CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Physiological stress represents a wide range of physical responses that occur as a direct effect of a stressor causing an upset in the homeostasis of the body (Koolhaas, 2011). Several stressors including exercise-induced stress have been associated with a shift in cytokine production towards the anti-inflammatory patterns with cortisol and catecholamine elevations as the possible mediators (Elenkov and Chrousos, 2002). It has been established that academic stress is inhibitory to the proliferation of $CD4^+$ T lymphocytes with the elevation of serum cortisol as a possible mediator (Ehiaghe *et al*., 2014). It has also been reported that academic stress significantly increases serum cortisol level of Nigerian students (Onyenekwe *et al*., 2014). It is well known that stress increases the activity of the hypothalamic-pituitary-adrenal axis and causes a decrease in reproductive hormones (e.g. luteinizing and follicle stimulating hormones) in order to preserve the host state alertness (Kamel *et al*., 2001).

Physical exercise is important for maintaining physical fitness and can contribute positively to maintaining a healthy weight, muscle strengthening, promoting physiological well-being and strengthening of the immune system (Stampfer *et al*., 2000; Hu *et al*., 2001; Gosker and Schol, 2008). The athlete's reaction to exercise is a coordinated response of multiple organ system, which include immune response modulation and generation of reactive oxygen and nitrogen species that, besides their tissue damaging potential, also play a crucial role in cellular signaling (Connolly *et al*., 2004; Zicker *et al*., 2005).

Studies have shown that exhaustive exercise can cause up-regulation of several genes in the human body including lymphocytic tumor necrosis factor (TNF) α, succinate dehydrogenase (SDH) genes which show increased expression patterns within 1 to 4 hours after stimulation, their expression pattern is transient with maximal mRNA accumulation of about 8 hours, before returning to their baseline level after 72 hours of withdrawal of the stressor (Abdalla *et al*., 2003; Stenberg *et al*., 2006; Cappell *et al*., 2007; Bermudez *et al*., 2011; Josef, 2012). Although tumor necrosis factor alpha (TNF-α) genes are the earliest to be expressed within 1 hour as compared to interleukin (IL) -6, and IL-10 genes (Cappell *et al*., 2007). The possible mechanism of the up-regulation of the lymphocytic gene expression patterns could be linked to adenosine triphosphate depletion, accumulation of adenosine diphosphate, and adenosine monophosphate due to consumption of adenosine triphosphate by the exercising muscles (Brandt and Pedersen, 2010).

1.2 Statement of problem

Stress can cause up-regulation or down-regulation of several genes in the human body depending on its intensity and duration (Pilegaard *et al*., 2000; Xiang *et al*., 2014). It has been established that stress is a common phenomenon amongst Nigerian students (Ehiaghe *et al*., 2014; Onyenekwe *et al*., 2014). Over production of cortisol during prolonged stress can inhibit the proliferation of immunological cells such as macrophages and leukocytes (De-Quevain *et al*., 2005). The biochemical markers of stress currently in use such as blood levels of cortisol, glucose and progesterone are not stable. Therefore, the expression of lymphocytic genes in stressed individuals may be better alternative markers to explore in confirming early and stable evidence of stress.

1.3 Significance of the study

Post exercise stress has been reported to cause up-regulation of several genes in the human body including lymphocytic genes which shows increased expression patterns within 1 to 4 hours after stimulation (Abdalla *et al*., 2003; Katia *et al*., 2008; Bermudez *et al*., 2011; Josef, 2012). Therefore, the up-regulatory patterns of these lymphocytic genes might be of diagnostic importance in establishing evidences of stress as studies have shown that exhaustive exercise activates the release of multiple cytokine genes which modulate the immune responses of stressed individuals (Golzari *et al*., 2010). Hence the present study.

1.4 Aim of the study

This study was designed to use some lymphocytic gene expressions to confirm evidence of stress in exercise exhausted apparently healthy young male undergraduates.

1.4 Specific objectives

i. To confirm the evidence of stress in exercise exhausted apparently healthy young male undergraduates using hormonal profile {progesterone (PG), luteinizing hormone (LH), follicle stimulating hormone (FSH), cortisol}, glucose, absolute neutrophils counts, absolute lymphocytes counts and cardiac proteins (troponin inhibitory and creatine kinase-3).

ii. To determine the different patterns of lymphocytic gene expressions such as interleukin (IL) -10, tumor necrosis factor (TNF) α, interferon (IFN) -γ, succinate dehydrogenase (SDH) and hypoxanthine guanine phosphoribosyl transferase (HGPT) in acute exercise using reverse transcriptase polymerase chain reaction method.

iii. To identify the most stable lymphocytic gene expressions in acute exercise.

1.5 Research questions

i. What are the patterns of hormonal profile {progesterone (PG), luteinizing hormone (LH), follicle stimulating hormone (FSH), cortisol}, glucose and cardiac proteins (troponin inhibitory (TI) and creatine kinase-3 (CK MM) in acute exercise?

ii. What are the different patterns of some lymphocytic gene expressions (such as interleukin (IL) -10, tumor necrosis factor (TNF) α, interferon (IFN) -γ, succinate dehydrogenase (SDH) and hypoxanthine guanine phosphoribosyl transferase (HGPT) in acute exercise?

iii. Which are the most stable lymphocytic genes?

1.6 Hypothesis for the study

Ho = Lymphocytic genes such as SDH, HGPT, TNF α, IFN-γ and IL 10 can be used to confirm evidence of stress in acute exercise.

Ha = Lymphocytic genes such as SDH, HGPT, TNF α, IFN-γ and IL 10 cannot be used to establish evidence of stress in acute exercise.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cells of the immune system

Leukocytes are divided into granulocytes (neutrophils, basophiles and eosinophils) and agranulocytes (monocytes and lymphocytes). The natural killer cells (NK cells) (which function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity) and B cells (for humoral, antibody-driven adaptive immunity) are all lymphocytes. They are the main type of cell found in lymph, which prompted the name lymphocyte. Under normal conditions, the generation of the T-cell repertoire begins with the differentiation of precursor T cells (pre-T cells) in the fetal liver (during early embryogenesis) or bone marrow (during late embryogenesis and after birth) (Ikuta *et al*., 1992). Pre-T cells repopulate the thymus to become mature T cells (Rothenberg and Lugo, 1995). During which the cells undergo several phenotypic changes (Zerrahn *et al.,* 1999). It is completed by only a small percentage. Intra thymic thymocyte selection process is crucial to avoid the presence of auto reactive T cells in circulation (Von-Boehmer *et al*., 1999).

T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity. The function of T cells and B cells are to recognize specific "non-self" antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells. B cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens some T cells, called T helper cells, produce cytokines that direct the immune

response, while other T cells, called cytotoxic T cells, produce toxic granules that contain powerful enzymes which induce the death of pathogen-infected cells (Janeway *et al*., 2001).

Activation of B cells and T cells leave a lasting legacy of the antigens they have encountered in the form of memory cells throughout the lifetime of the host, these memory cells will remember each specific pathogen encountered, and are able to mount a strong and rapid response if the pathogen is detected again. The T helper cell is a sub-type of the T cell that is able to activate all these lymphocytes. NK cells are a part of the innate immune system and play a major role in defending the host from both tumors and virally infected cells. NK cells distinguish infected cells and tumors from normal and uninfected cells by recognizing changes of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferon. Activated NK cells release cytotoxic (cell-killing) granules which then destroy the altered cells. They were named "natural killer cells" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I (Janeway *et al*., 2001).

Table 2.0: Typical recognition markers for lymphocytes

Class	Function	Proportion	Phenotypic marker(s)
NK cells	Lysis of virally infected cells and tumor cells	7% (2-13%)	CD16 and CD65
Helper T cells	Release cytokines and growth factors that regulate other immune cells	46% (28-59%)	TCRαβ, CD3 and CD4
Cytotoxic T cells	Lysis of virally infected cells, tumor cells and allografts	19% (13-32%)	TCRαβ, CD3 and CD8
γδ T cells	Immunoregulation and cytotoxicity	5% (2%-8%)	TCRγδ and CD3
B cells	Secretion of antibodies	23% (18-47%)	MHC class II, CD19 and CD21

Source: Berrington *et al.,* 2005

It is impossible to distinguish between T cells and B cells in a peripheral blood smear (Abbas and Lichman, 2003). Flow cytometry testing is used for specific lymphocyte population counts. This can be used to specifically determine the percentage of lymphocytes that contain a particular combination of specific cell surface proteins, such as immunoglobulin or cluster of differentiation (CD) markers (Janeway *et al.*, 2001).

2.2 Immune response to stress

Stress is known to alter components of cellular immunity in human within minutes of its onset (Bachen *et al.*, 1992; Herbert *et al.*, 2004). These changes are often attributed to processes such as lymphocyte migration from lymphoid organs to circulating blood (Van-Tits *et al.*, 2000). It is also possible that lymphocyte population do not expand under stress but instead rise only in their relative blood concentration due to a concomitant reduction in plasma volume (hemoconcentration), during physical stress, an increase in arterial pressure causes fluid to filter out of the circulation into the extra vascular spaces, which necessarily increase the concentration of all large and non diffusible constituents of blood (e.g., plasma proteins and blood cells, including lymphocytes (Jern *et al.*, 2001).

Different stimuli can activate T cells in several ways: by antigen binding to the T cell receptors-CD3 complex in association with histocompatibility complexes (Weiss *et al.*, 1996), by modulation of other surface molecules such as CD2 and CD4 (Bierer and Burakoff, 2000), directly activating protein kinase c via the generation of free radicals (Nishizuka,2000). The profile of cytokines gene expression depends at least partly on cell surface receptor binding of a ligands and activation of intracellular signaling pathways (Fraser *et al.*, 1993). Signals from separate cell surface receptors are integrated at the level of the responsive gene (Crabtree, 2000).

Previous data from T cell lines, T-cell clones and peripheral blood mononuclear cells indicate that these cells have the capacity to produce gene expression (Zhao *et al*., 1994). The production of multiple cytokines in stimulated T cell population might be regulated either by a common mechanism or by independent pathway (Mosmann *et al*., 1989). Quantitative differences in gene expression in $CD4^+$ and $CD8^+$ T cell subsets have been observed (Breen *et al*., 1997). In humans, the majority of $CD4^+$ T cell clone obtained from healthy donors produces interferon gamma (IFN γ), interleukin -2 (IL2), interleukin -4 (IL 4) and interleukin -10 (IL 10) upon stimulation (Paliard *et al.,* 2000). Besides their regulation at the transcriptional level, a posttranscriptional regulation that control the stability of mRNA exist (Wiskocil, 1990). This may depend on the mode of stimulation and the rate of IL 2 mRNA degradation (Mugge and Lopex, 1991; Sinisalo *et al*., 2000; Bill *et al*., 2004). The stimulated leucocytes as a result of the acute inflammatory response have an increased tendency to adhere to vascular endothelium which may cause capillary leucocytosis (Friedman *et al*., 1990; Ito *et al*., 1990; Kuchel, 1999).

If high-intensity exercise is viewed as a stress situation, it might well influence the type of the immune response. Stress situations could influence the expression of T helper 1 (TH1) and T helper 2 (TH2) response profiles (Webster *et al*., 1998). Epidemiological and experimental studies suggest that stress and stress-related hormones (glucocorticoids, epinephrine, and norepinephrine) influence the development, course, and pathology of certain allergic, autoimmune, inflammatory, infectious diseases by predominately stimulating a TH2 as opposed to a TH1 type response, that is, by enhancing humoral more than cellular immunity (Wilder, 1995). The elevation of cyclic adenosine monophosphate (cAMP) and glucocorticoids are potent inhibitors of IL-2 and interferon-γ (Wilder, 1995). It has been demonstrated that chronic

strenuous exercise leads to an increased basal cortisol and catecholamine levels which tends to favor the T helper-2 immunity profile (Elenkov *et al*., 1996).

Moderate exercise has been associated with a 29% decreased incidence of upper respiratory tract infections (URTI) (Geddes, 2007). The depression in the immune system following acute bouts of exercise may be one of the mechanisms for this anti-inflammatory effect (Gleeson, 2007). The inflammatory response that occurs after strenuous exercise is also associated with oxidative stress, especially in the 24 hours after an exercise session (Dekkers *et al.*, 1996; Tiidus, 1998). The immune system is merely responding to the alteration done by the exercise bout, during which most of the adaptation leads to greater fitness, if balance diet and proper resting are observed after exercise (Ehiaghe *et al*., 2013). The immune system response to exhaustive exercise peaks 2 to 7 days after exercise during this process, free radicals is produced by neutrophils to remove damaged tissue (Tiidus, 1998).

Antioxidant supplements may also prevent any of the health gains that normally come from exercise (Ristow *et al*., 2009). The evidence for benefits from antioxidant supplementation in vigorous exercise is mixed. There is strong evidence that one of the adaptations resulting from exercise is a strengthening of the body's antioxidant defenses (Leeuwenburgh *et al*., 1994). This effect may be to some extent protective against diseases which are associated with oxidative stress, which would provide a partial explanation for the lower incidence of major diseases and better health of those who undertake regular exercise (Leeuwenburgh and Heinecke, 2001).

2.3 Exercise-induced muscle soreness and cytokine patterns

Muscle soreness and injury typically result from endurance exercise leading to the development of an acute inflammatory response during and after physical activity (Nehlsen-Cannarella *et al*.,

1997). Prolonged and intensive endurance exercise significantly increases the magnitude of the inflammatory immune response (McFarlin *et al.*, 2004) and this inflammatory response includes the production of local and systemic inflammatory cytokines including (IL-1, IL-6, IL-8 and TNF-α) and acute-phase proteins (Nehlsen-Cannarella *et al.*, 1997; Nieman, 1997). Studies have shown that following prolonged and intensive endurance training, plasma cortisol, IL-6, epinephrine and norepinephrine, and white cell counts are all significantly elevated (Nehlsen-Cannarella *et al.*, 1997; Mitchell *et al.*, 1998; Nieman *et al.*, 1998; Nieman *et al.*, 2004 Scharhag *et al.*, 2006; Duclos *et al.*, 2007). These exercise-associated immune-enhanced and suppressive changes may last between 3 to 72 hours (Nieman, 2003).

Inflammation is an important component of the innate immune response. It is initiated by tissue damage, which then activates the innate immune cells (Janeway *et al.*, 2001). The inflammatory response has three critical roles in host defense and recovery (Janeway *et al.*, 2001). These roles include: 1) the recruitment of additional cells and molecules to the site of inflammation; 2) the creation of a physical barrier to prevent the spread of infectious agents; and 3) the removal of dead tissues and the repair of injured tissues (Janeway *et al.*, 2001). The inflammatory process has been well described and is characterized by pain, redness, heat and swelling at the active site. Injured and dying cells will release inflammation-inciting molecules, including cytokines, prostaglandins and pain effectors which together initiate a sequence of tissue responses. However, responding leukocytes subsequently produce and secrete more cytokines and chemokines enhancing and modifying the inflammation (Janeway *et al.*, 2001). This chemical gradient provides a mechanism by which the white blood cells can move towards the area of damaged tissue (Abbas *et al.*, 2007). Neutrophils are classically the first cell to migrate to the site

of inflammation followed by macrophages and other leukocytes, such as lymphocytes, which can also be drawn to the site during later stages of ongoing inflammation (Janeway *et al.*, 2001).

The combined release of inflammatory mediators from damaged cells (Newton and Dixit, 2012) and infiltrating immune cells allows these molecules to spill out into the larger circulatory system (Abbas *et al.*, 2007). Principally, the production of cytokines, including IL-1 and IL-6 from macrophages, stimulates the activation of a group of protein produced by the liver, called the acute-phase proteins including amyloid A, fibrinogen, Complement components C3 and C4 and C - reactive protein (Samols *et al.*, 2002). Increased circulating inflammatory cytokines enhance the production and release of these proteins from the liver, whose levels then rise in the blood and induce subsequent effects (Abbas *et al.*, 2007). For example, CRP plasma levels under normal conditions are generally less than 3mg/L; however, during acute stress responses including infections and exhaustive exercise, levels can increase up to a 1000-fold (Abbas *et al.*, 2007). One consequence of the production of these systemic proteins is the activation of the HPA axis (Nehlsen-Cannarella *et al.*, 1997; Tsigos and Chrousos, 2002).

2.4 The hypothalamic-pituitary adrenocortical axis and the immune system

In addition to CRP-activation of the HPA axis, the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), IL-1, and IL-6, are also potent stimulators of the HPA axis, synergistically or alone (Tsigos and Chrousos, 2002). Each cytokine induces a positive feedback loop causing enhancement of secretion of all cytokines; TNF-α and IL-1 stimulate each other and both induce IL-6 release (Mastorakos *et al.*, 2005). IL-6 is the predominant cytokine in activating the HPA axis, particularly in conditions of chronic inflammatory stress (Tsigos and Chrousos, 2002).

Cortisol induces its immunosuppressive effects by permeating immune cell membranes and binding to glucocorticoids receptors forming a cortisol-glucocorticoids receptor complex (Flaster *et al.*, 2007). The inflammatory response is subsequently inhibited by the following: cortisol down-regulates gene expression of the pro-inflammatory mediators; and up-regulates gene expression of anti-inflammatory agents (Pavlov *et al.*, 2003). The cytokines TNF-α, IL-1, IL-6, IL-8, IL-12 and INF-γ, and other inflammatory mediators are all inhibited at the level of gene transcription through suppression of AP-1 (activator protein-1) and NF-κ B (nuclear factor kappa B), the latter being the most notable since NF-κ B is essential for regulating the production of cytokines (Pavlov *et al.*, 2003; Flaster *et al.*, 2007). The synthesis of anti-inflammatory cytokines, including IL-4 and IL-10 is then activated (Pavlov *et al.*, 2003). Furthermore, cortisol suppresses the immune response through decreased migration of leukocytes to the site of infection or tissue injury by inhibiting the expression of cell adhesion molecules (Pavlov *et al.*, 2003; Flaster *et al.*, 2007).

2.5 Mobilization of energy reserves

Catecholamine, cortisol, and growth hormone stimulate the mobilization of energy by stimulating adipose, liver, and skeletal muscle cells (Plowman and Smith, 2008). These hormones bind to receptors on target cells causing the following: a) adipose cells: inhibiting fat storage and inducing lipolysis; b) liver cells: enhancing glycogenolysis; c) skeletal muscle cells: enhancing muscle glycogenolysis (Plowman and Smith, 2008). Circulating IL-6 enhances these effects by also stimulating lipolysis and oxidation of fatty acids (Petersen and Pedersen, 2005). It has been established that adenosine triphosphate generated from anaerobic metabolism (phosphocreatine, stored adenosine triphosphate and anaerobic glycolysis) and aerobic metabolism (cellular respiration) mediates the sliding of actin along the length of myosin to

achieve muscle contraction (Kenney *et al*., 2012). The essential role of anaerobic glycolysis during muscle contraction is manifested in the elevation of blood lactate level from 1.6 to 8.3 mM, the release of H^+ from the intensely active muscle concomitantly lower the blood pH from 7.42 to 7.24 (Jeremy *et al.*, 2001). The liver rapidly mobilizes glycogen to meet the glucose need of the contracting muscle; this pace cannot be sustained for more than 1.8 minutes for two reasons. First, creatine phosphate is consumed within 10 seconds. Second, the lactate produced would cause acidosis (Kenney *et al*., 2012). Moreover, aerobic metabolisms is the major source for adenosine triphosphate production from blood glucose, lactate and fats during exercise that last longer than 2 to 3 minutes. These changes are essential for producing ATP required for supporting muscle contraction and maintaining blood glucose levels (Plowman and Smith, 2008).

Catecholamines simultaneously act on pancreatic alpha- and beta-cells inducing the release of glucagon and suppressing the secretion of insulin respectively (Plowman and Smith, 2008). Insulin and glucagon induce antagonistic effects: insulin is responsible for converting glucose into glycogen in the liver and skeletal muscles, and triglycerides in the adipose tissues (Fox, 2009). Meanwhile, glucagon stimulates the breakdown of glycogen into glucose (glycogenolysis), and also, promotes the breakdown of non-carbohydrate molecules into glucose (gluconeogenesis) (Fox, 2009). Glycogenolysis and gluconeogenesis both result in glucose being secreted into the bloodstream to ensure that blood glucose levels do not drop below the fasting level; therefore, sustaining energy for physical activity (Fox, 2009). Thus, the suppression of insulin secretion and the release of glucagon are essential for the mobilization of energy to exert physical activity (Marliss and Vranic, 2002).

2.6 Lymphocytic gene expression

The category of lymphocyte house-keeping genes expressions consists of genes that are involved in the regulation of basic and ubiquitous cellular functions required for the survival of most cell types, due to their presumptive invariable expression, house-keeping genes have been used extensively as reference genes for normalization of gene expression data derived from a variety of cell types or experimental treatments using microarray or reverse-transcriptase polymerase chain reaction (Eisenberg and Levanon, 2003). A reference gene is necessary to correct for basic sample differences, including differences in cellular input, RNA quality, efficiency of reverse transcription and batch to batch variation in reagents. Some of the house-keeping genes commonly used as expression controls include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), β_2-microglobulin, cyclooxygenase 1, hypoxanthine phosphoribosyl transferase 1(HPRT), glucose-6-phosphate dehydrogenase, cyclophilin A, tubulin, transferrin receptor (TFRC) and 18S ribosomal RNA (R18S) (Lee *et al*., 2002; De-Kok *et al*., 2005). GAPDH is a key regulatory enzyme, which catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis and β-actin is a cytoskeleton protein involved in cell structure and motility. Based on these basic and ubiquitous cellular functions, GAPDH and β-actin are considered as house-keeping genes.

However, mounting evidence now recognizes GAPDH and β-actin as multifunctional proteins involved in diverse biological processes independent of their traditional "house-keeping" roles. Several studies have demonstrated the roles of GAPDH in regulation of membrane fusion and transport (Tisdale *et al*., 2004), apoptosis (Ishitani and Chuang, 1996; Hara and Snyder, 2006), DNA repair, DNA replication (Meyer-Siegler *et al.*,1991) and regulation of transcription and translation (Zheng and Roeder, 2003; Chuang *et al*., 2005). In addition, GAPDH has been

implicated in the pathophysiology of neurodegenerative diseases (Chuang *et al*., 2005). Both GAPDH and β-actin are differentially expressed in several cancers. Its expression has been shown to positively correlate with tumor invasiveness and metastatic potential (Le *et al*., 1998). The list of candidate genes is nevertheless far from being complete, as the athlete's reaction to exercise is a coordinated response of multiple organ systems e.g. induction of heat shock proteins, inflammatory response modulation (pro- and anti-inflammatory cytokines), generation of reactive oxygen and reactive nitrogen species (ROS and RSN), besides their damaging potential, play a crucial role in cellular signaling (Connolly *et al*., 2004; Zieker *et al*., 2005; Barrey *et al*., 2009; Cappelli *et al*., 2007).

