The Effect of Exercise in the Heat on Circulating Tumor Necrosis Factor-α Concentration

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Abstract

Mark Berry: The Effect of Exercise in the Heat on Circulating Tumor Necrosis Factor-\(\alpha\) Concentration
(Under the direction of Dr. Robert G. McMurray)

To examine the effect of a hot environment on the TNF-\(\alpha\) response to exercise, eight male subjects (24 ± 1.7 years of age, 1.80 ± 0.06 m, 78.3 ± 6.06 kg, 24.2 ± 1.9 mg/m2, 53.8 ± 3.7 ml/kg/min; mean ± SD) completed two, 40 min underwater cycling sessions at an intensity of 65% \(\text{VO}_2\text{peak}\). One session took place while submerged in cool water (25.5 ± 0.02°C) and the other in hot water (38.1 ± 0.06°C). TNF-\(\alpha\) concentration was determined by an enzyme-linked immunosorbent assay from blood samples obtained immediately prior to, immediately post and two-hours after each exercise session. The pre-exercise TNF-\(\alpha\) concentration for the cool and hot trials was 1.76 ± 0.94 and 1.58 ± 1.09, while the post-exercise concentration was 1.62 ± .81 and 1.54 ± 1.03, and the two hour post-exercise was 1.75 ± 0.80 and 1.63 ± 1.03, respectively. No significant differences were found between the pre, post-exercise or two hour post-exercise TNF-\(\alpha\) concentration within or between the exercise bouts (\(p > 0.05\)). No statistically significant relationship was found between change in core temperature and change in TNF-\(\alpha\) concentration (\(r = .366; p > 0.05\)). The results of the present study suggest that forty minutes of moderate-intensity exercise in a cool or hot environment does not elevate TNF-\(\alpha\) concentration immediately post or two hours post-exercise.
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Chapter I

Basis for Study

Researchers have found that exercise in the heat can be regarded as a classic physical stress, eliciting immunological responses that also occur in reaction to other stressors such as sepsis, surgery, or burns (Rhind et al. 2004). These immunological responses include increases in neutrophil concentration, increases in the recruitment of T-cells and natural killer cells and a rise in cytokine production (Pederson 2000, Pederson & Toft 2000). One cytokine that may increase in response to both exercise in the heat and other physiological stress is tumor necrosis factor-alpha (TNF-α).

TNF-α is a cytokine primarily produced by activated mononuclear phagocytes in response to infection, but can also be produced by T-lymphocytes, neural cells, Kupffer cells and endothelial cells (Abbas et al. 1994, Moldoveanu et al. 2001). Defined as a pro-inflammatory cytokine, TNF-α is principally involved with the recruitment of neutrophils and monocytes to areas of infection in an attempt to increase apoptosis and eliminate infectious bacteria (Abbas et al. 1994). TNF-α can also stimulate a cascade of inflammatory reactions involving the expression of other cytokines (Bemelmans et al. 1996). When significant amounts of TNF-α reach the systemic circulation it can take on an endocrine like role and stimulate increases in core body temperature (Abbas et al. 1994).

The evidence related to the TNF-α response to exercise is equivocal; with several studies reporting no change in TNF-α following exercise and other studies reporting
increased circulating TNF-α levels (Ullum et al. 1994, Rivier et al. 1994). The proposed mechanisms of an exercise induced TNF-α response have not been clearly defined, but multiple factors may be involved. These include, increased cell debris release from injured muscles, increased endotoxin release from the gut, and increases in the number of circulating leukocytes during exercise (Brenner et al. 1999). Changes in the mode, intensity and duration of exercise can increase the stress placed on the body during exercise and may lead to alterations in the mechanisms involved with TNF-α release during to exercise (Rhind et al. 2004). Environmental conditions may also affect the mechanisms involved in the TNF-α response to exercise (Rhind et al. 2004). Therefore, all of these factors must be considered when examining the degree to which exercise influences TNF-α concentration (Moldoveanu et al. 2001).

Passive exposure to heat without exercise is another form of physiological stress that has been shown to affect the circulating TNF-α concentration (Walsh & Whitman 2006). Research has shown that exposure to sixty minutes of high ambient temperatures (41.8°C) caused an average increase of 600% in circulating TNF-α concentration (Robbins et al. 1995). Furthermore, evidence suggests that passive exposure to heat can stimulate peripheral monocytes, cause the leakage of endotoxin from the intestine, and may augment endotoxin induced TNF-α release; all of which have been proposed as mechanisms leading to increased circulating TNF-α concentrations (Walsh & Whitman 2006, Bouchama & Knochel 2002, Zeller et al. 2000). The determining factors related to the TNF-α response to heat include, the temperature and length of exposure, and the associated increases in core body temperature; with warmer temperatures and longer exposures leading to larger increases in
core body temperature and a greater rise in circulating TNF-α concentration (Walsh & Whitman 2006).

In addition to their role as individual stressors, recent research suggests exercise and heat exposure combined may work to synergistically affect the mechanisms involved with control of the TNF-α response (Walsh & Whitman 2006, Bouchama & Knochel 2002). When compared to exercise in a cool environment, exercise in the heat leads to greater endotoxin release from the gut and an increased leukocyte response, both of which are involved in the mediation of the TNF-α response (Walsh & Whitman 2006, Bouchama & Knochel 2002). Although research has demonstrated the amplification of the mechanisms related to the controls of the TNF-α response, attempts to verify this model by examining the direct effect of exercise in the heat on circulating TNF-α concentration have been limited (Walsh & Whitman 2006).

Research regarding the TNF-α response to exercise in the heat have produced varying results (Rhind et al. 2004, Starkie et al. 2005, Peake et al. 2007). Some research has shown post exercise TNF-α concentration to be significantly higher (200%) than resting values, while other studies have shown much smaller increases in circulating TNF-α concentration (17%) following exercise in the heat (Starkie et al. 2005, Peake et al. 2007). The root of these divergent results may be related to differences in the methodologies used, including variations in the temperature of exposure, and discrepancies in the mode, intensity and duration of exercise. Thus, whether the changes in circulating TNF-α concentration observed during exercise in the heat are simply related to the rise in core temperature or whether exercise itself intensifies the immune response for a given amount of body heating is still unclear (Shephard 1998).
Purpose

The purpose of this study was to determine whether exercising in the heat affects the magnitude of change in circulating TNF-α concentration following a single forty-minute bout of exercise. This was investigated by comparing the circulating levels of TNF-α following exercise in a cool environment to the levels of TNF-α following exercise in the heat.

Research Hypotheses

1. Exercise in the heat will cause an increase in circulating concentrations of TNF-α immediately and two-hours post-exercise when compared to resting levels.

2. Exercise in the heat will elicit greater changes in circulating TNF-α concentration immediately and two-hours post-exercise when compared to exercise in a cool environment.

3. A significant positive relationship will exist between changes in TNF-α concentration and the changes in core temperature.

Definition of Terms

1. **Cytokine**: Proteins produced by many different cell types that mediate inflammatory and immune reactions. Cytokines are the principle mediators of communication between cells in the immune system (Abbas et al. 1994).

2. **Tumor Necrosis Factor-alpha (TNF-α)**: A cytokine produced primarily by activated mononuclear phagocytes that functions to stimulate the recruitment of neutrophils and monocytes to sites of infections (Abbas et al. 2004).

3. **Endotoxin**: A component of cell wall gram negative bacteria that is released from dying bacteria and stimulates many innate immune responses (Abbas et al. 2004).
4. **Phagocyte:** A cell, such as a white blood cell, that engulfs and absorbs waste material, harmful microorganisms, or other foreign bodies in the bloodstream and tissues (Abbas et al. 2004).

5. **Neutrophil:** The most abundant circulating white blood cell that is recruited to inflammatory sites and is capable of phagocytosing and digesting microbes (Abbas et al. 2004).

6. **Monocyte:** A type of bone marrow-derived circulating blood cell that is the precursor of tissue macrophages (Abbas et al. 2004).

7. **T Cell:** A type of lymphocyte that controls the cell mediated immune response in the adaptive immune system (Abbas et al. 2004).

8. **Natural Killer (NK) Cells:** A subset of bone-marrow derived lymphocytes that function in innate immune responses to kill microbe-infected cells by direct lytic mechanisms (Abbas et al. 2004).

**Assumptions**

1. Subjects abstained from additional exercise within the 24 hours prior to each exercise session.

2. Subjects had no prior history of any heat related illnesses.

3. Subjects were free of infection for the three weeks preceding participation within this study.

4. Subjects were not on anti-inflammatory medications during their participation within the study.

5. Forty-minutes of exercise was sufficient to increase circulating TNF-α concentration.

6. Exercise in 25°C water resulted in a smaller elevation in core temperature than the exercise in the 39°C water.
Delimitations

1. Male subjects between the ages of 18 and 40 were recruited from the University of North Carolina at Chapel Hill.

2. Subjects were healthy and physically active, participating in at least thirty minutes of exercise at a minimum of four times per week.

3. Subjects were exposed to 39°C water during the experimental trial and 25°C water for the control trial.

4. Subjects reported for each exercise session after fasting for four hours and having abstained from exercise during the preceding 24 hours.

Limitations

1. Results can be generalized only to healthy, physically active adult males between 18 and 40 years of age.

2. Although subjects were assumed to be free of infection during the study, and to have been so for three weeks prior to the study, newly developed infections may have been unknown to the subjects and could have resulted in the disruption of the baseline TNF-α concentration.

3. Results can be generalized to a hot trial exposure of 39°C only.

Significance of Study

Physical activity in high temperatures is a common practice for individuals involved with outdoor exercise training, as well as those whose professional obligations require activity in warmer environments (firefighters, military personnel, construction workers, etc.). In warm environments physical activity can be associated with multiple immunological changes, including alterations in the circulating levels of the cytokine TNF-α (Rhind el al. 2004, Starkie et al. 2005). TNF-α is a pro-inflammatory cytokine which is expressed in response to
infection and physiological stressors such as exercise and passive heat exposure (Ostrowski et al. 1999, Walsh & Whitman 2006). Although TNF-α is a vital component of the immune response, high concentrations can lead to pathological states such as sepsis and shock (Rhind et al. 2006, Abbas et al. 1994). Increased circulating concentrations of TNF-α may also cause changes in the hypothalamic thermoregulatory set point, causing an increase in core body temperature, and have been associated with an increased risk of developing heat related illnesses (Walsh & Whitman 2006). Because high levels of circulating TNF-α may have major health consequences, it is important to clearly understand the TNF-α response to exercise in the heat. Unfortunately, previous studies related to this topic have produced conflicting results, and the TNF-α response to exercise in the heat is not clearly defined (Rhind et al. 2004, Starkie et al. 2005, Peake et al. 2007).

Some evidence suggests that exercise in the heat may lead to larger increases in circulating TNF-α concentration when compared to exercise in a cool environment, but results have varied. For example, researchers reported large increases in TNF-α concentration (90% and 200%) immediately after exercise in a warm environment, while others have indicated smaller TNF-α increases (17%) (Rhind et al. 2004, Starkie et al. 2005, Peake et al. 2007). Because there have been discrepancies in previous reports, this study was designed to investigate the effect of exercise in the heat on circulating TNF-α levels. This study will help clarify the role of environment temperature on the TNF-α response to exercise. Collectively, the results of this study may help to determine appropriate environmental conditions for which it is safe for physical activity to occur without negatively affecting the immune system.
Chapter II
Review of Literature

Tumor necrosis factor-α (TNF-α) is classified as a pro-inflammatory cytokine and is principally involved with increasing the recruitment of neutrophils and monocytes to areas of infection (Abbas et al. 1994, Bemelmans et al. 1996). TNF-α can be produced by activated mononuclear phagocytes, T-lymphocytes, neural cells, Kupffer cells and endothelial cells (Abbas et al. 1994, Moldoveanu et al. 2001). In addition to the heightened concentration associated with infection, increases in TNF-α production are also be seen in response to physiological stressors such as sepsis, surgery, burns, passive heat exposure and exercise.

Recently there has been an increase in the amount of literature concerning the combined effect of heat exposure and exercise on the TNF-α response (Rhind et al. 2004, Starkie et al. 2005, Peake et al. 2007). However, conflicting results have been found in many of the studies, making the direction and magnitude of the TNF-α response difficult to understand. The inconsistencies in the literature may stem from discrepancies in the methodology used, including variations in environmental conditions and differences in the mode, intensity and duration of exercise. This review of literature will focus on how environmental conditions may be a key factor within exercise bouts which influence the TNF-α response. The first section will provide the basic information concerning cytokines, with the review focusing on TNF-α. Subsequent sections will highlight the TNF-α response...
to exercise, the TNF-α response to passive heat exposure, and lastly, the TNF-α response to exercise in the heat.

