INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

EFFECT OF ORAL GLUTAMINE SUPPLEMENTATION ON RESTING BLOOD AND SALIVA IMMUNE PARAMETERS IN ENDURANCE TRAINED ATHLETES

A Thesis
Submitted to the School of Kinesiology, Lakehead University
in Partial Fulfillment of the Requirements for the Degree
Master of Science
in
Kinesiology

by:

JOE QUADRILATERO ©
September, 2001

ACKNOWLEDGMENTS

I would like to take this opportunity to thank all the individuals involved with this thesis. Dr. Ian Newhouse and Dr. Norm LaVoie, for being excellent advisors. Your patience, guidance and ongoing support will always be appreciated. I would particularly like to thank you both for allowing me the freedom and opportunity to develop as an individual, student and researcher throughout the past few years. Dr. Jim McAuliffe for always making yourself available for statistical advice as well as devoting your time to the development of this manuscript. Dr. Gabriel Dakubo for providing your expertise in the area of immunology and providing advice during the writing of this document. Dr. John MacDougall for taking time to teach me the techniques needed to complete this thesis. I would also like to thank the Northwestern Ontario Regional Cancer Centre and all the staff. In particular, Dr. John Th'ng, Rucy Vergedis, Marissa Kubinic, and Christina Richard for allowing me the opportunity to be part of their lab for the past year. Donna Newhouse for providing your invaluable services as a phlebotomist during testing time and for making yourself available for answering any technical questions when they arose. I'd like to also thank all the graduate and undergraduate students who kept me sane for the past few years. I would also like to send out a special thank you to the following funding agencies that provided funding for both myself and this project. They include: the Lloyd Carr-Harris Foundation, the Gatorade Sports Science Institute, SportPharma Inc., Argonomics America Inc., and the Ontario Graduate Scholarship Program. Finally, I would like give a special thanks to all my family and friends for always having faith and sticking with me throughout the years. Without all these great people this thesis would have not been possible.

ABSTRACT

There is considerable evidence suggesting that frequent, intense, long duration exercise is associated with adverse effects on the immune system as shown by a decrease in immune cell numbers and function. This may ultimately be responsible for the increased rate of infection, in particular, upper respiratory tract infection, seen in athletes. It has been suggested that one possible mechanism for this immunosuppression is a decrease in plasma glutamine. Glutamine is a non-essential amino acid that is utilized at a high rate by immune cells. A decrease in plasma glutamine may then impair immune function and possibly lead to increased rates of infection. Recent research has shown that plasma glutamine levels are decreased during times of metabolic and catabolic stress, such as cancer, burns, surgery, endurance exercise and overtraining. This decrease in plasma glutamine coupled with the immunocompromised state of these individuals may be evidence of the important role of glutamine in immune function. Recent research has shown that glutamine supplementation results in increased immune cell counts and immune function, and decreased rates of infection in various populations. Therefore, the purpose of this study was to determine the effect of oral glutamine supplementation on resting blood and saliva immune parameters in endurance trained athletes. Fifteen endurance athletes (male = 9, female = 6, age = 27.9 ± 2.2 yrs, height = 177.0 ± 2.1 cm, weight = 68.5 ± 2.8 kg, running = 64.1 ± 3.2 km/wk) participated in a randomized double-blind glutamine/placebo cross-over study. Thirty grams of glutamine or placebo was provided for a duration of 2 weeks, separated by a 4 week washout. Resting blood and saliva was collected before and after each supplementation period and was analyzed for, total leukocytes, neutrophils, monocytes, lymphocytes, T cells, B cells, natural killer, CD4, CD8 cells, saliva IgA and plasma glutamine using a 2-way repeated measures factorial ANOVA. The effect of gender was analyzed by a 3-way mixed plot factorial ANOVA. As well, subjects completed a nutritional analysis, a life stress questionnaire and a training log during the supplementation periods in order to monitor any changes in these control variables. Statistical analysis revealed that leukocyte and neutrophil count as well as percent neutrophils for female subjects significantly increased by 13.7 %, 33.5 %, and 16.1 %, respectively following glutamine supplementation, while those receiving placebo decreased by 13.2 %, 23.2 %, and 10.5 %, respectively ($p<0.05$). There was no significant change ($p>0.05$) in any immune parameters of male athletes. This data suggests that under these conditions oral glutamine supplementation may be an effective method of increasing leukocyte and neutrophil counts in endurance trained females.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

Purpose of the Study

The purpose of this study was to determine the effect of oral glutamine supplementation on resting blood and saliva immune parameters in endurance trained athletes.

Significance of the Study

During exercise training the duration and the intensity of the program may ultimately dictate the effect that the training has on immune function and infection. It has generally been accepted that moderate intensity exercise may help improve immune function, which will therefore decrease the risk of developing infection. On the other hand high intensity exercise may be detrimental to immune function and lead to a higher rate of infection in athletes (Nieman and Pedersen, 1999). One possible mechanism for the decreased immune function and increased infection rates associated with intense exercise and overtraining is a decrease in glutamine stores. Recent studies have shown that athletes participating in high intensity exercise or those who are in a state of overtraining have lower glutamine stores than other athletes and control subjects (Parry-Billing et al., 1992; Rowbottom, Keast, Goodman, & Morton, 1995; Keast, Arstein, Harper, Fry, & Morton, 1995; Castell et al., 1997).

Glutamine is the most abundant amino acid in the blood and in the muscles. Glutamine is the primary fuel for rapidly dividing cells, including leukocytes. In particular,

glutamine is important for the proliferation of lymphocytes and macrophages. Therefore, glutamine is considered to be important in the maintenance of proper immune function (Ardawi & Newsholme, 1985; Hall, Heel, & McCauley, 1996; Walsh, Blannin, Robson, & Gleeson, 1998). Under normal conditions glutamine is considered to be a non-essential amino acid. Therefore, glutamine is readily synthesized in the body by other amino acids and intermediates (Lacey & Wilmore, 1990). However, during times of catabolic and metabolic stress the body's synthesis of glutamine does not meet the body's need for glutamine. This imbalance in glutamine homeostasis may then result in glutamine depletion and deficiency in these stressed individuals (Walsh et al., 1998).

During times of stress such as when overtrained or during cancer, the body may require between 20 to 40 grams of glutamine to maintain proper homeostasis (Krebs, 1935). However, most diets provide less than 10 grams of glutamine a day (Hall et al., 1996). Therefore, it has been suggested that this glutamine depletion can be corrected by supplementing with glutamine in the diet. Furthermore, glutamine supplementation may be a useful practice for those individuals experiencing impaired immune function due to various physiological stresses (Chandra, 1995).

Several studies involving athletes, individuals with cancer and those with critical illnesses have evaluated the role of glutamine supplementation on immune function, and the rate of infection. Both Castell et al. (1997) and Rohde, Asp, MacLean and Pedersen (1998) found that providing glutamine supplementation to athletes following a marathon did not show any beneficial results. However, Castell, Poortmans, & Newsholme (1996) found that athletes who supplemented with glutamine after running a marathon had a lower rate of

infection. Similarly, Castell and Newsholme (1997) found that athletes supplementing with glutamine after a marathon had a significantly higher CD4/CD8 ratio. Several animal studies have been conducted and have shown that glutamine supplementation may increase survival rate in rats with bacterial infections and those receiving chemotherapy (Adjei, Matsumoto, Oka, Hiroi, & Yamamoto, 1994; Klimberg et al., 1990). It has also been shown that glutamine supplementation in tumor bearing rats has a beneficial effect by reducing tumor growth (Fahr, Kornbluth, Blossom, Schaeffer, & Klimberg, 1994; Klimberg et al., 1992). Clinical investigations in humans have shown that glutamine supplementation has beneficial effects including, decreased hospital stays, increased immune function, decreased use of antibiotics and a decreased overall cost of patient care (Ziegler et al., 1992). Several authors have also found that glutamine supplementation increases immune cell proliferation in cancer patients receiving radiochemotherapy and in bone marrow transplants (Yoshida et al., 1998; Ziegler et al., 1998).

This recent research suggests that glutamine supplementation may be beneficial to individuals experiencing catabolic and metabolic stress. Therefore, the intent of this study is to examine the effect of oral glutamine supplementation on resting blood and saliva immune parameters in endurance trained athletes.

Definitions

Basophils - A type of circulating granulocyte derived from bone marrow with structural and functional similarities to mast cells. These cells play an important role in acute allergic and inflammatory reactions. They account for 0.5-1 % of the total leukocyte count. Normal blood concentrations range from 0.01 - 0.1 x 10^9/L of blood (Abbas, Lichtman, & Pober, 2000; Hoffbrand & Pettit, 1993; Shephard, 1997).

B lymphocytes - Also known as B cells. The only cell of the immune system capable of producing antibodies, therefore making it a central component of the humoral immune response. The B lymphocytes mature in the bone marrow, hence the term "B" cell. The B lymphocytes account for 5-15 % of the total lymphocytes (Abbas et al., 2000; Hoffbrand & Pettit, 1993; Shephard, 1997).

CD - Cluster of Differentiation. One or more cell surface molecules, detectable by monoclonal antibodies that define a particular cell line or state of cellular differentiation (Hoffbrand & Pettit, 1993).

Cytotoxic T lymphocytes- Also known as cytolytic T lymphocytes. Sub-class of the T-lymphocytes that express the CD8 cell surface antigen. Major function is to recognize and kill host cells infected with viruses or other intercellular microbes. The cytotoxic T cells comprise 30 - 40 % of the T lymphocytes population (Abbas et al., 2000; Shephard, 1997).

ECD - Phycoerythrin-Texas Red, excites at 486nm-580nm, and emits at 610nm-635nm.

ELISA - Enzyme linked immunosorbent assay. Assay used for the detection and quantitation of an antibody or antigen using an anti-immunoglobulin conjugated to and enzyme which changes color of a substrate (Roitt, 1994).

Eosinophils -These cells are found in the tissues in many diseases, but predominantly in two forms: allergy and parasitic infections. They account for 1-6 % of total leukocyte count. Normal concentrations range from 0.04 - 0.44 x 10^9/L of blood (Hoffbrand & Pettit, 1993; Shephard, 1997).

FITC - Fluorescein isothiocyanate, excites at 468nm-509nm, and emits at 504nm-541nm.

Glutamine - Five carbon amino acid with two amide moieties, an α-amino group and a terminal amide group. It is the most abundant amino acid in the blood and in the free amino acid pool of the body. Glutamine plays an important role in the transport of nitrogen between organs. Glutamine is also an important fuel for some cells of the immune system. Normal plasma levels of glutamine range from 500-700 μmol/L (after an overnight fast)

(Walsh et al. 1998; Hall et al., 1996; Lacey and Wilmore, 1990).

Helper T lymphocytes - A functional sub-class of the T lymphocytes that express the CD4 cell surface antigen. These cells main functions are to release cytokines which promote the proliferation of T and B cells, activate killer cells, and regulate inflammatory reactions. Helper T cells account for 60-70% of the total T lymphocytes (Shephard, 1997).

Immune Response - A complex sequence of events performed to discriminate between host cells and foreign cells and thereby eliminated the latter. It is dependent on three main cell types: macrophages, T lymphocytes and the B lymphocytes (Hoffbrand & Pettit, 1993).

Immunocompromised - This term will be used interchangeably with immunosuppression and immunodeficiency throughout this paper. It is a state of functional unresponsiveness, due in some circumstances to depletion of specific immune components, which may have been induced by drugs, physical agents, infection and cancer (Ziegler, 1991).

Immunoglobulin - Soluble factors of the immune defense system. They are glycoproteins secreted by B lymphocyte derived plasma cells (Shephard, 1997).

Immunoglobulin A - IgA is the principle immunoglobulin found on the mucosal surface and in various secretory fluids (Shephard, 1997).

LAK - Lymphokine-activated killer cells. Have a similar function to natural killer cells (Shephard, 1997).

Leukocytes - Also known as white blood cells. All leukocytes express the CD45 cell surface antigen. There are groups of cells that mediate the immune response. Normal circulating concentrations range from 4 -11 x 10^9/L of blood. The leukocytes are further differentiated into the neutrophils, eosinopils, lymphocytes and monocytes (Hoffbrand & Pettit, 1993; Shephard, 1997).

Lymphocytes - The only cell in the human body capable of recognizing and distinguishing different antigenic elements. These cells account for 20-50% of total leukocyte count. Normal concentrations range from 1.5-3.5 x 10^9/L of blood. The lymphocytes are further differentiated into the B lymphocytes and the T lymphocytes (Abbas et al., 2000; Hoffbrand & Pettit, 1993; Shephard, 1997).

Monocytes - These cells are involved in the central role of host defense against infection. These cells are phagocytotic. These cells are recruited into inflammatory sites, where they differentiate into macrophages. They account for 2-10% of total leukocyte count. Normal plasma concentrations range from 0.2 - 0.8 x 10^9/L of blood (Abbas et al., 2000;

Hoffbrand & Pettit, 1993; Shephard, 1997).

Natural Killer Cells - A sub-set of the lymphocytes that function in the innate immune response and are important in fighting virus infected tumor cells. These cells respond to kill microbe-infected cells by a lysis mechanism and by secreting interferon-Y, which activate macrophages to destroy these cells. They account for 10-20 % of total lymphocyte count (Abbas et al., 2000; Hoffbrand & Pettit, 1993; Shephard, 1997).

Neutrophils - Also called polymorphonucleur leukocytes (PMN). These cells are responsible in maintaining normal host defenses against microorganisms and involved in the early stages of the inflammatory response. These cells are phagocytotic. They account for 40-75 % of total leukocyte count. Normal plasma concentrations range from 2.5 -7.5 x 10^9/L of blood (Abbas et al., 2000; Hoffbrand & Pettit, 1993; Shephard, 1997).

Overtraining - A syndrome which usually occurs when the athlete's training exceeds the body's physiological and psychological capacity to cope with the stress. This often results in fatigue, muscle soreness, decreased performance, suppressed immune function and increased vulnerability to infection (Ketner & Mellion, 1995; Shephard, 1997).

PC5 - Phycoerythrin-Cy5, excites at 486nm-580nm, and emits at 660nm-680nm.

Phagocytosis - The engulfing of large parasites or other particles by certain cell of the immune system, including neutrophils and macrophages (Abbas et al., 2000).

RD1 - Phycoerythrin, excites at 486nm-580nm, and emits at 568nm-590nm.

Suppressor T lymphocytes - A sub-set of the T lymphocytes that secretes cytokines that block the activation and function of other effector T lymphocytes (Abbas et al., 2000).

T Lymphocytes - These cells are also known as the T cells. These cells are involved in the hosts defense against virus infections. The T lymphocytes mature in the thymus, hence the term "T" cell. These cells mediates cell-mediated immune responses in the adaptive immune system. The T lymphocytes account for 60-75% of the total lymphocytes. The T lymphocytes are further differentiated into the T helper and T cytotoxic cells (Abbas et al., 2000; Hoffbrand & Pettit, 1993; Mackinnon, 1999; Shephard, 1997).

Chapter 2

REVIEW OF LITERATURE

Immune System

The immune system is a unique and complex system of the body that has developed the ability to distinguish the body's own cells from foreign cells. The ability to perform this function allows the immune system to protect the body against foreign elements such as microorganisms, viruses, allergens, tumour growth and cell and tissue transplantation (Mackinnon, 1999).

The immune system can be simplified into two main components; innate immunity and adaptive immunity (Table 1). Innate immunity or natural immunity is the component of the system that occurs immediately. Although the innate defence is immediate, it has predetermined nonspecific protection against an immune challenge. However, this is the body's first line of host defence. Innate immunity includes physical barriers, chemical barriers, and cellular components (Mackinnon, 1999; Shephard, 1997). Adaptive immunity or acquired immunity occurs due to a response to a pathogen. The adaptive component of the immune system is specific to a given foreign micromolecule. The major components of adaptive immunity include, humoral factors and cellular factors. Both the innate and adaptive systems work together in order to provide optimal host defence (Mackinnon, 1999; Shephard, 1997).

Components of Innate and Adaptive Immunity

Innate Immunity	Adaptive Immunity
Physical Barriers Skin Mucus Epithelial Cell Barriers Chemical Barriers Complements Lysozymes pH Acute Phase Proteins Other Soluble Factors Cellular Components Monocytes/Macrophages Granulocytes (e.g., neutrophils) Natural Killer Cells	Humoral Factors Antibodies Immunologic Memory Cellular Factors T-lymphocytes B-lymphocytes LAK / Cytotoxic Cells Plasma Cells

Table 1.

Cells of the Immune System

Leukocytes or white blood cells (WBC) are found in various tissues in the body, as well as in the blood and lymph circulation. Leukocytes originate from stem cells in the bone marrow, but certain cell types mature in the thymus (T lymphocytes) and bone marrow (B lymphocytes). Leukocytes migrate between different lymphoid tissue via the circulation and lymphatic system. Only 1-2 % of the body's total leukocytes are present in the blood at any given time (Mackinnon, 1999).

The number of leukocytes in normal blood ranges between 4-11 x 10^9/L of blood.

Fluctuations occur during the day; with the lowest values occurring during rest. These leukocytes are further broken down into sub-populations with specific functions during the immune response. The major types of leukocytes found in the circulation are the granulocytes, monocytes, and lymphocytes. The granulocytes sub-set of the leukocytes includes the basophils, eosinophils, and neutrophils. The basophils are the least common of all the circulating granulocytes. They account for 0.5 - 1% (0.01-0.1 x 10^9/L of blood) of the total blood leukocytes in the body. These cells release histamine and other substances which play an important role in acute allergic and inflammatory reactions. The eosinophils account for approximately 1 - 6 % (0.04 - 0.44 x 10^9/L of blood) of the total blood leukocyte count in the body. These cells are involved in phagocytosis and are active against parasites that are too large to be engulfed by the other types of leukocytes. The eosinophil can also generate reactive oxygen species that attack the cell membranes of invading organisms. The neutrophils make up the largest portion of the leukocytes comprising approximately 40 - 75 % (2.5 - 7.5 x 10^9/L of blood) of the total blood leukocyte concentration. Neutrophils are phagocytic, and can engulf and digest bacteria, microorganisms and microscopic particles. They have the ability to enter tissues early in the inflammatory response and are also able to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). The monocytes form 2 - 10 % (0.2 -0.8 x 10^9/L of blood) of the total leukocyte concentration. The majority of the monocytes leave the blood and develop into macrophages in various tissues. These cells are involved in phagocytosis and secrete cytokines which activate other elements of the immune system (Mackinnon, 1999; Roitt, 1994; Shephard, 1997).

The lymphocytes account for 20 - 50 % (1.5 - 3.5 x 10^9/L of blood) of the total blood leukocyte concentration. The lymphocytes are further broken down into the T lymphocytes or T cells, B lymphocytes or B cells and the natural killer cells. The T-lymphocytes account for approximately 60 - 75 % of the total lymphocyte count and are involved in the defense against virus infections. All T lymphocytes carry the CD3 cell surface antigen. The T lymphocytes are differentiated further into two major groups, the T helper and T cytotoxic cells. The T helper cells account for 60-70% of the total T lymphocytes and carry the CD4 cell surface antigen. The main function of these cells are to release cytokines which promote the proliferation of T and B cell, activate killer cells, and regulate inflammatory reactions. The T cytotoxic cells carry the CD8 cell surface antigen and account for 30 - 40 % of the total T lymphocyte population. These cells are activated by cytokines, and directly destroy infected cells by recognizing the foreign surface constituents of these abnormal cells. Natural killer cells represent 10 - 20 % of the total circulating lymphocyte population and are characterized as being large granular lymphocytes. Natural killer cells are defined as a subset of the lymphocytes as cells expressing the CD16 and CD56 surface antigens but they lack the CD3 surface antigen. These cells function to recognize and kill tumor and other viral infected cells (Mackinnon, 1997; Shephard, 1997).

The B lymphocytes account for 5 -15 % of the total lymphocytes and carry the CD19 cell surface antigen. B lymphocytes are stimulated by T helper cells in response to an infection. These B cells then differentiate into plasma cells that produce and secrete antibodies. An antibody is an immunoglobulin (Ig) that reacts specifically with an antigen.

There are five classes of immunoglobulins (IgG, IgA, IgM, IgD, IgE), each with distinct properties. Immunoglobulin A (IgA) is the predominant Ig in mucosal secretions found in tears, breast milk, and fluids of the respiratory, genitourinary, and gastrointestinal tracts.

Exercise and Immunology

Throughout the fitness/sports world the general belief is that regular physical activity is beneficial and will help decrease an individual's risk of infection. However, recent literature has suggested that the duration and intensity of the exercise is very important when determining the effect of various exercise and training programs on the active individual (Nieman & Pedersen, 1999). Research has suggested that moderate intensity exercise may help increase immune function and therefore may reduce the risk of acquiring various infections. A number of studies have looked at this phenomenon and found that low to moderate intensity exercise is beneficial to immune function. Newsholme (1994) indicated that low intensity exercise enhances lymphocyte response and natural killer cell activity in the circulating blood. Nieman et al. (1993) found that the incidence of upper respiratory tract infection was less in active subjects compared to sedentary subjects. The results showed that the individuals participating in a walking program had a 21 % ($p < 0.042$) incidence of infection compared to a 50 % incidence of infection in the matched-pair control subjects. Furthermore, Nieman, Nehlsen-Cannarella, et al. (1990) found that individuals participating in a walking program reported fewer days of infection (5.1 days, $p < 0.039$) compared to the inactive control subjects (10.8 days).