Different approaches have been proposed to normalize measurements of expression levels but this is generally done using an internal control genes (house-keeping genes) (Huggett *et al*., 2005). In addition, the house-keeping genes and the target gene should have similar ranges of expression to avoid analytical problems. Widely expressed genes like ACTB, GAPDH *or* R18S are generally preferred, without preliminary analysis of their expression profiles under the specific study conditions (Vandesompele *et al*., 2002; Zicker *et al.*, 2005). Nevertheless, a number of studies report how commonly accepted HKGs do not always constitute reliable controls (Selvey *et al*., 2001), because of unexpected variation in their expression profiles. GAPDH, TFRC and RPL32 were classified as the least stable genes and they are not likely to be useful in this given experimental system. Notably, the expression of GAPDH, that has been used as HKG in a previous exercise induced stress study (Katia *et al*., 2008). Succinate dehydrogenase (SDHA) and hypoxanthine guanine phosphoribosyl transferase (HPRT) are the most stable reference genes with a very good statistical reliability. Moreover, the use of these genes appears to be sufficient for a reliable normalization of the genes of interest (Katia *et al*., 2008). Succinate

dehydrogenase is one of the major enzymes that control the generation of high energy electron to create the proton gradient for the generation of at 95% of ATP in the cells including lymphocytes (Jern *et al.,* 2001). It has been reported that a deficiency of hypoxanthine-guanine phosphoribosyl (HGPRT) transferase caused by HGPRT 1 mutation may lead to lesch-nyhan syndrome, which results in a compulsive self destructive behavior (Jeremy *et al*., 2001). HGPRT plays a central role in the generation of purine nucleotides which are the building block of nucleic acid (Finette *et al*., 2002). An ample supply of nucleotides is essential for many life processes. Nucleotides are the activated precursors of nucleic acid, as such, they are necessary for the replication of the genome and the transcription of the genetic information into ribonucleic acid (RNA) (Jeremy *et al*., 2001).

2.7 Cytokine genes expression

Different stimuli can activate T cells in several ways: by antigen binding to the T-cell receptor–CD3 complex in association with major histocompatibility complexes (Weiss *et al.,* 1996), by modulation of other surface molecules such as CD2 and CD4 (Bierer and Burakoff, 2000) and by bypassing the surface receptor signaling and directly activating protein kinase c on lymphocytes (Nishizuka, 2000). Signals from separate cell surface receptors are integrated at the level of the responsive gene (Crabtree, 2000). The coordinated production of cytokines following lymphocyte activation controls proliferation, differentiation, function of cells and is crucial for regulation of the immune response (Fraser *et al*., 1993). By using the highly sensitive RT-PCR, some gene shows increases within 1 to 4 hours after stimulation, thus, they all belong to the early gene group (Ullman *et al*., 1990), although IFN-γ, IL-2, and TNF-α genes were the earliest to be expressed within 1 hour as compared to IL-2, IL-6, and IL-10 genes. T-cell activation can be initiated by diverse agents such as antigens, plant mitogens, cytokines and monoclonal antibodies

(Weiss *et al*., 1996). These stimuli cause complex series of ordered interactions and events, including activation of transmembrane signaling pathways, cytokine gene expression, transcription and translation (Weiss *et al*., 1996).

Exercise increase the intracellular level of calcium ions, energy regulating molecules such as adenosine diphosphates (ADP), adenosine monophosphates (AMP), free phosphate group and reactive oxygen species, these substances are potent signal transducers and have been shown to activate protein kinase found on lymphocytes (Holloszy, 2008; Olesen *et al*., 2010; Baar, 2014). Increase in messenger ribonucleic acid (mRNA) expression is normally associated with increase transcription and protein contents, other factors that can affect lymphocyte genes expression includes; mRNA stability, mRNA transport, translation efficiency, amino acid availability, protein assembly and stability (Bento *et al*.,2008). The fundamental link between the immune and oxidative stress responses to exhaustive exercise is undeniable (Quindry *et al*., 2003; Tauler *et al*., 2004). Elevated reactive oxygen species production following exercise spreads to the nucleus and induces DNA damage in lymphocytes (Mars *et al*., 1998; Wang *et al*., 2005). Mitochondrial biogenesis is a complex process involving the replication of mitochondrial DNA and the expression of mitochondrial proteins encoded by both nuclear and mitochondrial genomes (Bourdon *et al*., 2007).

2.8 Antioxidants

Antioxidants are molecules that inhibits the oxidation of other molecules, oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent, oxidation reactions can produce free radicals, these radicals can start chain reactions, when the chain reaction occurs in a cell, it can cause damage or death to the cell, antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions, they

do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies, 1997). Plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidase. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases (Jha *et al*., 1995; Bjelakovic *et al*., 2007; Baillie *et al*., 2009).

2.9 Mechanism of oxidative challenge

A paradox in metabolism is that, while the vast majority of complex life on Earth requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (Davies,1995). Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani *et al*., 2004). In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell (Davies, 1995). However, reactive oxygen species also have useful cellular functions, such as redox signaling. Thus, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O_2^-) (Valko *et al*.,2007). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules (Stohs and Bagchi, 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing

DNA or proteins (Sies, 1997). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms (Valko *et al*.,2004; Nakabeppu *et al*.,2006) while damage to proteins causes enzyme inhibition, denaturation and protein degradation (Stadtman,1992).

2.10 Immune responses and oxidative stress

The fundamental link between the immune and oxidative stress responses to prolonged endurance or high-intensity exercise is undeniable (Quindry *et al*., 2003; Tauler *et al*., 2004). Elevated ROS production following exercise permeates the nucleus and induces DNA damage in lymphocytes (Mars *et al*., 1998; Wang *et al*., 2005). The immunosuppression observed following exercise of a strenuous and prolonged nature has been linked to a decrease in circulating lymphocytes and a blunted natural killer cell activity (Nieman, 1997; Pedersen and Toft, 2000).

Post exercise oxidative stress is associated with elevations in lymphocytes SOD and CAT activity in a manner appearing consistent with posttranscriptional regulation. Oxidative stress occurs when reactive oxygen species (ROS)/reactive nitrogen species (RNS) production and antioxidant defenses become imbalanced. Redox regulation via ROS/RNS and the antioxidant defenses represents a tightly controlled system that can have both deleterious and beneficial effects within the cellular environment. ROS up-regulate the expression of superoxide dismutase (SOD) and glutathione peroxidase (GPX) in skeletal muscle (Gomez *et al*., 2008).

Additionally, lymphocyte SOD, CAT and GPX activities increase in response to acute oxidant exposure in vitro and following exhaustive endurance exercise, while neutrophils exhibit decreased antioxidant enzyme activities following intense exercise (Tauler *et al*., 2004; Tauler *et al*., 2006), considering that acute exercise increases ROS production (Jha, 1995) and immune cells express antioxidants, it is important to understand the integrated dynamics between

oxidative stress and immune cell function. Increases in oxidative stress during exercise and the subsequent immune response during recovery from exercise provide a unique opportunity to examine the relationship between stress and immunity (Fisher *et al*., 2011). Lymphocyte superoxide dismutase (SOD) and catalase (CAT) activities have been shown to increase in response to acute oxidant exposure in vitro and following exhaustive exercise (Tauler *et al*., 2004; Tauler *et al*., 2006).

2.11 The stress system

The term "stress" describes the state of the organism under the influence of external or internal forces, or "stressors", which threaten to alter its dynamic equilibrium or homeostasis. The adaptive changes occurring in response to stressors are both behavioral and physical. Once a certain threshold has been exceeded, a systemic reaction takes place that involves the "stress system" in the brain along with its peripheral components, the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic sympathetic system (Chrousos, 2004).

2.12 Stress response

Stress has been a research topic for over 100 years and historically, the paramount early leader in the study of stress was Hans selye. This theory proposes an intimate involvement of the adrenal gland, specifically the cortex, in the adaptation and mal-adaptation process to all form of stress (Hackney, 2006). He published a model dividing stress into eustress and distress, where stress enhances function (physical or mental, such as through strength training or challenging work), it may be considered eustress, persistent stress that is not resolved through coping or adaptation, it may be considered distress which can lead to anxiety or withdrawal (depression) behavior (Keil, 2004). Neuroscientists such as Bruce McEwen and Jaap Koolhaas believe that stress should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity

of an individual (Koolhaas, 2011). Physiological stress represents a wide range of physical responses that occur as a direct effect of a stressor causing an upset in the homeostasis of the body (Koolhaas, 2011).

Physiologists define stress as how the body reacts to a stressor, real or imagined a stimulus that causes stress. Acute stressors affect an organism in the short term; chronic stressors over the longer term. General adaptation syndrome (GAS), developed by Hans Selye, is a profile of how individuals respond to stress; the general adaptation syndrome is characterized by three phases: a nonspecific mobilization phase, which promotes sympathetic nervous system activity; a resistance phase, during which the organism makes efforts to cope with the threat; and an exhaustion phase, which occurs if the organism fails to overcome the threat and depletes its physiological resources (Taylor and Sirois, 2012). The immune system is particularly sensitive to stress and specific effects of stress have been demonstrated by a number of studies (Dhabhar *et al*., 1995). Acute stress generally has positive effects, while chronic stress typically provokes immunosuppression and diverse associated pathologies, including autoimmune diseases (Dhabhar and McEwen, 1999).

Vertebrates respond to stress by activating cytokines producing cell to release both pro and anti inflammatory cytokines, the pro-inflammatory cytokines in turn activate the hypothalamus–pituitary–adrenal to release glucocorticoids into the blood-stream in attempt to regain homeostasis. These stress hormones mobilize energy by stimulating the release of glucose into the bloodstream and enhance delivery of glucose, fatty acids and triglycerides to skeletal muscle and the brain (Sapolsky, 1994; Wingfield *et al.*, 2002). Glucocorticoids also divert energy from various costly physiological processes that are not required for immediate survival, including

digestion, energy storage, growth, immunity and reproduction (Sapolsky, 2000). Thus, while adaptive in the short-term, chronic glucocorticoids elevation has serious negative effects on the organism, including reproductive failure and decreased resistance to disease (Sapolsky, 2000).

2.13 Neuroanatomy

2.14 Brain

The brain plays a critical role in the body's perception of and response to stress. However, pinpointing exactly which regions of the brain are responsible for particular aspects of a stress response is difficult and often unclear. Understanding that the brain works in more of a network-like fashion carrying information about a stressful situation across regions of the brain (from cortical sensory areas to more basal structures and vice versa) can help explain how stress and its negative consequences are heavily rooted in neural communication dysfunction (Ulrich and Herman, 2009).

2.15 Hypothalamus

The hypothalamus is a small portion of the brain located below the thalamus and above the brainstem. One of its most important functions is to help link together the body's nervous and endocrine systems. This structure has many bidirectional neural inputs and outputs from and to various other brain regions. These connections help to regulate the hypothalamus' ability to secrete hormones into the body's blood stream, having far-reaching and long-lasting effects on physiological processes such as metabolism. During a stress response, the hypothalamus secretes various hormones including corticotrophin-releasing hormone which stimulates the anterior pituitary gland to secrete adrenocorticotrophic hormone which in turn stimulate the adrenal cortex to release cortisol (Richard *et al*., 2006; O'Connor *et al*., 2009).

2.16 Amygdale

The amygdale is a small, "almond"-shaped structure located bilaterally, deep within the medial temporal lobes of the brain and is a part of the brain's limbic system, with projections to and from the hypothalamus, hippocampus, and locus coeruleus among other areas. Thought to play a role in the processing of emotions, the amygdale has been implicated in modulating stress response mechanisms, particularly when feelings of anxiety or fear are involved (Roozendaal *et al*., 2009).

2.17 Hippocampus

The hippocampus is a structure located bilaterally, deep within the medial temporal lobes of the brain, just below each amygdale. The hippocampus is thought to play an important role in memory formation. There are numerous connections to the hippocampus from the cerebral cortex, hypothalamus, and amygdale, among other regions. During stress, the hippocampus is particularly important, in that cognitive processes such as prior memories can have a great influence on enhancing, suppressing, or even independently generating a stress response. The hippocampus is also an area in the brain that is susceptible to damage brought upon by chronic stress (McEwen, 2012).

2.18 Prefrontal cortex

The prefrontal cortex, located in the frontal lobe, is the anterior-most region of the cerebral cortex. An important function of the prefrontal cortex is to regulate cognitive processes including planning, attention and problem solving through extensive connections with other brain regions. The prefrontal cortex can become impaired during the stress response (McEwen and Morrison, 2013).

2.19 Locus coeruleus

The locus coeruleus is an area located in the pons of the brainstem that is the principal site of the synthesis of the neurotransmitter norepinephrine, which plays an important role in the sympathetic nervous system's fight-or-flight response to stress. This area receives input from the hypothalamus, amygdale, and raphe nucleus among other regions and projects widely across the brain as well as to the spinal cord (Arnsten, 2009).

2.20 Raphe nucleus

The raphe nucleus is an area located in the pons of the brainstem that is the principal site of the synthesis of the neurotransmitter serotonin, which plays an important role in mood regulation, particularly when stress is associated with depression and anxiety. Projections extend from this region to widespread areas across the brain, namely the hypothalamus, and are thought to modulate an organism's circadian rhythm and sensation of pain among other processes (Arnsten, 2009).

2.21 Spinal cord

The spinal cord plays a critical role in transferring stress response neural impulses from the brain to the rest of the body. In addition to the neuroendocrine blood hormone signaling system initiated by the hypothalamus, the spinal cord communicates with the rest of the body by innervating the peripheral nervous system. Certain nerves that belong to the sympathetic branch of the central nervous system exit the spinal cord and stimulate peripheral nerves, which in turn engage the body's major organs and muscles in a fight-or-flight manner (Arnsten, 2009).

2.22 Pituitary gland

The pituitary gland is a small organ that is located at the base of the brain just under the hypothalamus. This gland releases various hormones that play significant roles in regulating homeostasis. During a stress response, the pituitary gland releases hormones into the blood stream including adrenocorticotropic hormone, which modulates a heavily regulated stress response system (Arnsten, 2009).

2.23 Adrenal gland

The adrenal gland is a major organ of the endocrine system that is located directly on top of the kidneys and is chiefly responsible for the synthesis of stress hormones that are released into the blood stream during a stress response. Cortisol is the major stress hormone released by the adrenal gland. In addition to the locus coeruleus existing as a source of the neurotransmitter norepinephrine within the central nervous system, the adrenal gland can also release norepinephrine during a stress response into the body's blood stream, at which point norepinephrine acts as a hormone in the endocrine system. Cortisol is one way the brain instructs the body to attempt to regain homeostasis by redistributing energy (glucose) to areas of the body that need it most, (the heart and brain) and away from the digestive and reproductive organs, in other to overcome the challenge at hand (Michael *et al.*, 2001).

2.24 Hypothalamic-pituitary-adrenal (HPA) axis

The stress system is active when the body is at rest, responding to many distinct circadian, neurosensory, blood-borne and limbic signals (Chrousos, 2004). These signals include cytokines produced by immune-mediated inflammatory reactions, such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6). Immune cells carry receptors for a number of

hormones, neuropeptides, and neurotransmitters. During stress responses, the three proinflammatory cytokines such as TNF-α, IL-1, IL-6 can stimulate hypothalamic glucocorticoids secretion (Mastorakos *et al*., 1999). The three proinflammatory cytokines are secreted in a cascade-like fashion, with TNF α appearing first, IL-1 second and IL-6 last. All three cytokines stimulate their own secretion, in an autocrine fashion, from the cells that produce them. Tumor necrosis factor-α and IL-1 stimulate each other's secretion and both promote the release of IL-6. The three proinflammatory cytokines activate the HPA axis independently as well as in combination (Mastorakos *et al*., 1993; Groschl *et al*., 2003). It is noteworthy that catecholamine and cortisol influence the development, course and pathology of certain allergic, autoimmune, inflammatory, infectious and neoplastic diseases as they favor the TH2 response profile (Webster *et al*., 1998; O'Connor *et al*., 2009).

Though progesterone is generally known as a gonadally released reproductive hormone, it is also produced in the brain by the adrenal gland, where progesterone is an indirect precursor to cortisol (Baulieu *et al.*, 2001). Like cortisol, progesterone is released in response to adrenocorticotropic hormone (ACTH) (Genazzani *et al*., 1998), and levels peak in the morning and decline over the course of the day (Groschl *et al*., 2003). Male mammals produce considerable amounts of progesterone. They have circulating levels of unbound progesterone roughly equal to women in the early follicular phase (Schultheiss *et al*., 2003). It is likely that the adrenals are the main source of progesterone in males. In females, both the ovary and the adrenal contribute to circulating progesterone levels (Paul and Purdy, 1992; Soderpalm *et al*., 2004). In rodents, progesterone and allopregnanolone down-regulate HPA axis responses to stress (Guo *et al*., 1995). Studies in animals have found increases in progesterone and its metabolite, allopregnanolone, in response to stress (Barbaccia *et al*., 1996; Genazzani *et al*., 1998; Girdler *et*

al., 2001; Klatzkin *et al*., 2006) and other motivation and emotion arousing stimuli (Schultheiss *et al*., 2003; Brambilla *et al*., 2005; Eser *et al*., 2006).

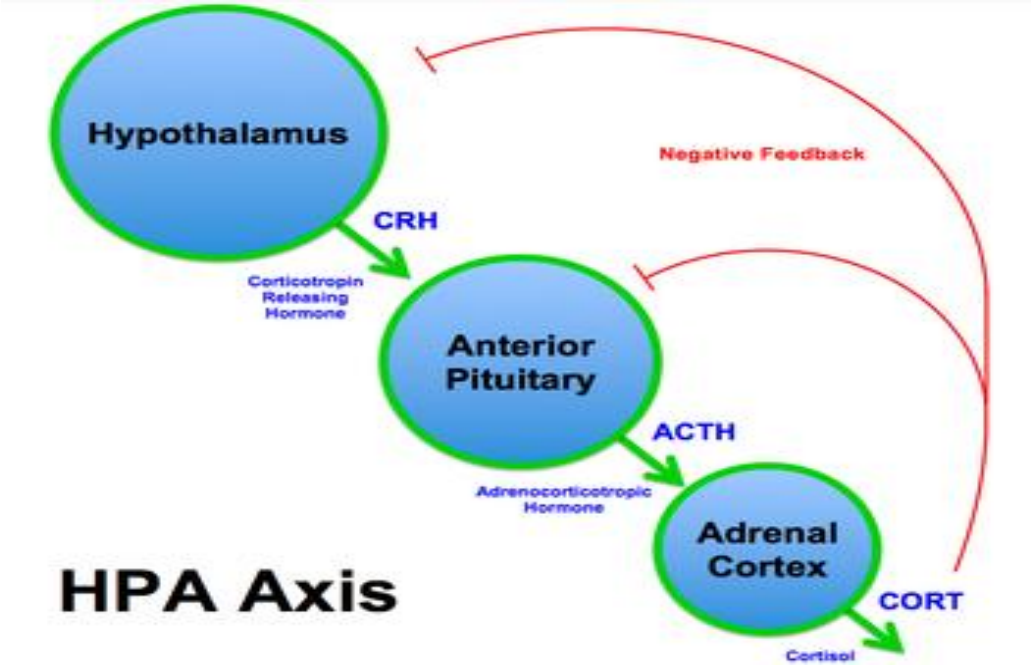


Figure 2.1: Basic hypothalamic–pituitary–adrenal axis summary: Source: Chrousos, 1992.

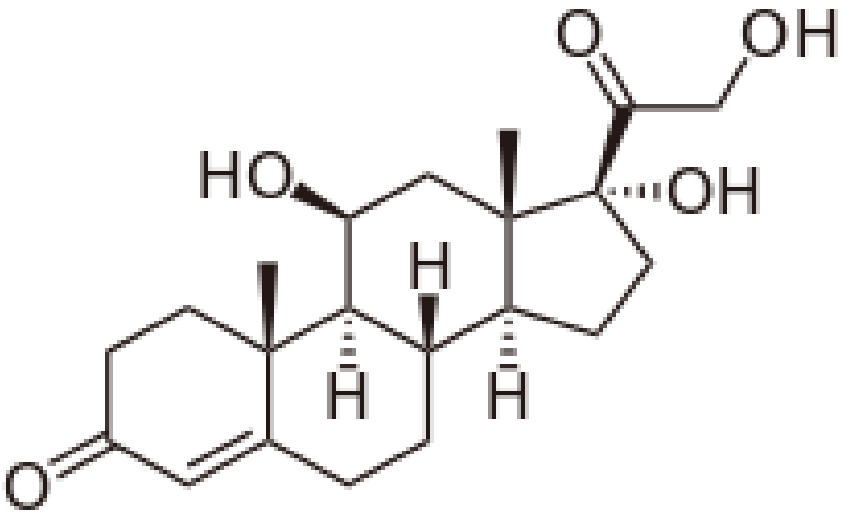


Figure 2.2: Molecular structure of cortisol: Source: Chrousos, 1992.

2.25 Stress and the reproductive system

The hypothalamic-pituitary-gonadal (reproductive) axis is inhibited at all levels by various components of the HPA axis (Figure 2.5). Either directly or through β-endorphin release, CRH suppresses the gonadotropin-releasing hormone (GnRH). Glucocorticoids exert inhibitory effects at the level of hypothalamic, pituitary and gonads (Ullman *et al*., 1990). It is well known that stress is accompanied by both an increase in the activity of the HPA axis and a decrease in reproductive functions in order to preserve the organism's state of alert at the expense of gonadal activity in case of emergency. In animals, the relationship between population density and reproductive efficiency has been studied in wild rodents (Kamel *et al*., 2001). There is also strong evidence that the immediate changes in LH secretion are mediated at the level of GnRH-secreting neurons, while long-term effects also involve peripheral mechanisms such as alteration in pituitary and gonadal responsiveness (Rivier and Rivest, 1991). Controlled trials involving men undergoing endurance training and control groups of sedentary men give the impression of generally lowered androgen levels in exercising men. Normal feedback regulation would require luteinizing hormone levels to rises with falling testosterone levels (Hackney *et al*., 1998). In addition, cortisol levels, as a prominent endocrine marker for physical or mental stress exposure, are elevated significantly in exercising men as compared with non exercising men (Vervoorn *et al*., 1991). The lowering effect of endurance training on testosterone levels may be seen as a part of a general response pattern of the mental and physical stress response on the hypothalamic-pituitary-adrenal (HPA) axis (Benton *et al*., 2008). An additional impact factor might be the increased glucocorticoids secretion which may be responsible for the down regulated testosterone biosynthesis in the Leydig cell (Benton *et al*., 2008).

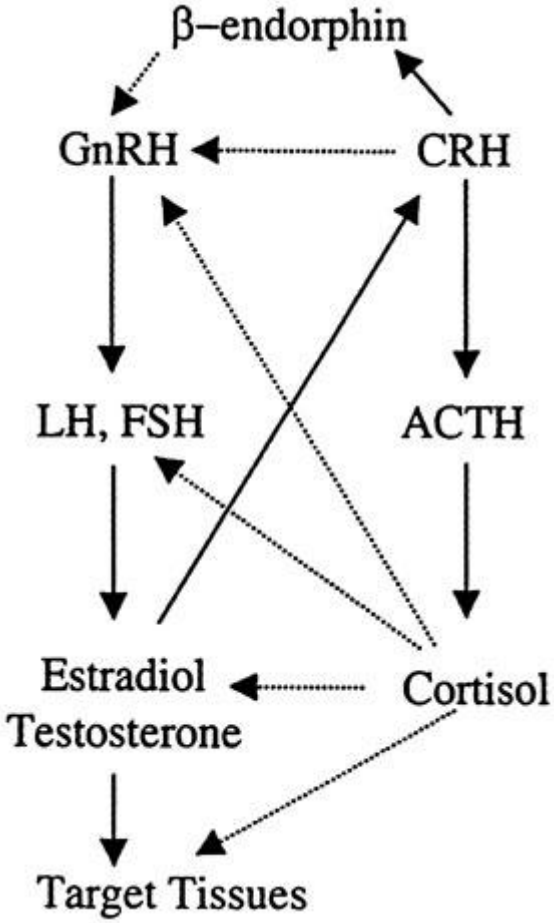


Figure 2.3: Heuristic representation of the interactions between the hypothalamic-pituitary-adrenal axis and the hypothalamic-pituitary-gonadal axis. Activation of the stress system leads to inhibition of the reproductive axis by direct or indirect inhibition of the GnRH neuron by CRH, beta endorphin, and glucocorticoids: Source: Hardy and Ganjam, 2000.

