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THE EFFECTS OF ACUTE CAFFEINE ADMINISTRATION ON INFLAMMATION
IN HEALTHY MEN AND WOMEN WITH A FAMILY HISTORY OF
HYPERTENSION

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Abstract

The relationship between caffeine consumption and cardiovascular disease (CVD) is controversial. However, chronic inflammation is associated with elevated CVD risk. Acute stress reliably increases proinflammatory markers such as IL-1β. We examined the effect of caffeine administration on the inflammatory stress response in 26 males and 26 females (age 18-29 years) with a confirmed family history of hypertension. Daily caffeine consumers were included following an intensive health screening to confirm health status. Following confirmation of parental hypertension, participants completed a 3.5-hr lab session to examine inflammatory responses to a speech and mental arithmetic stressor either without (N=26) or with (N=26) 3.3 mg/kg orally-administered caffeine. Blood samples were collected immediately before stress (20 min post-caffeine administration) and 60 min post-stress. Women completed their lab session during the luteal phase of their menstrual cycle. There was a significant time effect for interleukin-10 (F = 19.212, $p < 0.01$). Regardless of treatment group or gender, IL-10 decreased between baseline and recovery. Tumor necrosis factor-alpha showed a significant time by caffeine effect (F = 7.862, $p = 0.007$). There was an increase in TNF-α at follow-up as compared to baseline for the caffeine treated group. There were no significant findings for Il-4 or IL-1β. Results suggest that caffeine administration under stress may minimally influence the inflammatory response.

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Chapter 1

Introduction

Coffee and Cardiovascular Disease

The connection between coffee and cardiovascular disease (CVD) is
controversial. Some studies have shown coffee contributes to CVD. For example, one
epidemiological study found that boiled coffee consumption led to an increased risk of
first nonfatal myocardial infarction (Hammar et al., 2003). It has also been reported that
boiled, unfiltered coffee increases cholesterol levels, which may explain this increased
risk of CVD among coffee users (Rodrigues & Klein, 2006). While not controlling for
filtered vs. boiled coffee, other studies have also found an increased risk of CVD with
heavy coffee consumption (i.e., 5 or more cups of coffee a day) (Palmer, Rosenberg, Rao,
& Shapiro, 1995; Tavani, Bertuzzi, Negri, Sorbara, & Vecchia, 2001; Tofler, Foy, Ng,
Hickey, & Burke, 2001).

However, several studies have not found such a relationship. Lopez-Garcia et al.
(2006) found no association between coffee consumption and coronary heart disease,
even when separate analyses were done based on filtered vs. non-filtered coffee or
caffeinated vs. decaffeinated coffee (Lopez-Garcia et al., 2006). Another study that
looked at CVD risk in terms of myocardial infarction and stroke also found no
relationship (Floegel et al., 2012).

To complicate the picture further, multiple studies have shown that coffee
consumption may even be beneficial in terms of CVD. Several studies demonstrated an
inverse relationship; increasing coffee consumption led to a decreased risk for CVD
morbidity and mortality (Freedman, Park, Abnet, Hollenbeck, & Sinha, 2012; Woodward

& Tunstall-Pedoe, 1999). Others found a U-shaped relationship with those drinking a moderate amount of coffee showing the greatest benefit (Andersen, Jacobs, Carlsen, & Blomhoff, 2006; Mineharu et al., 2011) . One study showed gender differences with an inverse relationship between coffee consumption and CVD mortality among women but not men; the findings demonstrated a 70% lower risk of CVD mortality for women that drank at least 1 cup of coffee a day compared to those who did not drink any (Sugiyama et al., 2010).

Campos and Baylin (2007) noted that this apparent discrepancy on the effects of coffee on CVD may in part be due to the type of study conducted; case-control studies generally found coffee to increase the risk for CVD, whereas cohort studies have generally found a null or negative association (Campos & Baylin, 2007). Cardiovascular disease is a leading cause of death worldwide, and its prevalence is growing (Dahlöf, 2010). Therefore, it is imperative to further investigate the relationship, if any, between coffee and CVD.

Caffeine

Caffeine is the primary psychoactive ingredient in coffee (James, 1997). Worldwide, it is also the most commonly consumed psychostimulant drug (Chou, 1992). Caffeine is quickly absorbed into the bloodstream, reaching peak plasma concentrations in about 40-60 minutes (James, 1997). Its half-life elimination is typically between 3-7 hours, although factors such as age, pregnancy, or disease can affect this timing (James, 1997). Furthermore, sex may influence the metabolism of caffeine. Women might exhibit a faster clearance rate than men (Callahan, Robertson, Branfman, McComish, & Yesair, 1983), and the menstrual cycle may also influence metabolism with women having been

shown to have a slower rate of elimination in the luteal phase of their cycle, when sex hormones are highest, as compared to the follicular phase (Lane, Steege, Rupp, & Kuhn, 1992). Once in the liver, caffeine is broken down into paraxathine, theobromine, and the theophylline, with paraxanthine being the primary metabolite in humans (James, 1997).

Caffeine is a sympathomimetic that affects the body in multiple ways, such as increasing blood pressure, arterial stiffness, and epinephrine concentration (Riksen, Rongen, & Smits, 2009). It appears to exert many of its roles through the antagonism of adenosine A_1 and A_{2A} receptors (Riksen et al., 2009). Interestingly, adenosine is involved in anti-inflammatory responses; specifically, adenosine suppresses certain pro-inflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α), while it leads to the production of anti-inflammatory cytokines such as Interleukin-10 (IL-10) (Eigler et al., 1997; Le Moine et al., 1996). Thus, caffeine may alter immune function through antagonism of adenosine receptors which would result in inhibition of the anti-inflammatory response.

However, caffeine may exert immunological effects via a second pathway. It has been shown that caffeine leads to an increase in intracellular cyclic adenosine monophosphate (cAMP) (Horrigan, Kelly, & Connor, 2004). Similar to adenosine, cAMP also has suppressive effects on the immune system. Through a pathway involving protein kinase A (PKA), cAMP has been shown to inhibit pro-inflammatory cytokines, like TNF-α and IL-12, and promote the production of anti-inflammatory cytokine IL-10 (Eigler et al., 1998; Procópio et al., 1999). Thus, it is possible for caffeine to exert suppressive immunological effects through increasing the concentration of cAMP.

Inflammation

It is important to consider the immunological effects of caffeine because inflammation is a key component in the development of CVD. Triggers of atherosclerosis cause endothelial cells to express adhesion molecules. Because of these adhesion molecules, leukocytes are able to attach to the endothelial wall (Libby, 2006). These leukocytes then help monocytes to adhere to the lesions. These lesions then lead to the development of atherosclerotic plaques which are a signature of CVD (Libby, 2006). Thus, an indirect relationship between caffeine and CVD can be investigated through examining the effects of caffeine on inflammation.

There are a variety of inflammation markers that can be used. Only one of these markers, C -reactive protein (CRP), has a well-standardized assay with high sensitivity, making it currently the only marker that has clinical significance in regards to CVD (Willerson & Ridker, 2004). It has been shown to be a reliable marker of inflammation with predictive value of future vascular events (Koenig et al., 1999; P M Ridker, Hennekens, Buring, & Rifai, 2000; Paul M. Ridker, 2001). Furthermore, the American Heart Association and the Centers for Disease Control and Prevention have recommended it to be used in the clinical setting to identify those at risk (Pearson et al., 2003).

However, CRP may not be a comprehensive marker of inflammatory status and risk for future cardiovascular events. Pro-inflammatory cytokines also are associated with CVD risk (Kaptoge et al., 2013). While not as heavily researched as CRP, cytokines may even be better indicators for future coronary events. One study measured CRP, TNF-α, and IL-6 in an older population (70-79 years old) with no incidence of CVD at baseline.

While high levels of any one of the three markers predicted the onset of cardiovascular events, individuals that had high levels of all three markers were most at risk. Furthermore, this study actually found CRP to be less consistent in its predictive value of future vascular events compared to IL-6 or TNF-α (Cesari et al., 2003). Another study measured circulating levels of TNF-α in patients that had a myocardial infarction (MI), finding a 3-fold increase in the risk of recurrent MI or coronary death in those with the highest levels of TNF-α. Moreover, this increased risk was independent of CRP levels (Ridker et al., 2000). Furthermore, as pro-inflammatory cytokines help to regulate the inflammatory process and are more up-stream markers of inflammation than CRP, using them as markers may help to better understand the origin and development of CVD (Libby, 2006).

Caffeine and Cytokines

Epidemiological Studies

Few epidemiological studies have examined the relationship between coffee or caffeine intake and cytokine levels. Lopez-Garcia and colleagues (2006) examined the effects of caffeinated and decaffeinated filtered coffee on cytokine levels among women in a cross-sectional study. These women were free of cardiovascular disease at the point of time that blood was drawn and were all between 43 and 70 years old. Specifically, this study measured levels of soluble tumor necrosis factor α receptor 2 (sTNF-R2) (Lopez-Garcia et al., 2006). This soluble TNF receptor is related to TNF-α in that TNF-α and other cytokines induce this receptor; additionally, while it may limit the activity of TNF-α, this receptor also promotes inflammation (Aderka, 1996). This study found a slight

inverse association between coffee intake and sTNF-R2 in healthy women such that as coffee intake increased, sTNF-R2 levels decreased. While this finding was not statistically significant, it suggested that women do not appear to be at greater inflammatory risk compared to men (Lopez-Garcia et al., 2006).

A second cross-sectional survey also examined the relationship between coffee and inflammation included both men and women (Zampelas, Panagiotakos, Pitsavos, Chrysohoou, & Stefanadis, 2004). The inflammatory markers used in this study included IL-6 and TNF-α. Once again, participants had no evidence or history of CVD. The study found a linear dose-response relationship between coffee consumption and inflammatory markers. For instance, both men and women who consumed greater than 200 mL a day (approximately one cup) of coffee showed a 28% increase in TNF-α levels as compared to non-drinkers. Results approached statistical significance when greater than 200 mL of coffee a day was consumed (or about 1 cup of coffee). This study did not control for filtered vs. unfiltered coffee (Zampelas et al., 2004); however, other research has shown that unfiltered coffee, which contains compounds not found in filtered coffee, increases levels of lipoproteins, such as cholesterol and triglycerides (see Rodrigues & Klein, 2006 for review). Therefore, it is difficult to discern from this study what specific components in coffee led to these physiological differences.

