INFLAMMATION AND OXIDATIVE DAMAGE DURING EXAM STRESS

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Oxidative stress and resulting oxidative damage at the cellular level is thought to be a major factor in the aging process. Associated cellular dysfunction or loss of elasticity as a result of oxidative stress may increase the risk for age related diseases like cancer and atherosclerosis. Psychological stress may be influential in this process through altered behaviors and/or changes in biological systems that then affect oxidative stress. One source of oxidative stress is inflammation and inflammatory cells such as the neutrophil, which produce reactive oxygen species (ROS). Recent research suggests that stress may increase inflammation. These increases in inflammation during stress may lead to elevations in oxidative stress, which may be a pathway through which psychological stress increases risk for disease and possibly accelerates aging. To date, no study has examined the potential relationship between inflammation and oxidative stress during times of psychological stress. The present study investigates the interrelationships among stress, neutrophil activity, and oxidative damage, controlling for health behaviors, in a group of 18 healthy professional students taking exams. This study found increases in neutrophil activity during exams when compared to a month after the exam. However, oxidative damage did not increase during exams, and changes in alcohol consumption may partially account for this finding. Interestingly, oxidative damage was found to be highly correlated with neutrophil activity during exams after controlling for demographic and health behaviors. The implication and limitations of these findings are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Contributors

1.0   INTRODUCTION

There are many theories of how people age, and some popular models suggest that oxidative stress and resulting oxidative damage at the cellular level are major factors in the speed at which aging occurs. Aging is facilitated by elevated levels of oxidized proteins, lipids, and DNA and a decreased ability of defensive systems to prevent subsequent oxidation (Ames, Shigenaga, & Hagen, 1993). Associated cellular dysfunction or loss of elasticity may increase the risk for age related diseases like cancer and atherosclerosis (Klaunig & Kamendulis, 2004). Psychological processes may be influential in this process through altered behaviors and/or changes in biological systems that then effect oxidative stress.

One source of oxidative stress is inflammation and inflammatory cells, which produce reactive oxygen species (ROS). Current evidence points to increases in inflammatory markers during psychological stress (Black, 2003). Increases in inflammation during stress may lead to elevations in oxidative stress, which may be a pathway through which psychological stress increases risk for disease and possibly accelerates aging. To date, no study has examined the potential relationship between inflammation and oxidative stress. This study will address this void and test the hypothesis that neutrophil activity, a key element of inflammation, contributes to the association between psychological stress and oxidative stress. The present study will investigate the interrelationships among stress, inflammation and oxidative damage in a group of healthy professional students.

1.1   SOURCES OF OXIDATIVE STRESS

Oxidative stress is a state of imbalance in the body with more pro-oxidants than anti-oxidants, and higher levels of ROS from various sources and decreases or stable quantities of anti-oxidants. Sources of ROS in the body include inflammatory cells, transformation of organic compounds into cellular energy occurring in the mitochondria, and the break down of fatty acids occurring in peroxisomes (Klaunig & Kamendulis; Champ & Harvey, 1994). ROS can also be produced from microsomal cytochrome P450 enzyme activities, which is involved in the breakdown of toxic compounds, steroids, and alcohol (Klaunig & Kamendulis, 2004; Champe & Harvey, 1994). Another source of ROS production is the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) by cyclo-oxygenase (COX) (Chung, Jung, & Yu, 2005). Normal cellular processes produce ROS and these products are opposed by anti-oxidant systems in both intracellular and extracellular space that reduce these species back to harmless states. However, excess levels of ROS or decreased antioxidant activity could increase the chance of oxidative damage.

1.2   OXIDATIVE STRESS AND CANCER INITIATION

Oxidative stress has been long proposed to be involved in the pathogenesis of cancer (Hsie, Recio, Katz, Lee, Wagner, & Schenley, 1986). Excess levels of oxidative stress can result in genetic mutations and the development of malignancies; Oxidants, such as hydroxyl radicals and peroxynitrite (formed from nitric oxide and superoxide), cause DNA damage that can lead to mutations (Marnett, 2000). Although various checkpoints exist in the cell to prevent mutations, such as DNA damage detectors, cellular arrest, apoptosis, and DNA repair mechanisms,

2

damaged sites can go unrepaired prior to cellular replication and become part of the genome (Sompayrac, 2003).

Mutations in the genome are often innocuous, but specific mutations, particularly in areas responsible for regulating cell growth, have been linked to initiation of cancer (Sompayrac, 2003). Proto-oncogenes, genes involved in the regulatory process of the cell, can mutate into oncogenes, which contribute to cell transformation. Many cancers have mutations in the gene that encodes p53, the protein involved in suppression of tumor growth by activating cellular arrest (Sompayrac, 2003). When the cell is damaged by ROS, cellular arrest normally occurs, preventing replication before damage has been repaired. If the cell is unable to repair the insults incurred by ROS, it will usually activate apoptosis, programmed cell senescence. Malfunctions in these checkpoints result in permanent changes that may permit development of tumors (Fitzpatrick, 2001; Klaunig & Kamedulis, 2004; Sompayrac, 2003).

1.3   INFLAMMATION AND OXIDATIVE STRESS

The innate immune system is a complex network of cells and chemical messengers. Phagocytic cells, such as neutrophils, macrophages, and monocytes, are part of the innate immune system and play a crucial role during an inflammatory response. The predominant phagocyte in peripheral blood is the neutrophil, which makes up as much as 70% of circulating white blood cells and has a very short life (average of 5 days) (Sompayrac, 2003). The macrophages residing in tissue have a long life and upon tissue injury or encountering infectious agent become activated. Damaged tissue and macrophages release cytokines such as IL-6, TNF-α, and IL-1 and chemokines, which act as signals to stimulate the production and recruitment of

neutrophils to the site of infection or injury. Phagocytic cells produce toxic substances upon activation to destroy and remove bacteria, cells infected with bacteria, and apoptotic/necrotic cells (Paul, 2003). These toxic substances include proteolytic enzymes, nitric oxide, and reactive oxygen species (ROS) (Roitt & Delves, 2001). These substances act on nearby cells and pathogenic agents by damaging cell walls, proteins, lipids, and genetic material. ROS found in phagocytic cells include both hydrogen peroxide (H_2O_2) and superoxide anions. Thus inflammation and the phagocytic cells associated with inflammation are one source of oxidative stress (Yeo, Han, Nam, Kim, Cho, & Hahm, 2005).

Although typical production of ROS does not result in elevated oxidative stress, overproduction as a result of chronic inflammation that accompanies chronic infections, hypercholesterolemia, or other pathophysiological conditions does elevate levels of ROS. Excess production of ROS secondary to inflammation has been proposed to be involved in aging (Chung, et al., 2005), and in the etiology of some cancers (Fitzpatrick, 2001; Coussens & Werb, 2002). Inflammation and subsequent oxidative stress is thought to be involved in the pathogenesis of atherosclerosis, rheumatoid arthritis, and acute respiratory distress syndrome (Babior, 2000). In this regard, several studies have found increased levels of markers of oxidative stress in individuals with inflammatory diseases (e.g. rheumatoid arthritis, atopic dermatitis, cystic fibrosis, type II diabetes) (Blasiak, Arabski, Krupa, Wozniak, Zadrozny, Kasznicki, Zurawska, & Drzewoski, 2004; Brown, McBurney, Lunec, & Kelly, 1995; Buske-Kirschbaum, Gierens, Hollig, & Hellhammer, 2002; Krapfenbauer, Birnbacher, Vierhapper, Herkner, Kampel, & Lubec, 1998; Rall, Roubenoff, Meydani, Han, & Meydani, 2000).

In addition, there is growing evidence for increased risk for cancer in individuals with chronic inflammatory diseases (Yeo, et. al., 2005), such as bronchitis, inflammatory bowel

disease, chronic pancreatitis, gingivitis, and various infections (see Coussens & Werb, 2002 for a review). A recent epidemiologic study found that elevated markers of inflammation, such as C-reactive protein, IL-6, and TNF-alpha, were associated with increased risk of death from cancer (Il'yasova, D., Colbert, L. H., Harris, T. B., Newman, A. B., Bauer, D. C., Satterfield, S., et al., 2005). Taken together, current evidence suggests that inflammation is one pathway through which elevated oxidative stress may lead to cancer, atherosclerosis, and other age related diseases.

