EFFECTS OF EXERCISE ON AGE- AND STRESS- RELATED ATTENUATION OF VACCINATION RESPONSES IN MICE

BY

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ABSTRACT

Several published studies suggest that acute eccentric exercise and exercise training can improve vaccination responses in humans. Normal aging and chronic stress can lead to immunosenescence and immunosuppression, respectively, and there may be a role for exercise in augmenting immune responses under these conditions. However, the underlying mechanisms as to how such exercise might promote this effect is unclear. In order to understand the potential eccentric exercise-induced beneficial effect, verification is needed in an animal model. In the first cohort of experiments, we examined the effects of acute eccentric exercise on primary antibody and cell-mediated responses to vaccination in young mice and aged mice. First, we examined the effects of acute eccentric exercise on primary immune responses to ovalbumin (OVA) vaccination in young mice. Young mice were exercised at 17m/min speed at -20% grade for 45 minutes on a treadmill (ECC1) or remained sedentary (SED). Both ECC1 and SED mice were intramuscularly (IM) injected with 100μg of ovalbumin and 200μg of alum adjuvant immediately after exercise. At three weeks post-exercise, all mice were injected with OVA into the dorsal side of ear to determine the delayed-type hypersensitivity (DTH) response as a measure of cell-mediated immunity to the vaccination. Ear thickness was measured immediately before and every 24h after intradermal treatment. In the second experiment, two bouts of downhill treadmill running were performed on consecutive days (ECC2) and all young mice were vaccinated immediately after the second bout of exercise. In the third experiment, young mice were randomly assigned to an eccentric electrically-stimulated group (ECCstim) or a sham

group (Sham). Mice were then vaccinated 6 hours post-exercise. In these three experiments, plasma was collected prior to, and at one, two and four weeks post-vaccination. ELISA was performed to analyze anti-OVA IgG. In all three experiments, there was a significant time main effect indicating plasma anti-OVA IgG was significantly increased at one, two and four weeks relative to pre-immunization. However, there were no significant differences between ECC1, ECC2 or ECCstim and respective control groups, demonstrating that acute eccentric exercise does not improve primary antibody responses in young mice. Also, we did not find significant differences between ECC1 and SED in their DTH responses. Then we replicated the study to determine the effects of acute eccentric exercise on the immune responses to OVA vaccination in aged mice. Aged mice (27 months) in the eccentric exercise group (ECCaged) performed the same single bout of treadmill running as mentioned before. Both ECC and SED mice were IM vaccinated immediately after exercise. Plasma was collected prior to, and at one, two and four weeks post-vaccination. ELISA was performed to analyze anti-OVA IgG. In addition, DTH responses were measured at three weeks post-exercise as mentioned before. We found a significant difference between ECC and SED groups in ear DTH at 24h post-injection, indicating that eccentric exercise increased cell-mediated, but not antibody, responses in aged mice. In conclusion, we found acute eccentric exercise enhanced cell-mediated response in aged mice, but not antibody responses in either young or aged mice. It has been shown that chronic restraint stress suppresses immune responses to vaccination. Therefore, in the second cohort of experiments, we investigated the effects of acute eccentric exercise and voluntary wheel exercise training on antibody and cell-mediated immune responses to vaccination in chronically

stressed mice. Mice were randomized into four groups: No stress, Stress-ECC, Stress-VWR and Stress-SED. Mice in the three stressed groups received restraint stress for 6hours/day, 5 days/week for three weeks. Body weights were measured daily immediate after stress session. After one week of stress, Stress-ECC mice performed the same single bout of treadmill running as mentioned before. Stress-VWR mice voluntarily ran on a telemetered wheel for the entire period of experiment. All groups of mice were IM vaccinated immediately after the eccentric exercise. Plasma was collected prior to, and at one, two and four weeks post-vaccination. ELISA was performed to analyze anti-OVA IgG and anti-OVA IgM. In addition, all mice received ear injections after three weeks of stress and DTH responses were measured as mentioned before. We found that restraint stress significantly reduced body weight and caused adrenal hypertrophy. We also found there was a trend that both Stress- ECC and Stress-VWR groups elevated anti-OVA IgM and anti-OVA IgG responses compared to Stress-SED group. In conclusion, acute eccentric exercise and voluntary exercise training trends to alleviate the chronic stress- induced reductions in vaccination responses.

