ACUTE AND CHRONIC EFFECTS OF COMBINED ENDURANCE AND STRENGTH TRAINING ON BLOOD LEUKOCYTES IN UNTRAINED HEALTHY MEN

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Master's Thesis
Exercise Physiology
Spring 2014
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ABSTRACT

Lasmanova, Jevgenia 2014. Acute and chronic effects of combined endurance and strength training on blood leukocytes in untrained healthy men. Department of Biology of Physical Activity, University of Jyväskylä. Master's Thesis in Exercise Physiology. 71 pp.

The alterations in white blood cell (WBC) count are seen in individuals engaged in regular endurance training. Whereas no substantial changes in total or differential WBC count has been observed in strength trained individuals. For healthy adults it is recommended to participate in both endurance and strength training. The effects of independent endurance and strength exercises performed on separate occasions are studied profoundly. However, there are considerably less studies investigating the effects of combined endurance and strength (E+S) exercise training on WBCs. Therefore, the purpose of the present study was to examine the acute and chronic effects of combined E+S training on WBCs in healthy young men.

Twenty-two untrained subjects (30.5 ± 6.9 years) were selected to examine the acute effects of combined E+S exercise bout. The chronic effects of 12-week combined E+S training were examined using the data from 16 subjects (30.3 ± 6.7 years). Additionally, the cellular stress induced by the E+S exercise bout was studied by measuring 72 kDa heat shock protein transcripts (*HSPA1A*) prior to and following the 12-week training programme (n=6, 29.6 ± 9.1 years). Total and differential WBC count was determined using an automated haematology analyser. *HSPA1A* mRNA content in peripheral blood mononuclear cells (PBMC) was detected using a real-time quantitative PCR.

Combined E+S exercise bout induced substantial leukocytosis (48%, $p \leq 0.001$) in the circulation of untrained men. The greatest increase was observed in neutrophil count (65%, $p \leq 0.001$), followed by mixed cell count (monocytes, eosinophils, basophils) (24%, $p \leq 0.05$). Total WBC count decreased during the 12 week training period, from $6.61 \pm 1.24 \cdot 10^9$/L to $5.99 \pm 1.06 \cdot 10^9$/L ($p \leq 0.05$). Neutrophil count declined from $3.53 \pm 1.14 \cdot 10^9$/L to $2.85 \pm 0.58 \cdot 10^9$/L ($p \leq 0.05$) following the training period. The relative increase in leukocytes in response to acute E+S exercise bout was greater after the training period. After the 12-week training period *HSPA1A* mRNA content increased at rest and decreased in response to acute exercise bout ($p \leq 0.05$).

In conclusion, acute E+S exercise bout elicits leukocytosis in untrained men similar to leukocytosis seen in independent endurance or strength exercises. Twelve weeks of combined E+S training affected total and differential leukocyte count significantly. An adaptation to training was manifested in decrease in number of total WBCs and neutrophils at rest. The relative exercise-induced leukocytosis was greater following the combined E+S training when compared to pre-training exercise bout. In addition *HSPA1A* mRNA content increased at rest after 12-week combined E+S training period. According to the results of the study it can be said that regular combined E+S training alters peripheral immune system beneficially.

Keywords: combined training, leukocytosis, white blood cells, neutrophils, *HSPA1A*

CONTENTS

1 INTRODUCTION

The exercise immunology as a sub-discipline of exercise sciences appeared in early 1990s together with the progress in new technologies that enabled studying immunological parameters. Exercise has been accepted as a model of the general immune response to environmental stress, as there are many similarities between strenuous exercise and stress that are linked through the stress hormones. (Pedersen & Hoffman-Goetz 2000, Shephard 2010.) Nowadays the effects of physical exercise on immune system cell number and function have been studied widely.

To maintain a good health and decrease the risk of getting a chronic disease, adults should engage in regular physical training. Being habitually physically active influences the immune system favourably. In order to reduce the risk of a disease, an exercise training intensity and volume should remain moderate, as excessive intensive training may lead to suppression of immune system and increase the occurrence of diseases (Figure 1). (Nieman 2012, Neto et al. 2011.)

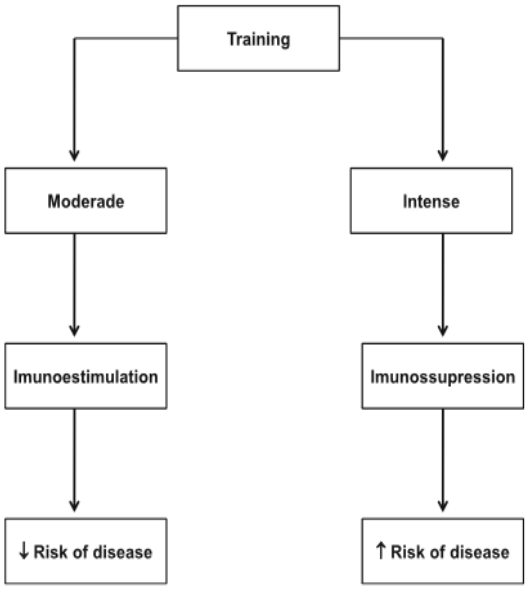

FIGURE 1. Training and immune system (adapted from Neto et al. 2011).

In order to stay in good health, it is recommended to participate in both endurance and strength type activities several times a week (Haskell et al. 2007). Therefore, the purpose of present thesis is to investigate how the combination of recommended physical activities performed in one session two times a week for 12 weeks influence immune system cells in peripheral circulation of healthy young recreationally active men.

2 THE IMMUNE SYSTEM

The immune system, one of the organ systems in human body, is crucial for survival (Parham 2009, 1). It is an organization of organs, molecules and cells that are dedicated to defend the organism against the invaders. In the absence of functioning defence system, minor infections can become fatal to the host organism. (Delves & Roitt 2000a, Parham 2009, 1, Wood 2006, 1.)

2.1 Innate immune system

Innate immune system reacts first when body's physical and chemical barriers have been breached. Innate i.e. natural immunity is determined completely by the genes that individual inherits from parents. This pre-existing mechanism is designed to prevent the entry of pathogens and eliminate trespassed invaders. The above mentioned barriers are part of innate immune system, and in addition white blood cells i.e. leukocytes participate in defending the organism. (Parham 2009, 9, Wood 2006, 19.) Innate immunity depends upon mononuclear phagocytes, such as macrophages, dendritic cells and monocytes, and polymorphonuclear phagocytes, including neutrophils, basophils and eosinophils (Beutler 2004). In addition, mast cells and natural killer (NK) cells also bear germline-encoded recognition receptors and contribute to innate immune defences (Janeway & Medzhitov 2002).

Innate immune defence against pathogens consists of two parts – recognition and destruction. First of all presence of the pathogen has to be recognized. (Parham 2009, 9.) For recognition, innate immune system relies on the particular molecules that are common to pathogens but are absent in the host (Alberts et al. 2002). Soluble receptors in the blood, members of complement and cell-surface receptors, such as members of Toll-like receptor family, bind to pathogen or to altered self-cells (Alberts et al. 2002, Parham 2009, 9). Detection of the pathogen triggers an immune response which is expressed as inflammation of the infected area. During the inflammatory response cells of the innate immune system become activated, differentiate into effector cells and try

to eliminate the invader. (Janeway & Medzhitov 2002.) The inflammatory response elicited by the innate immune system is characterized by pain, redness, heat and swelling of the site of infection (Alberts et al. 2002). These generally necessary symptoms are not due to infection caused by the pathogen but to organism's response to it (Parham 2009, 9).

Phagocytic cells such as macrophages, neutrophils and monocytes eliminate the pathogen via the engulfment (Delves & Roitt 2000a). The components of innate immune system release signalling molecules, e.g. cytokines and interferons that contribute to the inflammatory response. After the pathogen has been phagocytised, toxic substances are released into intracellular vesicle called phagosome to kill it. (Alberts et al. 2002.) Majority of infections are cleared efficiently by the innate immune system and do not lead to the disease. The minority of pathogens that escape innate immunity will face a third line of defence, i.e. adaptive immune system. (Parham 2009, 68.)

2.2 Cells of the innate immune system

Monocytes and macrophages. Monocytes (Figure 2) originate from common myeloid progenitor cell. They are released from the bone marrow into the peripheral circulation where they circulate for several days. Monocyte volume in peripheral circulation is 5-10% of all circulating leukocytes. After entering the tissue they develop into tissue-resident macrophages. In addition to macrophages, monocytes give rise also to dendritic cells and osteoclasts. (reviewed in Gordon & Taylor 2005.)

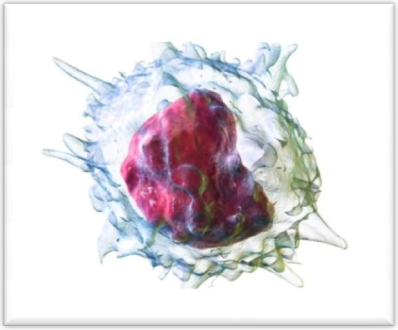

FIGURE 2. Illustration of monocyte, *Blausen Medical*, Web, May 2014

Macrophages are present in all tissues, where they patrol, engulf and kill microbes. In addition, they synthesize and release chemotactic substances that attract other immune cells, in particular neutrophils, to the site of infection. (Beutler 2004.) Other functions of macrophages include clearance of erythrocytes, recycling the iron and haemoglobin for reuse, removing cellular debris during tissue remodelling and clearing cells that have undergone apoptosis (Mosser & Edwards 2008).

Neutrophils. These polymorphonuclear leukocytes represent a majority (50-60%) of the total circulating leukocyte pool (Pedersen & Hoffman-Goetz 2000). Neutrophils (Figure 3) have essential role in the defence against bacterial and fungal infections (Monteseirin 2009) since they are one of the first cells to cross the endothelium and arrive at the site of an infection (Witko-Sarsat et al. 2000). Chemoattractants, which are present in the infected area attract neutrophils to the site (Delves & Roitt 2000a), where they phagocytize invaders (Gabriel & Kindermann 1997). Neutrophils are also involved in the pathology of several inflammatory conditions, since they cause tissue peroxidation due to incomplete phagocytosis (Pedersen & Hoffman-Goetz 2000).

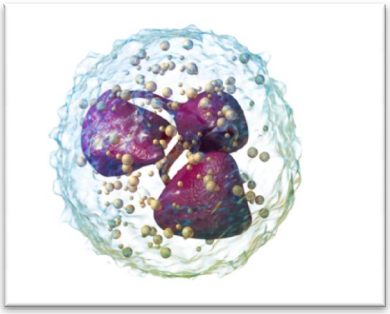

FIGURE 3. Illustration of neutrophil, *Blausen Medical*, Web, May 2014

Basophils and eosinophils. Less than 1% of leukocytes that circulate in human peripheral blood are represented by granulocytes called basophils (Figure 4, right). These cells are normally found in circulation and not in tissues. (Marone et al. 2005.) The role of basophils in innate immunity involves the defence of host against parasites. Basophils are activated via aggregation of surface-bound molecules by the antigen, which leads to degranulation and release of various mediators that promote elimination of the invader. (reviewed in. Stone et al. 2010.)

Another subpopulation of granulocytes, eosinophils (Figure 4, left), representing 1-6% of the leukocytes, is found in human peripheral circulation. Upon diverse stimuli these cells are recruited from blood into the site of inflammation. (Rothenberg & Hogan 2006, Parham 2009, 15.) Their main function is to produce cytotoxic granule proteins. An increase in eosinophil count refers to a presence of allergic diseases such as atopic asthma or a helminthic infection. (reviewed in Stone et al. 2010.)

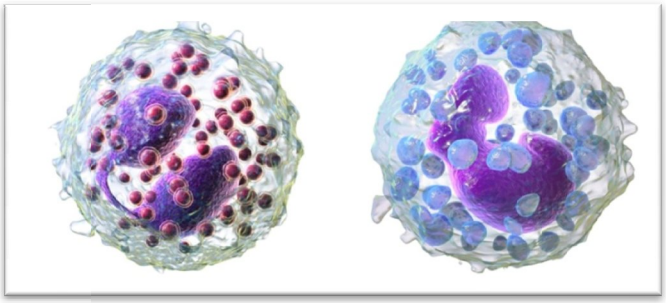

FIGURE 4. Illustration of eosinophil (*left*) and basophil (*right*), *Blausen Medical*, Web, May 2014

Mast cells. These large mononuclear immune cells are distributed in all vascularized tissues, especially at the interfaces with the external environment, such as skin, respiratory, gastrointestinal and genitourinary tracts (Marone et al. 2005). As mast cells are located in association with blood vessels and at epithelial surfaces they respond to signals of innate immunity with immediate and delayed release of inflammatory mediators. In addition, mast cells are involved in the pathogenesis of immediate hypersensitivity and autoimmune diseases. (reviewed in Stone et al. 2010.)

Dendritic cells. Dendritic cells constitute a family of various cells around the body that have one common role, i.e. initiation of immune response. In skin epidermal tissue, at least two members of dendritic cell family are present, Langerhans cells and dermal dendritic cells. Dendritic cells can also be found in thymus, liver and peripheral blood. They pick up antigens, phagocytize them and migrate to areas rich in cells from adaptive immune system; subsequently dendritic cells activate naïve adaptive immune system cells. (Chung et al. 2004.)

Natural killer cells. NK cells recognize and kill abnormal host cells. Their cell-mediated cytotoxicity is directed against virus-infected and tumour cells. (Delves & Roitt 2000a, Gabriel & Kindermann 1997.) NK cells are considered to be components of innate immune system as they lack specific surface receptors. However, in contrast to all other innate immune system cells which originate from myeloid progenitor cell, NK cells origin from lymphoid progenitor cell in bone marrow and express many lymphoid markers. Thus based on morphology, NK cells can be classified as subpopulation of lymphocytes. (Vivier et al. 2011.)

2.3 Adaptive immune system

There are several ways for pathogens to avoid elimination by innate immune system. For instance they do not express pathogen-specific or foreign molecules, and therefore may remain undetected. In this case, cells with more specific receptors, which are able to recognize particular invaders, are recruited. Receptors present on the lymphocytes have much better ability to detect trespassed pathogens or pathogen specific molecules (Wood 2006, 42), as they are not encoded in the germ-line but are created *de novo* in each organism (Iwasaki & Medzhitov 2010).

Innate immune system instructs the adaptive immune system about an ongoing pathogenic challenge (Janeway & Medzhitov 2002). Adaptive immune response comprises the proliferation and differentiation of antigen-specific lymphocytes, T and B cells. Specialized cells, known as antigen-presenting cell are able to display particles of the pathogen that has been killed to T lymphocytes in major histocompatibility complex molecules. (Iwasaki & Medzhitov 2010.) Further, T cell activation leads to B cell activation, which in turn increases the efficacy and focus of the immune response against specific pathogen (Parham 2009, 10).

