limitation in future studies, another approach would be to conduct a longitudinal study. A longitudinal study would assume that if there are no pre-existing differences in stress reactivity between the group of individuals to be trained and the untrained group then changes arising after training are a result of the exercise itself. Because of the limitations of this sample, it can not be ruled out that fitness has a role in protecting cells from the potentially harmful impact of psychological stress. Future studies using a wider range of fit and unfit individuals or a longitudinal design are needed to further examine this hypothesis.

Another limitation of this study is the small sample size. It is possible that the small effect size seen in the repeated measures ANCOVA examining the raw DNA damage scores in

response to psychological stress is a result of limited power due to the size of the sample. In addition, the small sample size may have hindered the ability to find a significant interaction effect between group and fitness in the hierarchical multiple regression. However, because of the limited range of fitness levels it is not expected that an increase in sample size would have improved the chances of finding a significant interaction in the analysis.

Lastly, it is of concern that over half of the recruited sample could not complete the study due to the inability to donate enough blood for analysis. This occurred for several reasons. The first reason was phlebotomy error. It was difficult for the phlebotomist to find the antecubital vein in participants where it was not prominent. This could be another contributing factor in the exclusion of unfit individuals, because their veins may have not have been as easy to locate. In addition, participants who were overly anxious about giving blood typically could not make it through the entire study. It is possible that the elimination of these individuals reduced the variability in stress reactivity responses. Finally, it was necessary to exclude individuals who could not give the required amount of blood because they formed blood clots too fast. This was also most likely caused by phlebotomy error. These reasons taken together mean that the sample left in this study only included individuals with prominent veins who were comfortable giving blood. Future studies will need to address these issues in order to avoid excluding individuals unnecessarily.

6.4 CONCLUSION

Despite the limitations, this study does contribute to the present knowledge of the effects of psychological stress on cellular functioning and health. However, this should be considered a

preliminary study that other researchers can expand and improve upon in order to make progress in investigating the beneficial health impact of exercise. Future studies are needed to broaden our understanding of how physical fitness may protect the human body from the potentially damaging effects of psychological stress.

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