**Cytokines**

Cytokines are one of the key mediators of the immune response, and can be produced not only from activated macrophages, but also from many other cells within the body (Abbas et al. 1994). Produced during the effector phase of natural and specific immunity, cytokines serve to regulate the intensity and length of the immune response by inhibiting or stimulating many of the cells involved within immunity (Abbas et al. 1994). In general, cytokines are water soluble proteins and glycoproteins, with a mass between 8 and 30 kDa (Abbas et al. 1994). Depending on their role within the immune systems, cytokines are classified as either pro- or anti-inflammatory (Abbas et al. 1994). Pro-inflammatory cytokines increase the recruitment and activation of leukocytes, enhancing inflammation, while anti-inflammatory cytokines hinder inflammatory responses by inhibiting both the production and the actions of the pro-inflammatory cytokines (Abbas et al. 1994). Overall, it is the cytokines ability to act in both an autocrine and paracrine like fashion and their pleiotopic qualities which allow them to have a profound overall effect on the immune response. One such cytokine with many biological actions that can have a profound effect on the immune response is TNF-α. Table 1 presents a selection of various cytokines and a summary of their roles.
Table 1. Selected cytokines and their biological effects (adapted from Abbas et al. 1994).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Principle Source</th>
<th>Target Cell</th>
<th>Biological Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin (IL)-1</td>
<td>Macrophages, endothelial cells, epithelial cells</td>
<td>Endothelial cells</td>
<td>Activation</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td></td>
<td>Fever</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td></td>
<td>Synthesis of acute phase proteins</td>
</tr>
<tr>
<td>Interleukin (IL)-2</td>
<td>T cells</td>
<td>T cells</td>
<td>Proliferation, increased cytokine synthesis, apoptosis</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td></td>
<td>Proliferation, activation</td>
</tr>
<tr>
<td></td>
<td>B cells</td>
<td></td>
<td>Proliferation, anti-body synthesis</td>
</tr>
<tr>
<td>Interleukin (IL)-4</td>
<td>CD4⁺ T cells, mast cells</td>
<td>B cells</td>
<td>Isotype switching to immunoglobulin E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cells</td>
<td>Cell differentiation, proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mast cells</td>
<td>Proliferation</td>
</tr>
<tr>
<td>Interleukin (IL)-5</td>
<td>CD4⁺ T cells</td>
<td>Eosinophils</td>
<td>Activation, increased production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cells</td>
<td>Proliferation</td>
</tr>
<tr>
<td>Interleukin (IL)-6</td>
<td>Monocytes, macrophages, endothelial cells, T cells</td>
<td>Liver</td>
<td>Synthesis of acute phase proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cells</td>
<td>Proliferation of anti-body producing cells</td>
</tr>
<tr>
<td>Interleukin (IL)-10</td>
<td>Macrophages, T cells</td>
<td>Macrophages</td>
<td>Inhibition of IL-12 production, expression of co-stimulators and class II MHC molecules</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cells</td>
<td>Proliferation</td>
</tr>
<tr>
<td>Interleukin (IL)-12</td>
<td>Macrophages, dendritic cells</td>
<td>NK Cells</td>
<td>IFN-γ synthesis, increased cytolytic activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cells</td>
<td>Cell differentiation</td>
</tr>
<tr>
<td>Interferon (IFN)-γ</td>
<td>T cells, NK cells</td>
<td>Macrophages</td>
<td>Activation (increased microbial functions)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelial cells</td>
<td>Activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Various cells</td>
<td>Increased expression of class I and II MHC molecules, increased antigen processing and presentation to T-cells</td>
</tr>
</tbody>
</table>

Abbreviations:
MHC: Major histocompatibility complex
NK cells: Natural killer cells
T cells: T lymphocytes
B cells: B lymphocytes
TNF-α

TNF-α is a pro-inflammatory cytokine produced by mononuclear phagocytes, T-lymphocytes, neural cells, Kupffer cells and endothelial cells (Abbas et al. 1994). Initially discovered during the 19th century, TNF-α has a large range of effects on a variety of cell types (Bemelmans et al. 1996). The overall effects of TNF-α may be beneficial or detrimental depending upon its concentration, duration of cell exposure and the presence of other immune system mediators that may act in concert with TNF-α (Camussi et al. 1991).

One way in which TNF-α benefits the immune system is through its ability to increase the recruitment of neutrophils and monocytes to areas of infection (Abbas et al. 1994). Immune cell recruitment increases in an attempt to eliminate infectious bacteria and can be brought on via multiple pathways (Abbas et al. 1994). TNF-α is able to increase immune cell recruitment via its effect on endothelial cells, as TNF-α stimulation causes endothelial cells to express adhesion molecules (Abbas et al. 1994). Enhanced expression of adhesion molecules from the endothelial cells permits neutrophils, monocytes and lymphocytes to bind to the endothelial surface with greater efficiency (Abbas et al. 1994, Camussi et al. 1991). A second pathway through which TNF-α is able to influence cell recruitment is through the stimulation of both endothelial cells and macrophages (Abbas et al. 1994). TNF-α stimulation of endothelial cells and macrophages enhances the secretion of chemokines that work to increase the total leukocyte recruitment to areas of infection (Abbas et al. 1994). A third way in which TNF-α can increase immune cell recruitment to areas of infection involves its role of in production of additional cytokines (Abbas et al. 1994). TNF-α increases the production of other cytokines, for example Interleukin-1, with functions similar to its own. An increased production of similar cytokines allows the immune
processes that TNF-α itself is involved with to be amplified (Abbas et al. 1994). By increasing the production of cytokines that also influence immune cell recruitment, TNF-α is able to further influence immune cell recruitment. The overall ability of TNF-α to increase recruitment of neutrophils and monocytes via the multiple pathways is an advantageous and vital immune response (Abbas et al. 1994). The importance of recruitment by TNF-α is supported by the finding that inadequate amounts of the cytokine have been associated with a diminished ability to contain infections (Abbas et al. 1994).

TNF-α is also involved with the coordination of tissue remodeling, as research has shown that TNF-α influences both cell growth and cell breakdown (Vicek et al. 1986, Bertolini et al. 1986, Saklatvala 1986). TNF-α is capable of stimulating fibroblast production, a cell type directly utilized within tissue synthesis and healing, and mesenchymal cell production, a stem cell involved with tissue synthesis that can give rise to a variety of other cell types (Vicek et al. 1986). TNF-α is also able to affect cell growth by increasing the production of other cytokines that are involved with increased cell proliferation (Vicek et al. 1986). In contrast to cell growth, evidence has also suggested that TNF-α can be involved in the increased breakdown in tissues such as of both bone and cartilage (Bertolini et al. 1986, Saklatvala 1986). The proposed mechanism through which bone breakdown is increased is related to the capacity of TNF-α to inhibit collagen synthesis, and decrease the amount of alkaline phosphate, which is an enzymatic marker of new bone formation (Bertolini et al. 1986). Cartilage growth is affected through the inhibition of proteoglycan synthesis, which is the component of cartilage tissue that allows for the resistance of compression during load bearing (Saklatvala 1986). The ability of TNF-α to stimulate both tissue growth and
breakdown provides evidence that it may be an important factor in the regulation of tissue remodeling.

A negative function of TNF-α relates to its involvement in the progression of heat illnesses (Walsh & Whitman 2006). TNF-α raises prostaglandin production within the hypothalamus, which increases the thermoregulatory set point (Abbas et al. 1994, Walsh & Whitman 2006). As the set point increases, the core body temperature at which sweating occurs rises, diminishing the body’s ability to dissipate heat effectively (Walsh & Whitman 2006). TNF-α may also increase vascular permeability, which can amplify plasma volume losses (Lim & Mackinnon 2006). Losses in plasma volume may decrease the amount of blood volume that can be sent to the skin, and this may further compromise the ability of the body to efficiently release heat (Lim & Mackinnon 2006). As the body loses the ability to dissipate heat effectively, core temperature rises rapidly and the risk of developing heat related illnesses is increased. Past research has suggested that a strong relationship exists between circulating TNF-α concentration and the severity of heat stroke, emphasizing the role that TNF-α may have in the development of heat related illnesses (Lim & Mackinnon 2006).

TNF-α also acts on hepatocytes within the liver to increase the production of acute phase plasma proteins (Bauman & Gauldie 1994). The acute phase response is an immediate set of reactions that are induced in an effort to prevent ongoing tissue damage, to destroy infected organisms and to activate repair processes (Bauman & Gauldie 1994). It has been found that TNF-α can enhance the expression of α-acid glycoprotein, serum amyloid A, C-reactive protein, complement component C3 and haptoglobin, all of which are major components of the acute phase response (Bauman & Gauldie 1994). The entire acute phase
reactive process is also known as inflammation. Therefore, it can be inferred that through its involvement in hepatic acute phase protein expression, TNF-α can lead to the progression of the body’s inflammatory response.

Increases in the circulating concentration of TNF-α also affect the coagulation properties within the endothelium of blood vessels (Abbas et al. 1994). TNF-α stimulates the expression of tissue factor, a potent activator of coagulation, and inhibits the production of thrombomodulin, an inhibitor of coagulation (Abbas et al. 1994). These changes in coagulation can lead to the development of an intravascular thrombus, causing a decrease in blood flow to the area in which the thrombus has formed (Abbas et al. 1994). The capacity of TNF-α to affect coagulation properties is what actually allows TNF-α to cause the necrosis of tumor cells, as changes in coagulation lead to decreases in blood flow to the tumor and ultimately cause tumor cell death (Abbas et al. 1994).

Prolonged high levels of TNF-α production may provoke cachexia, a state characterized by the wasting of muscle and fat cells (Abbas et al. 1994). It has been found that adipocytes and skeletal myocytes incubated with TNF-α increase both lipolysis and glycogenolysis processes, initiating a catabolic environment (Torti et al. 1985, Lee et al. 1987). Evidence has also suggested that high concentrations of TNF-α can increase the rate of whole body energy expenditure, protein turnover, and cause losses in body mass similar to metabolic alterations seen in association with chronic infections and critical illnesses (Tracey et al. 1988).

The systemic release of TNF-α is also involved in the progression of symptoms related to endotoxic shock (Camussi et al. 1991). Large sustained increases in TNF-α concentration are associated with the inhibition myocardial contractility and the loss of
vascular smooth muscle tone (Camussi et al. 1991). These cardiovascular changes can cause substantial decreases in blood pressure and further the development of shock (Camussi et al. 1991).

Although TNF-α is a vital component of a healthy immune response, evidence has shown that increases in the circulating concentration can also lead to a variety of health problems (Abbas et al. 1994). Examples of pathological states associated with an increased concentration of TNF-α include sepsis and shock (Rhind et al. 2004, Abbas et al. 1994). The collective evidence supporting the concept that TNF-α can contribute to health related issues may be of clinical importance in situations in which large increases in TNF-α are seen.

**TNF-α Response to Exercise**

Exercise is a form of physiological stress that can influence TNF-α concentration (Moldoveanu et al. 2001, Ostrowski et al. 1999). The literature related to the TNF-α response to acute and chronic exercise will be reviewed separately, as the two forms of exercise can affect the circulating concentration of TNF-α in different ways.

The evidence concerning the TNF-α response to acute exercise is equivocal. In those studies that have shown significant results the direction and magnitude of the change in TNF-α may be influenced by the mode, intensity and duration of the exercise (Rivier et al. 2007, Febbraio et al. 2003). Ostrowski et al. (1999) examined the effect of a marathon run (26.2 miles) on the circulating TNF-α concentration in ten healthy male subjects. During the marathon run the subjects were exercising at an intensity of 60-80% of their VO₂max (Sjodin & Svedenhag 1985). TNF-α measurements were made on blood samples taken one week prior to the race (baseline measurement), immediately after the race, and every 30 minutes during the four hour period directly following the marathon. The TNF-α concentration
peaked immediately after exercise (a two fold increase from the baseline measurement) then slowly decreased during the four hours after exercise. The circulating concentration of TNF-α stayed significantly elevated over the baseline concentration for three full hours after the marathon had been completed.

Moldoveanu et al. (2000) evaluated the TNF-α response to three hours of exercise at 60-65% of VO_2peak in ten healthy male subjects. Subjects exercised for one hour on a cycle, then performed one hour of treadmill running, and finished by returning back to the cycle for one hour. When compared to resting baseline measurements, circulating TNF-α was significantly elevated by 20% and 90% at 60 and 180 minutes of exercise, respectively. At the one hour mark of recovery the TNF-α concentration began to decrease from the peak values observed, but remained elevated over resting values for 24 hours after the exercise was concluded.

The effect of three different modes of exercise on the circulating TNF-α concentration was assessed by Brenner et al (1999). The exercise modes consisted of high intensity cycling (average intensity of 90% VO_2max) for five minutes, a standard circuit training routine (five different types of weight lifting exercises), and cycling at 60-65% of VO_2max for two hours. The only exercise mode to cause a significant change in the circulating TNF-α concentration was the continuous cycling, which saw an increase (~50%) in TNF-α concentration immediately post exercise when compared to baseline values.

Vassilakopoulos et al. (1998) studied the effect of antioxidant supplementation on the TNF-α response to 45 minutes of exercise at 70% VO_2max. Ten healthy male subjects performed two exercise sessions, one prior to 60 days of antioxidant supplementation (vitamins A, E and C for 60 days, allopurinol for 15 days, and N-acetylcysteine for 3 days)
and one directly after supplementation. Blood samples were collected at rest, immediately after exercise, and at 30 and 120 minutes into recovery. The TNF-α concentration was significantly elevated (60%) over resting values immediately after exercise in the non-supplementation trial, but not the supplementation trial. As the TNF-α response to exercise was nearly abolished after antioxidant supplementation; these results suggest that oxidative stress may have a role in the regulation of TNF-α expression during an acute bout of exercise.

Although the results of several studies suggest that the circulating concentration of TNF-α increases in response to acute exercise, a number of studies have also found no change in TNF-α concentration. Febbraio et al. (2003) investigated the effect of cycling at an intensity of 60% of VO$_2$peak for 25 minutes on TNF-α concentration in both healthy subjects and patients with type II diabetes. Blood samples were taken at rest and immediately before the cessation of cycling. No change in circulating TNF-α concentration was found following exercise in either the healthy subject or the type II diabetes patients. The lack of a significant TNF-α response may be due to the short duration of exercise that was used within this study, as the studies with significant findings employed longer exercise bouts. The 25 minutes of exercise utilized within this study may not have placed enough stress on the body to substantially affect the TNF-α response.

Likewise, Rivier et al. (1994) found no significant increase in TNF-α concentration in response to acute exercise. Sixteen healthy male subjects completed incremental exercise tests, and had blood drawn at rest, immediately post exercise, as well as 20 minutes into recovery. While it was found that TNF-α increased slightly in response to exercise, the change was not significant when compared to resting levels. All of the studies with
significant findings used continuous exercise protocols, while this study employed an incremental test. The differences in methodology may explain why exercise had no significant effect on the TNF-α response within this study.

Ullum et al. (1994) failed to detect a significant increase in the circulating TNF-α concentration in response to one hour of cycling at 75% VO$_2$max. Blood samples were taken from seventeen healthy male subjects at rest, during the last minutes of exercise, and two and four-hours post exercise. No increase in the circulating concentration of TNF-α was observed at any point during or post exercise. The author suggested that the lack of a significant finding could be due to the sensitivity of assay used, as the TNF-α concentrations were below detectable in many of the subject before the exercise began.

In summary, the collective literature concerning the TNF-α response to acute exercise has produced varying results. There have been multiple studies suggesting either increases or no change in the circulating TNF-α following acute exercise and during recovery (Ostrowski et al. 1999, Moldoveanu et al. 2000, Rivier et al. 1994, Ullum et al. 1994). As large variations in the in the mode, intensity, and duration of the exercise are present within these studies, these factors may greatly affect the TNF-α response to acute exercise. It may be the case that a certain intensity or duration threshold must be met before any significant change in the circulating TNF-α concentration can occur in response to acute exercise. In general, the studies which involved exercise intensities greater than 65% or a duration longer than one hour saw significant increases in the TNF-α concentration, while the studies that used shorter and less intense exercise bouts saw no significant change.