During the primary immune response, i.e. first encounter of adaptive immune system with the antigen, two types of T and B cells are generated, both effector and memory lymphocytes. Generation of memory cells enables the organism to have quantitatively and qualitatively better secondary immune response during subsequent encounter with the same pathogen. The secondary immune response is faster as relative number of

memory lymphocytes to naïve lymphocytes is greater, and they are also more easily activated. (Delves & Roitt 2000a.) Thus having an adaptive immune system and immunological memory allows responding to each encounter with pathogen on the basis of past interaction (Delves & Roitt 2000a, McFall-Ngai 2007).

2.4 Cells of the adaptive immune system

Lymphocytes. The lymphoid progenitor cell gives rise to lymphoid lineage of leukocytes. Two distinct lymphocyte subpopulations can be distinguished, large granular lymphocytes, i.e. NK cells and small lymphocytes. The small lymphocytes (Figure 5) comprise several subpopulations that have different cell-surface receptors and roles in adaptive immune response. (Parham 2009, 16.)

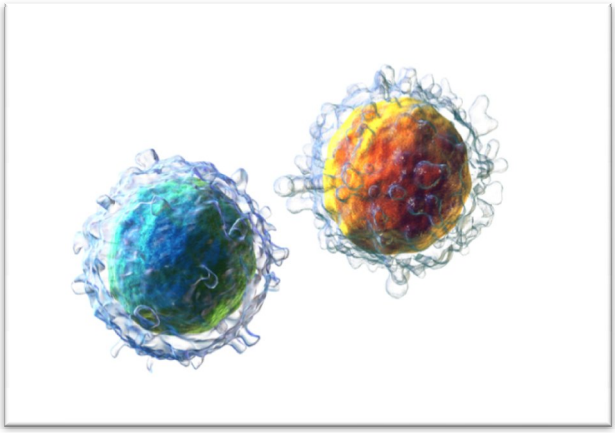

FIGURE 5. Illustration of small lymphocytes, *Blausen Medical*, Web, May 2014

Small lymphocytes develop from progenitor cells in the bone marrow. B cells remain within bone marrow for whole developmental period, whereas T cells leave at an immature stage, migrate to thymus and finish the maturation there. During development, both T and B cells produce antigen-specific receptors. As the region of antigen recognition, i.e. variable region is highly specific, production of T or B cell receptor requires process of random rearrangement and splicing of multiple DNA segments. (Parkin & Cohen 2001.) Each individual lymphocyte uses different combination of gene segments, thus about 10^{15} different variable regions can be produced from fewer than 400 genes (Delves & Roitt 2000a).

T cells can be divided into several subpopulations according to the markers on their surface and functions. For example CD4 marker positive cells function as helper cells and provide signals to enhance the functions of other lymphocytes and phagocytes. $CD8^+$ T cells are cytotoxic killer cells that have role in removal of pathogen by killing virally-infected cells. In addition, T cells regulate immune responses by limiting the scale of tissue damage during inflammation. (Bonilla & Oettgen 2010, Delves & Roitt 2000b.)

B cells require help from T cells to complete differentiation into effector plasma cells and acquire ability to produce and release antigen-specific antibodies (Delves & Roitt 2000b). Five different types of antibodies can be distinguished, namely IgG, IgA, IgM, IgD and IgE. Each type has different functions and can be secreted as circulating molecule or as stationary molecule on the membrane of B cell. (Delves & Roitt 2000a.) Antibodies can directly neutralize invader's toxins, prevent the pathogen adhering to host mucosal surfaces, activate other immune defences and tag bacteria for phagocytosis (Parkin & Cohen 2001).

3 STRESS RESPONSE

According to Hungarian scientist Hans Selye (1907–1982), the father of biological stress concept, the stress can be defined as nonspecific response of the body to any demand (Selye 1973).

Selye conducted several experiments with rodents using different noxious agents such as exposure to cold, surgical injury, spinal shock, excessive muscular exercise and intoxicants with sublethal doses. He noticed the appearance of typical pattern in response to different noxious agents, which was independent of the nature of the damaging agent. It led him to the development of "General Adaptation Syndrome" (GAS) concept, which describes three stages of an organism's response to applied stress (Figure 6). During the brief first stage, so called "alarm reaction stage", an organism tries to restore disturbed homeostasis. In the second, more prolonged stage, known as "resistance development stage", an organism resists the stress. In Selye's experiments rats built up resistance to damaging agents so that functions of their organs were practically normal. However, if stress's intensity or duration exceeds organism's resistance capabilities, the third stage – "exhaustion stage", occurs. In this terminal stage the organism cannot cope with the stress and death may ensue. (Locke & Noble 2002, 3, Selye 1998.) Because organisms responded to different stresses with similar pattern, the Selye's GAS paradigm may be applied to describe organisms' response to various stresses, including the exercise (Locke & Noble 2002, 3).

One of the cells survival promoting responses is heat shock response (HSR), an evolutionarily conserved mechanism designed to protect cells (Asea & Pedersen 2010, Fulda et al. 2010). This biochemical response is activated to counteract not only elevations in temperature but also many other potentially damaging stimuli, such as oxidative (Cajone et al. 1989) and psychological stress (Fleshner et al. 2004). HSR consists of transient modifications in gene expression and synthesis of different stress proteins, which further help the organism to cope with noxious stimuli (Asea & Pedersen 2010).

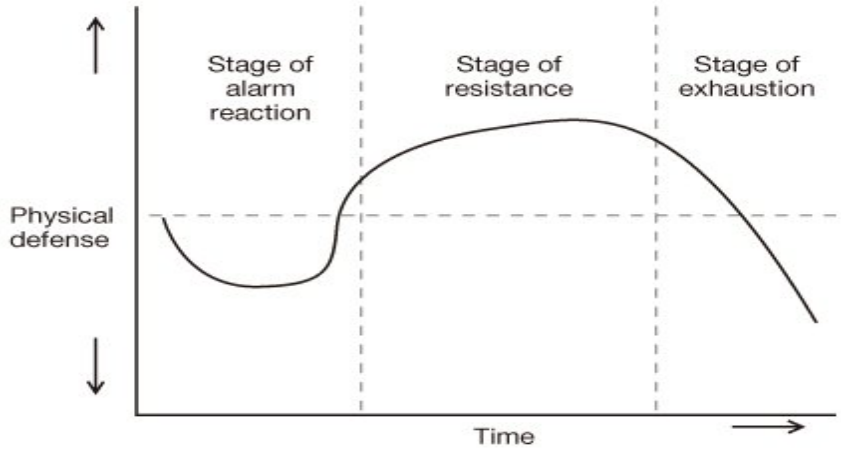

FIGURE 6. GAS stages (adapted from Selye 1956).

3.1 Stress proteins

The HSR, nowadays known as universal response to a great array of stresses, was discovered by Italian scientist, Feruccuio Ritossa, in 1962. Ritossa was studying nucleic acids synthesis in puffs of *Drosophila* salivary glands, and noticed unexpected transcriptional activity when cells were placed at too high temperatures in the incubator. In response to elevated temperatures the cells synthesized unknown factors, which later were identified as heat shock proteins (HSP). (De Maio et al. 2012.)

Currently it is known that at least two distinct groups of stress proteins are synthesized by cells, including immune system cells, during the HSR. In addition to HSPs a group of glucose-regulated proteins (GRP) is synthasized in endoplasmic reticulum where they function as molecular chaperons and assist in protein assambley and folding (Lee 2001). Although the term "heat shock protein" may be misleading, because other stressors besides the elevated temperatures are capable of inducing synthesis of these proteins. HSPs are still referred by their original name since heat was the initial stressor used to characterize them. (Locke & Noble 2002, 6, Whitley et al. 1999.)

HSPs are categorised into large and small HSP families. The major mammalian HSP families are those with molecular masses of 60, 70, 90 and 100 kDa. (Kiang & Tsokos 1998.) The low-molecular weight HSP family (sHSPs) comprise proteins with masses

20 to 30 kDa, such as heme oxygenase (Hsp32), αB-crystallin and HSP20, additionally there are very small weight proteins, for example ubiquitin – an 8 kDa protein (Kregel 2002, Whitley et al. 1999). Within each gene family there are members that are constitutively expressed, inducibly regulated, targeted to different subcellular compartments, or a combination of all of these (Schmitt et al. 2007).

3.2 The roles of HSPs

Stress proteins have a critical role in the maintenance of normal cellular homeostasis (Whitley et al. 1999). The members of HSP families are highly conserved between species from Eubacteria to humans and the inter-species homology varies between 50% and 90% (Pockley et al. 2008, Prohaszka & Fust 2004).

The functions of stress proteins depend on many factors, including the family it belongs to, the localization to the different cellular compartments inside the cell and in extracellular environment, as well as the regulation of the protein, e.g. constitutive or inducible (Schmitt et al. 2007). Housekeeping function is the main intrinsic activity of constitutive HSPs, as HSPs present in the cell in the absence of any stress ensure the correct folding of newly formed polypeptides (Whitley et al. 1999). In addition, HSPs have a role in the regulation of cellular redox state, cellular differentiation and growth. HSPs participate in cellular metabolism, apoptosis and activation of enzymes and receptors. (Prohaszka & Fust 2004, Richter et al. 2010.) The individual functions of HSP families and members are described in Table 1.

TABLE 1. Main functions of HSPs (Calderwood et al. 2007, Kregel 2002, Whitley et al. 1999, Pockley 2001, Morton et al. 2009).

Stress protein	Functions
Ubiquitin	part of non-lysosomal protein degradation pathway
HSP70	molecular chaperones, cytoprotection
HSP60	molecular chaperones, pro-apoptotic
HSP90	part of steroid receptor complex
HSP100	protein folding
αB-crystallin	molecular chaperone, stabilization of cytoskeleton
Hsp27	microfilament stabilization
Hsp32	haeme catabolism

HSPs synthesized by the cells in response to harmful event, i.e. inducible proteins, function as molecular chaperones (Morimoto 1998), and the stressors activating heat shock gene transcription are presented in Table 2. Predominantly five HSP families, members of which have molecular masses of 100, 90, 70, 60 kDa and sHSPs comprise a class of molecular chaperones (Richter et al. 2010). These HSPs prevent the unwanted interactions to occur by binding to the denatured proteins in energy dependent manner (Tavaria et al. 1996), and mediating the transport of these proteins to the target organelles for final packaging, degradation or repair (Kiang & Tsokos 1998). Molecular chaperones recognize non-native, partially or totally unfolded protein through increased exposure of hydrophobic amino acids (Richter et al. 2010).

The above-mentioned functions of HSPs are conducted inside the cell but besides strong cytoprotective effects HSPs also have some roles in extracellular environment. The members of HSP70 and 90 families have been found in extracellular medium where their functions are mainly immunogenic. (Schmitt et al. 2007.) Extracellular HSPs possess powerful immunological properties, and therefore can be perceived as being inflammatory mediators and "danger signals" for the immune system (Pockley et al. 2008, Prohaszka & Fust 2004). In addition, they serve as antigen carriers and stimulate subpopulations of leukocytes to secrete inflammatory cytokines (Moseley 2000).

TABLE 2. Stressors that activate heat shock gene transcription (Morimoto 1998, Kregel 2002, De Maio 1999, Whitley et al. 1999).

Environmental	**pathogenic**	**physiological/metabolic**
elevated temperature	viruses	glucose starvation
nicotine	bacteria	hyperthermia
heavy metals	parasites	hypothermia
ethanol		reactive oxygen species
surgical stress		reactive nitrogen species
ozone		acidosis
nitric oxide		hypoxia
restraint		hyperoxia
exercise		anoxia
		ischemia
		tissue injury and repair
		aging
		hypertrophy
		fever

3.3 HSP70 family

Genetic analyses carried out in lower organisms, such as *E. coli* have shown that HSP70 family members are essential for growth at all temperatures, which indicates a crucial role for these proteins in normal cellular physiology (Lindquist & Craig 1988). This is the one of the best characterized HSP families containing many of highly-related protein isoforms varying in size from 66 kDa to 78 kDa. In humans, there are at least 11 distinct genes encoding HSPs. (Tavaria et al. 1996.)

The most studied members are proteins with molecular masses of 73, 75, 78 and 72 kDa. These proteins are divided into constitutive and inducible isoforms (Tavaria et al. 1996). The first three members (73, 75 and 78 kDa proteins) are present in cell in the absence of stress. The fourth member a 72 kDa protein is synthesized in response to stressful stimuli, although 73 kDa protein levels may also slightly increase during stress. (De Maio 1999.) The nomenclature of HSP70 family proteins in the literature is inconsistent and several terms are used when describing a single protein. In this thesis the nomenclature guidelines provided by Kampinga et al. (2009) will be used to describe HSP70 family members, and HSPA1A term stands for the 72 kDa protein encoded by *HSPA1A* gene.

HSPA1A, a highly inducible isoform of HSP70 family has critical physiological functions (Yamada et al. 2008, Liu et al. 2006). The levels of this protein are very low in absence of stress, but they increase rapidly in response to potentially dangerous stimulation (Kiang & Tsokos 1998). The increase in HSPA1A content have been detected in response to stress in various human and rodent tissues, including brain, heart, liver (Campisi et al. 2003), skeletal muscle (Febbraio & Koukoulas 2000) and leukocytes (Fehrenbach et al. 2000a). The stress-induced synthesis of HSPA1A is generally cytoprotective (Asea 2007); it blocks the apoptotic pathway of the cell at different levels and is able to rescue cells in the later phase of apoptosis (Schmitt et al. 2007).

The induction of HSPA1A in leukocytes is a protective mechanism against heat and other stresses. Leukocyte subpopulates, monocytes and granulocytes synthesize

HSPA1A to protect themselves from noxious molecules they produce (Fehrenbach et al. 2000b). The content of this protein in leukocytes can be an indicator of the extent of previous stress and provide tolerance against subsequent stimuli. The concentration of HSPA1A in humans or rodents may also indicate an adaptation to stress in immunocompetent cells (Fehrenbach et al. 2001).

There are several factors that may affect HSP synthesis in the cell. Rao et al. (1999) demonstrated that age affects the ability of leukocytes subpopulation to synthesize HSPA1A. The HSPA1A induction in response to stress was substantially greater in lymphocytes of young (16-29 years) individuals as compared to old (76-84 years) individuals. In addition to age, nutrition has also been shown to affect HSP production. Nutritional antioxidant, such as vitamin C supplementation for 8 weeks suppressed lymphocytes' ability to express HSPA1A in response to exogenous oxidative stress (Khassaf et al. 2003). The gender has no effect on HSPA1A content or expression in leukocytes in response to physical stress (Simar et al. 2004).