Clinical Studies

The two available epidemiological studies report conflicting results. Additionally, due to the nature of cross-sectional studies, no causality can be determined. Therefore, it is important to review related clinical studies. Unfortunately, once again very little has been done in this area, and the few studies that do exist also provide inconclusive

evidence. One study administered instant caffeinated coffee (with 3 milligrams caffeine per kilogram of body weight), instant decaffeinated coffee, or water to healthy young men in a randomized crossover design. They found that coffee treatment had no significant effect on the pro-inflammatory cytokines measured (IL-6 and IL-18) (Gavrieli et al., 2011). A second study used a longitudinal design where participants spent one month abstaining from coffee, a second month consuming 4 cups of coffee/day, and a third month consuming 8 cups/day. All participants had an elevated risk of type 2 diabetes. Levels of IL-18 significantly decreased with the consumption of 8 cups/day as compared to the month of abstinence while no significant changes were found for IL-6 (Kempf et al., 2010). A third study involved participants that were overweight but otherwise healthy. Participants either consumed 5 cups of instant caffeinated coffee per day, decaffeinated coffee, or water for 8 weeks. IL-6 was found to have increased in the caffeinated coffee group as compared to the placebo group. Similar but non-significant results were found for the decaffeinated group (Wedick et al., 2011).

It is difficult to draw comparisons due to the variety of populations and study designs used in these three studies. For instance, it is possible that Kempf et al. (2010) found decreased IL-18 whereas Gavrieli et al. (2011) did not because participants consumed coffee over a much longer period of time in the first study (i.e., 2 months vs. 1 day). It is plausible that coffee only exerts effects on inflammation if it has been consumed chronically versus an acute administration. Furthermore, two out of three studies used populations at risk for Type 2 Diabetes (Kempf et al., 2010; Wedick et al., 2011); therefore, it is difficult to extrapolate the results to the general population. Finally, while the studies all used both caffeinated and decaffeinated coffee, none administered

caffeine alone. Thus, none of the available clinical studies to date have examined the pharmacological impact of caffeine on inflammation; other compounds in coffee could be responsible for the observed effects, or these other compounds could mitigate the potential effects of caffeine on inflammation.

<u>Unanswered Questions</u>

Caffeine is the most commonly used psychostimulant drug, while cardiovascular disease is the leading cause of death worldwide (Chou, 1992; Dahlöf, 2010); yet, the relationship between caffeine and CVD remains largely unknown. Inflammation is a key component in the development of cardiovascular disease; thus, understanding the impact of caffeine on inflammation is imperative as it can give insight into one potential mechanism of this disease. Additionally, if caffeine does affect inflammation, knowing specifically which components of the inflammatory response are affected by caffeine could be a first step in determining a biological mechanism. To date, few human studies have investigated the effects of caffeine on inflammation, and fewer still have specifically examined cytokines, which play an intricate role in the inflammatory response. Furthermore, none of these studies have directly examined the acute inflammatory effects of caffeine administration alone (i.e., without coffee). This present study fills this research gap by examining the effects of acute caffeine administration on pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-4 and IL-10) in both men and women.

It was hypothesized that caffeine in the presence of stress would increase levels of pro-inflammatory cytokines and would decrease levels of anti-inflammatory cytokines as

compared to the control group. Furthermore, no sex differences between the caffeine and control group were expected based on the limited available literature.

Chapter 2

Methods

<u>Participants</u>

Participants in this study included 52 healthy men (N = 26) and women (N = 26) who were between the ages of 18 and 29 years (21.4 $\pm$ 0.3 years). Participants were recruited in Penn State classrooms with permission from the course instructor, as well as through flyers that were posted around the Penn State campus and local community. Eligibility was determined through an initial telephone interview conducted by a trained researcher, which involved questions regarding current medications and significant health issues. Eligible participants were followed up with an initial screening lab session at the General Clinical Research Center on the Penn State Campus where a cholesterol screening was conducted. Participants were asked to fast for the 12 hours prior to this screening for a proper cholesterol reading (Craig, Amin, Russell, & Paradise, 2000). In order to confirm a family history of hypertension, questionnaires were completed and returned by a potential participant's biological parents. Family history of hypertension was regarded as present if at least one biological parent of the participant was (1) diagnosed with hypertension and, (2) currently taking or had taken in the past year prescription blood pressure regulation medication (al'Absi et al., 1998; Greenberg & Shapiro, 1987). Following confirmation of family history of hypertension and normal cholesterol levels (total cholesterol less than 240 mg/dl, high-density lipoprotein greater or equal to 40 mg/dl, low-density lipoprotein less than 130 mg/dl), participants were scheduled for a 3.5 hour laboratory session.

Individuals were excluded from the study if they had significant health issues that would affect the interpretation of blood pressure or blood marker data, including diabetes, hypertension, neurological disorders such as stroke, history of mental illnesses such as depression or anxiety, and cognitive or attentional disorders such as attention deficit/hyperactivity disorder, or for drug and medication use that would affect normal hormonal, metabolic and cardiovascular functioning, including oral and injected corticosteroids, psychostimulants, aspirin, and antioxidant vitamins. Participants also were excluded for the use of nicotine products like tobacco, body mass index (kg/m^2) greater than 30, laboratory resting systolic blood pressure greater than 140 mmHG or diastolic blood pressure greater than 90 mmHG.

Participants were regular consumers of caffeine such that daily caffeine consumption was between 100 mg and 500 mg a day (i.e., 1-5 cups of coffee/day). The lower limit of 100 mg of caffeine was set in order to ensure that participants would tolerate the caffeine administration (al'Absi & Wittmers, 2003; al'Absi et al., 1998), while the upper limit of 500 mg ensured that participants would still respond to the dosage of caffeine administered in this study (al'Absi et al., 1998).

Exclusionary criteria for women participants included the use of hormonal medication, if they were currently pregnant or had been in the past year, or if they were currently or recently breastfeeding. Women who were undergoing or had undergone menopause were also excluded. In order to maximize potential sex differences, female participants had their lab sessions scheduled during the late luteal phase of their menstrual cycle because this is when estrogen levels are at their highest (Elhadd et al., 2003). Additionally, it has been shown that caffeine metabolism may be reduced by 25%

in females during the luteal phase of the menstrual cycle, which may further emphasize the effects of caffeine in this study (Lane et al., 1992). Menstrual cycle phase was determined by the self-reported date of the last menstrual period and menstrual cycle length, and then confirmed through estradiol and progesterone levels assayed from baseline serum samples. All female participants in this study were in the luteal phase of their menstrual cycle. All procedures were reviewed and approved by the Pennsylvania State University Institutional Review Board prior to the initiation of this study. (IRB 19000) (See Appendix A)

Laboratory Session: Overview

Figure 1 presents a timeline of the experiment. This study had a mixed model experimental design. Sex (male or female) and drug administration (caffeine or placebo) were the between-subject factors. Stress was the within-subjects factor, with all participants undergoing the same stress protocol. The stress protocol consisted of a baseline rest period, a challenge period, and a recovery period. The challenge involved a 13.5 minute speech challenge and a 15 minute mental arithmetic task. Blood pressure and heart rate were recorded every 2 minutes throughout the laboratory session. Self-reported rating of stress using a 7-point Likert scale and blood samples were collected at the (1) end of baseline period right before the stress protocol, (2) 15 minutes after the end of the stress protocol, and (3) 45 minutes after the end of the stress protocol. In order to control for the diurnal rhythm of cortisol across all participants, all sessions were conducted in the afternoon, beginning at 1:00 PM (Van Cauter, 1990).

Baseline

Participants were asked to refrain from consuming caffeine for the 4 hours prior to the laboratory session. They were also instructed to consume a low-fat lunch because high-fat meals have been found to significantly increase serum CRP levels (Carroll & Schade, 2003). Once participants arrived at the GCRC, they provided informed consent and had their height and weight measured. A nurse practitioner confirmed eligibility by assessing each participant's health status, which included a pregnancy test for all women participants. Participants then completed questioners that helped to determine daily caffeine intake and mood. A blood pressure cuff (Dinamap Compact Blood Pressure Monitor, Critikon, Tampa, FL) was applied to the participant's dominant arm. Following this, a catheter was inserted by a trained nurse. Participants were then asked to sit and relax for 30 minutes. During this period, a blood pressure reading was taken every 2 minutes. At the end of this baseline period, a symptom report questionnaire was administered.

Caffeine Administration

Following the 30 minute baseline period, participants were administered either caffeine or the placebo. The caffeine (3.3 mg/kg body weight) (Spectrum Chemical Corporation, Gardena, CA) was mixed with refrigerated white grapefruit juice (Unsweetened White Grapefruit Juice, Giant® brand, Landover, MD), while the placebo consisted of the white grapefruit juice without caffeine. The participant's body weight, measured in the beginning of the laboratory session, was used to calculate the exact caffeine dosage. This study is consistent with others that have also used this caffeine dosage and use of grapefruit juice (e.g., Hartley et al., 2000; Lovallo et al., 1989, 1991).

Grapefruit juice has been shown to have no effect on caffeine's pharmacokinetics and no hemodynamic effects (Maish, Hampton, Whitsett, Shepard, & Lovallo, 1996), and additionally, it masks the bitter taste of the caffeine. The drink was prepared by a nurse who was given a sealed envelope indicating the drug condition. Thus, the experimenter was kept blind to the participant's drug condition. Following drug administration, participants were asked to rest for 20 minutes in order to allow the caffeine to be absorbed, after which a mood assessment questionnaire was administered and the first blood sample was taken. Blood pressure and heart rate were both taken every 2 minutes during this period.