Although low to moderate intensity exercise may be beneficial to immune function and possibly help decrease the risk of infection in these individuals, high intensity exercise has been shown to elicit the opposite effect. Several clinical and epidemiological studies have indicated that athletes are at a higher risk of infection after intense training or following competitive events (Nieman and Petersen, 1999). It has been suggested that prolonged cardiovascular endurance exercise may lead to a significant change in immune function therefore increasing the risk of infection in these athletes. After this type of exercise there may be an "open window" which may allow viruses and bacteria to gain a foothold, which may in turn increase these athletes' risk of infection following exercise (Nieman and Petersen, 1999). Newsholme (1994) also states that these changes in the immune system associated high intensity long duration exercise may have a adverse effect on the health of the athlete, in particular during times of increased training. This may result in the athlete being more susceptible to infection during crucial times in the season. There is considerable evidence that shows that athletes participating in high intensity exercise have decreased immune function and a higher incidence of infection compared to the general population. Newsholme (1994) reports that this type of athlete has been shown to have a higher incidence of upper respiratory tract infection (URTI) and intestinal problems than the general population. Nieman, Johanssen, Lee, Cermak, & Arabatzis (1990) reported that 12.9 % of individuals participating in the 1987 Los Angeles marathon experienced upper respiratory tract infections while only 2.2 % of the non-participating control subjects (who did not participate) experienced upper respiratory tract infections. It was also discovered that runners who had trained at a volume of 96 km/week (for a period of 2 months prior)

doubled their odds of getting an infection compared to those athletes who trained at a volume of 32 km/week. Peters and Bateman (1983) also found an increased infection rate in athletes participating in intense competition. The results from this investigation indicated that the incidence of upper respiratory tract infection was 33.3 % in runners after a 56 km marathon compared to only 15.3 % in the control subjects not participating in the marathon. These results, were in line with the finding of Peters (1990) who found a 28.7 % infection rate in marathon runners competing in a 56 km marathon as compared to a 12.9 % rate of infection in the control subjects not participating in the marathon. It has also been suggested that running mileage is a significant risk factor for developing URTI (Heath et al., 1991).

The relationship between upper respiratory tract infection and exercise has been explained by Nieman (1994) as being a J-shaped curve as depicted in Figure 1. This relationship proposes that sedentary individuals will have an average risk of developing an upper respiratory tract infection while subjects who are involved in moderate intensity and duration exercise will have a below average risk of developing an URTI. Furthermore, as training intensity and duration increases so will the risk of developing an URTI. Therefore, manipulating training intensity and duration will either increase or decrease an individual's chance of developing an URTI.

These increased infection rates may ultimately be due to a decrease in immune function in these individuals. There is considerable evidence that prolonged exhaustive type exercise has a detrimental effect on immune cells, in particular the natural killer cells, neutrophils, lymphocytes, T-lymphocytes, B-lymphocytes, CD4/CD8 ratio and

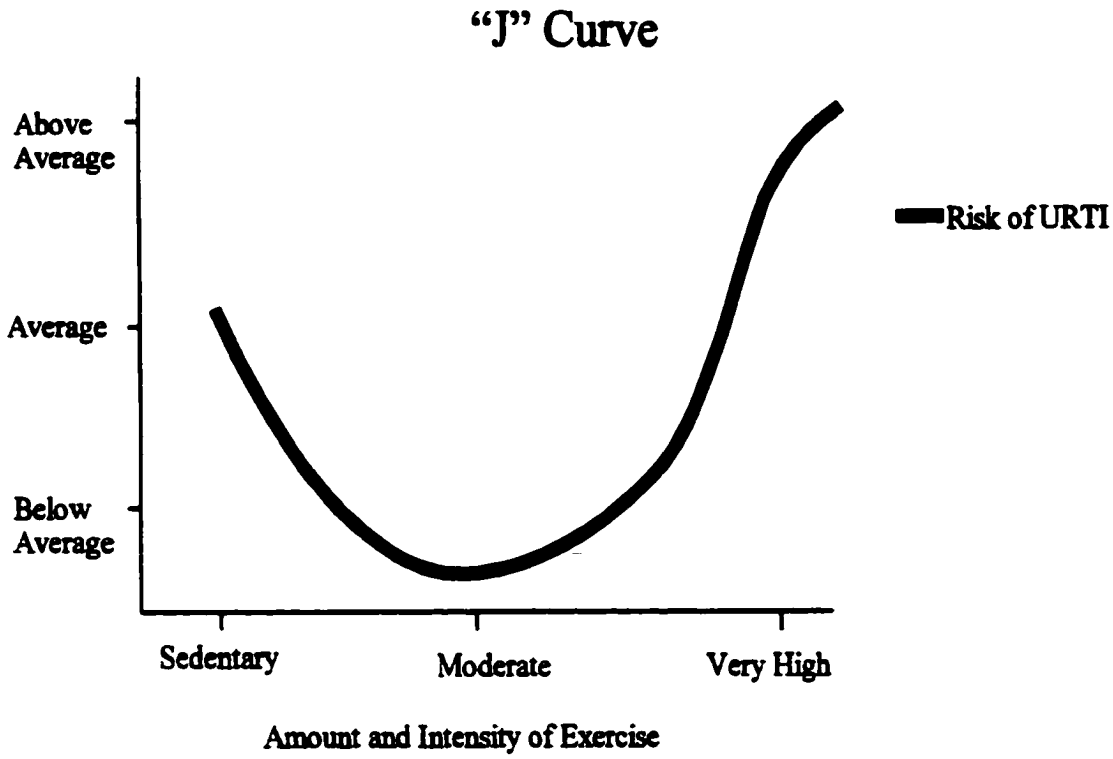

Figure 1 - "J" curve model on the relationship between amount and intensity of exercise and risk of upper respiratory tract infection (URTI) (Nieman, 1994).

immunoglobulins (Gleeson et al., 1995; Lehmann et al., 1992; Lehmann et al., 1996; Mackinnon & Hopper, 1996; Nieman and Pedersen, 1999; Newsholme, 1994; Northoff, Enkel, & Weinstock, 1995; Pyne et al., 1995). Mackinnon, Ginn, & Seymour (1993) showed that elite squash and hockey players had a lower salivary immunoglobulin A (s-IgA) preceding an upper respiratory tract infection. McDowell et al. (1992) also found that salivary immunoglobulin A was decreased by 24.4 % ($p < 0.05$) after subjects completed a maximum treadmill test.

Frisina, Gaudiere, Cable, Keast, & Palmer (1994) found that the percentage of T lymphocytes and B lymphocytes decreased by 14 % ($p < 0.01$) and 9 % ($p < 0.01$), respectively after subjects exercised at 112 % of their maximum aerobic work capacity. There was also a decrease of 47 % ($p < 0.05$) in the CD4/CD8 ratio associated with this exercise protocol. The authors state that a reduction in the CD4/CD8 ratio that occurred after exercise may have detrimental effects on the athlete.

Collectively, these data suggest that following prolonged exercise, immune function is suppressed and therefore it would make sense that athletes participating in these types of events or training regimens would have a higher incidence of infection than athletes participating in moderate intensity exercise (Nieman and Pedersen, 1999).

Glutamine

Proteins are important parts of our diet because they help support growth and repair various components in the body. These proteins are made up of smaller units called amino acids. Amino acids are commonly found in food and can also be synthesized in the body from other amino acids. The amino acids that cannot be synthesized in the body are termed "essential" amino acids. Therefore, these amino acids must be supplied from an exogenous source (our diet). The remaining amino acids can be synthesized in the body from other amino acid and intermediates. Thus, these amino acids are termed "non-essential" or "dispensable" amino acids. Therefore, under normal physiological conditions there is no real need for these non-essential amino acids in our diet (Lacey & Wilmore, 1990).

However, under certain physiological conditions the need for some of these non-essential amino acids may be greater than the endogenous (body's) rate of synthesis. Therefore, an amino acid that is usually considered to be non-essential becomes essential for this individual at that particular point in time. When this occurs, this amino acid is considered to be "conditionally" essential, therefore becoming necessary under that particular physiological condition (Lacey & Wilmore, 1990).

One example of a conditionally essential amino acid in human nutrition, is cysteine for newborns. This amino acid is essential because newborns are not mature enough to produce the enzyme (cystathionase) needed to synthesize cysteine in the body. Another example would be tyrosine and cysteine in individuals with liver problems. Due to the fact that these individuals livers have lost the ability to synthesis these non-essential amino acids, an exogenous source of tyrosine and cysteine is essential (Gaull, Rassin, Raiha, & Heinonen, 1977; Lacey & Wilmore, 1990). Recently, glutamine has been identified as possibly being a conditionally essential amino acid for individuals experiencing catabolic and metabolic stress (Lacey and Wilmore, 1990).

Glutamine is the most abundant amino acid in the blood and in human muscle (Calder, 1995; Lacey and Wilmore, 1990). The concentration of glutamine in the skeletal muscle contributes approximately 60 % of the total free amino acid pool (Calder, 1995). Normal plasma glutamine concentrations range from 500-700μmol/l (Castell & Newsholme, 1997). Glutamine is the most important nitrogen carrier and it is essential for protein synthesis and cell proliferation in the body (Rohde, MacLean, & Pedersen, 1998).

Skeletal muscle has the enzymatic capacity to synthesize glutamine. Therefore, skeletal muscle is the major tissue involved in glutamine production and is the major tissue that releases glutamine into the blood. It has been suggested that the skeletal muscle plays a vital role in the process that regulates glutamine utilization by the immune system in the blood. Therefore, any activity that involves the skeletal muscle may ultimately affect the immune system. Although the skeletal muscle can synthesize glutamine under normal conditions, it has been suggested that during times of metabolic stress, such as cancer, burns, sepsis, surgery and after exhaustive type exercise, the body's synthesis may not meet the body's requirements. This may result in a glutamine deficiency in these individuals and impair their immune function (Ardawi & Newsholme, 1985; Rohde, Asp et al., 1998).

Animal studies have shown that the concentration of glutamine in skeletal muscle is lower in stress situations, such as sepsis, cancer and after burn injuries (Calder, 1995). These findings are similar in humans where decreased plasma concentrations are also lowered by sepsis, injury, burns, surgery, endurance exercise and overtraining. Furthermore, research has shown that intramuscular glutamine stores are lowered by 50 % in these situations (Calder, 1995; Parry-Billing, Evans, Calder, & Newsholme, 1990).

The observations that lower plasma glutamine concentrations occur during these situations has led some to suggest that this is due to the increased demand by other tissues such as the kidney, liver, gut and the immune cells (Calder, 1995).

Glutamine and the Immune System

Glutamine is known to be an important fuel for some cells of the immune system, in particular lymphocytes and monocytes (Newsholme, 1994; Nieman and Petersen, 1999). Data has also suggested that the CD4 cells (T helper) have an above average dependence on glutamine when stimulated in vitro (Hack et al., 1997; Tvede et al., 1989). The high rate of glutamine utilization even at rest permits these cells to respond to an immune challenge without delay. For example, B and T lymphocyte proliferation occurs when a foreign microorganism is invading the body. When this occurs, these cells must respond as rapid as possible in order to defend the host. Any delay in the response to this microorganism caused by a low rate of fuel (glutamine) may result in a late response and may result in an ineffective control of the foreign agent (Newsholme, 1994).

Several immunostimulator actions of glutamine have been reported, including in vitro proliferation of lymphocytes in rats, mice and humans. Furthermore, when glutamine is absent these lymphocytes do not proliferate, but as glutamine concentrations in culture increase, lymphocyte proliferation also increases (Ardawi & Newsholme, 1983; Calder, 1994; Parry-Billing, Blomstrand, McAndrew, & Newsholme, 1990).

Due to the fact that some cells of the immune system are dependent on glutamine concentrations in vitro at a specific physiological range, immune cells in vivo may also be dependent on glutamine over this same range. Therefore, it would be likely that decreased plasma glutamine concentrations would at least partially lead to immunosuppression in the human host (Calder, 1994). Due to the importance of glutamine to the cells of the immune

system, there has recently been some evidence to support the hypothesis that decreases in immune function may be in part due to a decrease in plasma glutamine levels (Hack et al., 1997).

Glutamine and Exercise

After exhaustive type exercise glutamine concentrations have been shown to decrease below pretesting values. Newsholme, Parry-Billing, McAndrews, & Bidgett (1991) showed that plasma glutamine concentration decreased by 16 % after a marathon race and still remained decreased for several hours after the race. Athletes who were diagnosed as being overtrained also showed a significantly lower (9%) glutamine concentration compared to match paired control subjects (Parry-Billing et al., 1992). Rowbottom et al. (1995) found similar results when comparing 10 athletes diagnosed to be overtrained to match-paired controls not diagnosed as being overtrained. The plasma glutamine concentrations of the overtrained athletes were significantly lower (704 μmol/L, $p < 0.01$) compared to the control subjects (1000 μmol/L). Keast et al. (1995) found that when subjects ran at 90 % and 120 % of their VO_2max there was a 44 % ($p < 0.05$) and a 55 % ($p < 0.01$) decrease in plasma glutamine level compared to pretest values. Furthermore, Castell et al. (1997) found that after subjects participated in a marathon plasma concentrations decreased by 19 % ($p < 0.05$) coupled with a 40 % ($p < 0.01$) decrease in natural killer cell concentrations.

It is unclear what the underling mechanism is for the decreased glutamine concentration seen following long term exercise and overtraining. However, it is possible that these conditions interfere with the control of the rate of glutamine released from the

muscle and therefore cause a decreased glutamine concentration in these individuals (Newsholme, 1994). Parry-Billing et al. (1992) have also suggested that overtraining may decrease the rate of glutamine released by the muscle, therefore causing a decrease in plasma glutamine concentration and adversely affecting immune cell concentrations. Therefore, it has been suggested that the immunosuppression and increased infection rate associated with high intensity, long duration exercise and overtraining may be in part, a result of low plasma glutamine concentrations (Keast et al., 1995; Parry-Billing et al., 1992).

Rowbottom et al. (1995) have modified the J curve presented by Nieman (1994) by adding plasma glutamine concentrations to the model to explain the relationship between upper respiratory tract infection and glutamine concentration, as seen in Figure 2. It is proposed that sedentary individuals will have both an average glutamine concentration and an average risk of developing an upper respiratory tract infection, whereas individuals engaged in moderate amounts and intensity exercise will have a higher glutamine concentration and a lower risk of developing a URTI. Furthermore, as amount and intensity of exercise increases there will be a decrease in glutamine concentrations coupled with a higher risk of developing an URTI.

Because lower glutamine concentrations have been associated with decreased immune function and increased infection in intense training athletes, it has been suggested that plasma glutamine concentrations can be a helpful tool in determining overtraining status. Although some evidence indicates that symptoms occurring during

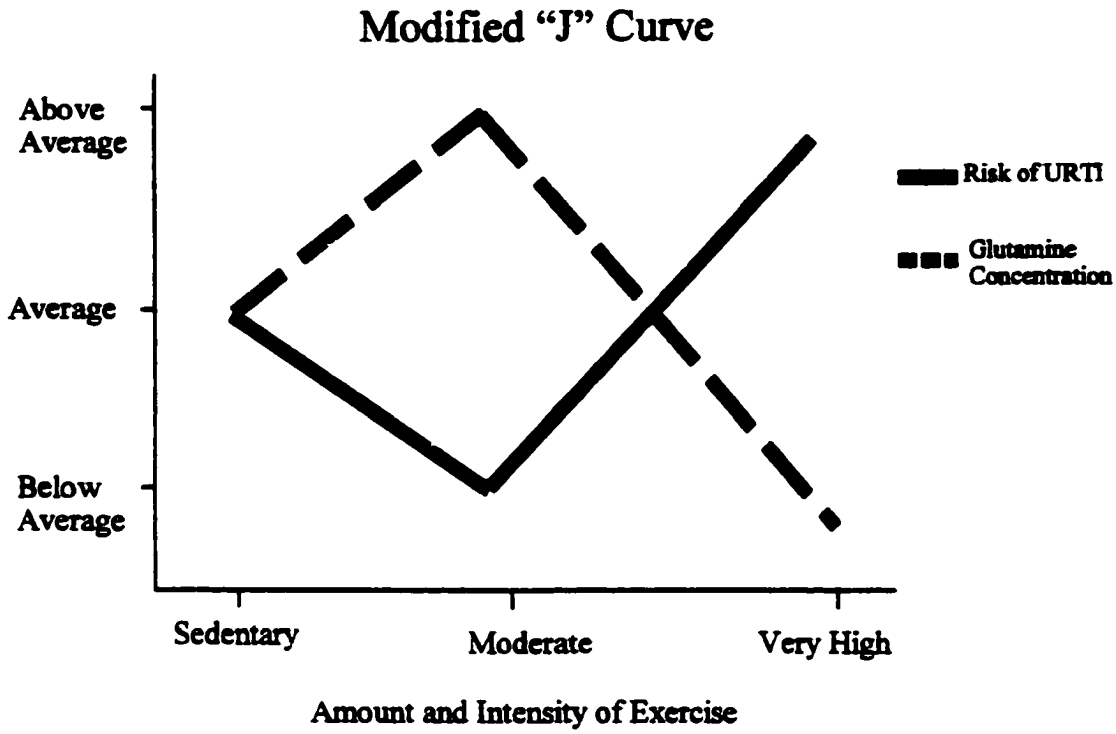

Figure 2 - Modified "J" curve of the relationship between various amounts of exercise and both upper respiratory tract infection (URTI) and plasma glutamine concentrations (Rowbottom et al., 1995).

falls in glutamine levels are parallel to those seen during overtraining syndrome, further research should be conducted to determine its validity as a physiological marker for overtraining (McKenzie, 1999; Rowbottom et al., 1995). However it has recently been suggested that glutamine concentrations are a useful tool in monitoring tolerance to training volume (Smith & Norris, 2000).

Glutamine Supplementation

Moderately severe protein energy malnutrition has been shown to impair a number of immune responses including decreased T lymphocyte levels and in particular the CD4 concentration. Therefore, a modest increase in the amounts of some nutrients such as glutamine may enhance immune function. This may be a useful practice in the management of individuals experiencing impaired immune function due to a particular physiological stress such as surgery, cancer, and overtraining (Chandra, 1995).

Due to the fact that plasma glutamine concentrations decline during catabolic illness in spite of the increased release from the muscle, Smith and Wilmore (1990) suggest that endogenous synthesis is not adequate to meet the increased demands of glutamine utilization and therefore, a deficiency may develop. During these times of physiological stress it may be important to consume 20 - 40 grams of glutamine per day to maintain proper homeostasis in the body (Krebs, 1935).

Most naturally occurring proteins contain 4 - 8 % of their amino acid composition as glutamine. However, the daily consumption of glutamine has been estimated to be less than 10 grams per day (Hall et al., 1996). If we examine the above value (4 - 8 %) and the Recommended Dietary Allowance (RDA) for protein (0.8 grams/kg of body weight), a 75 kg individual meeting their RDA would consume approximately 2.5 - 5 grams of glutamine a day. This value is consistent with that of Hall et al. (1996).

The high rate of glutamine utilization by the lymphocytes, macrophages and other immune cells coupled with the increased dietary need for glutamine during times of physiological stress, suggest that the provision of glutamine may be important in the

maintenance of immune function. Data has suggested that an optimal amount of glutamine is required for the immune system to function effectively (Calder, 1995). Therefore because of glutamine's stimulatory effect on immune function, it would seem appropriate to provide glutamine to individuals after surgery, burns, sepsis, injury and exhaustive training or exercise (Calder, 1995).

Safety of Glutamine Supplementation

Several studies have looked at the safety of glutamine supplementation in humans. Ziegler et al. (1990) found that glutamine at a dose of 0.57 grams•kg^{-1} body weight per day is well tolerated in healthy humans in the form of an oral or intravenous bolus, as short-term infusion or as a major part of intravenous formulas. The authors also state that glutamine supplementation appears to be safe in bone marrow transplant patients for a period of up to four weeks. Furthermore, glutamine was readily metabolized and cleared from the blood without significant generation of toxic end products. Lacey et al. (1996) also found that premature infants fed 20% of their total protein requirements as glutamine had no abnormal biochemical effects. Savy (1997) notes that common hospital feeding of glutamine to adults is prescribed at a dose of 30 grams daily divided into three 10 gram doses. This dose can be adjusted to 20 grams for smaller individuals and 40 grams for larger individuals.

Cancer and Critical Illness Studies

Several animal studies have evaluated the effect of glutamine supplementation on immune function, tumor size and mortality. Adjei et al. (1994) fed mice a standard casein diet or a diet supplemented with 4% (by weight) glutamine. The mice were then inoculated with *Staphylococcus aureus* (bacterial infection) and monitored for a period of 20 days. The results indicated that there was only a 20 % survival rate in the mice fed casein and a 66 % survival rate in the mice fed glutamine. Klimberg et al. (1990) found similar results in rats receiving whole abdominal radiation. Rats who received 3 % glutamine as part of their diet had a 100% survival rate whereas those rats fed 3% glycine as part of their diet had only a 45% ($p< 0.01$) survival rate.

In a study with tumor bearing rats, Fahr et al. (1994) fed half of the rats a glycine diet while the remaining rats received glutamine. The results showed that the rats fed a glutamine diet had a 40 % decrease in tumor growth coupled with a 30 % increase in natural killer cell activity. The authors suggest that glutamine supplementation may help reduce tumor size by promoting natural killer cell activity. Similarly, Klimberg et al. (1992) found that when rats receiving chemotherapy (methotrexate) were fed glutamine as part of their diet, they had nearly double the loss in tumor volume compared to those receiving glycine as part of their diet.

Clinical investigations indicate that glutamine supplementation in critically ill patients resulted in a decreased rate of infection, a decreased use of antibiotics, an increase in immune function, a decreased hospital stay and an overall decrease in the cost of care (Wilmore et al. 1999). Clinical investigations with cancer patients have also found positive

results following glutamine supplementation. Yoshida et al. (1998) examined the effect of glutamine supplementation in patients with advanced esophageal cancer receiving radiochemotherapy. Results indicated that glutamine supplementation in these patients prevented a reduction in the lymphocyte count (1007 ± 151 vs 567 ± 96 cell/mm^3, $p < 0.01$), T lymphocyte count (854 ± 106 vs 467 ± 80 cell/mm^3, $p < 0.05$) , and B lymphocyte count (86 ± 13 vs 45 ± 14 cell/mm^3, $p < 0.05$) compared to those not receiving glutamine after 7 days. The authors suggest the oral glutamine supplementation protects lymphocytes in patients with advanced esophageal cancer receiving radiochemotherapy.