2.26 Effect of chronic stress

Chronic stress is defined as a state of prolonged tension from internal or external stressors, which may cause various physical manifestations – e.g., asthma, back pain, arrhythmias, fatigue, headaches, hypertension, irritable bowel syndrome, ulcers and suppress the immune system. Chronic stress takes a more significant toll on your body than acute stress does. It can raise blood pressure, increase the risk of heart attack and stroke, increase vulnerability to anxiety and depression, contribute to infertility, and hasten the aging process (Jaremka *et al*., 2013). Studies revealing the relationship between the immune system and the central nervous system indicate that stress can alter the function of the white blood cells involved in immune function known as lymphocytes and macrophages.

People undergoing stressful life events, such as marital bereavement, have a weaker lymph proliferative response. People in distressed marriages have also been shown to have greater decreases in cellular immunity functioning over time when compared to those in happier marriages (Jaremka *et al*., 2013). After antigens initiate an immune response, white blood cells send signals, composed of cytokines and other hormonal proteins, to the brain and neuroendocrine system (Dantzer and Kelly, 1990). During chronic stress, cortisol is over produced, causing fewer receptors to be produced on immunological cells. Prolonged exposure to cortisol damages cells in the hippocampus; this damage results in impaired learning, it has been shown that cortisol inhibits memory retrieval of already stored information (Dantzer and Kelly, 1990).

2.27 Benefits of exercise

The benefits of exercise have been known since antiquity. Marcus Cicero, around 65 BC, stated: "It is exercise alone that supports the spirits, and keeps the mind in vigor." It has been reported that men of similar social class and occupation (bus conductors versus bus drivers) had markedly different rates of heart attacks, depending on the level of exercise they got. Bus drivers had a sedentary occupation and a higher incidence of heart disease, while bus conductors were forced to move continually and had a lower incidence of heart disease (Kimber *et al*., 2003).

Physical exercise is important for maintaining physical fitness and can contribute positively to maintaining a healthy weight, muscle strength, promoting physiological well-being and strengthening the immune system (Stampfer *et al*., 2000; Hu *et al*., 2001; Gosker and Schol, 2008). Developing research has demonstrated that many of the benefits of exercise are mediated through the release of myokines which promote the growth of new tissue and reduces the risk of developing inflammatory diseases (Pedersen, 2013). Endurance exercise before meals lowers blood glucose level (Borer *et al*., 2009). According to the World Health Organization, lack of physical activity contributes to approximately 17% of heart disease and diabetes, 12% of falls in the elderly and 10% of breast cancer and colon cancer (Wislett et al., 2009). There is evidence that vigorous exercise (90–95% of VO_2 Max) induces a greater degree of physiological cardiac hypertrophy than moderate exercise (40 to 60% of VO_2 Max) (Wislett *et al*., 2009). The lymphocyte concentration increase seen during moderate exercise is thought to be due to recruitment of all lymphocyte subpopulations into circulation, specifically from tissue pools such as the spleen, lymph nodes, and the gastrointestinal tract (Pedersen and Nieman, 1998). Additionally, animal models have shown a redistribution of lymphocytes from the circulation back into the tissue pools following exercise (Randall *et al*., 1999). Also, Ortega, (2003) in his

study observed that post exercise stress enhances white blood cells phagocytic capacity. Post exercise stress is associated with increased leukocytes migration from the lymphoid organs to the circulating blood (Herbert *et al*., 2004). It has been established that elevated cortisol level during exhaustive exercise enhances white blood cells recruitment capacity (Clinton *et al*., 2010).

2.28 Cardiovascular system and exercise

Most beneficial effects of physical activity on cardiovascular disease mortality can be attained through moderate-intensity activity (40% to 60% of maximal oxygen uptake, depending on age), persons who modify their behavior after myocardial infarction to include regular exercise have improved rates of survival. Persons who remain sedentary have the highest risk of cardiovascular disease mortality (Geddes, 2007). Multiple studies have examined the cardiac troponin{cardiac troponin tropomyosin (cTnT) and cardiac troponin inhibitory (cTn I) response to physical exercise, most from competitive athletic events that require a participant to maintain an elevated cardiac output, heart rate, and systolic blood pressure for several hours. This sustained increase in cardiac work stresses the myocardium, which in conjunction with the physiologic milieu of prolonged exercise (e.g., elevations in reactive oxygen species, altered pH, and increased core temperature) could hypothetically damage cardiomyocytes leading to elevated troponin levels from participants in marathon-distance (Fortescue *et al*., 2007; Mousavi *et al*.,2009; Mingels *et al*.,2009) and ultra-distance foot races (Shave *et al*.,2002), triathlons (sequential swimming, cycling, and running) (Tulloh *et al*.,2006), and dedicated cycling events (Neumayr *et al*.,2005;Scharhag *et al*.,2005). Also, Goett *et al*., (2009) and Marianne *et al*., (2012), reported in their studies that an increased mechanical stress on the contracting muscles during exhaustive exercise bout can significantly elevate the level of cardiac troponin inhibitory and creatine kinase-3 of the exercised subjects

Exercise-induced increases in myocardial permeability which facilitate the release of cytosolic cTn. Thus, it is possible that post-exercise cTn is due to passive diffusion of cTn from the intra- to extra-cellular compartment (Evans and Cannon, 1991; McNeil and Khakee, 1992; Goette *et al*., 2009). Mechanical stimuli might produce transient disruptions of the myocardial plasma membrane, termed "cell wounds" which can significantly elevate the level of cardiac troponin inhibitory and creatine kinase-3 (Clarke *et al*., 1995; Ross and Borg, 2011; Scharhag *et al*., 2006; Hessel *et al*., 2008; Koller and Schoberberger, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Subjects recruitment

The study was carried out in the Faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus and ethical approval was obtained from the ethics committee of the Faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus. The reason for the research was explained to the prospective participants and only those who gave their written consent were recruited for the study. This study was limited to apparently healthy young male undergraduate students of the Faculty of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus within 18 and 35 years of age who are willing to participate in the study. Those with no underlying history of illness e.g. Hypertension, irregular heart rate, glucose utilization disorders, asthma, sickle cell anemia and other forms of anemia were included. Of the forty-three (43) students recruited using the simple random sampling method during the first week of resumption of the first semester 2013/2014 academic year, eighteen (18) of the participants from Medical Laboratory Science Department dropped-out of the research due to time constrain. Twenty-five (25) healthy young male undergraduate students (12 students from Physiotherapy Department, 10 students from Radiological Science Department and 3 students from the Medical Laboratory Science Department) with an average age of 24.3 ± 3 years and body mass index of 22.7 ± 1.8 (kg/m^2) of the Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus participated fully in the study.

3.2 Exclusion criteria

Young male with an underlying history of illness e.g. Hypertension, irregular heart rate, glucose utilization disorders, asthmatics, sickle cell anemia and other forms of anemia were excluded. Those currently undertaking examination were excluded. Those that engage in strenuous activities such as (professional athletes, welders etc) were excluded. Subjects currently on antioxidant supplementation, alcohol and any antimicrobial agents were excluded to avoid any drug related gene expression inhibitions.

3.3 Relaxation period

The participants were placed on a bed rest in the comfort of their rooms and asked to continue similar dietary practices. They were instructed to report to the Laboratory unit of the Department of Medical Laboratory Science, Nnamdi Azikiwe University, Nnewi Campus for daily blood pressure, heart rate, fasting glucose level monitoring which lasted for 48-hours prior to the endurance race. The purpose of the wash-out period was to monitor the blood pressure, heart rate and fasting glucose level of the subjects.

3.4 Procedures for the pre-exercise stage data collection

Details of the longitudinal experimental design were explained to the participants. All participants were familiarized with the procedure of the endurance race using the treadmill and consent was obtained from the prospective participants. The participants were encouraged to eat balance diet two hours prior to the endurance race to avoid hypoglycemic shock during the endurance race and avoid any strenuous activity during the period of blood samples collections. Upon arrival at the Gymnastic studio of the Medical Rehabilitation Department, Nnamdi Azikiwe University, Nnewi Campus, Nigeria, the size of the bicep and thigh muscles of the

participants was measured using inelastic measuring tape. The participants were allowed to rest for at least ten minutes after which the blood pressure and pulse rate were measured from the left arm as described by Musa *et al.,* (2002) using an automated digital electronic BP monitor (Omron digital BP monitor, Model 11 EM 403c; Tokyo Japan).

Weight; The weights of the participants were obtained with the participant standing erect and bare-foot on the weight scale calibrated in kilogram with minimal clothing.

Height; The height of the participants was obtained using a calibrated height meter with the participant standing erect on the platform of the height meter with the back turned to the height meter; measurement was taken at the level of the vertex.

3.5 Procedures for the pre-exercise stage blood sample collection

Eight milliliters volume of venous blood sample were collected from the ante-cubital vein using standard laboratory collection technique and shared equally into ethylene diamine tetra acetic acid (EDTA) vacutainers for white blood count estimation, sodium fluoride-potassium oxalate (FO) vacutainers for plasma glucose estimation, ribonucleic acid-gard (RNAgard) vacutainers for total lymphocytes ribonucleic acid isolation and an anticoagulant free vacutainers, subsequently centrifuged at 750 x g for 15 minutes to obtain serum. The serum was immediately aliquoted into eppendorf tubes placed on ice and immediately stored at -80^0 C in an ultra refrigerator for immuno assays.

3.6 Exercise protocol

The acute exercise protocol commenced with a 5 minutes warm-up comprising of stretching exercise after which the subjects took part in an endurance race using the Bruce treadmill protocol for sub maximal exercise. The participants performed the exercise session within 8.00 am and 9.30 am. The exercise protocol started at 2.7 km/hr and a 10% grade and increased by

2% every 3 minutes in a step-like manner to a final stage at 9.6 km/hr with a 22% grade as described by Vanessa and Elizabeth, (2000). Table 3.1 shows details. The target heart rates on the treadmill were 60-80 percent of the heart rate reserve (HRR), defined as the difference between maximal heart rate (MHR) and resting heart rate (RHR).

The HRR were calculated using the formula:

HRR = MHR - RHR.

MHR = 220 - age in Years.

According to the American College of Sports Medicine (ACSM, 1998). The subjects continued until they are tired or when they attained twenty-one minutes which is regarded as the time to attain exhaustion using the Bruce treadmill protocol for sub-maximal exercise. Fourteen (14) out of twenty-five (25) subjects were exhausted before twenty-one minutes.

Table 3.1: Bruce treadmill protocol sub maximal exercise

Stage	Minutes	% grade	km/h
1	3	10	2.7
2	6	12	4.0
3	9	14	5.4
4	12	16	6.7
5	15	18	8.0
6	18	20	8.8
7	21	22	9.6

Source: Vanessa and Elizabeth, 2000.

3.7 Procedures for the post data and blood sample collections

These were obtained in a similar way described in the pre-stage but at three different time intervals:

i. One hour post exercise with adequate rest

ii. Four hours post exercise with adequate rest

iii. Twenty-four hours post exercise with adequate rest

3.8 Rest protocol

The subjects showered with warm water 10 minutes after the exercise bout and went on a bed rest for one hour and four hours in a comfortable environment before the 1 hour and 4 hours' data and blood sample collections respectively. Thereafter, the subjects took at least 6- 8 hours sleep on a comfortable bed before the 24 hours' data and blood sample collections.

3.9 Assay methodology

Serum glucose determination (Using Randox kit)

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator.

Assay procedure

Ten micro liters volume of the standard or test sample was added to a 1000 µl volume of Reagent 1, mixed and incubated for 25 minutes at 20° C. The absorbance of the standard or the sample was measured against the reagent blank within 60 minutes at a wavelength of 540 nm.

Hematological parameter estimation (As described by Samuel *et al.*, 2010)

Hematological parameter was determined using sysmex® Automated Hematology analyzer.

Quantitative troponin inhibitory estimation (**As described by Etievent *et al.*, 1995)**

Principle of the assay

The Troponin I (Human) ELISA Kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. This test kit operates on the basis of competition between the hormone conjugate and the troponin in the sample for a limited number of binding sites on the antibody coated plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of the stop solution. The absorbance is measured on a microtitre plate reader. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample.

Assay procedure

One hundred micro liters volume of standards, specimens, and controls were dispensed into appropriate wells. A 100 µl volume of enzyme conjugate reagent was added into each well. It was thoroughly mixed for 30 seconds and incubated at room temperature for 90 minutes. The wells were rinsed five times with wash solution. A 100 µl volume of TMB reagent dispense into each well and incubated at room temperature for 20 minutes. The reaction was stopped by adding

100 µl volume of stop solution to each well. It was gently mixed for 30 seconds. The absorbance was read at 450nm with a microtitre well reader.

Quantitative progesterone estimation (As described by Onyenekwe *et al*., 2014)

Principle of the assay

This test kit operates on the basis of competition between the hormone conjugate and the progesterone in the sample for a limited number of binding sites on the antibody coated plate. The washing procedures remove unbound materials. After the washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of the stop solution. The absorbance is measured on a microtitre plate reader. The intensity of the color formed is inversely proportional to the concentration of progesterone in the sample.

Assay procedure

Twenty-five micro liters volume of standards, specimens, and controls was dispensed into appropriate wells. A 100 µl volume of working progesterone conjugate reagent was dispensed into each well. A 50 µl volume of rabbit anti-progesterone reagent was dispensed to each well. It was thoroughly mixed for 30 seconds Incubated at room temperature for 90 minutes. The micro titers well were rinsed five times with wash solution. A 100 µl volume of TMB Reagent was dispensed into each well and gently mixed for 10 seconds. It was then incubated at room temperature for 20 minutes. The reaction was stop by adding 100 µl volume of stop solution to each well. The absorbance was read at 450 nm with a micro titer plate reader.

Serum cortisol estimation (As described by Ehiaghe *et al.*, 2013)

Principle of the test

This test kit operates on the basis of competition between an unlabeled antigen (present in standards, controls and samples) and an enzyme-labeled antigen for a limited number of antibody binding sites on the wells. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of the stop solution. The absorbance is measured on a microtitre plate reader. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample.

Assay procedure

Twenty micro liters volume of standard or sample(s) was added per microplates. A 200 µl volume of cortisol hormone conjugate was added to the standard or sample(s) and covered with a sealing tape. It was incubated at room temperature for 1 hour. The solution was discarded and microplates washed three times with a 300 µl of volume 1X wash solution. A 100 µl volume of tetramethylbenzidine one step substrate was added to each micro plate and incubated for 15 minutes at room temperature in the dark. A 100 µl volume of stop solution was added to each micro plate. The intensity of the color developed was measured at 450 nm wavelength using stat fax® 4700 micro strip reader.

Quantitative luteinizing hormone estimation (As described by Warren and Perroth, 2001)

Assay principle

The kit is intended for the quantitative determination of luteinizing hormone (LH) concentration in human serum. The assay system utilizes a mouse monoclonal anti-α-LH antibody for solid phase immobilization and a mouse monoclonal anti-β-LH antibody in the conjugate solution.

The test sample was allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45 minutes of incubation at room temperature, the wells are washed wash solution to remove unbound-labeled antibodies. A solution of tetramethylbenzidine (TMB) reagent was added and incubated for 20 minutes, resulting in the development of a blue color. The color development was stopped with the addition of stop solution and measured at 450 nm. The concentration of LH is directly proportional to the color intensity of the test sample.

Assay procedure

Fifty micro liters volume of standard, specimens, and controls were dispensed into appropriate wells. A 100 µl volume of enzyme conjugate reagent was dispensed into each well and gently mixed for 30 seconds and Incubated at room temperature for 45 minutes. The microtitre wells were rinsed five times with wash solution. A 100 µl volume of TMB reagents was dispensed into each well and gently mixed for 10 seconds. It was then incubated at room temperature in the dark for 20 minutes. A 100 µl volume of stop solution was added to stop the reaction in the wells. It was read at 450 nm with a microtitre plate reader.

Follicle stimulating hormone estimation (As described by Warren and Perroth, 2001)

Principle of the test

The kit is designed for the accurate quantitative measurement of follicle stimulating hormone in human serum. A 96-well plate has been pre coated with anti-follicle stimulating hormone antibodies. Samples and standards and are added to the wells, where follicle stimulating hormone in the sample and standards binds to the pre coated antibody. After incubation and washing, added anti-follicle stimulating hormone conjugate binds to the antibody-follicle stimulating hormone complex. After incubation, the wells are washed to remove unbound material and TMB

substrate was then added to produce blue coloration. The reaction is terminated by addition of stop solution which stops the color development and produces a color change from blue to yellow. The intensity of signal is directly proportional to the amount of follicle stimulating hormone in the sample and the intensity is measured at 450nm.

Assay procedure

Fifty micro liters volume of standards or samples was added into their respective wells. A 100 µl volume of the enzyme conjugate was added into each well and mixed for 30 seconds. The wells were covered with the foil supplied with the kit and incubated at room temperature for 45 minutes. The wells were washed five times with a 300 µl volume of wash solution. A 100 µl volume of TMB reagents was added into each well and incubated at room temperature for 20 minutes in the dark with gentle shaking. The reaction was stopped by adding 100 µl volume of stop solution to each well. The intensity of the color produced was directly proportional to the amount of follicle stimulating hormone present in the sample(s) and the intensity was measured at 450 nm.

Serum creatine kinase-3 (MM) estimation (Marianne *et al.*, 2012)

Principle of the test

The assay system utilizes a monoclonal antibody directed against a distinct antigenic determinant on the CK-MM molecule is used for solid phase immobilization on the microtitre wells. A goat anti-CK-MM antibody conjugated to horseradish peroxidase is in the antibody-enzyme conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the CK-MM molecules being sandwiched between the solid phase and enzyme-linked antibodies. After one-hour incubation at room temperature, the wells are washed with wash solution to remove unbound labeled antibodies. A solution of TMB reagent is added and

incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of stop solution changing the color to yellow. The concentration of CK-MM is directly proportional to the color intensity of the test sample.

Assay procedure

Twenty micro liters volume of standard or sample(s) was added per microplates. A 200 µl volume hormone conjugate was added to the standard or sample(s) and covered with a sealing tape. It was incubated at room temperature for one hour. The solution was discarded and microplates washed three times with 300 µl volume of 1X wash solution. A 100 µl volume of tetramethylbenzidine one step substrate was added to each micro plate and incubated for 15 minutes at room temperature in the dark with gentle shaking. A 100 µl volume of stop solution was added to each micro plate. The intensity of the color developed was read at 450 nm wavelength using stat fax® 4700 micro strip reader.

3.10 Polymerase chain reaction methods

Total RNA Extraction using the ZYMO RESEARCH Whole-Blood RNA MiniPrep

Total RNA was extracted using the ZR Whole–Blood RNA MiniPrep according to ZYMO RESEARCH specification. A 600 µl volume red blood cell lysis buffers were added to 200 µl volume of ribonucleic acid-gard (RNAgard) stored whole blood sample in an RNase-free tube and mixed by inverting. The mixture was incubated for 5 minutes at 25^0 C and centrifuge at ≥ 12,000 × g for 1 minute. The supernatant was removed. A 600 µl volume of blood RNA buffer was added to the cell pellet and mixed properly. The resultant mixture was transferred into the Zymo-Spin IIIC column in a collection tube and centrifuged at ≥ 12,000 × g for 2 minutes. The column was transferred into a new collection tube. A 400 µl volume of RNA pre wash buffer was added to the column and centrifuge at ≥ 12,000 × g for 30 seconds. A 400 µl volume RNA

wash buffer was added to the column and centrifuge at ≥ 12,000 × g for 30 seconds. The column was transferred into an RNase free tube. 100 µl RNA recovery buffer was added to the Zymo spin IIIC column and centrifuged at ≥ 12,000 × g for 30 seconds. A 100 µl volume ethanol (100%) was added to the flow- through in the RNase free tube and mixed by pipetting. The mixture was transferred into the Zymo spin IC column in a collection tube and centrifuged at ≥ 12,000 × g for 30 seconds. A 400 µl volume of the RNA prep buffer was added to the column and centrifuged at ≥ 12,000 × g for 1 minute, the flow through was discarded. A 800 µl volume of the RNA wash buffer was added to the column and centrifuged at ≥ 12,000 × g for 1 minute, the flow through was discarded. The wash step was repeated with 400 µl volume of RNA wash buffer. The Zymo-spin IC column was centrifuged in an empty collection tube at ≥ 12,000 × g for 2 minutes. It was then transferred into an RNase free tube. Total RNA was eluted by added 80 µl volume of DNase/RNase free water directly to the column matrix and centrifuged at 10,000 × g for 30 seconds. A 70 µl volume of the Total RNA extracted was transferred into an RNA stable tube supplied by Biomatrica with catalog number 93221-001 for storage of Total RNA at room temperature while 10 µl was used for quality control check on the Total RNA extracted.

3.11 Detection of the total RNA isolated

One gram of agarose powder was weighed and poured into 100 ml of Tris EDTA buffer in a Pyrex conical flask. It was heated using a microwave at 100^0 C for 5minutes. It was allowed to cool to 56^0 C and 6 µl volume of ethidium bromide was added to 100 ml of the gel mixture. The gel was poured into the electrophoresis chamber and allowed to solidify. A 3 µl volume of loading dye was added to 7 µl volume of the Total RNA from each subject, the molecular marker was loaded in the first lane, followed by the samples. Electrophoresis was performed at 90 volts

for 30 minutes. The gel was removed and viewed on the UV transilluminator; the picture of the gel was taken (Figure 3.1).

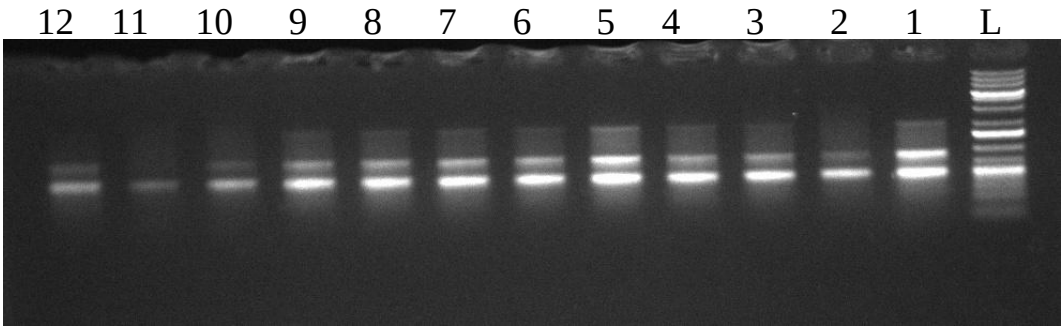


Figure 3.1: Image of Total RNA isolated from blood sample of exercise induced stress subjects analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. L is a 20bp molecular ladder. Lane 1- 12 are RNA isolated from the exercised subjects.