Similar to discrepancies reported within the results related to acute exercise, the effect of chronic exercise on the TNF-α response is not clearly defined. Although it has been
hypothesized that regular exercise may suppress resting TNF-α concentration, the results of the existing literature differ as to the effectiveness of the TNF-α suppression (Horne et al. 1997, Stewart et al. 2007, Adamopoulos et al. 2002). The divergent results may be due to variations in the populations used within each study; as many of the studies displaying decreases in resting TNF-α level in response to chronic exercise have examined diseased or overweight populations (Horne et al. 1997, Adamopoulos et al. 2002). Variations in the results may also stem from differences in the exercise intensities used within each study (Stewart et al. 2007, Straczkowski et al. 2001).

Horne et al. (1997) investigated the TNF-α response within forty five subjects (27-male, 18-females) involved in four different prolonged exercise training programs. The twelve week programs consisted of individual endurance and resistance training programs, a combined endurance and resistance program and a control group. An effort was made to equally distribute male and females into each group. The endurance program consisted of 30 minutes of continuous cycling at the subject’s ventilatory threshold for three days a week. The resistance training program involved three days a week of both machine and free weight training. The combined resistance and endurance program united the two individual programs for six days a week of exercise. The control group performed no exercise. Blood samples were taken prior to the initiation of, six weeks into, and at the end of the twelve week exercise program. The samples were taken after 48-72 hours of no training and in between the hours of 4pm and 6pm. All subjects had baseline TNF-α measurements that fell within the normal range (3-20 pg/ml). With the exception of one group (the females in the endurance training group), no significant changes in resting TNF-α concentration was observed in the groups after the exercise program. The results of this study may indicate that
chronic endurance and resistance training programs have a limited ability to suppress resting TNF-α concentration in healthy populations.

Stewart et al. (2007) observed no change in resting the TNF-α concentration in response to chronic exercise training. A group of 29 younger adults (18-35 years old) and a group of 31 older adults (65-85 years old) were recruited for this study, with relatively equal numbers of males and females being used. Subjects were asked to perform a twelve week combined endurance and resistance exercise training program. The program entailed three days a week of exercise, with each day consisting of 20 minutes of endurance exercise at an intensity of 70-80% heart rate reserve and two sets of eight different resistance exercises. Blood samples were taken after eight to ten hours of fasting between the hours of 6am-8am, and occurred before and after the twelve week exercise intervention. Normal resting TNF-α levels (3-20 pg/ml) were seen within all groups. No significant change in resting TNF-α concentration was observed in either the young or old adults following the twelve week program. Similar to the Horne et al. study, these findings may suggest that chronic exercise is not able to significantly alter resting TNF-α concentration in healthy subjects.

In contrast to the two studies involving healthy subjects, there has also been research involving diseased and overweight subjects that indicates chronic exercise can significantly influence resting TNF-α concentration (Adamopoulos et al. 2002, Straczkowski et al. 2001). In a study conducted by Adamopoulos et al. (2002), 24 patients with moderate to severe congestive heart failure (CHF) and 20 healthy control subjects performed a twelve week home-based bicycle exercise training program. The program consisted of 30 minutes of cycling five days a week. Subjects were asked to continually monitor their heart rate during exercise and to keep it within 60-80% of their previously determined maximal heart rate.
Blood samples were obtained from the subjects at baseline, after the twelve weeks of training, and during the detraining stage when subjects were no longer exercising. Although both groups fell within the normal resting TNF-α range at baseline (3-20 pg/ml), the CHF group began the training program with a significantly higher TNF-α concentration than the control group (7.4 ± 0.8 pg/ml vs. 3.6 ± 0.2 pg/ml). A significant decrease in resting TNF-α concentration was observed in the CHF patients, but not the healthy subjects, after the twelve weeks of training. The findings also indicated that during the detraining period the resting TNF-α concentration of the CHF subjects began to return to near baseline levels, further emphasizing the finding that exercise may have had a role in suppressing resting TNF-α in the CHF patients.

Straczkowski et al. (2001) examined the effect of a twelve week exercise program on the resting TNF-α concentration in obese women (BMI >30 kg/m²) with normal fasting glucose (NGT), and obese women with impaired fasting glucose tolerance (IGT). A group of women with a BMI < 25 kg/m² and normal fasting glucose was used as a control. All groups contained eight subjects. The endurance exercise program involved the subjects cycling for thirty minutes at an intensity equal to 70% of their maximal heart rate five days a week. Blood samples were obtained prior to the initiation and after the exercise program was completed. Samples were taken at 8 am after a twelve hour fast, and 48 hours after the last bout of exercise. While the baseline TNF-α measurements of all the groups fell within the normal range (3-20 pg/ml), the resting concentrations of the obese (IGT and NGT) groups were significantly higher than the levels observed in the control group (6.59 ± 2.31 pg/ml and 3.88 ± 0.49 pg/ml vs. 3.27 ± 0.39 pg/ml). After the twelve week exercise intervention there
was a significant decrease in the resting TNF-α concentration in both obese groups, but not the control group.

The findings of the studies involving chronic exercise in unhealthy populations contradict the results that were reported in studies investigating healthy subjects. In the studies using the healthy subjects, it seems likely that the subjects would have already had a low resting concentration of TNF-α, which may not have been able to be decreased any further by the exercise program. This may have been the case in the studies involving healthy populations, as all baseline TNF-α measurements fell within a normal range (3-20 pg/ml). Therefore, it is reasonable that these studies found that chronic exercise had no effect on resting TNF-α level. In contrast, the persistent activation of the immune system associated with CHF and obesity may raise the resting TNF-α concentration to a high enough point where chronic exercise can influence the TNF-α level. This idea is supported by the findings of the Adamopoulos et al. (2002) and the Straczkowski et al (2001) studies, which observed significantly higher baseline TNF-α concentrations in the CHF and obese subjects when compared to healthy controls. Discrepancies in the results may also stem from differences in the intensities of exercise used with the healthy and unhealthy populations. It appears as though the studies of chronic exercise involving higher exercise intensities (ventilatory threshold and 70-80% of HRR), seen in association with the healthy populations, had no effect on the resting TNF-α concentration. Conversely, the studies of chronic exercise utilizing lower intensities (60-80% of heart rate max) effectively lowered resting TNF-α concentration. The possibility of a heightened immune response combined with the differences in the exercise protocols used may explain why chronic exercise caused a decrease in resting the TNF-α concentration in the unhealthy, but not the healthy populations.
Passive exposure to heat is another form of physiological stress that may alter the circulating TNF-α concentration (Walsh & Whitman 2006). Similar to the responses observed with acute and chronic exercise, results relating to the effect of passive heat exposure on TNF-α concentration have varied. When determining the effect that heat exposure has on the TNF-α response multiple factors must be taken into account, including the environmental temperature and length of the exposure, as well as the core temperature increases that are associated with the heat exposure (Rivier et al. 1994). Warmer environmental temperatures and longer exposure are associated with larger increases in core temperature, which may elicit greater increases in the circulating TNF-α concentration (Rivier et al. 1994).

Robins et al. (1995) investigated the effect of whole body hyperthermia, induced by passive heat exposure, on the circulating TNF-α concentration. Subjects were exposed to a temperature of 41.8°C for 60 minutes. Blood samples were obtained fifteen minutes before heat exposure, during exposure, and 2.5, 5.5 and 23.5 hours after exposure. It was found that a significant increase in TNF-α occurred immediately post heat exposure for a portion (43%) of the subjects. Blood analysis for the other subjects revealed no detectable concentration of TNF-α prior to or after heat exposure. The researchers hypothesized that the inability to detect TNF-α in some of the samples was related to the sensitivity of the assay used and the biological differences between the subjects.

Likewise, D’ Oleire et al. (1995) observed an increase in the TNF-α concentration in response to heat exposure in several (22%) of the subject examined. Blood samples were obtained from nine subjects fifteen minutes prior to and immediately after one hour of heat exposure.
exposure (41.8°C). Samples were also taken three hours, six hours and twenty-four hours post exposure in order to track the prolonged progress of the TNF-α response. A significant (600%) increase in TNF-α was observed in two of the nine subjects, with peak concentrations occurring three hours after the heat exposure. Similar to the Robins et al. study, the inability to determine a detectable TNF-α concentration in a portion of the blood samples before and after the heat exposure was attributed to the sensitivity of the assay used, as well as the biological differences within each subject. It may be the case the local production of TNF-α increased in response to the heat exposure, but that the increase was not large enough to cause a systemic increase in TNF-α concentration that would be picked up by that assay used.

Using a different protocol, Bouchama et al. (1991) observed significantly elevated TNF-α levels in seventeen patients already experiencing non-exertional (classic) heatstroke. All patients involved had a rectal temperature greater than 40.1°C upon admission to the hospital. Samples were taken from patients immediately after admission and after a cooling treatment had been administered. Results indicated that the circulating TNF-α concentration was significantly elevated in all subjects experiencing heatstroke when compared to control subjects (199 ± 25 pg/ml vs. (31.4 ± 8.4 pg/ml). TNF-α concentration decreased in response to the cooling treatments, and the corresponding drop in rectal temperature, but still remained above the levels observed within the control subjects.

Contradicting the previous studies, Dubose et al. (2002) observed no significant change in the circulating TNF-α concentration in response to passive heat exposure. Blood samples were taken from ten male subjects and exposed to heat alone (42.4°C), endotoxin alone, and heat plus endotoxin. Endotoxin is a component of cell wall gram negative
bacteria that stimulates TNF-α production and naturally increases in concentration in response to heat exposure (Bouchama & Knochel 2002). After the exposures, a significant increase (~300%) in TNF-α concentration occurred in response to the combination of heat and endotoxin, but not the individual exposures. These results suggest that exposure to heat alone may not be enough to cause a change in the circulating concentration of TNF-α. It may be that a concomitant increase in endotoxin is needed with the heat exposure before the TNF-α concentration can be effected.

In summary, the results of previously conducted research suggest that the circulating TNF-α concentration may or may not increase in response to passive heat exposure. In general, it appears that exposure to high environmental temperatures may lead to a greater TNF-α response, but the exact magnitude and direction of response is not clearly defined. Discrepancies in the results of previously conducted studies may stem from differences in the temperature and duration of heat exposure, as well as the exposure techniques (environmental chamber vs. water immersion) that were used.

**TNF-α Response to Exercise in the Heat**

In addition to their roles as individual physiological stressors, research suggests that when combined, exercise and heat exposure may additively affect the TNF-α response. Similar to the studies investigating exercise and passive heat exposure separately, a variety of exercise and heat methodologies have been used to examine the combined effect of the stressors. Due to the differences within these studies, the extent to which exercise and heat combined influence the TNF-α response has not been clearly defined.

A significant increase in the circulating TNF-α concentration in response to exercise in the heat can be seen a study conducted by Peake et al (2007). This study involved ten
trained male subjects and two separate exercise trials. Subjects were exposed to environmental temperatures of 18°C and 32°C during the two exercise trials. The temperature was controlled through the use of an environmental chamber, and each exercise trial consisted of 90 minutes of steady state cycling (60% VO₂max), directly followed by a 16.1 km performance time trial. Blood samples were taken prior to exercise, after the 90 minutes of steady state exercise, immediately after the time trial, and 45 and 90 minutes into recovery. Rectal temperature was measured continuously during the exercise and recovery, with the highest temperatures (~39.0°C) occurring at the end of exercise in the 32°C heat. The circulating TNF-α concentration significantly increased over baseline levels after exercise in heat (32°C), but not the cold (18°C). Results also indicated that the TNF-α concentration was significantly higher (17%) at minute 90 during exercise in the 32°C heat when compared exercise in the 18°C cold (1.5 ± 0.2 pg/ml vs. 1.2 ± 0.1 pg/ml).

Rhind et al. (2004) observed an increased TNF-α response during exercise in the heat (39°C) when compared exercise in a cold environment (18°C). The exercise trials entailed ten healthy male subjects cycling at 65% of their previously established VO₂peak for forty-minutes while immersed in water up to the chest. Blood samples were taken immediately before the exercise began, after 20 and 40 minutes of exercise, and 30 and 120 minutes into recovery. Rectal temperature was continuously monitored during exercise and recovery periods, with peak temperatures (39.1 ± 0.2°C) being reached immediately post exercise in the hot environment. A significant increase in the circulating TNF-α concentration was observed immediately after exercise in the hot, but not the cold environment (~1.4 pg/ml vs. 0.1 pg/ml). The concentration of TNF-α began to increase after twenty minutes of exercise in the heat, and peaked above resting levels by the end of exercise (>90%). Results also
showed that the increase in the TNF-α concentration was positively correlated with the rise in rectal temperature \( r = 0.493 \).

Starkie et al. (2005) investigated the TNF-α response to 90 minutes of exercise when subjects were exposed to 15°C and 35°C. The exercise trials involved seven endurance trained males cycling at 70% of VO\(_2\text{peak}\). Rectal temperature and blood samples were collected prior to exercise, at fifteen minute intervals during exercise, and two hours post exercise. Rectal temperature was significantly elevated between minutes 65-90 during exercise in the heat when compared to the cooler environment (~39°C vs. 38°C). Peak rectal temperatures (>39.0°C) occurred 90 minutes into hot exercise trial. Circulating TNF-α concentration peaked immediately post exercise in both trials, but was significantly higher in the hot exercise trial than during the cooler trial (~3.3 pg/ml vs. 2.1 pg/ml).

Similar to the studies related to exercise alone and heat, the literature concerning the TNF-α response to exercise in the heat has produced a wide range of results. Although research has established that these stressors can influence the mechanisms involved with the control of the TNF-α response, attempts to confirm this model by examining the direct effect of exercise in the heat on the circulating TNF-α concentration have been limited (Walsh & Whitman 2006). When the research related to this topic is reviewed, differences in the magnitude of change in the circulating TNF-α concentration caused by exercise are evident (Rhind et al. 2004, Starkie et al. 2005, Peake et al. 2007). The differing results may be related to discrepancies in the methodologies used, including variations in environmental conditions, and in the mode, intensity and duration of the exercise bouts. A comparison of the methods and results of the relating to exercise in the heat and the TNF-α response can be seen in Table 2.
Table 2. A comparison of the studies examining the effect of exercise in the heat on resting TNF-α concentration.