Ziegler et al. (1992) found that bone marrow transplant patients who supplemented with glutamine developed less infection (3 vs 9, $p = 0.041$), had shorter hospital stays (29 ± 1 days vs 36 ±2 days, $p = 0.017$), and that hospital charges were $21 095 less per patient compared to individuals receiving placebo. Ziegler et al. (1998) found that bone marrow transplant patients supplemented with glutamine as part of their parenteral nutrition had improved immune plasma profiles compared to those receiving standard parenteral nutrition formulas. The glutamine supplemented patients had an increased lymphocyte count (590 ± 71 vs 332 ± 50 cells/μL, $p = 0.01$), greater T lymphocyte count (229 ± 70 vs 54 ± 19 cells/μL, $p = 0.03$), greater CD4 (44 ± 10 vs 19 ± 6 cells/μL, $p = 0.048$), and increased CD8 count (101 ± 15 vs 53 ± 10 cells/μL, $p = 0.015$). The authors suggest that glutamine supplementation may support lymphocyte recovery after bone marrow transplants.

Schloerb and Amare (1993) found that bone marrow transplant patients who supplemented with glutamine had a significantly shorter hospital stay (26.9 ± 1.3 days) compared to those standard parenteral nutrition formulas (32.7 ± 2.1, $p < 0.05$). The authors

suggest that this decrease in hospital stay had significant implications towards overall patient care and hospital economics. Care for these patients was conservatively estimated at $1000 per patient per day. The authors further suggest that if the hospital had 30 patients a year, these results would lead to $180 000 savings a year. Furthermore, a saving of approximately 1 week (5.8 days) in hospital stay would make available 270 extra bed days per year. This is all at the cost of less than $3 a day for 40 grams of glutamine. Jensen, Miller, Talabiska, Fish, & Gianferante (1996) studied intensive care unit patients and found improvements in immune function with glutamine supplementation. Patients who supplemented with glutamine enriched nutritional formulas showed a higher CD4/CD8 ratio compared to baseline values of those who were supplement with a standard nutritional formula. These results seem very promising, however, additional, research is needed to determine the effect of glutamine supplementation on the immune function of individuals with cancer. Furthermore, the effect of oral glutamine supplementation should be evaluated to determine its effectiveness as a nutritional therapy.

Training Studies

Few studies have evaluated the effect of glutamine supplementation on athletes and the few that have been conducted have shown mixed results. Castell et al. (1997) found that glutamine supplementation provided to male athletes after a marathon had no effect on lymphocyte distribution. Rohde, Asp, et al. (1998) also found that there was no benefit when male athletes were provided glutamine. Subjects in that study were provided with glutamine or placebo after a marathon to determine the effect on leukocyte subsets. Results

indicated that glutamine supplementation did not have any effect on any leukocyte subsets in subjects after a marathon. In a similar study Rohde, MacLean, et al. (1998) had male subjects perform three bouts of cycle ergometry lasting 60, 45, and 30 minutes at 75 % VO_2max separated by two hours rest. Although glutamine concentrations were higher in the glutamine supplemented group there was no difference in circulating lymphocytes between groups. Furthermore, Walsh, Blannin, Bishop, Robson, & Gleeson (2000) had male subjects cycle for 2 hours at 60 % VO_2max, while receiving a sugar free drink every 15 minutes for the first 90 minutes of exercise followed by a glutamine or placebo drink for the remainder of the exercise protocol and for 2 hours of recovery. Analysis revealed that glutamine supplementation had no effect on the magnitude of post-exercise leukocytosis, and neutrophil function as assessed by oxidative burst activity.

Some authors have found that glutamine supplementation results in positive effects on immune function following exercise. Castell, Poortmans, & Newsholme (1996) had male and female subjects supplement with either glutamine or a placebo after a marathon or ultra-marathon. Subjects were then required to report their incidence of infection for a period of seven days. The results indicated that subjects reporting no infections were significantly higher in the athletes receiving glutamine (80.8 ± 4.2 %, $p< 0.001$) compared to the athletes receiving placebo (48.8 ± 7.4 %). It has also been reported that a mixed group of male and female athletes receiving glutamine following a marathon or ultra-marathon had a significantly higher CD4/CD8 ratio then athletes receiving a placebo (Castell & Newsholme, 1997).

To this author's knowledge, all studies to date involving athletes and glutamine supplementation have looked at the effect of glutamine following a one day (acute) exercise session. In these studies blood sampling is often performed during or immediately following exercise. This may be a problem because leukocyte count may be increased from the normal values of 4500-11000 cells per mm^3 to an excess of 20000 cells per mm^3 following physical exertion (IIR, 1999). Therefore, a major increase in leukocyte count after exercise may mask any effect (either positive or negative) that may occur by providing glutamine supplementation. If blood sampling was performed at times when leukocyte count was near normal daily baseline values, the effect of glutamine supplementation could be better evaluated. Also, no study to date has evaluated the effect of long term (more than 1 day) glutamine supplementation on the immune function of athletes. The supplementation period, as well as the time of blood sampling may be important points to consider when trying to determine the effect of glutamine supplementation on the immune system of athletes. These points will be addressed during this study. Also, by using a longer supplementation period, a larger dose and evaluating the effect of the supplementation during chronic training the author feels that this may be more applicable to most athletes.

In summary, there is considerable evidence that during times of metabolic and catabolic stress, glutamine concentrations in the body become depleted. Due to the fact that many immune cells are dependent on glutamine as their primary fuel source, immune function may become impaired under these conditions. During these particular conditions glutamine may then be considered to be a conditionally essential amino acid because the

body's synthesis cannot meet it's own needs. Therefore, supplementation with glutamine may be an effective way of improving glutamine status, facilitating immune function, decreasing infection, and improving the overall health of athletes after intense prolonged exercise or those experiencing overtraining syndrome.

Chapter 3

METHODS

Subjects

All subjects for this study participated on a volunteer basis and were free to withdraw from the study at any time. Prior to commencement of the study, all subjects were informed about the purpose of the study before giving written consent to participate. All ethical guidelines established by Lakehead University Ethics Advisory Committee were followed.

Fifteen males and females (male = 9, female = 6, age = 27.9 ± 2.2 yrs, height = 177.0 ± 2.1 cm, weight = 68.5 ± 2.8 kg, running = 64.1 ± 3.2 km/wk) endurance athletes from the Thunder Bay District currently active in aerobic training were selected to participate in the study. Selection criteria was determined by using a questionnaire which allowed the researchers to determine the subjects health status, training volume, training intensity, and dietary practices (supplements taken). To participate in the study subjects meet the following criteria:

Selection Criteria

1) Having no known physical or mental conditions that may effect their participation in the study.

2) Training at a volume of at least 50 km per week.

3) The expectation and willingness to maintain their regular training routine for the duration of the study (8 weeks).

4) The expectation and willingness to maintain their regular dietary intake throughout the duration of the study, while including the appropriate supplement (placebo or glutamine) as part of their regular diet.

5) The willingness to provide a 3 day dietary intake summary before and after each supplementation period.

6) Not currently taking any other supplement (eg. creatine, protein drinks etc.) throughout the entire study.

7)The expectation and willingness to maintain a regular training log, outlining training volume frequency and intensity during each of the 2 week supplementation periods.

8) Willingness to provide a blood and saliva sample during four separate occasions throughout the study.

Exclusion Criteria after Selection

Subjects selected to take part in the study were excluded from analysis if any of the following conditions occurred:

1) Not providing a blood and saliva sample at each of the four sample collection periods.

2) Not ingesting at least 90 % of the supplement during each of the 14 day supplementation periods. Instructions will be given to subjects to return all unused supplement and calculations will be made.

3) Not maintaining their training at a level of ± 20% of their regular training volume.

4) Not providing a 3 day dietary intake summary before and after each supplementation period.

Experimental Design

The study was a randomized double-blind glutamine/placebo cross-over design study which took place over a period of 8 weeks. Subjects acted as their own control as they supplemented with both placebo and glutamine for 2 weeks (14 days) with a 4 week washout period between supplementation periods. There is a lack of research examining the time needed for an adequate washout when supplementing with glutamine. Walsh et al. (2000)

and Rhode, MacLean et al. (1998) had subject supplement with glutamine for a single day and utilized a 7 day washout. Although the supplementation period in this study is longer than these previous studies the authors feel that this will be adequate. Furthermore, the effectiveness of the washout will be tested prior to further analysis. Seven of the subjects (5 males, 2 females) were randomly assigned to supplement their diet with glutamine for 2 weeks and after a 4 week washout period they then supplemented with placebo for an additional 2 weeks (GT/PL). The remaining eight subjects (4 males, 4 females) supplemented their diet with placebo for 2 weeks and then after a 4 week washout period they then supplemented with glutamine for an additional 2 weeks (PL/GT). Each subject was required to visit the laboratory on four separate testing sessions (T1, T2, T3, and T4). Test one (T1) was performed on day 1, whereas test two (T2) was performed on day 15. Test one (T1) and test two (T2) were separated by supplementation period one (S1) which started on day 1 and ended on day 14. This is the supplementation period before the four week washout period (W). Test three (T3) was performed on day 44 of the study (after the washout period) whereas test four (T4) was performed on day 58 of the study (after washout period). Test 3 (T3) and test 4 (T4) was separated by supplementation period two (S2), which started on day 44 and ended on day 57 of the study (Figure 3).

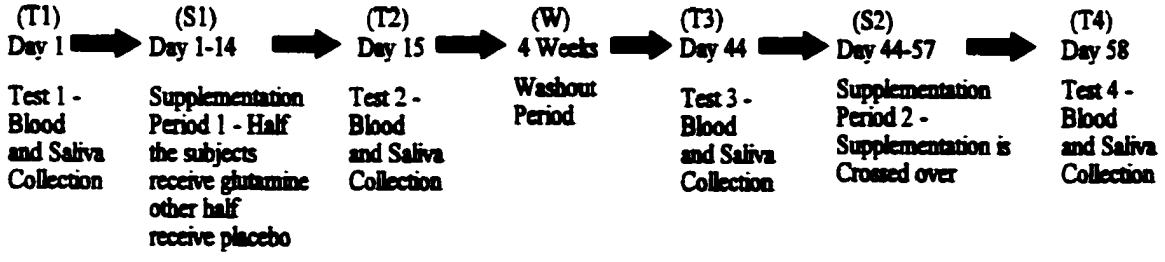

Figure 3.

Haematological and Saliva Collection

Subjects reported to the laboratory in the morning (between 7-9 am) before and after each supplementation period (T1, T2, T3, T4) in a fasted state. Subjects were also instructed to avoid physical activity for a period of 12 hours. Subjects provide both a blood and saliva sample during each of their visits to the laboratory. All blood sampling was performed by a certified Phlebotomist after subjects had rested (seated) in the laboratory for a period of 30 minutes. Blood was collected in Vacutainer[c] tubes by using an antecubital venipuncture technique. Two blood samples were collected from each subject on each visit to the laboratory. A 2.5 ml blood sample was collected in a Vacutainer[c] tube containing the anticoagulant ethlyenediaminetetraacetic acid (EDTA). This sample was used for all haematological analysis (Complete Blood Count and Lymphocyte Differentials). A second 8 ml blood sample was collected in a Sodium Heparin Vacutainer[c] tube. This sample was immediately placed on ice until centrifugation. This sample was centrifuged at 3000g for 10 minutes and then plasma was extracted and transferred to a 5 ml Falcon tube. This sample was then frozen at -70°C until further analysis for plasma glutamine concentrations.

Haematological Analysis

Blood samples were sent to a health unit laboratory where Complete Blood Counts (CBC) were performed within 6 hours using the CELL-DYN[c] 3500, (Abbott Laboratories, Santa Clara, CA). This apparatus discriminates cells according to volume as they induce resistance while passing through an electrical potential (Field, 1996). This analysis yielded the following dependant variables; total leukocytes, neutrophils, eosinophils, basophils,

monocytes, and total lymphocyte counts. Following, the CBC the same sample was used for analysis of lymphocyte differentials, using the EPICS XL MCL™ (Beckman Coulter, Fullerton,CA) flow cytometery. Lymphocyte differentials including T lymphocytes (CD3+), T helper (CD3+CD4+), T suppressor/cytotoxic (CD3+CD8+), B lymphocytes (CD3-CD19+) and natural killer (CD3-CD16+56+) cell differentials were analysed using a flow cytometer. The CD3+CD4+ and CD3+CD8+ counts were also used to develop a ratio score from these two variables (CD4/CD8 ratio). In flow cytometric analysis, cell size is determined through the analysis of its light scatter characteristics, and cell immunophenotyping is defined by specific antibodies tagged with fluorescent dyes. Briefly, this apparatus detects single cell characteristics (size, granularity, fluorescence) by using an optical to electronic coupling system that detects light scatter and fluorescence emissions after cell staining (Field, 1996). In order to achieve accurate and reliable results it is important that light scatter and fluorescence parameters on a flow cytometer are standardized to ensure optimal instrument performance on a daily basis. The use of fluorospheres to standardize light scatter intensity, fluorescence intensity and optimal hydrodynamics focussing is widely accepted.

Before analysis could be performed, calibration and compensations procedures were conducted. Calibration procedures consisted of Flow Check, and Flow-Set. Flow-Check Fluorospheres™ (Beckman Coulter™; PN 6605359) consist of 10 μm (nominal diameter) polystyrene fluorescent microspheres suspended in an aqueous medium containing surfactants and preservatives at 1 x 10^6 fluorospheres/ml (nominal concentration). The fluorescence emission of the dye contained within the fluorospheres range from 525 nm to

700 nm when excited at 488 nm. Flow-Check fluorescence are used to verify instrument alignment and fluidics. Flow-Set Fluorospheres™ (Beckman Coulter™; PN 6607007) are a suspension of fluorospheres (3.6 μm) used as an aid in optimizing a flow cytometer for quantitative analysis of human leukocytes. Each fluorospheres contains a dye which has a fluorescent emission range of 525 nm to 700 nm when excited at 488 nm. They have a uniform size and fluorescence intensity allowing the standardization of light scatter, fluorescence intensity and optimal hydrodynamic focusing instrument settings. Performing both Flow-Check and Flow-Set procedures allows adjustment and/or verification of the alignment of the optical and fluidics system of the flow cytometer. In this study a 500 µl sample of Flow-Set Fluorospheres as well as 500 µl of Flow-Check Fluorospheres were dispensed into two separate tubes labeled Set and Check, and run to ensure calibration of the instrument.

Due to the fact that a four color staining procedure was used during this experiment, compensation measures were also conducted. The CYTO-COMP™ Reagents kit (Beckman Coulter™; PN 6607021) consist of four two color flourescent reagents comprised of two monoclonal antibodies combinations making up each of the reagents. The antibodies are labeled with specific combinations of four fluorochrome; FITC (Fluorescein isothiocyanate), RD1 (Phycoerythrin), ECD (Phycoerythrin-Texas Red), and PC5 (Phycoerythrin-Cy5). The CYTO-COMP™ Reagents are used in conjunction with the CYTO-COMP™ Cells (Beckman Coulter™; PN 6607023), a preparation of lyophilized human lymphocytes and reconstitution buffer used to adjust color compensation settings on a flow cytometer prior to multi-color analysis with FITC, RD1, ECD, and PC5 conjugated monoclonal antibody

reagents.

Briefly, 100 µl of reconstituted CYTO-COMP™ cells were added to four tubes (labeled C1, C2, C3, C4). Then 10 µl of CYTO-COMP™ Reagent 1 (FICT/RD1), CYTO-COMP™ Reagent 2 (RD1/ECD), CYTO-COMP™ Reagent 3 (RD1/PC5), and CYTO-COMP™ Reagent 4 (ECD/PC5) was added to tube C1, C2, C3, and C4, respectively. One millilitre of PBS was then added to each tube and vortexed. After calibration and compensation procedures were complete, samples and control cells were stained for analysis. 100 µl of IMMUNO-TROL™ whole blood control cells (Beckman Coulter™; PN 4238091-E) or sample whole blood from each subject was then added to two separate tubes. IMMUNO-TROL™ Control Cells are a positive process control for flow cytometry assays for lymphocytes, granulocytes, and monocytes specific antigens and single platform absolute counts. This preparation is used to verify the process of sample staining, lysing, and analysis. Light scatter, population distribution, fluorescence intensity, and antigen density mimic those of whole blood. To the tubes containing IMMUNO-TROL™ or test sample whole blood, 5 µl of CYTO-STAT® tetraCHROME™ Reagent CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 Monoclonal Antibody Reagent (Beckman Coulter™ ; PN 6607013) was added to the first tube. Also, 5 µl of CYTO-STAT® tetraCHROME™ Reagent CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5 Monoclonal Antibody Reagent (Beckman Coulter™; PN 6607073) as well as 5 µl of IOTest® Conjugated CD16-PE (ImmunoTech™; PN IM1238) was added to the second tube. All sample and control tubes were then vortexed gently and incubated for 15 minutes at room temperature. 500 µl of

OptiLyse C ® (Beckman Coulter™; PN IM1401) was then added to all sample and control tubes, vortexed and allowed to incubate for 10 minutes. OptiLyse C© lysing solution is an erythrolytic reagent for lysing human red blood cells following direct immunofluorescence staining of whole blood. The final step was to add 500 µl of PBS (Phosphate Buffered Saline) to all samples and control tubes, vortex, and incubate for 20 minutes. These tubes were then loaded on the auto loader and analysed on the EPICS XL MCL™ for the previously mentioned lymphocyte differentials. See Table 2 for sample preparation during flow cytometer analysis.

Sample Preparation for Flow Cytometer Analysis

Reagents	Test Tube Label									
	Check	Set	C1	C2	C3	C4	T Panel	NK-B Panel	T Control	NK-B Control
Flow-Check Fluorospheres	500µl									
Flow-Set Fluorospheres		500µl								
CYTO-COMP Cells			100µl	100µl	100µl	100µl				
CYTO-COMP 1 FITC/RD1			10µl *							
CYTO-COMP 2 RD1/ECD				10µl *						
CYTO-COMP 3 RD1/PC5					10µl *					
CYTO-COMP 4 ECD/PC5						10µl*				
tetraCHROME CD45/CD4/ CD8/CD3							5µl		5µl	
tetraCHROME CD45/CD56/ CD19/CD3								5µl		5µl
IOTEST CD16								5µl		5µl
Test Sample Whole Blood							100µl†	100µl†		
IMMUNO-TROL									100µl†	100µl†
OptiLyse C							500µl‡	500µl‡	500µl‡	500µl‡
PBS			1ml† †	1ml† †	1ml† †	1ml††	500µl††	500µl††	500µl††	500µl† †

Table 2.

* Add CYTO-COMP reagent, vortex and incubate for 15 minutes.

† Add test or control blood, vortex and incubated for 15 minutes.

‡ Add OptiLyse C, vortex, and incubation for 10 minutes.

†† Add PBS, vortex and incubate for 20 minutes.

Plasma Analysis

The determination of glutamine is a two step reaction which first deaminates glutamine to L-glutamate (Reaction A) and then continues with the dehydrogenation of the L-glutamate to α-ketogluterate accompanied by the reduction of NAD^+ (Nicotinamide Adenine Dinucleotide) to NADH (Reaction B). The conversion of NAD^+ to NADH is then measured spectrophotometrically and is proportional to the amount of glutamate that is oxidized, therefore giving the amount of glutamine converted to glutamate in the sample (Lund, 1986).

A standard curve was constructed of known concentrations of L-glutamine (see Table 3). These preparation were then used in Reaction B. All plasma samples were thawed and initially deproteinized with an equal volume of 10 % perchloric acid. These were then centrifuged at 3000g for 3 minutes. The sample was then neutralized with 20 % KOH and allowed to stand for 10 minutes in an ice bath and then centrifuged. The precipitate was then discarded.

Due to the fact that the plasma samples contain both L-glutamine and L-glutamate the samples must be measured for both endogenous L-glutamate and L-glutamate derived from the deamination of L-glutamine. Therefore, it is essential that we measure glutamate levels before the deamination of glutamine to account for this endogenous source. Therefore, two separate tubes were prepared for each sample. Acetate buffer, Glutaminase (Sigma[c], G8880), water, and plasma test samples were used as the reagents for this reaction. See Table 4 for sample preparation for the determination of glutamate (both endogenous

Glutamate and deaminated Glutamine). Following this all tubes were incubated at 37 °C for 60 minutes.

Dilutions for Glutamine Standard Curve Determination

Glutamine Standard Concentration (mM/l)	2 mmol/l Stock Glutamine Solution (µl)	Water (µl)	10 kU/l Glutaminase Solution (µl)	0.5 mol/l Acetate Buffer (µl)
0	0	790	10	200
0.1	50	740	10	200
0.2	100	690	10	200
0.3	150	640	10	200
0.4	200	590	10	200
0.5	250	540	10	200
0.6	300	490	10	200
0.7	350	440	10	200
0.8	400	390	10	200
0.9	450	340	10	200
1	500	290	10	200

Table 3.

Reaction A - Deamination of Glutamine

	Tube	
Solutions	Endogenous Glutamate	Endogenous Glutamate & Deaminated Glutamine
0.5 mol/l Acetate Buffer	200 µl	200 µl
10 kU/l Glutaminase	—	10 µl
Test Sample	250 µl	250 µl
Water	550 µl	540 µl

Table 4.

Following Reaction A (Deamination of Glutamine), the standard curve preparations as well as the two tubes from each sample (one analysed for endogenous glutamate and the other analysed for endogenous glutamate and deaminated glutamine) were used for Reaction B (Dehydrogenation of Glutamate). As well, Tris-EDTA-Hydrazine Buffer, NAD^- Solution, ADP Solution, and water were used in Reaction B. See Table 5 for sample preparation for the Dehydrogenation of Glutamate (Reaction B).