4 IMMUNE SYSTEM AND EXERCISE STRESS

4.1 Exercise as a stress model

Muscular exercise as a prototype of stress was already used by H. Selye. It was one of the nonspecific aversive stimuli and elicited the same symptoms as did surgical injury and exposure to cold. (Selye 1998.) Additionally, exercise represents a quantifiable model of physical stress as the patterns of hormonal and immunological responses have many similarities with other clinical stressors (Hoffman-Goetz & Pedersen 1994). Physical exercise diverts many organ systems to adapt to a new state (Mastorakos & Pavlatou 2005).

The cellular stress response during an exercise is induced by several factors, e.g. increase in metabolic by-products and stress hormone levels, hyperthermia, energy depletion, oxidative phosphorylation, ischemia and pH alterations (McNaughton et al. 2006, Yamada et al. 2008). These events lead to transcriptional activation of heat shock transcription factor (HSF) which then undergoes stress-induced oligomerization, i.e. transits from a monomer to a trimer and acquires DNA-binding activity (Ali & Imperiali 2005, Sarge et al. 1993). Subsequently, formed trimers bind to heat shock element (HSE) on the promoter site of the gene. The binding of a trimer to HSE results in transcription of messenger RNA (mRNA) which is translated eventually into HSPA1A (Figure 7) (Kregel 2002, Yamada et al. 2008).

In vivo (exercise stress) and *in vitro* (exogenous heat shock) stress induces HSP transcription and translation in leukocytes of individuals with different physical activity background. However, exercise stress induces more prolonged elevation in HSPA1A protein expression, suggesting that besides increase in body temperature, other endogenous factors are related to exercise-induced *HSPA1A* expression. (Schneider et al. 2002.)

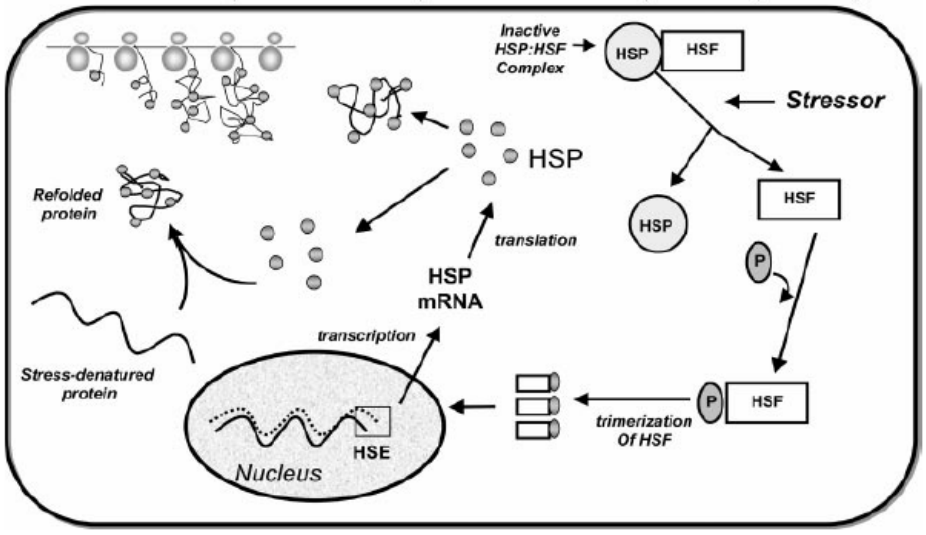

FIGURE 7. Illustration of heat shock response (Kregel 2002)

In peripheral leukocytes *HSPA1A* expression is up-regulated and HSPA1A synthesis increased during the exercise bout. This process functions as a defence against exercise-induced stress. (Fehrenbach et al. 2000b.) Several factors (Figure 8), including subject's age and training status, affect *HSPA1A* mRNA and protein induction during the exercise (Yamada et al. 2008).

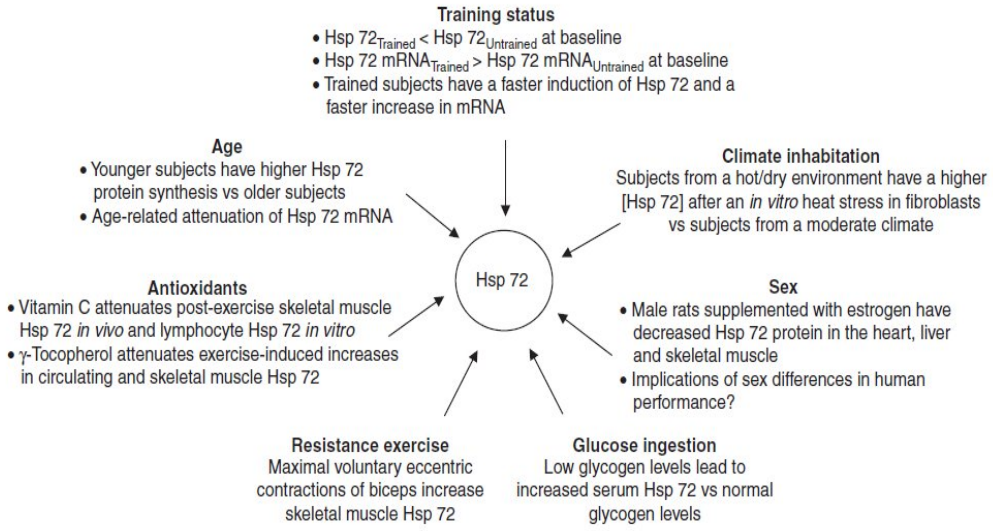

FIGURE 8. Factors affecting HSPA1A (Hsp 72) protein and mRNA content (adapted fromYamada et al. 2008).

The biological tissues display five qualitative adaptive responses to physical stress including decreased tolerance (e.g. atrophy), maintenance, increased stress tolerance (e.g. hypertrophy), injury or death. The physical stress levels that are in the range of tissues' ability to cope with applied stimuli, e.g. maintenance zone, result in no apparent changes. Whereas physical stress levels lower than maintenance range result in decreased tolerance to subsequent stress, and higher stress levels will increase tissue tolerance to subsequent stresses. These principles describe the relative effects of regular exercise training, in which appropriate overloading stimulates musculoskeletal, cardiovascular/pulmonary and neuromuscular systems to adapt to physical demands and increase their tolerance. (Mueller & Maluf 2002.)

In general it is believed that regular moderate intensity exercise training has beneficial effects on immune system and excessive amounts of exercise may rather have negative consequences (Walsh et al. 2011b). Physical activity is associated with lower concentration of inflammatory markers, e.g. circulating leukocyte levels. Individuals who are little to moderately active during their leisure time frequently have 10% lower white blood cell (WBC) counts than sedentary individuals. (Pitsavos et al. 2003.)

It has been shown that monocyte and neutrophil counts are associated with individual's maximal oxygen uptake (VO_{2max}) (Michishita et al. 2008), which is a parameter defining the ability of person's cardiorespiratory system to transport oxygen from the air to the tissues at given level of physical conditioning and oxygen availability (Hawkins et al. 2007). Michishita et al. (2008) reported an association between cardiorespiratory fitness and leukocyte content, as monocyte and neutrophil counts were higher in women with low VO_{2max} compared to those with higher physical fitness.

Similar findings were observed by Metrikat and colleagues (2009), who analysed the associations between inflammatory markers and physical fitness in more than 10 000 young men. The cardiorespiratory fitness was determined by the physical working capacity at a heart rate of 170 bpm (PWC 170) and subjects were divided into three groups according to heart rate results. Leukocyte count was inversely associated with fitness level, as men in high physical fitness group had lower levels of WBCs than men with poorer aerobic capacity.

Kim et al. (2005) obtained similar result in apparently healthy Korean men. Researchers classified 8241 men in range of 16-79 years (median 48) dividing them into three groups based on their WBC counts. Subjects also performed graded exercise test on the treadmill to determine their peak oxygen uptake. An inverse association between leukocyte count and cardiorespiratory fitness was found. The results of the study suggest that greater physical fitness has a beneficial effect by reducing the subclinical inflammation, as men with higher cardiorespiratory fitness had lower total WBC counts.

These cross-sectional studies suggest that good physical fitness is associated with lower levels of inflammatory markers, represented by the number of circulating leukocytes, in very different populations, as both overweight older women and healthy men confirmed beneficial effects of physical conditioning.

4.2 Acute responses to endurance and strength exercise

Acute exercise affects the number and the function of circulating cells of both innate and adaptive immune system (Walsh et al. 2011b). In 1932, Edwards and Wood described leukocytosis, an increase in total white blood cell count, in response to hard muscular work and increase seemed to be proportional to intensity and duration of the exercise. They described 200-300% leukocytosis in American football players immediately after the match. Authors also reviewed the results of the other researchers, who detected leukocytosis in athletes after marathon run and shorter distance runs. (Edwards & Wood 1932.)

The response of leukocyte subpopulations to an acute exercise bout is very stereotyped and can be divided into two phases (Figure 9). The rise of the neutrophil, lymphocyte and monocyte counts starts during the first minutes of endurance type exercise. Immediately after cessation of the exercise neutrophil count continues to increase, whereas lymphocyte and mature monocyte values drop below the pre-exercise levels and they stay low for 2h after the exercise. The leukocyte values return to resting levels within 24 hours after the end of the exercise bout that lasts less than two hours. (Gabriel & Kindermann 1997, Moyna et al. 1996, Pedersen & Hoffman-Goetz 2000.) The first increase in neutrophil count is caused by demargination due to shear stress and increase

in catecholamine concentration (McCarthy et al. 1992a). Increase in stress hormone levels such as cortisol during the exercise may also be responsible for monocytosis in peripheral circulation (Okutsu et al. 2008). Lymphocytosis observed during and immediately after the exercise is caused by the actions of catecholamines, especially epinephrine (Walsh et al. 2011b).

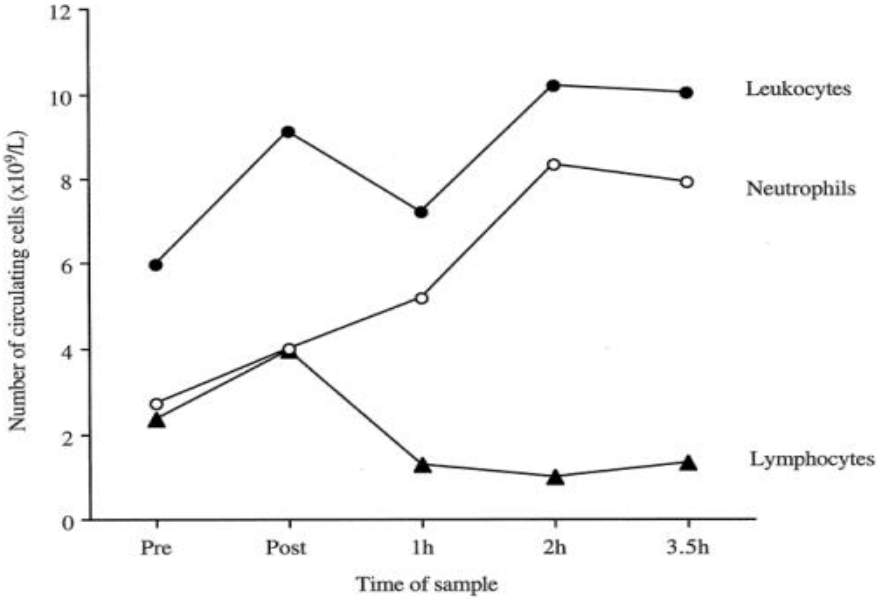

FIGURE 9. Exercise-induced leukocytosis (adapted from Rowbottom & Green 2000).

Similarly to acute endurance exercise bout, resistance exercise induces rapid leukocytosis in healthy men (Hulmi et al. 2010). Total leukocytes, lymphocyte, monocyte and neutrophil numbers increase during and immediately after the resistance exercise (Kraemer et al. 1996). Monocyte and in particular neutrophil counts may remain elevated up to 2 hours post-exercise, whereas lymphocytes, including NK cells decline rather quickly to baseline or below baseline values (reviwed in Freidenreich & Volek 2012). The major contributors to resistance exercise induced leukocytosis are lymphocytes, especially NK cells (Ramel et al. 2003, Simonson & Jackson 2004).

It seems that previous resistance training background does not affect the response to an acute resistance exercise bout in young and older men. For instance, Hulmi et al. (2010) did not find substantial differences in WBC counts in response to acute resistance exercise bout in subjects who underwent 21-week total-body resistance training programme. Likewise, previous regular endurance training (running, cycling,

swimming) for a minimum of 30 minutes three times a week does not affect the magnitude and composition of leukocytosis in response to acute aerobic exercise bout (Moyna et al. 1996).

4.3 HSPA1A and acute exercise

Fehrenbach and co-workers (2000b) have studied the expression of HSPA1A in circulating leukocyte subpopulations of endurance trained athletes after a half-marathon, and showed increased HSPA1A content in the cytoplasm of leukocytes immediately after the half-marathon until the 24h post-competition. Additionally, applying a heat-shock *in vitro* (2 h, 42°) to the leukocytes of trained and untrained subjects at rest showed more pronounced increase in *HSPA1A* mRNA in leukocytes of trained athletes (Fehrenbach et al. 2000a).

The effects of half-marathon on HSPA1A protein and mRNA levels were studied also by Schneider et al. (2002) and compared to effects of heat-shock *in vitro.* An increase in protein and mRNA levels was observed after both stresses, but HSPA1A expression remained up-regulated for longer time in leukocytes after physical stress *in vivo* (half-marathon).

Leukocytes of trained men and women were also studied by Shastry and others (2002)**,** who did not detect the increase in HSPA1A levels in response to endurance exercise bout. They examined expression of HSPA1A in trained individuals after moderate-to-heavy exercise. 11 trained subjects ran on a treadmill for one hour at 70% of VO_{2max}. HSPA1A levels were measured prior to the exercise, 15 and 24 hours following the exercise. The lack of significant increase was explained by individual variation: seven of 11 subjects showed an increase in HSPA1A levels, two showed no changes and two subjects showed a decrease; suggesting a genetic variability in *HSPA1A* expression. Also it is possible that duration and intensity of the exercise was not sufficient enough to stimulate the increase in endurance trained subjects' leukocytes.

Shin et al. (2004) investigated *HSPA1A* mRNA and protein expression in response to endurance type exercise in not only trained but also untrained subjects' leukocytes. Ten endurance-trained and 10 untrained young men ran on a treadmill for one hour at 70%

of their heart rate reserve. Immediately after and 30 minutes post exercise HSPA1A protein concentration increased significantly in both groups, slightly more in untrained subjects compared to endurance trained men. *HSPA1A* mRNA in the other hand was expressed more in trained men compared to untrained.

The three studies described above used exercise of relatively long duration. In contrast, Sakharov and his group (2009) used short duration exercise test to determine *HSPA1A* expression in trained skiers. Four young trained skiers performed an incremental treadmill test lasting approximately 15 minutes. This highly intense physiological stress was sufficient to increase mRNA levels on average 1.5 times.