<table>
<thead>
<tr>
<th>Researcher</th>
<th>Methods</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peake et al (2007)</td>
<td>1) 90 min @ 60% VO2peak</td>
<td>Peak Tre = 38.1°C (C)</td>
</tr>
<tr>
<td></td>
<td>18°C (C) 32°C (H)</td>
<td>38.8°C (H)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post Exercise: 17% &gt; in H</td>
</tr>
<tr>
<td>Rhind et al. (2004)</td>
<td>2) 40 min @ 65% VO2max</td>
<td>Peak Tre = 37.6°C (C)</td>
</tr>
<tr>
<td></td>
<td>18°C H2O (C) 39°C H2O (H)</td>
<td>39.3°C (H)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post Exercise:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 pg/ml (C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 pg/ml (H)*</td>
</tr>
<tr>
<td>Starkie et al. (2005)</td>
<td>1) 90 min @ 70% VO2peak</td>
<td>Peak Tre = ~ 38.0°C (C)</td>
</tr>
<tr>
<td></td>
<td>15°C H2O (C) 35°C H2O (H)</td>
<td>~ 39.0°C (H)*</td>
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<tr>
<td></td>
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<td>Post Exercise:</td>
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<tr>
<td></td>
<td></td>
<td>2.1 pg/ml (C)</td>
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<tr>
<td></td>
<td></td>
<td>3.3 pg/ml (H)*</td>
</tr>
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</table>

1 = Chamber; 2 = Water

* Indicates significant difference between trials

Summary

When reviewing the literature related to acute exercise it appears that both exercise intensity and duration have an effect on the ability of exercise to influence the TNF-α response. In general, increases in the TNF-α response to acute exercise appear to be greater when the exercise bout is continuous and greater than 30 minutes in duration, and when the intensity of the exercise exceeds 60% of VO2peak. This relationship may be related to a threshold effect, in that a certain, but presently unknown, amount of stress must be placed on the body by the exercise before the mechanisms controlling the TNF-α response can be affected. Similarly, there have been variations in the results concerning the capacity of chronic exercise to influence resting TNF-α concentration. When the chronic exercise studies are examined, it becomes evident that the population used (healthy vs. unhealthy), as well as the exercise intensity, may be critical factors related to the ability of chronic exercise
to influence the resting TNF-α response. Discrepancies are also observed in the results of studies concerning passive heat exposure and the TNF-α response. Much like the exercise studies, the direction and magnitude of the TNF-α response to heat exposure is unclear. The findings suggest that warmer temperatures and longer exposures lead to greater increases in core temperature, and a larger rise in the circulating TNF-α concentration, but this finding is not uniform across the studies. Differences in the length and temperature of heat exposure, as well as disparities in the method of heating, may work to explain some of the variation in the results concerning passive heat exposure. The results of the studies investigating the TNF-α response to exercise in the heat also differ in the degree to which they suggest the physiological stressors can affect circulating TNF-α concentration. The divergent results may be due to differences in the length and temperature of the heat exposure and in the duration and intensity of the exercise used within the studies.

Based on these discrepancies, the proposed study will examine the effect of exercise in the heat on the circulating concentration of TNF-α by using two, forty-minute cycling bouts at an intensity of 65% of VO₂peak. One exercise trial will take place with the subjects cycling while immersed in 25°C water, and the other will involve the subjects cycling while immersed in 39°C water. The aim of the present study is to evaluate the direction and magnitude of the TNF-α response to exercise in the heat. A secondary goal of the study it to examine the relationship between increases in core temperature associated with exercise in the heat and changes in the circulating concentration of TNF-α.
Chapter III

Methodology

The purpose of this study was to determine the effect of exercise in the heat on the circulating concentration of tumor necrosis factor-α (TNF-α). This purpose was achieved by comparing changes in TNF-α concentration following exercise in a cool environment with the changes following exercise in the heat. This chapter explains the methodology by describing the subjects and instrumentation that will be used during the investigation, as well as by detailing the protocol and physiological data testing techniques that will be employed. Lastly, this chapter will describe the statistical methods that will be used to analyze the data.

Subjects

Eight physically active male subjects between the ages of 18-40 years participated in this investigation. In order to be considered physically active, the subjects must have been engaged in a minimum of 30 minutes of moderate activity at least four times a week. Exclusion criteria for this study included: known infection within three weeks prior to the initiation of the study, any major history of medical or orthopedic concerns, any history of heat related maladies, being female, claustrophobic tendencies, hydrophobic tendencies, any use of anti-inflammatory medications, any history of heart disease, diabetes, or lung disease, and a fear of blood draws.

Instrumentation

The subjects’ height was measured using a portable stadiometer (Perspectives Enterprises, Portage, MI). Body mass was determined by a mechanical balance beam scale
During all exercise trials subjects had their oxygen consumption measured by a Parvo Medics TrueMax 2400 metabolic system while cycling on a modified mechanically braked cycle ergometer (Monark, Varberg, Sweden). Subjects were fitted with a head set (Hans Rudolph, Wyandotte, MI) which held a mouthpiece in place (Vacumed, Ventura, CA), allowing for the collection of oxygen consumption data during exercise. The exercise bouts took place in a 1790 liter submersion tank. During testing core temperature ($T_{re}$) was monitored by a YSI rectal probe (YSI, Daytona, OH) which was connected to a thermistor thermometer (Cole-Palmer, Vernon Hill, Ill). Skin temperature ($T_{sk}$) measurements were obtained via YSI skin sensors, which were also connected to a thermistor thermometer. Heart rate (HR) was monitored by a Polar telemetry system (Polar Electro, Inc., Finland). During exercise testing subjects reported rating of perceived exertion (RPE) according to Borg’s Original 6-20 RPE scale (Borg 1982).

All blood samples were drawn by venipuncture of an antecubital vein using a 3 cubic centimeter (cc) Vanishpoint syringe with a 22 gauge, 1” needle (Retractable Technologies, Inc. TX). Blood was then immediately transferred equally into three “3ml” Vacutainer® tubes (Sterile 13 x 75 millimeters) treated with 5.4 mg of K$_2$ EDTA. Whole blood samples were centrifuged using a Centra 8 refrigerated centrifuge (International Equipment Company, Needham Heights, MA).

Protocol

The subjects visited the Applied Physiology Laboratory three separate times within a two week span. Each session was separated by at least 48 hours. The first session consisted of the subjects reading and signing the informed consent form, providing their characteristics (height, weight and age), filling out a training history form, supplying information for the
Department of Exercise and Sport Science Medical History form, undergoing a physical examination and performing a graded peak exercise test on a cycle ergometer to determine peak oxygen uptake (VO\textsubscript{2peak}). During the two experimental trials (sessions two and three), which occurred on separate days, subjects performed forty-minutes of submaximal cycling at 65\% of their VO\textsubscript{2peak} while submerged in water up to the xyphoid process; one session occurred with the subjects immersed in 25°C water, while in the other session subjects were immersed in 39°C water. Blood samples were taken immediately prior to exercise, immediately post-exercise, and two-hours post-exercise during sessions two and three. The circulating concentration of TNF-\textalpha was determined from the blood samples taken during each session.

Session One

Prior to arriving to the Applied Physiology Laboratory for the first session, subjects were contacted via email to alert them to be ready for VO\textsubscript{2peak} testing. Within this email a detailed description of the exercise test was provided, and subjects were asked to refrain from eating for four hours, from consuming caffeine for twelve hours, and from ingesting alcohol 24 hours prior to the testing. Subjects were asked to bring the proper attire to the Laboratory to allow for underwater testing (athletic shorts and foot apparel of their choice i.e. water, kayaking, sandals, running, tennis shoes, or barefoot). Upon arrival subjects were given the opportunity to read the informed consent form, and ask any questions they may have related to participation within the study. After all questions are answered, subjects who were still interested in participation signed the consent form. Subjects then filled out a standard Department of Exercise and Sport Science medical history and a training history form. The medical history questionnaire was completed in order to rule out any contraindications to the exercise performed in this study. The training history form was utilized to ensure that the
subjects have the minimum training background necessary for participation in the study. After providing their age, the subject’s height and body mass was measured. Following the completion of the forms, subjects underwent a physical examination screening. The physical examination was used to identify any contraindications to exercise.

The subjects were then asked to change into appropriate attire for exercise in the water, consisting of athletic shorts, no shirt, and foot apparel of their choice (water or kayaking shoes, sandals, running or tennis shoes, or barefoot). At this time the subjects also secured a heart rate monitor around their chests. Next, the subjects were asked to rest in a seated position for ten minutes, with resting oxygen consumption (VO$_2$), heart rate and blood pressure measurements being taken at the end of the ten minute period. Resting VO$_2$ measurements were taken for three minutes. After the resting measures were obtained, five minutes was allotted for subjects to warm-up on a land based cycle ergometer at a self selected workload. Time was also given to allow the subjects to stretch prior to entering the tank. Following the warm up, subjects secured a weighted belt around their waists to overcome buoyancy while immersed and were then guided to a tank filled with 25°C water. After entering the tank the water level was adjusted to reach the subjects xyphoid process after they were seated on the cycle ergometer. Subjects were then allowed to get situated on the cycle ergometer, while making changes in the seat height and the pedal straps as they saw fit (seat height and pedal strap position were recorded to allow for standardization during the subsequent trials). When subjects were comfortable they were fitted with the head set and mouthpiece.

After becoming comfortable on the cycle the subjects started pedaling and completed the graded peak exercise test. The first stage of the exercise test involved the subjects
pedaling at a rate of 40 revolutions per minute (RPM) for a period of five minutes. At the end of the first stage, the pedal rate was increased to 45 rpm and subjects pedaled at the new rate for three minutes. Following the second stage the pedal rate was increased by 5 rpm every three minutes until subjects reached volitional fatigue. VO$_2$, heart rate and rate of perceived exertion (RPE) were monitored minute-by-minute throughout the exercise test. The test was considered valid if at least two of the following criteria are met: RPE > 18, a respiratory exchange ration (RER) >1.10, or a plateau in VO$_2$ with an increase in workload (Powers & Howley 2001). Following termination of the test, the subjects stopped cycling, were escorted out of the tank, were dried and began an active recovery consisting of cycling at a workload of their choice on a land based cycle ergometer. Subjects continued the active recovery on the cycle ergometer until their heart rate fell below 120 beats per minute (BPM). If heart rate failed to fall below 120 bpm in three to five minutes, subjects stopped cycling and completed their recovery lying on a cot located within the Applied Physiology Laboratory. Subjects were released from the Applied Physiology Laboratory when their heart rates were within 20 beats of the resting rates and blood pressures were within 10 mmHg of their resting values. From the graded peak exercise test, a pedal rate equal to 65% of the subjects’ VO$_2$peak was computed using the Karvonen method: (0.65 * (VO$_2$peak - VO$_2$rest)) + VO$_2$rest (Karvonen et al. 1957). VO$_2$peak was equal to the maximal oxygen consumption achieved during the exercise test, while VO$_2$rest was equal to the mean of the values obtained during the last minute of the resting VO$_2$ measurement.

**Experimental Trials**

The two experimental trials followed identical protocols, with the only difference being that one session entailed cycling while submerged in 25°C water (cool trial), while the other consisted of cycling while submerged in 39°C water (hot trial). Water temperature was
verified immediately prior to and after each exercise bout. A counter balanced design was used to determine the order of exercise water temperature. Subjects were asked arrive to the Applied Physiology Laboratory having refrained from alcohol consumption for 24 hours, from caffeine intake for twelve hours and food intake for four hours. Instructions were given indicating that the subjects could drink water any time prior to or after arriving to the laboratory. Subjects were directed to eat their standard pre work out meal prior to arriving to the Applied Physiology Laboratory. The composition and amount of food consumed was recorded by the subjects, and a similar meal was consumed prior to the next experimental trial. The arrival time of the subjects was held constant for the experimental trials. For both trials, subjects cycled, while submerged in water up to the xyphoid process, for forty-minutes at a workload equal to 65% of their VO$_2$peak.

The first experimental trial was scheduled at least two days after session one. Prior to beginning the exercise test subjects changed into the same attire, athletic shorts, no shirt, and a choice of shoe apparel, used during the peak test performed during session one. During the changing process subjects also self-inserted a rectal thermometer approximately ten centimeters into the rectum and strapped a Polar heart rate monitor around their chests. Skin thermisters were then be placed on the subjects’ anterior mid-thigh ($T_{th}$), chest ($T_{ch}$), arm ($T_{a}$), and head ($T_{h}$), with surgical tape being used to secure each thermister. At this point subjects were asked to rest quietly in an upright-seated position for twenty minutes. During minutes 10-13 resting VO$_2$ measurements were taken as the subjects are seated. When the nineteenth minute of rest was reached, baseline heart rate, blood pressure, and temperature measurements were obtained. At the end of the twenty minute rest period a 10 milliliter (mL) blood sample was drawn via sterile venipuncture of an anticubital vein. Blood samples
were immediately transferred from the syringe into three Vacutainer® tubes (Sterile 13 x 75 millimeters (mm) treated with 5.4 mg of K₂EDTA) and stored on ice during the session.

The subjects then secured a weighted belt around their waist and were escorted to the tank filled with water which contained the cycle ergometer. After being immersed up to the xyphoid process, baseline temperature and heart rate measurements were recorded with the subject seated on the underwater cycle. After the baseline measurements were made, subjects began cycling at a workload estimated to be equal to 65% of their VO₂peak. At minute three of the cycling session the subjects’ VO₂ data was monitored to ensure they were working at 65% of their VO₂peak. If subjects were not working at 65% of their VO₂peak, the pedal rate was adjusted accordingly. Following the initial pedal rate adjustments, no more changes in pedal rate occurred. The timing and the magnitude of any changes in pedal rate was recorded in order standardize the workloads for the remaining session. Oxygen consumption was sampled via the mouthpiece for the last two minutes of each ten-minute interval (minutes 8-10, 18-20, 28-30, 38-40). Heart rate and RPE was measured and recorded during the last minute of each five minute interval. Core body temperature (Tₘₑ) and all skin temperatures (Tₛₖ) were monitored during each minute of the exercise session and recorded at five minute intervals. If during the exercise session the subjects’ core body temperature rose above 39°C, the subject had an RPE > 19, or if the supervising faculty member advised so, the trial was stopped. If the test was stopped on account of unsafe core temperature increases, the subject immediately stopped cycling and was escorted out of the tank. Subjects had access to drinking water at their own discretion during the exercise bout.

At the end of the forty-minutes of cycling the subjects were escorted out of the tank and were dried. The subjects were then guided to an upright chair located within the Applied
Physiology laboratory where another 9 ml blood sample was obtained via venipuncture of an antecubital vein. Again, the blood sample was be transferred into three K$_2$ EDTA treated Vacutainers® and stored on ice for later analysis. Following the second blood sample subjects were allowed to perform an active cool down, cycling at a workload of their own choice, on a land-based cycle ergometer. The active recovery continued until the subject felt comfortable and water was available at all times during the recovery from exercise.