2.0   PSYCHOLOGICAL STRESS

The stress response consists of psychological and biological changes that accompany perceptions of threat, harm, or loss and are characterized by the activation of the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal axis (HPA) (Lazarus & Folkman, 1984). The activation of the SNS results in nonspecific physiological changes including the release of norepinephrine (NE) via sympathetic nerve endings and epinephrine (E) from the adrenal gland which then affect many organs of the body, including the vasculature. NE has high affinity to α-adrenergic receptors, while E binds avidly with β-adrenergic receptors. Activation of α-adrenergic receptors in the systemic arterioles generally causes constriction, raising peripheral resistance and diastolic blood pressure, and β-adrenergic receptor binding results in dilation of muscle and coronary vessels, increased cardiac volume and contraction frequency, elevating systolic blood pressure (Schurmeyer & Wickings, 1999; Rushmer, 1989). The HPA axis, when activated by the presence of stress, causes release of corticotropic releasing hormone (CRH) from the hypothalamus. CRH acts on the pituitary and stimulates the release of adrenocorticotropic hormone (ACTH) that travels to the adrenal gland and stimulates the release of glucocorticoids, primarily cortisol in humans. (Baum & Grunberg, 1997; Schurmeyer & Wickings, 1999)

Baum, Cohen, and Hall (1993) suggest defining stressors by the duration of the stressful event, duration of threat, and the duration of the response. Chronic stressors are thought of as either reoccurring acute stressors with frequent elicitation of the characteristic stress response without habituation, or chronic prolonged elevation in arousal. Assessments of stress severity must include an evaluation of the degree to which the individual perceives threatened loss or harm, and not just an assessment of the presence or absence of stressful events (Baum, Cohen, & Hall, 1993).

Exam stressors can be very potent stressors, especially when the exam is perceived as important to the individual's future career objectives. Failure on the exam threatens the loss of this objective, the esteem of others, and confidence in self. Examples of these types of exams include medical students taking their board exams, nursing students taking their certification exams, and law students taking their bar exams. Each of these exams requires months of preparation, long hours of studying, and weeks of anxiety about performance success. Passing the exam is critical because success qualifies the individual to perform their selected career, while failure prevents this from happening. Continued general tension about preparing for the upcoming exam is interspersed with acute reactions to the perceived threat of the exam when an intrusive thought about the importance of the exam occurs.

Chronic stressors have been associated with poorer health, and some of this association is thought to be a result of alterations in levels of stress hormones and their subsequent effects on the immune system (Dougall & Baum, 2003). It is also possible that stressors partially exert their effect on health through changes in health behaviors. During times of stress humans often adopt poorer health behaviors, such as decreased physical activity and increased alcohol consumption (Steptoe, Wardle, Pollard, Canaan, & Davies, 1996). Many health behaviors could potentially

influence the immune system and levels of oxidative stress, including vitamin use, nutrition, alcohol use, smoking, regular physical activity, and sleep quality (Moller, Wallin, & Knudsen, 1996; Kiecolt-Glaser & Glaser, 1988). Thus it would be important to assess these behaviors when studying the effect of stress on health.

2.1 PSYCHOLOGICAL STRESS AND OXIDATIVE STRESS

One of the first studies to examine the potential relationship between psychological factors and oxidative stress focused on DNA damage and repair among 28 psychiatric patients scoring either high or low on the depression scale of the MMPI (Kiecolt-Glaser, Stephens, Lipetz, Speicher, and Glaser, 1985). Cellular repair of DNA damage after x-irradiation of cells taken from participants in each group was monitored over a 5 hour period. High distress was found to be associated with lower repair at the 5 hour time point, but not at 0 or 2 hours post x-irradiation (Kiecolt-Glaser, et. al., 1985). Conclusions from this study about the impact of stress on DNA damage and repair are limited by the psychiatric sample, but research since this ground breaking study provides support for a role of stress in DNA damage.

Forlenza, Latimer, and Baum (2000) and Cohen, Marshall, Cheng, Agarwal, and Wei (2000) both investigated the DNA repair capacity of peripheral blood lymphocytes during exam stress. Forlenza and colleagues (2000) used the unscheduled DNA synthesis (UDS) assay to determine DNA repair capacity. In this assay, cells were either damaged by exposure to UV-C light or not and were examined after 2 hours to assess amount of repair. When compared with low stress periods, exam stress was associated with greater repair of DNA damage. However,

8

this was observed for all cells, whether exposed to UV-C light or not, leading the authors to suggest that stress is an endogenous source of DNA damage.

Similar findings were reported by Cohen et al (2000) who also investigated the effect of exam stress on repair capacity of immune cells. In this case, a different assay was employed to assess repair capacity. The host-cell reactivation (HRC) assay involves infecting the host cell with strands of DNA that have been damaged by UV light and monitoring the cells ability to repair this damaged DNA. Using HRC, Cohen and colleagues (2000) found greater DNA repair capacity during an exam period than after vacation. Again, the authors suggest that DNA damage as a result of stress may be a possible cause for the observed increase in DNA repair. Stress-related increases in DNA repair were not associated with change in diet or alcohol use; however, other potential lifestyle factors, such as BMI, were not controlled.

Irie, Asami, Nagata, Miyata, and Kasai (2001) assessed a more direct marker of oxidative damage to DNA by measuring levels of 8-hydroxydeoxyguanosine (8-OH-dG) in blood leukocytes. This measure determines the level of 8-OH-dG, a product of DNA damage, in leukocytes using high performance liquid chromatography. Findings demonstrate a positive relationship between perceived psychological stress and workload and levels of oxidative damage in female subjects only. Additionally, females who perceived that they could alleviate the stress had lower levels of oxidative damage than those who perceived that they could not ease the stress. There were no significant relationships between stress and this measure of DNA damage among males. The authors attribute these sex differences to the possibility that females experience more perceived overwork and more domestic demands and stressors outside of work than males, however there were no differences between males and females reports of stress. The measures of stress used in this study were one item indices with no known reliability, and make

interpretation difficult (Irie, Asami, Nagata, Miyata, & Kasai, 2001). This study measured sleep quality, physical activity, diet, and BMI and found no association with 8-OH-dG values.

Another cross-sectional study conducted by the same group assessed blood leukocyte levels of 8-OH-dG among male and female workers (Irie, Asami, Nagata, Ikeda, Miyata, & Kasai, 2001). Regression analysis was used to identify various psychosocial predictors of oxidative damage, controlling for alcohol, smoking, age, and BMI, but not diet. In males (n = 274), hours of work positively predicted oxidative stress, while in females (n = 85) predictors of oxidative damage included the following negative affect subscales on the POMS: tension-anxiety, depression-rejection, anger-hostility, fatigue, and confusion. Additionally, the wishful thinking coping style in females and the self-blame coping style in males predicted higher 8-OH-dG levels. Interestingly, a subset of males (n = 15) reporting loss of a close family member within the last year showed higher levels of oxidative damage than their non-bereaved counterparts.

More recently Irie, Asami, Ikeda, and Kasai (2003) published a third study assessing blood leukocyte levels of 8-OH-dG and found a positive relationship with depressive symptoms in women. They also found a positive correlation (r = .38) between the percentage of circulating neutrophils and depression scores in females, and a positive correlation between the percentage of neutrophils and 8-OH-dG levels in females (r = .31). They hypothesized that the increases in DNA damage may be a result of neutrophil activity, but did not statistically assess this as a mediator. This study controlled for BMI, smoking and alcohol use, but did not report measuring diet.

In a study of parents of a child with cystic fibrosis and controls, the stress of caregiving appeared to make lymphocytes more susceptible to damage inducing agents (Dimitroglou,

Zafiropoulou, Messini-Nikolaki, Doudounakis, Tsilimigaki, & Piperakis, 2003). In this study, lymphocytes were isolated from blood, damaged by exposure to hydrogen peroxide or γ-irradiation, and assessed immediately and after 2 hours for levels of DNA damage using the comet assay. The comet assay is a technique that uses electrophoresis to pull out DNA strand breaks and quantifies the degree of damage in 100 visualized cells per sample. Levels of endogenous damage were not different between stressed parents and controls. However, the stressed group showed significantly higher levels of induced damage which remained after cells were allowed to repair, suggesting psychological stress may make individual's lymphocytes more susceptible to damage. Unfortunately, this study did not report recording any relevant health behaviors and the sample of control parents were selected if their stress scores were low rather than matching controls by age and income.

The only study to date that specifically looks at markers of oxidative stress during exam stress found increased levels of DNA damage in peripheral blood lymphocytes, measured using the comet assay, at the time of exams when compared to a less stressful time period (Sivonova, Zitnanova, Hlincikova, Skodacek, Trebaticka, & Durackova, 2004). Sivonova et al, also found an increased speed of lipoproteins to oxidize and a decrease in antioxidant levels during stress. This study reports that diet habits did not differ between the two time periods, and researchers requested participants to avoid taking antioxidant containing vitamins or food, and avoid excessive alcohol consumption. Other factors such as sleep quality, physical activity, and BMI were not evaluated. Thus, it is possible that the increase in DNA damage observed during exams was the result of health behaviors.

A recent study by Epel, Blackburn, Lin, Dhabhar, Adler, Morrow, and Cawthon (2004) investigated the effects of caring for a chronically ill child on telomere length, telomerase

activity, and oxidative stress in peripheral blood lymphocytes. The oxidative stress marker used was a measure of oxidized lipids. Telomeres are the binding site for primers at the end of every strand of DNA, and are involved in chromosomal stability. Telomeres shorten with each cell division and could possible be shortened as a result of oxidative damage to DNA (von Zglinicki, 2002). The lengths of telomeres are a marker of cellular age, and once the telomere is too short, the cell will die. Epel and collegues (2004) sampled mothers of healthy children (n = 19) and mothers of ill children (n = 39), and assessed perceived stress (PSS), length of time as a caregiver, and telomere length, telomerase activity, and oxidative stress. Within the group with an ill child, telomere length, telomerase activity, and oxidative stress where all related to scores on the PSS and to years of caregiving, suggesting that chronic stress may contribute to shortening of telomeres, reductions in telomerase activity, and increases in oxidative stress. These relationships remained after controlling for age, BMI, smoking, and vitamin use[1] (Epel, Blackburn, Lin, Dhabhar, Adler, Morrow, & Cawthon, 2004) In contrast, between group analyses comparing mother of an ill child with mothers of healthy children revealed no significant differences in telomere length, telomerase activity, or oxidative stress.