Table 3.2: Assay condition for each lymphocyte genes evaluated and purchased from Inqaba Biotechnology Industries, Hartfield, South Africa.

Gene Name	Primers 5^1-3^1 (forward, reverse)	Molecular weight	Melting temperature (Tm)(^{0}C)	Gene bank accession number
Tumor necrosis factor alpha	TGTTGTAGCAAACCCTCAAGC AGTCGGTCACCCTTCTCCA	6389.6 5699.4	60.61 62.32	AFO43342
Interferon gamma	TCTGCATCGTTTTGGGCT GCAGGAGGACAACCATTACT	6096 6423	58.35 62.57	X13274
Interleukin-10	ATGCACAGCTCAGCACTGC TCAGTTTCGTATCTTCATTGTC	5757.2 6657.3	62.32 57.08	M57627
Hypoxanthine phosphoribosyl transferase	AATTATGGACAGGACTGAACGG ATAATCCAGCAGGTCAGCAAAG	6831 6760	60.81 60.81	AY372182
Succinate dehydrogenase	GAGGAATGGTCTGGAATACTG GCCTCTGCTCCATAAATCG	6549 5723	60.61 60.16	DQ402987

Preparation of the 100 µM stock solution of the primers

The lyophilized primers were spun down with the aid of the microcentrifuge before opening it to ensure that the primer pellets are at the bottom of the tubes.

The primers were diluted as follows;

Tumor necrosis factor alpha F (TNFα-F): A 409.46 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Tumor necrosis factor alpha R (TNFα-R): A 276.57 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Interferon gamma F (IFN-F): A 430.81 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Interferon gamma R (IFN-R): A 401.69 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Interleukin-10 F (IL-10-F): A 326.01 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Interleukin-10 R (IL-10-R): A 463.52 µl of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Hypoxanthine phosphoribosyl transferase F (HPRT-F): A 346.16 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Hypoxanthine phosphoribosyl transferase R (HPRT-R): A 359.17 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Succinate dehydrogenase F (SDH-F): A 294.09 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

Succinate dehydrogenase R (SDH-R): A 357.12 µl volume of nuclease free water was to resuspend the lyophilized primer and further diluted 1:10 with nuclease free water (10 µM) (i.e. 10 µl volume of the resuspended primer to 90 µl volume of nuclease free water).

3.12 One Taq One-Step reverse transcriptase polymerase chain reaction for lymphocytic gene expressions

The extracted Total RNA was retro-transcribed and amplified using One Taq One Step RT-PCR kit with catalog number NEB E5315S by NEW ENGLAND BioLabs incorporation according to the manufacturer's specification. Selected primers were used to target lymphocyte genes using MJ research peltier thermal cycler polymerase chain reaction machine at the Lahor Research Laboratory and Medical Centre, 121, Old Benin-Agbor Road, Benin City, Edo state, Nigeria. Table 3.0 shows details of the primers used. The PCR were performed in a 50 µl volume reaction mixture containing 25 µl volume of one Taq one-step reaction master mix (2x), 2 µl volume of One Taq one- step enzyme mix (2x), 2 µl volume of each gene-specific forward primer (10 µM), 2 µl volume of each gene-specific reverse primer (10 µM), 9 µl volume of nuclease-free water

and 10 µl volume of the RNA template was added. Negative controls samples for the RT-PCR consisted of a mixture to which all reagents added except RNA. The PCR was started immediately as follows: Reverse transcriptase at 48^0 C for 30 seconds, initial denaturation at 94^0 C for 1 minute, denaturation at 94^0 C for 15 seconds, annealing at Tm^0 C-5(The lowest melting temperature of each set of lymphocyte primer minus five) for 30 seconds, extension at 68^0 C for 1 minute, Go to the denaturation step for 39 cycles, final extension at 68^0 C for 5 minutes and final holding at 4^0 C forever.

3.13 Preparation of agarose gel

One percent agarose gel was prepared by dissolving 1.0 g of LE Agarose powder in 100 ml volume of Tris Borate EDTA Buffer. The mixture was then heated in a microwave at 100^0 C for 5 minutes, it was then allowed to cool to 56^0 C and 6 µl volume of ethidium bromide was added to it. The agarose gel was poured into the electrophoresis chambers with gel comb, and allowed to solidify.

3.14 Electrophoresis

Five micro liters volume of the amplified PCR products and DNA ladder were analyzed on 1% agarose gel containing ethidium bromide in 1X Tris EDTA buffer. Electrophoresis was performed at 90 volts for 30 minutes with the EDVOTEK tetra source electrophoresis machine, Bethesda, USA. After electrophoresis TNF-α, IFN-γ, IL-10, HPRT and SDH were visualized with Wealtec Dolphin-Doc UV transilluminator and photographed. Molecular weights were estimated using molecular weight of the DNA ladder. Gene expression level are regarded as negative, if it is below the ethidium bromide detection level (< 0.1 pg) as reported by NEW ENGLAND BIOlabs incorporation.

3.15 Statistical analysis

All numerical results were analyzed with one-way ANOVA with least significance difference post hoc test to compare all pairs of columns while Student's t- test was used to compare independent variables. Gene expression results were analyzed with Chi-Square test using SPSS version 20.0 statistical program. P values < 0.05 were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 The baseline characteristics of the participants

Twenty-five male undergraduates of average age of 24.3 ± 3.0 years with an average body mass index of 22.7 ± 1.8 participated fully in the study (Table 4.1).

4.2 The glucose level

The mean ± S.D of glucose level of subjects who were exhausted before 21 minutes of the exercise bout were 94.2 ± 8.20 mg/dl pre-exercise, 80.0 ± 8.78 mg/dl one hour post- exercise, 82.3 ± 8.66 mg/dl four hours post-exercise and 95.5 ± 8.00 mg/dl twenty-four hours post exercise. The glucose levels was significantly lower at one hour and four hours post exercise when compared with pre-exercise (P= 0.000). However, there was no significant difference when compared with the twenty-four hours post exercise (P = 0.678). The mean ± S.D of glucose level of subjects who were exhausted at twenty-one minutes of the exercise bout were 94.4 ± 10.61 mg/dl pre-exercise, 80.5 ± 8.06 mg/dl one hour post exercise, 83.6 ± 7.59 mg/dl four hours post exercise and 95.6 ± 6.69 mg/dl twenty-four hours post exercise. The glucose levels was significantly lower at one hour and four hours post exercise when compared with pre-exercise (P = 0.000). Similarly, there was no significant difference when compared with the twenty-four hours post exercise (P = 0.687) (Table 4.2).

4.3 The cortisol level

The mean ± S.D of cortisol level of subjects who were exhausted before the 21 minutes of the exercise bout were 293.1 ± 150.86 nmol/L pre-exercise, 411.0 ± 169.71 nmol/L one hour post exercise, 246.0 ± 155.12 nmol/L four hours post exercise and 183.4 ± 72.83 nmol/L twenty-four hours post exercise. The cortisol levels was significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). However, there was no significant difference when compared with the four and twenty-four hours post exercise (P = 0.222). The mean ± S.D of cortisol level of subjects who were exhausted at twenty-one minutes of the exercise bout were 226.4 ± 67.95 nmol/L pre-exercise, 355.6 ± 153.62 nmol/L one hour post exercise, 190.1 ± 64.05 nmol/L four hours post exercise and 183.6 ± 58.11 nmol/L twenty-four hours post exercise. The cortisol levels was significantly higher at one hour post exercise when compared with pre exercise (P = 0.000). Similarly, there was no significant difference when compared with the four hours post exercise (P = 0.494) and twenty-four hours post exercise (P = 0.420) (Table 4.2).

4.4 The progesterone level

The mean ± S.D of progesterone level of subjects who were exhausted before the 21 minutes of the exercise bout were 5.4 ± 1.06 nmol/L pre-exercise, 3.1 ± 1.24 nmol/L one hour post exercise, 2.9 ± 1.21 nmol/L four hours post exercise and 2.3 ± 1.29 nmol/L twenty-four hours post exercise. The progesterone levels was significantly lower at one hour post exercise when compared with pre exercise stage (P = 0.05). The mean ± S.D of progesterone level of subjects who were exhausted at twenty- one minutes of the exercise bout were 5.2 ± 1.28 nmol/L pre-exercise, 3.0 ± 1.91 nmol/L one hour post exercise, 2.8 ± 1.27 nmol/L four hours post exercise and 2.3 ± 1.29 nmol/L twenty-four hours post exercise. The progesterone level was significantly lower at one hour post exercise when compared with pre exercise (P = 0.012) (Table 4.2).

Table 4.1: Baseline characteristics of the participants

Variables	All subjects (N=25)	Subjects exhausted before 21 minutes (N=14)	Subjects exhausted at 21 minutes (N=11)	t- values	P-values
Age (yrs)	24.3 ± 3.0	23.50 ± 3.0	25.36 ± 3.0	0.191	0.666
Height (m)	1.7 ± 0.07	1.73 ± 0.07	1.72 ± 0.06	0.177	0.678
Weight (kg)	67.6 ± 6.2	68.6 ± 6.67	66.3 ± 6.55	1.083	0.309
Size of bicep muscle (cm)	28.3 ± 2.5	28.43 ± 2.5	28.03 ± 2.6	0.325	0.574
Size of thigh muscle (cm)	49.4 ± 6.5	48.21 ±7.6	51.00 ± 4.6	0.692	0.414
Body mass index (kg/m^2)	22.7 ± 1.8	22.90 ±1.8	22.43 ± 1.8	0.002	0.968

Keys:

SD: = Standard deviation
X = Mean value
P = $P < 0.05$ were considered significant
N = Number of subjects

Note: All values are presented as mean ± SD

Table 4.2: Levels (mean ± SD) of glucose (mg/dl), cortisol (nmol/L) and progesterone (nmol/L) in exercised participants

Time intervals	**Glucose level**		**Cortisol level**		**Progesterone level**	
	Subjects exhausted before 21 minutes (n = 14)	**Subjects exhausted at 21 minutes (n = 11)**	**Subjects exhausted before 21 minutes (n = 14)**	**Subjects exhausted at 21 minutes (n = 11)**	**Subjects exhausted before 21 minutes (n = 14)**	**Subjects exhausted at 21 minutes (n = 11)**
Pre-exercise (A)	94.2 ± 8.2	94.4 ±10.61	293.1 ± 150.86	226.4 ± 67.95	5.4 ± 1.06	5.2 ± 1.28
1hour post exercise(B)	80.0 ± 8.78	80.5±8.06	411.0 ± 169.71	355.6 ± 153.62	3.1 ± 1.24	3.0 ± 1.19
4 hours post exercise(C)	82.3 ± 8.66	83.6 ±7.59	246.0 ± 155.12	190.1 ± 64.05	2.9 ± 1.21	2.8 ± 1.27
24 hours post exercise(D)	95.5 ± 8.00	95.6 ± 6.69	183.4 ± 72.83	183.6 ± 58.11	2.3 ± 1.29	2.3 ± 1.29
F Value	9.286		6.032		2.351	
P Value	0.000*		0.000*		0.030*	
A vs. B	0.000*	0.000*	0.013*	0.016*	0.050*	0.012*
A vs. C	0.000*	0.002*	0.316 (ns)	0.494 (ns)	0.166 (ns)	0.135(ns)
A vs. D	0.678 (ns)	0.687 (ns)	0.222(ns)	0.420 (ns)	0.063 (ns)	0.052(ns)
A_{21} Vs. $A_{<21}$	0.956(ns)		0.184(ns)		0.141(ns)	
B_{21} Vs. $B_{<21}$	0.466(ns)		0.001*		0.731(ns)	
C_{21} Vs. $C_{<21}$	0.690(ns)		0.265(ns)		0.832(ns)	
D_{21} Vs. $D_{<21}$	0.989(ns)		0.987(ns)		0.963(ns)	

Keys:
<21 = exhaustion before 21 minutes
21 = exhaustion at 21 minutes
ns= non-significant
*= significant

4.5 The luteinizing hormone level

The mean ± S.D of luteinizing hormone level of subjects who were exhausted before the 21 minutes of the exercise bout were 6.1 ± 1.84 mIU/ml pre-exercise, 2.7 ± 1.6 mIU/ml one hour post exercise, 5.1 ± 2.02 mIU/ml four hours post exercise and 6.6 ± 2.0 mIU/ml twenty-four hours post exercise. The luteinizing hormone levels was significantly lower at one hour post exercise when compared with pre-exercise (P = 0.000). However, there was no significant difference when compared with the four hours post and twenty-four hours post exercise (P = 0.121 and P = 0.490) respectively. The mean ± S.D of luteinizing hormone level of subjects who were exhausted at twenty- one minutes of the exercise bout were 6.0 ± 1.49 mIU/ml pre-exercise, 3.0 ± 2.10 mIU/ml one hour after exercise, 4.5 ± 1.52 mIU /ml four hours post exercise and 6.2 ± 1.34 mIU/ml twenty-four hours post exercise. The luteinizing hormone levels was significantly lower at one hour post exercise and four hours post exercise when compared with pre-exercise (P = 0.000). However, there was no significant difference when compared with the twenty-four hours post exercise (P = 0.971). It was also observed that there were no significant difference between subjects who were exhausted before the end of the exercise bout and those who were exhausted at the end of the exercise bout (Table 4.3).

4.6 The follicle stimulating hormone level

The mean ± S.D of follicle stimulating hormone level of subjects who were exhausted before the 21 minutes of the exercise bout were 4.1 ± 2.27 mIU/ml pre-exercise, 2.9 ± 2.30 mIU/ml one hour post exercise, 3.6 ± 2.27 mIU/ml four hours post exercise and 4.0 ± 2.21 mIU/ml twenty-four hours post exercise. The follicle stimulating hormone levels was significantly lower at one hour post exercise when compared with pre-exercise (P= 0.000). The mean ± S.D of

luteinizing hormone level of subjects who were exhausted at the end of the exercise bout were 4.1 ± 1.29 mIU/ml pre-exercise, 2.0 ± 1.14 mIU/ml one hour post exercise, 2.0 ± 1.19 mIU /ml four hours post exercise and 3.2 ± 1.22 mIU/ml twenty-four hours post exercise. The follicle stimulating hormone levels was significantly lower at one hour post exercise when compared with pre exercise (P = 0.002) (Table 4.3).

4.7 The troponin I level

The mean ± S.D of troponin I level of subjects who were exhausted before the 21 minutes of the exercise bout were 1.7 ± 1.83 ng/ml pre-exercise, 5.7 ± 3.04 ng/ml one hour post exercise, 4.5 ± 3.00 ng/ml four hours post exercise and 3.9 ± 2.89 ng/ml twenty-four hours post exercise. The troponin I levels was significantly higher at one hour post exercise, four hours post exercise and twenty-four hours post exercise when compared with pre-exercise (P= 0.000). The mean ± S.D of troponin I level of subjects who were exhausted at twenty- one minutes of the exercise bout were 3.4 ± 1.87 ng/ml pre-exercise, 6.7 ± 2.91 ng/ml one hour post exercise, 4.2 ± 2.48 ng/ml four hours post exercise and 3.2 ± 2.02 ng/ml twenty-four hours post exercise. The troponin levels was significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). However, it was also observed that there were no significant difference between subjects who were exhausted before the end of the exercise bout and those who were exhausted at the end of the exercise bout (Table 4.3).

4.8 The creatine kinase- MM level

The mean ± S.D of creatine kinase MM level of subjects who were exhausted before the 21 minutes of the exercise bout were 3.7 ± 1.12 ng/ml pre-exercise, 6.4 ± 1.55 ng/ml one hour post exercise, 4.4 ± 1.77 ng/ml four hours post exercise and 3.3 ± 1.18 ng/ml twenty-four hours post exercise bout. The creatine kinase MM levels was significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). The mean ± S.D of creatine kinase MM level of subjects who were exhausted at twenty- one minutes of the exercise bout were 3.5 ± 1.20 ng/ml pre-exercise, 5.9 ± 1.73 ng/ml one hour post exercise, 4.0 ± 1.57 ng/ml four hours post exercise and 2.7 ± 1.13 ng/ml twenty-four hours post exercise. The creatine kinase MM levels was significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). However, it was also observed that there were no significant difference between subjects who were exhausted before the end of the exercise bout and those who were exhausted at the end of the exercise bout (Table 4.3).

4.9 Absolute lymphocyte count

The mean ± S.D of the absolute lymphocyte count of subjects who were exhausted before the 21 minutes of the exercise bout were 1.5 ± 0.34 cells/µl × 10^3 pre-exercise, 2.0 ± 0.44 cells/µl × 10^3 one hour post exercise, 2.0 ± 0.47 cells/µl × 10^3 four hours post exercise and 1.6 ± 0.36 cells/µl twenty-four hours post exercise. The mean ± S.D of the absolute lymphocyte count of subjects who were exhausted at twenty- one minutes of the exercise bout were 1.1 ± 0.29 cells/µl × 10^3 pre-exercise, 1.6 ± 0.43 cells/µl × 10^3 one hour post exercise, 1.5 ± 0.37 cells/µl × 10^3 four hours post exercise and 1.2 ± 0.24 cells/µl twenty-four hours post exercise. The absolute lymphocyte counts was significantly higher at one hour and four hours post exercise when compared with the pre-exercise stage (P = 0.000) (Table 4.4).

4.10 Absolute neutrophils count

The mean ± S.D of the absolute lymphocyte count of subjects who were exhausted before the 21 minutes of the exercise bout were 2.4 ± 0.53 cells/µl × 10^3 pre-exercise, 3.0 ± 0.59 cells/µl × 10^3 one hour post exercise, 3.3 ± 0.73 cells/µl × 10^3 four hours post exercise and 2.7 ± 0.59 cells/µl twenty-four hours post exercise. The mean ± S.D of the absolute lymphocyte count of subjects who were exhausted at twenty- one minutes of the exercise bout were 1.7 ± 0.36 cells/µl × 10^3 pre-exercise, 2.3 ± 0.43 cells/µl × 10^3 one hour post exercise, 2.5 ± 0.63 cells/µl × 10^3 four hours post exercise and 1.9 ± 0.38 cells/µl twenty-four hours post exercise. The absolute lymphocyte counts was significantly higher at one hour and four hours post exercise when compared with pre-exercise stage (P = 0.000) (Table 4.4).

Table 4.3: Levels (mean ± SD) of luteinizing hormone (mIU/ml), follicle stimulating hormone (mIU/ml), troponin I (ng/ml) and creatine kinase – MM (ng/ml) in exercised participants

Time intervals	Luteinizing hormone		Follicle stimulating hormone		Troponin I		Creatine kinase MM	
	Subjects exhausted before 21 minutes (n =14)	**Subjects exhausted at 21 minutes (n =11)**	**Subjects exhausted before 21 minutes (n =14)**	**Subjects exhausted at 21 minutes (n =11)**	**Subjects exhausted before 21 minutes (n =14)**	**Subjects exhausted at 21 minutes (n =11)**	**Subjects exhausted before 21 minute (n =14)**	**Subjects exhausted at 21 minutes (n =11)**
Pre-exercise(A)	6.1 ± 1.84	6.0 ± 1.49	4.1 ± 2.27	4.2 ±1.29	1.7 ± 1.83	3.4 ± 1.87	3.7 ± 1.12	3.5 ± 1.20
1hour post exercise(B)	2.7 ± 1.60	3.0 ± 2.10	2.9 ± 2.30	2.0 ± 1.14	6.8 ± 3.04	6.7 ± 2.91	6.4 ± 1.55	5.9 ±1.73
4 hours post exercise(C)	5.1 ± 2.02	4.5 ± 1.52	3.6 ± 2.27	2.0 ± 1.19	4.5 ± 3.00	4.2 ± 2.48	4.4 ± 1.77	4.0 ± 1.57
24 hours post exercise(D)	6.6 ± 2.00	6.2 ± 1.34	4.0 ± 2.21	3.2 ± 1.22	3.0 ± 2.89	3.2 ± 2.02	3.3 ± 1.18	2.7 ± 1.13
F Value	9.153		2.344		4.551		10.282	
P Value	0.000*		0.000*		0.000*		0.000*	
A vs. B	0.000*	0.000*	0.000*	0.002*	0.000*	0.003*	0.000*	0.000*
A vs. C	0.121 (ns)	0.120(ns)	0.515 (ns)	0.166 (ns)	0.555(ns)	0.468 (ns)	0.206 (ns)	0.414 (ns)
A vs. D	0.490 (ns)	0.971 (ns)	0.928 (ns)	0.946 (ns)	0.223(ns)	0.856 (ns)	0.453 (ns)	0.198 (ns)
A_{21} Vs. $A_{<21}$	0.887 (ns)		0.754 (ns)		0.096 (ns)		0.757 (ns)	
B_{21} Vs. $B_{<21}$	0.637(ns)		0.248 (ns)		0.332 (ns)		0.423 (ns)	
C_{21} Vs. $C_{<21}$	0.416 (ns)		0.052(ns)		0.796 (ns)		0.529 (ns)	
D_{21} Vs. $D_{<21}$	0.587(ns)		0.249 (ns)		0.498 (ns)		0.332 (ns)	

ns = non significant * = significant

Table 4.4: Levels (mean ± SD) of lymphocyte count (%), neutrophils count (%), total white blood cell count (Cells/ul), absolute lymphocyte count (cells/ul) and absolute neutrophils count (cells/ul) in exercised participants.