Following the completion of the cool down, subjects changed into a Laboratory provided sweat suit and remained in the Applied Physiology Laboratory for two hours. Subjects were allowed to move around the Laboratory and participated in activities requiring minimal exertion (i.e. reading). Blood pressure measurements were taken every five minutes for the first 20 minutes after the end of exercise to ensure subject safety. At the end of the two hour period, a third 9 ml blood sample was obtained via venipuncture of an antecubital vein. The blood sample were then transferred into three K$_2$ EDTA Vacutainers® and stored on ice. After the final blood sample was obtained, final measurements will be taken for heart rate, blood pressure, skin temperature and core temperature. At this point the subjects were escorted to a private area within the Applied Physiology where they were allowed to self-extract the rectal thermometer and change into their regular attire. If the subject’s heart rate had returned to within 20 beats of their resting value, blood pressure had returned to within 10 mmHg of their resting value, and core temperature had declined to within 1°C of their resting value they were released from the Applied Physiology Laboratory. If the vital measurements had not returned to their resting values, subjects were required to remain in the Laboratory until the measurements returned to resting values.
Biochemical Analysis

Hemoglobin and Hematocrit

Hemoglobin and hematocrit measurements were obtained using the Advia 2120 hematology system at the Clinical Chemistry Laboratory located within the University of North Carolina Memorial Hospital.

TNF-α Analysis

TNF-α concentration was determined in duplicate, following the manufacturers recommended procedures for an enzyme-linked immunosorbent assay kit (R & D Systems, Minneapolis, MN; See Appendix C). The sensitivity information for the assay used is stated in the manufacturers’ assay description.

Plasma Volume Shifts

Hematocrit and hemoglobin concentrations were used in order to calculate plasma volume shifts (Dill & Costill 1974). TNF-α concentration was corrected for any shifts in plasma volume that may have occurred in order to ensure that any changes observed in circulating TNF-α concentration were not due to hemoconcentration.

Statistical Analysis

Data analysis was performed using Statistical Package for the Social Sciences (SPSS) software, version 15.0 (Chicago, Ill). The α-level was set a priori at 0.05 for all analyses. Descriptive statistics (mean ± standard error) was computed for subjects’ age, height, body mass, and VO₂peak. Statistical analyses were performed in order to examine the questions posed within each research hypothesis.

Research Hypotheses
1. Exercise in the heat will cause an increase in circulating concentrations of TNF-α immediately and two-hours post-exercise when compared to resting levels.
a) In order to compare the resting TNF-$\alpha$ concentration and the concentration measured immediately and two hours post-exercise a one way repeated measures analysis of variance (ANOVA) was used. If the ANOVA produced significant results, a Tukey post hoc analysis was performed to detect at which time points during exercise and recovery the TNF-$\alpha$ concentration was significantly different then resting values.

2. Exercise in the heat will elicit greater changes in circulating TNF-$\alpha$ concentration immediately and two-hours post-exercise when compared to exercise in a cool environment.

   a) In order to compare the TNF-$\alpha$ response to exercise in a warm environment and exercise in a cool environment, changes ($\Delta$ values) in TNF-$\alpha$ concentration were calculated for pre-post and pre-two hours post exercise measurements. A 2x2 totally-within subjects ANOVA was then used to compare the changes in TNF-$\alpha$ concentration that occurred during each trial. If a significant difference was found, a Tukey post hoc analysis was used to determine at which time points during the experimental trials the differences in TNF-$\alpha$ concentration occurred.

3. A significant positive relationship will exist between changes in TNF-$\alpha$ concentration and the changes in core temperature.

   a) A Pearson Product Moment Correlation was used to examine the relationship between changes in circulating TNF-$\alpha$ concentration and changes in core temperature.
Chapter IV

Results

Subjects

Eight healthy men served as subjects. At the time of participation all subjects were engaging in a minimum of 30 minutes of moderate physical activity at least four times a week. The subjects were 24 ± 1.7 (mean ± SD) years of age, 1.80 ± 0.06 m in height, 78.3 ± 6.06 kg in body mass, and had an average body mass index (BMI) of 24.2 ± 1.9 kg/m². Subjects had a VO₂peak of 52.8 ± 3.7 ml/kg/min or 4.1 ± 0.4 L/min. One subject did not meet the previously established criteria set to produce a valid VO₂peak, as the VO₂peak testing was stopped due to an inability to maintain pedal cadence. Statistical analysis of the VO₂ data involved the results of all eight subjects.

Exercise Trials

Subjects completed two underwater cycling exercise trials, one in a cool temperature of 25.5 ± 0.02°C and the other trial in a hot temperature of 38.1 ± 0.06°C. These two exercise trials will be referred to as cool and hot throughout this document. Three of the eight subjects were not able to complete the full forty minutes of exercise in the hot environment. Two subjects stopped the exercise trial due to volitional fatigue, at minutes thirty and thirty five, respectively, and a third subject was stopped at minute thirty due to safety concerns involving increases in core temperature. Data from all eight subjects was used during statistical analysis. For the subjects who did not complete the full forty minutes of exercise,
the last measurement taken immediately prior to the end of exercise was used as the final measurement.

Table 3 shows the VO$_2$ (ml/kg/min) values for each exercise trial. For both trials, the average VO$_2$ (ml/kg/min) was not significantly different (p > 0.05) from the prescribed workload of 65% of VO$_2$peak. No significant difference (p > 0.05) was observed between the average VO$_2$ (ml/kg/min) values when the two trials were compared. At all time points during exercise, heart rates were found to be significantly higher (p < 0.05) during exercise in the heat when compared to exercise in the cool environment. Rate of perceived exertion (RPE) values were significant higher (p < 0.05) at minutes 20, 30 and immediately prior to the end of exercise in the hot trial when compared the cool trial.

Table 3. Mean (± SD) Heart rate (HR), rate of perceived exertion (RPE) and oxygen uptake (VO$_2$) responses to moderate intensity exercise in a cool (25°C) and hot (39°C) environment. * (p < 0.05, Hot vs. Cool)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>HR (bpm)</th>
<th>RPE</th>
<th>VO$_2$ (ml/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cool</td>
<td>Hot</td>
<td>Cool</td>
</tr>
<tr>
<td>Rest</td>
<td>66 ± 5</td>
<td>66 ± 5</td>
<td>--</td>
</tr>
<tr>
<td>10</td>
<td>135 ± 22</td>
<td>166 ± 26 *</td>
<td>13 ± 2.0</td>
</tr>
<tr>
<td>20</td>
<td>141 ± 21</td>
<td>177 ± 20 *</td>
<td>13 ± 2.3</td>
</tr>
<tr>
<td>30</td>
<td>143 ± 19</td>
<td>181 ± 18 *</td>
<td>14 ± 2.0</td>
</tr>
<tr>
<td>Final</td>
<td>144 ± 8</td>
<td>180 ± 12 *</td>
<td>14 ± 1.9</td>
</tr>
</tbody>
</table>

Core temperatures measurements were recorded at rest, minutes 10, 20, 30 and immediately prior to the end of the exercise bouts (Figure 1). Core temperature was found to
be significantly higher (p < 0.05) at minutes 20, 30 and at the end of exercise in the hot trial when compared to the cool trial.

![Graph showing Core temperature responses to moderate intensity exercise in a cool (25°C) and hot (39°C) environment.](image)

**Figure 1:** Mean (± SD) Core temperature responses to moderate intensity exercise in a cool (25°C) and hot (39°C) environment. * (p < 0.05, Hot vs. Cool)

Hematocrit and hemoglobin measurement were made on all pre-, post-, and two hours post-exercise blood samples to calculate plasma volume shifts during the exercise and the two hours of rest post exercise. For the cool exercise trial, the average plasma volume loss during exercise was -4.8 ± 4.4%, and the average loss pre-exercise to two hours post exercise was -2.3 ± 3.8%. In the hot exercise trial, the average plasma volume loss during exercise was -8.8 ± 5.3%, and the average loss pre-exercise to two hours post exercise was -1.8 ± 3.7%. All TNF-α measurements were corrected for plasma volume changes prior to statistical analysis.
**TNF-α Responses**

Plasma TNF-α concentration was determined from blood samples taken immediately prior to, immediately post and two hours after each exercise bout. The plasma TNF-α concentration for all samples is illustrated in Figure 2. No significant effect of time (p > 0.05) on the circulating TNF-α concentration was evident during either the hot or cool trials. The results of a 2x3 within subjects ANOVA revealed no significant difference (p > 0.05) in TNF-α concentration at rest, immediately post or two hours post-exercise when the hot and cool trials were compared.

![Figure 2: Mean (± SD) Plasma TNF-α concentrations observed in response to moderate intensity exercise in a cool (25°C) and hot (39°C) environment.](image)

Changes (Δ) in TNF-α concentration were calculated by subtracting the pre-exercise concentration from the post-exercise concentration and subtracting the pre-exercise...
concentration from the two hour post-exercise concentration. The change values are illustrated in Figure 3. The results of a 2x2 within subjects ANOVA revealed no statistically significant differences (p > 0.05) between changes in TNF-α concentration in response to exercise in the cool environment when compared to the changes induced by exercise in the heat.

![Figure 3: Mean (± SD) Changes in TNF-α concentration in response to exercise in a cool (25°C) and hot (39°C) environment.](image)

**Correlation Analysis**

A correlation coefficient (Pearson) was calculated between the change in TNF-α concentration and the exercise-induced change in core temperature. The correlation coefficient, $r = 0.366$, was not statistically significant (p > 0.05). A correlation coefficient
was also calculated between the change in TNF-α concentration and the exercise-induced change in heart rate values and it was also not statistical significant ( \( r = 0.209; p > 0.05 \)).
Chapter V
Discussion

The purpose of the present study was to determine whether exercising in a warm environment affects the magnitude of change in circulating TNF-α concentration. Eight male subjects performed two moderate-intensity cycling exercise sessions; one immersed in a water temperature of 39°C and the other in 25°C water. Plasma TNF-α concentration was determined from blood samples taken immediately prior, immediately post and two hours after each forty-minute exercise session.

Exercise Trials

Core temperature was significantly higher ($p < 0.05$) during the hot trial when compared to the thermoneutral trial. During each of the trials subjects were submersed in water up to the mid-chest level, which is equal to approximately 65% of the total surface of the body being underwater (Hardy & DuBois 1938). Convective heat gain would be greater in the hot trial because the 39°C temperature of the water is well above average core body temperature, while the 25°C temperature of cool trial is below average core body temperature (Powers & Howley 2001). The increased convective heat gain, coupled with an increase in metabolic heat production due to exercise, caused a very rapid increase in core temperature during the hot trial. It may be of interest to note that large increases in core temperature ($2.28 \pm 0.40^\circ C$) can be induced very quickly via an acute bout of moderate-intensity exercise and partial submersion of the body in 39°C water.
Significant differences ($p < 0.05$) were noted in the heart rates between the two trials, as higher rates were seen in the hot trial when compared to the cool trial. Since the two trials had similar VO$_2$ values, the increased heart rates observed during the hot trial were not due to an increased workload, but were due to the increased heat stress that was placed on the body (Powers & Howley 2001). Stroke volume is compromised during exercise in the heat due to a decrease in blood flow back to the heart, which stems from a need to divert more blood flow to the periphery in order to dissipate heat (Powers & Howley 2001). As previous research suggests, heart rate would have to be increased in the hot trial in order to maintain cardiac output in response to the decreased stroke volume (Powers & Howley 2001).

RPE was also significantly higher ($p < 0.05$) during the hot trial when compared to the cool trial. This finding is similar to those observed within previous research published by Nybo and Nielsen (2001), in which elevated RPE values were reported in response to a 40°C exercise trial when compared to an 18°C trial. Additional findings from the Nybo and Nielsen (2001) study suggest that during exercise in the heat perceived exertion is associated with increases in core temperature and central fatigue, but not muscular fatigue (Nybo & Nielsen 2001). This may imply that the increased RPE observed during the hot trial of the present study was not an indicator of an increased muscular workload, but rather a gauge of the stress being placed on the central nervous system by the hot environment.

**TNF-α Responses**

The resting TNF-α concentrations observed in the present study were similar for all testing sessions and are comparable to those measured in similar studies (Peake et al. 2007, Starkie et al. 2005). Resting measures of hematocrit and hemoglobin were also within a
normal range, indicating that the subjects possessed normal physiological fluid characteristics (Tietz 1990).

No significant changes (p > 0.05) were detected in plasma TNF-α concentration immediately-post or two-hours post-exercise following exercise in a cool environment. This finding is supported by previous research (Febbraio et al. 2003, Smith et al. 1992, Ullum et al. 1994). Febbraio et al. (2003) examined the effect of cycling at an intensity of 60% of VO$_2$peak for 25 minutes and observed no significant change in TNF-α concentration post exercise. Smith et al. (1992) also reported no significant change in circulating TNF-α concentration following an acute exercise bout consisting of sixty minutes of exercise at 60% of VO$_2$max. Similar results were observed by Ullum et al. (1994), which found no significant increase in TNF-α concentration in response to sixty minutes of cycling at 75% of VO$_2$max. The results of the present study, as well as other studies, suggest that acute exercise in a cool environment has no affect on circulating TNF-α concentration.

No significant changes (p > 0.05) in TNF-α concentration were observed immediately-post or two hours post-exercise following exercise in the heat. This finding is not supported by the studies published by Rhind et al. (2004), Starkie et al. (2005), and Peake et al. (2007). In a study by Rhind et al. (2004), that used a similar exercise protocol as the present study, significant increases in TNF-α concentration were reported immediately following exercise. Starkie et al. (2005) measured circulating TNF-α concentration following ninety minutes of cycling at 70% of VO$_2$peak in an air temperature of 35°C and reported elevated TNF-α levels immediately following exercise. Significant increases in TNF-α concentration immediately after exercise were also observed in a study published by
Peake et al. (2007), in which subjects cycled for ninety minutes at 60% of VO\textsubscript{2}max in an air temperature of 32°C.

One possible factor that may have contributed to the conflicting results of the present study and previously published research could be the duration of the exercise sessions. Rhind et al. (2004) suggests that the extent of cytokine changes during exercise is related to the magnitude of the stress that is placed on the body, which is directly influenced by the duration of the exercise bout. Two of the three studies reporting increases in circulating TNF-α concentration immediately following exercise, involved at least ninety minutes of exercise (Starkie et al. 2005, Peake et al. 2007). In may be the case that the forty minutes of exercise performed in the present study did not place sufficient stress on the body and therefore did not induce significant changes in the circulating TNF-α concentration. The effect of exercise duration on TNF-α concentration may be a point that future research needs to address.