Taken together, these findings support the theory that psychological stress may increase oxidative stress, raising the possibility that over long periods of time this accumulation of oxidative damage may tax the body's capacity to function. Permanent mutations in the genome and elevations in oxidized lipids and proteins imbedded in cell walls and tissue ultimately reduce the life of the organism by reducing the functionality of the cell and increase the risk of cancerous growth. Less clear, is whether the increases in oxidative stress during times of psychological stress are directly attributed to the physiologic arousal that accompanies stress or

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[1] Exception to this: The correlation between PSS and oxidative stress lost significance (r = 27 to r = .22) when BMI, smoking and vitamin use were included in the analysis.

12

are the result of health behavior changes that increase oxidative damage, such as poor nutrition (Thompson, Heimendinger, Haegele, Sedlacek, Gillette, O'Neill, et. al. 1999) and alcohol consumption (Purohit, Khalsa, & Serrano, 2005).

2.2   PSYCHOLOGICAL STRESS AND INFLAMMATION

Recent literature has considered the potential role stress may play in the activation of the inflammatory response (Black, 2003). Investigations into inflammatory cytokines suggest that chronic stressors may increase circulating pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and other downstream markers of inflammation such as C-reactive protein (Kiecolt-Glaser, McGuire, Robles, & Glaser, 2002). Exam stress has been associated with the production of inflammatory cytokines. For example, medical students taking an examination were found to have increased IL-1β and decrease INF-gamma cytokine levels (Dobbin, Harth, McCain, Martin, & Cousin, 1991). In a review of the literature, Segerstom and Miller (2004) reported fairly consistent findings linking exam stress to noticeable decreased stimulated production of IL-2 and IFN-γ, with concomitant increases in stimulated production of IL-6 and IL-1β, supporting a shift to a proinflammatory state (Segerstrom & Miller, 2004). Inflammatory cytokines recruit phagocytes to the sites of infection or injury, and this noticeable increase in circulating levels of inflammatory cytokines during stress could cause increased activation of phagocytes. The combination of IL-6 and platelet activating factor (PAF) have been show to prime neutrophils for release of ROS (Biffl, Moore, Moore, Carl, Kim, & Franciose, 1994). In sum, evidence supports an activation of inflammatory innate immunity during times of exam stress and suggests associated increases in oxidative stress.

2.3 PYCHOLOGICAL STRESS AND NEUTROPHIL ACTIVITY

In support of an association between stress and neutrophil activity, stress has been associated with increase numbers of circulating phagocytes and neutrophils bearing activation markers in rats and humans, and this increase appears to be dependant on either or both catecholamines and gluccocorticoids (Dhabhar, Miller, McEwen, & Spencer, 1996; Engler, Dawils, Hoves, Kurth, Stevenson, Schauenstein, & Stefanski, 2004). For example, the chronic stress of living near Three Mile Island was associated with elevated levels of neutrophils in peripheral blood, and these higher levels of circulating neutrophils were positively correlated (r = .57) with urinary eprinephrine levels (McKinnon, Weisse, Reynolds, Bowles, & Baum, 1989). Infusion with either norepinephrine or epinephrine appears to increase neutrophil numbers in circulation, while cortisol increases the survival rate of neutrophils (Gader, & Cash, 1975; Davis, Albert, Tracy, Calvano, Lowry, Shires, et al., 1991; Boomershine, Wang, & Zwilling, 2001). These studies suggest that stress-related increases in catecholamines and cortisol stimulate an increase in circulating levels of phagocytes.

Stress also appears to increase phagocyte production of ROS (Kang, Coe, & McCarthy, 1996; Kihara, Teshima, Sogawa, & Nakagawa, 1992; Wadee, Kuschke, Kometz, & Berk, 2001). Here, Kang, et al. (1996) show increased stimulated neutrophil production of ROS in both healthy and asthmatic adolescent students during and 2-3 weeks after examinations as compared to a period of lower stress. Similarly, Kihara, et al (1992) report more production of ROS by stimulated neutrophils a day before a nursing examination when compared to a month prior. Observed increases in the production of ROS by neutrophils during exams do not appear to be the result of increased numbers in circulation (Kihara et al.,1992; Kang, et al., 1996). However, it remains unclear whether stress-realted changes in health behaviors accounted for these findings.

14

Wadee, et al. (2001) investigated the impact of trait anxiety and exam stress on the ability of stimulated and unstimulated neutrophils to produce ROS. Findings show higher production of ROS by unstimulated neutrophils during exams, when compared with a time of lower stress. Further analysis revealed that elevated neutrophil activity occurred only among individuals who endorse high trait anxiety, while those endorsing low trait anxiety did not show the increased neutrophil activity during exams. In this study, Wadee and colleagues employed the nitro blue tetrazolium (NBT) test to assess ROS production. The NBT provides a percentage of cells that produce ROS and thus controls for cell number. Once again, this study did not assess the role of health behaviors or BMI. However, despite this limitation, the findings suggest that neutrophil production of ROS is increased during times of stress, especially in individuals with high levels of anxiety.

2.4   SOURCES OF INFLAMMATION

There are many possible sources of inflammatory stimuli during times of stress including macrophages, endothelial cells, and adipose tissue. Increases in inflammation may result from elevated levels of NE and E, which act directly on macrophages, neutrophils, and adipocytes. In this regard, NE has been demonstrated to increase and E to decrease the activity of macrophages and neutrophils (Boomershine, Wang, & Zwilling, 2001). In addition, β-adrenergic receptors, which have a high affinity for E, on adipocytes have been shown to stimulate the production of IL-6 (Mahamed-Ali, Flower, Sethi, Hotamisligil, Gray, Humphries, York, & Pinkney, 2001). Inflammatory cytokines could activate phagocytes directly (Biffle, et al., 1994), or a secondary

signal may be necessary for these cells to become active, such as from a low lying infection or tissue injury (Black, 2003).

Alternatively, elevations in SNS may indirectly activate inflammation by increasing blood pressure, causing shear stress that results in tissue injury and an associated inflammatory response (Black, 2003; Manuck, Marsland, Kaplan, & Williams, 1995). Previously damaged sites are repaired with fibrous caps and harbor resident macrophages which ingest oxidized LDL and form foam cells and fatty streaks, causing additional inflammation and production of reactive oxygen species (ROS) (Gordon & Libby, 2003; Adams, 1994). The coagulation pathway can be activated at the site of an existing fibrous plaque formation when an additional breach in the cap occurs, often caused by sheer stress to the vessel wall. Release of thrombin increases expression of P-selectin (an adhesion molecule) by platelets and endothelium, which allows adhesion of platelets and leukocytes to the endothelium. Expression of CD40 by T cells and platelets increases coagulation in the endothelium, activates endothelium expression of adhesion molecules (i.e. ICAM-1), increases production of inflammatory cytokines (IL-6, IL-1β) by monocytes, and increases secretion of granulocyte-monocyte colony stimulating factor (GM-CSF) and metalloproteinase from smooth muscle cells of the vasculature and monocytes. This cascade of events recruits additional neutrophils, monocytes and platelets to the site of damage. (Gordon & Libby; Yamada & Topol, 2000). Thus sheer stress may play a role in the activation of inflammation during times of psychological stress.

3.0   CONCLUSION

Psychological factors, such as stress, are thought to increase risk for age-related diseases through both alterations in health behaviors, and through repeated or excessive elevations in the hypothalamus-pituitary-adrenal system and the sympathetic-adrenomedullary system, which then act on tissue and intracellular processes directly and alter components of the immune system (Cohen & Herbert, 1996; Manuck, Marsland, Kaplan, & Williams, 1995; Antoni, Lutgendorf, Cole, Dhabhar, Sephton, McDonald, Stefanek, & Sood, 2006).

Research has investigated the possible association between psychological stress and oxidative stress, as well as inflammation. These studies generally support the hypothesis that stress increases levels of oxidative damage. Furthermore, stress has been associated with elevated inflammatory markers and increased neutrophil production of ROS. Although both increases in oxidative stress and increases in neutrophil production of ROS have been found independently during exam stress, the analysis of the potential relationship between these variables has not yet been conducted.

Many factors may be involved in these relationships, and changes in health behaviors during times of stress may contribute a great deal to these findings. Health behaviors that could potentially influence levels of oxidative stress include vitamin use, nutrition, alcohol use, smoking, and physical activity (Moller, Wallin, & Knudsen, 1996). In addition, these health behaviors, as well as sleep quality, may impact immune function (Kiecolt-Glaser & Glaser,

1988). Many of the reviewed studies failed to control for these health factors. The present study attempts to control for such factors when assessing the impact of stress on oxidative stress and neutrophil activity.