Time intervals	Lymphocyte count		Neutrophils count		Total white cell count x 10^3		Absolute lymphocyte count x 10^3		Absolute neutrophils count x 10^3	
	Subjects exhausted before 21 minutes (n = 14)	Subjects exhausted at 21 minutes (n = 11)	Subjects exhausted before 21 minutes (n = 14)	Subjects exhausted at 21 minutes (n = 11)	Subjects exhausted before 21 minutes (n = 14)	Subjects exhausted at 21 minutes (n = 11)	Subjects exhausted before 21 minutes (n = 14)	Subjects exhausted at 21 minutes (n = 11)	Subjects exhausted before 21 minutes (n = 14)	Subjects exhausted at 21 minutes (n = 11)
Pre-exercise(A)	30.9 ± 1.23	30.9 ± 1.64	51.1 ± 1.21	50.5 ± 1.04	4.71 ± 1.08	3.3 ± 0.72	1.5 ± 0.34	1.1 ±0.29	2.4 ± 0.53	1.7 ± 0.36
1hour post exercise(B)	36.7 ± 1.90	36.6 ± 1.63	54.7 ± 1.77	54.2 ± 1.66	5.4 ± 1.14	4.3 ±0.99	2.0 ± 0.44	1.6 ± 0.43	3.0 ± 0.59	2.3 ±0.6
4 hours post exercise(C)	34.4 ± 1.15	34.2 ± 1.40	57.4 ± 1.07	57.4 ± 1.57	5.7 ± 1.30	4.3 ± 1.05	2.0 ± 0.47	1.5 ± 0.37	3.3 ± 0.73	2.5 ± 0.63
24 hours post exercise(D)	33.0 ± 1.18	33.0 ± 1.55	53.2 ± 0.89	52.6 ± 1.21	5.0 ± 1.09	3.6 ± 0.71	1.6 ± 0.36	1.2 ± 2.24	2.7 ± 0.59	1.9 ± 0.38
F Value	29.191		41.424		7.663		9.394		10.037	
P Value	0.000		0.000		0.000		0.000		0.000	
A vs. B	0.000*	0.000*	0.000*	0.000*	0.071(ns)	0.035*	0.000*	0.002*	0.012*	0.010*
A vs. C	0.000*	0.000*	0.000*	0.000*	0.015*	0.025*	0.001*	0.011*	0.000*	0.001*
A vs. D	0.000*	0.001*	0.000*	0.001*	0.428(ns)	0.503(ns)	0.181(ns)	0.401(ns)	0.363(ns)	0.392(ns)
A_{21} Vs. $A_{<21}$	0.930(ns)		0.283(ns)		0.002*		0.015*		0.003*	
B_{21} Vs. $B_{<21}$	0.000*		0.354(ns)		0.008*		0.006*		0.008*	
C_{21} Vs. $C_{<21}$	0.769(ns)		0.910(ns)		0.002*		0.003*		0.001*	
D_{21} Vs. $D_{<21}$	1.000(ns)		0.314(ns)		0.001*		0.006*		0.001*	

ns = non significant * = significant

4.11 Lymphocytic succinate dehydrogenase genes expression

Plates 4.1 - 4.10: The reverse transcriptase PCR results for lymphocytic succinate dehydrogenase genes detected at 91 base pair (bp) analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, $P = 0.000$) respectively.

NOTE:

Subjects 1- 14 were exhausted before 21 minutes of the exercise bout

Subjects 15- 25 were exhausted at 21 minutes of the exercise bout

A = Pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

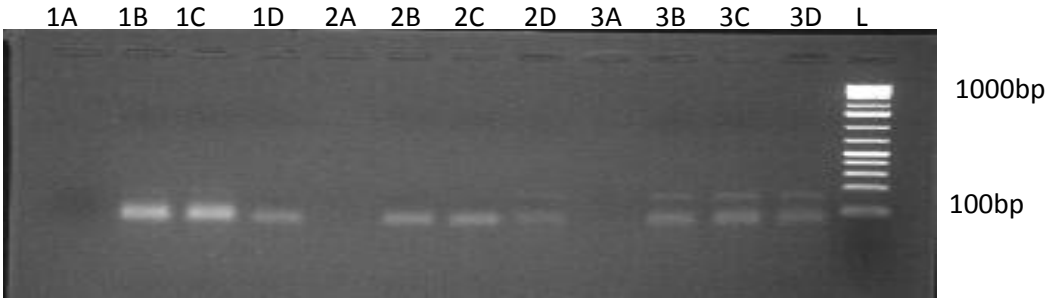


Plate 4.1: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1B, 1C, 1D, 2B, 2C, 2D, 3B, 3C and 3D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 1-3. Lanes 1A, 2A and 3A are negative bands from the pre stage of the exercise.

Note: 2D, 3C and 3D had heterozygous gene expression patterns.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

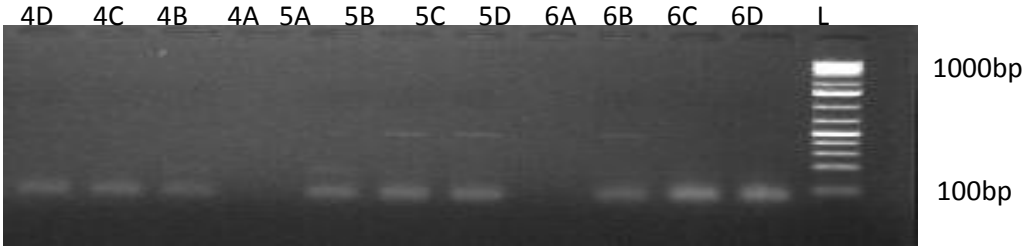


Plate 4.2: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 4B, 4C, 4D, 5B, 5C, 5D, 6B, 6C and 6D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 4- 6. Lanes 4A, 5A and 6A are negative bands from the pre stage of the exercise.

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

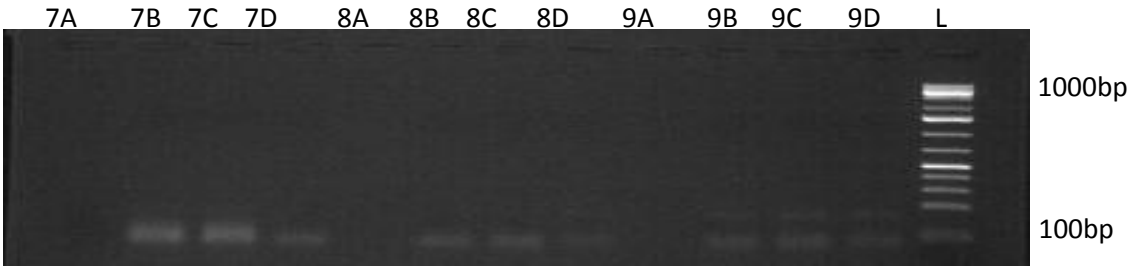


Plate 4.3: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 7B, 7C, 7D, 8B, 8C, 8D, 9B, 9C and 9D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 7- 9. Lanes 7A, 8A and 9A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

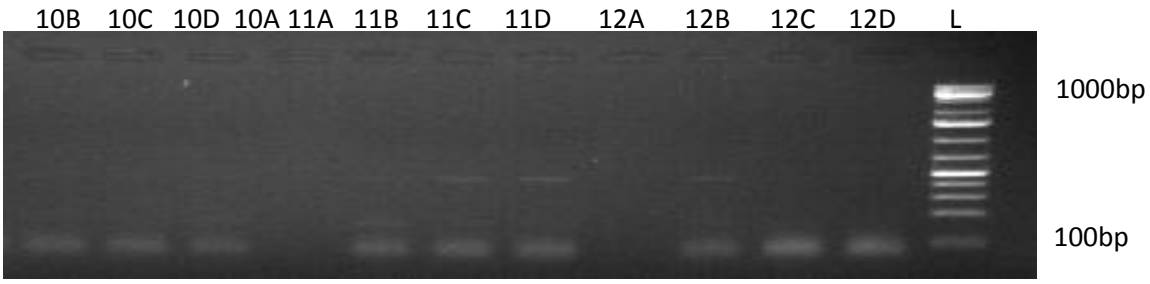


Plate 4.4: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 10B, 10C, 10D, 11B, 11C, 11D, 12B, 12C and 12D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 10-12. Lanes 10A, 11A and 12A are negative bands from the pre stage of the exercise.

Note: 11B, 11C and 11D had heterozygous gene expression patterns.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

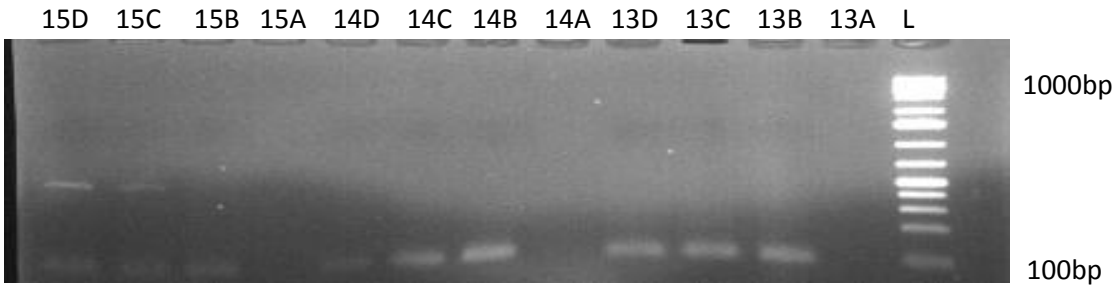


Plate 4.5: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 13B, 13C, 13D, 14B, 14C, 14D, 15B, 15C and 15D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 13- 15. Lanes 13A, 14A and 15A are negative bands from the pre stage of the exercise.

Note: 15C and 15D had heterozygous gene expression patterns.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

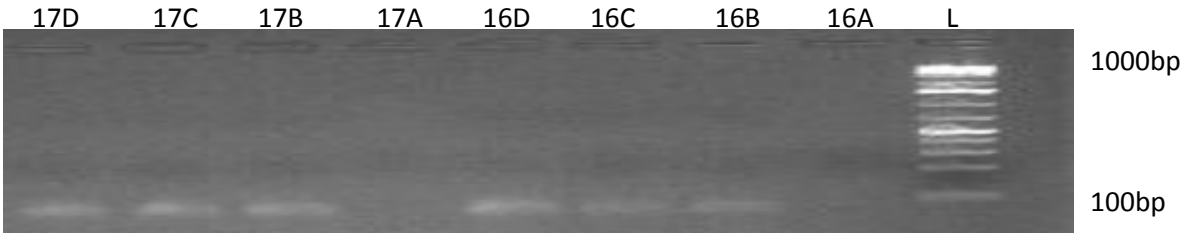


Plate 4.6: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 16B, 16C, 16D, 17B, 17C and 17D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 16 -17. Lanes 16A and 17A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

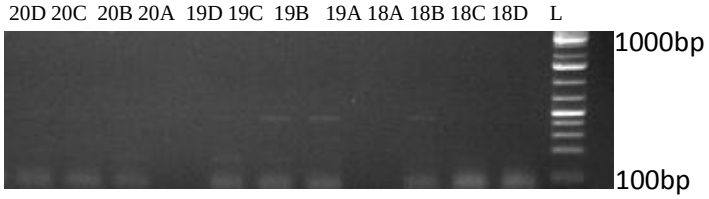


Plate 4.7: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 18B, 18C, 18D, 19B, 19C, 19D, 20B, 20C and 20D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 18- 20. Lanes 18A, 19A and 20A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

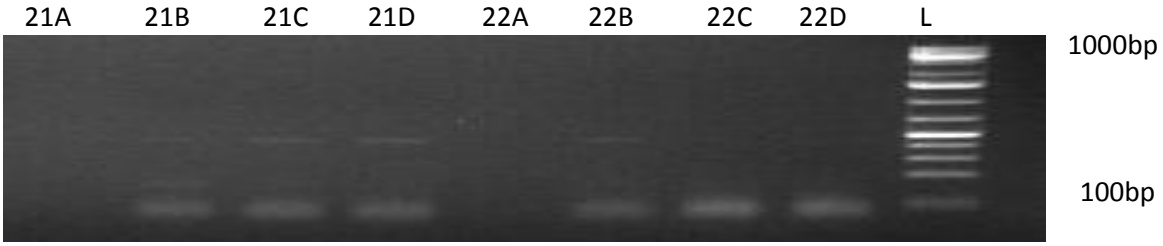


Plate 4.8: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 21B, 21C, 21D, 22B, 22C and 22D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 21- 22. Lanes 21A and 22A are negative bands from the pre stage of the exercise.

Note: 21C and 21D had heterozygous gene expression patterns.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

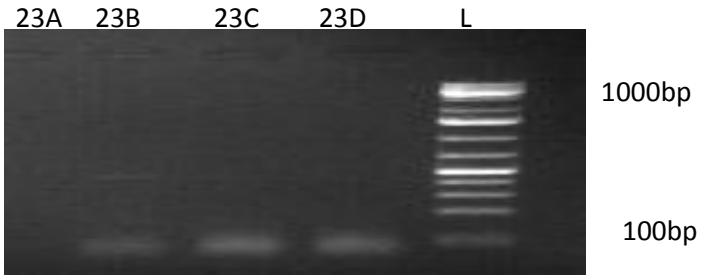


Plate 4.9: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 23B, 23C and 23D are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subject 23. Lanes 23A are negative bands from the pre stage of the exercise.NC is a no template control.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

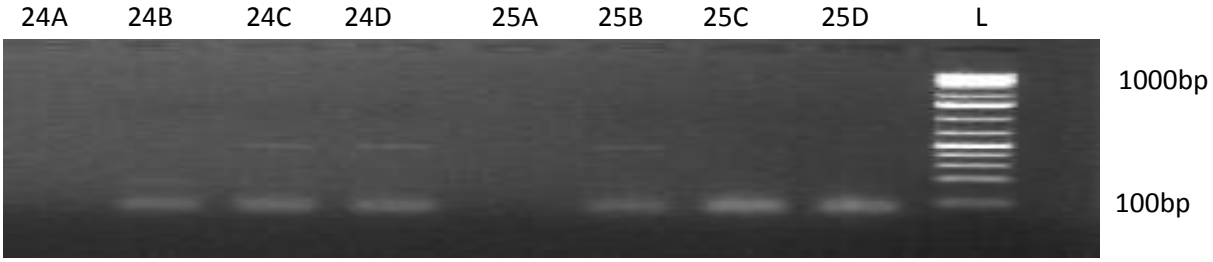


Plate 4.10: Reverse transcriptase PCR results for succinate dehydrogenase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 24B, 24C, 24D, 25B and 25C are positive bands for the expressed succinate dehydrogenase genes at 91bp from the exercised subjects 24- 25. Lanes 24A and 25A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

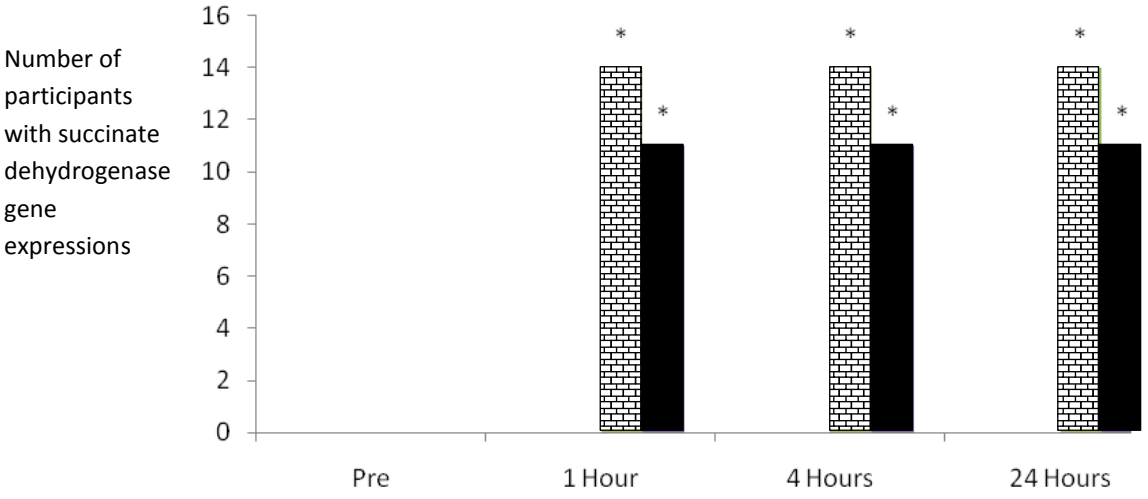


Figure 4.1: An overall multiple bar chart representation of the up-regulation of SDH gene detected in exercised subjects at different time intervals. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

Keys:

Pre = Gene not expressed in exercised participants

Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals

Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals

***= Significant P < 0.05**

4.12 Lymphocytic hypoxanthine phosphoribosyl transferase genes expression

Plates 4.11 - 4.19: The reverse transcriptase PCR results for lymphocytic hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

NOTE:

Subjects 1- 14 were exhausted before 21 minutes of the exercise bout

Subjects 15- 25 were exhausted at 21 minutes of the exercise bout

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

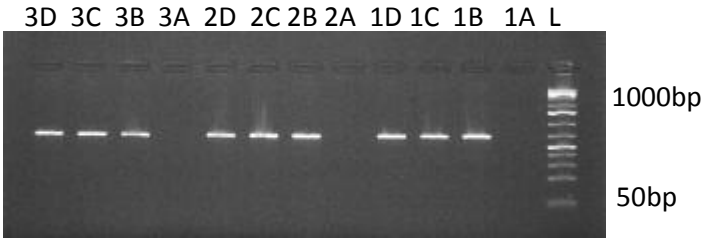


Plate 4.11: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 1B, 1C, 1D, 2B, 2C, 2D, 3B, 3C and 3D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 1- 3. Lanes 1A, 2A and 3A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = -1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

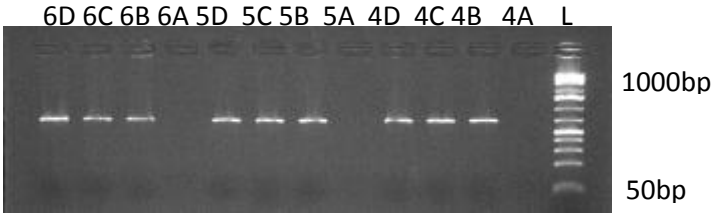


Plate 4.12: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 4B, 4C, 4D, 5B, 5C, 5D, 6B, 6C and 6D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 4 - 6. Lanes 4A, 5A and 6A are negative bands from the pre stage of the exercise.

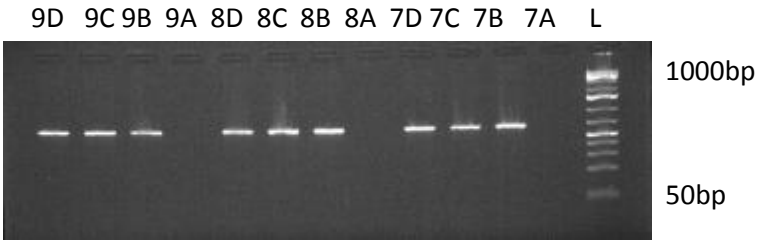
Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise



Plates 4.13: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 7B, 7C, 7D, 8B, 8C, 8D, 9B, 9C and 9D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 7- 9. Lanes 7A, 8A and 9A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

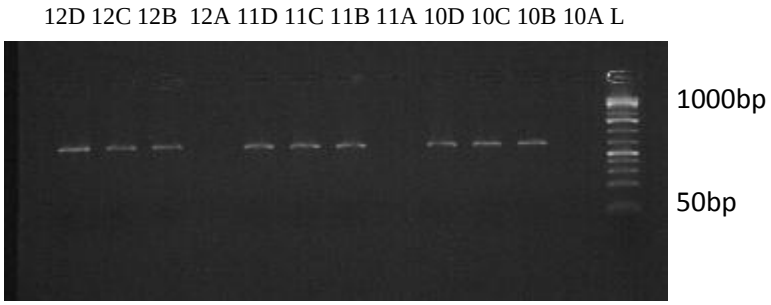


Plate 4.14: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 10B, 10C, 10D, 11B, 11C, 11D, 12B, 12C and 12D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 10 - 12. Lanes 10A, 11A and 12A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

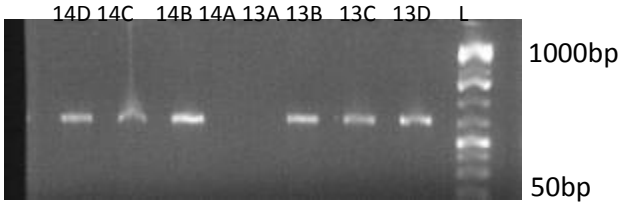


Plate 4.15: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 13B, 13C, 13D, 14B, 14C and 14D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 13- 14. Lanes 13A and 14A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

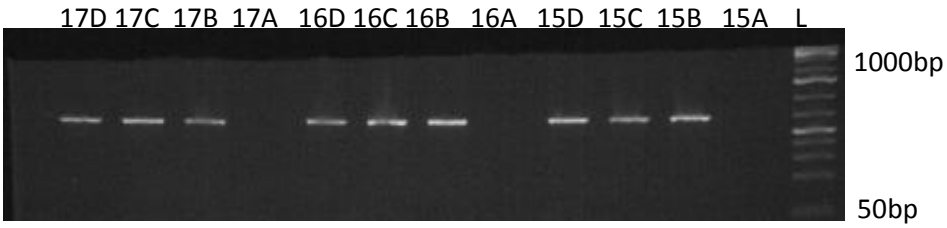


Plate 4.16: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 15B, 15C, 15D, 16B, 16C, 16D, 17B, 17C and 17D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 15 - 17. Lanes 15A, 16A and 17A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B =1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

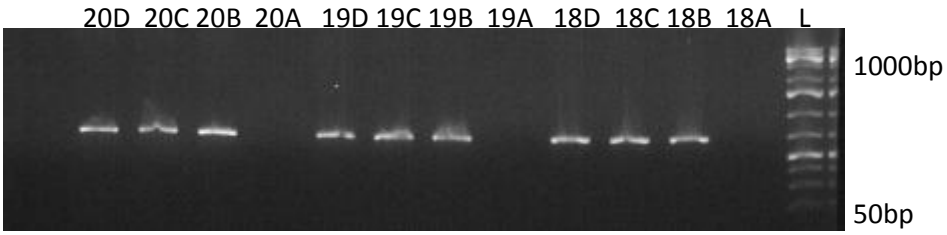


Plate 4.17: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 18B, 18C, 18D, 19B, 19C, 19D, 20B, 20C and 20D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 18- 20. Lanes 18A, 19A and 20A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

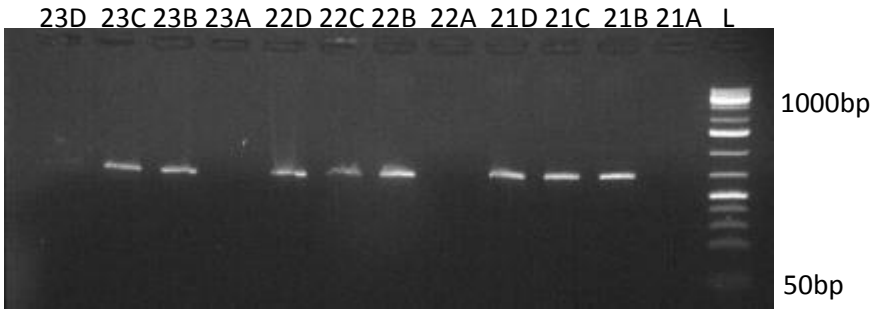


Plate 4.18: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 21B, 21C, 21D, 22B, 22C, 22D, 23B, 23C and 23D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 21- 23. Lanes 21A, 22A and 23A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

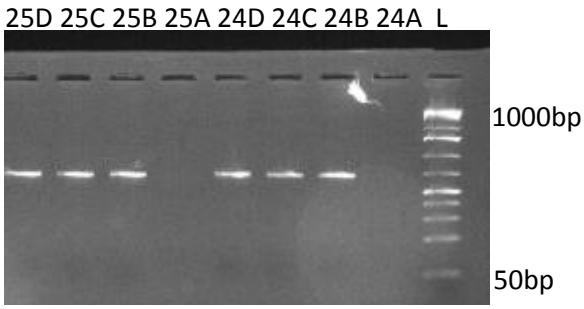


Plate 4.19: Reverse transcriptase PCR results for hypoxanthine phosphoribosyl transferase genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 24B, 24C, 24D, 25B, 25C and 25D are positive bands for the expressed hypoxanthine phosphoribosyl transferase genes at 275bp from the exercised subjects 24- 25. Lanes 24A and 25A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

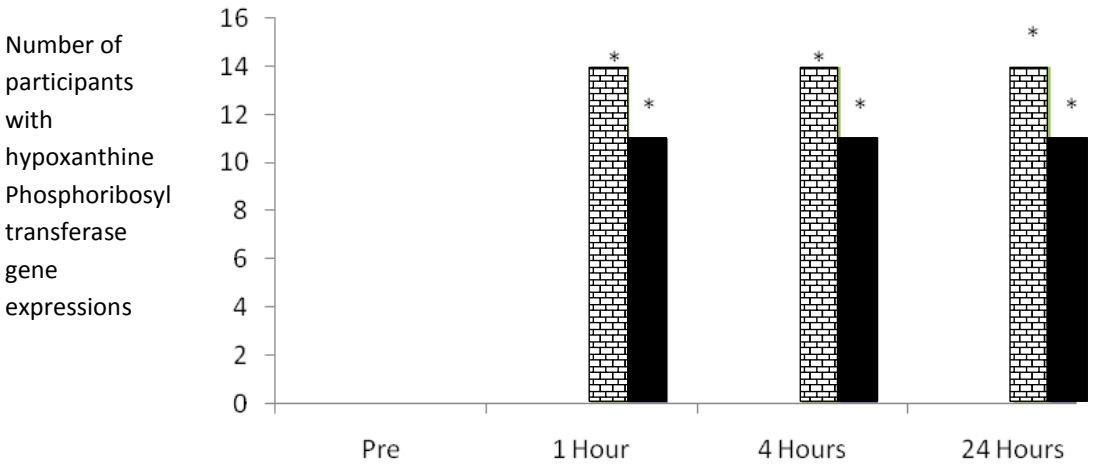


Figure 4.2: An overall multiple bar chart representation of the up-regulation of HPRT gene detected in exercised subjects at different time intervals. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

Keys:

Pre = Gene not expressed in exercised participants

Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals

Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals

***= Significant P < 0.05**

4.13 Lymphocytic interleukin 10 genes expression

Plates 4.20 - 4.29: The reverse transcriptase PCR results for lymphocytic interleukin 10 genes analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. The genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise (χ^2 = 50, P = 0.000) respectively.