Training status may influence the TNF-α response to acute exercise (Moldoveanu et al. 2001), such that highly active, endurance trained individuals have lower TNF-α responses during moderate exercise. In contrast to this finding, the study published by Rhind et al. (2004) reported significant elevations of TNF-α in all subjects (n = 8) in response to acute exercise in the heat, but utilized a subject pool with a wide range of VO\textsubscript{2}peak values (39.3 to 75.4 ml/kg/min). The physical activity levels of the subjects were not reported. The results of the present study would disagree with the Rhind et al. findings. The lack of physical activity data presented may play a role in the differing findings, as aerobic power (VO\textsubscript{2}peak) may not be representative of the training status of the subjects. Since aerobic power can be influenced by multiple factors, i.e. genetics, it may not necessarily indicate the actual training
status of the subjects. The subjects used for the present study were active at least for times a week, and may have been more homogeneous in their training status than the subjects of the Rhind et al. study. If the subjects in the present study were more endurance trained, it may have resulted in a different TNF-α response to exercise in the heat when compared to the Rhind et al. study.

A third factor that may have played a role in the conflicting results is the mode of exercise that was used within the present study. Two of the three studies that found significant increases in TNF-α concentration following exercise in the heat used a land based cycling protocol (Starkie et al. 2005, Peake et al. 2007), while the present study used a water based protocol. A previously published study investigating muscle activity in land based exercise compared to submerged exercise reported less muscle activity and lower percentage of maximal voluntary contraction during the submerged activity (Kaneda et al. 2007). If submerged exercise requires lower levels of muscular activity, it may not induce the same amount of muscular damage as a similar amount of land based exercise would. Since increased cellular debris release from injured muscles is one of major mechanisms that can lead to increased TNF-α concentrations, it would follow that lower muscular damage would lead to a reduced TNF-α response. In this way the submerged exercise used in the present study may have led to a decreased TNF-α response when compared to the studies that utilized land based cycling protocols.

The small sample sizes used in the studies could have also influenced the divergent results. Of the three studies that reported a significant increase in TNF-α post exercise, the largest subject pool was seen in the Peake et al. (2007) study, which had ten male participants. As the two other studies with significant results, and the present study, had
subject pools less than ten, individual variability in the TNF-α response to exercise could have largely influenced the differing results.

Limitations

Although the sample size of eight subjects used is comparable to the sizes used in previously published investigations, it still very small and may have limited the present study (Rhind et al. 2004, Starkie et al. 2005, and Peake et al. 2007). A second limitation stems from three of eight subjects failing to complete exercise during the warm trial. Further inspection of their individual results shows that even though they did not finish the hot trial, their responses were similar to subjects who completed the full trial.

Summary

The present study was designed to examine the change in circulating TNF-α concentration in response to a forty minute moderate-intensity exercise session in a warm environment. Eight male subjects performed two cycling sessions at 65% VO_{2}peak; one while immersed in a water temperature of 39°C and the other in 25°C water. Plasma TNF-α concentration was determined from blood samples taken immediately prior to, immediately post and two hours after each exercise session. Neither of the two experimental trials elicited a significant increase in TNF-α concentration over resting values immediately post or two hours post-exercise. No significant differences were revealed in TNF-α concentration at rest, immediately post or two-hours post-exercise when the two trials were compared.

Conclusion

The results of the present study indicate that for moderately trained men, forty minutes of moderate-intensity exercise in a cool or hot environment does not elevate TNF-α concentration immediately post or two hours post-exercise. Therefore, research hypothesis
one, exercise in a warm environment will cause an increase in circulating concentrations of TNF-α immediately and two hours post-exercise when compared to resting levels, was rejected. No difference in circulating TNF-α concentration was observed between the cool and hot trials immediately post or two hours post-exercise. As a result of this finding, research hypothesis two, exercise in a warm environment will elicit greater changes in circulating TNF-α concentration immediately and two hours post-exercise when compared to exercise in a cool environment, was also rejected. Additionally, no relationship was observed between change in core temperature and change in TNF-α concentration, which led to rejection of hypothesis three, which stated that a significant positive relationship will exist between changes in TNF-α concentration and the changes in core temperature.

**Recommendations**

Based upon the results of the present study, further research examining additional exercise durations is recommended to provide a clearer outline of the effect of exercise in the heat on the TNF-α response to exercise. The effect of training status on the TNF-α response is another area that warrants further investigation. Lastly, it is recommended that future studies involve the use of larger sample sizes in order to increase the power of statistical analyses and enhance external validity.
Appendix A

Informed Consent Form
University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects age 18-40
Biomedical Form

IRB Study # 07-1827
Consent Form Version Date: 12.5.2007

Title of Study: The Effect of Exercise in a Warm Environment on Tumor Necrosis Factor-α Concentration and Circulating Neutrophil Count

Principal Investigator: Mark Berry and Erica Cooper
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Co-Investigators: Faculty Advisor: Robert G. McMurray, Ph.D.
Funding Source: University of North Carolina-Chapel Hill Exercise and Sport Science Department

Study Contact telephone number: (919)-609-8915, (757) 642-3670
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What are some general things you should know about research studies?
You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason.

Research studies are designed to obtain new knowledge that may help other people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?
Background: Stress, either physical or mental, has been shown to alter immune function. Two stressors that are known to alter immune function individually include exercise and heat exposure. While many studies have examined the effects of heat and exercise individually
on immune function, little research has been devoted to exploring the combined effect of exercise and heat on immune function. Any disruption of immune function due to exercise in the heat may be of particular importance to coaches, athletes, physicians, and individuals whose professions require activity in warm environments (firefighters, military personnel). The current study will focus on the effect of exercise in the heat on two markers of immune function: Tumor Necrosis Factor-α (TNF-α) and Neutrophils.

The purpose of this research study is two fold; 1) to determine how exercise in the heat effects the TNF-α concentration in the blood and 2) to determine how exercise in the heat effects the number of circulating neutrophils in the blood.

You are being asked to be in the study because you are a recreationally active male between the ages of 18 and 40 who does not have a history of heart disease, a history of infection within the past three weeks, an injury hindering your ability to exercise, claustrophobic tendencies, or a fear of blood draws or water.

**Are there any reasons you should not be in this study?**
You should not be in this study if you are female, have a history of heat related problems, heart disease, diabetes or diseases affecting your lungs (such as asthma), are not between the ages of 18-40, have an orthopedic injury that would hinder your ability to exercise, are not comfortable with water, have claustrophobic tendencies, if you are currently on anti-inflammatory medications, or if you are not comfortable with blood draws. Also, you should not be in this study if you have had an infection within the past three weeks.

**How many people will take part in this study?**
If you decide to be in this study, you will be one of approximately eight people in this research study.

**How long will your part in this study last?**
Your participation in the study will consist of three sessions. The first session will take approximately 1.5 hours. The second and third sessions will take approximately 3.5 hours. The first session will be used to conduct a physical exam, to determine your peak aerobic capacity, and to establish the exercise intensity for the following two sessions. The two exercise sessions will involve sub-maximal exercise. They will be separated by at least 24 hours, and must occur within 2 weeks of the first test session.

**What will happen if you take part in the study?**
You will be asked to complete three exercise sessions. Each session will consist of approximately 40 minutes of actual exercise. The first session will be used to obtain your consent, weight and height measurements, to complete a physical exam, and to complete a peak exercise test.

You will arrive to the Applied Physiology Laboratory for the first appointment having refrained from alcohol consumption for the past 24 hours. Immediately after arriving to the lab you will read and sign the informed consent form. Once you have agreed to participate and sign the consent form, you will fill out a medical history form and have your weight and
height measured. You may elect not to answer any of the questions on the medical history form; however, this may result in your exclusion from the study. You will complete the standard Applied Physiology Laboratory medical screening history and undergo a physical examination administered by Dr. McMurray.

Next, you will be given the opportunity to practice cycling on a stationary bike, while also taking time to stretch and warm up before the exercise test. You will then strap a battery operated heart rate monitor around your chest. Before beginning the test, you will practice breathing through a mouthpiece apparatus. When comfortable, you will move to a different cycle ergometer that is immersed into chest high water of a cool temperature (25°C Celsius, 77°F Fahrenheit). You will climb down large no-slip stairs into the tank with assistance. Before entering the tank, you will change into appropriate underwater attire: your own athletic/swimming trunks and no shirt. You may wear shoes (water, kayaking, sandals, running or tennis shoes) or perform the underwater cycling barefoot. Your clothing and shoe choice must remain consistent throughout all 3 exercise trials. You will then sit on the cycle ergometer for two minutes while your oxygen consumption is measured. After two minutes, the graded peak cycling test will begin. The first stage of the test will last 5 minutes, and you will cycle at 40 rpm. At the end of the five-minute stage, the pedal rate will increase to 50 rpm. You will cycle at the new pedal rate for three minutes. From then on, you will increase your pedal rate by 5 rpm every three minutes until you are too tired to continue. At this point, the exercise test will be stopped. You will recover by cycling at a very low pedal rate until your heart rate drops below 120 beats per minute. If your heart rate does not drop below 120 beats per minute within 3-5 minutes, you will stop cycling, be escorted out of the tank, dried off, and continue recovery lying down on a cot until your heart rate does decline below 120 beats per minute. Once your heart rate has slowed to within 20 beats of your resting rate, and blood pressure has returned to within 10 mmHg of your resting pressure, the next exercise trial will be scheduled, and you will be released. If you become uncomfortable at any point, you may stop the test. The test may also be stopped if the situation becomes unsafe or if any member of the research team decides to stop the test.

The procedures for the experimental trial and the control trial are the same, except for the water temperature in which you will be immersed. In the control trial, you will cycle while immersed in cool water (25°C Celsius; 77°F Fahrenheit) and in the experimental trial, you will cycle while immersed in warm water (39°C Celsius; 102°F Fahrenheit). The temperature of water for the warm trial is lower than the temperature of most hot tubs. Water temperature for the exercise sessions will be randomly assigned. For 24 hours leading up the trials, you must abstain from exercise and alcohol consumption. Approximately 4 hours prior to arriving at the Applied Physiology Laboratory, you will eat a meal typical to what you might eat prior to a regular workout. After this meal you will not be allowed to consume any food until the end of the trial. Please avoid alcohol for 24 hours and caffeinated drinks for a minimum of 4 hours before the exercise trials. During the trial, you will be allowed to drink water at your discretion.

After arriving at the Applied Physiology Laboratory for exercise trials 2 and 3, you will change into appropriate attire (athletic/swimming shorts and no shirt) and self-insert a battery operated rectal thermometer. The rectal thermometer consists of a small circular electric
temperature sensor (approximately the size of a small button) connected to a wire. The other end of the wire will be connected to a thermister thermometer, allowing the core temperature readout to be seen. The rectal thermometer will allow for the accurate and continuous monitoring of internal temperature changes during exercise and will ensure your safety during the exercise sessions. Battery powered skin thermisters will be attached to your mid-thigh and chest to monitor changes in skin temperature. Then, you will rest quietly, sitting in an upright position for twenty minutes. At minute 19 of the rest period, your heart rate and blood pressure will be measured. At the end of the 20 minutes of rest, a trained individual will use a sterile technique to draw a 10 ml (~2 teaspoons) blood sample. Your blood sample will be labeled with your subject identification number, and stored for analysis.

After the first blood draw, you will enter the water tank. You will be immersed in water to the mid-chest level during exercise. When you are fully submerged, you will begin a 5-minute warm-up period, cycling at a self-selected pace. During this time, a member of the research team will ensure that you are comfortable with the cycle ergometer, headset and mouthpiece apparatus used to measure oxygen consumption, and water submersion.

After the warm-up, you will begin the exercise test. You will cycle for 40 minutes at 65% of your peak exercise capacity, while submersed up to the mid-chest in water. After four-minutes of exercise, your expired air will be monitored for one minute to ensure that you are exercising at the 65% of your peak capacity. If you are not exercising at the correct intensity, pedal rate will be adjusted as needed. Pedal rates in the second session will be exactly the same as the first session. Your expired air will be sampled for the last 4 minutes of each ten-minute interval (minutes 6-10, 16-20, 26-30, 36-40). Throughout the 40 minutes of exercise, your heart rate and rating of perceived exertion will be recorded during the last 15 seconds of each five-minute interval. Also, your internal temperature (via rectal thermometer) and skin temperature (via skin thermisters) will be monitored every minute and recorded at five-minute intervals to ensure your safety. During the exercise test, you will have access to water at any point. Again, if you become uncomfortable at any point, or if the research team deems in necessary, the test may be stopped.

After 40 minutes, you will stop cycling and your headset and mouthpiece apparatus will be removed. You will be assisted walking up the stairs and out of the water tank, and dried off. A research assistant will escort you to an upright chair where again, a trained individual will use a sterilized procedure to obtain another 10 ml (~2 teaspoons) blood sample. You will then move to a stationary bicycle located in room air to cool down at a self-selected pace until you are comfortable. Again, you may drink water at any time.

After the cool down period, you will put on a sweat suit (provided by the Applied Physiology Laboratory) to keep warm. You will remain in the laboratory for two hours, during which you may walk around the laboratory, use the bathroom, read or study. Magazines will be made available for you to read during this time. After two hours, a trained individual will take a third 10 ml (~2 teaspoons) blood sample using a sterile procedure. The location of puncture will be cleaned and bandaged. You will schedule the next experimental trial and
ask any questions you may have. You will be allowed to leave the Applied Physiology Laboratory when your heart rate is within 20 beats/minute of your resting rate, your blood pressure is within 10 mmHg of your resting blood pressure, and your core temperature drops below 38°C (100°F).

**What are the possible benefits from being in this study?**

Research is designed to benefit society by gaining new knowledge. Upon completion of this study, athletes, coaches, physicians, and individuals engaged in activity in heat will have a greater understanding of the immune system’s response to activity in the heat, specifically, how exercise in the heat may or may not increase the risk of illness. You will also have the benefit of knowing your sub-maximal and peak aerobic capacity and heart rates, which may be valuable for your training; however, you may not benefit directly from participating in this study.

**What are the possible risks or discomforts involved with being in this study?**

There should be no risk of psychological harm from completing the research; therefore there should be no emotional distress.

You will be asked to complete very strenuous exercise bouts during participation within this study. When completing strenuous exercise some common side effects may include, muscle soreness, fatigue. As you are accustomed to regular exercise the muscle soreness and fatigue should be minimal. Uncommonly, strenuous exercise has also been associated with the risk of heart attack or stroke, as there is a 1 in 10,000 chance that a healthy subject may experience an adverse event during aerobic fitness testing. However, the following precautions are in place to minimize the risk including: close supervision, pulse rate monitoring, periodic blood pressure monitoring, pre-physical examinations and extensive medical screening. The supervising faculty (Dr. McMurray) and the exercise research test assistants are trained in exercise stress testing, cardio-pulmonary resuscitation, and the use of an automated defibrillator device. Dr. McMurray, PhD., has maintained his certification with the American College of Sports Medicine (originally certified in 1982), which ensures current knowledge/expertise in exercise testing special populations, including older adults and those with disease processes. Supplemental oxygen is available in the lab along with a telephone with posted emergency call instructions for notifying campus security to arrange medical transport for the subject to UNC hospital. Further, a fully equipped Sports Medicine Rehabilitation facility is located 100 yards away.