The present study uses an examination stress model to examine the relationship between neutrophil activity and oxidative stress and psychological stress. This approach allows for control of individual differences by within-subject comparison of times of higher and lower levels of stress. This quasi-experimental design is considered a valid, ecologically relevant method of proxy manipulation of the stressor. Briefly, healthy students taking their law bar exam, medical board exam, or nursing board exam were recruited and seen just prior to taking their exam and again a month later. Primary outcome variables include the percentage of stimulated neutrophils testing positive for ROS production and levels of oxidative stress in a morning spot urine sample.

3.1 HYPOTHESIS

Hypothesis 1: There will be poorer health behaviors during exam stress when compared to a month later.

Hypothesis 2: There will be higher levels of oxidative damage and increased numbers of stimulated neutrophils testing positive for ROS production during exam stress when compared to a month later, even after controlling for health behaviors.

Hypothesis 3: The elevations in oxidative damage during the stressful time point are expected to be correlated with measures of psychological stress and with higher neutrophil activity, after controlling for health behaviors.

Hypothesis 4: The relationship between exam stress and oxidative damage will be partially mediated by neutrophil activity, after controlling for health behaviors.

4.0   METHODS

4.1   PARTICIPANTS

A total of 20 participants were recruited from the Pittsburgh area. Of this sample, one participant failed to complete the second visit and one participant was ruled out after indicating medical use of a gluccocorticoid (known immune altering drugs). Thus, the final sample included 18 participants who completed both time points. Participants' ages ranged from 25 to 36, with an average age of 28. About 67% of participants were female, and the majority of participants (78%) designated themselves as Caucasian. Participants included 14 law students taking their bar exam, 3 medical students taking their board exams, and 1 nursing student taking the nursing board exam. (See Table 1 for descriptive statistics).[2]

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[2] To maximize recruitment we collaborated with a colleague in the recruitment of participants. As a result, five of the participants reported having a diagnosis of asthma, however, all reported mild symptoms. Major dependant variables were analyzed with asthma as a between subjects factor and trends remained consistent with reported results, although significance was lost do to decreased degrees of freedom.

4.2   DESIGN

The study used a repeated measures design with two sampling time points. We recruited participants to come in during the week preceding an important exam, when participants were expected to be studying ardently and levels of anxiety about the exam were expected to be high. The second meeting was scheduled a month or more (range 4-7 weeks) after their exam, when anxiety about the exam was expected to have dissipated. At each time point blood samples, blood pressure, and urine samples were collected and participants filled out a series of questionnaires.

4.3   PROCEDURES

Healthy medical, nursing, and law students who were planning to take their board exams and bar exams were recruited from the Pittsburgh community through advertisements in newspapers and flyers posted and distributed on campuses. Participants were initially screened for eligibility criteria on the phone. Due to the immune and oxidative stress measures in this investigation, we ruled out participants reporting any of the following behaviors and/or health problems that may alter immune or oxidative stress measures: regular use of tobacco, excessive consumption of alcohol (defined by reporting 15 or more drinks a week), illegal drug use, or diagnosis of severe asthma, arthritis, diabetes, cancer, heart disease, hypertension, autoimmune disorder, major depression/anxiety disorder, or other major psychiatric disorders. In addition, participants were screened on the day of the study for the presence of an infectious illness (Jackson, Dowling, Anderson, Riff, Saporta, & Turck, 1960; Cohen, Doyle, Turner, Alper, and Skoner, 2003).

At Time 1, students who were eligible for participation were asked to come to the lab in the morning hours between 7 and 11 am within a week of their reported exam or upon return from a reported break. Two participant was unable to come in the morning, and so their second visit was scheduled at the same time of day as their first visit. The morning was selected to make time of day of blood draw and urine collection as consistent as possible across participants. Upon arrival at the lab informed consent was obtained, blood samples were drawn, height and weight recorded, and blood pressure monitored every 5 minutes for 15 minutes. Participants were given a questionnaire to complete. Participants were then instructed in urine collection procedures, and were asked to use the nearby bathroom to collect their urine. The Time 2 appointment was scheduled around the participant's schedule, between four and seven weeks later, adjusting for any planned vacations, etc.

Time 2: To reduce attrition, participants were contacted by either mail or phone to remind them of their second appointment. Upon arrival for the second appointment, we repeated the procedures used at time one. Participants were paid $20-25 for each time point completed.

Urine collection procedure. Participants were asked to urinate in a sterile collection container. The urine sample labeled (ID, date, and time) and refrigerated immediately. They were then frozen at -20 degrees in the Behavioral Medicine Department Laboratory

Blood sample procedures. A needle was placed in an antecubital vein of the participant's arm and 6ml of blood was collected. 3 mls collected in an EDTA coated tube was sent to the clinical laboratory for complete blood counts (CBC). The remaining 3 ml of blood was collected in a heparin coated tube and used in the Nitro Blue Tetrazolium (NBT) reduction assay.

Demographics. Gender, age, ethnicity, and type of exam were recorded.

4.4   STRESS MEASURES

Impact of Event Scale-Intrusive Thoughts subscale (IES-IT) (Exam Specific). To assess frequency of intrusive thoughts about the exam stress, the intrusive thoughts subscale of the IES was administered (Horowitz, Wilner, & Alvarez, 1979). The IES-IT is considered a reliable measure of intrusive thoughts in relation to a specific trauma/stressful event, and this eight item subscales has reported good internal reliability (alpha = .87 to .91) and test-retest reliability of r = .57. (Weiss & Marmar, 1997). The Cronbach's alpha for this scale in our study was .66 at Time 1, and .91 at Time 2.

Exam Appraisal. Appraisal was assessed by a series of questions designed to determine the extent to which the participant appraises the exam as a threat. The items are as follows: 1) how important is your board exam to your future career objectives, 2) how worried are you about getting a good score on the exam, 3) how nervous do you feel about taking the exam, and 4) how concerned are you about getting the score you need. Possible answers are on a scale from 0 to 4, reflecting "not at all" to "very". Cronbach's alpha for this scale at Time 1 = .71 and Time 2 = .75.

Perceived Stress Scale (PSS). The PSS was administered at both time points and is considered to be a good measure of perceived stress (Cohen, Kamarck, & Mermelstein, 1983). Cronbach's alpha for the PSS at Time 1 = .76 and Time 2 = .89.

State Trait Anxiety Inventory (STAI). The STAI-state version was administered at both time points to evaluate current levels of anxiety (Spielberger, 1983). The STAI-state is a 20-item measure widely used to assess state anxiety. Cronbach's alpha for this scale at Time 1 was .95, and Time 2 was .93.

Blood Pressure. To evaluate a physiological indicator of stress, blood pressure was measured. Systolic and Diastolic blood pressure was taken three times over a fifteen minute period. Because of possible adaptation to the blood pressure cuff, the first measurement was disregarded. The second and third measurements were averaged together to create one value at each time point.

4.5   HEALTH BEHAVIORS

To determine whether health behaviors affected the biological outcomes, and to determine whether these behaviors were different at the two time points, we measured the following:

Fruit and vegetable consumption: A one item measure of fruit and vegetable consumption was administered. Participants were asked to indicate an estimate of the number of fruits and vegetables they had consumed in the last week.

Alcohol Consumption-Weekend and Weekdays: Participants were asked to indicate on average how many alcoholic drinks they consume on the weekend day and on week days.

Body Mass Index (BMI). BMI was determined by dividing weight (lbs.) by the square of height (inches) and multiplying by 703.

Physical Activity. Paffenberger's Physical Activity Index (Paffenberger, Wing, & Hyde, 1978) was administered to evaluate and control for subjects average physical activity level. Physical activity level was assessed by requesting an estimate of type and frequency of activity engaged in over the prior week. From this information we then calculated average kilocalories burned per

week. This measurement tool is commonly used to assess physical activity levels, and considered reliable and valid.

Sleep Quality. The Pittsburgh Sleep Quality Index (PSQI) (Buysse, Reynolds, Monk, Berman, *&* Kupfer, 1989) is a well established and reliable measure of recent sleep quality (Cronbach's alpha = .83) and is considered a valid measure of sleep. This measure was administered to participants to control for poor sleep that may disrupt biological measures. As outlined in Buysee, et al. (1989), a global sleep quality score was calculated from the PSQI. Higher scores are taken to reflect poorer sleep quality on this measure.

4.6   BIOLOGICAL MEASURES

Oxidative damage. The amount of oxidative damage to cells of the body was estimated by determination of 8-OH-dG levels in a morning spot urine sample. Spot urine sampling was chosen over an overnight collection to avoid subject attrition, compliance problems, and reduce burden. Moreover, research suggests that 8-OH-dG in urine offers a stable measure of oxidative damage, and is considered a reflection of the amount of oxidative damage occurring throughout the body (Cutler & Mattson, 2003). Recent reports have found natural diurnal variations in 8-OH-dG levels in urine, with morning values consistently the lowest (Kanabrocki, Murray, Hermida, Scott, Bremner, Ryan, Ayala, Third, Shirazi, Nemchausky, & Hooper, 2002). Additional research has established a strong relationship (R^2 = .75) between 8-OH-dG levels in a morning spot sample and a 24 hr sample of urine, thus the morning spot sample can be considered a valid assessment of the average amount of oxidative damage occurring in that individual (Miwa, Matsumaru, Akimoto, Naito, & Ochi, 2004).