4.4 Chronic effects of endurance and strength exercise training

The effects of chronic exercise training on the cells of immune system are not well known. Horn and colleagues (2010) analysed blood tests of more than 1000 elite male athletes which were collected over a 10-year period in 14 different sports disciplines (Figure 10). The lowest total WBC and neutrophil counts were found in athletes who were engaged in individual, highly aerobic sports such as cycling and triathlon. The highest WBC counts were observed in team sports, namely rugby and water polo. Significant relationship between aerobic content of a sport and WBC counts was detected. Authors of the article did not consider plasma volume expansion as a cause for low neutrophil count due the fact that neutropenia was rather substantial (20%) in endurance disciplines compared to other sports.

Sport	*n*	Total white blood cell count, 10^9/L		Neutrophil count, 10^9/L	
		Mean	95% Reference range	Mean	95% Reference range
Archery	21	6.7	4.1–10.9	3.7	1.8–7.6
Athletics	113	6.3	3.9–10.2	3.5	1.7–7.1
Basketball	101	6.6	4.3–10.3	3.7	1.9–7.2
Boxing	59	7.2	4.5–11.6	4.0	2.0–8.2
Canoeing	25	6.2	3.7–10.2	3.5	1.7–7.2
Cycling	173	5.7	3.7–8.8	2.8	1.5–5.5
Rowing	195	6.1	3.7–10.1	3.4	1.6–6.9
Rugby Union, AFL	150	7.4	4.7–11.6	4.2	2.3–7.9
Swimming	127	6.7	4.3–10.3	3.4	1.8–6.5
Soccer	165	6.9	4.1–11.7	3.7	1.7–8.0
Triathlon	48	5.9	3.5–9.9	2.9	1.3–6.4
Volleyball	50	6.9	4.2–11.4	4.0	1.9–8.1
Winter sports	32	6.4	4.2–9.8	3.5	1.7–7.0
Water polo	51	7.4	4.3–12.5	4.3	1.9–9.4
All sports	1,310	6.6	3.9–11.1	3.6	1.7–7.7
Normal reference range			4.5–11.0		2.0–8.0

FIGURE 10. Total WBC and neutrophil counts in athletes, adapted from Horn et al. (2010).

Saygin et al. (2006) compared total WBC values in sedentary individuals, volley ball players and long distance running athletes. The highest total WBC count was found in volley ball players, followed by sedentary individuals, while the endurance athletes had the lowest leukocyte count. Michishita et al. (2010) recruited overweight and previously sedentary women to an endurance exercise study. Result of this longitudinal study showed that six weeks of training lowered fasting total WBC count. Data from these longitudinal and cross-sectional studies show that endurance type athletes tend to have lower total leukocyte levels and aerobic training may decrease WBC in previously sedentary individuals.

WBC counts of young resistance trained men were compared to untrained male subjects in study of Ramel et al. (2003). Men who performed resistance type exercise training at least three times a week for six months or more, had slightly lower total leukocyte,

neutrophil, monocyte and lymphocyte levels compared to not resistance trained men. However, 70% of the resistance trained men also took part in aerobic exercise 2-3 times per week, which may have affected the resting leukocyte levels.

Chronic strength training alone without endurance exercise does not seem to elicit significant changes in basal leukocyte count. Hulmi et al. (2010) studied the effects of 21-week resistance training in which young and middle-aged healthy men participated in total-body heavy workouts twice a week. Likewise, Cardoso et al. (2012) compared basal levels of WBC at rest in women who took part in resistance training at local fitness centre as a minimum two times per week for last two years and sedentary controls. No differences were found in WBC counts at rest between sedentary and resistance trained women.

4.5 HSPA1A and chronic exercise training

Adaptations to endurance type exercise down-regulate HSPA1A protein expression at the baseline and in response to exercise in humans (Yamada et al. 2008). Shastry et al. (2002) found that endurance trained athletes have significantly lower levels of HSPA1A in leukocytes at rest. The same pattern was observed in several other studies (Fehrenbach et al. 2000a, Selkirk et al. 2009, Shin et al. 2004) where the counts of HSPA1A-positive leukocytes were lower in trained athletes compared with untrained subjects' leukocytes. Trained subjects had slightly lower HSPA1A levels in response to same relative intensity exercise bout than untrained subjects.

While trained subjects tend to have lower HSPA1A protein levels at rest, the mRNA levels are usually higher at the baseline in leukocytes of endurance trained athletes compared with sedentary (Yamada et al. 2008). The content of mRNA measured in two studies yielded the same results – trained individuals have higher mRNA levels compared to untrained (Fehrenbach et al. 2000a) or sedentary controls (Shastry et al. 2002).

One explanation to differences in HSPA1A protein and mRNA content in relation to training background can lay in thermoregulation. Endurance trained athletes may have better thermoregulation, they sweat more than untrained subjects and have lower

increase in body temperature which leads to lower HSPA1A protein content (Shin et al. 2004). Additionally, higher mRNA content at rest in athletes provides enough transcripts for immediate translation into protein whenever necessary (e.g. after stress exposure) (Fehrenbach et al. 2000a). Thus the increased mRNA levels allow athletes to have lower protein levels at rest; as mRNA can be translated rapidly into protein (Yamada et al. 2008).

4.6 Exercise and hormones

In response to exercise stress the release and concentration of numerous hormones, including norepinephrine, epinephrine, growth hormone (GH) (Kindermann et al. 1982) cortisol, β-endorphin, adrenocorticotropic hormone (ACTH), (Gabriel & Kindermann 1997) and testosterone (Meckel et al. 2009) is altered. Several of these stress-induced hormones elicit substantial changes in total number and relative proportions of blood leukocytes (Dhabhar 2008).

Catecholamines. Epinephrine and norepinephrine have roles in mediating body's physiological responses to physical stressors such as endurance or strength exercise bout. Catecholamines stimulate cardiac, respiratory, thermoregulatory functions (Zouhal et al. 2008), as the activation of sympathetic nervous system results in increased heart rate, cardiac output and systemic arterial pressure, and bronchodilation (Zouhal et al. 2013). The correlation between exercise-induced catecholamine increase and leukocytosis is strong (Shephard 2003). Increase in circulating neutrophil, monocyte and lymphocyte, in particular NK cell, number in response to an acute exercise bout can be caused due to shear stress and release of catecholamines (McCarthy et al. 1992b, Walsh et al. 2011a).

Anabolic hormones. GH secretion by acidophilic cells of anterior pituitary is increased during physical activity from to two to ten-fold (Kraemer & Ratamess 2005; Ranabir & Reetu 2011). GH levels in plasma increase significantly in response to strength exercise bout (Vissing et al. 2011) and acute aerobic exercise session (Wideman et al. 2002) promoting tissue anabolism (Kraemer & Ratamess 2005). An increase in GH

concentration, in addition to catecholamines, mediates the acute exercise effects on blood neutrophils (Pedersen & Toft 2000).

Testosterone is an anabolic hormone produced primarily by Leydig cells located in testes, it is important for muscle mass development and maintenance. It serves as a major promoter of muscle hypertrophy and increase in strength in response to strength training. (Vingren et al. 2010.) Testosterone levels in serum increase also in response to prolonged endurance exercise bout (Daly et al. 2005). The relationship between exercise-induced testosterone increase and leukocytosis has not been studied extensively. At the baseline, Brand et al. (2012) found an inverse association between total WBC, in particular granulocyte count and testosterone levels in middle-aged and older men. In addition, higher sex hormone values were associated with lower inflammatory markers.

Stress hormone cortisol activates catabolic and anti-anabolic processes which are essential for adaptation in stress situations. Its' metabolic effects include creation of free pool of amino acids for protein synthesis. (Viru & Viru 2004.) Moderate to high intensity 30 minutes endurance exercise bout elicits a significant increase in circulating cortisol levels (Hill et al. 2008). Likewise, high intensity resistance exercise bout causes greater cortisol response than moderate intensity strength exercise session (Raastad et al. 2000). Cortisol exerts its effects on circulating leukocytes with a time lag and do not play a major role in acute exercise leukocytosis (Pedersen & Hoffman-Goetz 2000). More likely cortisol is responsible for sustained post-exercise lymphopenia and neutrophilia (Pedersen & Toft 2000). In the study of Risøy et al. (2003) strength exercise-induced increase in cortisol had no correlation with post-exercise neutrophilia, but endurance exercise bout induced increase showed association with late neutrophilia.

5 PURPOSE OF THE STUDY

The main purpose of the present study was to examine and describe acute and chronic effects of single session combined endurance and strength (E+S) training on peripheral blood leukocytes in healthy untrained men. Additionally, the evaluation of acute and chronic strain of concurrent training by means of studying the expression of *HSPA1A* mRNA in peripheral blood mononuclear cells (PBMC) was planned.

Research questions:

1) Is the total WBC count affected by the combined E+S training (acute and chronic responses)?

2) Is the differential WBC count, i.e. neutrophil, lymphocyte and mixed cell counts, affected by combined E+S training (acute and chronic responses)?

3) Are there changes in *HSPA1A* mRNA levels after 12 weeks of training and in response to an acute E+S exercise bout in PBMC?

4) Are there associations between physical fitness parameters such as endurance or strength, exercise-induced hormones and circulating WBCs?

Research hypothesis:

The hypotheses to the proposed research question are as follows:

Hypothesis 1: Independently, acute endurance or strength exercise bout induces leukocytosis, thus analogous increase in immune system cells will be seen in response to combined E+S bout. While regular endurance training is associated with lower leukocyte counts, regular strength training does not elicit the same changes. As combined training contains both endurance and strength exercises at equal proportion,

there will be no change or slight decrease in total leukocyte count after 12 weeks of training.

Hypothesis 2: Neutrophil, lymphocyte and mixed cell counts increase during endurance and strength exercise bouts. Combined E+S exercise session will increase the levels of these leukocyte subpopulations.

Hypothesis 3: Endurance-trained individuals have higher *HSPA1A* mRNA levels at rest, thus combined E+S exercise training will increase baseline *HSPA1A* mRNA in subjects' PBMC. *HSPA1A* mRNA levels increase in PBMC in response to an acute exercise bout.

Hypothesis 4: Serum concentration of anabolic and stress hormones increases in response to an acute exercise bout. Anabolic and stress hormone levels will increase during the combined E+S exercise bout and show an association with circulating leukocytes.

6 METHODS AND MATERIALS

6.1 Subjects and recruitment

Twenty-two healthy men aged 18-40 from the Jyväskylä region were recruited to participate in the study. Subjects were recruited via advertisements in newspapers and public places, e.g. libraries and gyms. Ads were posted on the website of University of Jyväskylä, university staff and student e-mail lists and the city of Jyväskylä. To participate in the experiment, subjects needed to be recreationally active but without prior participation in systematic and progressive endurance or strength exercise training for a minimum of 12 months' prior to the start of the study. Recreational activity included light walking, occasional ball games (football) or cycling. In addition, requirements for participation included the subjects to be free of acute illnesses and injuries, abstain from smoking for at least 12 months prior to the study as well as to have the body mass index (BMI) below 31kg/m^2. Individuals with metabolic syndrome, glucose tolerance impairments, cardiovascular or musculoskeletal diseases that may limit the participation in exercise training were excluded.

All participant candidates completed a phone interview concerning their general health aspects and attended resting electrocardiogram (ECG) screening as well as blood pressure testing conducted by project staff. The ECG and blood pressure results were analysed and approved by cardiologist. Approved subjects participated in a meeting with project staff where study design, measurements, procedures, possible benefits and risks were carefully explained. All subjects who agreed to participate signed the informed consent forms prior to the start of the study. Study was approved by the Ethical Committee at the University of Jyväskylä and conducted in accordance with the Declaration of Helsinki.

6.2 Study design

The combined E+S training study started in October and November of 2011. After completing the whole study, 22 subjects who had all necessary data were selected to study the acute response of untrained men to an acute loading containing endurance and strength exercise. To study the chronic effects of 12-week combined training the data from 16 subjects was used. To investigate the stress response to acute E+S loading prior to and after 12 weeks of combined training six subjects were selected.

All subjects were familiarized with the training and measurement protocols and equipment prior to proceeding with basal measurements of maximal endurance and strength. After the baseline measurements subjects were assigned into training group, performing an endurance exercise session followed by strength exercise session. During the 12 weeks period subjects participated in training sessions in the gym twice a week. After the 12 weeks of training subjects participated in post-intervention measurements where the same relative loads were used. The overview of the intervention is described in the Figure 11. The data used in present study was obtained from larger Ph.D projects of Moritz Schumann, M.Sc. and Enni-Maria Hietavala, M.Sc.

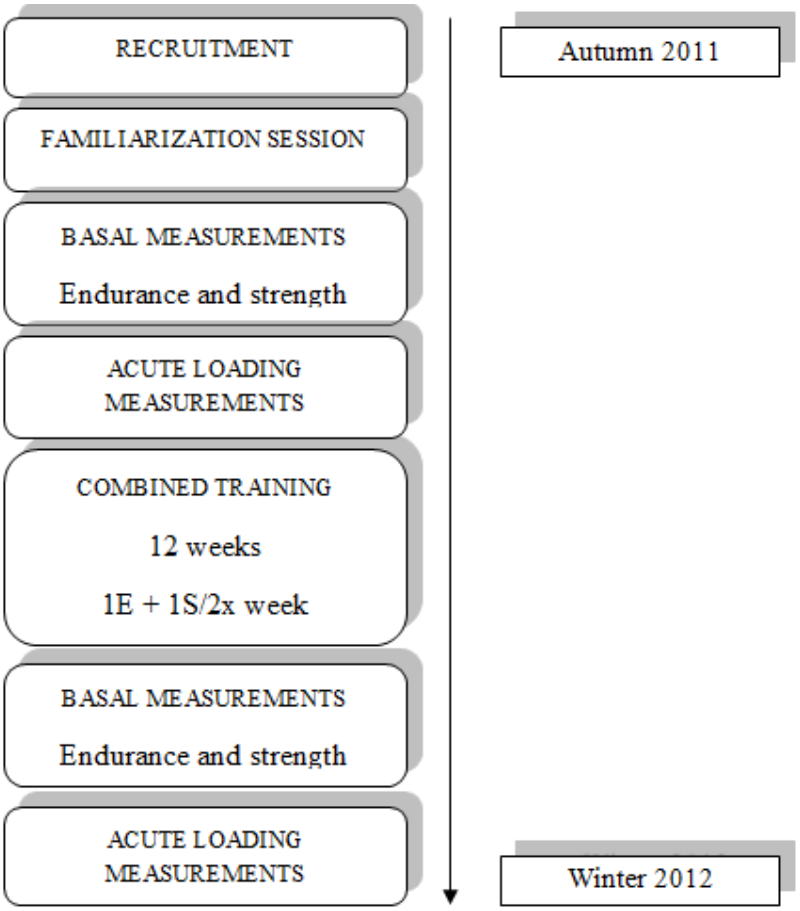

FIGURE 11. Scheme of the study.