You will also be asked to provide blood samples during the experimental trials. The blood draws will be conducted by a trained individual, Dr. McMurray, from the University of North Carolina-Chapel Hill Applied Physiology Laboratory. All standard safety and sterile procedures will be used during all blood draws. If minor bruising does develop as a result of the blood draws, ice and compression will be applied to the area. Also, the total amount of blood drawn (approximately 30 ml) is small and should have no adverse effects.

Upon entering and exiting the water tank, there is a small risk of slipping or falling. The water tank in which the cycle will be immersed is equipped with large no-slip steps that
provide easy access for you. You will also be provided assistance to aid your balance as you enter and exit the tank. Absorbent towels will cover the floor area surrounding the water tank to prevent accidental slips or falls. Additionally, the tank provides easy access in the event that the investigators need access to you during your exercise sessions. Although the water comes up only to your chest, there is a small risk of accidental submersion. In order to minimize this risk, a flotation device will be available while you are in the water. Also, once the cycle ergometer is in place in the tank, there is little space for you to move around the tank, reducing the potential for submersion.

There is the minor risk of developing a degree of temperature related stress, including heat cramps, heat exhaustion, or heat stroke. Constant monitoring of core temperature via a battery operated rectal thermometer, which is the most reliable method of monitoring core temperature, minimizes this risk. Additionally, studies involving exercise combined with heat exposure have been performed in UNC Chapel Hill’s Applied Physiology Laboratory since the mid-1980s with no adverse effects reported. You will be carefully monitored during all exercise sessions, and testing will stop if core temperature exceeds 39°C (102°F).

In the case that your core temperature does rise above 39°C (102°F) during exercise, immediate action will be taken in order to cool you off. You will be escorted out of the tank, dried off, and guided to a separate area of the Applied Physiology Laboratory. Once completely dry, all uncovered areas of your skin will be sprayed with cool water, while a fan blows air across your body. Evaporative cooling will be increased and your internal body temperature will begin to go down. This method of cooling is regularly used to treat heat related illnesses and has been shown to cause rapid drops in internal body temperatures. Another member of the team will also apply ice to your neck in order to augment the cooling process. The entire cooling protocol will continue until your internal temperature drops to within one degree of your resting temperature. Monitoring your body temperature during exercise and use of these cooling protocols in case your internal temperature exceeds 39°C (102°F) will help to avoid any large increases in internal temperature.

Although the use of rectal thermometry is considered a safe method of internal temperature measurement, it has been associated with a small increase in the risk of infection. In order to combat this risk all thermometers will be stored within disinfected area of the laboratory, and will be cleaned with an alcohol solution following every use. Taking these steps to sterilize the thermometers should negate any increased risk of infection.

If you fall, strain a muscle, or in any way injure yourself during the exercise testing process, first aid will be provided.

Although no radioactive materials are being used for this study, the UNC Chapel Hill Applied Physiology Laboratory, where you will be performing exercise tests, houses radioactive materials. The amount of radioactive material is very small and should pose no health threat to you. All radioactive material is stored in a contained biochemistry section of the Applied Physiology Laboratory, and is kept in compliance with the UNC Office of Environmental, Health & Safety regulations.
In addition, there may be uncommon or previously unknown risks that might occur. You should report any problems to the researchers.

**What if we learn about new findings or information during the study?**
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

**How will your privacy be protected?**
No subjects will be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

Upon agreement to participate in the study, each participant will be assigned an ID number. All data will be stored by ID number in a computer that is password protected. Only the researchers and the faculty adviser will have access to this information. Once you have completed all three trials, your name, telephone number, and email address will be destroyed. The data will be kept by ID with no other identifiers. All data collection sheets and questionnaires used for data collection will be kept in a locked filing cabinet located in room 25B Fetzer Gym (Dr. McMurray’s office). After every trial, a research team member will transfer all data to the computer.

**What will happen if you are injured by this research?**
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

**What if you want to stop before your part in the study is complete?**
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped.

**Will you receive anything for being in this study?**
You will be allowed to retain a copy of your personal test data to aid you in planning your training. Otherwise, you will not be compensated for participation in this study.

**Will it cost you anything to be in this study?**
It will not cost you anything to take part in this study. If you enroll in this study, you will have to pay for your own transportation. Parking behind Fetzer Gym will be provided for those coming from off campus.

**What if you are a UNC student?**
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

**What if you are a UNC employee?**
Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

**Who is sponsoring this study?**
There is no outside funding for this study, as the study will be sponsored by the University of North Carolina - Chapel Hill Applied Physiology Laboratory. The researchers do not have a direct financial interest in the final results of the study.

**What if you have questions about this study?**
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

**What if you have questions about your rights as a research subject?**
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

**Title of Study:** The Effect of Exercise in a Warm Environment on Tumor Necrosis Factor-α Concentration and Circulating Neutrophil Count

**Principal Investigators:** Mark P. Berry and Erica S. Cooper

**Subject’s Agreement:**
I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

_________________________________________   _________________
Signature of Research Subject     Date

_________________________________________
Printed Name of Research Subject
Appendix B

Training History, Medical History and Physical Exam Forms

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Department of Exercise and Sport Science

Training History Form

Are you currently involved a regular exercise program?   ____ yes ____ no

Do you regularly walk or run one or more miles continuously?  ____ yes ____ no
How often per week? _____________

Do you participate in other forms of regular aerobic exercise?  ____ yes ____ no
If yes, average number of miles covered per workout or day:  ____miles____?

Do you regularly lift weights?     ____ yes ____ no
______# times/wk

Do you consider yourself: please circle

Sedentary    lightly active   moderately active   highly active

How long (years) have you been consistently training?

Overall, how many days are you active each week?

Besides those listed, are there any other types of activities do you participate in on a regular basis? How much time do you spend each week in these activities?

Please describe your average typical weekly workout schedule over the last six months (i.e. types of workouts/amount per day)

When was the last time you had an injury that stopped you from exercising for more than three days, and what was the injury?

How long were you injured?
Department of Exercise and Sport Science
Medical History

Subject:__________________________ ID: ___________  Telephone:______________

Address:________________________________________________________________

Occupation:__________________________ Age:__________________________

Patient History
1. How would you describe your general health at present?  
   Excellent_____ Good_____ Fair_____ Poor_______
2. Do you have any health problems at the present time?   _____ _____
3. If yes, please describe:__________________________________________
4. Have you ever been told you have heart trouble?   _____ _____
5. If yes, please describe:__________________________________________
6. Do you ever get pain in your chest?           _____ _____
7. Do you ever feel light-headed or have you ever fainted?   _____ _____
8. If yes, please describe:__________________________________________
9. Have you ever been told that your blood pressure has been elevated? _____ _____
10. If yes, please describe:__________________________________________
11. Have you ever had difficulty breathing either at rest or with exertion? _____ _____
12. If yes, please describe:__________________________________________
13. Are you now, or have you been in the past 5 years, under a doctor’s care for any reason? _____ _____
14. If yes for what reason?__________________________________________
15. Have you been in the hospital in the past 5 years?   _____ _____
16. If yes, for what reason?__________________________________________
17. Have you ever experienced an epileptic seizure or been informed that you have epilepsy? _____ _____
18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year? _____ _____
19. If yes, name the disease:__________________________________________
20. Have you ever been treated for or told you might have diabetes? _____ _____
21. Have you ever been treated for or told you might or low blood sugar? _____ _____
22. Do you have any known allergies to drugs?   _____ _____
23. If so, what?__________________________________________
24. Have you ever been “knocked-out” or experienced a concussion? _____ _____
25. If yes, have you been “knocked-out” more than once? _____ _____
26. Have you ever experienced heat stroke or heat exhaustion? _____ _____
27. If yes, when? _____ _____
28. Have you ever had any additional illnesses or operations? (Other than childhood diseases) _____ _____
29. If yes, please indicate specific illness or operations: _____ _____
30. Are you now taking any pills or medications? _____ _____
31. If yes, please list: _____ _____
32. Have you had any recent (within 1 year) difficulties with your:
   a. Feet _____ _____
   b. Legs _____ _____
   c. Back _____ _____

Family History
33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
   a. Sudden death _____ _____
   b. Cardiac disease _____ _____
   c. Marfan’s syndrome _____ _____

Mental History
34. Have you ever experienced depression? _____ _____
35. If yes, did you seek the advice of a doctor? _____ _____
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? _____ _____
37. If yes, please list condition and if you are currently taking any medication.
   Condition                Medication

____________________________________________________________________

Bone and Joint History
34. Have you ever been treated for Osgood-Schlatter’s disease? _____ _____
35. Have you ever had any injury to your neck involving nerves or vertebrae? _____ _____
36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? _____ _____
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? _____ _____
38. Have you ever experienced any injury to your arms, elbows, or wrists? _____ _____
39. If yes, indicate location and type of injury: __________________________________________

40. Do you experience pain in your back? _____ _____
41. Have you ever had an injury to your back? _____ _____
42. If yes, did you seek the advice of a doctor? _____ _____
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____ _____
44. Do you think you have a trick knee? _____ _____
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint surgery that presently limits your physical capacity? _____ _____
46. If yes, indicate where: ________________________________________________________

47. Have you ever had a bone graft or spinal fusion? _____ _____

Activity History
48. During your early childhood (to age 12) would you say you were:
   Very active ____ Quite active____ Moderately active____ Seldom active____
49. During your adolescent years (age 13-18) would you say you were:
   Very active ____ Quite active____ Moderately active____ Seldom active____
50. Did you participate in:
   a. Intramural school sports? _____ _____
   b. Community sponsored sports? _____ _____
   c. Varsity school sports? _____ _____
   d. Active family recreation? _____ _____
51. Since leaving high school, how active have you been?
   Very active ____ Quite active____ Active____ Inactive____
52. Do you participate in any vigorous activity at present? _____ _____
53. If yes, please list:
   Activity Frequency Duration Intensity

54. How would you describe your present state of fitness?
   Excellent_____ Good_____ Fair_____ Poor_____
55. Please list the type(s) of work you have been doing for the previous ten years:
   Year Work Indoor/Outdoor Location (city/state)

56. Whom shall we notify in case of emergency?
   Name: ____________________________________________
   Phone: (Home)____________________ (Work)____________________
   Address: ___________________________________________________
57. Name and address of personal physician: ____________________________________________
_____________________________________________________________________________

All of the above questions have been answered completely and truthfully to the best of my knowledge.

Signature: ___________________________________________ Date: ________________

Examination status:  Approved          Disapproved
Department of Exercise & Sport Science
Physical Examination Screening

Name: ________________________   ID: ______   Age: _____ Gender: _____

Please respond to each of the following in writing.

Pulse rate and regularity: ________________  ECG Interpretation: _____________

Blood Pressure:
Supine: __________   Sitting: __________  Standing (Left side): __________
Squat: __________   Standing (Right side): __________

Marfan Syndrome evaluation: (Δ BP, Physical Char.) __________________________

Palpation of Pulses:  Carotid: __________  Radial: __________  Pedal: __________

Auscultation of the Lungs:
   Back:    Lower: __________  Middle: __________  Upper: __________
   Front:    Middle: __________  Upper: __________

Auscultation of Heart Sounds:(Supine, Standing, Squatting)
Non-Specific HS: _____ / _____ / _____
Murmur: ___  Gallop: ____  Click: ___  Rub: ___  Click w/ Murmur: ___

Bruit:     Carotid: __________  Abdominal: __________
Edema:     Abdominal: __________  Calf: __________  Pedal: _____
Tenderness: Abdominal: __________  Other: __________
Xanthoma or xanthelasm: __________

Medical / Family History:
High Blood Pressure: _________  Diabetes: _________  CHD/CAD: _______

Last examination w/ physician: _________

Medications (prescription / counter): __________________________________________

Examiner: ___________________________  Date: ______________
Quantikine HS, Human TNF-α/TNFSF1A Immunoasasay

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Quantikine® HS

Human TNF-α/TNFSF1A Immunoassay

Catalog Number HSTA00D
  SSTA00D
  PHSTA00D

For the quantitative determination of human tumor necrosis factor alpha (TNF-α) in serum and plasma.

This package insert must be read in its entirety before using this product.

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

The prototype ligand of the TNF superfamily, TNF-\(\alpha\)/TNFSF1A, is a pleiotropic cytokine that plays a central role in inflammation and apoptosis (1 - 4). It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length (4, 5). It contains a 30 amino acid (aa) cytoplasmic domain, a 26 aa transmembrane segment, and a 177 aa extracellular region (6, 7). TNF-\(\alpha\) is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 aa residue soluble form of TNF-\(\alpha\) (sTNF-\(\alpha\)) is released from the C-terminus of the transmembrane protein through the activity of TNF-\(\alpha\)-converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase (8, 9). Human cells known to express TNF-\(\alpha\) include B cells (10), colonic columnar epithelial cells (11), NK and CD3\(^+\)CD56\(^+\) hepatic natural T cells (12), macrophages (13), monocytes and monocyte-derived dendritic cells (14), CD4\(^+\) and CD8\(^+\) T cells (15), mast cells (16), neutrophils (17), keratinocytes (18), plasma cells (19), and adipocytes (20).