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CHAPTER 1

INTRODUCTION

1.1 Significance

Vaccination is one of the most successful public health interventions in preventing infectious diseases and reducing the mortality and morbidity rates associated with these diseases . However, there is a significant variation in vaccine efficacy between young and older adults [2]. The Centers for Disease Control and Prevention reported that influenza vaccination efficacy in the young population is 70-90%, whilst only 17-53% in the aged population [3]. In order to promote vaccine responses, we need to either improve vaccines themselves or find behavioral interventions that alter host factors to augment immune responses to vaccination.

Exercise has been proposed to be an effective, cost-efficient behavior intervention to enhance immune function and has a potential beneficial effect on immune responses to vaccination. Several human studies have suggested that acute eccentric exercise enhances vaccination responses [4-6]. Eccentric exercise causes the exercising muscle to produce continuous force while it lengthens, leading to damage of the internal structure of the muscle fibers and connective tissue [7]. The substantial increase in plasma creatine kinase indicates this damage is greater than that caused by concentric or shortening muscle contractions [8]. This muscle damage results in a localized inflammatory response and delayed onset muscle soreness when conducted in naive participants [9]. It is hypothesized that the influx of immune cells and the release

of inflammatory mediators caused by this eccentrically-induced muscle damage creates a pro-inflammatory environment in a muscle that may activate dendritic and other cells to augment the immune response to vaccination when given intramuscularly in the damaged muscle [10]. This heightened inflammatory environment may be a particularly effective behavioral adjuvant as eccentric exercise localizes muscle damage to the specific site of intramuscular vaccine administration. This strategy could be easily used to augment IM vaccination responses in aging populations.

Exercise training has also been proved to augment either humoral or cell-mediated immunity to vaccination, especially in the elderly. Our lab has conducted two experiments comparing 10 months of aerobic moderate exercise intervention (30-60 min/session, 3 sessions/week) to flexibility/balance training in older adults [11, 12]. We found that cardiovascular exercise resulted in a longer lasting seroprotection to influenza vaccine when measured 24 weeks post-vaccination, whereas flexibility/balance training did not [12]. Similarly, aerobic exercise training induced higher primary IgG1 and IgM antibody responses compared to the sedentary controls [11]. However, not all studies reported beneficial effects of exercise training in young and middle-aged adults. Long et al. showed a life-style physical activity intervention had an increase in walking behavior and quality of life, but not antibody responses to pneumococcal vaccination compared to the control group [13]. Therefore, exercise training may be favorable to improve vaccine responses in an immunosuppressive setting.

Besides aging, chronic stress also suppresses immune function including DTH responses and antibody responses to vaccination. Dhabhar et al. has reported that chronic restraint stress with 6h/day for three to five weeks significantly reduced DTH responses in rats and increasing stress exposure was also associated with reduction of peripheral blood lymphocytes redistribution and decreased glucocorticoid responsivity [14]. Other stressors, such as social stressor, have also been shown to reduce antibody responses to vaccination [15].

Despite the potential beneficial role of acute eccentric exercise on immune responses to vaccination, the underlying mechanisms remain understudied. Examination of the mechanisms are needed in order to verify and understand if initial findings support the idea of using this strategy to enhance vaccination responses. Therefore, we propose to use an animal model to study the mechanisms behind the purported acute eccentric exercise-induced augmentation of immune responses to vaccination. **The purpose of this study was to examine the effects of exercise on age- and stress- related attenuation in mice.** We will use a completely novel benign protein antigen ovalbumin (OVA) to investigate primary immune responses in all experiments. We hypothesized that eccentric exercise will improve immune responses in young and aged mice; exercise will attenuate chronical stress- induced reductions in vaccination responses.

1.2 Specific Aims

1. **Determine the effects of eccentric forced downhill running exercise**

on the immune response to vaccination in young mice. We hypothesize that eccentric exercise will augment both antibody and cell-mediated immune responses in young mice.