Reaction B - Dehydrogenation of Glutamate

	Tube		
Solutions	Standard	Endogenous Glutamate	Endogenous Glutamate & Deaminated Glutamine
0.1 mol/l Tris - 2 mmol/l EDTA- 0.63 mol/l Hydrazine Buffer	1 ml	1 ml	1 ml
30 mmol/l β-NAD^- Solution	100 µl	100 µl	100 µl
100 mmol/l ADP Solution	10 µl	10 µl	10 µl
Water	390 µl	390 µl	390 µl
Standard*	500 µl	—	—
Endogenous Glutamate*	—	500 µl	—
Endogenous Glutamate & Deaminated Glutamine*	—	—	500 µl

Table 5. * from Reaction A

Absorbance was then read at 339 nm using the Spectronic 1001 Plus (Milton Roy® Inc., Ivyland, PA) to obtain background reading. Then 20 µl of 1200 kU/l Glutamate Dehydrogenase (L-GLDH) (Roche Biochemicals®, 0127078) was added to each cuvet and mixed by inversion an allowed to incubate at room temperature for 40 minutes. Absorbance was then read at 339 nm. Background absorbance was subtracted from sample absorbance to give net absorbance. The concentration of L-glutamine in a sample was then calculated by dividing the absorbance result by 250 µl and multiplied by any dilutions that were made. The concentration of glutamine for each subject was then determined by subtracting the glutamine concentration derived from the endogenous glutamate from the glutamine concentration derived from the endogenous glutamate and the deamination of glutamine.

Saliva Analysis

Subjects were asked to dribble saliva, unstimulated, into a sterile collection cup. Saliva samples were immediately place on ice and frozen at -70°C until further analysis. Saliva samples were analyzed for salivary immunoglobulin A (IgA) using an enzyme linked immunosorbent assay (ELISA). Briefly, 10µl anti-human IgA (Anti-human IgA, α-chain specific, developed in rabbit; Sigma®, I8760) was added to 8ml of coating buffer and mixed. 100µl was then added to all wells of a 96 well microtiter plate. The plate was then incubated for at least an hour (this procedure was done the day before, in which case the plates were coated and refrigerated overnight). The saliva samples and standards (Purified human colostrum IgA 2500mg/L; Sigma® I2636) were taken out of the freezer to thaw.

Saliva samples were initially diluted 10 times by adding 9 volumes of water (100µl + 900µl). 100µl of this was then diluted with 900µl water, of which 100µl was diluted with 900µl of water. This resulted in three dilutions of x10, x10 and x10 to give a total dilution factor of 1000. The stock standard was diluted by 25 (50µl + 1200µl water) to give a standard of 100mg/L. This was then diluted by 100 (50µl + 4950µl water) to give a working standard of 1000µg/L. Further dilutions (Table 6) of this working standard were required to make the appropriate range (0-500 µg/L).

Dilutions for IgA Standard Curve Determination

IgA Standard Concentration (µg/L)	1000 µg/L IgA Stock Solution (µl)	Water (µl)
0	0	2000
25	50	1950
50	100	1900
100	200	1800
150	300	1700
200	400	1600
250	500	1500
300	600	1400
350	700	1300
400	800	1200
450	900	1100
500	1000	1000

Table 6.

After incubation the plate was blotted and washed with washing buffer. This was repeated 3 times. 150µl of blocking protein (2% Bovine Serum Albumin-PBS solution) was then added to all the wells. The plate was then incubated at room temperature for 20 minutes. The plate was then blotted and washed with washing buffer. This was repeated 3 times. After this wash, 100µl of either standard or saliva was added to wells in quadruplicate. The plate was then incubated at room temperature for 90 minutes. After incubation the plate was then blotted and washed with washing buffer. This was repeated 3 times. 10µl of anti-human IgA peroxidase conjugate (Anti-human IgA, α-chain specific peroxidase conjugate $F(ab')_2$ developed in goat; Sigma® A4165) was added to 10ml of PBS, mixed and 100µl added to all wells. The plate was then incubated at room temperature for 90 minutes. After incubation the plate was blotted and washed with washing buffer. This was repeated 3 times. 100µl of o-Phenylenediamine Dihydrochloride (OPD Sigma® Fast Tablets; A4165) was then added to each well. The plate was incubated at room temperature for 45 minutes before absorbance was measured by dual wave method at 405nm and 650nm using the UVmax Kinetic Microplate Reader (Molecular Devices Inc., Sunnyvale,CA).

Supplementation

During the glutamine trials, subjects supplemented their diet with 10 grams of L-glutamine (SportPharma™ Inc., Concord, Florida) three times daily, while during the placebo trials, subjects supplemented their diet with 10 grams of Inula Pure© (Ergonomics America™, Wyoming) three times daily. This resulted in 30 grams of glutamine or placebo

being ingested daily by each subject. This supplementation regimen continued for a period of 14 days. Each subject was given their own supplementation package which contained the required doses for the duration of the 2 week supplementation period. All assignments of supplementation were allocated on a double-blind basis. During each supplementation period subject were asked to keep track of how many supplement doses were missed during that period.

Training Log, Dietary Journal and Life Stress Questionnaire Control Factors

All subjects were required to keep a training diary that outlines their daily training volume, frequency and intensity for each day throughout the supplementation period. Subjects were also required to write down their entire dietary intake for a period of three days (two week days and one weekend) before and after each supplementation period. Dietary intake was analysed using a computerized software package (FoodWorks[c] College Edition Version 1.0). All subjects also completed a life stress questionnaire (The Social Readjustment Rating Scale; Holmes & Rahe, 1967) which computes a stress score depending on changes in biological, sociocultural, environmental, and individual factors. The main purpose for all three of these measures is to serve as control measure to ensure that training, diet and overall stress remain fairly consistent for each individual subject throughout the study.

Statistical Analysis

Due to the use of a cross-over design data was initially analysed for carry-over effects using the procedure outlined by Hills and Armitage (1979). For this procedure difference scores before and after the washout period were calculated and analysed using an independent sample t-test. If no significant differences were found it was deemed that no carry-over effect had occurred. To make sure there was no carry-over effect data was analysed further using a paired sample t-test between pre-supplementation values (Test 1 vs Test 3). This was to insure that pre-test values returned to normal resting values following the washout period. If tests for washout revealed that no carry-over effect had occurred, data from before and after the washout period were pooled and used for the subsequent analysis. Both of these analyses for carry-over effect were used on male and female pooled data and on gender specific data. Total leukocytes, neutrophil, basophils, eosinophils, total lymphocyte, T lymphocytes (CD3+), B lymphocytes (CD19+), Helper T Lymphocytes (CD3+CD4+), Cytolytic T lymphocytes (CD3+CD8+), and natural killer (CD3-CD16+CD56+) cell counts as well as the CD4/CD8 ratio for grouped data was analysed using a 2 (pre vs post) x 2 (glutamine vs placebo) repeated measure 2-way analysis of variance (ANOVA). To determine if gender differences played a role in the overall analysis, a 2 (pre vs post) x 2 (glutamine vs placebo) x 2 (male vs female) mixed plot 3-way ANOVA was utilized. Dietary intake and stress scores for each of the four test periods were analysed using a One-way ANOVA. If a significant interaction was present in any of the ANOVA results Duncan's Multiple Range Test was performed to determine where the significance lies. Training parameters before and after the washout period were

analysed using a student's t-test. On all occasions, statistical significance was accepted at a level of $p < 0.05$. Statistical analysis was performed using SPSS© Version 10.0 statistical computer package (SPSS© Inc., Chicago, IL) and Stastica (StatSoft© Inc., Tulsa, OK). All data is presented as means ± SEM, unless otherwise stated.

Chapter 4

RESULTS

Washout Analysis

Data was initially analysed for carry-over effects for pooled data on males and females. Both the paired t-tests and independent t-tests revealed that there was no significant ($p<0.05$) different between all variables for male and female pooled data. Furthermore, data was analysed for carry-over effects for gender specific data. The paired t-tests on female and male data revealed no significant difference ($p<0.05$) between all variables. Independent t-tests on male and female data revealed no significant difference ($p<0.05$) on all variables except for percent monocytes of leukocytes for male specific data ($p= 0.046$). It was concluded from these analyses that no carry-over effect had occurred and therefore data from before and after the washout period were pooled and used in the subsequent analysis.

Leukocyte and Lymphocyte Sub-Populations

The mean resting values for leukocyte and lymphocyte sub-populations before and after glutamine or placebo supplementation for all subjects is presented in Tables 7 and 8, respectively. Separate 2 (treatment: glutamine or placebo) by 2 (time: pre or post) analyses of variance (ANOVA) were conducted on leukocyte and lymphocyte sub-populations. Complete ANOVA source tables can be found in Appendix J. For leukocyte count, the main effect for supplement and time were not significant ($p <0.05$), however there was a trend towards a significant treatment by time interaction, $F(1, 14) = 3.63$, $p = 0.08$.

Analysis of data indicated that there was a trend for mean leukocyte count to increase from 4.90 ± 0.30 to 5.19 ± 0.34 cells x10^9/L following glutamine supplementation, while there was a trend for values to decrease from 5.28 ± 0.30 to 4.97 ± 0.31 cells x10^9/L following placebo supplementation, $F(1, 14) = 3.63$, $p = 0.08$. When these changes are expressed as a percent change of baseline values, there was a 5.92 % increase and a 5.87 % decrease in leukocyte count following glutamine and placebo supplementation, respectively (Figure 4).

A 2 (treatment: glutamine or placebo) by 2 (time: pre or post) ANOVA was conducted on neutrophil count. For neutrophil count the main effect for treatment was significant $F(1,14) = 5.14$, $p = 0.04$, with glutamine values being higher throughout the study period. The main effect for time was not significant, however, there was a trend towards a significant treatment by time interaction, $F(1, 14) = 3.79$, $p = 0.07$. Analysis indicated that neutrophil count increased from 2.33 ± 0.17 to 2.59 ± 0.21 cells x10^9/L following glutamine supplementation, and decreased from 2.78 ± 0.21 to 2.49 ± 0.19 cells x10^9/L following placebo supplementation. Expressed as a percent change of baseline values, there was a 11.16 % increase and a 10.43 % decrease in neutrophil count following glutamine and placebo supplementation, respectively (Figure 5). There were no significant ($p > 0.05$) main effects or interaction for all other leukocyte and lymphocyte sub-populations data (See Figures 6-13).

Absolute Values for Leukocyte Sub-Populations

Measure (Mean ± SEM)			
Leukocyte Count (x10⁹/L)			
PreGlu	4.90 ± 0.30	PostGlu	5.19 ± 0.34
PrePla	5.28 ± 0.30	PostPla	4.97 ± 0.31
Neutrophil Count (x10⁹/L)			
PreGlu	2.33 ± 0.17*	PostGlu	2.59 ± 0.21*
PrePla	2.78 ± 0.21	PostPla	2.49 ± 0.19
Basophil Count (x10⁹/L)			
PreGlu	0.051 ± 0.003	PostGlu	0.051 ± 0.007
PrePla	0.048 ± 0.004	PostPla	0.049 ± 0.005
Eosinophil Count (x10⁹/L)			
PreGlu	0.286 ± 0.111	PostGlu	0.310 ± 0.136
PrePla	0.224 ± 0.052	PostPla	0.228 ± 0.058
Monocyte Count (x10⁹/L)			
PreGlu	0.446 ± 0.033	PostGlu	0.437 ± 0.033
PrePla	0.466 ± 0.038	PostPla	0.433 ± 0.025
Lymphocyte Count (x10⁹/L)			
PreGlu	1.78 ± 0.097	PostGlu	1.80 ± 0.117
PrePla	1.76 ± 0.098	PostPla	1.76 ± 0.119

Table 7: Immunologic parameters for subjects before and after supplementation with glutamine or placebo. Data is presented as grouped data for gender (n=15). * Indicates significant ($p<0.05$) main effect for supplementation, with glutamine values being higher throughout the study. All values are presented as means ± SEM.

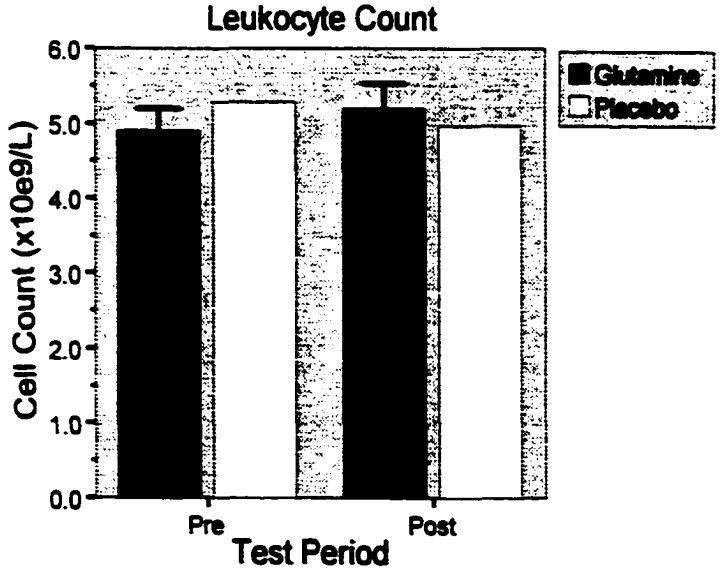

Figure 4: Leukocyte cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

Absolute Values for Lymphocyte Sub-Populations

Measure (Mean ± SEM)			
T lymphocyte (CD3+) Count (x10⁹/L)			
PreGlu	1.37 ± 0.087	PostGlu	1.37 ± 0.091
PrePla	1.34 ± 0.086	PostPla	1.34 ± 0.097
Helper T lymphocyte (CD3+CD4+) Count (x10⁹/L)			
PreGlu	0.865 ± 0.070	PostGlu	0.852 ± 0.069
PrePla	0.839 ± 0.070	PostPla	0.842 ± 0.072
Cytolytic T lymphocytes (CD3+CD8+) Count (x10⁹/L)			
PreGlu	0.438 ± 0.026	PostGlu	0.447 ± 0.031
PrePla	0.429 ± 0.027	PostPla	0.426 ± 0.034
B lymphocyte (CD3-CD19+) Count (x10⁹/L)			
PreGlu	0.191 ± 0.016	PostGlu	0.203 ± 0.022
PrePla	0.189 ± 0.015	PostPla	0.187 ± 0.014
Natural Killer Cell (CD3-CD16+CD56+) Count (x10⁹/L)			
PreGlu	0.195 ± 0.022	PostGlu	0.217 ± 0.032
PrePla	0.203 ± 0.033	PostPla	0.213 ± 0.036

Table 8: Immunologic parameters for subjects before and after supplementation with glutamine or placebo. Data is presented as grouped data for gender (n=15). All values are presented as means ± SEM.

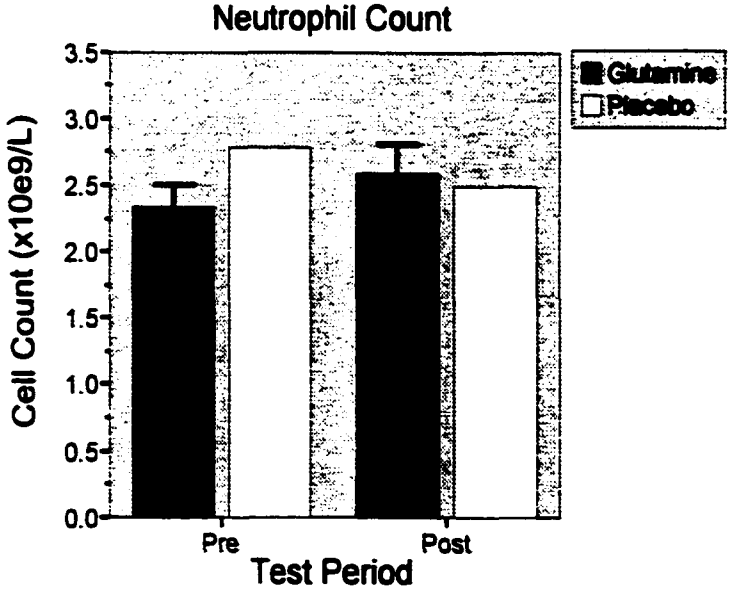

Figure 5: Neutrophil cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM

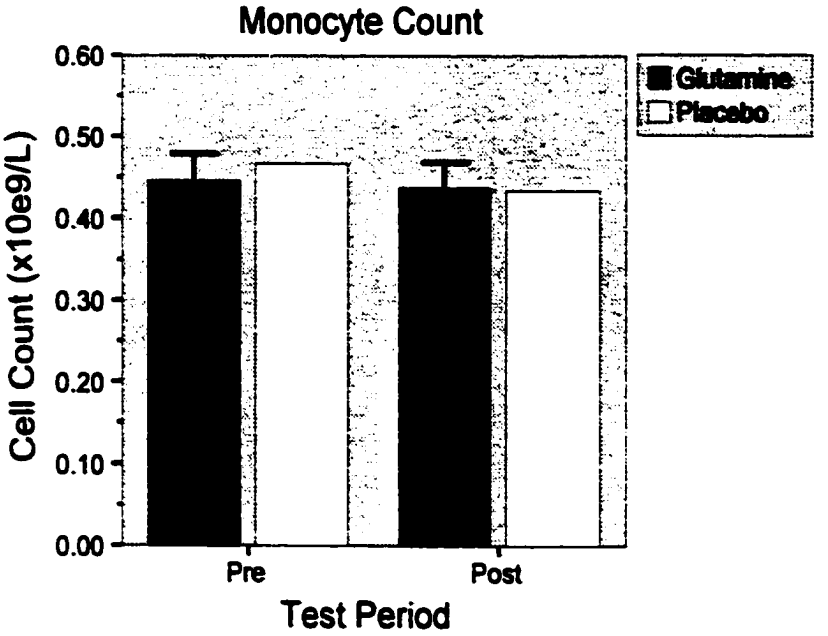

Figure 6: Monocyte cell count before and after supplementation with glutamine or placebo. All values are presented as means ±SEM.

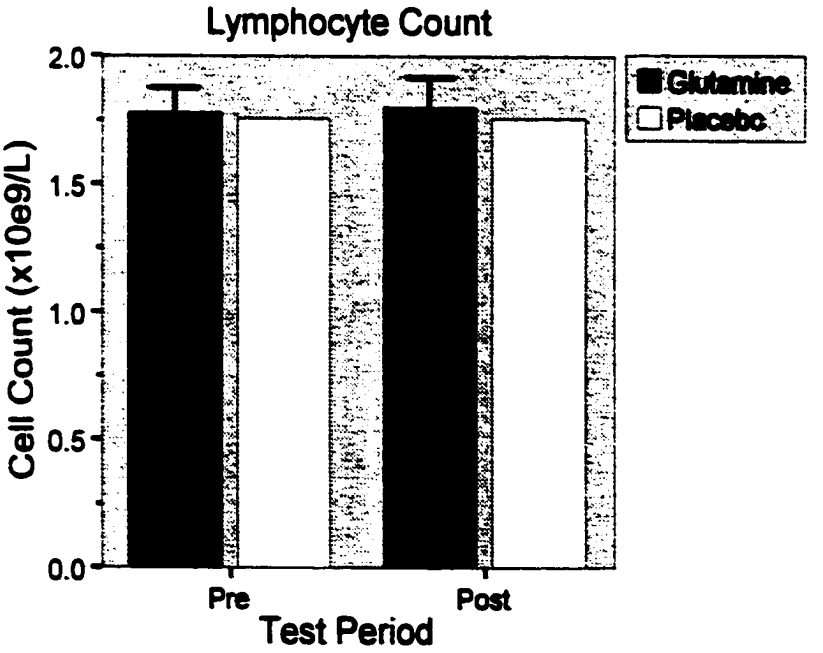

Figure 7: Lymphocyte cell count before and after supplementation with glutamine or placebo. All values are presented as means = SEM.