Stressor

The next portion of the study involved each participant undergoing a psychosocial stressor, which was based upon the Trier Social Stress Task protocol (Kirschbaum, Pirke, & Hellhammer, 1993). This challenge involved the preparation and delivery of a brief speech, followed by a mental arithmetic task consisting of serial subtraction. During the speech task, participants were given 10 minutes to prepare a speech regarding an experience of personal failure, and then had 3.5 minutes to deliver it in front of an experimenter and a video camera. During the mental arithmetic tasks, participants were asked to count backwards from a four-digit number, first by 7's and then by 13's, as accurately and quickly as possible. This portion lasted 15 minutes. The participants were told the experimenter was a psychologist who would evaluate his or her performance on the tasks and that the videotape would later be evaluated by a panel of psychologists. However, the tasks were not videotaped and were not evaluated by psychologists. Mood assessment questionnaires were administered before and after the stress task and blood

pressure and heart rate were taken every 2 minutes. A second blood draw was taken 15

minutes following the stressor in order to allow stress hormone levels to rise.

<u>Recovery</u>

Participants were asked to sit quietly for an additional 45 minutes after the second

blood draw. This delay was to allow enough time for rises in cytokine levels to occur

(Steptoe, Hamer, & Chida, 2007). A series of questionnaires were administered during

this recovery period, including a symptom report questionnaire, a mood assessment, and

a caffeine recognition questionnaire. Blood pressure and heart rate were taken every 2

minutes during this period. At the end of the recovery period, a third and final blood

sample was drawn, after which the catheter was removed. Participants were then

debriefed about the procedure and compensated $50 for their time.

<u>Blood Sample Handling</u>

For each of the three blood draws, blood was drawn into anticoagulant-free

(serum) tubes. Serum samples were allowed to sit at room temperature for 15 min prior to

centrifugation at 4ºC at $1500 \times g$ for 15 min and then were frozen at -80ºC for later TNF-

α, IL-1β, IL-4, and IL-10 assessment.

<u>Assays</u>

Interleukin-1 beta, tumor necrosis factor alpha, and interleukin-4 were assessed

using separate enzyme linked immunosorbent assay (ELISA) kits developed in-house at

the GCRC (see Appendices B, C, D). The IL-1B kit had a minimum sensitivity of 0.452

pg/ml and upper range of 4000 pg/ml. The TNF-a kit had a minimum sensitivity of 8.2

pg/mL and upper range of 30000 pg/mL. The IL-4 kit had a minimum sensitivity of 16.4

pg/mL and upper range of 25000. Interleukin-10 levels were assessed using a

commercially available enzyme immunometric assay (EIA) kit (Assay Designs, Inc., Ann Arbor, Michigan) that had a minimum sensitivity of 1.512 pg/ml and upper limit of 500 pg/ml (see Appendix E). All samples were run in duplicate with mean values being reported and used for analyses. Duplicate test values that varied by more than 5% error were subject to repeat testing.

Design and Data Analyses

The study design comprised a 2 (sex) x 2 (caffeine, placebo) design. Separate repeated-measures analyses of variances (ANOVA) were conducted to test the effects of gender and caffeine on IL-4, IL-10, IL-1β, and TNF-α across the laboratory session. Separate ANOVAs were used to test any significant group X time interactions. All cytokines were natural log transformed to normalize their distribution. All tests were two-tailed, and criterion for statistical significance was set at alpha = 0.05.

Chapter 3

Results

Twenty-six men and 26 women, all between the ages of 19 and 29 years old, participated in a study with a mixed model experimental design. Half of the participants were administered caffeine while the other half were administered a placebo. All participants then underwent the Trier Social Stress Test. Blood samples were taken at baseline (20 minutes after drug administration) and an hour after the stressor. Table 1 presents mean cytokine levels. A mood assessment survey that was administered directly before and after the Trier Social Stress Test confirmed that the protocol was successful in increasing how stressed participants felt [F $(1, 47) = 38.91$, $p < 0.01$].

Figure 2 presents mean IL-4 levels among men and women exposed to placebo or caffeine. There was a marginally significant caffeine by time effect for interleukin-4 [F $(1, 47) = 3.23$, $p = 0.08$] such that Il-4 levels among individuals in the control group declined whereas IL-4 levels among individuals exposed to caffeine did not. However, time effects within each group are not statistically significant.

Figure 3 presents mean IL-1β levels among men and women exposed to placebo or caffeine. There was a marginally significant time by gender effect for interleukin 1-β [F $(1, 47) = 3.26$, $p = 0.08$]. Specifically, women's levels did not significantly change. However men's IL-1β levels showed a moderate time effect where their levels decreased between baseline and recovery [F $(1, 24) = 3.64$, $p = 0.07$]. There were no difference in this gender effect between the treatment groups [F $(1, 47) = 0.65$, $p = $ NS].

Figure 4 presents mean IL-10 levels among men and women exposed to placebo or caffeine. There was a significant time effect for interleukin-10 [F $(1, 48) = 19.21$, $p < $

0.01]. Regardless of treatment group or gender, IL-10 decreased between baseline and recovery.

Figure 5 presents mean TNF-α levels among men and women exposed to placebo or caffeine. Tumor necrosis factor-alpha showed a significant time by caffeine effect [F $(1, 47) = 7.86$, $p < 0.01$]. There was an increase in TNF-α at follow-up as compared to baseline for the caffeine treated group. There was no difference in this caffeine effect between males and females [$F (1, 47) = 0.01$, $p = $ NS].

Chapter 4

Discussion

The role of coffee and caffeine in the development of cardiovascular disease has been debated. There has been some research to suggest that those who consume coffee are more likely to develop CVD whereas other research has suggested the opposite. It is known that inflammation plays a key role in the development of CVD. Therefore, the purpose of this study was to further examine this relationship through investigating the effect of caffeine on inflammation. Specifically, two anti-inflammatory cytokines (IL-4 and IL-10) and two pro-inflammatory cytokines (IL-1β and TNF-α) were measured.

Stress

Psychological stress has been known to dampen the immune system. For instance, studies have shown that higher levels of stress increase the time for wounds to heal and an individual's susceptibility to illness (Broadbent, Petrie, Alley, & Booth, 2003; Cohen, Tyrrell, & Smith, 1991; Kiecolt-Glaser, Marucha, Malarkey, Mercado, & Glaser, 1995; Takkouche, Regueira, & Gestal-Otero, 2001). In this study, acute psychological stress decreased circulating levels of IL-10, an anti-inflammatory cytokine, in both men and women. This finding supports prior findings that stress dampens the immune system (see Godbout & Glaser, 2006 for review), and specifically supports findings from additional studies that have found psychological stress to decrease the production of IL-10 (Buske-Kirschbaum, Kern, Ebrecht, & Hellhammer, 2007; Maes et al., 1998). However, in the current study, circulating levels of IL-4, also an anti-inflammatory cytokine, were not affected by stress alone, which is in contrast to previous findings which also found

production levels of this cytokine to decrease (Buske-Kirschbaum et al., 2007; Maes et al., 1998).

This study also found some sex-dependent effects of stress on inflammation. For pro-inflammatory cytokine IL-1β, stress decreased circulating levels in men but not in women. Previous findings in regards to the effect of psychological stress on IL-1β are mixed. While one all-male study found an oral presentation stressor to be associated with an increase in IL-1β levels (Heinz et al., 2003), another study found no effect of stress on IL-1β in healthy men. Our findings are contrary to another study that only involved female participants, which found IL-1β levels to significantly increase following an interview stress protocol (Altemus, Rao, Dhabhar, Ding, & Granstein, 2001). The current study adds to the literature in that it is the only one that tests the effects of psychological stress on IL-1β in both men and women. Therefore, more studies are needed to understand how sex may moderate the effects of stress on inflammatory cytokines. As women underwent the stress protocol during the luteal phase of their menstrual cycles, when levels of estrogen and progesterone hormones are at their highest, it is possible that these hormones may have counteracted the slight anti-inflammatory properties of stress observed in men. Further studies are needed to test this hypothesis.

Previous research on the effects of stress on pro-inflammatory cytokine TNF-α also are mixed. Stress has been found to increase production of TNF-α in stimulated blood (Ackerman, Martino, Heyman, Moyna, & Rabin, 1998). Furthermore, a study with healthy women that examined circulating cytokine levels also found a significant increase 40 minutes post-task (Altemus et al., 2001). However, several other studies that

examined circulating levels of TNF-α in both men and women found no effect of stress

(Miller, Cohen, & Kim, 2002; Owen & Steptoe, 2003), and a meta-analysis concluded

that the effects of stress on TNF-α were not significant (Steptoe et al., 2007). Our study

also found no significant effects of stress on TNF-α, thus adding to this growing body of

literature that suggests stress alone may not have an effect on this particular cytokine in

men or women.

Together, these findings suggest that acute psychological stress does impact the

immune system. However, it does not appear to do so in a consistent manner; acute

psychological stress significantly decreased levels of one anti-inflammatory cytokine

(i.e., IL-10) for both sexes and slightly decreased one pro-inflammatory cytokine (i.e., IL-

1β) in men. Therefore, while both pro-inflammatory and anti-inflammatory cytokines

were not uniformly affected, stress did appear to exert a net inflammatory effect. Our

findings thus support the conclusion of a meta-analysis conducted by Steptoe, Hamer,

and Chida (2007), which also found acute psychological stress to moderately increase

inflammatory markers. Acute stress may affect circulating levels of inflammatory

cytokines through several pathways (Steptoe et al., 2007). For instance, stress leads to

changes in plasma volume, which may affect cytokine concentrations. Other mechanisms

may include stress causing an upregulation of inflammatory marker synthesis through

sympathoadrenal processes, or stress leading to changes in the number of immune cells

that release cytokines (Steptoe et al., 2007). The complexity of the various mechanisms

involved, and the possibility that individual cytokines may be affected differently by each

mechanism, is a possible explanation for our observation that stress did not impact the immune system in a consistent manner.