8-OH-dG is an established measure of oxidative stress and a known product of DNA damage (Wu, Chiou, Chang, & Wu, 2004), and was assessed by use of an enzyme linked immunosorbent assay (ELISA; Oxis Corporation). Samples were run in duplicate, and the assays CV% average was 12.7%, with a range from 0-66.8%. The high CV% values were due to assay technology. Specifically, although within sample variance of raw values was very small, adjusted values less than 2ng/ml or greater than 80ng/ml fell out of the linear portion of the standard curve. This can result in large variances in these particular samples after adjustment by the curve. The mean CV% for sample values (n = 23) falling within the linear portion of the curve, values ranging from 2-80ng/ml, was 7.5% with a range of 0-25%, while the sample values falling out of the linear portion of the standard curve (n = 7) mean CV% was 29.6% with a range of 2-67%.

In addition, 10 of the 36 adjusted values fell below the lower level of detection of the ELISA kit, these values were removed prior to calculating mean values. Of the total 18 participants, 15 had 8-OH-dG values at Time 1 and 17 at Time 2, with 15 having values at both time points. All 8-OH-dG values were adjusted by creatinine concentration in urine.

Neutrophil Activity. Neutrophil production of reactive oxygen species was determined through use of a nitro blue tetrazolium (NBT) reduction assay acquired from Sigma-Aldrich, Inc. (Procedure No. 840). Briefly, whole blood was transferred to siliconized glass viles containing NBT Solution. Stimulant (nonviable bacterial extract) was added per manufacturer's suggestions (unstimulated NBT methods do not include addition of a stimulant), and the sample was gently mixed before incubation at 37º C for ten minutes. Samples were removed and allowed to sit for another ten minutes. Samples were then gently mixed and smeared onto a clean glass slide and allowed to dry. Smears were then flooded with 1 ml of Accustain Wright Stain, allowed to stand for thirty seconds, and rinsed with distilled water. Smears were allowed to air dry, and cover

slips were then placed over the smears. Images of cells were taken using a 20X magnification on a microscope, and then saved to disk. Images were coded so that a trained counter was blind to the treatment condition. One-hundred neutrophils from each slide were counted. Those testing positive for NBT reduction appeared with a dark blue crystalline formation. Normal human values range from 2-17% positive cells.

Number of Neutrophils in Circulation: The absolute neutrophil count (ANC) was derived from a CBC performed by the Clinical Hematology Laboratory at the UPMC Presbyterian Hospital of Pittsburgh.

4.7 STATISTICAL ANALYSIS[3]

All variables were checked for normal distribution, and, if skewed or high kurtosis, the variable was transformed using square-root or log-linear transformations. Variables requiring transformations included the measure of alcohol consumption on week days and weekend, the Paffenbarger Activity Questionnaire, the Perceived Stress Scale, and the Speilberger's State Anxiety Inventory, the intrusive thoughts subscale from the IES, and 8-OH-dG values. In addition, variables were checked for outliers that were 3 or more standard deviations from the mean, and removed. Analysis of the distribution of diastolic blood pressure at Time 2 revealed one outlier who was subsequently removed from the analyses. The distribution of absolute neutrophil count revealed two outliers at time 2, who were also removed from the analyses. To avoid making type 1 errors with multiple tests, we employed Dunn's (1961) Bonferonni adjustment for multiple comparisons method that adjusts the criterion for significance.

The following statistical analyses were performed:

1. To determine if any of the health behavior variables differ between Time 1 and Time 2, a repeated measures multivariate analysis of variance (MANOVA) was performed using the Bonferonni method for correction for multiple tests.

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[3] All statistical tests will be considered significant at the $p < .05$ level.

2. We used repeated measures MANOVA to determine whether there are significant differences between Time 1 and Time 2 scores on the following stress variables: STAI, PSS, IES-IT, Exam Appraisal, systolic blood pressure, and diastolic blood pressure, using the Bonferonni method for correction of multiple tests.

3. Correlation analyses were calculated at Time 1 and at Time 2 between 8-OH-dG and demographic and health behavior variables. In addition, correlation analyses were conducted to calculate the relationship between Time 1 and Time 2 neutrophil activity and demographic and health behaviors. Control variables with medium and large effect size correlations in the above analyses were entered as covariates in a partial correlation analyses between the two outcome variables and psychological stress indices.

4. Using repeated measures analysis of covariance (ANCOVA), we examined whether there were differences between Time 1 and Time 2 on 8-OH-dG and neutrophil activity, with demographic and health behaviors showing a trend towards significance ($p < .10$) in analyses #3 entered as covariates.

5. To test whether neutrophil activity mediates the relationship between elevated stress and oxidative damage, a mediation model using repeated measures ANCOVA was used. To test this mediation pathway, the following must first be met:

   a. Neutrophil activity levels must be higher during exam stress than during low stress.

   b. Oxidative damage levels must be higher during exams than during low stress.

   c. Neutrophil activity must be correlated with oxidative damage.

Once these tests yield required values, the mediation analysis can be performed using an ANOVA design. The hypothesized mediator, neutrophil activity, is entered as a covariate, exam stress/low stress is entered as the independent variable, and the dependant measure is 8-OH-dG. In order to claim partial mediation there must be a partial loss of significant main effect of stress when the covariate is entered. Thus, the difference in oxidative stress levels at times of exams vs. low stress should be reduced when phagocyte productivity is entered into the ANCOVA as a covariate.

5.0   RESULTS

5.1   DIFFERENCES IN HEALTH BEHAVIORS

Health behaviors were entered into a repeated measure MANOVA to test for differences between Time 1 and Time 2. The multivariate test for the overall model was significant, $F (5, 10) = 4.40$, $p = .022$. Univariate tests revealed no significant differences from Time 1 to Time 2 in alcohol consumption on the weekdays, $F (1, 14) = .217$, n.s., or average fruit and vegetable consumption, $F (1, 14) = .493$, n.s. The univariate test found a trend for differences between Time 1 and Time 2 physical activity, $F (1, 14) = 3.20$, $p = .095$, and alcohol consumption on the weekend, $F (1, 14) = 4.42$, $p = .054$, with higher alcohol consumption on the weekends and more physical activity at Time 2. Participants reported significantly improved sleep quality at Time 2, univariate, $F (1, 14) = 5.24$, $p = .038$. See Table 1 for means and standard deviations of health behaviors at both time 1 and time 2.

5.2   DIFFERENCES IN PSYCHOLOGICAL AND PHYSIOLOGICAL STRESS

To test whether levels of stress and anxiety were higher just prior to taking exams, as compared to a month later, a repeated measures MANOVA with the Bonferonni correction was performed using Time 1 and Time 2 scores on the PSS, the STAI, the IES-IT, and the Exam Appraisal, as well as systolic and diastolic blood pressure measures. The multivariate test of the overall model was significant, $F(6, 10) = 5.05$, $p = .012$. Univariate analyses supported our hypotheses. Participants reported significantly higher levels of perceived stress and state anxiety at Time 1 than at Time 2, $F(1, 15) = 25.56$, $p < .001$ and $F(1, 15) = 13.91$, $p = .002$, respectively. Participants also reported significantly more intrusive thoughts about their exam the week before taking the exam than a month later, $F(1, 15) = 29.71$, $p < .001$, and higher scores on Exam Appraisal at Time 1 than Time 2, $F(1, 15) = 7.74$, $p = .014$. Increases in psychological stress were not accompanied by significant elevations in peripheral resistance, as measured by blood pressure. The univariate tests revealed a trend towards higher diastolic blood pressure at Time 1 compared to Time 2 levels, $F(1, 15) = 3.97$, $p = .065$, but no difference in systolic blood pressure, $F(1, 15) = 1.13$, n.s. Overall, these findings support the hypothesis that taking exams increases perceived stress, anxiety, and intrusive thoughts about the exam.

Table 1. Demographics and Psychosocial Characteristics

Characteristics	Time 1				Time 2		
	M	SD	%		M	SD	%
Age (years)	28.4	3.8					
Gender							
Male			33.3				
Female			66.7				
Ethnicity							
Caucasian			77.8				
African American			11.1				
Asian American			11.1				
Type of Exam							
Medical Boards			16.7				
Nursing Boards			5.5				
Bar Exam			77.8				
BMI	26.7	6.0			26.5	5.9	
Health Behaviors							
Avg. Exercise (Cal/wk)	1712.9	1425.6			2588.8	2266.3±	
Fruit & Vegetable	3.0	1.4			2.7	1.5	
Alcohol/day (weekday)	.79	1.2			.63	.84	
Alcohol/day (weekend)	1.9	2.2			2.6	2.0±	
Sleep Quality (PSQI)	5.1	1.8			3.4	2.0*	
Multi-Vitamin Use			61.1%				50%
State Anxiety (STAI)	2.2	.62			1.6	.44**	
Perceived Stress (PSS)	1.7	.61			1.0	.72**	
Intrusive thoughts (IES-IT)	2.6	.51			1.7	.73**	
Exam Appraisal	3.0	.70			2.7	.87*	
Diastolic BP	68.0	7.7			64	6.0±	
Systolic BP	116.5	11.7			114.1	12.8	

Notes: Significant differences between Time 1 and Time 2 noted as ±p<.10, *p<.05 or **p<.005. Values in table are means and standard deviations prior to transformations. Statistical tests used transformed data.