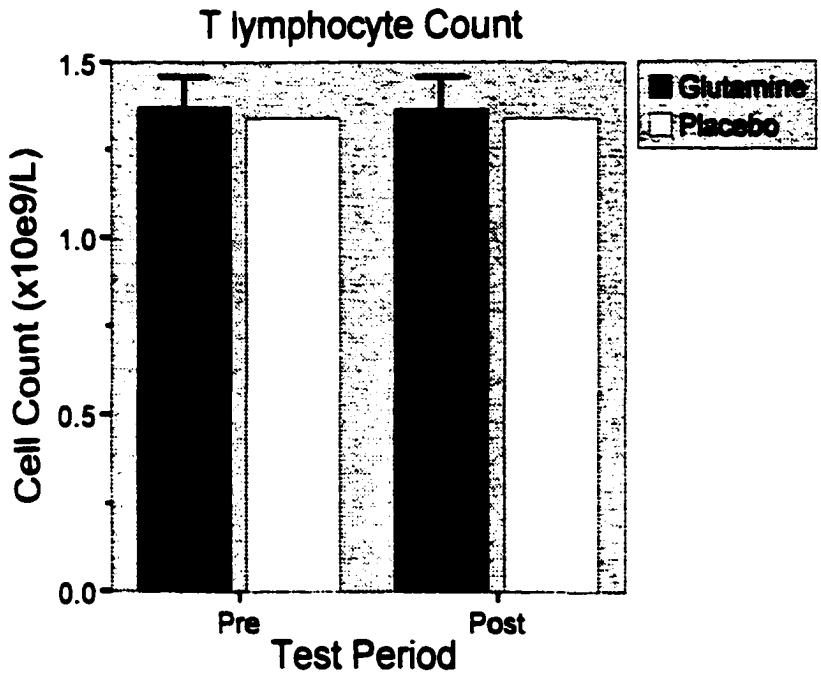

Figure 8: T lymphocyte (CD3+) cell count before and aftersupplementation with glutamine or placebo. All values are presented as means ± SEM

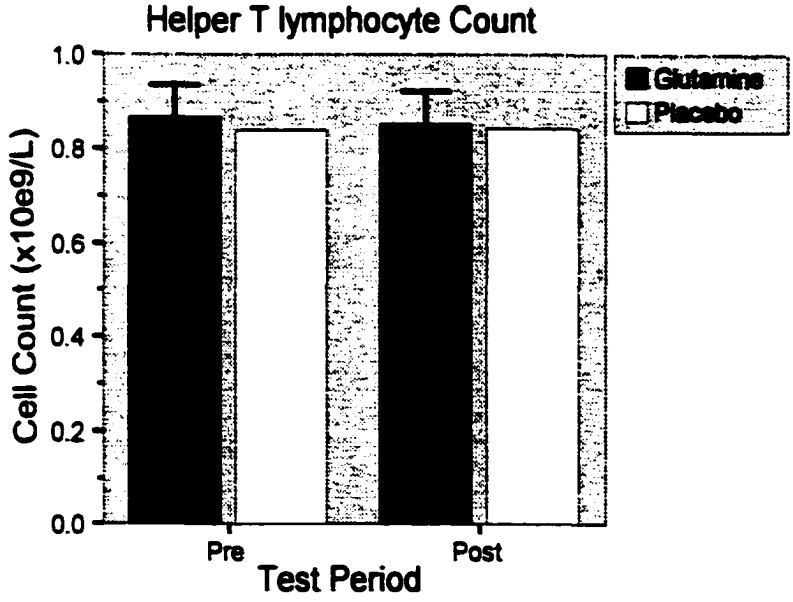

Figure 9: Helper T lymphocyte (CD3+CD4+) cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

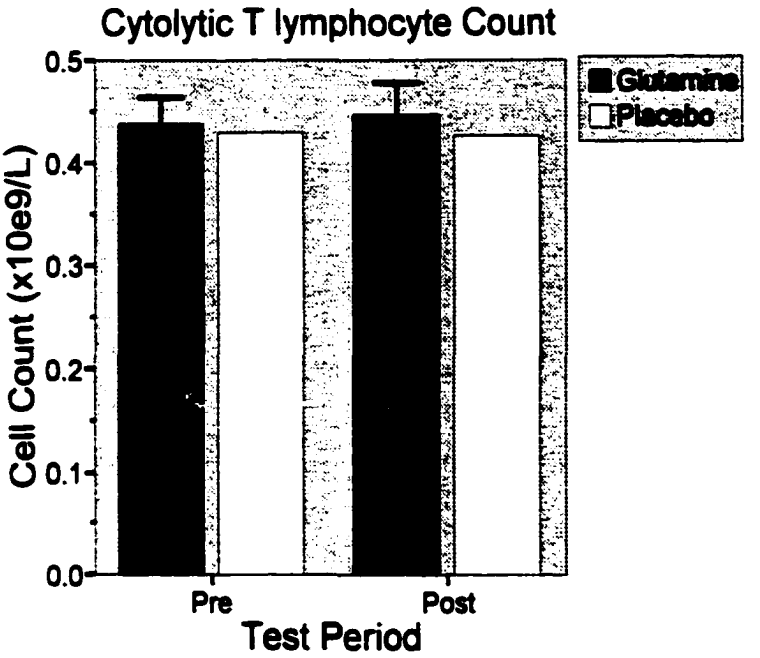

Figure 10: Cytolytic T lymphocyte (CD3-CD8-) cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

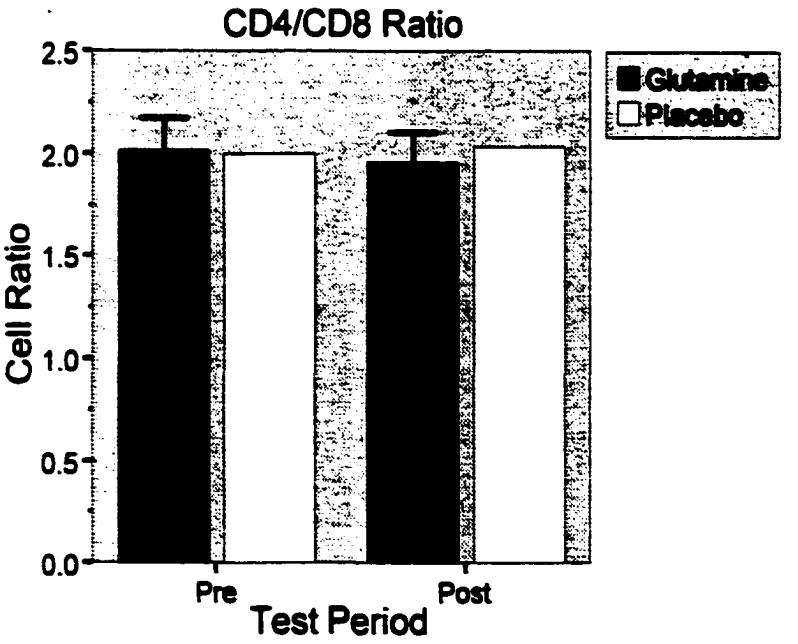

Figure 11: CD4/CD8 ratio before and after supplementation with glutamine or placebo. All values are presented as means = SEM.

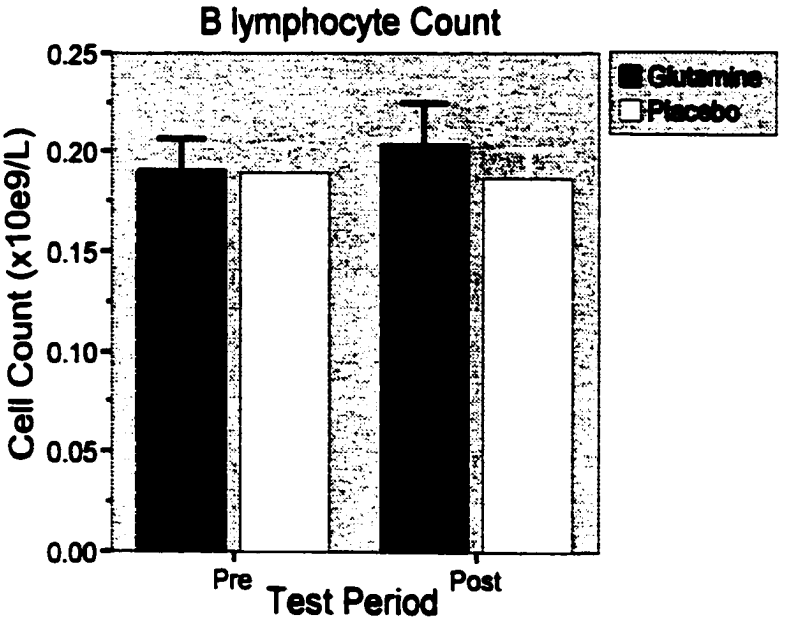

Figure 12: B lymphocyte (CD3-CD19+) cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

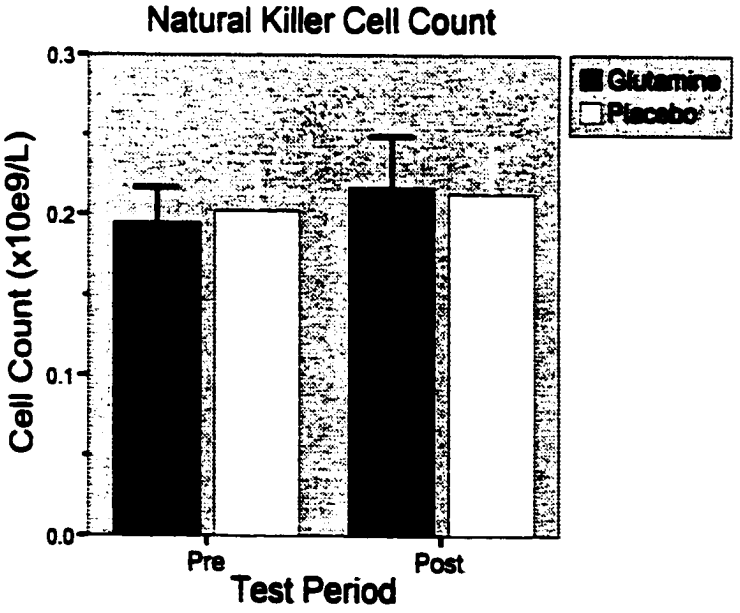

Figure 13: Natural killer (CD3-CD16+CD56+) cell count before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

Due to the fact that the 2-way factorial ANOVA revealed the there were trends in the data further investigation was conducted on determine if other factors could account for these findings. Therefore data was differentiated by initial glutamine levels (high vs low), initial cell counts (high vs low). Furthermore, due to the fact that physiological differences in males and females may have an effect on the results, data was also discriminated by gender. A 3-way ANOVA on this data indicated that there was a significant effect of gender on the results. Therefore separate 2 (treatment: glutamine or placebo) by 2 (time: pre or post) by 2 (gender: male or female) ANOVA were conducted on the data[1].

Absolute leukocytes, neutrophils, and percent neutrophils of leukocytes were found to have a significant effect by gender (Table 9). All other immunological parameter were not significantly different ($p > 0.05$) when differentiated by gender.

Absolute Values for Leukocyte Sub-Populations Differentiated by Gender

Leukocyte Count ($x10^9/L$)								
Females					Males			
PreGlu	4.90 ± 0.49	PostGlu	5.57 ± 0.55*		PreGlu	4.91 ± 0.45	PostGlu	4.94 ± 0.45
PrePla	5.47 ± 0.55	PostPla	4.75 ± 0.50*		PrePla	5.15 ± 0.39	PostPla	5.11 ± 0.41
Neutrophil Count ($x10^9/L$)								
Females					Males			
PreGlu	2.12 ± 0.27	PostGlu	2.83 ± 0.33*		PreGlu	2.47 ± 0.22	PostGlu	2.43 ± 0.27
PrePla	2.89 ± 0.34	PostPla	2.22 ± 0.30*		PrePla	2.70 ± 0.28	PostPla	2.67 ± 0.25
Percent Neutrophils of Leukocytes								
Females					Males			
PreGlu	44.25 ± 2.80	PostGlu	51.37 ± 3.29*		PreGlu	50.13 ± 2.28	PostGlu	48.61 ± 2.68
PrePla	52.57 ± 3.06	PostPla	47.03 ± 2.70*		PrePla	51.86 ± 2.49	PostPla	52.04 ± 2.21

Table 9: Immunologic parameters for subjects before and after supplementation with glutamine or placebo. Data is differentiated by gender (Females n=6; Males n=9). * Indicates a significant ($p<0.05$) difference compared to baseline. All values are presented as means ± SEM.

[1]Although significant findings were found it should be noted that this analysis was limited by relatively small and unequal numbers per cell (Females n=6, Males n=9).

For leukocyte count differentiated by gender the 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction was significant, (p <0.05). More importantly the 2 (gender: male or female) by 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction for leukocyte count was significant, F (1, 13) = 5.69, p = 0.03, therefore Duncan's post hoc tests were performed to determine the nature of the interaction. There was an increase for females receiving glutamine from 4.90 ± 0.49 to 5.57 ± 0.55 cells x10^9/L, p <0.05, while those receiving placebo decreased from 5.47 ± 0.55 to 4.75 ± 0.50 cells x10^9/L, p <0.05. Values for male subjects receiving glutamine showed virtually no change from 4.91 ± 0.45 to 4.94 ± 0.45 cells x10^9/L, p >0.05, while those on placebo changed from 5.15 ± 0.39 to 5.11 ± 0.41 cells x10^9/L, p >0.05. When these changes are expressed as a percentage change of baseline values, there was a 13.67 % increase and a 13.16 % decrease in female leukocyte count following glutamine and placebo supplementation, respectively. Males showed a 0.61 % increase and 0.78 % decrease in leukocyte count following glutamine and placebo supplementation, respectively (Figure 14). There were no significant main effects or other interaction for leukocyte count data when differentiated by gender (p >0.05).

For neutrophil count differentiated by gender the 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction was significant, (p <0.05). More importantly the 2 (gender: male or female) by 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction for neutrophil count was significant, F (1, 13) = 9.87, p = 0.008), therefore Duncan's post hoc tests were performed to determine the nature of the interaction. Absolute neutrophil count significantly increased for females receiving glutamine from 2.12

± 0.27 to 2.83 ± 0.33 cells x10^9/L, $p < 0.05$, while those receiving placebo decreased from 2.89 ± 0.34 to 2.22 ± 0.30 cells x10^9/L, $p < 0.05$. Values for males subjects receiving glutamine decreased from 2.47 ± 0.22 to 2.43 ± 0.22 cells x10^9/L, $p < 0.05$, while those on placebo decreased from 2.70 ± 0.28 to 2.67 ± 0.25 cells x10^9/L, $p < 0.05$. When these changes are expressed as a percent change of baseline values, there was a 33.49 % increase and a 23.18 % decrease in female leukocyte count following glutamine and placebo supplementation, respectively. Males showed a 2.99 % decrease and 0.19 % decrease in leukocyte count following glutamine and placebo supplementation, respectively (Figure 15). It should be noted that the treatment effect seen in the female subject may partially be due to the groups varying pretest values before supplementation and not solely due to the supplementation treatment. There were no significant main effects or other interaction for neutrophil count data when differentiated by gender ($p > 0.05$).

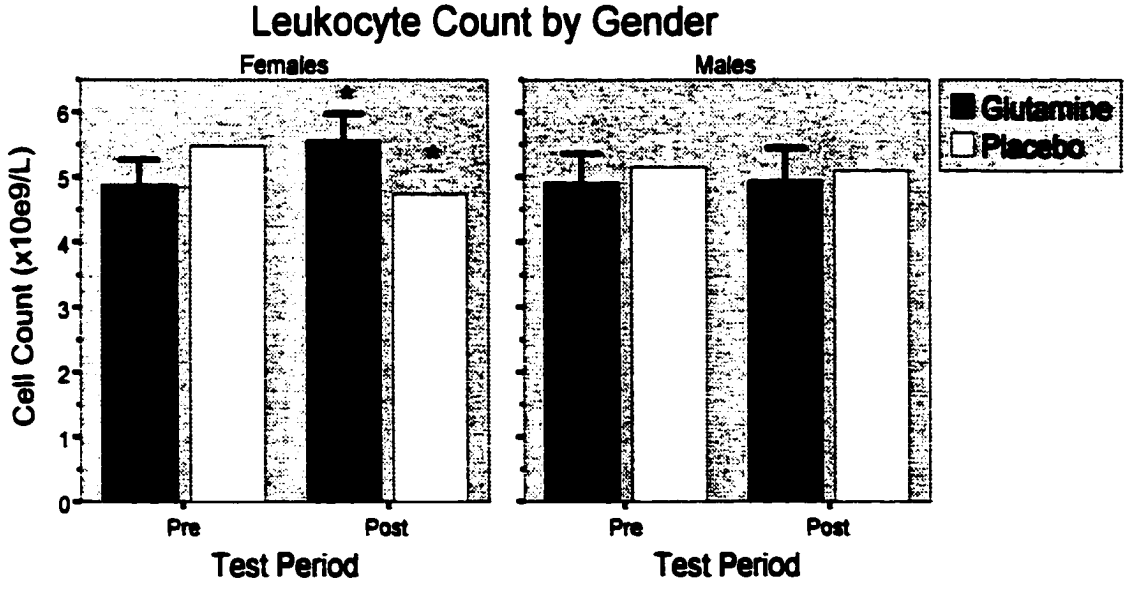

Figure 14: Absolute leukocyte count differentiated by gender before and after supplementation with glutamine or placebo. All values are presented as means ± SEM. *Indicates a significant ($p < 0.05$) difference compared to baseline values.

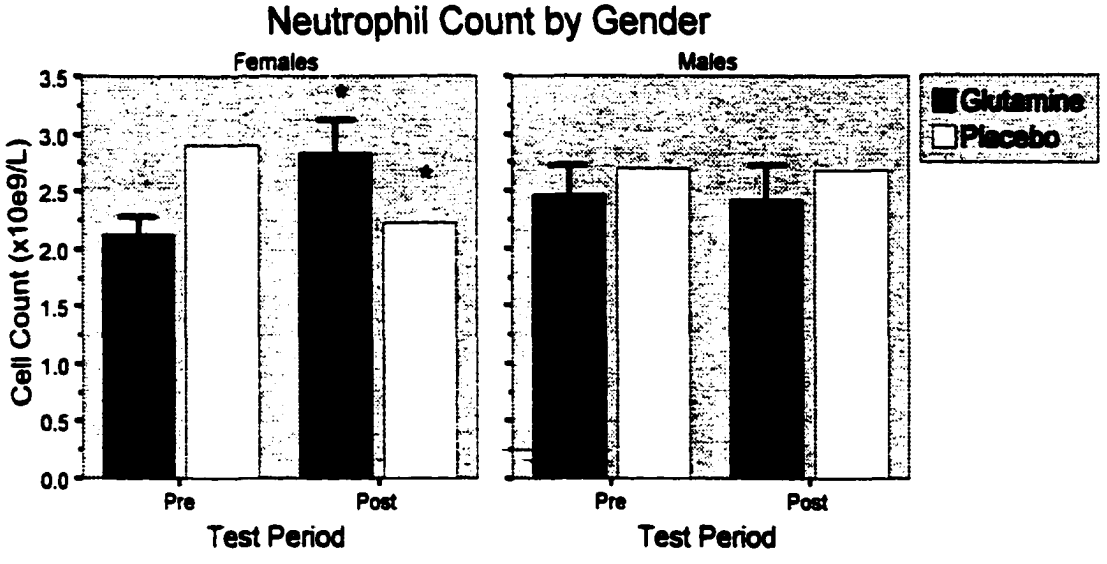

Figure 15: Absolute neutrophil count differentiated by gender before and after supplementation with glutamine or placebo. All values are presented as means ± SEM. *Indicates a significant difference compared to baseline values.

Proportion of Cell Type

The mean resting values for the proportion of leukocyte and lymphocyte sub-populations are shown in Tables 10 and 11, respectively. A 2 (treatment: glutamine or placebo) by 2 (time: pre or post) on percent neutrophils of leukocytes. For percent neutrophils the main effect for treatment was significant ($p < 0.05$), with glutamine values being higher throughout the study period. The main effect for time was not significant, however, there was a trend towards a significant treatment by time interaction,

$F(1, 14) = 2.87$, $p = 0.11$. Analysis of data indicated that there was a trend for the glutamine supplemented group to increase the proportion of neutrophils from 47.78 ± 1.87 % to 49.71 ± 2.03 %, while the placebo group decreased from 52.14 ± 1.86 % to 50.04 ± 1.77 %. When these changes are expressed as a percent change of baseline, there was a 4.04 % increase and a 4.03 % decrease in leukocyte count following glutamine and placebo supplementation, respectively as shown in Figure 16. There were no significant main effects or interactions for all other proportion of cell type data ($p > 0.05$).

Proportion of Cell Type (Leukocyte Sub-Populations)

Measure (Mean ± SEM)			
Percent Neutrophils of Leukocytes			
PreGlu	47.78 ± 1.87*	PostGlu	49.71 ± 2.03*
PrePla	52.14 ± 1.86	PostPla	50.04 ± 1.77
Percent Basophils of Leukocytes			
PreGlu	1.05 ± 0.06	PostGlu	0.99 ± 0.11
PrePla	0.92 ± 0.05	PostPla	1.00 ± 0.07
Percent Eosinophils of Leukocytes			
PreGlu	5.11 ± 1.66	PostGlu	5.28 ± 1.99
PrePla	3.97 ± 0.75	PostPla	4.18 ± 0.88
Percent Monocytes of Leukocytes			
PreGlu	9.17 ± 0.53	PostGlu	8.57 ± 0.52
PrePla	8.93 ± 0.64	PostPla	8.96 ± 0.54
Percent Lymphocytes of Leukocytes			
PreGlu	36.89 ± 1.50	PostGlu	35.47 ± 1.92
PrePla	34.05 ± 1.88	PostPla	35.83 ± 1.84

Table 10: Immunologic parameters for subjects before and after supplementation with glutamine or placebo. Data is presented as grouped data for gender (n=15). * Indicates significant ($p<0.05$) main effect for supplementation, with glutamine values being higher throughout the study. All values are presented as means ± SEM.

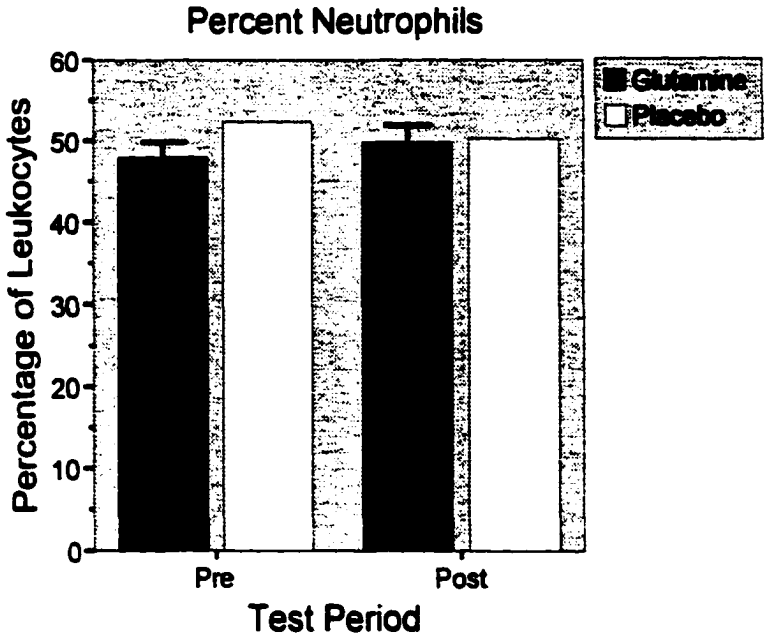

Figure 16: Proportion of neutrophils of leukocyte before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

Proportion of Cell Type (Lymphocyte Sub-Populations)

Measure (Mean ± SEM)			
Percent of T lymphocytes (CD3) of Lymphocytes			
PreGlu	76.67 ± 1.67	PostGlu	76.25 ± 1.76
PrePla	76.11 ± 1.75	PostPla	75.86 ± 1.73
Percent CD3+CD4+ of Lymphocytes			
PreGlu	47.94 ± 2.32	PostGlu	47.38 ± 2.25
PrePla	47.26 ± 2.28	PostPla	47.49 ± 2.21
Percent CD3+CD8+ of Lymphocytes			
PreGlu	24.79 ± 1.03	PostGlu	25.05 ± 1.00
PrePla	24.65 ± 1.12	PostPla	24.30 ± 1.05
CD4/CD8 Ratio			
PreGlu	2.01 ± 0.16	PostGlu	1.96 ± 0.14
PrePla	2.00 ± 0.16	PostPla	2.04 ± 0.16
Percent CD19 of Lymphocytes			
PreGlu	10.95 ± 0.82	PostGlu	11.21 ± 0.78
PrePla	10.89 ± 0.79	PostPla	10.83 ± 0.76
Percent CD3-CD16+CD56+ of Lymphocytes			
PreGlu	11.23 ± 1.21	PostGlu	11.85 ± 1.46
PrePla	11.51 ± 1.85	PostPla	11.89 ± 1.52

Table 11: Immunologic parameters for subjects before and after supplementation with glutamine or placebo. Data is presented as grouped data for gender (n=15). All values are presented as means ± SEM.