<u>Caffeine</u>

It was hypothesized that caffeine would also have a net inflammatory effect by increasing pro-inflammatory cytokines and decreasing anti-inflammatory cytokines. The results of this study were inconclusive. Levels of one pro-inflammatory cytokine, TNF-α, did significantly increase from baseline to recovery for the caffeine group compared to the control group; yet, caffeine alone had no effect for IL-1β, also a pro-inflammatory cytokine. The only studies to specifically measure the effect of coffee/caffeine on TNF-α in humans were epidemiological in nature and reported conflicting results (Lopez-Garcia et al., 2006; Zampelas et al., 2004). However, previous studies that have measured alternate biomarkers of inflammation, such as Il-6 and fibrinogen, have also found caffeine to increase inflammation in both men and women (Bennett, Rodrigues, & Klein, 2013; Wedick et al., 2011). Other results have reported that coffee/caffeine does not affect other pro-inflammatory agents, such as IL-18 and CRP (Bennett et al., 2013; Gavrieli et al., 2011). One clinically controlled study reported that chronic coffee consumption (about 2 months) decreased levels of IL-18 in men and women (Kempf et al., 2010). However, it is difficult to compare results across these studies because the methods used varied widely, as did the markers of inflammation measured.

Furthermore, as previously discussed, stress has been shown to increase TNF-α levels, but this increase was not observed in this study. However, caffeine in the presence

of stress did affect TNF-α. It is possible that stress and caffeine had an additive effect on this cytokine such that both were required in order to observe a significant change.

Data suggests that if caffeine increased pro-inflammatory cytokines, it would decrease anti-inflammatory cytokines (Horrigan, Kelly, & Connor, 2006). Interestingly, however, while caffeine had no effect on IL-10 (an anti-inflammatory cytokine), caffeine appeared protective against a decrease in IL-4 levels (also an anti-inflammatory cytokine) following stress. While the results were only marginally statistically significant, levels among individuals in the control group declined while those exposed to caffeine did not. While no statistically significant effects of stress were observed for IL-4 in our study, previous literature suggests that IL-4 levels should decrease in response to stress (Buske-Kirschbaum et al., 2007; Maes et al., 1998). This expected result was observed in the control group, but not in the caffeine group, suggesting that caffeine may have mitigated the effect of stress on inflammation for this particular cytokine.

Our study indicates that caffeine may have varying effects on inflammation depending on the specific biomarker observed. Caffeine has been shown to intensify the stress response (Lane, Adcock, Williams, & Kuhn, 1990); thus, some of caffeine's effect on inflammation, such as the increase in TNF-α levels observed in this study, may be via the mechanisms in which stress affects inflammation. Furthermore, caffeine is a known antagonist for adenosine, which is anti-inflammatory; thus, by antagonizing adenosine, caffeine would be expected to increase inflammation (Riksen et al., 2009). However, caffeine may exert anti-inflammatory properties via an alternative pathway; caffeine causes an increase in cAMP, which exerts suppressive effects on inflammation (Horrigan et al., 2004). Consequently, the inconsistent effects of caffeine on inflammation as

observed in this study may be a result of caffeine working through these complex and competing mechanisms.

<u>Limitations and Future Directions</u>

The time course of cytokine responses to acute stress has not been established (Steptoe et al., 2007). The present study only measured cytokine levels out to one hour following the stressor. It is possible that this time frame was not long enough to fully capture the immune response; future studies should involve longer monitoring periods. This study also involved the administration of a single dose (3.3 mg/kg body weight) of caffeine. Epidemiological studies of the effects of coffee on CVD found differing results depending on the amount of coffee consumed per day. Future studies should investigate any possible dose-resposne relationship between caffeine and inflammation by utilizing an acute administration with varying doses of caffeine.

It is possible that caffeine does influence the immune system, but only over a longer period of time. Two of three clinical studies that studied caffeine's effect on inflammation utilized this chronic model where effects were measured after caffeine had been consumed regularly for at least one month (Kempf et al., 2010; Wedick et al., 2011). Ours was an acute study and more similar in design to the study conducted by Gavrieli and colleagues (2011). Therefore, more studies are needed in order to understand whether acute or chronic consumption of caffeine has differing effects on inflammation. It is equally possible that caffeine affects the immune system, but the effects were not adequately captured in this study. For instance, circulating levels of cytokines were measured, thus providing an indication for immune status. However, the source of the cytokines in the body cannot be determined through this method (Calder, 2007). Future

studies should investigate the effect of acute caffeine administration on immune functioning through the use of assays that can detect the level of cytokine production in response to a particular mitogen or bacterial lipopolysaccharide, or studies could instead measure the production of cellular mRNA levels (Calder, 2007). These types of analyses could give further insight into a mechanism in which caffeine may impact inflammation. Furthermore, only four cytokines were analyzed in this study, and thus it is possible that caffeine and stress have effects on cytokines other than the ones tested.

Our study adds to the literature because we administered caffeine alone and not in the form of coffee, as had previous studies that examined the relationship between caffeine and inflammation through administering caffeinated versus decaffeinated coffee (Gavrieli et al., 2011; Kempf et al., 2010; Lopez-Garcia et al., 2006; Wedick et al., 2011; Zampelas et al., 2004). Our study design is beneficial in order to learn more about the pharmacological effects of what is often considered to be the primary active ingredient in coffee. However, it is also possible that compounds in coffee other than caffeine impact inflammation. For instance, a review by Rodrigues and Klein (2006) highlighted diterpenes, oils found in coffee, as compounds responsible for increasing cholesterol levels. To our knowledge, no studies have examined the effect of these compounds on inflammation in humans; however, results of several animal studies have suggested kahweol, a coffee diterpene, to have anti-inflammatory effects (Cárdenas, Quesada, & Medina, 2011; Kim, Kim, & Jeong, 2006). More studies are needed to fully understand what compounds in coffee are or are not responsible for affecting inflammation.

While research on other components of caffeine is also needed, it is still important to continue research on the effects of caffeine as this is a primary ingredient in beverages

other than coffee. For instance, energy drinks have become increasingly popular; these drinks can be a high source of caffeine with up to 505 mg per can or bottle, compared to 77-150 mg in 6 oz. cup of coffee (see Reissig, Strain, & Griffiths, 2009 for review). These drinks have been increasingly implicated as a source of caffeine toxicity, which may result in issues such as seizures and cardiac ischemia (Gunja & Brown, 2012). While a growing field, no research to date has been done regarding the relationship between energy drinks and traditional inflammatory markers. However, a study by Worthley and colleagues (2010) does suggest energy drinks may affect the immune system; they found that acute consumption of a sugar-free energy drink increased platelet aggregation and impaired endothelial function in healthy men and women. However, the energy drink contained only 80 mg of caffeine. Future studies should investigate the effects of energy drinks on the higher end of the caffeine spectrum and should include additional immune markers.

Our study investigated potential sex differences in the effects of caffeine and inflammation by recruiting women participants in the luteal phase of their menstrual cycles, where levels of sex hormones would be at their highest. Women are an important population to study as their risk for cardiovascular disease increases once they reach menopause as a result of a subsequent decrease in their estrogen levels (Rosano, Vitale, Marazzi, & Volterrani, 2007). Furthermore, previous research has suggested that caffeine is metabolized differently in women; several studies reported slower rates of caffeine elimination during women's luteal phase (Balogh, Irmisch, Klinger, Splinter, & Hoffmann, 1987; Lane et al., 1992). Our study only found one marginally significant effect of sex in regards to stress, but found no sex differences in regards to the effects of

caffeine on inflammatory markers. Future studies need to include women in various phases of their menstrual cycles and compare effects of caffeine and stress on inflammation in pre- and post-menopausal women.

Furthermore, our study was composed of men and women with a family history of hypertension. This is significant to note because this population has been shown to respond differently to caffeine and stress. For instance, one study showed that men with a family history of hypertension had the highest levels of stress hormones (adrenocorticotropic hormone and cortisol) following administration of caffeine and a stress task compared to those with no family history (al'Absi et al., 1998), while another study showed that men with a family history of hypertension had the highest blood pressures following caffeine administration and stress protocol as compared to those with no family history (Lovallo et al., 1989). This is an important population to study because these individuals may be more vulnerable to the development of CVD as they are already at higher risk for hypertension, a risk factor for CVD. Our study is also unique because it included women with a family history of hypertension as well. However, because this population appears to be more sensitive to the effects of caffeine and stress, the results of our study may not be generalizable to the general public. Future studies regarding the effects of caffeine and stress on inflammation should continue to look at this population, but should also include participants without a family history of hypertension.

Conclusion

This study was the first to examine the effects of stress and caffeine that was not in the form of coffee on inflammation in both men and women with a family history of hypertension. Furthermore, it was the first to measure several cytokines that had not been

previously reported on in this particular area of research. While it is difficult to draw definitive conclusions, the results of this study do suggest that both stress and caffeine interact with the immune system to alter the state of inflammation. Given the role that inflammation plays in the development of cardiovascular disease as well as the widespread use of caffeine, this is an important area of research that deserves further attention.

Tables

Table 1: Mean (± SEM) raw values for cytokine levels across the laboratory session by sex and caffeine treatment groups.