5.3   CORRELATIONAL ANALYSES

To test whether demographics, health behaviors, and stress indices were related to neutrophil activation, zero-order correlation analyses were run (See Tables 4 through 7). Demographic and health behaviors that were related to the outcome variables were then controlled for using partial correlations. To test whether associations were maintained after controlling for demographic and health behaviors that showed correlations with a medium or large effect size in the zero-order correlation analysis, variables with an r value equal to or larger than .243 (established by Cohen, 1988, as a medium effect size) were entered as covariates in a second partial correlation analysis.

5.4   CORRELATIONS WITH NEUTROPHIL ACTIVITY TIME 1

Zero-order correlations (see Table 2) revealed a trend for positive correlations between stimulated neutrophil activation and alcohol consumption on the weekdays, and multi-vitamin use. Stimulated neutrophil activation at Time 1 was also correlated with scores on the exam appraisal scale, suggesting that the higher the appraised threat of the exam the more activated the neutrophils. No other variables were significantly correlated with stimulated neutrophil activation. However, some correlations were considered medium and large according to Cohen's effect size table for r values (Cohen, 1988). Stimulated neutrophil activity showed a medium negative correlation with BMI, a positive correlation with sleep quality, and a positive correlation with Fruit and Vegetable consumption.

Table 2. Correlations with Stimulated Neutrophil Activity Time 1

Variables	Zero Order correlations		Partial Correlations	
	r	*p*	r	*p*
Age	-.121	.645		
Gender	-.087	.740		
BMI	-.302	.239		
Physical Activity	.225	.385		
Alcohol (Weekends)	.151	.564		
Alcohol (Weekdays)	.403	.108		
Sleep Quality (PSQI)	.382	.160		
Fruit & Vegetable	.397	.115		
Multi-Vitamin Use	.418	.095±		
Systolic BP	-.169	.517	.421	.226
Diastolic BP	-.121	.644	.505	.137
PSS	-.148	.572	.065	.859
STAI	-.161	.537	.068	.852
IES	.146	.577	.263	.462
Exam Appraisal	.610	.009*	.581	.078±
8-OH-dG	.236	.417	.895	.003**

Partial correlations performed controlling for BMI, Alcohol Weekdays, Sleep Quality, Fruit & Vegetable, and Multi-Vitamin use. ±p<.10, *p<.05 or **p<.005

To determine if correlations between stimulated neutrophil activity and physiological and psychological indicators of stress changed after controlling for demographic and health behaviors that were correlated with the neutrophil activity measure, we ran partial correlation analyses controlling for variables with a medium or larger effect size (See Table 2). After controlling for BMI, alcohol use on weekdays, sleep quality, fruit and vegetable consumption,

and multi-vitamin use, 8-OH-dG and neutrophils testing positive for ROS became highly significantly correlated. In addition, the Exam Appraisal scale remained positively correlated with neutrophil activation after adding covariates.

5.5 CORRELATIONS WITH 8-OH-DG TIME 1

Zero-order correlations with 8-OH-dG at Time 1 revealed no significant correlations (See Table 3). Medium to large correlations that were not significant were obtained between 8-OH-dG and demographic and health behaviors, including a correlation with gender, indicating males had moderately higher oxidative damage than females, a positive correlation with BMI, a negative correlation with alcohol consumption on the weekends, a negative correlation with sleep quality, and a negative correlation with fruits and vegetable consumption.

To control for health behaviors, partial correlations were run entering variables with a medium or large effect size to the matrix (See Table 3). After adjusting for gender, BMI, alcohol use on the weekend, sleep quality, and fruit and vegetable consumption, the relationship between systolic blood pressure and 8-OH-dG values dropped. Also, an increased positive correlation between 8-OH-dG and neutrophil activity was again evident after entering covariates.

Table 3. Correlations with 8-OH-dG Time 1

Variables	Zero Order correlations		Partial Correlations	
	r	*p*	*r*	*p*
Age	-.175	.534		
Gender	.458	.086±		
BMI	.375	.168		
Physical Activity	-.043	.878		
Alcohol (Weekends)	-.294	.288		
Alcohol (Weekdays)	-.014	.959		
Sleep Quality (PSQI)	-.259	.392		
Fruit & Vegetable	-.314	.254		
Multi-Vitamin Use	.103	.714		
Systolic BP	.450	.092±	.124	.769
Diastolic BP	.494	.061±	.220	.601
PSS	-.061	.828	-.295	.478
STAI	.130	.659	-.114	.788
IES	.410	.129	.586	.127
Exam Appraisal	.153	.586	.283	.497
% Neutrophils in Circulation	.464	.095±	.393	.335
Stimulated Neutrophil Activity	.268	.315	.655	.078±

Partial correlation controlling for Gender, BMI, Alcohol (weekend), PSQI and Fruit & Vegetable consumption. ±p<.10, *p<.05 or **p<.005

5.6   CORRELATIONS WITH STIMULATED NEUTROPHIL ACTIVITY TIME 2

Initial zero-order correlations revealed no significant correlations between neutrophil activity at Time 2 and health behaviors (See Table 4). Two health behaviors met the criterion for medium to large effect sizes. Both alcohol use on weekends and sleep quality showed modest negative correlations with neutrophil activity at Time 2.

Neutrophil activity at Time 2 showed a positive significant association with diastolic blood pressure, and a positive significant correlation with IES-IT. These correlations remained strong after controlling for health behavior variables with medium to large correlations in a partial correlation analyses, although the correlation with diastolic blood pressure dropped below significance. Interestingly, a positive correlation between neutrophil activity and Exam Appraisal approached significant after adding the covariates to the model.

Table 4. Correlations with Stimulated Neutrophil Activity Time 2

Variables	Zero Order correlations		Partial Correlations	
	r	*p*	r	*p*
Age	-.130	.620		
Gender	-.006	.982		
BMI	.023	.929		
Physical Activity	-.196	.451		
Alcohol (Weekends)	-.452	.069±		
Alcohol (Weekday)	.111	.672		
Sleep Quality (PSQI)	-.52	.849		
Fruit & Vegetable	-.114	.673		
Multi-Vitamin Use	-.225	.386		
Systolic BP	.175	.501	.142	.627
Diastolic BP	.643	.007**	.510	.075±
PSS	-.091	.727	-.342	.231
STAI	.221	.394	.189	.518
IES-IT	.506	.038*	.536	.048*
Exam Appraisal	.383	.129	.473	.088±
8-OH-dG	.268	.315	.340	.255

Partial correlations were run controlling for Alcohol use on weekends and Sleep Quality.

±p<.10, *p<.05 or **p<.005

5.7   CORRELATION ANALYSES WITH 8-OH-DG VALUES TIME 2

Zero-order correlations between 8-OH-dG values at Time 2 and health behaviors revealed no significant relationships (See Table 5). Using the evaluation of effect sizes to determine health behaviors to enter as covariates in the partial correlation, we identified a modest positive correlation between alcohol consumption on weekdays and 8-OH-dG values at Time 2, and a negative correlation between fruit and vegetable consumption and 8-OH-dG values at Time 2. After controlling for alcohol and fruit and vegetable consumption, 8-OH-dG was not significantly correlated with any of the variables.

Table 5. Correlations with 8-OH-dG Time 2

Variables	Zero Order correlations		Partial Correlations	
	r	*p*	*r*	*p*
Age	.134	.609		
Gender	-.043	.869		
BMI	.187	.471		
Physical Activity	-.099	.706		
Alcohol (Weekends)	.026	.920		
Alcohol (Weekday)	.474	.055±		
Sleep Quality (PSQI)	-.062	.820		
Fruit & Vegetable	-.364	.166		
Multi-Vitamin Use	-.221	.429		
Systolic BP	.250	.332	.217	.456
Diastolic BP	.244	.362	.271	.348
PSS	-.025	.924	.061	.836
STAI	.011	.968	.048	.870
IES	.099	.705	.155	.597
Exam Appraisal	-.123	.650	-.300	.298
% Neutrophils in Circulation	.049	.858	.268	.354

++Partial correlation controlling for Fruit and Vegetable consumption and Alcohol consumption on weekdays. ±$p<.10$, *$p<.05$ or **$p<.005$

5.8   NEUTROPHIL COUNT AND ACTIVITY ANALYSES

The difference in the number of neutrophils in circulation and the percentage of neutrophils in circulation at Time 1 compared to Time 2 was tested using a repeated measure MANOVA (See Table 6 for means and standard deviations). The multivariate test for the overall model was not significant, $F (2, 13) = 2.45$, $p = .145$.