2. **Determine the effects of electrically-stimulated eccentric contractions on primary antibody responses to vaccination in young mice**. While forced downhill running promotes eccentric damaging contractions, it also induces a significant stress response that could confound or mask the effects of eccentric exercise. The use of electrically-stimulated eccentric contractions in anesthetized mice (vs. sham control) overcomes this limitation allowing us to determine the direct role of exercise-induced muscle damage on immune responses to vaccination. We hypothesize that electrically-stimulated eccentric contractions will result in greater antibody immune responses to vaccination when compared to forced downhill running.
3. **Determine the effects of eccentric forced downhill running exercise on the immune response to vaccination in aged mice.** We hypothesize that eccentric exercise will improve both antibody and cell-mediated immune responses in immunosenescent aged mice.
4. **Determine the effects of acute eccentric exercise on immune response to vaccination in chronically stressed mice.** It has been reported that chronic restraint stress can suppress immune function in mice. We hypothesize that acute eccentric exercise will alleviate the chronic stress suppressive antibody and cell-mediated immune response

to vaccination in young mice.

5. **Determine the effects of voluntary wheel training on immune response to vaccination in chronically stressed mice.** We hypothesize that voluntary exercise training will alleviate the chronic stress suppressive antibody and cell-mediated immune response to vaccination in young mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Exercise and Vaccination

Exercise has been proposed to act as an adjuvant to vaccination in some settings to enhance vaccine efficacy. Exercise can be categorized into exercise training (months to years) and acute exercise (minutes/hours) based on the exercise duration. Here I summarize the current literature for effects of both chronic and acute exercise on vaccination in young and middle aged adults and the hypothesized mechanisms including animal studies. Effects of exercise on vaccination in older adults and the potential mechanisms will be reviewed in the next section.

Exercise training and Vaccination

There have been three studies that have examined the effects of exercise training on vaccination in young and middle aged adults [13, 16, 17]. Long et al. conducted a randomized controlled trial (RCT) to investigate whether a life-style physical activity intervention improved antibody response to pneumococcal vaccination in sedentary middle-aged women [13]. Women in the exercised group, who completed a 16-week training program, had an increase in walking behavior and quality of life compared to the control group. However, there was no differences in antibody responses to pneumococcal vaccination between groups. In addition to the RCT study, there were two cross-sectional studies that reported the effects of exercise training on vaccination in young adults [16, 17].

Schuler et al. examined the effect of moderate physical activity/fitness on immune response to influenza vaccine in college students [16]. Participants were classified into groups according to self-reported physical activity and measured physical fitness (VO_{2max}). They found neither physical fitness nor physical activity were associated with the antibody response to influenza vaccination. Another cross-sectional study compared active young adults and sedentary controls and found a positive relationship between exercise training and stronger antibody responses to influenza vaccination [17]. Participants were assigned to an exercise group and completed heavy training for three weeks or to a control group that maintained normal activity based on measured aerobic capacity. Though there were no differences in the immunoglobulin G (IgG) response to influenza vaccine between groups at 14 days after vaccination, there was a significant difference in baseline IgG between groups and the heavy training group had a higher IgG concentration maintenance at 12 months post-vaccination. Given the mixed findings in young and middle-aged adults, exercise training may not be effective at enhancing immune responses in younger cohorts that already exhibit strong responses.

Kapasi et al. compared different duration of moderate exercise training on antibody immune responses in young mice [18]. Female C57BL/6 mice were randomized into 2-week exercise training, 8-week exercise training or sedentary control group. Mice conducted a 2-week exercise had a significant increase in secondary antibody levels, which were similar to the extent of 8-week exercise

group. Studies on effects of chronic stress on immune responses to vaccination in animals will be reviewed in a later section.

Acute Exercise and Vaccination

There have been two studies that have examined the effects of acute prolonged, intense exercise on vaccination in young adults [19, 20]. The “Open window hypothesis” states that prolonged, intense exercise will temporarily suppress immune function at 2-24h after exercise because of an increase in plasma cortisol level, a decrease in blood lymphocyte numbers, natural killer cell numbers and neutrophil phagocytic function [21]. Bruunsgaard et al. found that a half-ironman competition did not elevate antibody responses to diphtheria, tetanus toxoid and pneumococcal vaccination compared with either sedentary adults or resting athletes [19]. Eskola et al reported that participants (n=4) that completed a marathon had a higher antibody responses to tetanus toxoid vaccination compared to a sedentary control group, results contrary to the “open window hypothesis”[20].

Contrary to prolonged intense exercise, acute moderate exercise is thought to be beneficial to immune functioning [5].There were several studies exploring the effects of acute moderate exercise on vaccination in young adults. Edwards and colleagues recently conducted a series of experiments using eccentrically-biased exercise to examine the effects on vaccination responses [4-6]. For all three studies, the participants in exercise groups performed the eccentric portions of the bicep curl and lateral raise exercise, contracting the biceps brachii and deltoid muscles of the non-dominant arm, respectively.