The two identified high affinity receptors for human TNF-\(\alpha\) are TNF RI/TNFRSF1A and TNF RII/TNFRSF1B. While each possess a typical TNF R structure, these receptors have relatively little aa identity and exhibit differences in receptor/ligand kinetics and cellular signaling. The dissociation rate of sTNF-\(\alpha\) from TNF RII is significantly faster than that of TNF RI (21), and while the membrane (mTNF-\(\alpha\)) and soluble forms of TNF-\(\alpha\) activate TNF RI equally well, TNF RII is activated more effectively by mTNF-\(\alpha\) (22). TNF RI is a 55 - 60 kDa, 415 aa, type I transmembrane glycoprotein (23 - 25). The cytoplasmic domain is notable for the presence of an 80 aa "death domain" motif known for its capacity to mediate apoptosis (26). TNF-\(\alpha\) activation of TNF RI, and internalization of the ligand/receptor complex, leads to the recruitment of adaptor proteins including TNF receptor-associated death domain (TRADD) (27) and Fas-associated death domain (FADD) (28), ultimately leading to the activation of downstream effectors and apoptosis. In addition, TRADD interaction with TNF receptor-associating factors (TRAFs) can initiate anti-apoptotic NF-\(\kappa\)B signaling pathways (28 - 30). TNF RII is a 75 - 80 kDa, transmembrane glycoprotein that lacks the cytoplasmic death domain (31, 32). However, the receptor retains the ability to recruit TRAF2, leading to downstream NF-\(\kappa\)B signaling (33), and in some cells, TNF RII-mediated apoptotic pathways that may involve crosstalk between TNF RI and TNF RII receptors (34, 35). Although TNF RI and TNF RII can individually mediate TNF-\(\alpha\) functions, some physiological activities apparently require the presence and interaction of both receptors (36 - 39). A novel TNF RII isoform, generated via alternative splicing, results in an intracellularly expressed 50 kDa receptor termed icp75TNFR (40). Over-expression of icp75TNFR results in TRAF2-mediated NF-\(\kappa\)B activation suggesting the potential for intracellular TNF-\(\alpha\) activity (40).

The role of TNF-\(\alpha\) and/or its receptors in host defense and inflammatory responses has been well documented. TNF-\(\alpha\) is reported to promote inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, serve as a chemotactic agent for monocytes, and activate phagocyte killing mechanisms (30). Deficiencies in either TNF-\(\alpha\) or its receptors can increase susceptibility to infection by intracellular pathogens (41 - 44). TNF-\(\alpha\) may also play a role in lymphoid tissue development. Knockout mice lack splenic B cell follicles and the ability to form germinal centers (45, 46). Other potential physiological roles for TNF-\(\alpha\) and its receptors include regulating the differentiation of hematopoietic stem and progenitor cells (47 - 49).
TNF-α has been implicated in a number of pathophysiological processes. It is associated with unregulated pro-inflammatory activity and is thought to be a critical mediator of endotoxin-induced septic shock (50). Cachexia (or whole body wasting) has also been associated with long-term circulating TNF-α (51, 52). Other disorders with potential TNF-α involvement include asthma (53), type 2 diabetes (54), Crohn’s disease (55), and rheumatoid arthritis (56).

The Quantikine HS TNF-α Immunoassay is a 6.5 hour solid phase ELISA designed to measure TNF-α in serum and plasma. It contains E. coli-derived recombinant human TNF-α and antibodies raised against the recombinant factor. It has been shown to accurately quantitate recombinant human TNF-α. Results obtained with naturally occurring TNF-α samples showed linear curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that the Quantikine HS Immunoassay kit can be used to determine relative mass values for natural TNF-α. Since the measurement of TNF-α is insensitive to the addition of recombinant forms of either of the two types of soluble receptors, it is probable that this measurement detects the total amount of TNF-α in samples, i.e., the total amount of free TNF-α plus the amount of TNF-α bound to soluble receptors.

**PRINCIPLE OF THE ASSAY**

**DUE TO THE HIGH SENSITIVITY OF THIS KIT, PLEASE NOTE THE FOLLOWING PRECAUTIONS.**

- Alkaline phosphatase is detectable in saliva. Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).
- Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.
- Use separate pipettes and glassware for dispensing all reagents to avoid cross-contamination.
- Careful washing of the microplate is essential to minimize nonspecific binding of the conjugate.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added to the wells and color develops in proportion to the amount of TNF-α bound in the initial step. The color development is stopped and the intensity of the color is measured.
LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other sources or lots.
- Although this kit has been designed to eliminate matrix problems, some samples may exist that give falsely elevated values when assayed neat. If samples generate values that are suspiciously high or higher than the highest standard, dilute the samples in Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

MATERIALS PROVIDED

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<td>Stop Solution</td>
<td>895032</td>
<td>1 vial</td>
<td>6 vials</td>
</tr>
<tr>
<td>Plate Covers</td>
<td></td>
<td>8 strips</td>
<td>48 strips</td>
</tr>
</tbody>
</table>

HSTA00D contains sufficient materials to run an ELISA on one 96 well plate.
SSTA00D (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PHSTA00D). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary.
Please refer to the literature accompanying your order for specific vial counts.
### STORAGE

<table>
<thead>
<tr>
<th>Unopened Kit</th>
<th>Store at 2 - 8°C. Do not use past kit expiration date.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted Wash Buffer</td>
<td></td>
</tr>
<tr>
<td>Stop Solution</td>
<td></td>
</tr>
<tr>
<td>Assay Diluent RD1F</td>
<td>Store for up to 1 month at 2 - 8°C.*</td>
</tr>
<tr>
<td>Calibrator Diluent RD6-13</td>
<td></td>
</tr>
<tr>
<td>Conjugate</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>Aliquot and store at ≤ -20°C for up to 1 month in a manual defrost freezer. Avoid multiple freeze-thaw cycles.</td>
</tr>
<tr>
<td>Substrate Solution</td>
<td>Store in an upright position for up to 1 month at ≤ -20°C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.</td>
</tr>
<tr>
<td>Amplifier Solution</td>
<td></td>
</tr>
<tr>
<td>Microplate Wells</td>
<td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8°C.*</td>
</tr>
</tbody>
</table>

*Provided this is within the expiration date of the kit.

### OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 490 nm, preferably with dual wavelength correction set at 650 nm or 690 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 1000 mL graduated cylinder for preparation of Wash Buffer.
- Human TNF-α Controls (optional; available from R&D Systems).
- Polypropylene tubes.

### PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Alkaline phosphatase and TNF-α is detectable in saliva. Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).
SAMPLE COLLECTION AND STORAGE

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

**Note:** Allowing samples to sit on the clot for more than 30 minutes may result in higher TNF-α levels.

Plasma - Collect plasma using EDTA, heparin or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Note:** Alkaline phosphatase and TNF-α is detectable in saliva. Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Step Solution).

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer.

**Substrate Solution** - Reconstitute the lyophilized Substrate with 6.0 mL of Substrate Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

**Amplifier Solution** - Reconstitute the lyophilized Amplifier with 6.0 mL of Amplifier Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

**TNF-α Standard** - Reconstitute the TNF-α Standard with the volume of Calibrator Diluent RD6-13 printed on the Standard vial label to produce a stock solution of 32 pg/mL. This reconstitution produces a 1X stock solution. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

**Use polypropylene tubes.** Pipette 500 μL of Calibrator Diluent RD6-13 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (32 pg/mL). Calibrator Diluent RD6-13 serves as the zero standard (0 pg/mL).
ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

Note: Alkaline phosphatase and TNF-α is detectable in saliva. Take precautionary measures to protect reagents (e.g. wear a mask and gloves).

1. Prepare all reagents, samples, and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 50 μL of Assay Diluent RD1F to each well. Assay Diluent RD1F contains a precipitate. Mix well before and during use.
4. Add 200 μL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature.
5. Wash
   a. Remove liquid from the wells by aspirating or inverting the plate and decanting the contents.
   b. Remove excess liquid by grasping the plate firmly and smartly rapping the inverted plate on a clean paper towel at least 5 times.
   c. Fill each well with 400 μL of Wash Buffer using a squirt bottle, manifold dispenser, or autowasher.
   d. Remove liquid from the wells by aspirating or inverting the plate and decanting the contents.
   e. Repeat steps b, c, and d 5 times for a total of 6 washes. After the last wash, smartly rap the inverted plate on a clean paper towel at least 10 times to remove excess Wash Buffer.
6. Add 200 μL of TNF-α HS Conjugate to each well. The TNF-α HS Conjugate may contain a precipitate. Mix well before and during use. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the wash as in step 5.
8. Add 50 μL of Substrate Solution to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature. **Do not wash the plate.**
9. Add 50 μL of Amplifier Solution to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature. **Note:** Addition of Amplifier Solution initiates color development.
10. Add 50 μL of Stop Solution to each well. Addition of Stop Solution does not affect color in the wells.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.
ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as instructed.

2. Add 50 μL Assay Diluent RD1F to each well.

3. Add 200 μL Standard, control, or sample to each well. Incubate 3 hours at RT.

4. Wash 6 times.

5. Add 200 μL Conjugate to each well. Incubate 2 hours RT.

6. Wash 6 times.

7. Add 50 μL Substrate Solution to each well. Incubate 1 hour RT.

8. Add 50 μL Amplifier Solution to each well. Incubate 30 minutes RT.

9. Add 50 μL Stop Solution to each well. Read at 490 nm within 30 min. λ correction 650 or 690 nm
**CALCULATION OF RESULTS**

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the TNF-α concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding TNF-α concentration.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

**TYPICAL STANDARD CURVE**

The following standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.
TECHNICAL HINTS

- Neither the addition of the Substrate Solution nor the Stop Solution will result in a color change.
- Addition of the Amplifier Solution will result in a color change (colorless to shades of maroon).
- Amplifier Solution and Stop Solution should be added to the plate in the same order as the Substrate Solution.
- To ensure accurate results, bring liquids to room temperature and mix to homogeneity prior to pipetting or aliquoting.
- When mixing protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECISION

**Intra-assay Precision** (Precision within an assay)
Three samples of known concentration were tested in replicates of twenty to assess intra-assay precision.

**Inter-assay Precision** (Precision between assays)
Three samples of known concentration were evaluated in forty-one separate assays to assess inter-assay precision.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-Assay Precision</th>
<th>Inter-Assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean (pg/mL)</td>
<td>1.96</td>
<td>11.5</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.17</td>
<td>0.49</td>
</tr>
<tr>
<td>CV (%)</td>
<td>8.5</td>
<td>4.3</td>
</tr>
</tbody>
</table>
RECOVERY
The recovery was determined by spiking four samples with mTNF-α to three different levels throughout the range of the assay in various matrices.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average % Recovery</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>93</td>
<td>85 - 98%</td>
</tr>
<tr>
<td>EDTA plasma</td>
<td>99</td>
<td>92 - 111%</td>
</tr>
<tr>
<td>Citrate plasma</td>
<td>95</td>
<td>88 - 105%</td>
</tr>
<tr>
<td>Heparin plasma</td>
<td>93</td>
<td>87 - 103%</td>
</tr>
</tbody>
</table>

LINEARITY
To assess linearity of the assay, samples were spiked with high concentrations of TNF-α in various matrices and diluted with Calibrator Diluent RD6-13 to produce samples with values within the dynamic range of the assay.

<table>
<thead>
<tr>
<th></th>
<th>Serum (n=4)</th>
<th>EDTA plasma (n=4)</th>
<th>Citrate plasma (n=4)</th>
<th>Heparin plasma (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average % Recovery</td>
<td>104 97 - 107 98 92 - 102 104 97 - 109 100 97 - 106</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (%)</td>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>Average % Recovery</td>
<td>108 97 - 115 101 98 - 104 102 102 - 103 98 91 - 103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (%)</td>
<td>1:8</td>
<td></td>
<td>1:16</td>
<td></td>
</tr>
<tr>
<td>Average % Recovery</td>
<td>106 97 - 114 104 100 - 109 101 95 - 104 102 95 - 106</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (%)</td>
<td>1:16</td>
<td></td>
<td></td>
<td>100 97 - 105 99 93 - 107 104 95 - 110 101 95 - 108</td>
</tr>
</tbody>
</table>

SENSITIVITY
Seventy-four assays were evaluated and the minimum detectable dose (MDD) of TNF-α ranged from 0.038 pg/mL to 0.191 pg/mL. The mean MDD was 0.106 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.
CALIBRATION
This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human TNF-α produced at R&D Systems.

The NIBSC/WHO TNF-α First International Reference Standard 87/650, which is intended as a potency standard, was evaluated in this kit. This standard is an *E. coli*-expressed recombinant human TNF-α.

The dose response curve of this First International Standard parallels the Quantikine HS standard curve. To convert sample values obtained with the Quantikine HS TNF-α kit to equivalent NIBSC 87/650 nominally assigned mass values, use the equation below.

NIBSC/WHO (87/650) equivalent value (IU/mL) = 0.03 x Quantikine HS TNF-α value (pg/mL).

SAMPLE VALUES
Serum and plasma samples were evaluated in this assay.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Mean of Detectable (pg/mL)</th>
<th>% Detectable</th>
<th>Range (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (n=33)</td>
<td>1.206</td>
<td>100</td>
<td>0.550 - 2.816</td>
</tr>
<tr>
<td>EDTA plasma (n=22)</td>
<td>1.036</td>
<td>91</td>
<td>ND - 2.139</td>
</tr>
<tr>
<td>Citrate plasma (n=14)</td>
<td>0.739</td>
<td>79</td>
<td>ND - 1.195</td>
</tr>
<tr>
<td>Heparin plasma (n=26)</td>
<td>0.873</td>
<td>85</td>
<td>ND - 1.411</td>
</tr>
</tbody>
</table>

ND = Non-detectable
SPECIFICITY

This assay recognizes recombinant and natural human TNF-α. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

<table>
<thead>
<tr>
<th>Recombinant human:</th>
<th>Recombinant mouse:</th>
<th>Recombinant rat:</th>
<th>Recombinant porcine:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG</td>
<td>IL-17</td>
<td>CNTF</td>
<td>IL-1α</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Leptin</td>
<td>GDNF</td>
<td>IL-1β</td>
</tr>
<tr>
<td>AR</td>
<td>LIF</td>
<td>IFN-γ</td>
<td>IL-2</td>
</tr>
<tr>
<td>BDNF</td>
<td>MIF</td>
<td>IL-1α</td>
<td>IL-4</td>
</tr>
<tr>
<td>CD4</td>
<td>NT-3</td>
<td>IL-1β</td>
<td>IL-6</td>
</tr>
<tr>
<td>CD40</td>
<td>NT-4</td>
<td>IL-2</td>
<td>IL-8</td>
</tr>
<tr>
<td>CD40 Ligand</td>
<td>OPG</td>
<td>IL-4</td>
<td>IL-10</td>
</tr>
<tr>
<td>CNTF</td>
<td>OSM</td>
<td>IL-10</td>
<td>TNF-α</td>
</tr>
<tr>
<td>CT-1</td>
<td>PTN</td>
<td>Leptin</td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>SCF</td>
<td>MK</td>
<td></td>
</tr>
<tr>
<td>Epo</td>
<td>SLPI</td>
<td>Tpo</td>
<td></td>
</tr>
<tr>
<td>Fas</td>
<td>SMDF</td>
<td>TRAIL</td>
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</tr>
<tr>
<td>GDNF</td>
<td>TNF-β</td>
<td>TRANCE</td>
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<tr>
<td>GITR</td>
<td>sTNF RI</td>
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<td>sTNF RII</td>
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REFERENCES

References