NOTE:

Subjects 1- 14 were exhausted before 21 minutes of the exercise bout

Subjects 15- 25 were exhausted at 21 minutes of the exercise bout

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

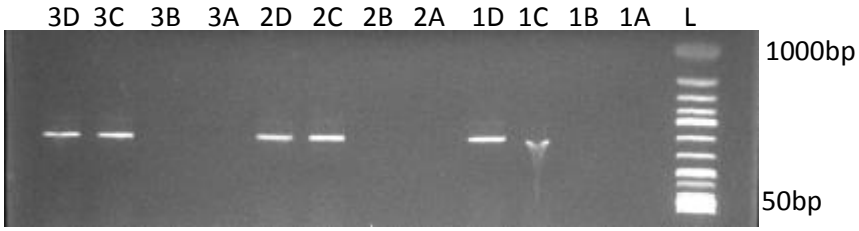


Plate 4.20: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 1C, 1D, 2C, 2D, 3C and 3D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 1 - 3. Lanes 1A, 1B, 2A, 2B, 3A and 3B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

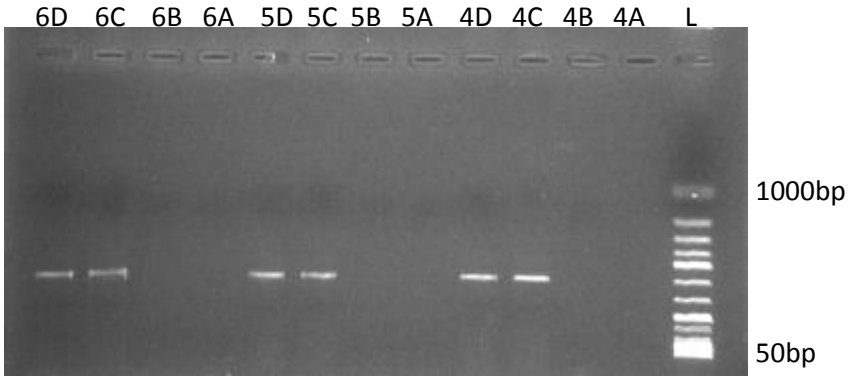


Plate 4.21: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 4C, 4D, 5C, 5D, 6C and 6D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 4 - 6. Lanes 4A, 4B, 5A, 5B, 6A and 6B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

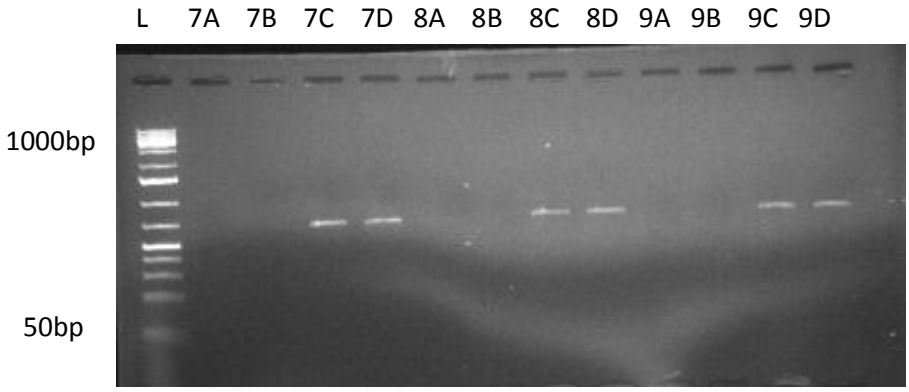


Plate 4.22: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 7C, 7D, 8C, 8D, 9C and 9D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 7 - 9. Lanes 7A, 7B, 8A, 8B, 9A and 9B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

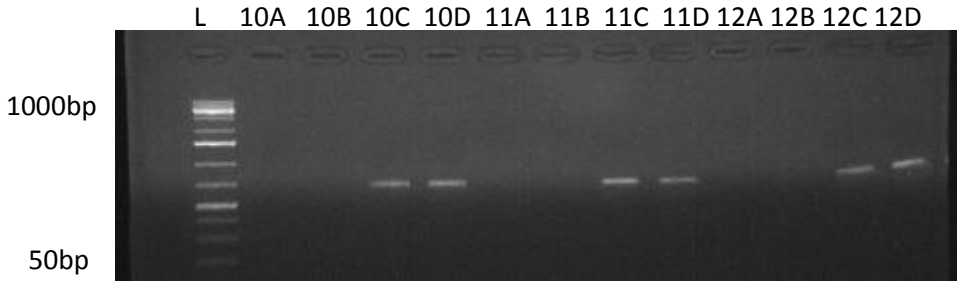


Plate 4.23: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 10C, 10D, 11C, 11D, 12C and 12D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 10 - 12. Lanes 10A, 10B, 11A, 11B, 12A and 12B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

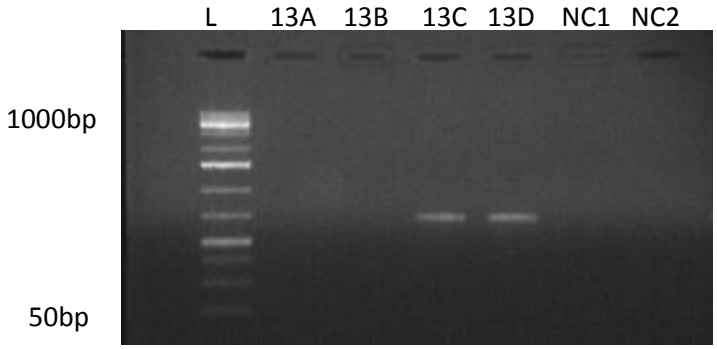


Plate 4.24: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 13C and 13D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subject 13. Lanes 13A and 13B are negative bands from the pre stage and 1 hour post exercise respectively while NC1 and NC2 are no template control.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

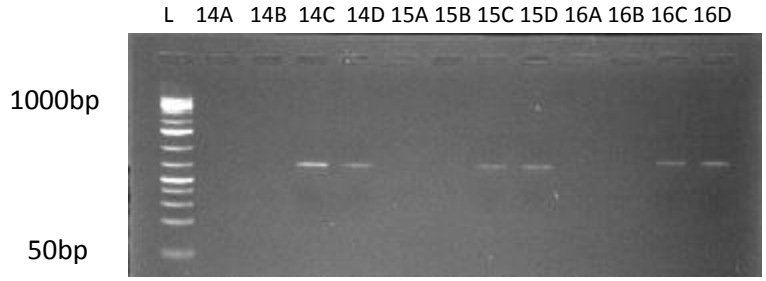


Plate 4.25: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 14C, 14D, 15C, 15D, 16C and 16D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 14 - 16. Lanes 14A, 14B, 15A, 15B, 16A and 16B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

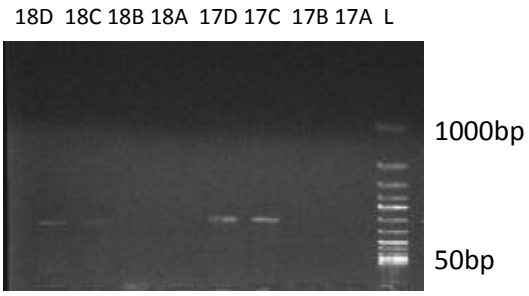


Plate 4.26: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 17C, 17D, 18C and 18D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 17 - 18. Lanes 17A, 17B, 18A and 18B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

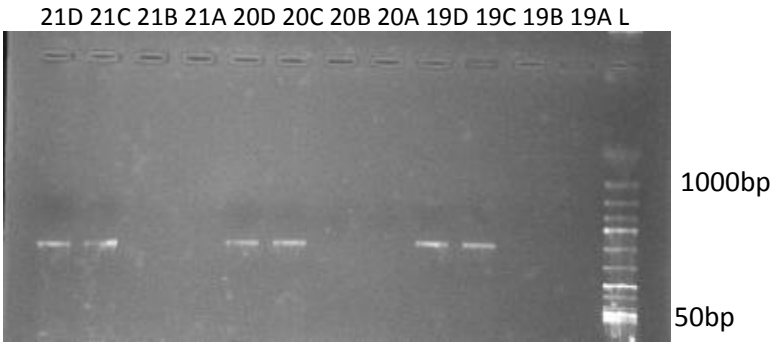


Plate 4.27: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 19C, 19D, 20C, 20D, 21C and 21D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 19 - 21. Lanes 19A, 19B, 20A, 20B, 21A and 21B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

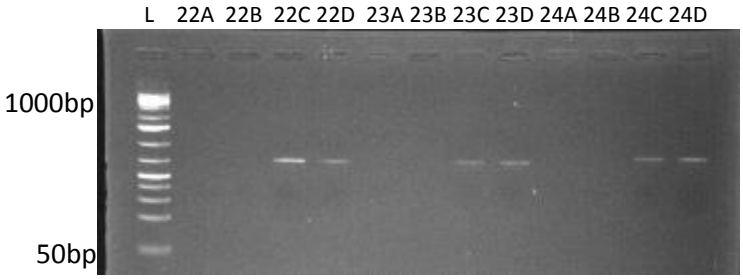


Plate 4.28: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 22C, 22D, 23C, 23D, 24C and 24D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subjects 22 - 24. Lanes 22A, 22B, 23A, 23B, 24A and 24B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

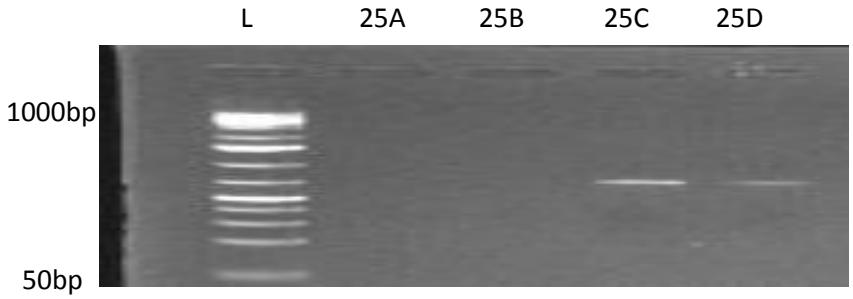


Plate 4.29: Reverse transcriptase PCR results for interleukin 10 genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 25C and 25D are positive bands for the expressed interleukin 10 genes at 250bp from the exercised subject 25. Lanes 24A and 25B are negative bands from the pre stage and 1 hour post exercise.

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

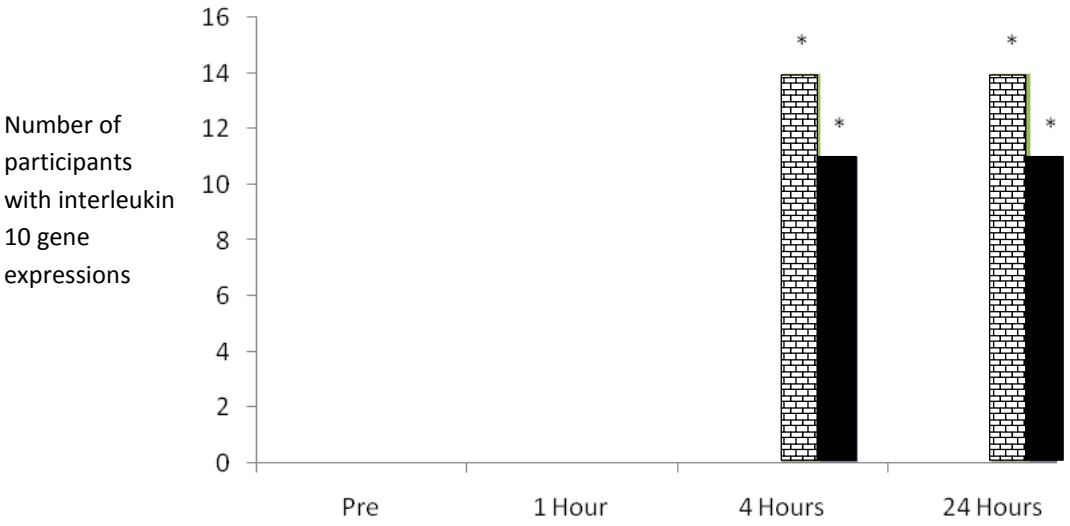


Figure 4.3: An overall multiple bar chart representation of the up-regulation of IL-10 gene detected in exercised subjects at different time intervals. The genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

Keys:

Pre = Gene not expressed in exercised participants

Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals

Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals

***= Significant P < 0.05**

4.14 Lymphocytic tumor necrosis factor alpha genes expression

Plates 4.30 - 4.39: The reverse transcriptase PCR results for lymphocytic tumor necrosis factor alpha genes analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

NOTE:

Subjects 1- 14 were exhausted before 21 minutes of the exercise bout

Subjects 15- 25 were exhausted at 21 minutes of the exercise bout

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

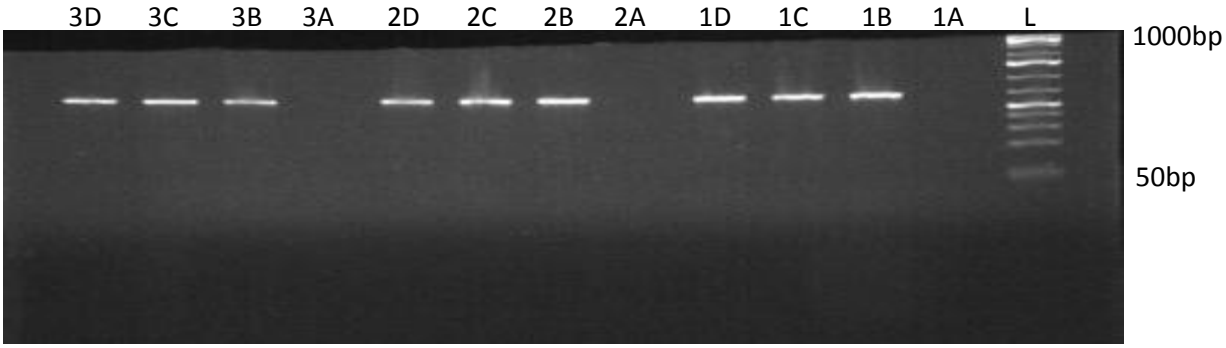


Plate 4.30: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 1B, 1C, 1D, 2B, 2C, 2D, 3B, 3C and 3D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 1 - 3. Lanes 1A, 2A and 3A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

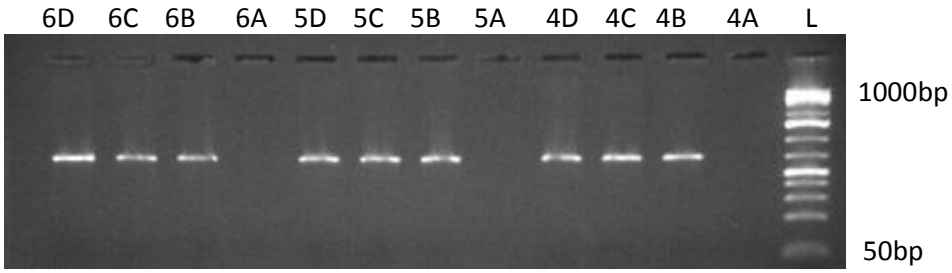


Plate 4.31: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 4B, 4C, 4D, 5B, 5C, 5D, 6B, 6C and 6D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 4 -6. Lanes 4A, 5A and 6A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

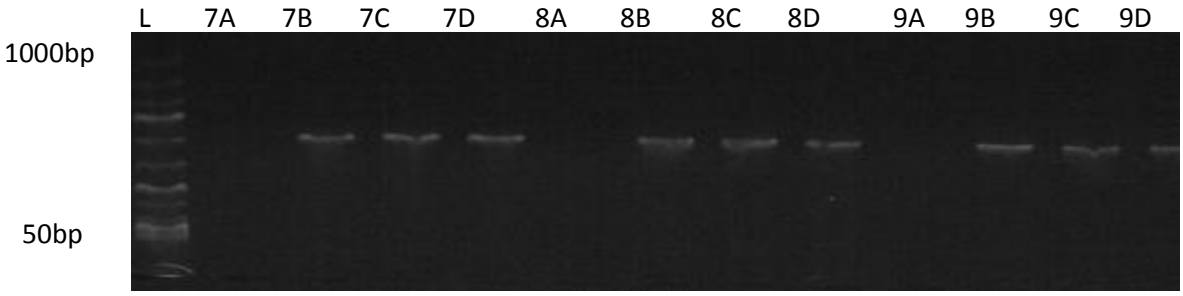


Plate 4.32: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 7B, 7C, 7D, 8B, 8C, 8D, 9B, 9C and 9D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 7 - 9. Lanes 7A, 8A and 9A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

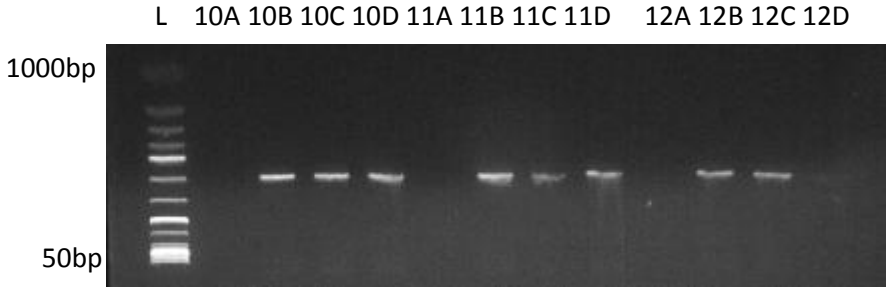


Plate 4.33: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lane 10B, 10C, 10D, 11B, 11C, 11D, 12B, 12C and 12D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 10 - 12. Lanes 10A, 11A and 12A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

Plate 4.34: Reverse transcriptase PCR results for lymphocytic tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 13B, 13C and 13D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subject 13. Lanes 13A is a negative band from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

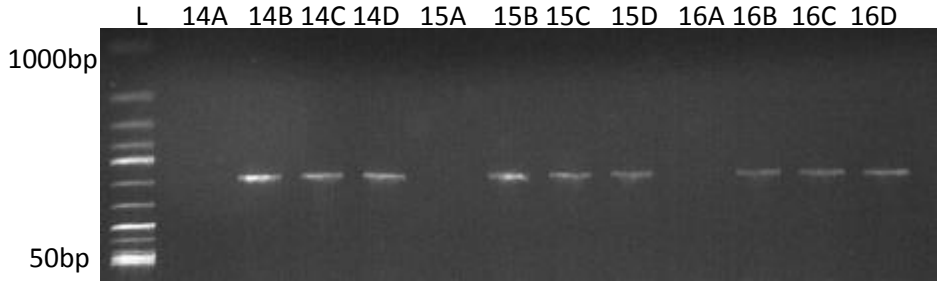


Plate 4.35: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 14B, 14C, 14D, 15B, 15C, 15D, 16B, 16C and 16D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 14 - 16. Lanes 14A, 15A and 16A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

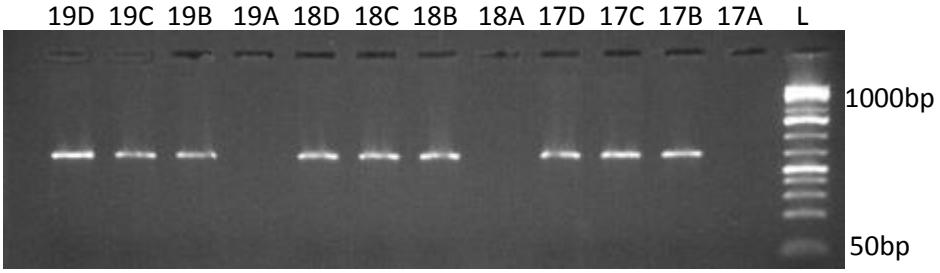


Plate 4.37: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 17B, 17C, 17D, 18B, 18C, 18D, 19B, 19C and 19D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 17 - 19. Lanes 17A, 18B and 19A are negative bands from the pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

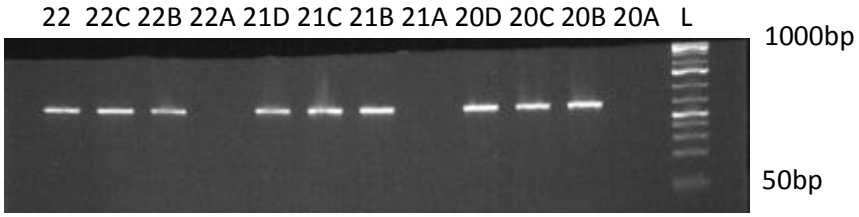


Plate 4.38: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 20B, 20C, 20D, 21B, 21C, 21D, 22B, 22C and 22D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 20 - 22. Lanes 20A, 21A and 22A are negative bands from subjects at pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

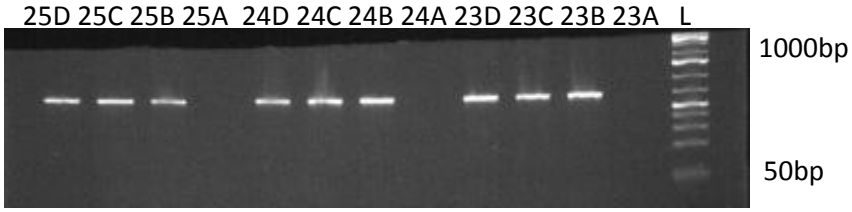


Plate 4.39: Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 23B, 23C, 23D, 24B, 24C, 24D, 25B and 25D are positive bands for the expressed tumor necrosis factor alpha genes at 300bp from the exercised subjects 23 - 25. Lanes 23A, 24A and 25A are negative bands from the subjects at pre stage of the exercise.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

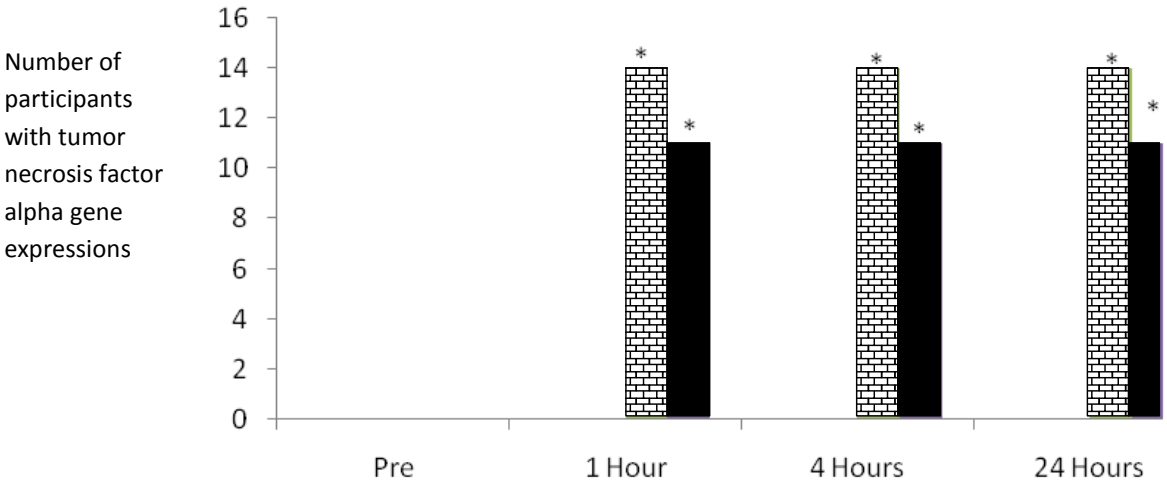


Figure 4.4: An overall multiple bar chart representation of the up-regulation of TNF α gene detected in exercised subjects at different time intervals. The genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, P = 0.000) respectively.