Table 6. Means and Standard Deviations in the Dependant Variables

	Time 1		Time 2	
Characteristics	M	SD	M	SD
Absolute Neutrophil # (10^4 cells/μl)	3.53	.4	2.9	.2
% WBC that are Neutrophils	55.5	2.8	50.4	1.9
Stimulated Neutrophil Activity (% Positive)	19.8	9.4	15.1	6.8
Unstimulated Neutrophil Activity (% Positive)	13.9	7.3	10.9	7.5
Oxidative Damage (8-OH-dG ng/mg creatinine)	2.2	.9	4.3	2.6

It was hypothesized that there would be differences in the number of active neutrophils, testing positive for ROS. Analyses of unstimulated neutrophil activity revealed a trend towards higher amounts at Time 1 compared to Time 2, but this was not significant, $F (1, 16) = 3.14$, $p = .096$. On the other hand, stimulated neutrophil activity at Time 1 was significantly higher than at Time 2, $F (1,16) = 5.15$, $p = .037$; See Table 6 for means and standard deviations. To determine if the stimulated neutrophil activity effect remained after controlling for health behaviors, a repeated measures analysis of covariance (ANCOVA) was used. First the two dependent measures (stimulated neutrophil activity Time 1 and stimulated neutrophil activity Time 2) were

tested for differences, and then the covariate alcohol consumption on weekends Time 2 and multivitamin use Time 1 were then entered to determine change in significance. These analysis, summarized in Table 7, revealed stimulated neutrophil activity still showed a trend towards being higher during examination after controlling for weekend alcohol consumption at Time 2 and multi-vitamin use at Time 1, $F(1, 16) = 4.011$, $p = .065$.

Table 7. Repeated Measures ANCOVA for Neutrophil Activity from Time 1 to Time 2

	F	*p*
Neutrophil Activity	5.15	.037*
Neutrophil Activity (With Covariates)[a]	4.01	.065

[a] Covariate entered: Alcohol weekends T2 & Multi-vitamin T1

5.9   8-OH-DG ANALYSES

Differences were not found between levels of 8-OH-dG at Time 1 and Time 2 (See Table 6 for means and standard deviations). To determine if health behaviors may account for the lack of significant finding, we ran repeated measures ANCOVA, entering gender and alcohol use on the weekdays Time 2 as covariates. As can be seen in Table 8, Time 1 and Time 2 8-OH-dG values were not significantly different, $F(1, 13) = .479$, n.s., and entering gender and alcohol use on the weekdays as covariates resulted in a non-significant increase of the F value to $F(1, 12) = 2.38$, n.s. 8-OH-dG did not significantly change from Time 1 to Time 2, even after controlling for gender and alcohol use on the weekdays Time 2.

Table 8. Repeated Measures ANCOVA for 8-OH-dG from Time 1 to Time 2

	F	p
8-OH-dG change	.479	.500
8-OH-dG (With Covariates)[a]	2.38	.149

[a] Covariate entered: Alcohol weekdays T2 & Gender

5.10 INTERACTION OF TIME WITH ALCOHOL CONSUMPTION ON 8-OH-DG VALUES

Previously mentioned analyses revealed increases in alcohol consumption on the weekend from Time 1 to Time 2. In a post-hoc analysis, we hypothesized that a lack of significant change in 8-OH-dG values may be due to changes in alcohol consumption, a health behavior that is known to increase oxidative damage (Purohit, Khalsa, & Serrano, 2005). Changes in alcohol consumption may account for the lack of decrease in the 8-OH-dG measure from Time 1 to Time 2 in those who increased consumption. We used a repeated measures ANOVA to test whether the change in alcohol consumption on the weekend moderated the relationship between Time 1 and Time 2 8-OH-dG. Change in alcohol consumption on weekends was determined by subtracting Time 1 from Time 2 values. To conserve power in the analysis, these scores were then dummy coded to categorize participants as either "no increase = 0" or "increase = 1" in alcohol consumption on the weekend at Time 2. Although power was too low to find significance using a moderation model, effect sizes can be used to cautiously interpret results. The results (displayed in Table 9) suggest that increases in alcohol consumption moderated the relationship between 8-OH-dG values at Time 1 and Time 2. Partial eta squared (η_p^2), the calculated effect size in the repeated measures ANOVA design, was .208 for the

interaction term. Figure 1 graphs the means in 8-OH-dG for the two groups at the two time points. Post-hoc simple contrasts between Time 1 and Time 2 8-OH-dG values in the group with increased alcohol consumption at Time 2 reveals marginally significant differences ($t = -1.867$, $p = .104$) with increases in 8-OH-dG values at Time 2, Time 1 mean (SD) = .65 (.13) and Time 2 mean (SD) = 1.05 (.17), with an n = 8. The group that did not increase in alcohol consumption at Time 2 failed to show any significant differences ($t = .808$, $p = .450$), with a mean (SD) 8-OH-dG value at Time 1 of .79 (.19), and Time 2 mean (SD) = .59 (.19).

Table 9. Repeated Measuers ANCOVA for Interaction of Alcohol on 8-OH-dG from Time 1 to Time 2

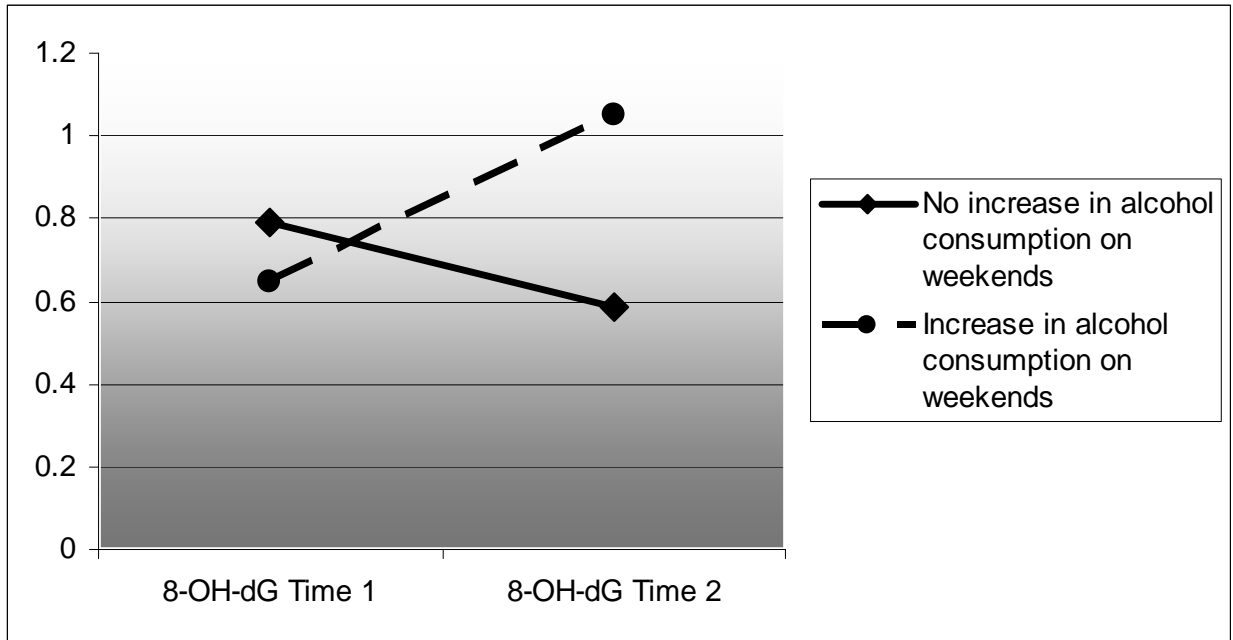|	F	p	η_p^2
8-OH-dG change	.390	.543	.029
8-OH-dG X Change in Alcohol (Weekend)	3.41	.088	.208

Figure 1. Interaction of Weekend Alcohol Consumption Change on 8-OH-dG Values By Time

5.11   MEDIATION ANALYSES

As outlined in the statistical analyses section, the test of mediation requires 1: significant differences between neutrophil activity at exam stress compared to low stress, 2: significantly higher levels of oxidative damage during exams as compared to low stress, and 3: neutrophil activity must be correlated with oxidative damage. Once these three have been found, the test for mediation can be performed. However, criterion 2 was not met in this study, and thus planned mediation analyses were not performed.

6.0   DISCUSSION

This study was designed to test the hypothesis that exam stress would be associated with both higher levels of neutrophil activity and oxidative damage when compared to a time with less stress. In addition, it was hypothesized that increases in neutrophil activity would mediate the relationship between psychological stress and oxidative damage. Unfortunately, we were unable to test this proposed mediation due to a lack of significant differences over time in the oxidative damage measure. This could be due to a number of factors, including the extent to which exams cause stress, increased alcohol consumption by participants at the second assessment, or a general lack of association between 8-OH-dG and exam stress. Differences in exam status were as predicted, so other explanations are possible, including a lack of power to detect an effect in those not increasing alcohol consumption at Time 2, or limitations due to the poor reliability in the 8-OH-dG values that fell out of the linear portion of the curve.