Influenza vaccine was administered to the deltoid muscle, where the muscle damage and inflammatory response occurred. In the first study, the weight used for the exercise group were 85% of each participant's repetition maximum (moderate intensity) and all participants in both the exercise and control group received vaccination 6h post-exercise [5]. They found that exercised women had an increased antibody response whilst men showed enhanced cell-mediated responses compared to the control group [5]. The second study using this eccentric exercise task examined the effects of different exercise intensities on vaccine efficacy [6]. They found all intensity groups (light, moderate and heavy) demonstrated significantly higher antibody responses compared to the control group when vaccinated immediately after exercise, with no differences between the three intensity groups [6]. The third study using this eccentric exercise model compared the effects of vaccine timing on vaccine efficacy [4]. They found all exercise groups regardless of their vaccine timing (immediately, 6h or 48h post exercise) showed similar antibody and cell-mediated responses when compared to the control group [4]. They argued that exercise-induced immunoenhancement was only observed when the control group had relatively poor responses, but not strong responses [4].

Besides these studies using eccentric exercise, Edwards et al reported that an acute bout of cycling exercise or mental stress task had an immunoenhancing effect only in women in response to influenza vaccine [22] and only in men in response to meningococcal vaccine [23]. Another study demonstrated that participants that completed a 15-min arm exercise task using

elastic bands and that received a half dose of pneumococcal vaccine had significantly higher antibody responses compared to the sedentary group, whilst the exercise group that received a full dose vaccine did not have an improvement in immune responses [24]. These studies further suggest that when control responses were weaker (rather than sex differences), exercise could enhance the immune responses to vaccination. Based on this evidence, it is importance to perform mechanistic experiments in aged mice or use less than full dose vaccination in young mice, where there is weaker control response, to determine whether eccentric exercise can improve sub-optimal immune responses to vaccination.

Acute stress, such as acute bout of exercise, has been shown to enhance both antibody and cell-mediated immune responses in animals. Acute restraint stress, which is a commonly used psychological stress model, has been shown to improve antibody titers to sheep red blood cell (SRBC) vaccination in rats [25]. However, when extending the stress exposure to 6h/day for 4 days, immunosuppressive effect was observed. Similarly, acute exposure to foot shock stress elevated antibody responses to KLH in rats [26]. Other than immunoenhancement in humoral responses, acute stress also improves cell-mediated responses. Dhabhar has reported acute restraint stress promoted DTH responses to various vaccination in rodent models [14, 27-32]. Another group also reported acute psychological stress enhanced DTH responses by mediating skin dendritic cells [33].

Hypothesized Mechanisms

The mechanisms by which acute or exercise training may alter immune responses to vaccination remains to be determined. Based on the studies above, it is generally accepted that prolonged intense exercise (e.g. marathon) is detrimental, whilst acute moderate exercise is beneficial to immune function. The potential mechanisms of high-intensity endurance exercise associated with transient immunosuppression are described in the “open window hypothesis” [21], these include reduced neutrophil phagocytic function, total lymphocyte and natural killer cell numbers [34]. The temporary suppression of immune function causes athletes to be more susceptible to infection. However, acute moderate exercise would not reach the threshold to arouse these immunosuppressive effects, but instead result in immunoenhancing effects. [9]. It is hypothesized that the influx of immune cells and the release of inflammatory mediators caused by this eccentrically-induced muscle damage creates a pro-inflammatory environment in a muscle that may activate dendritic and other cells to augment the immune response to vaccination when given intramuscularly in the damaged muscle [10]. This heightened inflammatory environment may be a particularly effective behavioral adjuvant as eccentric exercise localizes muscle damage to the specific site of intramuscular vaccine administration. This strategy could be easily used to augment IM vaccination responses in aging populations.