	Males		Females	
	0 mg/kg (N=13)	3.3 mg/kg (N=13)	0 mg/kg (N=13)	3.3 mg/kg (N=13)
Interleukin 1-beta (pg/mL)				
Baseline	419.09 ± 191.70	147.39 ± 103.67	287.54 ± 203.06	192.26 ± 69.87
Stress	358.44 ± 147.51	144.62 ± 101.70	247.51 ± 166.71	205.86 ± 76.71
Recovery	386.11 ± 166.01	131.56 ± 89.22	255.69 ± 170.28	214.96 ± 79.06
Tumor Necrosis Factor- alpha (pg/mL)				
Baseline	1336.44 ± 671.35	529.91 ± 337.48	1410.16 ± 920.65	714.77 ± 206.25
Stress	1374.36 ± 708.29	501.63 ± 301.14	1418.12 ± 936.46	731.45 ± 215.77
Recovery	1420.31 ± 746.61	524.12 ± 312.78	1309.97 ± 850.25	766.57 ± 224.20
Interleukin 4 (pg/mL)				
Baseline	3820.42 ± 2046.13	547.01 ± 381.00	2562.67 ± 1757.14	1000.28 ± 331.66
Stress	3605.24 ± 1990.58	558.02 ± 378.80	2743.38 ± 1925.59	898.95 ± 306.67
Recovery	3763.82 ± 2030.12	558.42 ± 390.06	2596.73 ± 1932.66	1010.84 ± 316.53
Interleukin 10 (pg/mL)				
Baseline	12.82 ± 7.41	7.58 ± 3.07	9.19 ± 3.10	3.72 ± 0.41
Stress	13.15 ± 7.81	6.44 ± 2.84	8.75 ± 3.37	2.95 ± 0.43
Recovery	10.45 ± 6.32	6.14 ± 2.85	8.57 ± 3.58	2.90 ± 0.57

Figures

Figure 1: Experimental Timeline

Figure 2: Mean serum IL-4 levels (pg/mL) at baseline and 60-min after stressor exposure among men and women exposed to caffeine (N = 26) or placebo (N = 26). Means ± SEM.

Figure 3: Mean IL-1β serum levels (pg/mL) at baseline and 60-min after stressor exposure among men and women exposed to caffeine (N = 26) or placebo (N = 26). Means ± SEM.

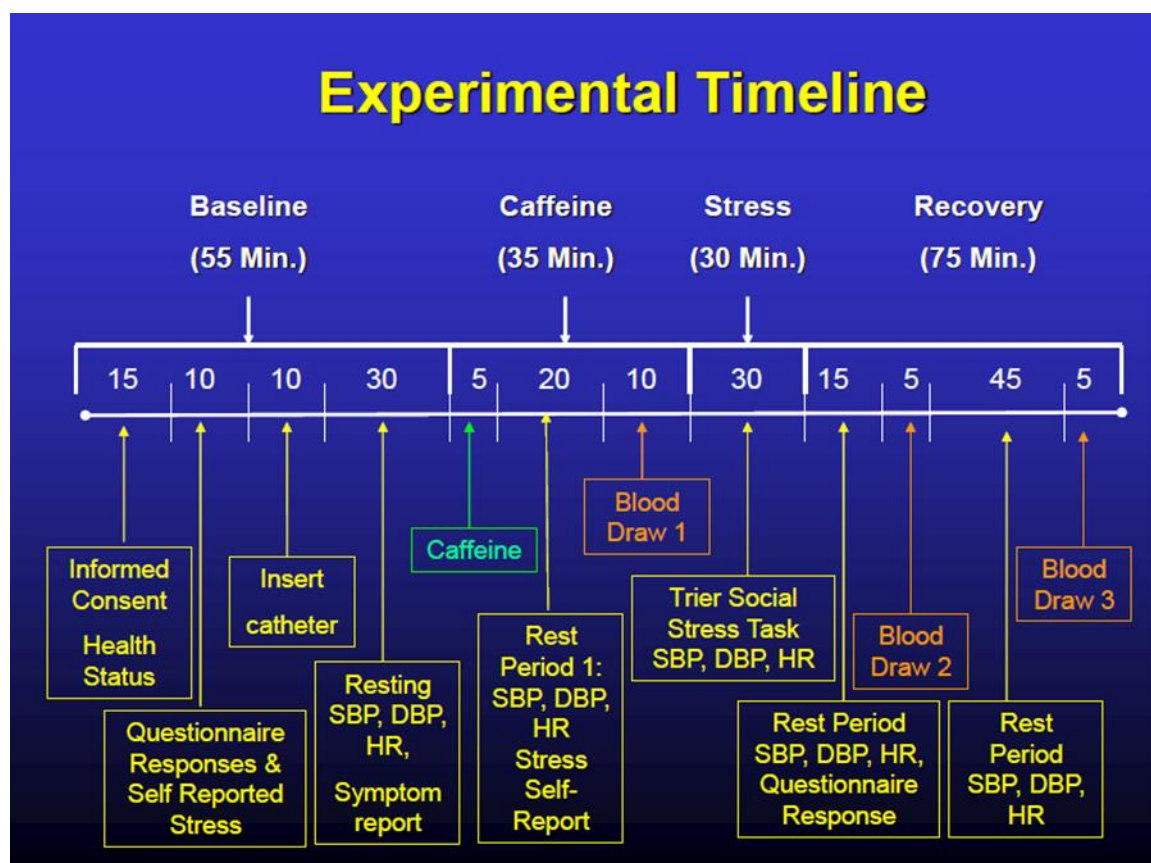**Figure 4: Mean serum IL-10 levels (pg/mL) at baseline and 60-min after stressor exposure among men and women exposed to caffeine (N = 26) or placebo (N = 26). Means ± SEM.**
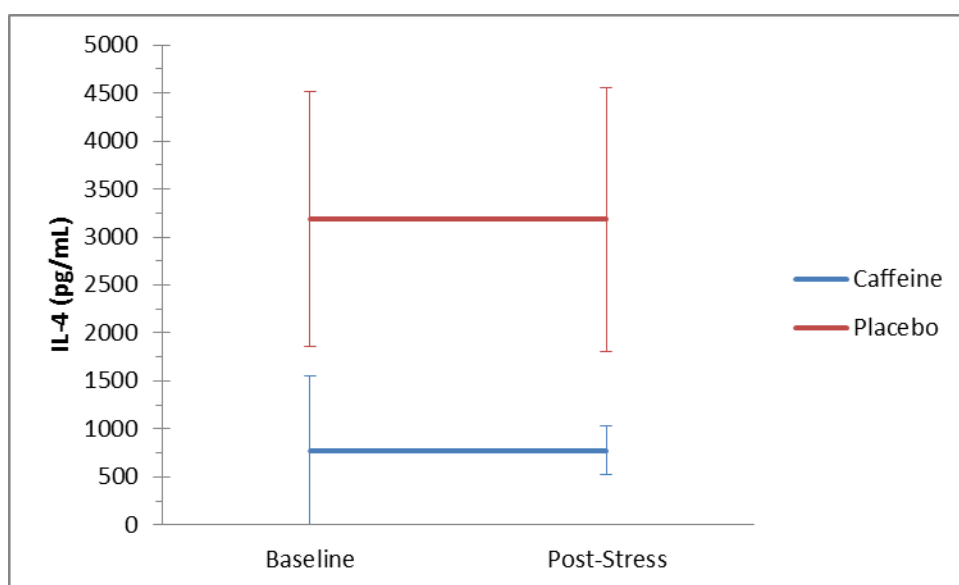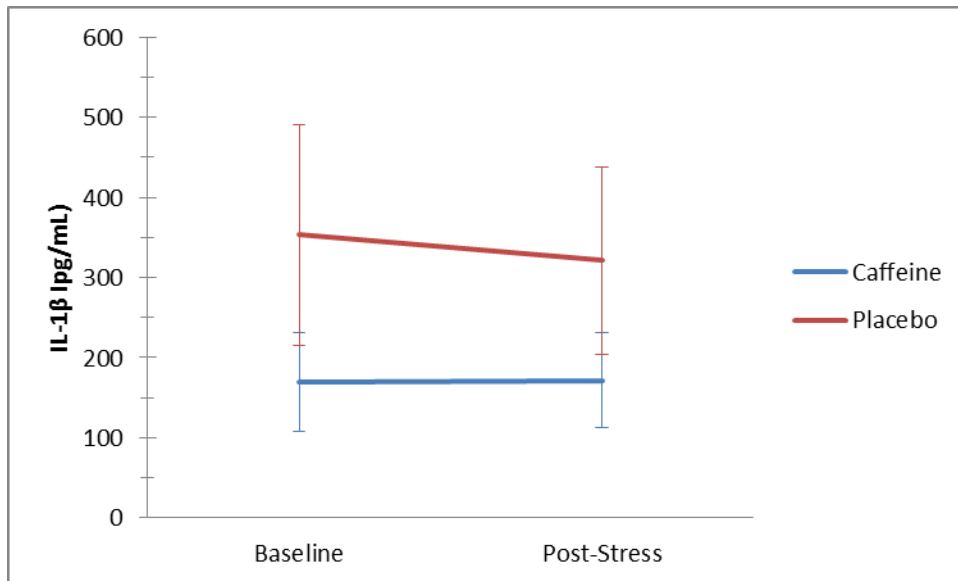
Figure 5: Mean serum TNF-α levels (pg/mL) at baseline and 60-min after stressor exposure among men and women exposed to caffeine (N = 26) or placebo (N = 26). Means ± SEM.