Keys:

Pre = Gene not expressed in exercised participants

Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals

Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals

***= Significant P < 0.05**

4.15 Lymphocytic interferon gamma genes expression

Plates 4.40 - 4.50: The reverse transcriptase PCR results for lymphocytic interferon gamma genes analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. The genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise in 20(80%) of the subjects when compared with the pre-exercise stage (χ^2 = 39, P = 0.000; χ^2 = 50, P = 0.000) while an up-regulation were also observed at 1 hour post exercise and sustained till 24 hours post exercise in 5(25%) of the subjects (χ^2 = 5.6, P = 0.000; χ^2 = 50, P = 0.000) respectively.

NOTE:

Subjects 1- 14 were exhausted before 21 minutes of the exercise bout

Subjects 15- 25 were exhausted at 21 minutes of the exercise bout

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

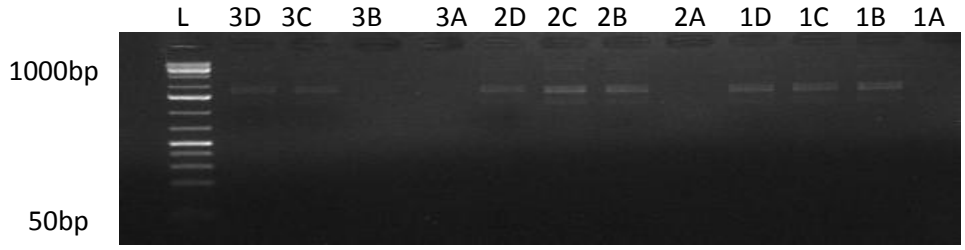


Plate 4.40: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 1B, 1C, 1D, 2B, 2C, 2D, 3C and 3D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 1 - 3. Lanes 1A, 2A, 3A and 3B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

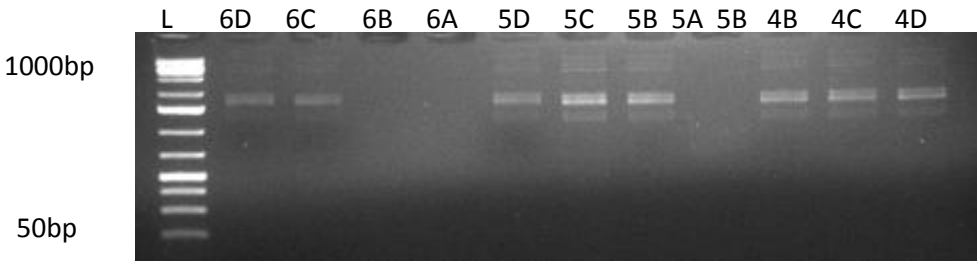


Plate 4.41: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 4B, 4C, 4D, 5B, 5C, 5D, 6C and 6D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 4 - 6. Lanes 4A,5A,6A and 6B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

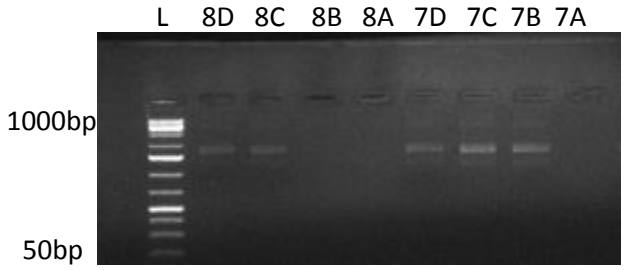


Plate 4.42: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 7B, 7C, 7D, 8C and 8D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 7 - 8. Lanes 7A, 8A and 8B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

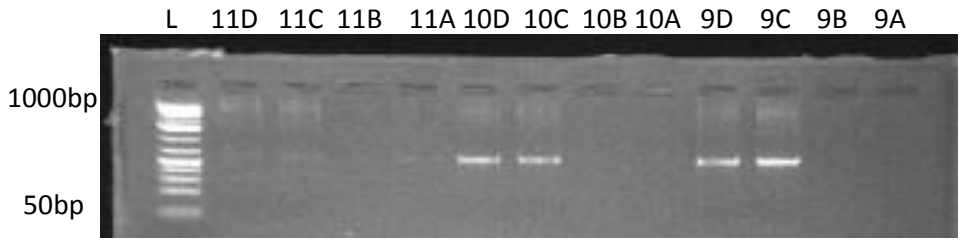


Plate 4.43: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 9C, 9D, 10C, 10D, 11C and 11D positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 9 - 11. Lanes 9A, 9B, 10A, 10B, 11A and 11B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

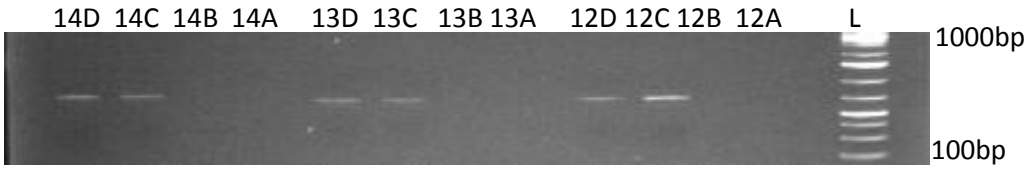


Plate 4.44: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 12C, 12D, 13D and 14D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 12 - 14. Lanes 12A, 12B, 13A, 13B, 13C, 14A, 14B and 14C are negative bands from the pre stage, 1 hour and 4 hours post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

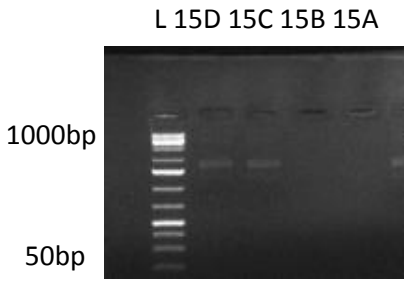


Plate 4.45: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lane 15D is positive band for the expressed interferon gamma genes at 375bp from the exercised subject 15. Lanes 15A, 15B and 15C are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

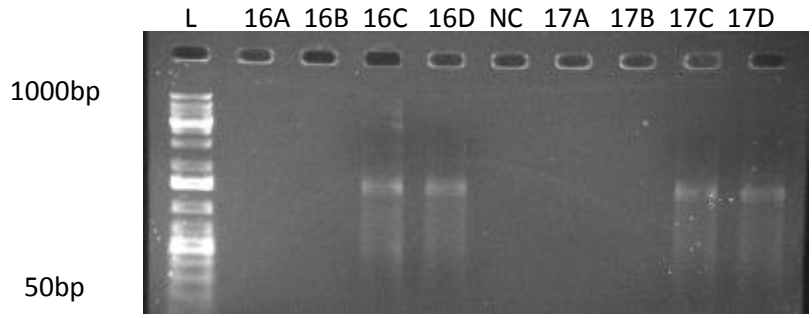


Plate 4.46: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 16C, 16D, 17C and 17D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 16 - 17. Lanes 16A, 16A, 17A and 17B are negative bands from the pre stage and 1 hour post exercise respectively.NC is a no template control.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

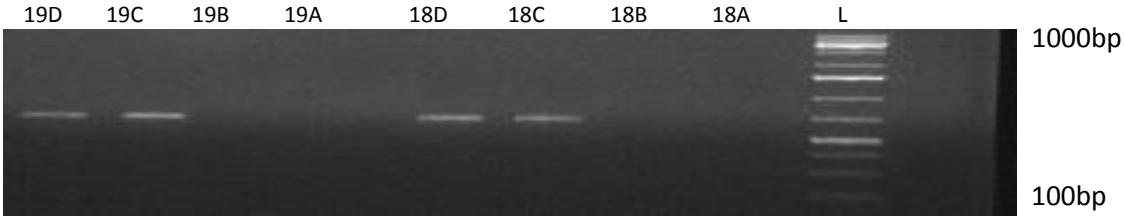


Plate 4.47: Reverse transcriptase PCR results for interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 18D, 19C and 19D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 18 - 19. Lanes 18A, 18B, 19A and 19C are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

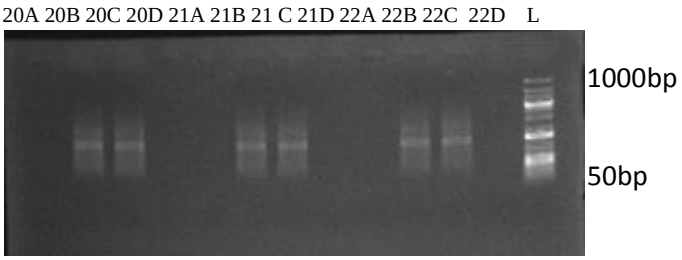


Plate 4.48: Reverse transcriptase PCR results for lymphocyte interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 20C, 20D, 21C, 21D, 22C and 22D are positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 20 - 22. Lanes 20A, 20B, 21A, 21B, 22A and 22B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

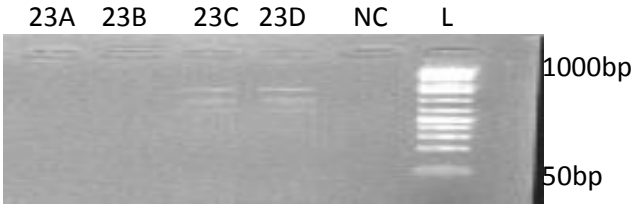


Plate 4.49: Reverse transcriptase PCR results for lymphocyte interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 23C and 23D positive bands for the expressed interferon gamma genes at 375bp from the exercised subject 23. Lanes 23A, 23B are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

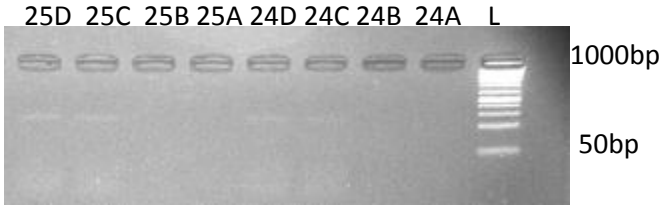


Plate 4.50: Reverse transcriptase PCR results for lymphocyte interferon gamma genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 50bp-1000bp DNA ladder (molecular marker). Lanes 24C, 24D, 25C and 25D positive bands for the expressed interferon gamma genes at 375bp from the exercised subjects 24 - 25. Lanes 24A, 24B, 25A and 25b are negative bands from the pre stage and 1 hour post exercise respectively.

Keys:

A = pre-exercise

B = 1-hour post exercise

C = 4-hours post exercise

D = 24-hours post exercise

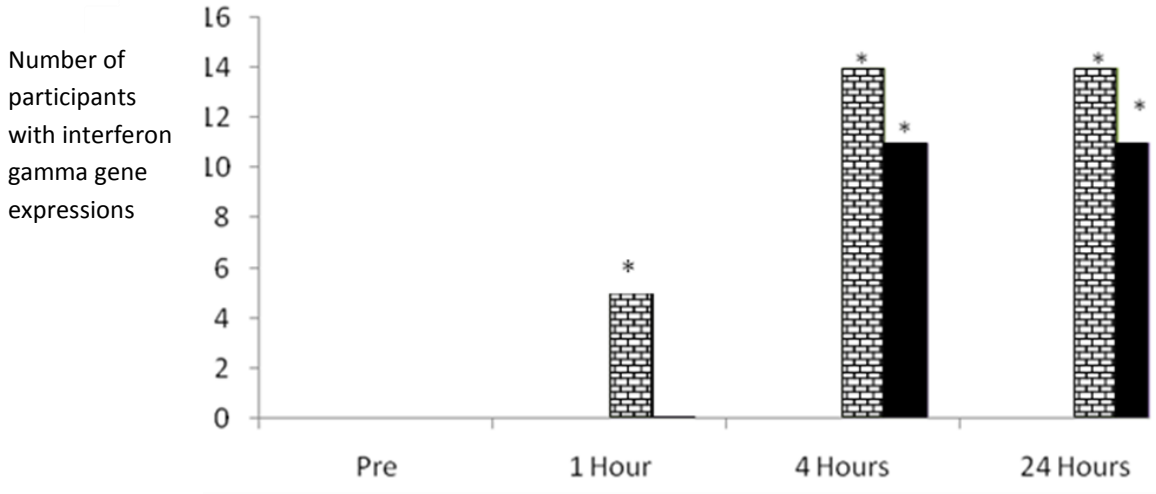


Figure 4.5: An overall multiple bar chart representation of the up-regulation of IFN ϒ gene detected in exercised subjects at different time intervals. The genes were up-regulated at 4 hour post exercise and sustained till 24 hours post exercise in 20 (80%) of the subjects when compared with the pre-exercise stage (χ^2 = 39, P = 0.000; χ^2 = 50, P = 0.000) while an up-regulation were observed at 1 hour post exercise and sustained till 24 hours post exercise in 5 (25%) of the subjects (χ^2 = 5.6, P = 0.000; χ^2 = 50, P = 0.000) respectively.

Keys:
Pre = Gene not expressed in exercised participants
Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals
Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals
***= Significant P < 0.05**

CHAPTER FIVE

5.1 DISCUSSION

This study used some lymphocytic genes to confirm evidence of stress in some young male undergraduate students that passed through exhaustive exercise. The subjects took part in an endurance race using the Bruce treadmill protocol for sub maximal exercise. The target heart rate on the treadmill was 60-80 percent of the heart rate reserve. In response to stressors some genes are known to be either up-regulated while others are down-regulated. The duration of expression of such genes may be an indication of possible role in mediating response to stress. Some group of lymphocyte genes have been shown to be either up-regulated or down regulated in different physiologic or pathologic conditions (Bermudez *et al*., 2011).

In the present study lymphocyte succinate dehydrogenase genes, lymphocyte hypoxanthine-guanine phosphoribosyl transferase genes and lymphocyte tumor necrosis factor alpha genes were up-regulated for 24 hours post exhaustive exercise. Other studies have shown that lymphocyte genes including succinate dehydrogenase genes and hypoxanthine-guanine phosphoribosyl transferase genes were up-regulated within 1 to 4 hours after stimulation with a stressor, their expression patterns were transient with maximal mRNA accumulation at about 8 hours, before returning to their baseline level after 72 hours of withdrawal of the stressor (Abdalla *et al*., 2003; Katia *et al*., 2008; Bermudez *et al*., 2011; Josef, 2012). However, in the present study it was evident that these genes were still up-regulated by 24 hours post exhaustive exercise suggesting that decline in up-regulation may not commence after 24 hours of removal of the stressor.

Tumor necrosis factor alpha is known to mediate immune responses via the up-regulation of transcription genes such as nuclear factor kappa B genes (Pavlov *et al*., 2003; Flaster *et al*., 2007; Rastogi, 2008). Tumor necrosis alpha is also a potent activator of the hypothalamus-pituitary-adrenal axis by stimulating the release of corticotropin releasing hormone from the hypothalamus during stress responses (Tsigos and Chrousos, 2002). Thus, it is possible that the expression of tumor necrosis factor alpha genes for 24 hours post exhaustive exercise enhances the synthesis of multiple cytokine genes which modulate the immune responses to stress. Denguezli *et al*., (2006); Flaster *et al*., 2007 and Xiao *et al*., (2012) in their studies reported that post exercise stress induces the release of tumor necrosis factor alpha genes which enhances the production of multiple cytokines during exhaustive exercise. Also, Golzari *et al*., (2010), reported that moderate exercise induces the up-regulation of tumor necrosis factor alpha, interferon gamma and interleukin- 12 genes expression. Tumor necrosis factor alpha genes have been suggested to mediate immune response to stress during exhaustive exercise (Pedersen *et al.*, 2001; Robson-Ansley *et al*., 2007; Ramson *et al.,* 2008).

Succinate dehydrogenase genes control the generation of high energy electron in the Kreb cycle to create the proton gradient for the generation of at least 95% of adenosine triphosphate in most cells including lymphocytic cells (Jeremy *et al.,* 2001) while hypoxanthine-guanine phosphoribosyl transferase genes play a central role in the Salvage pathway by recycling purine nucleotides, such as adenosine triphosphate which provide energy required by all living cells including lymphocytes to maintain mitochondrial membrane potential, cell viability and immune cell functions (Finette *et al.,* 2002; Evanna and Luke, 2013). The 24 hours post exhaustive exercise expression of succinate dehydrogenase and hypoxanthine-guanine phosphoribosyl transferase genes might be an indication

of an enhanced adenosine triphosphate generation within these study periods. It has been reported that succinate dehydrogenase and hypoxanthine-guanine phosphoribosyl transferase genes play important roles in the generation of adenosine triphosphate which provide energy needed by the lymphocytic cells to maintain cellular homeostasis and immune cell functions (Jeremy *et al.,* 2001; Evanna and Luke, 2013). It has also been observed that the up-regulation of the lymphocytic hypoxanthine-guanine phosphoribosyl transferase genes by exhaustive exercise is strongly associated with an improved potential for recycling purine nucleotides via the salvage pathway (Zielinski *et al*., 2013).

In the present study, lymphocyte interferon gamma genes were not expressed before 4 hours post exercise in about 75% of the participating students while about 25% of the participants had the gene expressed as from 1 hour post exercise. However, the lymphocyte interferon gamma genes were expressed in all the participating students as from 4 hours post exercise and the expressions were sustained in all the participants for 24 hours post exercise. Similarly, the interleukin 10 genes were expressed as from 4 hours post exercise and sustained for 24 hours post exercise. The delayed expression of the lymphocyte interferon gamma genes in about ¾ of the studied population calls for concern and might be an indication of individual susceptibility or resistance to stress. It further indicates that there may be other regulatory factors that may be protecting the expression of these genes. Intense studies are required in this direction to unravel the reason behind these bivalent observations at early state of post induction of stress.

Interleukin 10 is known to play a significant role in immune regulation involving both T helper 2 (Th2) and T helper 1 (Th1) responses (Shankar and Genhong, 2012). The expression of the interleukin 10 genes for 24 hours post exhaustive exercise might be an indication that there is an

effective control of the immune responses to stress within these periods. Bente, (2006) and Rodrigo *et al.,* (2012) reported that post exercise stress enhances the up-regulation of interleukin-10 genes which in turn inhibits the production of IL-1, TNF-α and IFN-γ in attempt to regain cellular homeostasis during muscle contractions. Stenberg *et al*., (2000) opined that humans respond to post exercise stress by activating cytokine producing cells to induce the production of cytokine genes such as tumor necrosis factor alpha, interferon gamma; it simultaneously releases interleukin-10 to effectively control immune response to stress. However, other work demonstrated that interleukin-10 levels are elevated during strenuous exercise (Ostrowski *et al*., 2000). Although Peake *et al*., (2005) reported that exercise induced muscle fatigue can up-regulate interleukin-10 genes. It has been observed that 30 minutes' walk increases the level of interleukin-10 genes in young male students (Nieman *et al*., 2005).

Interferon-gamma is known to induce the release of interleukin-1 from monocytes to amplify immune responses (Kate *et al*., 2004 and Rastogi, 2008). Thus, the expression of the interferon gamma genes for 24 hours post exhaustive exercise might be an indication of enhanced immune responses to stress within these study periods. Ambarish *et al*., (2014) observed that post exercise elevation of interferon gamma acts as a positive feedback mechanism on the leukocytes by enhancing their ability to produce additional cytokines such as tumor necrosis factor alpha, and interleukin-10 during muscle contractions. Liburt *et al*., (2010) opined that the immunological responses to post exercise stress are characterized by a significant increase in the expression of interferon-gamma, tumor necrosis factor alpha, and interleukin-10 genes within the blood and contracting muscle cells.

The possible mechanism of the up-regulation of the lymphocytic gene expression patterns observed in this study could be linked to adenosine triphosphate depletion, accumulation of adenosine diphosphate, and adenosine monophosphate due to consumption of adenosine triphosphate by the exercising muscles (Brandt and Pedersen, 2010). It has been reported that the post exercise immune responses to stress are induced by local accumulation of energy regulatory molecules such as adenosine monophosphate, adenosine diphosphate, calcium ions and free radicals in the contracting muscle cells (Vamshi, 2012).

The blood glucose level was significantly lower at 1 hour and 4 hours post exhaustive exercise when compared with the pre-exercise stage. This is indicative of enhanced blood glucose utilization for the generation of adenosine triphosphate which is needed for muscle contractions. Studies have reported that exhaustive exercise enhances the rate of blood glucose delivery and utilization for the generation of adenosine triphosphate which are needed for muscle contractions (Adam *et al*., 2005, Mery and McConell, 2009). Also, it has been reported that post exercises stress enhances blood glucose up-take into skeletal muscles which are needed for adenosine triphosphate generation during muscle contractions (Kristin and Laurine, 2014; Dos-Santos *et al*., 2015). Blood glucose levels were measured to rule out diabetes mellitus and hypoglycemia among the exercised subjects. However, the absence of hypoglycemia pre- and post-exercise stages establishes the fact that the 1 hour and 4 hours post exercise blood glucose reduction are due to stress associated with the exhaustive exercise bout. However, there was no significant difference with the 24 hours post exercise blood glucose level when compared with the pre-exercise stage. This is suggestive of a restored blood glucose homeostasis during the recovery period of the exhaustive exercise bout.