6.3 Basal measurements

The anthropometric characteristics of the subjects are represented in Table 3. Height was measured by tape measure fastened on the wall (accuracy 0.1 cm) with subject standing upright, feet shoulder with apart, heel against the wall and chin in neutral position during the measurement. Weight was measured with a digital scale (accuracy 0.1 kg) after the 12 hours fast, heavy clothing and shoes removed. BMI was calculated as body mass in kilograms divided by the square of the height in meters.

TABLE 3. Anthropometric data of the subjects.

	Acute	Chronic	Stress
Number (*n*)	22	16	6
Age (yrs)	30.5 ± 6.9	30.3 ± 6.7	29.6 ± 9.1
Weight (kg)	82.9 ± 10.2	82.7 ± 11	88.9 ± 9.7
Height (m)	1.78 ± 0.06	1.77 ± 0.07	1.79 ± 0.07
BMI (kg/m^2)	26.1 ± 2.97	26.2 ± 2.9	27.7 ± 3.2

Data is presented as mean ± SD. Acute= acute effects of E+S loading, Chronic= chronic effects of combined training, Stress= stress response by means of *HSPA1A* expression in PBMC in response to acute E+S loading before and after 12 weeks of training.

Cardiorespiratory measurements. Cardiorespiratory fitness (VO_{2max}) was determined using graded maximal aerobic cycling test until volitional exhaustion. The mechanically braked bike ergometer (Ergomedic 839E, Monark Exercise AB, Sweden) was used with initial load of 50 W for all subjects, intensity increased every two minutes by 25 W. Pedalling frequency was kept at 70 rpm throughout the test. Heart rate was monitored continuously (Polar S410; Polar Electro Oy, Kempele, Finland). Oxygen uptake was measured continuously breath-by-breath using a gas analyser (SensorMedics® Vmax 229, SensorMedics Corporation, Yorba Linda, California, USA).

Strength measurements. Dynamic one repetition maximum (1RM) measurement was used to determine maximal bilateral concentric strength of leg extensors using a dynamic leg press (David 210, David Sports Ltd., Helsinki, Finland). Subject was seated so that the angle of the knee was less than 60º, instructed to grasp the handles located by the seat of dynamometer and to keep constant contact with the seat. Prior to real 1RM testing a short warm-up was performed. It consisted of several repetitions at estimated maximal loads, starting from 70% of estimated 1RM, followed by 80-85% and finally one repetition at 90-95% of 1RM was performed prior to actual 1RM. After the warm up, on the verbal command subjects extended legs until complete extension to 180º, the greatest weight that was successfully lifted was accepted as one 1RM. No more than five attempts were allowed. In addition to dynamic 1RM, maximal isometric bilateral leg strength was measured by a horizontal leg press dynamometer (Department of Biology of Physical Activity, University of Jyväskylä, Finland).

6.4 Acute E+S loadings

Endurance loading (E) consisted of 30 minutes of continuous cycling on the bike ergometer with electrical resistance (Ergomedic 839E, Monark Exercise AB, Sweden). Subject pedalled at the pace of 70 rpm, the workload was 65% of maximal workload determined during basal measurement. A 5-minute light intensity warm-up was performed before actual endurance loading. Strength loading (S) followed endurance loading and was conducted using a dynamic leg press (David 210, David Sports Ltd., Helsinki, Finland). Explosive, maximal and hypertrophic strength was measured using the 1RM loads determined during basal strehgth measurements. The time between two loadings did not exceed 15 minutes and subjects were allowed to drink 2dl of water.

6.5 Training intervention

Training consisted of progressive and periodized combined single session E+S training. All subjects completed a session of combined training prior to the beginning of the experimental training period. During this preparatory training phase subjects trained with light resistance and low intensity. In actual training intervention endurance training, performed on the cycle ergometer was followed by whole body strength training. Total training duration averaged between 60 and 120 minutes, and all training sessions were supervised by project staff.

Endurance training intensity was based on heart rate zones calculated from subject's aerobic and anaerobic thresholds. Thresholds were determined during graded maximal cycling test. Polar® heart rate monitors were used to control the intensity of the endurance training which was performed on a bicycle ergometer. Continuous and interval training modes were used in cycling sessions lasting 30-50 minutes.

Strength training was initiated with light resistance and performed as circuit training to prepare subjects for subsequent resistive exercise training and to verify correct technique. Following the initial phase, strength training programme focused on muscle hypertrophy. Subsequently, mixed hypertrophic and maximal strength training was performed. During the last phase subjects completed maximal strength and explosive

strength sessions. Strength exercises were performed for all major muscle groups; knee extensors and flexors, extensor and flexor muscles of the arms as well as trunk. As the knee extensors and flexors were main focus of the resistance training, each strength training session included three leg exercises, i.e. leg press, leg extension and leg curl. In addition, biceps curls, lateral pulldown, dumbbell fly, military press and triceps pushdown exercises were performed for upper body muscles. Core exercises comprised abdominal crunches and back extension on the bench.

6.6 Blood sampling and analysis

6.6.1 Venous blood sampling

Blood was drawn into serum tubes (Venosafe, Terumo Medical Co., Leuven, Belgium) by qualified laboratory technician. Samples for WBC count analysis (Figure 12) were collected at the fasted state before the standardized breakfast and endurance loading (PRE); in the middle of the loadings, i.e. after the endurance and before the strength (MID); after the loadings (POST), 24- and 48 hours after loadings. Blood samples at these time points were collected twice, before and after 12-weeks of combined training.

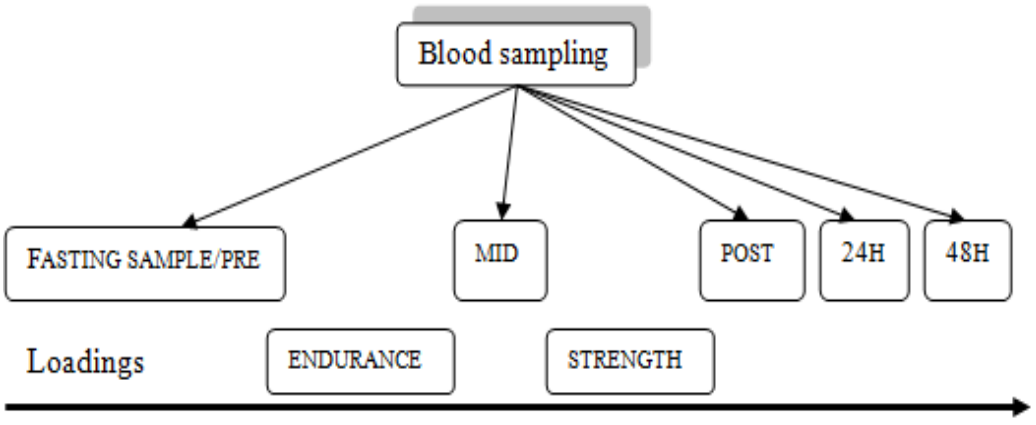

FIGURE 12. Scheme of blood sampling time points for WBC analysis. PRE= Sampole at the fasting state; MID=sample between the loadings (arter the endurance and before the strength loading); POST=sample after the acute E+S loading; 24H=sample 24 hours after acute E+S loading; 48H= sample 48 hours after the acute E+S loading.

WBC count was determined with Sysmex KX-21N (TOA Medical Electronics Co., Ltd, Kobe, Japan). Of the WBC neutrophils, lymphocytes and mixed cells (monocytes, basophils, eosinophils, immature precursor cells) were analysed. Whole blood was centrifuged at 2500 rpm (Megafuge 1.0R, Heraeus, Germany) for 10 minutes after which serum was removed and stored at −80°C until further hormonal analysis. Serum testosterone, cortisol, GH levels were measured using chemical luminescence techniques (Immulite 1000, DCP Diagnostics Corporation, Los Angeles, California, USA) and hormone specific immunoassay kits (Siemens, New York, NY, USA). The sensitivity for testosterone, cortisol and GH assays were: 0.5 $nmol \cdot l^{-1}$, 5.5 $nmol \cdot l^{-1}$, 0.026 $mlU \cdot l^{-1}$respectively.

6.6.2 Separation of PBMCs from whole blood

Blood from antecubital vein for *HSPA1A* expression analysis was collected into CPT tubes (BD Vacutainer ®CPT™ 8ml Sodium Citrate/Ficoll, Becton Dickinson Vacutainer®Systems, N.J. USA) at various time points (Figure 13). Blood was mixed immediately 8-10 times by inverting the tube. Samples were centrifuged within 30 minutes (Megafuge 1.0 R; Heraeus, Germany) at 1700 x g for 30 minutes at room temperature. PBMCs were collected with Pasteur pipette and transferred to 15 ml conical tube. 10 ml of PBS was added to tube, and the cells were mixed by inverting the tube 5 times. Samples were centrifuged at 300 x g for 15 minutes at room temperature. Supernatant was discharged, cell pellet resuspended by tapping the tube with index finger. 10 ml of PBS was added, cells were mixed by inverting the tube 5 times. Samples were centrifuged at 300 x g for 10 minutes at room temperature. Supernatant was discharged and the pellet was pipetted into an Eppendorf tube containing 1 ml of TRIzol®Reagent and stored at −80°C for further analysis.

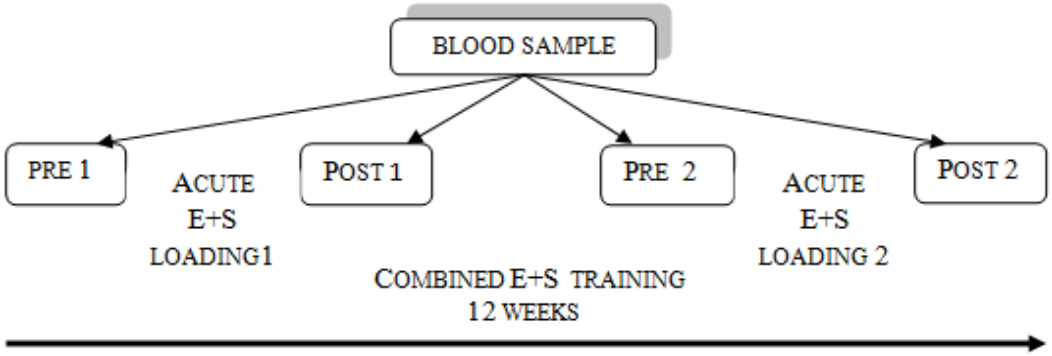

FIGURE 13. Scheme of blood sampling time points for *HSPA1A* expression analysis. PRE 1= sample before first acute E+S loading (at the fasting state) at week 0; POST 1= sample after first acute E+S loading at the week 0; PRE 2= sample before second acute E+S loading (at the fasting state) at week 12; POST 2= sample after second acute E+S loading at the week 12.

6.6.3 RNA extraction, cDNA synthesis and real-time quantitative PCR

RNA extraction was done according to TRIzol®Reagent manual. Samples were defrosted at the room temperature. 200 µl of chloroform per 1 ml of TRIzol®Reagent (Invitrogen, Carlsbad, USA) was added into the sample tube. Samples were shaken vigorously for 15 seconds, and then incubated for 2-3 minutes at room temperature. Samples were centrifuged at 12,000 x g for 15 minutes at 4ºC. The aqueous phase was removed from the tubes by pipetting. 500 µl of 100% isopropanol was added to aqueous phase and then incubated for 10 minutes at room temperature. To precipitate the RNA the samples were centrifuged at 12,000 x g for 10 minutes at 4ºC. Supernatant was removed from the tube, and the remaining RNA pellet was washed with 1 ml of 75% ethanol. Samples were centrifuged at 7500 x g for 5 minutes at 4ºC, and ethanol was discarded. RNA pellet was air dried for 5-10 minutes and then resuspended in 30 µl of RNase-free water and shaken vigorously. RNA concentration and purity was measured with nanodrop spectrophotometer.

TABLE 4. Concentration and purity of the RNA samples and dilution for cDNA synthesis.

Subject	PRE	RNAc (ng/µl)	A260 /280	RNA (µl)	H_2O (µl)	POST	RNAc (ng/µl)	A260 /280	RNA (µl)	H_2O (µl)
1	**w0**	172.7	1.86	5.21	4.79		378.8	1.94	2.38	7.62
	w12	413.6	1.96	2.18	7.82		237.5	1.98	4.21	5.79
2	**w0**	189.3	1.87	4.75	5.25		173.4	1.82	5.19	4.81
	w12	178.8	1.86	5.03	4.97		115.9	1.90	8.62	1.38
3	**w0**	296.6	1.95	3.03	6.97		237.6	1.96	3.79	6.21
	w12	287.2	1.93	3.13	6.87		290.6	1.99	3.44	6.56
4	**w0**	76.2	1.72	3.94	6.06		32.4	1.70	9.26	0.74
	w12	174.4	1.90	5.73	4.27		408.7	1.97	2.20	7.80
5	**w0**	395.9	1.96	2.53	7.47		227.2	1.99	4.40	5.60
	w12	348.5	1.94	2.58	7.42		496.9	1.96	1.81	8.19
6	**w0**	254.0	1.92	3.54	6.46		347.9	1.95	2.87	7.13
	w12	242.7	1.97	3.71	6.29		181.6	1.97	5.51	4.49

w0=week 0, w12=week 12; PRE= prior to the combined training programme; POST= following the combined training programme. RNAc= RNA concentration (ng/µl); A260/280= ratio of the absorbance at 260 and 280nm; RNA= RNA amount in one microliter.

For the cDNA synthesis one microgram of total RNA was reverse transcribed according to the manufacturer's instructions using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). The dilution of RNAs is shown in Table 4 and the synthesis reaction mix in Table 5. cDNA synthesis was performed in automated Eppendorf thermal cycler using the following conditions: 10 min at +25°C, 120 min at +37°C, 5 min at +85°C, ∞ at +4°C. Finally the cDNAs were stored at -20°C until further use.

Real-time PCR analysis was performed using in-house designed primers iQ SYBR Supermix and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA). The primer sequences for *HSPA1A* were Fwd 5'GGGGAGGACTTCGACAACAGG'3, rev 5'GGAACAGGTCGGAGCACAGC'3, and for housekeeping gene *β-actin* Fwd 5'AGAGCTAGCTGCCTGAC'3, Rev 5'GATGCCACAGGACTCCA'3. The annealing temperature was 56ºC. Each sample was analyzed in duplicate and PCR cycle parameters were as follows: +95ºC for 10

min, 40 cycles at +95ºC for 10 s, at +56ºC for 30 s and at +72ºC for 30 s, followed finally by 5 s at +65ºC . Relative expression levels for *HSPA1A* were calculated with the $\Delta\Delta C_t$ method and normalized to the expression of *β-actin*.

TABLE 5. *Left* the master mix for cDNA synthesis. *Right* master mix for real-time PCR reaction.