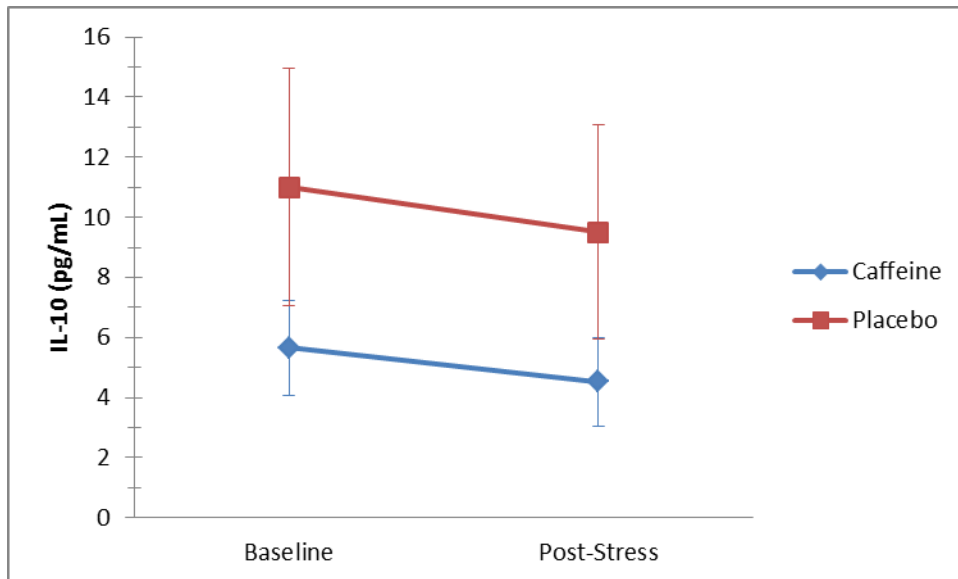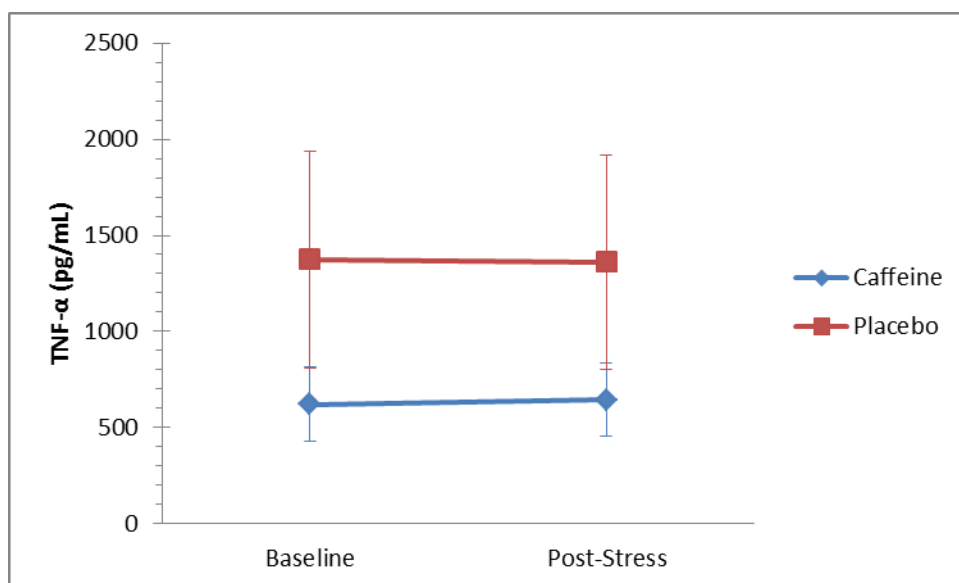**Appendix A: Institutional Review Board Approval and Consent Forms**

PENNSTATE #39744

	Vice President for Research	The Pennsylvania State University	(814) 865-1775
	Office for Research Protections	212 Kern Graduate Building	Fax: (814) 863-8699
		University Park, PA 16802-3301	www.research.psu.edu/orp/

Date: July 13, 2004

From: Mary B. Becker, IRB Administrator

To: Isabella M. Rodrigues

Subject: Results of Review of Proposal - Full Review **(IRB #19000)**
             Approval <u>Expiration</u> Date: June 16, 2005
             "Effects of Caffeine on Cardiovascular Risk Markers"

The Biomedical Institutional Review Board has reviewed and approved your proposal for use of human participants in your research. By accepting this decision, you agree to obtain prior approval from the IRB for any changes to your study. Unanticipated participant events that are encountered during the conduct of this research must be reported in a timely fashion.

Enclosed is/are the dated, IRB-approved informed consent(s) to be used when recruiting participants for this research. Participants must receive a **copy** of the approved informed consent form to keep for their records.

If signed consent is obtained, the principal investigator is expected to maintain the original signed consent forms along with the IRB research records for this research <u>at least three (3) years</u> after termination of IRB approval. For projects that involve protected health information (PHI) and are regulated by HIPAA, records are to be maintained for <u>six (6) years</u>. The principal investigator must determine and adhere to additional requirements established by the FDA and any outside sponsors.

If your study will extend beyond the above noted approval expiration date, the principal investigator must submit a completed Continuing Progress Report to the Office for Research Protections (ORP) to request renewed approval for this research.

On behalf of the committee and the University, thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of human participants.

MBB/slk
Enclosure
cc: Laura C. Klein

Please Note: The ORP encourages you to subscribe to the ORP listserv for protocol and research-related information. Send a blank email to: <u>L-ORP-Research-L-subscribe-request@lists.psu.edu</u>.

#39744

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY

Title of Project: The Effects of Caffeine on Cardiovascular Risk Markers

Principal Investigator: Isabella M. Rodrigues
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814) 863-5845
Email: imr104@psu.edu

Other Investigator: Laura Cousino Klein, Ph.D.
315 East Health and Human Development
Penn State University
University Park, PA 16802
Telephone: (814)-865-8813
Email: Lklein@psu.edu

This is to certify that you, _____, have been given the following information regarding your participation as a volunteer in a program of investigation under the supervision of Isabella M. Rodrigues and Laura Cousino Klein in the Department of Biobehavioral Health.

Purpose of the study:
The purpose of this study is to see how stress and caffeine affect blood markers of heart disease. Changes in hormones and blood markers of heart disease will be measured in blood samples collected during the study. We also are examining these changes in men and women to see how they compare in their responses to challenge and caffeine. These changes will provide important information about men and women's vulnerability to stress and caffeine.

You understand that you are being asked to participate in this study because you are a healthy adult (at least 18 years old) who does not have any of the following health conditions or problems:

1. high blood pressure;
2. a history of angina (chest pain due to heart problems) or arrhythmia (fast, slow, or irregular heart beats that require medication);
3. diabetes (excessive amounts of sugar in your blood);
4. any neurological disorder (e.g., stroke);

#39744

Date

Dear NAME

Your son/daughter, _____, is participating in a research project in the department of Biobehavioral Health at Penn State University, under the supervision of Dr. Laura Klein and approved by the Penn State University Office of Research Protections. This project is examining the relationship among caffeine, stress, blood pressure, and risk for development of heart disease. Your son/daughter has sent you this letter because one important aspect of our research is to verify the cardiovascular health of our research volunteer's parents. Specifically, we are interested in knowing about whether or not you have ever been diagnosed or treated for hypertension or high blood pressure. If this is okay with you, then please take a couple of minutes to complete the enclosed form and return it in the postage-paid envelope.

> *Your completion of this form is voluntary and you may withdraw at any time or refuse to answer any questions; there are no risks involved by participating in this survey. Your assistance with this information will be useful in helping us understand factors related to hypertension and cardiovascular disease.* **Completion of this form implies your consent to answer these questions, and to keep a copy of this letter for your records.**

Please know that the information you provide is completely confidential. This information is identified by a code number and your responses to these questions will remain anonymous. Thank you for helping us with our research.

If you have questions about your rights as a participant, please contact the Office for Research Protections in reference to IRB #19000 at (814) 865-1775.

Sincerely,

_____

Laura C. Klein, Ph. D.
Assistant Professor and Investigator
The Biobehavioral Health Studies Laboratory
Phone: (814) 865-8813
Email: lcklein@psu.edu

Isabella Rodrigues, B.A, B.S.
Graduate Researcher and Investigator
The Biobehavioral Health Studies Laboratory
Phone: (814) 863-5845
Email: imr104@psu.edu

#2

Appendix B: IL-1 β ELISA Protocol

IL-1β ELISA Protocol

Capture Ab

1. Reconstitute mouse anti-human IL-1β mAb (R&D Systems #MAB601) to 500 μg/mL as per R&D's instructions and store in 40 μL aliquots at -70 °C.; <u>for the assay</u>, dilute each aliquot to 4 μg/mL in 5.0 mL of Ngai's coating buffer. Add 50 μL to each well of the ELISA plate.
2. Seal plate, place inside a humidified container, and incubate overnight at 4 °C.

3. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Blocking

4. Block plate with 200 μL per well of a 1:4 Block Ace solution.
5. Seal plate, place inside a humidified container, and incubate for at least 2 hours at room temperature.

6. Wash plate 3x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Standards and Samples

7. Prepare standards (R&D Systems #201-LB) as per manufacturer's instructions, ranging from 8,000 to 7.8 pg/mL (1:1 dilution series), by diluting the 40 μL aliquot in 460 μL of a matrix similar to the samples or in a 1:10 Block Ace solution.
8. Add samples, standards, and blanks at 100 μL per well.
9. Seal plate, place inside a humidified container, and incubate overnight at 4 °C.

10. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Detection Ab

11. Reconstitute the biotinylated affinity purified goat anti-human IL-1β pAb (R&D Systems #BAF201) to 50 μg/mL in Tris-buffered saline as per R&D instructions and store in 40 μL aliquots at −70 °C; <u>for the assay</u>, dilute each aliquot to 0.2 μg/mL in 10.0 mL of a 1:10 Block Ace solution and apply 100 μL to each well of the plate.
12. Seal plate, place inside a humidified container, and incubate for 2 hours at room temperature.

13. Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Streptavidin Peroxidase

14. Dilute streptavidin peroxidase to 1 μg/mL in PBST (10 μL in 10 mL); add 100 μL per well.
15. Seal plate, place inside a humidified container, and incubate for 30 minutes at room temperature.

16.   Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Substrate

17.   Add 100 μL per well of the ABTS/peroxide substrate solution (see solutions).

18.   Incubate the plate for 60 – 90 minutes at room temperature in the dark, unsealed.

Read

19.   Set the microtiter plate reader to read at a wavelength of 405 nm and read the plate 60 to 90 minutes after substrate addition.