For percent neutrophils differentiated by gender the 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction was significant, ($p < 0.05$). More importantly the 2 (gender: male or female) by 2 (time: pre or post) by 2 (treatment: glutamine or placebo) interaction for leukocyte count was significant, $F(1, 13) = 21.51$, $p = 0.001$, therefore Duncan's post hoc tests were performed to determine the nature of the interaction. Post hoc analysis indicated that percent neutrophils significantly increased for females receiving glutamine from 44.25 ± 2.80 % to 52.57 ± 3.06 %, $p < 0.05$, while values for those subjects receiving placebo decreased from to 47.03 ± 2.70 % to 51.37 ± 3.29 %, $p < 0.05$, as shown in Table 9. Percent neutrophil values for males subjects receiving glutamine decreased from 50.13 ± 2.28 % to 48.61 ± 2.68 %, $p > 0.05$, while values for those subjects receiving placebo increased slightly from 51.86 ± 2.49 % to 52.04 ± 2.21%, $p > 0.05$. When these changes are expressed as a percentage change of baseline values, there was a 16.03 % increase and a 10.65 % decrease in females neutrophil percentage following glutamine and placebo supplementation, respectively. Male subjects showed a 2.99 % decrease and 0.19 % increase in neutrophil percentage following glutamine and placebo supplementation, respectively (Figure 17). It should be noted that the treatment effect seen in the female subject may partially be due to the groups varying pretest values before supplementation and not solely due to the supplementation treatment. There were no significant main effects or other interaction for percent neutrophils data when differentiated by gender ($p > 0.05$).

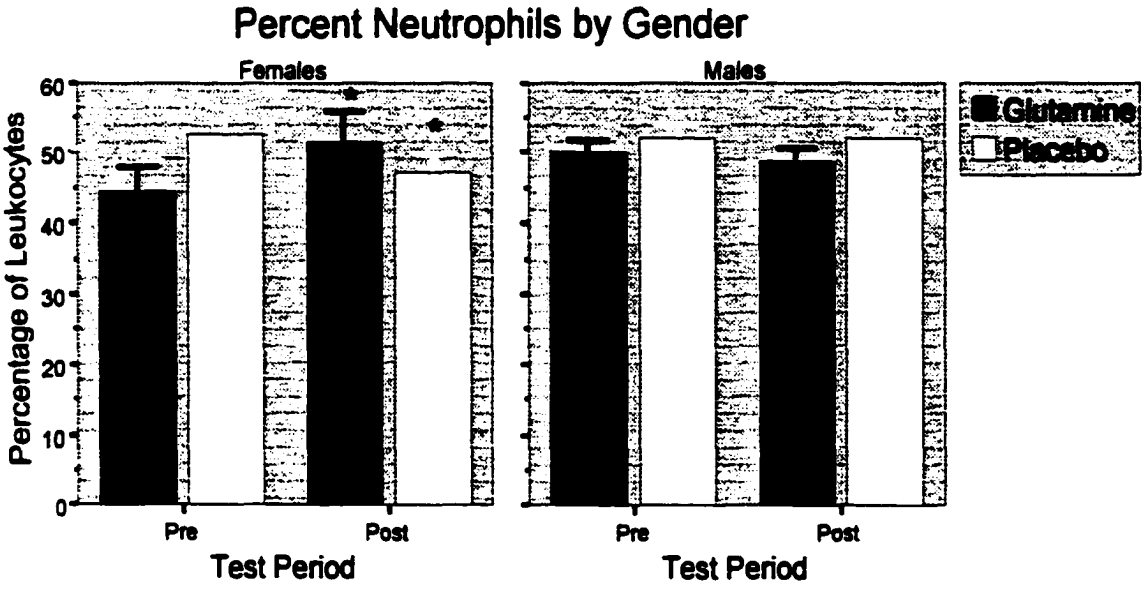

Figure 17: Proportion of neutrophil differentiated by gender before and after supplementation with glutamine or placebo. All values are presented as means ± SEM. *Indicates a significant ($p<0.05$) difference compared to baseline values.

Saliva IgA, & Plasma Glutamine

Saliva Immunoglobulin A (IgA) and plasma glutamine concentrations are presented in Table 12. For saliva IgA, the main effects for treatment and time as well as the interaction were not significant ($p>0.05$) (Figure 18). For plasma glutamine, the main effects for treatment and time as well as the interaction were not significant ($p>0.05$) (Figure 19).

Saliva IgA and Plasma Glutamine

Measure (Mean ± SEM)			
Saliva IgA (mg/l)			
PreGlu	175.44 ± 18.97	PostGlu	179.18 ± 27.82
PrePla	154.19 ± 19.60	PostPla	176.15 ± 18.75
Plasma Glutamine (μmol/l)			
PreGlu	681.11 ± 28.22	PostGlu	688.84 ± 56.52
PrePla	635.74 ± 35.22	PostPla	654.57 ± 34.31

Table 12: Saliva IgA and plasma glutamine concentrations for subjects before and after supplementation with glutamine or placebo. Data is presented as grouped data for gender (n=15). All values are presented as means ± SEM.

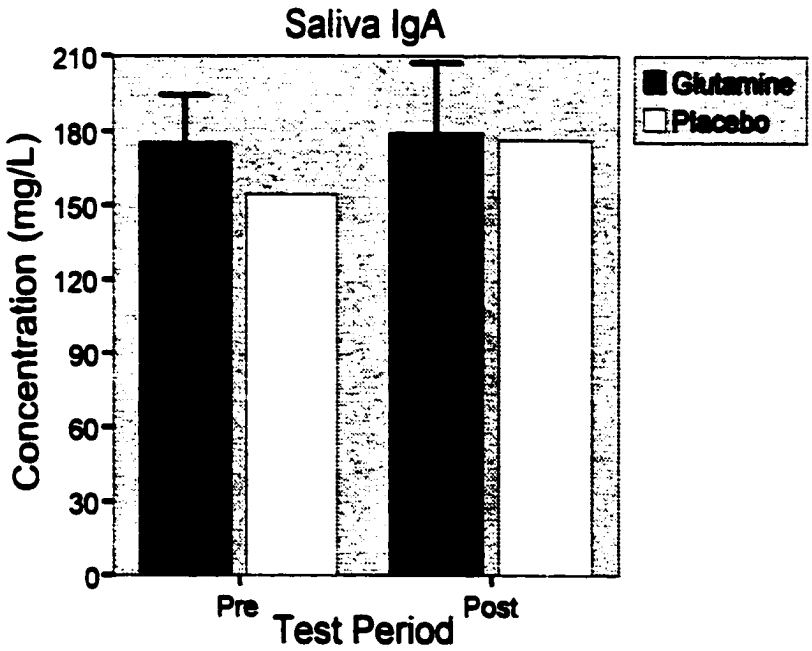

Figure 18: Saliva IgA concentration before and after supplementation with glutamine or placebo. All values are presented as means ±SEM.

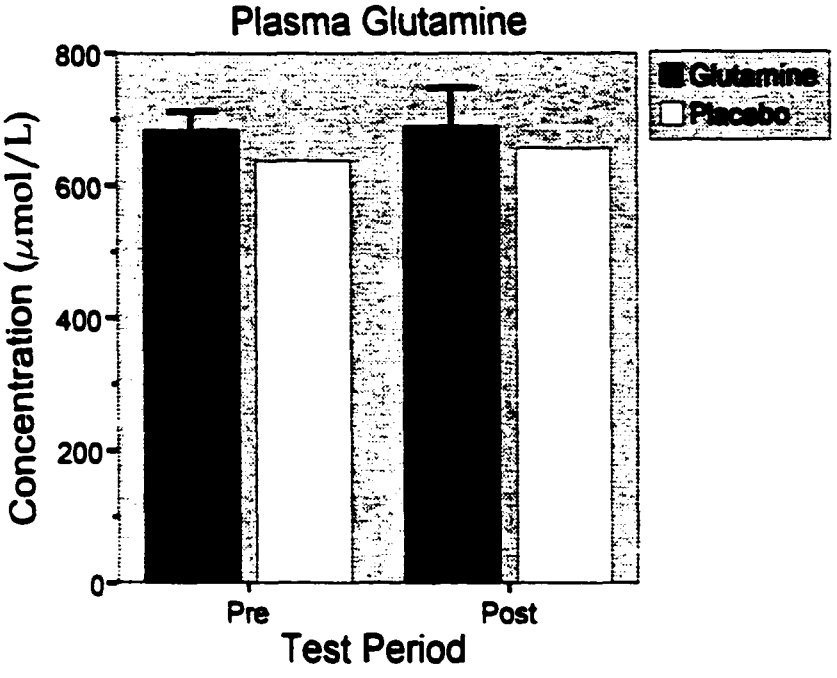

Figure 19: Plasma glutamine concentration before and after supplementation with glutamine or placebo. All values are presented as means ± SEM.

Control Factors (Endurance Training, Psychological Stress, & Nutritional Intake)

Endurance training, psychological stress, and nutritional intake are presented in Table 13. There was no significant change ($p > 0.05$) in endurance training during the two test periods before and after washout. There was also no significant difference ($p > 0.05$) in psychological stress and 3 day nutritional intake values during the four different testing periods.

Control Factors

Measure (Mean ± SEM)				Statistics
Endurance Training (Km/wk)				t = - 0.44
Pre Washout	64.07 ± 3.20	Post Washout	65.37 ± 4.51	df (14)
				p = 0.67
Psychological Stress Score				F = 0.44
Period 1	57.70 ± 11.03	Period 2	41.33 ± 10.68	df (3,59)
Period 3	46.93 ± 12.43	Period 4	51.53 ± 12.18	p = 0.73
Caloric Intake (kcal/day)				F = 0.50
Period 1	3043 ± 247	Period 2	2692 ± 204	df (3,59)
Period 3	2854 ± 241	Period 4	2714 ± 224	p = 0.69
Carbohydrate Intake (grams/day)				F = 0.24
Period 1	429.5 ± 38.9	Period 2	385.7 ± 31.3	df (3,59)
Period 3	405.2 ± 37.5	Period 4	405.2 ± 38.3	p = 0.87
Protein Intake (grams/day)				F = 0.41
Period 1	115.8 ± 12.6	Period 2	100.8 ± 9.3	df (3,59)
Period 3	109.4 ± 6.8	Period 4	105.6 ± 10.4	p = 0.75
Fat Intake (grams/day)				F = 0.94
Period 1	98.0 ± 8.5	Period 2	85.7 ± 7.5	df (3,59)
Period 3	89.7 ± 8.4	Period 4	79.5 ± 7.4	p = 0.43
Iron Intake (mg/day)				F = 0.63
Period 1	22.6 ± 2.2	Period 2	23.8 ± 2.3	df (3,59)
Period 3	23.4 ± 2.8	Period 4	27.5 ± 3.4	p = 0.60
Magnesium Intake (mg/day)				F = 0.31
Period 1	439.1 ± 41.8	Period 2	403.3 ± 37.7	df (3,59)
Period 3	396.2 ± 40.3	Period 4	389.7 ± 38.1	p = 0.82
Zinc Intake (mg/day)				F = 0.12
Period 1	14.3 ± 1.6	Period 2	13.5 ± 1.5	df (3,59)
Period 3	14.7 ± 5.2	Period 4	13.7 ± 0.7	p = 0.95
Vitamin A Intake (RE/day)				F = 0.52
Period 1	1310 ± 188	Period 2	1349 ± 220	df (3,59)
Period 3	1346 ± 146	Period 4	1635 ± 264	p = 0.67
Vitamin C Intake (mg/day)				F = 0.36
Period 1	345.5 ± 92.4	Period 2	300.5 ± 58.0	df (3,59)
Period 3	246.2 ± 60.3	Period 4	279.6 ± 61.1	p = 0.78
Vitamin E Intake (mg/day)				F = 0.72
Period 1	9.1 ± 1.5	Period 2	7.2 ± 1.1	df (3,59)
Period 3	6.7 ± 0.6	Period 4	8.5 ± 1.6	p = 0.55
Vitamin B6 Intake (mg/day)				F = 0.53
Period 1	3.07 ± 0.27	Period 2	2.86 ± 0.36	df (3,59)
Period 3	2.96 ± 0.28	Period 4	3.52 ± 0.60	p = 0.67

Table 13: Control factor data (nutritional intake, psychological stress, and training) for subjects before each of the four different test periods throughout the study. Data is presented as grouped data for gender (n=15). All values are presented as means ± SEM.

Chapter 5

DISCUSSION

Recent research has suggested that high intensity long duration exercise may be detrimental to immune function and lead to a higher rate of infection in athletes (Nieman and Pedersen, 1999). One possible mechanism for the decreased immune function and increased infection rates associated with intense long duration exercise and overtraining is a decrease in plasma glutamine concentrations. Recent studies have shown that athletes participating in high intensity exercise or those athletes who are in a state of overtraining have lower plasma glutamine concentrations than other athletes and control subjects (Parry-Billing et al., 1992; Rowbottom, et al., 1995; Castell et al., 1997).

Glutamine is the most abundant amino acid in the blood and in the muscles and is the primary fuel for rapidly dividing cells, including the cells of the immune system. In particular, glutamine is important for the proliferation of lymphocytes and macrophages, and therefore may be important in the maintenance of proper immune function (Ardawi & Newsholme, 1985; Hall, et al., 1996; Walsh, et al., 1998).

During times of metabolic stress such as when athletes are overtrained or during critical illnesses such as cancer, the body may require between 20-40 grams of glutamine to maintain proper homeostasis (Krebs, 1935). Due to the fact that a normal mixed protein diet has been estimated to provide less than 10 grams per day, an exogenous source of glutamine may be appropriate (Hall et al., 1996). Therefore, glutamine supplementation may be a useful practice for those individuals experiencing impaired immune function due to various physiological stresses (Chandra, 1995).

Collectively, this research suggests that glutamine supplementation may be beneficial to individuals experiencing catabolic and metabolic stress. Therefore, the intent of this study was to examine the effect of oral glutamine supplementation on resting blood and saliva immune parameters in endurance trained athletes.

Currently there has been a handful of studies examining the role of glutamine supplementation on the immune function of athletes following acute exercise (Castell et al., 1997; Castell & Newsholme, 1997; Castell, Poortmans, & Newsholme, 1996 Rohde, Asp, et al.,1998; Rohde, MacLean, et al., 1998; Walsh et al., 2000). However, to the knowledge of the author, this is the first study of its kind to examine the effect of oral glutamine on resting immune parameters of athletes participating in chronic, high intensity, endurance training. Furthermore, this is the first study to use a therapeutic dose of 30 grams daily in an athletic population. This dosage, which is commonly used in a hospital setting was administered for fourteen consecutive days.

The main finding of this study was that a glutamine supplementation regimen of 30 grams daily for 14 days resulted in an increase in leukocytes, neutrophils and percent neutrophils in female athletes but did not have any positive effect on any immune parameters of male athletes. When data was differentiated by gender the trends observed in absolute leukocyte count, absolute neutrophil count and precent neutrophil showed a significant gender effect. When compared to baseline values, absolute leukocyte count significantly increased for females endurance athletes receiving glutamine by 13.67 % and decreased by 13.16 % following placebo supplementation. Absolute leukocyte counts for male endurance athletes showed virtually no change with values increasing by 0.61 % following glutamine

supplementation and decreasing by 0.78 % following placebo supplementation.

Absolute neutrophil count significantly increased for females receiving glutamine by 33.49 % and decreased by 23.18 % following placebo supplementation. Again, males showed virtually no change with neutrophil counts increasing by 1.62 % following glutamine supplementation and decreasing by 1.11 % following placebo supplementation. Percent neutrophil values significantly increased for females receiving glutamine by 16.09 %, while those subjects receiving placebo decreased by 10.54 %. Male subjects showed non significant increases by 3.03 % and 0.35 % following glutamine and placebo supplementation, respectively.

There was also a significant main effect for supplementation in neutrophil cell count and percent neutrophils, with glutamine values being higher through the study. Furthermore, there was a trend for resting levels for leukocytes and neutrophils as well as percent neutrophils to increase over the period of study, while placebo supplementation resulted in a decrease in these variables. The results from this study show that when data was pooled and not differentiated for gender there was a trend for absolute leukocyte, absolute neutrophil and percent neutrophil values to increase following glutamine supplementation, while placebo supplementation resulted in a decrease from resting values. There was a 5.92 % increase in leukocyte count following glutamine supplementation coupled with a 5.87 % decrease in leukocyte count following placebo supplementation. Neutrophil count followed a similar trend with glutamine supplementation resulting in an 11.16 % increase while placebo supplementation resulted in 10.43 % decrease. Also, percent neutrophils followed this same trend with glutamine supplementation resulting in an 4.04 % increase while the

placebo group showed a 4.03 % decrease.

This study has demonstrated that endurance trained female athletes participating in chronic endurance exercise may benefit from glutamine supplementation. It is unclear why these changes in immune parameters were only seen in females and not in males, but it is possible that hormonal differences may have played a role in the efficacy of glutamine supplementation during chronic, high intensity, endurance training. However, there is a lack of research to support this hypothesis.

The results from this study on male athletes are in agreement to those of Castell et al. (1997) and Rohde, Asp, et al. (1998) who found that male athletes who supplemented with glutamine following a marathon had no effect on their leukocyte and lymphocyte distribution. These results are also in agreement to findings by Rohde, MacLean, et al. (1998) who found that glutamine supplementation had no significant effect on circulating lymphocytes after male subjects performed three bouts of cycle ergometry lasting 60, 45, and 30 minutes at 75 % VO_2max, separated by two hours rest. Although glutamine concentrations were higher in the glutamine supplemented group there was no difference in circulating lymphocytes between groups. These findings are in agreement with Walsh et al. (2000) who found that glutamine supplementation had no effect on the neutrophil function of male subjects following a 2 hour cycling bout at 60 % VO_2max.

The results of this study from female athletes are in agreement with findings from clinical trials with critically ill individuals. Clinical investigations with cancer patients have found positive results following glutamine supplementation. Yoshida et al. (1998) found that glutamine supplementation in patients with esophageal cancer receiving chemotherapy

prevented a reduction in the lymphocyte count, T-lymphocyte count, and B-lymphocyte count compared to those not receiving glutamine after 7 days. The authors suggest that oral glutamine supplementation protects lymphocytes in patients with advanced esophageal cancer receiving radiochemotherapy. Furthermore, Ziegler et al. (1998) found that bone marrow transplant patients, supplemented with glutamine as part of their parenteral nutrition, had an increased lymphocyte count, greater T-lymphocyte count, greater CD4, and increased CD8 count compared to those receiving standard parenteral nutrition formulas. The authors suggest that glutamine supplementation may support lymphocyte recovery after bone marrow transplants. Some other authors have also found that glutamine supplementation results in positive effects on immune function following exercise. Castell, et al. (1996) had subjects supplement with either glutamine or a placebo after a marathon or ultra-marathon. The results indicated that subjects reporting no infections was significantly higher (80.8 ± 4.2 %, $p < 0.001$) following glutamine supplementation compared to placebo supplementation (48.8 ± 7.4 %). It has also been reported that athletes receiving a glutamine drink following a marathon or ultra-marathon had a significantly higher CD4/CD8 ratio then athletes receiving a placebo drink (Castell & Newsholme, 1997).

The contrasting results reported in the literature and in this present study may be due to a difference in dose and length of the supplementation period. A larger dose and longer supplementation period may have provided ample glutamine to significantly affect immune cell counts in the female athletes. It is also possible that a one bout exercise protocol followed by a single day of supplementation that has been used in previous research may not have been sufficient to cause decrements in immune parameters that can be alleviated by

glutamine supplementation. Furthermore, the chronic training undertaken by the females in this study may have had a more detrimental effect on their immune system as seen by the decrease in both leukocyte and neutrophil count over the two week period during placebo supplementation. This decrease was not evident when females were provided with supplemental glutamine. In this case not only did glutamine significantly correct or stop the decline in overall leukocyte and neutrophil counts in the female athletes, but it also led to an increase above resting levels.

An interesting finding discovered during the review of literature revealed that there was a gender bias in the previous studies examining the effect of glutamine supplementation on the immune system. Previous research (Castell et al., 1997; Rohde, Asp, et al.,1998; Rohde, MacLean, et al., 1998; Walsh et al., 2000) that had found no positive results on immune function following glutamine supplementation had used only male subjects as part of their design. However, the two previously reported studies (Castell & Newsholme, 1997; Castell, et al., 1996) that found significant positive effects in immune function following glutamine supplementation utilized both male and female subjects, although these findings were not examined for gender differences. It is unclear if these findings are due to coincidence or due to a causal relationship. However, these current findings support previous findings indicating that female subjects may cause the majority of the changes seen in immune parameters following glutamine supplementation, whereas males do not experience any changes.