This study also observed that the cortisol level of the exercised subjects was significantly higher at 1 hour post exhaustive exercise stage (approximately 9:30 am) when compared with pre-exercise stage (approximately 8: 30 am). Under stress, hypothalamus is stimulated to release corticotrophin releasing hormone. The hormone is transported to its target, the anterior pituitary gland, via the hypophyseal portal system to which it binds and causes the anterior pituitary gland to secrete adrenocorticotrophic hormone which in turn stimulate the adrenal cortex to release cortisol (Richard *et al*., 2006). These results support the fact that stress of any origin, whether physical or mental can greatly enhance the secretion of adrenocorticotrophic hormone and consequently cortisol. It has been reported that elevated cortisol inhibits the action of insulin thereby encouraging higher blood glucose level via gluconeogenesis and glycogenolysis (Carl and Edward, 2006; Raddatz and Ramadori, 2007). Thus, it is possible that the 1 hour post exhaustive exercise cortisol elevation observed in this study inhibits the action of insulin thereby enhancing the production of blood glucose via gluconeogenesis and glycogenolysis in an attempt to regain glucose homeostasis, hence the 4 hours post exercise blood glucose level elevation. Tortora *et al*., (2006) observed that post exercise cortisol elevation enhances the production of blood glucose from non-carbohydrate substrates such as amino acids and glycerol from triglyceride breakdown. This is known as gluconeogenesis.

Sang-Hoon *et al*., (2007) reported that post exercise stress increases blood glucose utilization by enhancing both glycogenolysis and gluconeogenesis in the cell of the liver and skeletal muscles. It has been reported that glycogenolysis and gluconeogenesis enhances the release of glucose into the bloodstream during physical activity thereby ensuring that blood glucose levels do not drop below the fasting blood glucose level (Fox, 2009). Also, Holloszy, (2003) reported that gluconeogenesis

accounts for approximately 20% of blood glucose production during low to moderate intensity exercise in human subjects. Furthermore, post exercise cortisol elevation has been reported to stimulate the breakdown of triglycerides in adipocytes thereby enhancing the release of free fatty acid which are needed for the generation of adenosine triphosphate via the beta-oxidative pathway for muscle contractions (Brooks *et al*., 2005). However, there was a significant decrease of cortisol level at 4 hour post exhaustive exercise stage (approximately 1: 30 pm) and 24 hours post exercise stage (approximately 8: 30 am) when compared with the pre exercise stage (approximately 8: 30 am). The significant decrease in cortisol level could be attributed to a restored homeostasis as the subjects feel relieved of the stress induced by the exhaustive exercise bout.

It has been reported that adenosine triphosphate generated from anaerobic metabolism (phosphocreatine, stored adenosine triphosphate and anaerobic glycolysis) and aerobic metabolism modulates the sliding of actin along the length of myosin to achieve muscle contraction (Kenney *et al*., 2012). The essential role of anaerobic glycolysis during muscle contraction is manifested in the elevation of blood lactate level from 1.6 to 8.3 mM, the release of H^+ from the intensely active muscle concomitantly lower the blood pH from 7.42 to 7.24 (Jeremy *et al.*, 2001). Enhanced muscle contractions cannot be sustained with anaerobic metabolism for more than 1.8 minutes for two reasons. First, creatine phosphate and stored adenosine triphosphate are consumed within 10 seconds. Second, the lactate produced would cause acidosis (Kenney *et al*., 2012).

Moreover, aerobic metabolism is the major source for adenosine triphosphate production from blood glucose, glycogen and triglycerides during exercise that last longer than 2 to 3 minutes. Unlike anaerobic adenosine triphosphate production, the aerobic metabolism system is slow to turn on; but it has a much larger adenosine triphosphate producing capacity, this places considerable

demands on the cardiovascular and respiratory system to deliver oxygen to the active muscles (Kenney *et al*., 2012). These changes are essential for producing adenosine triphosphate required for supporting muscle contractions. Goett *et al*., (2011) and Marianne *et al*., (2012), reported in their studies that an increased mechanical stress on the contracting muscles during exhaustive exercise can significantly elevate the level of troponin inhibitory and creatine kinase-3. Thus, it is probable that the 1 hour post exercise elevations of troponin inhibitory and creatine kinase-3 are indicative of an increased mechanical stress on the contracting muscle during the exhaustive exercise bout. However, there were no significant differences observed at 4 hours and 24 hours post exercise elevation of troponin inhibitory and creatine kinase-3 when compared with the pre-exercise stage. This is suggestive of a restored homeostasis as the subjects feel relieved of the mechanical stress induced by the exhaustive exercise bout.

The present study also sub-categorized the participants into those that concluded the mandatory 21 minutes exhaustive exercise and those that were exhausted before the 21 minutes. It was interesting to note that the eleven (11) participants that concluded the mandatory 21 minutes exhaustive exercise had their lymphocytic genes up-regulated at 1 hour post exercise and sustained till 24 hours post exercise. These genes are succinate dehydrogenase, hypoxanthine guanine phosphoribosyl transferase and tumor necrosis factor α genes. Moreover, Interleukin-10 and interferon gamma genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise.

However, fourteen (14) of the subjects were exhausted before the 21 minutes of the exhaustive exercise bout. Their lymphocytic genes were up-regulated at 1 hour post exercise and sustained till 24 hours post exercise. The lymphocytic genes were succinate dehydrogenase, hypoxanthine

guanine phosphoribosyl transferase and tumor necrosis factor α genes. Interleukin-10 genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise, while interferon gamma genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise except for about 25% of the participating subjects who had an up-regulation at 1 hour post exercise and sustained till 24 hours post exercise. The up-regulatory patterns of these lymphocytic genes are of diagnostic importance in establishing evidences of stress as the immune responses to stress are characterized by leukocytes infiltration and production of multiple cytokines genes within the blood and contracting muscle cells (Josef, 2012). Thus, these lymphocytic gene expression patterns might be predicting the patterns of stress of individuals exposed to stressors. In terms of directions for future research, these ideas seem worthy of further investigation.

The serum progesterone level in this study was significantly lower at 1 hour post exhaustive exercise when compared with the pre exercise stage. This observed decrease is an indication that progesterone production is inhibited when the body is faced with stress associated with exhaustive exercise. Nelson, (2011), in his research reported an inhibitory effect of elevated cortisol secretion on the level of progesterone post exercise. The secretion of progesterone is inhibited during exhaustive exercise bout to conserve energy for the imminent fight or flight response (Mastorakos *et al.,* 2005; Maryam *et al*., 2014). Also, Onyenekwe *et al*., (2014), reported that progesterone level was found to be inversely proportional to serum cortisol level in examination stress which indicates that progesterone production is inhibited when the body is faced with stress. Furthermore, there was no significant difference at 4 hour post exercise stage and 24 hours post exhaustive exercise stage when compared with the pre exercise stage. The possible reason could be attributed to a restored homeostasis during the recovery period of the exhaustive exercise bout.

In this study, the serum luteinizing hormone level and follicle stimulating hormone were significantly lower at 1 hour post exercise when compared with the pre exercise stage. The observed decrease is suggestive that luteinizing hormone level and follicle stimulating hormone production are inhibited when the body is faced with stress associated with exhaustive exercise. Kamel *et al*., (2001) and Warren and Perlroth, (2001), in their research reported an inhibitory effect of elevated cortisol secretion on serum luteinizing hormone level and follicle stimulating hormone post exercise. The post exercise-induced reduction of luteinizing hormone level and follicle stimulating hormone are stimulated by the inhibitory effect of the elevated cortisol during exhaustive exercise (Vaamonde *et al.,* 2005). However, there was no significant difference at 4 hour post exercise stage and 24 hours post exercise stage when compared with the pre exercise stage. This is suggestive of a restored homeostasis.

The findings with absolute lymphocytes and neutrophils counts showed that they were significantly higher at 1 hour and 4 hours post exhaustive exercises when compared with pre-exercise. This is suggestive that post exercise stress enhances lymphocyte and neutrophil cells recruitment from tissue pools such as spleen, lymph nodes and the gastrointestinal tract, thus, suggesting a reduced risk of infection for those who engages in regular exercise. Ortega, (2003) in his study observed that post exercise stress enhances white blood cells phagocytic capacity. Post exercise stress is associated with increased leukocytes migration from the lymphoid organs to the circulating blood (Herbert *et al*., 2004). It has been reported that elevated cortisol level during exhaustive exercise enhances white blood cells recruitment capacity (Clinton *et al*., 2010). However, there was no significant difference at 24 hours post exhaustive exercise stage when compared with the pre exercise stage. This is indicative of a restored homeostasis during the recovery period of the exhaustive exercise bout.

The findings of this present study clearly confirmed that lymphocytic succinate dehydrogenase, hypoxanthine guanine phosphoribosyl transferase and tumor necrosis factor α genes had early and stable expression patterns within the 24 hours of investigation when compared with the standard stress biomarkers such as cortisol, progesterone and glucose. Thus, indicating that these lymphocytic genes are better markers to establish early and stable evidence of stress in the exercised subjects.

Post-exercise stress was associated with up-regulation of energy regulatory lymphocytic genes such as succinate dehydrogenase and hypoxanthine guanine phosphoribosyl transferase coupled with an enhanced lymphocytic cell functions which are evidence in the up-regulation patterns of both lymphocytic pro-inflammatory cytokine genes (tumor necrosis factor α and interferon gamma genes) and anti-inflammatory cytokine gene (interleukin-10 gene). In light of the fact that sub-maximal exercise using the Bruce protocol did not impair the lymphocytic cell functions in vivo, this model may be an ideal form of exercise to improve health and performance without overstressing the lymphocytic cell functions.

5.2 Conclusion

These findings demonstrated that some lymphocytic genes such as succinate dehydrogenase, hypoxanthine guanine phosphoribosyl transferase and tumor necrosis factor α genes had early and stable gene expression patterns that lasted for 24-hours suggesting that these lymphocytic genes can be used to confirm early and stable evidence of stress.

Post exercise stress activates the release of cortisol, and interleukin-10 genes to reinstate homeostasis through modulation of the immune response. Furthermore, post exercise stress

inhibits the production of luteinizing hormone, follicle stimulating hormone and progesterone level of the exercised subjects.

5.3 Contribution to knowledge

Lymphocytic gene expressions such succinate dehydrogenase, hypoxanthine guanine phosphoribosyl transferase and tumor necrosis factor α can be used as reference genes to confirm early and stable evidence of stress.

Acute exercise using the Bruce treadmill protocol did not impair the lymphocytic cell functions; this model may be an ideal form of exercise to improve health and performance.

Limitations

The cost implication of lymphocytic genes expression study limited the research to a 24 hours post exercise study period.

5.4 Recommendation for further studies

Grant should be given to fund similar research using quantitative polymerase chain reaction for improved lymphocytic gene expression patterns. Also, the grant will create opportunity to expand the study to include other parameters viz-a-viz adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, lactic acid and free-radicals which are the possible stimulator of lymphocytic genes expression in acute exercise.

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APPENDIX A

INFORMED CONSENT FORM

Dear Participant,

My name is **EHIAGHE FRIDAY ALFRED.** I am a postgraduate student of the department of Medical Laboratory Science (Immunology), Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus.

You are invited to participate in a research project entitled: "Molecular detection of lymphocytes genes associated with exercise-induced stress in male undergraduate students" purpose of this survey is to contribute to knowledge by providing empirical evidence on stress as it relates to human activity.

There are no identified risks from participating in this research. Participation in this research is completely voluntary and you may refuse to participate without any consequence. The questionnaire and all its information will be treated strictly confidential. Please understand that your acceptance to participate in this study is absolutely priceless. You can contact me by email or phone number of fredleo2547@yahoo.com or 08063327432.Thank you for your consideration.

Your signature below indicates that has read the above information and agrees to participate in this study.

_____ _____

Signature **Date**

APPENDIX B

QUESTIONNAIRE (BIODATA)

(Please tick or fill appropriately)

SECTION A

1. Identification Number: _____
2. Phone Number:_____
3. Age Range: 18-20 [ ] 21-23 [ ] 24-26 [ ] 27-30 [ ] 31-35 [ ]
4. Status: Single [ ] Married [ ] Divorced [ ]
5. Occupation:_____
6. Tribe:_____
7. Level of Education: SSCE [ ] B.Sc [ ] M.Sc [ ] Ph.D [ ] others

   _____

SECTION B

8. Size of the bicep muscle _____
9. Size of the thigh muscle _____
10. Height _____
11. Weight _____
12. Blood pressure BE ☐ 1hr AE ☐ 4hrs AE ☐ 24hrs AE ☐
13. Body temperature BE ☐ 1hr AE ☐ 4hrs AE ☐ 24hrs AE ☐

14. Pulse rate BE 1hr AE 4hrs AE 24hrs AE

15. Total distance covered _____

16. Time of exhaustion _____

17. Signs of stress

☐ Tension

☐ Headache

☐ Muscle aches

☐ Change of mode

☐ Exhaustion

Others _____

Keys; BE (before exercise), AE (immediately after exercise), 1hrs AE (1 hour after exercise), 4hrs (4 hours after exercise)

APPENDIX C

FACULTY OF HEALTH SCIENCES AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS
P.M.B. 5001 NNEWI ANAMBRA STATE, NIGERIA

OFFICE OF THE DEAN

Our Ref: _____ *Your Ref:* _____ *Date:* 22nd April, 2014

Ehiaghe Friday Alfred (Reg. No. 2012617003F)
c/o The Head
Department of Medical Laboratory Science
Faculty of Health Sciences and Technology
Nnamdi Azikiwe University
Nnewi Campus.

Re: Application for Ethical Approval to carry out Ph.D Research Project

Your corrected Ph.D proposal has been vetted, and you are granted ethical approval to carry out research work on "Molecular Detection of Some Lymphocyte Genes Expressing Biomakers Associated with Exercise Induced Stress Amongst Young Male Athletes in Eastern Nigeria".

Thank you.

Yours Sincerely,

Dr. Chiejina EN
(Chairman)
For FHST Ethical Committee.

APPENDIX D

Bruce protocol for sub maximal exercise and definition of rest

Bruce protocol (Sub Maximal Table)

Stage	Minutes	% grade	km/h	MPH	METS
1	3	10	2.7	1.7	5
2	6	12	4.0	2.5	7
3	9	14	5.4	3.4	10
4	12	16	6.7	4.2	13
5	15	18	8.0	5.0	15
6	18	20	8.8	5.5	18
7	21	22	9.6	6.0	20

VO2Max= 15 x Maximal heart rate/ Heart rate at rest.

Definition of adequate rest used in the study

Immediately after the exercise bout, the subjects took a warm shower and went on a bed rest for one hour and four hours in a comfortable environment before the one hour and four hours data and blood sample collections respectively. Thereafter, the subjects took at least 6- 8 hours sleep on a comfortable bed before the twenty four hours data and blood sample collections.

Average total distance covered by the exercised subjects was approximately 2.00 km

APPENDIX E

Sample Size

The sample size was obtained using the formula as described by John, (2003)

$$N = 4(Zcrit)^2 \times P\ (1-P)/d^2$$

Where:

N = Minimum sample size

d = The expected change in the measurement (0.2)

Z = Confidence interval (1.96)

P = Prevalence rate of stress (7%) (Sahoo and Khess, 2010)

$$N = \frac{4\times1.96^2\times0.07\times(1-0.07)}{0.2^2} = \frac{4\times3.8416\times0.07\times0.93}{0.04} = 25$$

APPENDIX F

Quality control data

Parameters	1st Reading	2nd Reading	3rd Reading	X	SD	CV (%)
SBP	120	120	119	119.70	0.58	0.49
BDP	80	80	81	80.30	0.58	0.72
BT	36.4	36.3	36.4	36.40	0.06	0.17
PR	60	61	60	60.33	0.58	0.96

Quality control sample results

Parameters	1st reading Ab Conc.	2nd reading Ab Conc.	3rd reading Ab Conc.	4th reading Ab Conc.	X of Conc.	SD of Conc.	CV (%)
Glucose (mg/dl)	0.320 95	0.319 94.6	0.320 95	0.320 95	94.9	0.2	0.2
LH (miu/ml)	0.470 6.2	0.496 6.19	0.471 6.21	0.470 6.2	6.2	0.0082	0.13
FSH (miu/ml)	0.296 4.9	0.297 4.92	0.295 4.88	0.296 4.9	4.89	0.03	0.61
Prog. (nmol/L)	0.994 1.4	0.993 1.5	0.994 1.4	0.993 1.5	1.45	0.06	4.0
Cortisol (nmol/L)	0.410 299	0.411 298	0.409 300	0.411 298	298.8	0.96	0.32
Troponin (ng/ml)	0.007 2.0	0.008 2.3	0.007 2.0	0.008 2.3	2.15	0.17	8.0
CK MB (ng/ml)	0.093 2.80	0.094 2.83	0.093 2.80	0.094 2.83	2.82	0.017	0.6
ALC (cells/ul) $X10^3$	1.20	1.21	1.21	1.20	1.20	0.005	0.4
ANC (cells/ul X 10^3	2.0	2.01	2.0	2.0	2.00	0.005	0.25

Keys

Ab - Absorbance
Conc- Concentration
X - Mean
SD - Standard deviation
CV - coefficient of variation
ANC- Absolute neutrophils count
ALC - Absolute lymphocyte count

APPENDIX G

Table 4.5: Mean (±SD) values of the systolic blood pressure (mm Hg), diastolic blood pressure (mm Hg), body temperature (^{o}C) and pulse rate (beats/minutes) of the exercised participants.

Time intervals	Systolic blood Pressure		Diastolic blood pressure		Body temperature		Pulse rate	
	Subjects that were exhausted before 21 minutes	Subjects that were exhausted at 21 minutes	Subjects that were exhausted before 21 minutes	Subjects that were exhausted at 21 minutes	Subjects that were exhausted before 21 minutes	Subjects that were exhausted at 21 minutes	Subjects that were exhausted before 21 minutes	Subjects that were exhausted at 21 minutes
Baseline(A)	116.1 ± 6.59	117.8 ± 8.65	74.1 ±5.17	74.3 ± 8.53	36.6 ±0.029	36.7 ± 0.20	66.1 ± 2.85	62.7 ± 3.10
Immediately post ex (B)	161.1 ± 9.19	170.8±18.24	84.4 ± 8.75	84.9 ±7.61	37.7 ±0.59	37.6 ± 0.43	151.4 ±5.14	149.0 ±6.53
1hr post ex(C)	118.5 ±7.04	118.3 ± 9.34	73.2 ±10.36	76.2 ± 7.72	36.7 ± 0.38	36.7 ± 0.76	93.3 ±12.95	88.6 ± 12.46
4hrs post ex(D)	116. 1 ±7.01	115.5 ± 8.14	75.0 ±7.81	77.0 ± 6.39	26.5 ± 0.26	36.4 ± 0.23	80.6 ±14.14	79.2 ±10.87
24hrs post exercise (E)	116.8 ± 5.13	113.9 ±7.77	72.0 ± 7.40	73.6 ± 9.98	36.4 ± 0.28	36.4 ± 0.25	74.4 ± 16.54	75.6 ± 13.4
F- Value	65.020		3.972		18.733		106.084	
P – Value	0.000		0.000		0.000		0.000	
A vs B	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
A vs C	0.468(ns)	0.851(ns)	0.727(ns)	0.582(ns)	0.573(ns)	0.339(ns)	0.000*	0.000*
A vs D	0.983(ns)	0.591(ns)	0.727(ns)	0.431(ns)	0.707(ns)	0.154(ns)	0.0001*	0.000*
A vs E	0.819(ns)	0.350(ns)	0.486(ns)	0.834(ns)	0.399(ns)	0.102(ns)	0.0500*	0.010*
A_{21} vs $A_{<21}$	0.674(ns)		0.968(ns)		0.504(ns)		0.441(ns)	
B_{21} vs $B_{<21}$	0.09(ns)		0.883(ns)		0.562(ns)		0.595(ns)	
C_{21} vs $C_{<21}$	0.951(ns)		0.365(ns)		0.888(ns)		0.295(ns)	
D21 vs $D_{<21}$	0.867(ns)		0.58(ns)		0.622(ns)		0.754(ns)	
E21 vs $E_{<21}$	0.434(ns)		0.637(ns)		0.782(ns)		0.789(ns)	

APPENDIX H
PREPARATION OF REAGENTS

10X Tris Borate EDTA Buffer

Dissolve 108 g of Tris base,55 g boric acid and mix 20 ml of 0.5M EDTA (pH 8) in 500 ml of distilled water. Make up the volume up to 1 liter.For use 1X concentration was prepared by taking 1 volume of 10X stock TBE buffer and 9 volume of distilled water.

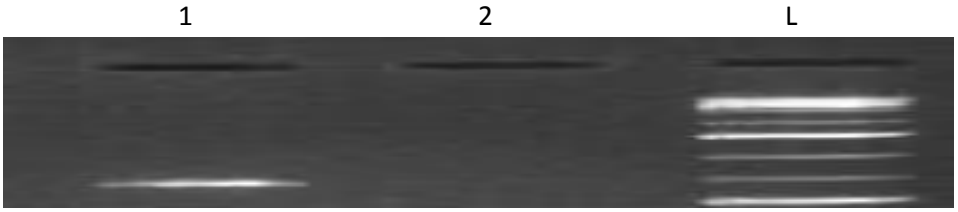
0.5 M EDTA

Dissolve 186g of EDTA in 800ml of distilled water and add 20g of NAOH.

Ethidium bromide stain

Dissolve 1g of ethidium bromide powder in 100 ml of water, stir on a magnetic stirrer for 6 hours to completely dissolve the dye. Wrap the container in aluminium foil or transfer the solution to a dark bottle. Store indefinitely at room temperature.

APPENDIX I



Reverse transcriptase PCR results for the β- actin positive control and no template control analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lane 1 is the targeted β-actin gene at 200bp while lane 2 is a no template control.

APPENDIX J

LAHOR RESEARCH & DIAGNOSTIC LABORATORIES

No. RC:498653

121, Old-Agbor Road, Near Chief Eduwu Ekhator's Palace,
Ohovbe-Ogbeson Quarters, Benin City, Edo Sate. Nigeria.
Tel: 08154273850, 08060138954, 08059000504.

E-mail: deagbonlahor@yahoo.com Website: www.lahor.org.ng

10th December, 2014

TO WHOM IT MAY CONCERN

LETTER OF ATTESTATION FOR
ALFRED FRIDAY EHIAGHE

I confirm that the above named Ph.D student of Nnamdi Azikiwe University, Akwa with Reg. No. 2012617003F carried out his Biochemical and Molecular Research investigation at Lahor Research Laboratories and Medical Centre, Benin City, Edo State.

The analysis comprises of lymphocyte gene expression studies, cortisol level estimation, progesterone level, follicle stimulating hormone estimation, Luteinizing hormone estimation, creatinine kinase estimation, troponin estimation and full blood count estimation.

I declare in my honour that the above statement is true and accurate.

Warm regards.

Prof. D.E. Agbonlahor, PhD, FMLSCN, FRCPath(UK), FAS.
MD/CEO, Lahor Research Laboratories
and Medical Center

Routine Medical Laboratory Diagnosis Specialised Immunoassays