The reaction mix for cDNA synthesis		**qPCR reaction mix**	
10xRT buffer	2.0 µl	iQ SYBR	12.5 µl
100 mM dNTP mix	0.8 µl	10 mM Fwd primer	1.25 µl
10 x Primers	2.0 µl	10 mM Rev primer	1.25 µl
RT enzyme	1.0 µl	H_2O	5.0 µl
H_2O	4.2 µl		

RT buffer – reaction buffer; dNTP - deoxynucleotide triphosphates; RT enzyme - reverse transcriptase enzyme; Fwd - forward; Rev - reverse

6.7 Statistics

Statistical analysis was done using IBM SPSS Statistics version 20 (IBM Corporation, Armonk, New York, USA) and graphed on Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA). Data is presented as mean ± standard deviation (SD). The Shapiro-Wilk Test was used to determine whether the data was normalyl distributed. Normally distributed leukocyte subsets were compared at different time points using paired-sample t-tests, with significance level set at *$p \leq 0.05$ and **$p \leq 0.001$. Wilcoxon signed-ranks test as non-parametric test was used to compare not normally distributed leukocyte subset values, with significance values set at *$p \leq 0.05$ and **$p \leq 0.001$. The relationships between hormones, physical parameters, leukocytes and *HSPA1A* expression were analysed according to distribution, normal values were analysed using Pearson correlation coefficient and for not normally distributed values Spearman's rank correlation coefficient was used, significance level was set at *$p < 0.05$.

7 RESULTS

7.1 Physical fitness parameters

12-week combined E+S training did not cause significant changes in weight or BMI of the study participants (Table 6). Significant increase in dynamic 1RM ($p=0.003$) and decrease in maximal oxygen consumption VO_{2max} ($p=0.028$) was observed after 12 weeks of training.

TABLE 6. Combined E+S training effects on physical parameters.

Time	weight (kg)	BMI (kg/m^2)	1RM (kg)	VO_{2max} (ml/kg/min)
Week 0	81.2 ± 9.8	26.1 ± 2.9	167 ± 28	33.2 ± 4.6
Week 12	80.7 ± 8.6	25.9 ± 2.5	178 ± 23*	31.9 ± 5.1*

Data presented as mean ± SD; 1RM – dynamic one repetition maximum, *$p \leq 0.05$ week 12 vs. week 0; *n*=16.

7.2 Immediate effects of acute E+S loading

The baseline values of total blood leukocytes, lymphocytes, neutrophils and mixed cells (monocytes, basophils, eosinophils, immature precursor cells) were within normal clinical range (Table 7). Acute loading, containing endurance and strength exercise, induced substantial leukocytosis in untrained healthy men. The greatest increase was observed in neutrophil subpopulation. Lymphocytes increased in the first phase of the loading, which was followed by the decrease. All leukocyte subsets returned to the baseline level 24 hours after the acute loading.

TABLE 7. Blood leukocyte count of untrained men before and after acute loading.

Leukocyte	PRE	POST	% of ↑
Total WBC count	6.35 ± 1.02	9.39 ± 2.44**	48%
Lymphocyte count	2.46 ± 0.71	2.79 ± 0.87	13%
Neutrophil count	3.24 ± 1.03	5.34 ± 2.15**	65%
Mixed cell count	0.66 ± 0.21	0.81 ± 0.19*	24%

The values given are means ± SD in 10^9/L, PRE – before acute loading, POST – after acute loading; **p≤0.001, *p≤0.05 PRE vs POST; % of ↑ - percent of increase; *n*=22.

Total WBC count increased 33% (p≤0.001) after the cycling part of the acute loading when compared to baseline (Figure 14). The WBC count elevation continued with strength protocol resulting in total increase of 48% (p≤0.001). Total leukocyte count increased 11%, from 8.44 ± 2.10 · 10^9/L measured after the endurance loading (AFTER E) to 9.39 ± 2.44 · 10^9/L measured after the strength loading (AFTER S) (p≤0.05). Total WBC count returned to baseline level 24 hours after the acute loading and did not change 48 hours after the loading, 6.26 ± 0.98 · 10^9/L and 6.10 ± 0.93 · 10^9/L respectively.

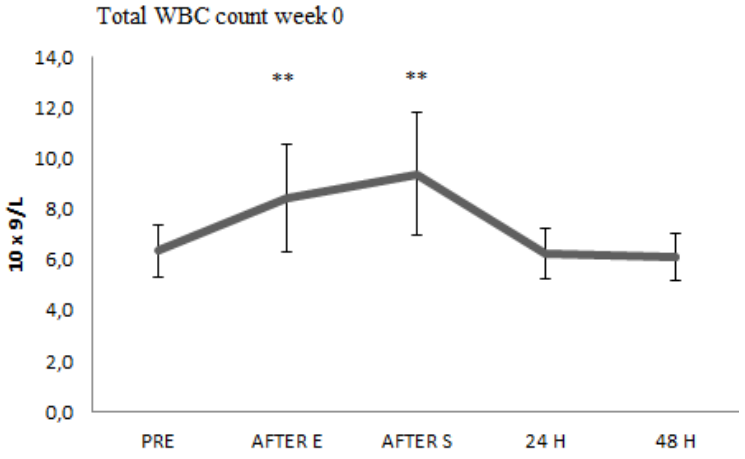

FIGURE 14. Total WBC count measured before (PRE) acute loading, after the endurance protocol (AFTER E), after strength protocol (AFTER S), 24 and 48 hours post acute loading prior to the 12-week training period, **p≤0.001 vs PRE, *n*=16.

Lymphocyte count increased 25% from the baseline following the endurance protocol, 2.46 ± 0.71 · 10^9/L and 3.07 ± 1.01· 10^9/L ($p \leq 0.05$) (Figure 15). Lymphocyte levels started to decline during the strength loading, when compared to levels measured after the cycling. Lymphocyte count returned near baseline level 24 hours after the acute loading (2.26 ± 0.63 · 10^9/L) and remained unchanged 48 hours after the loading (2.24 ± 0.43 · 10^9/L)

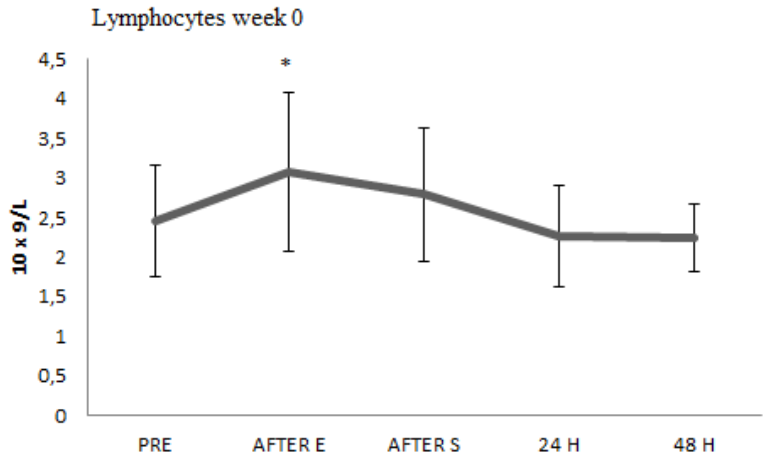

FIGURE 15. Changes in lymphocyte count measured before (PRE) acute loading, after the endurance protocol (AFTER E), after strength protocol (AFTER S), 24 and 48 hours post acute loading prior to the 12-week training period, *$p \leq 0.05$ vs PRE, *n*=16.

Blood neutrophils (Figure 16) increased 42% during the endurance loading when compared to baseline, from 3.24 ± 1.02 · 10^9/L to 4.61 ± 1.80 · 10^9/L ($p \leq 0.001$). Elevation continued, reaching 65% measured after the strength phase of the loading (5.34 ± 2.15 · 10^9/L) ($p \leq 0.001$). Neutrophil count dropped to the baseline level 24 hours (3.23 ± 0.87 · 10^9/L) after the loading with no further changes.

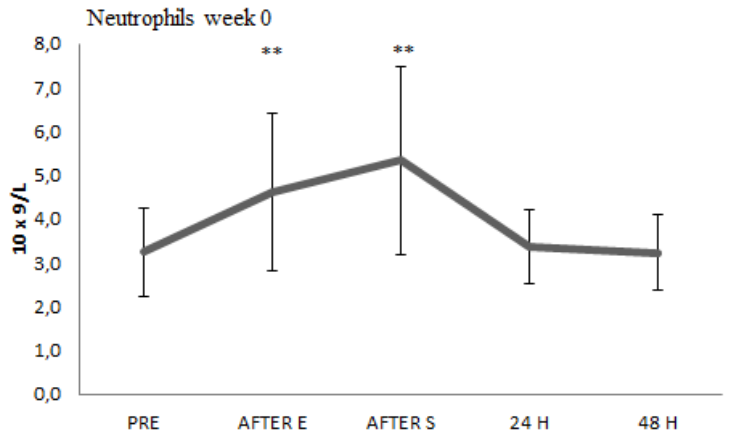

FIGURE 16. Changes in neutrophil count measured before (PRE) acute loading, after the endurance protocol (AFTER E), after strength protocol (AFTER S), 24 and 48 hours post acute loading prior to the 12-week training period, **p≤0.001 vs PRE, *n*=16.

Mixed cell count which included monocytes, eosinophils, basophils and immature precursor cells increased from pre acute E+S loading level 0.65 ± 0.21 · 10^9/L to 0.76 ± 0.24 · 10^9/L (p≤0.05) measured after endurance loading and then further to 0.81 ± 0.19 · 10^9/L (p≤0.05) measured after strength loading (Figure 17). In total mixed cell levels increased 24% with acute E+S loading (p≤0.05). Mixed cell levels measured 24 (0.64 ± 0.18 · 10^9/L) and 48 (0.63 ± 0.17 · 10^9/L) hours after the loading were at pre-loading level.

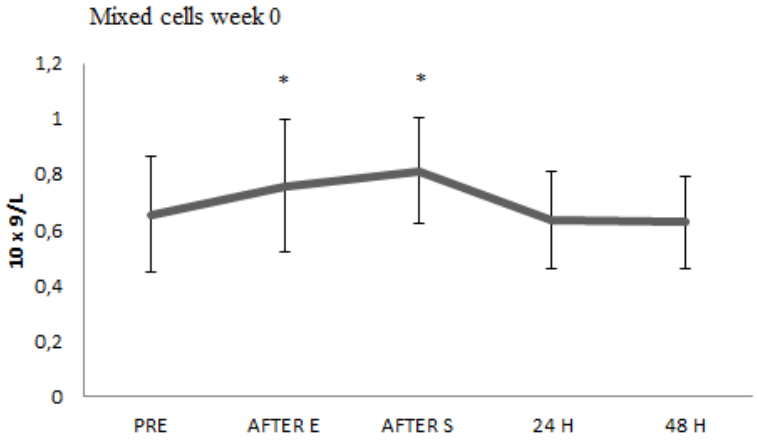

FIGURE 17. Changes in mixed cell count measured before (PRE) acute loading, after the endurance protocol (AFTER E), after strength protocol (AFTER S), 24 and 48 hours post acute loading prior to the 12-week training period, *p≤0.05 vs PRE, *n*=16.

7.3 *HSPA1A* expression in PBMCs

The acute response to E+S loading at the week 0 was represented by decrease in *HSPA1A* mRNA in four subjects, mRNA increased in PBMCs of two subjects. The response to acute loading using the same relative loads following 12 weeks of training was characterized by significant decrease ($p \leq 0.05$) in PBMCs' *HSPA1A* mRNA in all six subjects.

HSPA1A mRNA levels in PBMCs before the acute E+S loading in untrained men prior to the 12 week of combined training were in the range of 0.14–1.74. Following 12 weeks of training *HSPA1A* mRNA levels were between 0.70 and 1.63. The baseline values of *HSPA1A* mRNA following the combined training programme (week 12) were less scatter when compared to mRNA levels measured at week 0 (Figure 18). The post-loading *HSPA1A* expression after the training period (POST, week 12) was more constant and decreased in all subjects ($p \leq 0.05$).

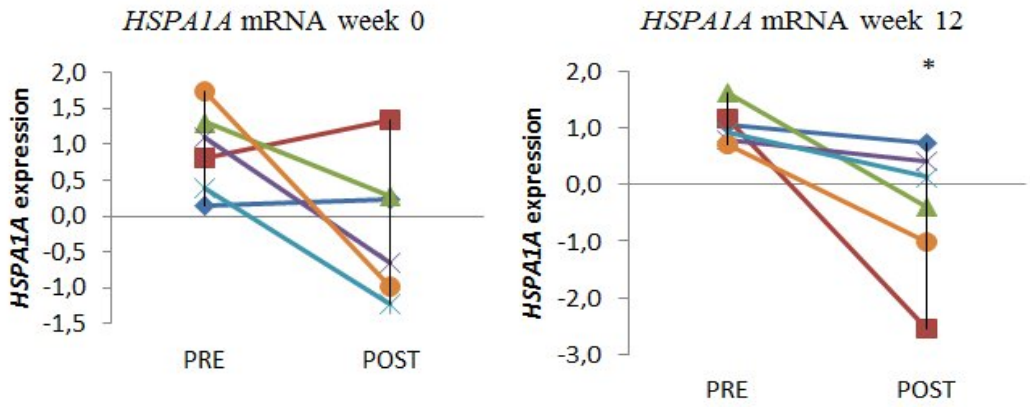

FIGURE 18. *Left: HSPA1A* mRNA expression before (PRE) and after (POST) an acute E+S loading prior to the training intervention. *Right: HSPA1A* mRNA expression before (PRE) and after (POST) an acute E+S loading following training programme; *$p \leq 0.05$ vs PRE, n=6.

Several correlations between *HSPA1A* expression and leukocytosis, leukocyte subsets and hormone values measured at the same time points were detcted. A tendency towards negative correlation was observed between *HSPA1A* mRNA levels and total leukocyte count following the acute loading prior to the 12 week training (r= -0.772, p= 0.72). Very strong correlation was observed also between *HSPA1A* mRNA levels and testosterone levels measured immediately after acute loading prior to 12 weeks of

training (r= 0.874, p= 0.023). After 12 weeks of training *HSPA1A* mRNA levels correlated strongly with growth hormone levels measured before acute loading (r= 0.886, p= 0.019)

7.4 Immediate responses to acute E+S loading following training

The total and differential WBC count changed in response to acute E+S loading before and after 12 weeks of training, although the differences in relative percent of increase were not significant.

TABLE 8. Changes in WBC in response to acute E+S loading.