Resting plasma glutamine levels were not significantly different after supplementation with glutamine or placebo and remained at physiologically normal resting

levels (500-700μmol) (Castel and Newsholme, 1997; Walsh et al., 1998). It is suggested that even though resting plasma glutamine levels were not affected by glutamine supplementation in this study, glutamine supplementation may have corrected the post-exercise fall in plasma glutamine following exercise. However the non-responsive change seen in glutamine profiles is not surprising considering the time course for plasma glutamine following glutamine supplementation. Plasma glutamine concentrations will increase sharply after administration, reaching peak values within 30 minutes and remain elevated for 1-2 hours and then return back to resting values within 3 hours (Castell and Newsholme, 1997). Considering that our subjects were asked to provide a blood sample 8-10 hours following their final glutamine dose, the results found in this study are not surprising.

Therefore, although post-exercise glutamine measures were not possible it is suggested that glutamine supplementation corrected post-exercise changes in plasma glutamine. This correction in plasma glutamine levels may have then led to increased resting level of leukocytes and neutrophils in female athletes. Two possible explanations for these changes are evident. First glutamine supplementation may have decreased the fall in plasma glutamine during actual training bouts. Second, changes in plasma glutamine may not reflect intramuscular glutamine stores following long term supplementation when plasma glutamine stores are at the physiologically high range.

Although this present study has shown that glutamine supplementation provided to females participating in chronic, high intensity, endurance training improved resting leukocyte and neutrophil counts, further research should be conducted to determine the mechanism for this change. Future considerations should include determining the gender

differences associated with these present changes. Recommendations include: 1) to determine the pattern of plasma glutamine over the menstrual cycle; 2) to determine if plasma glutamine values show a correlation with hormone profiles; 3) to determine if a causal relationship exists between sex hormone profiles and plasma glutamine levels following supplementation. Furthermore, it is suggested that future research should include: 1) an examination and comparison of intramuscular glutamine stores compared to plasma concentrations; 2) plasma glutamine time analysis at various points following specific exercise bouts, 3) examination of the efficacy of glutamine supplementation on the immune system of athletes participating in higher level of exercise (e.g. triathletes, cross-country skiers, etc.), and in different type of exercise training (e.g. anaerobic type exercise); 4) replication of this present design with a larger sample size of both male and female athletes.

In summary, the results of this present study show that glutamine supplementation at a dose of 30 grams daily for a period of fourteen days, not only stopped the decline in leukocyte and neutrophil count experienced by the placebo supplemented group, but also resulted in a rise in these variables above baseline levels in female subjects. Furthermore, the changes in the leukocyte and neutrophil counts seen in female athletes were not evident in male athletes. This may suggest that glutamine may play a role in other systems, such as the endocrine system, which can ultimately affect immune function. Therefore glutamine supplementation may be an effective way of improving leukocyte and neutrophil count in female athletes after intense prolonged exercise or those experiencing overtraining syndrome. Further research is required to determine if glutamine supplementation will correct the immunosuppressive state of female athletes during chronic endurance training.

References

Abbas, A. K., Lichtman, A. H., & Pober, J. S. (2000). Cellular and Molecular Immunology (4th ed.). Pittsburgh, PA: W. B. Saunders.

Adjei, A. A., Matsumoto, Y., Oku, T., Hiroi, Y., & Yamamoto, S. (1994). Dietary argenine and glutamine combination improves survival in septic mice. Nutrition Research, 14, 1591-1599.

Ardawi, M. S. M., & Newsholme, E. A. (1985). Metabolism in lymphocytes and its importance to the immune function. Essays in Biochemistry, 21, 1- 44.

Calder, P. C. (1994). Glutamine and the immune system. Clinical Nutrition, 13, 2-8.

Calder, P. C. (1995). Glutamine action in cells of the immune system. In L. Cynober, P. Furst, P. & Lawin, P. (Eds.), Pharmacological Nutrition Immune Nutrition (pp. 10-20). New York: W. Zuckschwerdt Verlag.

Castell, L. M. & Newsholme, E. A. (1997). The effects of oral glutamine supplementation on athletes after prolonged, exhaustive exercise. Nutrition, 13, 738-742.

Castell, L. M., Poortmans, J. R., Leclercq, R., Brausseur, M., Duchateau, J., & Newsholme, E. A. (1997). Some aspects of the acute phase response after a marathon race, and the effects of glutamine supplementation. European Journal of Applied Physiology and Occupational Physiology, 75, 47-53.

Castell, L. M., Poortmans, J. R., & Newsholme, E. A. (1996). Does glutamine have a role in reducing infection in athletes? European Journal of Applied Physiology and Occupational Physiology, 73, 488-490.

Chandra, R. K. (1995). Nutrition and immune response. In L. Cynober, P. Furst, P.

& Lawin, P. (Eds.), Pharmacological Nutrition Immune Nutrition (pp. 5-9). New York: W. Zuckschwerdt Verlag.

Fahr, M. J., Kornbluth, J., Blossom, S., Schaeffer, R., & Klimberg, V. S. (1994). Glutamine enhances immunoregulation of tumor growth. Journal of Parenteral and Enteral Nutrition, 18, 471-476.

Frisina, J. P., Gaudieri, S., Cable, T., Keast, D., & Palmer, T. N. (1994). Effect of acute exercise on lymphocyte subsets and metabolic activity. International Journal of Sports Medicine, 15, 36-41.

Gaull, G. E., Rassin, D. K., Raiha, N. C. R., & Heinonen, K. (1977). Milk protein quantity and quality in low-birth weight infants: Effects of sulfur amino acids in plasma urine. Journal of Pediatrics, 90, 3448-355.

Gleeson, M., McDonald, W. A., Cripps, A. W., Pyne, D. B., Clancy, R. L. & Fricker, P. A. (1995). The effect of immunity of long term intense training in elite swimmers. Clinical and Experimental Immunology, 102, 210-216.

Hall, J. C., Heel. K., & McCauley, R. (1996). Glutamine. British Journal of Surgery, 83, 305-312.

Hack, V., Weiss, C., Friedmann, B., Suttner, S., Schykowski, M., Erbe, N., Benner, A., Bartsch, P., & Droge, W. (1997). Decreased plasma glutamine level and CD4+ T cell number in response to 8 wk of anaerobic training. American Journal of Physiology, 272, E788-E795.

Heath, G. W., Ford, E. S., Craven, T. E., Macera, C. A., Jackson, K. L., & Pate, R. R. (1991). Exercise and the incidence of upper respiratory tract infections. Medicine and

Science in Sports and Exercise, 23, 152-157.

Hills, M., & Armitage, P. (1979). The two-period cross-over clinical trial. British Journal of Clinical Pharmacology, 8, 7-20.

Hoffbrand, A. V., & Pettit, J. E. (1993). Essential Haematology (3rd ed.). Cambridge, MA: Blackwell Scientific.

Holmes, R. H., & Rahe, R. H. (1967). The social readjustment rating scale. Journal of Psychosomatic Research, 11, 213-218.

Institute of Inflammation Research. (1996, January). The Human Immune System. [On-line article]. Retrieved November 20, 1999 from the World Wide Web: http://inet.uni2.dk/~iirrh/IIRhome.htm

Jensen, G. L., Miller, R. H., Talabiska, D. G., Fish, J., & Gianferante L. (1996). N A double-blind, prospective, randomized study of glutamine-enriched compared to standard peptide-based feeding in critically ill patients. American Journal of Clinical Nutrition, 64, 615-621.

Keast, D. Arstein, D., Harper, W., Fry, R. W., & Morton, A. R. (1995). Depression of plasma glutamine concentrations after exercise stress and its possible influence on the immune system. The Medical Journal of Australia, 162, 15-18.

Ketner, J. B., & Mellion, M. B. (1995). The overtraining syndrome: A review of presentation, pathophysioilogy, and treatment. Medicine, Exercise, Nutrition, and Health, 4, 136-145.

Klimberg, V. S., Nwokedi, E., Hutchins, L. F., Pappas, A. A., Lang, N. P., Broadwater, J. R., Read, R. C., & Westbrook, K. C. (1992). Glutamine facilitates

chemotherapy while reducing toxicity. Journal of Parenteral and Enteral Nutrition, 16, 835-875.

Klimberg, S. V., Salloum, R. M. Kasper, M., Plumley, D. A., Dolson, D. J., Hautamaki, R. D., Mendenhall, W. R., Bova, F. C., Bland, K. I., Copeland, E. M., & Souba, W. W. (1990). Oral glutamine accelerates healing of the small intestine and improves outcome after whole abdominal radiation. Archives of Surgery, 125, 1040-1045.

Krebs, H. A. (1935). Metabolism of amino acids. IV. The synthesis of glutamine from glutamic acid and ammonia and the enzymatic hydrolysis of glutamine in animal tissues. Biochemistry Journal, 29, 1951-1969.

Lacey, J. M., Crouch, J. B., Benfell, K., Ringer, S. A., Wilmore, C. K., Maguire, D., & Wilmore, D. W. (1996). The effects of glutamine supplemented parenteral nutrition in premature infants. Journal of Parenteral and Enteral Nutrition, 20, 74-80.

Lacey, J. M. & Wilmore D. W. (1990). Is glutamine a conditionally essential amino acid? Nutrition Reviews, 48, 297-309.

Lehmann, M., Baummartl, P. Wiesenack, Baumann, H., Fisher, S., Spori, U., Gendrisch, G., Kaminski R., & Keul, J. (1992). Training-overtraining: Influence of a defined increase in training volume vs intensity on performance, catecholamines and some metabolic parameters in experienced middle- and long-distance runners. European Journal of Applied Physiology and Occupational Physiology, 64, 169-177.

Lehmann, M., Mann, H., Gastmann, U., Keul, J., Vetter, D., Steinacker, J. M., & Haussinger, D. (1996). Unaccustomed high-milage vs intensity training-related changes in performance and serum amino acid levels. International Journal of Sports Medicine, 17,

187-192.

Lund, P. (1986). L-Glutamine and L-Glutamate: UV Method with Glutaminase and Glutamate Dehydrogenase (pp. 357-363). In H. U. Bergmeyer, (Ed), Methods of Enzymatic Analysis. Verkagsgesellshaft, Weinheim: VCH.

Mackinnon, L. T. (1999). Advances in exercise immunology. Human Kinetics: Champaign, IL.

Mackinnon, L. T., Ginn, E. M., Seymour, G. J. (1993). Temporal relationship between decreased salivary IgA and upper respiratory tract infection in elite athletes. Australian Journal of Science and Medicine in Sport, 25, 94-99.

Mackinnon, L. T., & Hooper, S. L. (1996). Plasma glutamine and upper respiratory tract infection during intensified training in swimmers. Medicine and Science in Sports and Exercise, 28, 285-290.

McDowell, S. L., Hughes, R. A., Hughes, R. J., Housh, D. J., Housh, T. J., & Johnson, G. O. (1992). The effect of exhaustive exercise on salivary immunoglobulin A. The Journal of Sports Medicine and Physical Fitness, 32, 412-415.

McKenzie, D.C. (1999). Markers of excessive exercise. Canadian Journal of Applied Physiology, 24, 66-73.

Newsholme, E. A. (1994). Biochemical mechanism to explain immunosuppression in well-trained and overtrained athletes. International Journal of Sports Medicine, 15, S142-S147.

Newsholme, E. A., Parry-Billing, N., McAndrew, N., & Bidgett, R. (1991). A biochemical mechanism to explain some characteristics of overtraining. Medicine and

Science in Sports and Exercise, 32, 79-93.

Nieman, D. C. (1994). Exercise, upper respiratory tract infection and the immune response. Medicine and Science in Sports and Exercise, 26, 128-139.

Nieman D. C., Henson, D. A., Gusewitch, G., Warren, B. J., Dotson, R. C., Butterworth, D. E., & Nehlsen-Cannarella, S. L. (1993). physical activity and immune function in elderly women. Medicine and Science in Sports and Exercise, 25, 823-831.

Nieman, D. C., Johanssen, L. M., Lee, L. M., Cermak, J. W., & Arabatzis K. (1990). Infectious episodes in runners before and after the Los Angeles marathon. Journal of Sports Medicine and Physical Fitness, 30, 316-328.

Nieman, D. C., Nehlsen-Cannarella, S. L., Markoff, P. A., Balk-Lamberton, A. J., Yank, H. Chritton, D. B. W., Lee, J. W., & Arabatzis, K. (1990). The effect of moderate exercise training on natural killer cells activity and acute upper respiratory tract infection. International Journal of Sports Medicine, 11, 467-473.

Nieman, D. C. & Pedersen, B. K. (1999). Exercise and immune function: Recent developments. Sports Medicine, 27, 73-80.

Northoff, H. Enkel, S., & Weinstock. C. (1995). Exercise, injury and immune function. Exercise Immunology Review, 1, 1-25.

Parry-Billing, M., Blomstrand, E., McAndrew, N., & Newsholme, E. A. (1990). A communicational link between skeletal muscle, brain, and cells of the immune system. International Journal of Sports Medicine, 11, S122-S128.

Parry-Billing, M. Budgett, R., Koutedakis, Y., Blomstraid, E., Brooks, S., Williams, C., Calder, P. C., Pilling, S., Baigrie, R., & Newsholme, E. A. (1992). Plasma

amino acid concentrations in the overtraining syndrome: Possible effects on the immune system. Medicine and Science in Sports and Exercise, 24, 1353-1358.

Parry-Billing, M., Evans, J., Calder, P. C., & Newsholme, E. A. (1990). Does glutamine contribute to immunosuppression after major burns? The Lancet, 336, 523-525.

Peters, E. M. (1990). Altitude fails to increase susceptibility of ultramarathon runners to post-race upper respiratory trat infection. South African Journal of Sports Medicine, 5, 4-8.

Peters, E. M. & Bateman, E. D. (1983). Respiratory tract infections: An epidemiological survey. South African Medical Journal, 64, 582-584.

Pyne, D. B., Baker, M. S., Fricker, P. A., McDonald, W. A., Telford, R. D., &Weidemann, M. J. (1995). Effects of an intense 12-wk training program by elite swimmers on neutrophil oxidative activity. Medicine and Science in Sports and Exercise, 27, 536-542.

Rohde, T., Asp, S., MacLean, D. A., & Pedersen B. K. (1998). Competitive sustained exercise in humans, lymphokine activated killer cell activity, and glutamine-an intervention study. European Journal of Applied Physiology and Occupational Physiology, 78, 448-453.

Rohde, T., MacLean, D. A., & Pedersen B. K. (1998). Effect of glutamine supplementation on changes in the immune system induced by repeated exercise. Medicine and Science in Sports and Exercise, 30, 856-862.

Roitt, I. (1994). Essential Immunology (8th Ed). Blackwell Scientific: London.

Rowbottom, D. G, Keast, D., Goodman, C., & Morton, A. R. (1995) The

hematological, biochemical and immunological profile of athletes suffering from the overtraining syndrome. European Journal of Applied Physiology and Occupational Physiology, 70, 50-509.

Savy, G. K. (1997). Enteral glutamine supplementation: Clinical review and practical guidelines. Nutrition and Clinical Practice, 12, 259-262.

Schloerb, P. R., & Amare, M. (1993). Total parenteral nutrition with glutamine in bone marrow transplant and other clinical applications (A randomized, double-blind study). Journal of Parenteral and Enteral Nutrition, 17, 407-413.

Shephard, R. J. (1997). Physical activity, training and the immune response. Cooper: Carmel, IN.

Smith, D.J. & Norris, S. R. (2000). Changes in glutamine and glutamate concentrations for tracking training tolerance. Medicine and Science in Sports and Exercise, 32, 684-689.

Smith, J. R., & Wilmore, D. W. (1990). Glutamine nutrition and requirements. Journal of Parental and Enteral Nutrition, 14, S 94-S 99.

Tvede, N., Pedersen, B. K., Hansen, F. R., Bendix, T., Christensen, L. D. Galbo, H., & Halkjaere-Kristensen (1997). Effect of physical exercise on blood mononuclear cell subpopulations and in vitro proliferation responses. Scandinavian Journal of Immunology, 29, 383-389.

Walsh, N. P., Blannin, A. K., Robson, P. J. & Gleeson, M. (1998). Glutamine, exercise and immune function. Sports Medicine, 26, 177-191.

Wilmore, D. W., Schoerb, P. R., & Ziegler, T. R. (1999). Glutamine in the support

of patients following bone marrow transplantation. Current Opinion in Clinical Nutrition and Metabolic Care, 2, 323-327.

Yoshida, S., Matsui, M., Shirouzau, Y., Hiromasa, F., Yamana, H., & Shirouzy, K. (1998). Effects of glutamine supplementation and radiochemotherapy on systemic immune and gut barrier function in patients with advanced esophageal cancer. Annals of Surgery, 227, 485-491.

Ziegler, J. L. (1991). Cancer in the immunocompromised host. In D. P. Stites, & A. I. Terr, (Eds.), Basic and Clinical Immunology (7th ed., pp. 588-598). East Norwalk, CT: Appleton & Lange.

Ziegler, T. R., Benfell, K., Smith, R. J., Young, L. S., Brown, E., Ferrari-Baliviera, E., Lowe, D. K., & Wilmore, D. W. (1990). Safety and metabolic effects of L-glutamine administration in humans. Journal of Parenteral and Enteral Nutrition, 14, S137-S146.

Ziegler, T. R., Bye, R. L., Persinger, R. L., Young, L. S., Antin, J. H., & Wilmore, D. W. (1998). Effects of glutamine supplementation on circulating lymphocytes after bone marrow transplantation: A pilot study. American Journal of the Medical Sciences, 315, 4-10.

Zeigler, T. R., Young, L. S., Benfell, K., Scheltinga, M., Hortos, K., Bye, R., Morrow, F. D., Jacobs, D. O., Smith, R. J., Antin, J. H., & Wilmore, D. W. (1992). Clinical and metabolic efficacy of glutamine-supplemented parenteral nutrition after bone marrow transplantation: A randomized, double-blind, controlled study. Annals of Internal Medicine, 116, 821-828.

Appendix A - Cover Letter

"The Effect of Oral Glutamine Supplementation on Immune Parameters in Endurance Trained Athletes"

Dear Participant:

I would like to begin by thanking you for considering to be a participant in this research study. Furthermore, feel free to approach me at anytime if you have any questions or concerns related to this study.

The intent of this research project is to evaluate the role of dietary glutamine supplementation on the immune system of endurance trained athletes. It is being conducted by the School of Kinesiology, Lakehead University in collaboration with the Northwestern Ontario Regional Cancer Centre and the University of Ottawa Hospital Research Institute, Centre for Molecular Medicine. Very little is known about glutamine's effect on the immune system of athletes, however research with critically ill patients has shown that glutamine supplementation is beneficial.

Potential volunteers will be screened for continued participation in the study. Criteria for inclusion include health status, and personal training habits. Therefore, all subjects will be required to complete a preliminary questionnaire, which outlines your training habits and any past or current medical problems. In order for the researcher to achieve accurate responses from this questionnaire, the specific selection criteria will not be disclosed. After completion of the preliminary questionnaire those individuals that fit the specific criteria will be asked to volunteer and those who don't will have fulfilled their role in this research project.

Those individuals fitting the specific selection criteria will continue with the procedures outlined below. These procedures will allow us to assess the role of glutamine on athletes immune system. During the study you will be asked to complete a questionnaire outlining your physical training habits, a life stress questionnaire, and a 3 day dietary intake log on four separate occasions. You will also be required to provide a blood and saliva sample on four separate occasions during this study. The utmost care and safety will be taken when handling blood and saliva and will be performed by a certified phlebotomist. We also ask that you maintain your regular training, and dietary schedule throughout the entire study. The entire study will last a period of two months, however you will only be required to visit with the researchers (the laboratory) for approximately 1 hour on 4 separate occasions. Throughout this two month period you will supplement your diet with two different treatments (glutamine and a placebo). You will supplement for two weeks will glutamine and two weeks with a placebo. These two supplementation periods will be separated by a one month washout period, consisting of no treatment. Glutamine is a natural occurring amino acid found in various plant and animal sources, and has been shown to have no known side effects when taken as an oral or intravenous

solution in various populations. The placebo will consist of a powder which is use as a sugar substitute.

As a volunteer, you have the right to refuse any test and to withdraw from the study at any time. All the information gathered during this study will remain confidential through the use of a coding system. No reference to any participant will be made at anytime in any report of the results. The data will be securely stored by Joe Quadrilatero and Dr. Ian Newhouse within the Faculty of Kinesiology at Lakehead University for seven years. However, the findings of this project will be made available to you at your request upon completion of the project.

If you have any questions concerning the study, I may be reached by phone at home (807) 577-9541 or at work (807) 346-7815. I may also be reached by means of email at (*quadrilj@air.on.ca*).

Thank you for your cooperation.

Sincerely,

Principal Investigator: Joe Quadrilatero
Advisors: Dr. Ian Newhouse and Dr. Norm LaVoie

Appendix B - Consent Form

1. I _____consent to take part in a study which examines the effect of oral glutamine supplementation on the immune system of endurance trained athletes and understand that the entire study will last a period of 8 weeks.

2. I agree to attend an information session, at which time all pertinent information regarding my participation in the study will be outlined, and all questions and concerns will be addressed. Also, instructions on how to properly preform the following will be provided:

    1) recording daily nutritional intake
    2) recording training routine
    3) completing life stress questionnaire
    4) administering treatment (glutamine or placebo)

3. I agree to record all my daily nutritional intake for 3 days on four separate occasions (before and after each of the two supplementation periods). That is, everything that I consume (food and drink) will be written down in a log book according to the procedures outlined in the information session. Furthermore, I agree to maintain my regular diet through the duration of the study.