6.1   OXIDATIVE DAMAGE AND STRESS

Correlation analyses did not reveal associations between 8-OH-dG and psychological stress. In addition, 8-OH-dG values were not significantly higher during exam stress when compared to a month or so later. In this study, with this small and select sample, exam stress was not related to oxidative damage.

One clue about the reasons for lack of effects of exams on 8-OH-dG is the observation that it increased in a subsample of participants at Time 2. This unexpected increase in 8-OH-dG values in some of the participants at Time 2 may have been the result of increased consumption of alcohol after taking the exam. Indeed, there are many possible reasons why alcohol consumption increased a month after taking exams, including the possibility that many students increase alcohol consumption after completion of their exams in an act of celebration or tension relief, or simply increased available time to socialize, which often includes alcohol consumption. At the same time, students likely reduce their alcohol consumption when studying for their exams, and the increase a month after taking the exam may reflect a return to normal activity. Our test of an interaction between increased alcohol consumption at Time 2 and 8-OH-dG values suggested that the change in this behavior may partially explain the lack of anticipated findings in oxidative damage. The observed interaction was not significant, but the effect size did increase with the interaction. A much larger sample size is necessary to confirm these results, but other studies have found relationships between alcohol use and oxidative damage (Nakajima, Takeuchi, Takeshita, & Morimoto, 1996; Purohit, Khalsa, & Serrano, 2005), which would mitigate against the predicted post-exam decrease.

In addition, there is the possibility that some of the 8-OH-dG values obtained were not reliable estimates of the levels of 8-OH-dG due to the fact that they fell on the non-linear portion of the curve used in this assay. The measurement error may prevent us from detecting the effects of stress on 8-OH-dG in urine. Previous research that has found relationships between 8-OH-dG and stress, assessed 8-OH-dG in DNA extracted from leukocytes in peripheral blood using HPLC methods (Irie, et al., 2001; 2001; 2003). Thus it is difficult to compare our findings to these other research results because their values are derived from particular cells in blood while our values are derived from the byproduct of damage to cells throughout the body found in urine.

6.2   NEUTROPHILS AND STRESS

Our hypothesis that neutrophil activity would be higher during stress was supported. Exam stress was associated with a higher percentage of neutrophils testing positive for ROS production after stimulation. However, the significance of this finding became marginal (from $p < .05$ level to $p < .065$) when covariates (alcohol consumption at Time 1 and multi-vitamin use at Time 2) were added. With a larger sample size, these findings may have remained significant after controlling for these correlated health behaviors.

Stimulated neutrophil activity was also correlated with stress indices. Exam appraisal was significantly positively related with stimulated neutrophil activity, indicating that during the week prior to taking a major exam, neutrophil activity increased as the appraised threat of the exam increased. Exam appraisal was measured using questions about how important was the exam to their career objectives, how nervous they felt about taking the exam, and how concerned

they were about getting the score they needed. This measure tapped participant's stress appraisal specific to the exam, and suggested that stressful appraisals increased neutrophil activity.

Interestingly, at Time 2, intrusive thoughts about the exam were significantly correlated with neutrophil activity, a relationship that remained after partialing out health behaviors. The measure of exam specific intrusive thoughts assesses the frequency of worries and concerns about the exam. After taking the exam, the frequency significantly decreased, but remained present to some degree, probably due to remaining uncertainty about their performance on the exam. A month after the exam, more intrusive thoughts were associated with more neutrophil activity.

Positive correlations at Time 1, although not significant, were found between neutrophil activity and systolic blood pressure and diastolic blood pressure after controlling for health behavior variables. Similarly, stimulated neutrophil activity at Time 2 was significantly positively correlated to diastolic blood pressure and intrusive thoughts, and this relationship remained strong after controlling for health behaviors. Positive correlations with blood pressure and intrusive thoughts suggest stress increases neutrophil activity. Physiological and psychological stress may work in concert with one another, ruminations and intrusive thoughts about the exam increase sympathetic nervous system arousal which in turn elevates blood pressure. The signal to activate neutrophils during times of stress may originate from increased SNS arousal which acts directly on the inflammatory pathway, or indirectly by elevating blood pressure that causes micro-tears in the vascular walls and subsequently activate the acute phase response which signals the recruitment of neutrophils to the sight of damage. Future work is needed to clarify if these causal pathways are involved.

Overall, the findings that neutrophil activity was higher during exam stress than a month later, and that neutrophil activity was positively related to intrusive thoughts, exam appraisal, and blood pressure support the hypothesis that stress increases inflammation. Previous research has reported elevated neutrophil activity after stimulation during examination stress (Kihara, Teshima, Sogawa, & Nakagawa, 1992; Kang, Coe, & McCarthy, 1996). Alternatively, Wadee, et al. (2001) report increased neutrophil activity in unstimulated cells during examination stress. The later study utilized the NBT assay, as this study has used, but the stimulated samples procedure included a longer incubation period than our stimulation procedure, which may account for their null findings in stimulated neutrophil activity (ie. Longer stimulation may result in maximal activation). Indeed, Wadee, et al. (2001) reported roughly 60% of neutrophils were positive for activation after stimulation, while our stimulated neutrophil activity values were between 10-30%. In addition, although our unstimulated neutrophil activity measure was not significantly higher during examination, there was a trend towards increased activity at Time 1 which may have become significant with a larger sample size. Overall, our findings are in line with previous literature that suggests exam stress increases neutrophil activity.

6.3   NEUTROPHILS AND OXIDATIVE DAMAGE

The finding that 8-OH-dG was highly correlated with neutrophil activity after controlling for numerous health behaviors and BMI at Time 1 lends support to the hypothesis that increases in inflammation during times of stress may also be related to increases in oxidative damage. However, any interpretation of these results must be made cautiously. First, we measured only one aspect of neutrophil activity; chemotactic and adherence-related functions were not

51

measured and could also reflect neutrophil contributions to oxidative stress. Secondly, although the correlation between these two variables at Time 1 are strong, Time 2 correlations are not as convincing. This may be a result of increases in alcohol consumption at Time 2, as mentioned earlier, and this could contribute to higher 8-OH-dG values. However, controlling for this did not significantly alter the correlation value at Time 2. These findings are consistent with the hypothesized association between inflammation and oxidative damage, but more research that offers better controls for factors that affect oxidative damage is needed before definitive conclusions can be made.

6.4   IMPLICATIONS

The implications of these findings are limited by the sample size; however, a number of findings are worth mentioning. Similar to previous research, neutrophil activity was elevated during exam stress (Kihara, et al. 1992; Kang, et al. 1996; Wadee, et al., 2001). In addition, measures of stress were correlated with neutrophil activity including exam appraisal prior to the exam, and intrusive thoughts and blood pressure after having taken the exam, suggesting that individual differences in appraisals of stress are related to individual differences in inflammation. The greater the perceived threat of the exam prior to taking the exam, and the more intrusive thoughts that remained about the exam a month after taking it, the more active the neutrophils. Exam stress may be marked by repeated acute stress reactions to the threat, and although at the second time point participants had already taken the exam, they had not obtained their results and thus many of the worries (threats) about their performance still remained.

A strong positive correlation between neutrophil activity and oxidative damage, after controlling for demographic and health behaviors, lends support for the hypothesis that increases in inflammation during stress is associated with increases in oxidative damage during stress. These two outcome measures have been found to be related to psychological stress in a number of studies, as noted earlier, yet whether they are also related to each other is still not clear. The positive correlation gives inspiration for future research to continue to address this hypothesis.

6.5   LIMITATIONS

This study had limitations that should be addressed in future research of this nature. First, sample size was low, which prevented us from detecting significance of any effect that was not large. However, our analysis included effect sizes to facilitate future work revisiting these potential relationships. The strengths of a within subjects design include the ability to control for many individual differences that tend to be diverse in a cross-sectional sample. However, despite this, we found differences over time in related variables, such as alcohol use, that may have obscured some effects. Had the sample been larger, this may not have been an issue. A number of factors that affected outcome measures did change overtime. Future research utilizing a within subject design to investigate oxidative damage may seek to increase sample size to allow for better control for these changing behaviors.

A primary prediction from this study was not tested, namely the hypothesis that increases in neutrophil activity at times of exam stress would mediate the relationship between exam stress and oxidative damage. This study was unable to test this hypothesis because oxidative damage

was not elevated during exam stress compared to a month later. The lack of difference in oxidative damage may have been a result of changes in health behaviors that washed out the effect of stress. Thus, although it is possible that stress does not increase oxidative damage to DNA, findings from other studies suggest that stress increases damage and the results of this study should not be taken as support for the conclusion that stress does not increase oxidative damage. Future research needs to address this hypothesis using a study design that better controls for alcohol consumption by either increasing sample size or matching participants with stress against a control population. The high correlation between neutrophil activity and oxidative damage at Time 1 should spur addition research, and suggests that more reliable results may be obtained in future work using larger samples.

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