Time point	tWBC	Neutrophils	Lymphocytes	Mixed cells
PRE w0	6.62±1.18	3.56±1.14	2.37±0.73	0.69±0.20
POST w0	9.63±2.10**	5.65±2.02**	2.60±0.89	0.82±0.15*
% of ↑	45%	59%	10%	19%
PRE w12	6.08±1.12	2.96±0.85	2.39±0.65	0.73±0.19
POST w12	9.28±3.08**	5.57±2.68**	2.86±0.89	0.86±0.24*
% of ↑	53%	88%	20%	18%
p value	p=0.343	p=0.197	p=0.224	p=0.776

Data is presented as mean±SD in 10^9/L; tWBC=total WBC; w0= week 0, w12=week 12; % of ↑= percent of increase; **p≤0.001 POST vs PRE; *p≤0.05 POST vs PRE; *p* value= difference between percent of increase in week 0 vs week 12; *n*=18.

7.5 Chronic effects of combined E+S exercise training

The absolute numbers of total WBCs decreased significantly in two time points out of the five (p≤0.05) with 12 weeks of combined E+S training (Figure 19). The baseline levels of WBC in blood of 16 men decreased from 6.61 ± 1.24 · 10^9/L prior to the training to 5.99 ± 1.06 · 10^9/L following the training programme (p≤0.05). Total WBC count decreased also significantly with 12 weeks of training in 24 hour time point, dropping from 6.69 ± 1.26 · 10^9/L to 5.90 ± 0.79 · 10^9/L (p≤0.05).

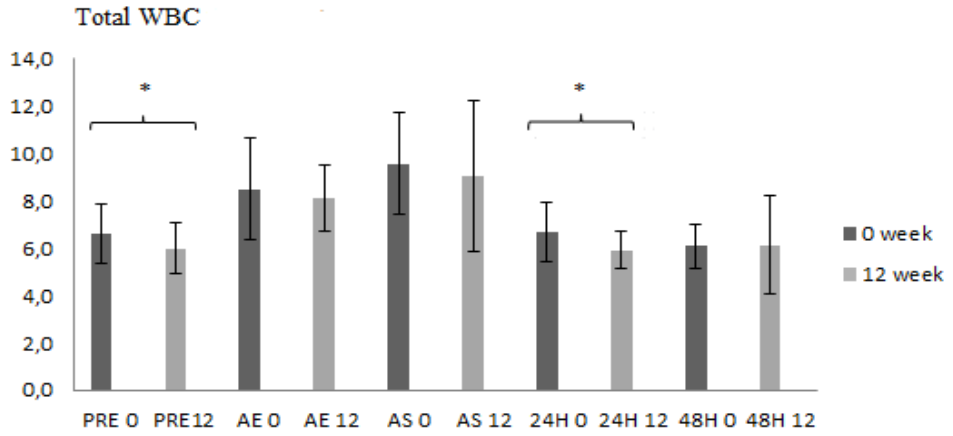

FIGURE 19. Changes in WBCs with 12-week of combined training; PRE= before acute E+S loading; AE – after endurance, AS – after strength; 0= week 0, 12=week 12; *p≤0.05 = week 0 vs week 12; *n*=16.

The absolute numbers of neutrophils decreased significantly in three time points following 12 weeks of combined E+S training (Figure 20). The basal neutrophil values dropped from 3.53 ± 1.14 · 10^9/L at week 0 to 2.85 ± 0.58 · 10^9/L at week 12 (p≤0.05). Neutrophil values were significantly lower also after the cycling part of the loading, declining from 5.03 ± 1.90 · 10^9/L to 4.14 ± 0.88 · 10^9/L (p≤0.05). Significantly lower neutrophil count was observed in 24 hour post-loading time point at week 12 (2.99 ± 0.62 · 10^9/L) versus week 0 (3.82 ± 1.20 · 10^9/L) (p≤0.05).

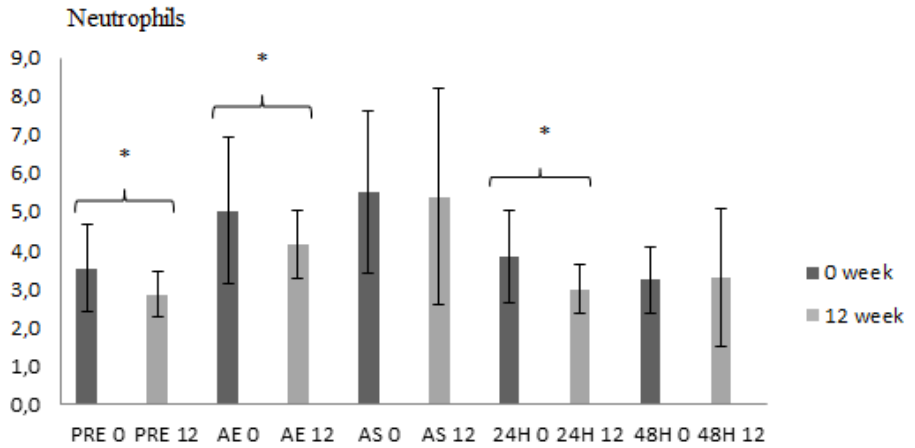

FIGURE 20. Changes in neutrophils with 12-week of combined training; PRE= before acute E+S loading; AE – after endurance, AS – after strength; 0= week 0, 12=week 12; *p≤0.05 = week 0 vs week 12; *n*=16.

Although the absolute changes in blood lymphocyte numbers did not reach significance level, an increase in response to the acute loading was observed at week 12 (Figure 21). Both, the endurance and strength phases of loading resulted in slightly greater lymphocyte count at week 12 when compared to pre training values; $3.16 \pm 1.04 \cdot 10^9$/L (week 12) versus $2.71 \pm 0.77 \cdot 10^9$/L (week 0) (p= 0.180) and $2.78 \pm 0.8 \cdot 10^9$/L (week 12) versus $2.59 \pm 0.85 \cdot 10^9$/L (week 0) (p= 0.215).

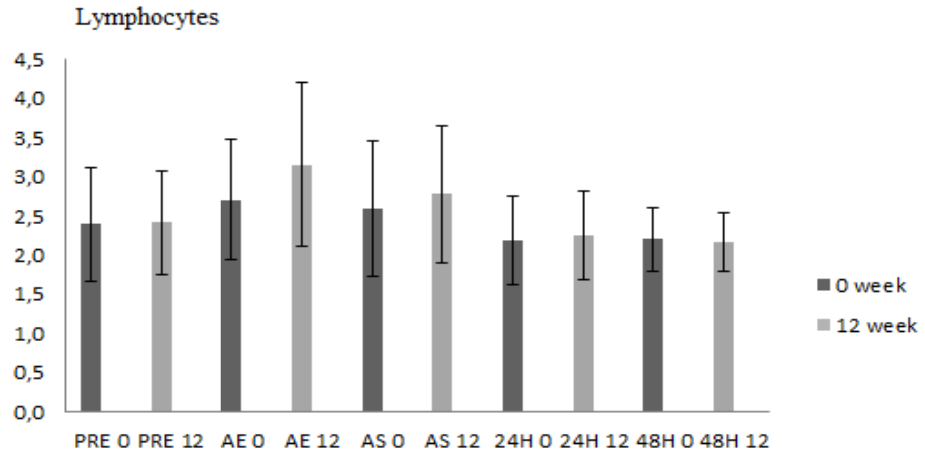

FIGURE 21. Changes in lymphocytes with 12-week of combined training. PRE= before acute E+S loading; AE – after endurance, AS – after strength; 0= week 0, 12=week 12; *n*=16.

Mixed cell count, similarly to lymphocyte count increased non-significantly after 12 weeks of training (Figure 22).

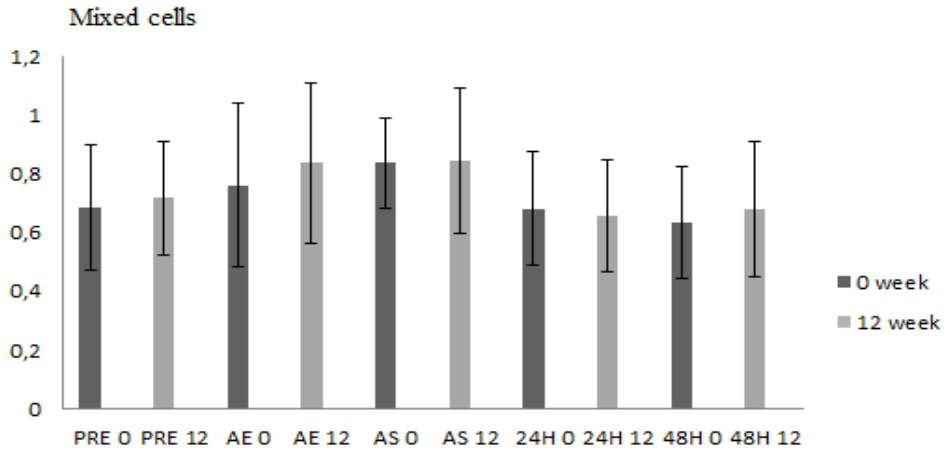

FIGURE 22. Changes in mixed cells with 12-week of combined training. PRE= before acute E+S loading; AE – after endurance, AS – after strength; 0= week 0, 12=week 12; *n*=16.

7.6 Hormones and leukocytes

GH concentration increased significantly in response to acute E+S loading at week 0 and week 12 ($p \leq 0.001$), whereas cortisol and testosterone levels did not change significantly with exercise bout (Table 10). Testosterone levels increased at the baseline with 12 weeks of combined training ($p \leq 0.001$).

TABLE 10. Serum hormone concentration.

	w0 PRE	**w0 POST**	**w12 PRE**	**w12 POST**
GH (µg/L)	0.89 ± 0.35	10.29 ± 2.19**	2.17 ± 1.05	7.34 ± 1.21**
Cortisol (nmol/L)	496 ± 24	496 ± 56	500 ± 21	497 ± 36
Testosterone (nmol/L)	13.82 ± 1.07	14.60 ± 1.28	15.38 ± 0.98#	16.70 ± 1.15

Data is presented as means ± SD; GH – growth hormone, w0= week 0, w12= week 12, PRE= before acute loading, POST= after acute loading, **$p \leq 0.001$ pre vs. post; # $p \leq 0.001$ pre at week 0 vs. pre at week 12; *n*=16.

Several significant correlations between GH and blood leukocytes were detected at the same time points during the acute loadings before and after 12-week combined E+S training. The insignificant correlation data is not presented. Prior to the beginning of 12-week training intervention a negative correlation was observed between mixed cell count and GH at the fasting state ($r = -0.527$, $p = 0.025$). Tendency towards positive correlation was detected between total WBC count and GH concentration after the acute E+S loading ($r = 0.417$, $p = 0.108$). Following 12 weeks of training a negative correlation was detected between mixed cell count and GH at the fasting state ($r = -0.558$, $p = 0.025$). Positive correlation was detected between total WBC count and GH levels following the acute loading ($r = 0.612$, $p = 0.012$).

8 DISCUSSION

The purpose of the current study was to examine and describe the effects of acute E+S loading and combined E+S training on the total and differential leukocyte count. The main finding of the study demonstrated that acute E+S loading induced substantial leukocytosis in the blood of untrained healthy men and 12 weeks of combined E+S training caused a decrease in basal leukocyte values. Additionally, enhanced response to an acute E+S loading was observed in several leukocyte subsets following the training programme. The levels of *HSPA1A* mRNA transcripts at the baseline increased after combined E+S training. Noteworthy, combined E+S training programme did not change substantially either the weight or BMI of the subjects, but significant improvement in strength parameters was observed.

8.1 Immediate responses to acute E+S loading in untrained men

Acute exercise bout induces general leukocytosis (Gabriel & Kindermann 1997) and affects differential counts of immune system cells, as changes in leukocyte subsets are often seen already during and immediately after the exercise bout (Pedersen & Hoffman-Goetz 2000). Independently, endurance (Natale et al. 2003) and strength (Simonson 2001) exercise bouts induce leukocytosis in untrained subjects, whereas the effects of concurrent endurance and strength exercise bouts on immune system parameters are less known. Combined exercise bout results in dual (endurance and resistance) responses that may interact and interfere with each other (Arazi et al. 2011).

Arazi et al. (2011) reported a leukocytosis in strength-trained male university students that was induced by concurrent exercise bout. These findings are in agreement with the findings of the present study, where substantial leukocytosis was detected in untrained men following an acute E+S loading protocol. In present study blood samples were obtained also between the endurance and strength phases of the loading. If performed on separate occasions, continuous moderate intensity endurance exercise induces greater leukocytosis than the circuit of resistance exercises (Natale et al. 2003). In current

combined bout, cycling exercise at 65% of the maximal workload for 30 minutes increased leukocyte levels at the greater extent than did subsequently performed strength protocol. Although, the impact of strength loading phase on blood leukocytes can not be assessed separately and the second phase of the loading comprises the combined effects of both exercise modes. Therefore, it can be said that cycling-induced leukocytosis continued into the strength loading but the initial elevation of total and differential WBC counts was greater.

The data concerning the effects of the mode, duration and intensity of the exercise on leukocytosis is controversial. Gimenez et al. (1986) reported that endurance exercise-induced leukocytosis was more related to the intensity than duration; in contrast Natale et al. (2003) found that prolonged exercise caused greater leukocytosis than high intensity short exercise and resistance exercise bout. In present study, both protocols lasted about 30 minutes and the relative physical strain of the protocols were analogous, thus seems that mode of the exercise (continuous endurance versus intermittent resistance) affected the leukocytosis.

Physical exercise is characterized by biphasic alterations in circulating leukocyte counts where instant mobilization of leukocytes is followed by transient lymphopenia below the resting levels and prolonged neutrophilia. Leukocyte numbers return to baseline values within couple of hours (2-4 hours) after cessations of the exercise, although the homeostasis may not be achieved after the high intensity long duration exercise. (Hansen et al. 1991, Rowbottom & Green 2000.) However, in present study the blood sampling time points did not allow to examine whether the acute E+S loading elicits the phenomenon of biphasic leukocytosis. Blood samples obtained 24 and 48 hours after the acute E+S loading demonstrated that homeostasis in immune system cells was achieved, as total and differential leukocyte count returned to the baseline values.

Prolonged aerobic exercise, and to the slightly lesser extent resistance exercise, induce substantial neutrophilia (Natale et al. 2003). In current study, cycling was performed first and induced great netrophilia, strength loading performed afterwards added to cycling-induced neutrophilia. Neutrophils contributed to total leukocytosis the most when compared to other immunocompetent cells, and this finding is in agreement with the results from other studies (McCarthy & Dale 1988, Natale et al. 2003). In Natale et

al. (2003) study prolonged aerobic exercise, short peak aerobic exercise and circuit resistance exercise induced significant leukocytosis due to increase in circulating neutrophil and monocyte quantity.