4. I agree to complete a training log during each of the two supplementation periods outlining my daily routine according to the procedures outlined in the information session. Furthermore, I agree to maintain my regular training volume through the duration of the study.

5. I agree to complete a life stress questionnaire (The Social Readjustment Rating Scale) on four separate occasions (before and after each of the two supplementation periods) according to the procedures outlined in the information session.

6. I agree to provide a blood and a saliva sample on four separate occasions (before and after each of the two supplementation periods). I understand that the blood and saliva samples will be taken by a certified phlebotomist which will perform the testing in a safe and sterile environment. I am aware that the chance of infection or serious complications is minimal, however some individuals may experience a feeling of light headedness upon removal of blood. I also, understand that there may be some slight bruising and discomfort from the needle, but should disappear within a few days.

7. I agree to participate in two separate supplementation periods each lasting a

duration of 2 weeks according to the procedures outlined in the information session. One supplementation period will involve the addition of glutamine to my regular diet while the other supplementation period will involve the addition of a placebo (Inula Pure® - a sugar substitute) to my regular diet. I understand that both supplementation periods will be separated by a 4 week period that involves no treatment (maintain regular diet). I am also aware that the treatment (glutamine or placebo) will be administered in a random fashion, therefore I will not be aware of which treatment I am receiving during each supplementation period.

8. I understand that as a volunteer, I have the right to refuse any test and to withdraw from the study at any time. All the information gathered during this study will remain confidential through the use of a coding system. No reference to any participant will be made at anytime in any report of the results. The data will be securely stored by Joe Quadrilatero and Dr. Ian Newhouse within the Faculty of Kinesiology at Lakehead University for seven years. However, the findings of this project will be made available to me at my request upon completion of the project. I understand that if I should have any questions concerning the study, I should contact Joe Quadrilatero by phone at home (807) 577-9541, at work (807) 346-7815 or by means of email at (*quadrilj@air.on.ca*).

Signature of Participant Date

Signature of Witness Date

9. I have explained the nature of the study to the participant and believe he/she has understood it fully.

Signature of Researcher Date

Appendix C - Preliminary Questionnaire

Name _____ Age_____ Sex _____

For all questions please be as specific as possible. Thank You.

1. What type of endurance training are you currently participating (eg. cross-country running, track, cross-country skiing) ?

2. On average, how many days per week do you participate in endurance type training?

3. On average, how many kilometers or miles do you perform during your endurance training per week?

4. On average, what is the duration (minutes) of your endurance training sessions?

5. On average, at what intensity are your endurance training sessions ? (eg. 50% of max, 75% of max).

6. Do you have any injuries or medical illnesses that have affected your training in the past ? If yes, how did it affected your training?

7. Periodically throughout the study you will be asked to provide a blood/saliva samples. Please list any events/competitions (or other obligations) during the months of September, October, November that require you to be away from Thunder Bay and that may affect your ability to provide samples ?

8. Have you ever supplemented with glutamine?

9. Are you currently taking any type of nutritional supplement (eg. vitamins, protein drink, creatine etc.) ?

10. Are you current participating in any other type of physical activity during an average week (eg. weight training, squash, soccer etc.)? Please describe in detail the time spent on these activities on an average week.

11. Will there be any changes to your training routine from now until mid November?

Appendix D - Reagents for Flow Cytometer Analysis

1) Flow-Check Fluorospheres™ (Beckman Coulter™; PN 6605359)

2) Flow-Set Fluorospheres™ (Beckman Coulter™; PN 6607007)

3) CYTO-COMP Reagent 1 (FICT/RD1) (Beckman Coulter™; PN 6607021)

4) CYTO-COMP Reagent 2 (RD1/ECD)

5) CYTO-COMP Reagent 3 (RD1/PC5)

6) CYTO-COMP Reagent 4 (ECD/PC5)

7) CYTO-COMP™ Cells (Beckman Coulter™; PN 6607023)

8) IMMUNO-TROL™ whole control blood (Beckman Coulter™; PN 4238091-E)

9) CYTO-STAT® tetraCHROME™ Reagent CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 Monoclonal Antibody Reagent (Beckman Coulter™ ; PN 6607013)

10) CYTO-STAT® tetraCHROME™ Reagent CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5 Monoclonal Antibody Reagent (Beckman Coulter™; PN 6607073)

11) IOTest® Conjugated CD16-PE (ImmunoTech™; PN IM1238)

12) OptiLyse C® (Beckman Coulter™; PN IM1401)

13) Phosphate Buffered Saline (PBS)

- ➢Dissolve one PBS package into 1 L of deionized water.
- ➢Adjust pH to 7.2.

Stability and Storage of Solutions:

- ➢Store antibodies, calibration and compensation reagents (1-11) in refrigerator at 2-8 °C.
- ➢Store OptiLyse (12) and PBS (13) at room temperature.

Appendix E - Reagents for Glutamine Analysis

1) Acetate Buffer (0.5 mol/l, pH 5.0):

a) ➢ Dissolve 6.8 g $C_2H_3O_2Na \cdot 3\ H_2O$ (Sodium Acetate Trihydrate) in deionized water and make up to 100 ml.
b) ➢ Dilute 2.9 ml glacial acetic acid to 100 ml with deionized water.

➢ Mix 67.8 ml (1a) with 32.3 ml (1b).

2) L-glutamine standard solution (2 mmol/l):

a) ➢ Dissolve 292 mg L-glutamine in 10 ml deionized water.
b) ➢ Dilute 0.1 ml solution (a) to 10 ml with deionized water.

3) Glutaminase (10 kU/l):

➢ Dissolve 10 U glutaminase powder (from E. coli, lyophilized powder, 20-30 U per mg protein at 37°C; essentially free from glutamate decarboxylase; commercial preparation Grade V from Sigma) in 1 ml acetate buffer (use 0.1 ml solution (1) diluted with 0.9 ml deionized water).

4) Tris/Hydrazine Buffer [0.1 mol/l Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol); 2 mmol/l EDTA; 0.63 mol/l hydrazine]:

➢Dissolve 1.2 g Tris and 74 mg EDTA-$Na_2H_2 \cdot 2\ H_2O$ in approximately 60 ml deionized water.
➢Add 5 ml hydrazine hydrate (62%, w/v).
➢Adjust to pH 9 with 5 mol/l HCL, and make up to 100ml with deionized water.

5) Nicotinamide-adenine Dinucleotide (β-NAD, 30 mmol/l):

➢Dissolve 200 mg NAD, free acid, in 10 ml deionized water.

6) Adenosine 5'-diphospate (ADP, 100mmol/l):

➢Dissolve 42.7 mg ADP, free acid, in water, neutralize with 0.1 ml 2mol/l NaOH and make up to 1 ml with deionized water

7) Glutamate Dehydrogenase (GIDH, 1200 kU/l)

➢Use stock solution from beef liver, 10 mg/ml in glycerol, 120 U/mg at 25 °C

8) Perchloric Acid (10 % v/v):

➢Dilute 10 ml perchloric acid (60 %, w/w, sp. gr. 1.54) to 60 ml with deionized water.

9) Potassium Hydroxide (KOH, 20 % w/v):

➢Dissolve 20 g KOH in 100 ml deionized water

Stability and Storage of Solutions:

➢Store buffer solutions (1), and (4) in refrigerator (2-8°C).
➢The Tris/hydrazine buffer (4) is stable for 7 days.
➢Store solutions (2), (3), (5), and (6) in deep freeze (-20 °C). Make working aliquots.
➢The Glutamate Dehydrogenase is stable for at least 12 months at 4°C.

Calculations

➢C1 = sum of glutamine concentrations after deamination of endogenous glutamate.
➢C2 = sum of glutamate concentration.
➢C3 = sum of glutamine concentration.
➢V = volume of sample in ml.
➢D = dilution factor

Δabsorbance in C2 - Δabsorbance in C1 = C3 ÷ V x D

Appendix F - Reagents for Saliva IgA Analysis

1) Anti-human IgA (α-chain specific) developed in Rabbit.

2) Purified Human Colostrum IgA 2500mg/L.

3) Anti-human IgA (α-chain specific) Peroxidase Conjugate $F(ab')_2$ developed in goat.

4) Coating buffer:

- ➢Dissolve 0.8g Na_2CO_3 (Sodium Carbonate)
- ➢Dissolve 1.45g $NaHCO_3$ (Sodium Bicarbonate)
- ➢Adjust pH to 9.6. Make up to final volume of 500 ml with deionized water.

5) Washing Buffer:

- ➢Dissolve 16 g NaCl (Sodium Chloride)
- ➢Dissolve 0.4 g KCl (Potassium Chloride)
- ➢Dissolve 0.4 g KH_2PO_4 (Monopotassium Phosphate)
- ➢Dissolve 5.8 g Na_2HPO_4 (Monosodium Phosphate)
- ➢Dissolve 2 g Tween-80
- ➢Adjust to pH 7.2. Make up to final value of 2L with deionized water.

6) Phosphate Buffered Saline (PBS):

- ➢Dissolve 1 PBS package into 1 L of deionized water.
- ➢Adjust pH to 7.2

7) Blocking Protein (2% w/v):

- ➢Dissolve 0.8g of Bovine Serum Albumin in 40 ml PBS.

8) OPD (o-Phenylenediamine Dihydrochloride) Peroxidase Conjugate Substrate:

- ➢Dissolve one OPD tablet and one urea hydrogen peroxide/buffer tablet into 20 deionized water.
- ➢Protect from light by wrapping in foil.

Stability and Storage of Solutions:

➢Store reagents (1-3) in deep freeze (-20 °C) in working aliquots.
➢Coating Buffer (4) is stable in refrigerator (2-8 °C) for 14 days.
➢PBS (6) and Washing Buffer (5) are stable at room temperature.
➢Prepare Blocking Protein (7) and Substrate (8) before each procedure.

Appendix G - Subject Instructions

Supplementation Instructions

1) Please do not compare treatments (supplement) with other participants in the study.

2) Please start supplementation TODAY. Remove a three dose package each day. Take one bag of supplement on three separate occasions (4-8 hrs apart) for each of the next 14 days (November 17th - November 30th).

3) Empty all the contents of one bag into a flavoured drink of your choice, stir the contents and drink immediately. **PLEASE NOTE: There are staples at the top of each three bag package. Please take care to ensure that a staple does not fall into your drink.**

Nutritional Intake Instructions

1) Record your dietary intake for October 16th, 17th & 18th and for November 30th, December 1st & 2nd.

2) Please be as specific as possible. Your description should include the type of food as well as the quantity. For example - if you ate a tuna sandwich with a glass of milk for lunch your description could be something like this -- 2 slices of white bread, 2 teaspoons of light mayo, 100 grams of tuna (in water), 2 cups (500ml) of 1% milk.

Training Log Instructions

1) Please start a training log TODAY.

2) Outline the type of training (running, weights etc.), volume (reps/sets, mileage), duration, and intensity (% of max) for each of the next 14 days (November 17th - November 30th).

3) Please bring a copy of your training log with you on the next test session.

Next Test Session

Your next test session will be December 1st at _____.

If you have any questions about these procedures please contact Joe by phone at 346-7815 (office), 577-9541 (home) or e-mail at jquadril@mail.lakeheadu.ca.

Appendix H - Social Readjustment Rating Scale

Rank	Life Event	Value
1	Death of spouse	100
2	Divorce	73
3	Marital separation	65
4	Jail term	63
5	Death of a close family member	63
6	Personal injury or illness	55
7	Marriage	50
8	Fired at work	47
9	Marital reconciliation	45
10	Retirement	45
11	Change in health of a family member	44
12	Pregnancy	40
13	Sex difficulties	39
14	Gain of a new family member	39
15	Business readjustment	39
16	Change in financial state	38
17	Death of a close friend	37
18	Change to different line at work	36
19	Change in the number of arguments with spouse	35
20	Mortgage over $10,000	31
21	Foreclosure of mortgage or loan	30
22	Change in responsibilities at work	29
23	Son or daughter leaving home	29
24	Trouble with in-laws	29
25	Outstanding personal achievement	28
26	Spouse begins or stops work	26
27	Begin or end of school	26
28	Change in living conditions	25
29	Revision of personal habits	24
30	Trouble with boss	23
31	Change in work hours or conditions	20
32	Change in residence	20
33	Change in school	20
34	Change in recreation	19
35	Change in church activities	19
36	Change in social activities	18
37	Mortgage or loan less than $10,000	17
38	Change in sleeping habits	16
39	Change in number of family get-togethers	15
40	Change in eating habits	15
41	Vacation	13
42	Christmas	12
43	Minor violation of the law	11

Holmes and Rahe, 1967

Appendix I - Social Validation Questionnaire

1) Did you observe any differences in the two different supplements that you received? If so explain.

2) Do you have any idea which supplement was the **glutamine** and which was the **placebo**?

Yes_____ or No _____ If yes, please answer below.

Supplement Period #1 _____

Supplement Period #2 _____

3) Did the first or second supplements give you any problem? If so explain.

4) Did you feel any positive effects from either of the supplements (ie. Increased performance, increased health status etc.)?

5) Would you participate in a study of this nature again? Please explain.

6) Would you change anything about this study? Explain.

Name_____ Date_____

Appendix J - ANOVA Source Tables

2 Way ANOVA TABLE
Leukocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0024	14	0.07859	0.03	0.86
Treatment (Glutamine vs Placebo)	1	0.08513	14	0.206	0.41	0.53
Time x Treatment	1	1.33206	14	0.367	3.63	0.08

2 Way ANOVA TABLE
Neutrophil Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0034	14	0.0631	0.05	0.82
Treatment (Glutamine vs Placebo)	1	0.4455	14	0.08672	5.14	0.04
Time x Treatment	1	1.1016	14	0.2906	3.79	0.07

2 Way ANOVA TABLE
Basophil Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0	14	0	0.1	0.75
Treatment (Glutamine vs Placebo)	1	0.001	14	0	0.45	0.51
Time x Treatment	1	0	14	0	0.01	0.93

2 Way ANOVA TABLE
Eosinophil Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0028	14	0.0061	0.45	0.51
Treatment (Glutamine vs Placebo)	1	0.07884	14	0.11706	0.67	0.43
Time x Treatment	1	0.0016	14	0.001	1.76	0.21

2 Way ANOVA TABLE
Monocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0066	14	0.0043	1.52	0.24
Treatment (Glutamine vs Placebo)	1	0.001	14	0.0048	0.21	0.66
Time x Treatment	1	0.0023	14	0.0033	0.7	0.42

2 Way ANOVA TABLE
Lymphocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0016	14	0.03139	0.05	0.82
Treatment (Glutamine vs Placebo)	1	0.01485	14	0.03832	0.39	0.54
Time x Treatment	1	0.001	14	0.02105	0.28	0.87

2 Way ANOVA TABLE
T lymphocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0	14	0.01854	0.02	0.9
Treatment (Glutamine vs Placebo)	1	0.01493	14	0.02289	0.65	0.43
Time x Treatment	1	0	14	0.01359	0.01	0.95

2 Way ANOVA TABLE
Helper T Lymphocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0	14	0.0085	0.05	0.83
Treatment (Glutamine vs Placebo)	1	0.0048	14	0.01115	0.43	0.52
Time x Treatment	1	0.0011	14	0.006	0.18	0.68

2 Way ANOVA TABLE
Cytolytic T lymphocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0	14	0.0019	0.06	0.81
Treatment (Glutamine vs Placebo)	1	0.0034	14	0.0023	1.45	0.25
Time x Treatment	1	0.001	14	0.0013	0.43	0.52

2 Way ANOVA TABLE
B lymphocyte Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0	14	0.001	0.68	0.42
Treatment (Glutamine vs Placebo)	1	0.0013	14	0.0013	1.02	0.33
Time x Treatment	1	0.001	14	0	2.07	0.17

2 Way ANOVA TABLE
Natural Killer Cell Count

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0038	14	0.002	1.92	0.19
Treatment (Glutamine vs Placebo)	1	0	14	0.0046	0.01	0.91
Time x Treatment	1	0.001	14	0.002	0.27	0.61

2 Way ANOVA TABLE
Percent Neutrophils

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.1042	14	8.886	0.01	0.92
Treatment (Glutamine vs Placebo)	1	82.368	14	15.452	5.33	0.04
Time x Treatment	1	61	14	27.271	2.87	0.11

2 Way ANOVA TABLE
Percent Basophils

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.0016	14	0.05301	0.03	0.87
Treatment (Glutamine vs Placebo)	1	0.0525	14	0.03741	1.4	0.26
Time x Treatment	1	0.0758	14	0.06418	1.18	0.3

2 Way ANOVA TABLE
Percent Eosinophils

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.5487	14	1.58144	0.35	0.57
Treatment (Glutamine vs Placebo)	1	18.98	14	23.7195	0.8	0.39
Time x Treatment	1	0.0059	14	0.31811	0.18	0.89

2 Way ANOVA TABLE
Percent Monocytes

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	1.22123	14	1.4337	0.85	0.37
Treatment (Glutamine vs Placebo)	1	0.08067	14	1.2521	0.06	0.8
Time x Treatment	1	1.4415	14	1.46666	0.98	0.34

2 Way ANOVA TABLE
Percent Lymphocytes

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.486	14	9.675	0.05	0.83
Treatment (Glutamine vs Placebo)	1	23.064	14	17.729	1.3	0.27
Time x Treatment	1	38.4	14	18.555	2.07	0.17

2 Way ANOVA TABLE
Percent T lymphocytes

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	1.70017	14	2.9803	0.57	0.46
Treatment (Glutamine vs Placebo)	1	3.40817	14	7.09585	0.48	0.5
Time x Treatment	1	0.10417	14	3.65577	0.03	0.87

2 Way ANOVA TABLE
Percent CD3+CD4+ lymphocytes

Source	dfEffect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.4167	14	4.4495	0.09	0.76
Treatment (Glutamine vs Placebo)	1	1.23267	14	5.6741	0.22	0.65
Time x Treatment	1	2.3207	14	3.1749	0.73	0.41

2 Way ANOVA TABLE
Percent CD3+CD8+ lymphocytes

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.02817	14	0.341	0.08	0.78
Treatment (Glutamine vs Placebo)	1	2.94817	14	0.7267	4.06	0.06
Time x Treatment	1	1.38017	14	1.298	1.06	0.32

2 Way ANOVA TABLE
CD4/CD8 Ratio

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.001	14	0.01121	0.08	0.78
Treatment (Glutamine vs Placebo)	1	0.01457	14	0.01685	0.87	0.37
Time x Treatment	1	0.03558	14	0.01949	1.83	0.2

2 Way ANOVA TABLE
Percent CD19 lymphocytes

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	0.15	14	0.423	0.35	0.56
Treatment (Glutamine vs Placebo)	1	0.726	14	1.1421	0.64	0.44
Time x Treatment	1	0.384	14	1.0994	0.35	0.56

2 Way ANOVA TABLE
Percent CD3-CD16+CD56+ lymphocytes

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	3.80017	14	5.22588	0.73	0.41
Treatment (Glutamine vs Placebo)	1	0.40017	14	12.5373	0.03	0.86
Time x Treatment	1	0.20417	14	5.5556	0.04	0.85

2 Way ANOVA TABLE
Saliva IgA

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	2475.41	14	2770.23	0.89	0.36
Treatment (Glutamine vs Placebo)	1	2211.85	14	2449.43	0.9	0.36
Time x Treatment	1	1244.6	14	2385.63	0.52	0.48

2 Way ANOVA TABLE
Plasma Glutamine

Source	df Effect	MS Effect	df Error	MS Error	F-Ratio	p level
Time (Pre vs Post)	1	2642.72	14	11139.99	0.24	0.63
Treatment (Glutamine vs Placebo)	1	23784.49	14	12108.47	1.96	0.18
Time x Treatment	1	462.15	14	9663.31	0.05	0.83

3 Way ANOVA TABLE
Leukocyte Count by Gender

Source	dfEffect	MS Effect	df Error	MS Error	F - Ratio	p level
Gender (Male vs Female)	1	0.31447	13	5.5314	0.06	0.82
Time (Pre vs Post)	1	0.0031	13	0.0845	0.04	0.85
Treatment (Glutamine vs Placebo)	1	0.02601	13	0.19169	0.14	0.72
Gender x Time	1	0.0015	13	0.0845	0.18	0.9
Gender x Treatment	1	0.38809	13	0.19169	2.03	0.18
Time x Treatment	1	1.90678	13	0.27472	6.94	0.02
Gender x Time x Treatment	1	1.56288	13	0.27472	5.69	0.03

3 Way ANOVA TABLE
Neutrophil Count by Gender

Source	dfEffect	MS Effect	df Error	MS Error	F - Ratio	p level
Gender (Male vs Female)	1	0.03969	13	2.00218	0.02	0.89
Time (Pre vs Post)	1	0.0014	13	0.0672	0.02	0.89
Treatment (Glutamine vs Placebo)	1	0.35469	13	0.0868	4.09	0.06
Gender x Time	1	0.01003	13	0.67217	0.15	0.71
Gender x Treatment	1	0.08525	13	0.0868	0.98	0.34
Time x Treatment	1	1.67281	13	0.17789	9.4	0.01
Gender x Time x Treatment	1	1.75561	13	0.17789	9.87	0.01

3 Way ANOVA TABLE
Percent Neutrophils by Gender

Source	dfEffect	MS Effect	df Error	MS Error	F - Ratio	p level
Gender (Male vs Female)	1	49.6547	13	177.339	0.28	0.61
Time (Pre vs Post)	1	0.0562	13	8.9802	0.01	0.94
Treatment (Glutamine vs Placebo)	1	75.1674	13	16.546	4.54	0.05
Gender x Time	1	7.6563	13	8.9802	0.85	0.37
Gender x Treatment	1	1.2367	13	16.546	0.08	0.79
Time x Treatment	1	107.693	13	8.6293	12.48	0
Gender x Time x Treatment	1	185.617	13	8.6293	21.51	0