SOLUTIONS:

PBS: 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl; q.s. to 1 L with ddH_2O, pH 7.0

COATING BUFFER: (Ngai): 15 mM Na_2CO_3 (0.0318 g/20mL ddH_2O), 34.8 mM $NaHCO_3$ (0.0585 g/20 mL ddH_2O), ph 9.6

WASH BUFFER: 0.5 mL TWEEN-20 in 1L PBS

BLOCKING BUFFER: 1:4 dilution of the stock Block Ace solution

SUBSTRATE: Add 150 mg 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma) to 500 mL of 0.1 M citric acid (21 g per 1 L ddH_2O, Sigma monohydrate citric acid) pH to 4.35 with NaOH. Aliquot into 10 mL per vial and store at -20^oC to -70^oC. <u>For the assay,</u> thaw an aliquot of ABTS substrate about 15 minutes before use. Just before use add 10 μL of 30% H_2O_2 per 10 mL of substrate and vortex.

Appendix C: TNF-α ELISA Protocol

TNF-α ELISA Protocol

Capture Ab

1. Reconstitute mouse anti-human TNF-α mAb (R&D Systems #MAB610) to 500 μg/mL as per R&D's instructions and store in 40 μL aliquots at -70 °C.; <u>for the assay</u>, dilute each aliquot to 4 μg/mL in 5.0 mL of coating buffer. Add 50 μL to each well of the ELISA plate.
2. Seal plate, place inside a humidified container, and incubate overnight at 4 °C.

3. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Blocking

4. Block plate with 200 μL per well of a 1:4 Block Ace solution.
5. Seal plate, place inside a humidified container, and incubate for at least 2 hours at room temperature.

6. Wash plate 3x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Standards and Samples

7. Prepare standards (R&D rh TNF-α, #210-TA), ranging from 10,000 to 9.8 pg/mL (1:1 dilution series), by diluting the 50 μL aliquot in 450 μL of a matrix similar to the samples or in a 1:10 Block Ace solution. Stock standards are 50μL of 100 ng/mL, so add 450 μL of a 1:10 Block Ace solution to the vial to get the 10 ng/mL first standard.
8. Add samples, standards, and blanks at 100 μL per well.
9. Seal plate, place inside a humidified container, and incubate overnight at 4 °C.

10. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Detection Ab

11. Reconstitute the biotinylated affinity purified goat anti-human TNF-α pAb (R&D Systems #BAF210) to 50 μg/mL in Tris-buffered saline as per R&D instructions and store in 20 μL aliquots at −70 °C; <u>for the assay</u>, dilute each aliquot to 0.1 μg/mL in 10.0 mL of a 1:10 Block Ace solution and apply 100 μL to each well of the plate.
12. Seal plate, place inside a humidified container, and incubate for 2 hours at room temperature.

13. Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Streptavidin Peroxidase

14. Dilute streptavidin peroxidase to 1 μg/mL in PBS (10 μL in 10 mL); add 100 μL per well.

15. Seal plate, place inside a humidified container, and incubate for 30 minutes at room
    temperature.

16. Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove
    any excess wash solution.

Substrate

17. Add 100 µL per well of the ABTS/peroxide substrate solution (see solutions).
18. Incubate the plate for 60 – 90 minutes at room temperature in the dark, unsealed.

Read

19. Set the microtiter plate reader to read at a wavelength of 405 nm and read the
    plate 60 to 90 minutes after substrate addition.

SOLUTIONS:

PBS: 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl; q.s. to 1 L with ddH_2O, pH
7.0

COATING BUFFER: (Ngai): 15 mM Na_2CO_3 (0.0318 g/20mL ddH_2O), 34.8 mM
$NaHCO_3$ (0.0585 g/20 mL ddH_2O), ph 9.6

WASH BUFFER: 0.5 mL TWEEN-20 in 1L PBS

BLOCKING BUFFER: a 1:4 dilution of the stock Block Ace solution

SUBSTRATE: Add 150 mg 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid,
Sigma) to 500 mL of 0.1 M citric acid (21 g per 1 L ddH_2O, Sigma monohydrate
citric acid) pH to 4.35 with NaOH. Aliquot into 10 mL per vial and store at -20^oC
to -70^oC. <u>For the assay</u>, thaw an aliquot of ABTS substrate about 15 minutes
before use. Just before use add 10 µL of 30% H_2O_2 per 10 mL of substrate and
vortex.

Appendix D: IL-4 ELISA Protocol

IL-4 ELISA Protocol

Capture Ab

1. Reconstitute mouse anti-human IL-4 mAb (R&D Systems #MAB604) to 500 µg/mL as per R&D's instructions and store in 40 µL aliquots at -70 $^{\circ}$C.; <u>for the assay</u>, dilute each aliquot to 4 µg/mL in 5.0 mL of coating buffer. Add 50 µL to each well of the ELISA plate.

2. Seal plate, place inside a humidified container, and incubate overnight at 4 $^{\circ}$C.

3. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Blocking

4. Block plate with 200 µL per well of a 1:4 Block Ace solution.

5. Seal plate, place inside a humidified container, and incubate for at least 2 hours at room temperature.

6. Wash plate 3x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Standards and Samples

7. Prepare standards (rh IL-4, R&D #204-IL), ranging from 10,000 to 9.8 pg/mL (1:1 dilution series), by diluting the 40 µL aliquot in 460 µL of a matrix similar to the samples or in a 1:10 Block Ace solution.

8. Add samples, standards, and blanks at 100 µL per well.

9. Seal plate, place inside a humidified container, and incubate overnight at 4 $^{\circ}$C.

10. Wash plate 4x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Detection Ab

11. Reconstitute the biotinylated affinity purified goat anti-human IL-4 pAb (R&D Systems #BAF204) to 50 µg/mL in Tris-buffered saline as per R&D instructions and store in 40 µL aliquots at −70 $^{\circ}$C; <u>for the assay</u>, dilute each aliquot to 0.2 µg/mL in 10.0 mL of a 1:10 Block Ace solution and apply 100 µL to each well of the plate.

12. Seal plate, place inside a humidified container, and incubate for 2 hours at room temperature.

13. Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Streptavidin Peroxidase

14. Dilute streptavidin peroxidase to 1 µg/mL in PBST (10 µL in 10 mL); add 100 µL per well.

15. Seal plate, place inside a humidified container, and incubate for 30 minutes at room temperature.

16.	Wash plate 6x1 min. with PBST, pH 7.0, and tap plate on paper towels to remove any excess wash solution.

Substrate

17.	Add 100 μL per well of the ABTS/peroxide substrate solution (see solutions).

18.	Incubate the plate for 60 – 90 minutes at room temperature in the dark, unsealed.

Read

19.	Set the microtiter plate reader to read at a wavelength of 405 nm and read the plate 60 to 90 minutes after substrate addition.

SOLUTIONS:

PBS:	8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl; q.s. to 1 L with ddH_2O, pH 7.0

COATING BUFFER: (Ngai): 15 mM Na_2CO_3 (0.0318 g/20mL ddH_2O), 34.8 mM $NaHCO_3$ (0.0585 g/20 mL ddH_2O), ph 9.6

WASH BUFFER: 0.5 mL TWEEN-20 in 1L PBS

BLOCKING BUFFER: a 1:4 dilution of the stock Block Ace solution

SUBSTRATE: Add 150 mg 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma) to 500 mL of 0.1 M citric acid (21 g per 1 L ddH_2O, Sigma monohydrate citric acid) pH to 4.35 with NaOH. Aliquot into 10 mL per vial and store at -20^oC to -70^oC. <u>For the assay</u>, thaw an aliquot of ABTS substrate about 15 minutes before use. Just before use add 10 μL of 30% H_2O_2 per 10 mL of substrate and vortex.

Appendix E: IL-10 ELI Protocol

New Wash Buffer™

assay designs
SIMPLIFY YOUR SCIENCE®

TiterZyme ®EIA

human IL-10

Enzyme Immunometric Assay Kit

Catalog No. 900-036

96 Well Kit

Table of Contents

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Description

Assay Designs' human IL-10 TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determintation of human IL-10 in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human IL-10 immobilized on a microtiter plate to bind the human IL-10 in the standards or sample. A recombinant human IL-10 Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a biotinylated monoclonal antibody to human IL-10 is added. This antibody binds to the human IL-10 captured on the plate. After a short incubation the excess antibody is washed out and Streptavidin conjugated to Horseradish peroxidase is added, which binds to the biotinylated human IL-10 antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of human IL-10 in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

Interleukin-10 (IL-10) was initially identified as the 'Cytokine Synthesis Inhibitory Factor' based on the observation that mouse Th1 cytokine production was suppressed by a Th2-derived factor.[3] IL-10 is a macrophage deactivating factor, acting on macrophage-monocyte accessory cells to produce its inhibitory effects on T cells and natural killer cells. It also regulates growth and/or differentiation of B cells, mast cells, granulocytes, dendritic cells, karatinocytes and endothelial cells.[4] The ability of IL-10 to suppress Th1 activities while stimulating Th2 and humoral immune responses has been circumvented by a variety intracellular pathogens known to target macrophages. A number of parasites, bacteria, fungi and viruses depress host immune responses by either inducing host IL-10 production or encode their own IL-10 homologs.[5] Besides being a potent immunosuppressant, IL-10 is also an antipyretic and functions in mast cell homeostasis.[6,7] Circulating levels of IL-10 are increased in allergic asthma, systemic sclerosis, a variety of cancers, post-transplantation patients, and in sepsis.[8-12] The therapeutic potential of IL-10 includes rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and HIV infections.[13-17]

Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution 2 is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The human IL-10 Standard provided, Catalog No. 80-0658, should be handled with care because of the known and unknown effects of human IL-10.
5. The human IL-10 Standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **human IL-10 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0369**
   A plate using break-apart strips coated with monoclonal antibody specific to human IL-10.
2. **human IL-10 EIA Antibody, 5 mL, Catalog No. 80-0371**
   A yellow solution of biotinylated monoclonal antibody to human IL-10.
3. **Assay Buffer 13, 55 mL, Catalog No. 80-1500**
   Tris buffered saline containing proteins and detergents.
4. **human IL-10 EIA Conjugate, 5 mL, Catalog No. 80-0889**
   A blue solution of Streptavidin conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **human IL-10 Standard, 2 each, Catalog No. 80-0658**
   Two vials containing 2,000 pg each of lyophilized recombinant human IL-10.
7. **TMB Substrate, 10 mL, Catalog No. 80-0350**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
8. **Stop Solution 2, 10 mL, Catalog No. 80-0377**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **human IL-10 Assay Layout Sheet, 1 each, Catalog No. 30-0105**
10. **Plate Sealer, 3 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Standards**, are stable at 4 °C until the kit's expiration date. The Standards **must** be stored at or below -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 100 μL and 1,000 μL.
3. Repeater pipet for dispensing 100 μL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
9. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with human IL-10 samples in a wide range of matrices. Samples diluted sufficiently into the proper diluent can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Culture fluids, serum and plasma are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer 13. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of human IL-10 in the appropriate matrix.