Lymphocyte numbers increased during the first phase of acute E+S loading, but following the cessation of cycling exercise lymphocyte count started to decline. The initial lymphocytosis can be due to recruitment of all lymphocyte subsets to the blood (Pedersen & Hoffman-Goetz 2000) and the subsequent decline, already during the exercise, can be caused by the increase in cortisol levels (Shinkai et al. 1996). In present study the correlation analysis did not reveal any associations between lymphocyte count variations and cortisol levels. Lymphocyte values started to decline during the strength exercise but they did not drop below the baseline level by the end of the acute E+S loading. The fall below the pre-values can not be excluded as the blood samples were not obtained in recovery period, i.e. 2-4 hours after the exercise when lymphopenia is the most prominent (McCarthy & Dale 1988, Pedersen et al. 1998).

Mixed cell count, including monocytes, eosinophils and basophils, increased continuously during the acute E+S loading. Likewise, Hulmi et al. (2010) as well as Risøy et al. (2003) reported the increase in mixed cell count following a resistance exercise bout in men. Natale et al. (2003) described substantial increase in monocyte count in response to the prolonged and short aerobic and resistance type exercise. Gabriel and Kindermann (1997) observed great increase in eosinophil count in response to anaerobic and short-term highly intensive exercise and mild increase in response to aerobic exercise. The number of basophils in peripheral circulation is very low and this cell type seems to be unresponsive to exercise stress (Simonson & Jackson 2004). Thus, in present study it might have been that eosinophils and monocytes contributed to exercise-induced increase seen in mixed cell count.

8.2 Immediate responses to acute E+S loading following exercise training

The effects of an acute exercise bout following regular exercise training on circulating leukocytes are not studied much. In current study, the acute E+S loading was used to

examine the effects of combined E+S training programme on blood leukocytes, as the same relative load in acute E+S loading was used before and after the 12-week training programme. Even though the difference in relative percent of increase did not reach significance levels, neutrophil count increased more in response to acute E+S loading following the training than it did prior training intervention. Lymphocytosis and general leukocytosis was also slightly more substantial after the training, whereas there were almost no changes in mixed cells response. Neutrophils contributed to general leukocytosis during acute E+S loading in both time points the most, whereas the contribution of other cells changed. Namely, lymphocytes increased more during the second acute E+S loading and mixed cell count increase diminished at the same time point. In contrast, longitudinal endurance and resistance studies examining blood leukocytes redistribution before and after the training interventions did not report any substantial changes. In Rhind et al. (1996) study 12 weeks of endurance training did not alter the proportion of the cells contributing to leukocytosis. Similarly, no changes were detected in previously untrained men after 21 weeks of resistance training (Hulmi et al. 2010).

The increased neutrophilia in response to second acute E+S loading can not be explained by increase in GH concentration, which is known to affect neutrophil count (Pedersen & Toft 2000), as GH increase was lower than in the first acute E+S loading. In addition to GH, catecholamine levels during the exercise affect the neutrophil count (McCarthy et al. 1992b). Although norepinephrine and epinephrine levels were not measured in present study, it can be speculated that concentration of these hormones may have altered the exercise-induced neutrophilia.

8.3 Chronic effects of combined E+S training

The effect of the chronic exercise training on immune cell number is rather modest (Mackinnon 2000, Gleeson 2007). Nevertheless, in cross-sectional study of Horn et al. (2010) lower basal total leukocyte count in elite athletes who are engaged in aerobically oriented sports such as cycling or triathlon were reported. In longitudinal study conducted by Michishita et al. (2010) individuals participating regularly in endurance training had lower baseline total WBC count. The results of current study are in

agreement with above mentioned studies, as 12 weeks of combined E+S training lowered subjects' resting total WBC count.

As reported by Hulmi et al. (2010) and Cardoso et al.(2012) participating regularly in strength training does not affect resting leukocyte values. In the present study strength training was combined with endurance training in one session, and therefore it can be speculated that endurance part of the combined E+S training might be responsible for lowering basal leukocyte values in healthy men. Similar tendency was observed in Ramel et al. (2003) study, where the subjects who participated in aerobic training in addition to resistance training (on separate occasions) had lower resting WBC values. The lower resting WBC counts may represent an anti-inflammatory adaptation induced by regular exercise training rather than pathological response (Horn et al. 2010).

In addition to lower total WBC count in this study, combined E+S training resulted in lower resting neutrophil values. Similar finding was reported in elite endurance athletes in Horn et al. (2010) study. In a contrary, volley ball players and long-distance runners in Saygin et al. (2006) study and orienteers in Risøy et al. (2003) study had higher neutrophil values at rest than sedentary controls. Interestingly, 21 weeks of strength training did not induce any changes in resting neutrophil count in young or old men (Hulmi et al. 2010), but results of the current study show that strength training in combination with endurance training decreases neutrophils values in healthy men at rest. Similarly to decrease observed in total WBC count, decrease in neutrophil count may reflect adaptive anti-inflammatory response to exercise training (Horn et al. 2010).

Regular endurance type exercise training does not affect the number of lymphocytes at rest (Moyna et al. 1996). In the present study, lymphocyte count did not change noticeably following 12 weeks of combined E+S training. Likewise, two weeks of strength training did not change lymphocyte numbers at rest in recreationally active men and blood lymphocyte count in young orienteers did not differ from sedentary controls (Risoy et al. 2003). Resting lymphocyte concentration did not differ between physically active men and women and sedentary subjects in Moyna et al. (1996) study. Only in Ferry et al. (1990) study young cyclists had higher lymphocyte count compared to sedentary controls at rest. Similarly to lymphocyte count, mixed cell count including monocytes, eosinophils and basophils, did not change with 12 weeks of training. No

changes in mixed cell count or independent monocyte count in response to training were observed in the other longitudinal resistance (Hulmi et al. 2010, Risoy et al. 2003) or endurance (Ferry et al. 1990) exercise studies.

8.4 *HSPA1A* responses to combined E+S loading and training

During the exercise, several biophysical and biochemical stimuli work together to induce rapid up-regulation in immediate early genes, including *HSPA1A*. The changes in this group of genes are already seen as early as 10 minutes after the stimulus. (Simon et al. 2006). In present study, the *HSPA1A* gene expression in response to an acute E+S loading containing cycling and strength exercises yielded unexpected results. In contrast to elevation of *HSPA1A* mRNA content reported in literature, levels of *HSPA1A* transcripts decreased in response to acute exercise bout in present study.

The increase in *HSPA1A* expression in leukocytes or PBMC has been reported mainly after endurance type of exercise of different durations and intensities (Connolly et al. 2004, Fehrenbach et al. 2000a, Sakharov et al. 2009, Schneider et al. 2002, Shin et al. 2004). *HSPA1A* expression response to resistance exercise has mostly been studied in skeletal muscle tissue (Liu et al. 2000, Liu et al. 2004), and results of the studies implying strength protocols describe an increase in gene expression. Additionally, the strain of the exercise plays a role in *HSPA1A* expression, as moderate and intense exercise induces greater up-regulation (Yamada et al. 2008). Collectively, sufficient intensity endurance and resistance exercise protocols performed separately elicit an increase in *HSPA1A* expression.

In this study, *HSPA1A* mRNA content in PBMCs increased in two subjects and decreased in four subjects in pre training measurements. Following 12 weeks of training *HSPA1A* mRNA levels decreased in all six subjects in response to acute loading. Although an up-regulation in *HSPA1A* expression was expected, long and inconsistent nature of the acute loading may have been the reason for such results. The timing of the blood samples did not allow to examine whether there was an increase in *HSPA1A* mRNA after the cycling part of the loading and if the strength loading phase was the reason of the *HSPA1A* down-regulation. In addition, the overall intensity of the acute

loading may have been insufficient to elicit an elevation in *HSPA1A* expression. The exact reason for *HSPA1A* mRNA decrease in present study is unknown. It can be speculated that transcripts of *HSPA1A* were used for translation into the protein at that time point. The doubts related to housekeeping gene (*β-actin*) unsuitability were unfounded, as acute loading did not change endogenous control levels and *β-actin* has been used as a control in leukocytes in previous exercise studies (Fehrenbach et al. 2001, Fehrenbach et al. 2000a). Undoubtedly, the lack of clear and uniform changes in *HSPA1A* mRNA can be due the the small sample size, since only PBMCs from six subjects were used for analysis.

Regular exercise training results in higher resting *HSPA1A* mRNA values (Yamada et al. 2008). In present study, baseline values of *HSPA1A* mRNA changed with 12 weeks of combined E+S training and subjects had higher mRNA content at rest following the training programme. Similar observations are found in cross-sectional studies (Fehrenbach et al. 2000a, Fehrenbach et al. 2000b) where endurance trained athletes have higher mRNA content if compared to untrained controls. It is unknown whether the regular resistance exercise training elicits the same changes, as there are no studies reporting baseline mRNA values of *HSPA1A* in leukocytes or PBMC. Nevertheless, the combined E+S training seems to induce increase in resting *HSPA1A* mRNA levels that may suggest an adaptation to exercise-stress, as higher mRNA content may assure faster translation of HSPA1A protein (Yamada et al. 2008).

8.5 Leukocyte and hormone interactions

In response to exercise the concentration of several hormones (e.g. GH, cortisol, catecholamines and testosterone) that have immunomodulatory effects increases (Gleeson 2007, Pedersen & Hoffman-Goetz 2000). In present study positive relationship between increase in GH concentration and general leukocytosis after the acute E+S loading at week 0 and week 12 was detected. GH modulates neutrophil count and function during the exercise (Hoffman-Goetz & Pedersen 1994), and as neutrophils increased the most during acute E+S loading it can explain this exercise-induced neuroimmune interaction. Similar findings were reported in men after the marathon race

where concurrent elevation in GH and neutrophilia were suggested to be related (Suzuki et al. 2000).

GH concentration measured in the morning after 12 hours of fasting correlated negatively with mixed cell count prior to and following the training programme. The negative relationship between these variables has not been reported elsewhere in literature so far. It is known that GH is released into circulation at the pulsatile manner and GH secretion increases at night during the sleep (Muller et al. 1999). Mixed cell count comprises primarily monocytes, eosinophils and basophils, therefore it can be speculated that one or more of the three cell types might be affected by GH concentration at this specific time point.

In cross-sectional studies (Kim et al. 2005, Metrikat et al. 2009, Michishita et al. 2008) the association between physical parameters, such as VO_{2max}, and leukocyte count was seen, as higher VO_{2max} values were associated with lower total or differential WBC counts. In this longitudinal study no such association was detected, and the number of immune cell decreased but no significant improvement in VO_{2max} was observed. The lack of improvement in cardiorespiratory parameter may be the reason why no relationship between leukocyte counts and physical parameters established. Up to date there is no information about leukocyte and strength parameter associations. In current study increase in 1RM was substantial but now correlations between strength and WBCs were observed.

8.6 Strengths and weaknesses of the study

Description of the combined E+S exercise bout (acute E+S loading) induced leukocytosis can be considered the main strength of the present study. In literature predominantly separate endurance and strength type exercise bouts have been employed to evaluate the effects on peripheral leukocytes. Thus for the first time, as known to the author of this thesis, effects of concurrent exercise modes performed in one session by recreationally active young men have been studied and defined.

The potential weakness of the present study was the absence of a control group, which would have allowed excluding other possible factors that may have affected leukocyte

count during the study period. One reason for the absence of the control group is the fact that data used in this thesis was a part of the larger research project, and control subjects' data was not collected during specific time period used in current study. Although, the total WBC count seems to relatively stable across the seasons, with exception during the time of an acute infections which leads to the increase in leukocytes (Maes et al. 1994), the rise in WBCs count may occur during the winter months (Gidlow et al. 1983). In current study, training intervention ended in the winter and decrease in WBC count during the winter months provides the support to the finding of exercise-induced adaptations in immune system cells. However, in the future a control group should be included into experiments to confirm the results of a present study.

The absence of catecholamine measurements can be seen as a shortcoming, it is known from other studies that these hormones do affect leukocyte count during the exercise. However, similarly the reason for this shortcoming was the fact that no catecholamine data was needed in the main project. In subsequent combined training studies, the concentration of these hormones should be measured to examine the interaction between leukocytes and catecholamines. Additionally, the number of the subjects used for *HSPA1A* mRNA measurements was small, as only six participants had the blood sample drawn at corresponding time points. In the prospect experiments more subjects should be included to measure *HSPA1A* mRNA expression in response to combined exercise bout, plus additional samples in post-exercise time point should be collected for HSPA1A protein measurements. Larger sample size would have resulted in stronger correlations between immune system cells, *HSPA1A* mRNA, hormones or physical performance parameters.

Total and differential WBC count in present study was measured using automated haematology analyser. Generally, the estimates of WBC counts obtained using automated analysers are accurate (Buttarello 2004). In addition, automated haematology analyser provides fast, accurate and reliable readings when compared to manual microscopic blood examination (Ike et al. 2010). However, in the literature a flow cytometry method has been used to accurately measure differential WBC counts. For instance, the automated haematology analysers are not as precise in differentiating basophils when compared to fluorescence-based flow cytometry (Ducrest et al. 2005).

The use of haematology analyser in present study can be explained by the fact that total and differential WBC count analysis was not the main purpose of the larger research project. In prospective exercise immunology studies a flow cytometry should be employed for precise differential leukocyte measurements.

8.7 Summary, conclusions and practical applications

In this thesis the impact of combined E+S exercise and training on the number of immune system cells was examined. The acute E+S exercise bout elicited leukocytosis in untrained men similar to leukocytosis seen in independent endurance or strength exercises. The decrease in number of total WBCs and neutrophils at rest following the combined E+S training period was observed. The exercise-induced leukocytosis was greater following the 12-week combined E+S training when compared to pre-training exercise bout. In addition *HSPA1A* mRNA content increased at rest after the combined E+S training period. Two noteworthy associations between GH and leukocytes were observed, namely total WBC count correlated positively with GH levels measured after the exercise bout, and an inverse correlation appeared between GH and mixed cell count prior to the acute E+S loadings.

According to the results of the study it can be said that regular combined E+S training alters peripheral immune system beneficially, since the number of total leukocytes and neutrophils decreased during the 12-week combined E+S training period. An amplified response of blood leukocytes to second acute E+S loading performed following combined E+S training period was detected. Together these alterations may suggest an adaptation of immune system cells to regular physical exercise, as cells can be mobilized rapidly in response to stressful stimuli and maintained low in absence of the stress. The content of stress protein *HSPA1A* transcripts at rest in PBMC increased during combined E+S training period which refers to an adaptation to physical stress at the intracellular level, since higher level of transcripts permits rapid stress protein synthesis. The exact reason for *HSPA1A* mRNA decrease in response to the exercise bout is unknown. It can be speculated that subjects' interindividual variation in response to an exercise stress might have affected the results.

In practical terms it can be concluded that intensity, mode and volume of the exercise training used in current study altered peripheral immune system beneficially and combined E+S training can be recommended to previously untrained individuals. The alterations in total and differential leukocyte counts were within clinical range and increase in *HSPA1A* mRNA at the baseline is consistent with adaptation to regular training reported in literature.

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