Samples must be stored frozen to avoid loss of bioactive human IL-10. If samples are to be run within 24 hours, they may be stored at 4 °C. Otherwise, samples must be stored frozen at or below -70 °C to avoid loss of bioactive human IL-10. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37 °C incubator. Do not vortex or sharply agitate samples.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**
9. **It is important that the matrix for the standards and samples be as similar as possible. Human IL-10 samples diluted with Assay Buffer 13 should be run with a standard curve diluted in the same buffer. Serum and plasma samples and samples with a protein concentration greater than 10% should be evaluated against a standard curve run in Assay Buffer 13 while tissue culture samples should be read against a standard curve diluted in the same complete but non-conditioned media. See Reagent Preperation, step #2.**

Reagent Preparation

1. **Wash Buffer**

   Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **human IL-10 Standards**

   Reconstitute one vial of human IL-10 Standard with 2 mL standard diluent (Assay Buffer 13 or Tissue Culture Media). Mix thoroughly without foaming. Label seven 12x75 mm glass tubes #1 through #7. Pipet 500 μL of standard diluent into tubes #1 through #7. Add 500 μL of the 1,000 pg/mL Standard to tube #1. Vortex thoroughly. Add 500 μL of tube #1 to tube #2 and vortex thoroughly. Add 500 μL of tube #2 to #3 and vortex thoroughly. Continue this for tubes #4 through #7.

   The concentration of human IL-10 in tubes #1 through #7 will be 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 pg/mL, respectively. See human IL-10 Assay Layout Sheet for dilution details. STORE STANDARD AT -20 °C, avoid repeated freeze-thaws.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

2. Pipet 100 μL of standard diluent (Assay Buffer 13 or Tissue Culture Media) into the S0 (0 pg/mL standard) wells.

3. Pipet 100 μL of Standards #1 through #7 into the appropriate wells.

4. Pipet 100 μL of the Samples into the appropriate wells.

5. Tap the plate gently to mix the contents.

6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.

7. Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

8. Pipet 100 μL of yellow Antibody into each well, except the Blank.

9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.

10. Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 100 μL of blue Conjugate to each well, except the Blank.

12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.

13. Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

14. Pipet 100 μL of Substrate Solution into each well.

15. Incubate for 15 minutes at room temperature on a plate shaker at ~500 rpm.

16. Pipet 100 μL Stop Solution 2 to each well. This stops the reaction and the plate should be read immediately.

17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

Calculation of Results

Several options are available for the calculation of the concentration of human IL-10 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of human IL-10 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus human IL-10 concentration in each standard. Approximate a straight line through the points. The concentration of human IL-10 in the unknowns can be determined by interpolation.

Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	h IL-10 (pg/mL)
Blank	0.073		
S0	0.087	0.014	**0**
S1	1.978	1.905	**500**
S2	0.981	0.908	**250**
S3	0.528	0.455	**125**
S4	0.302	0.229	**62.5**
S5	0.193	0.120	**31.25**
S6	0.139	0.066	**15.62**
S7	0.103	0.030	**7.81**
Unknown 1	1.699	1.626	**430.0**
Unknown 2	0.427	0.354	**97.2**

<u>Typical Standard Curve</u>

Typical standard curves are shown below. These curves **must not** be used to calculate human IL-10 concentrations; each user must run a standard curve for each assay.

human IL-10 Conc. (pg/mL)
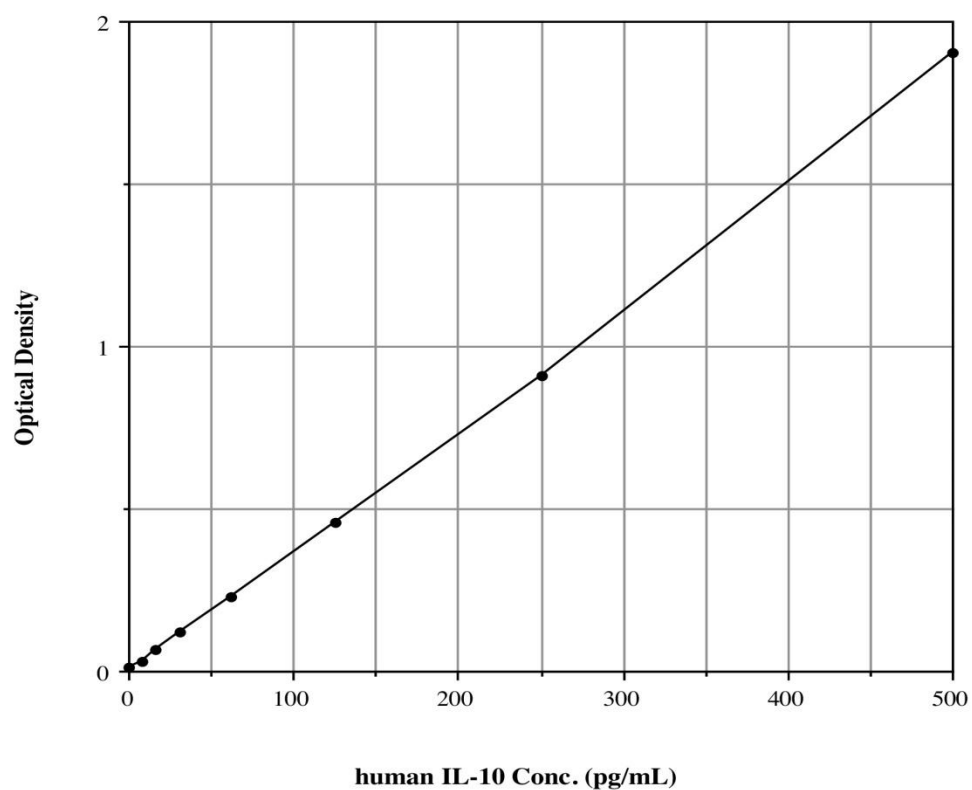
Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[18].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of human IL-10 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 =	0.009 ± 0.006 (14.61%)
Mean OD for Standard #7 =	0.034 ± 0.005 (13.21%)

Delta Optical Density (7.81 - 0 pg/mL) =   0.034 - 0.009 =   0.025

2 SD's of 0 pg/mL Standard = 2 x 0.006 =   0.012

Sensitivity = $\dfrac{0.012}{0.025}$ x   7.81 pg/mL =   **3.75 pg/mL**

Linearity

A sample containing 297.4 pg/mL human IL-10 was serially diluted 5 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual human IL-10 concentration versus measured human IL-10 concentration.

The line obtained had a slope of 1.01 with a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human IL-10 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human IL-10 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of human IL-10 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	h IL-10 (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	102.7	5.4	
Medium	194.3	3.9	
High	435.9	2.2	
Low	106.1		4.7
Medium	203.2		4.5
High	459.4		3.7

Cross Reactivities

The TiterZyme® human IL-10 EIA kit is specific for bioactive human IL-10. It is unaffected by the presence of recombinant mouse IL-10 and the following recombinant human proteins: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-11, TNF-α, TNF-β, GM-CSF and SCF.

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Human IL-10 concentrations were measured in human plasma and serum, and tissue culture media. Human IL-10 was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Human heparin Plasma	103.0	None
Human Serum	86.7	None
Tissue Culture Media	95.2	None

* See Sample Handling instructions on page 4 for details.

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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EDUCATION

The Pennsylvania State University   **University Park, PA**
    Bachelor of Science, Biobehavioral Health   May 2014
    Schreyer Honors College Scholar
    Minors: Global Health, Health Policy and Administration

RESEARCH EXPERIENCE

Klein Laboratory, Pennsylvania State University   **University Park, PA**
Research Assistant   August 2009–December 2010
- Assisted in longitudinal human studies examining various biobehavioral aspects of stress
- Assisted in a human study regarding the biobehavioral effects of psychological stress in smokers with Type 2 Diabetes
- Investigated the association between caffeine and inflammation in healthy men and women for honors thesis

Summer Translational Cardiovascular Sciences Institute   **University Park, PA**
Research Intern   Summer 2012
- Researched the relationship between caffeine, stress, and inflammation
- Attended seminars pertaining to cardiovascular disease

Tan Research Laboratory, Pennsylvania State University   **University Park, PA**
Research Assistant   January 2011–May 2011
- Developed Western Blot molecular weight marker
- Acquired skills in recombinant DNA practices

POSTERS AND PRESENTATIONS

Bennett, J. M., Bayly, J. E., & Klein, L. C. (2013). Caffeine and sex influence inflammatory responses to acute stress. Presented at the annual meeting of the American Psychosomatic Society, Miami, FL. Psychosomatic Medicine,75(3), A-146.

Bayly, J.E., Bennett, J.M., & Klein, L.C. (2013). Inflammatory responses to acute stress: Effects of caffeine and sex. Presented at The Pennsylvania State University Undergraduate Research Exhibition, University Park, PA.

HONORS AND AWARDS

- Student Marshal, Department of Biobehavioral Health, Spring 2014 Commencement
- Evan Pugh Scholar Award, Spring 2014
- Lane Family Scholarship, Fall 2012-Spring 2014
- Schreyer Honors College Academic Excellence Scholarship, 2010-2014
- Dean's List Academic Achievement, Fall 2010-Fall 2013
- President's Freshman Award, 2011