Though there has been some research describing these immune changes, the underlying mechanisms of the immunoenhancing effects of acute exercise remain debatable. Dhabhar et al have proposed that acute stress (minutes to hours) is immunoenhancing, which is considered adaptive for survival from the

evolutionary viewpoint (see Figure 2.1) [27]. He proposed three main mechanisms of acute stress-induced immunoenhancement: changes in stress hormones and leukocyte redistribution, which are systemic mediators; and changes in cytokines and chemokines, which may be local mediators at the site of antigen entry [27]. Leukocytes circulate from the blood, to organs (e.g. skin) of the body and back to the blood. The first reaction of an organism to a stressor is central nervous system detection of the stressor and the release of stress hormones to warn and prepare the body. These stress hormones will increase the affinity and expression of adhesion molecules (selections/integrins) on leukocytes and endothelial cells, which causes the selective margination of leukocytes within the vasculature of organs. This response would occur in response to an acute stress, regardless of antigen presence. When the stress is an acute bout of moderate exercise, another important factor that increases hemodynamics is the mechanical force of the exercise-induced increase in cardiac output, vascular vasodilation and blood flow [35]. In the absence of immune challenge, leukocytes will demarginate and join the circulating leukocyte pool when stress stops. However, when there is an immune challenge (wounding or infection), it will result in an inflammatory response and release of chemokines and cytokines, which recruit and activate the leukocytes into the local tissues. Therefore, acute stress may boost immune response by having higher numbers of leukocytes available for recruitment (Figure 2.1) [27].

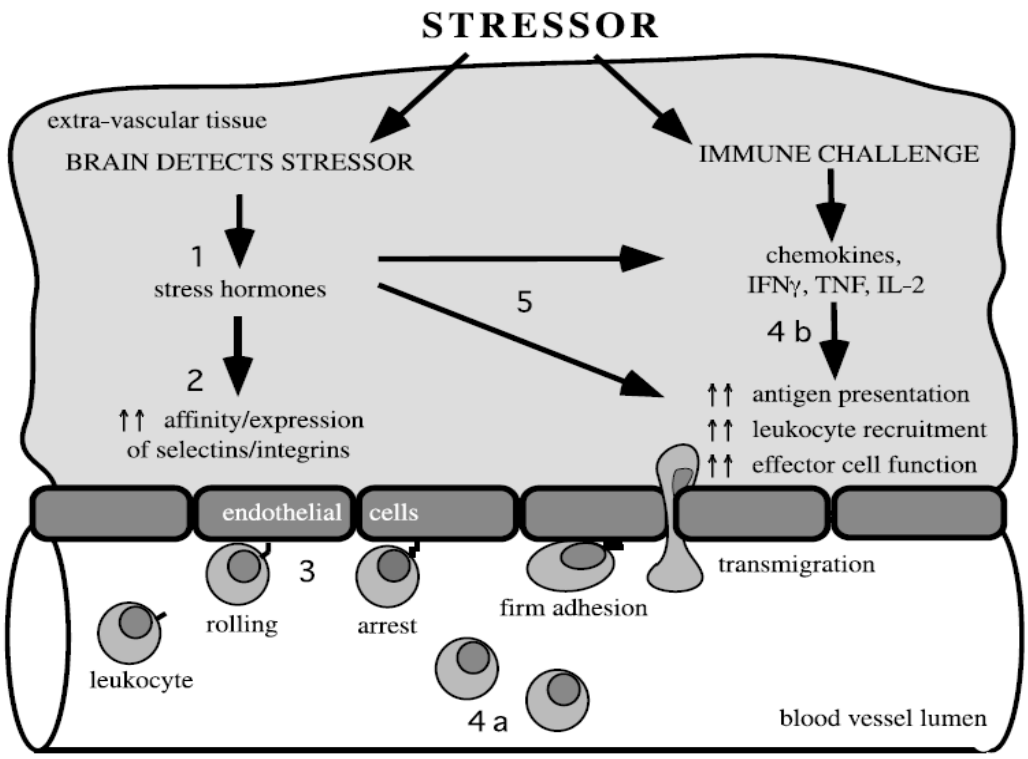

Figure 2.1: Hypothesized mechanisms of immunoenhancing effects of acute stress. Dhabhar et al 1999.

The two major classes of stress hormones that affect the kinetics of leukocytes in response to stress are glucocorticoids and catecholamines. Stress activates the hypothalamic–pituitary–adrenal (HPA) axis which results in stress hormone release (corticotrophin releasing hormone, adrenocorticotrophic hormone, and cortisol/corticosterone). Dhabhar et al measured the effects of acute stress (restraint stress, foot shock) at close temporal proximity to antigen administration on cell-mediated responses using the model of delayed type hypersensitivity (DTH) [27]. Skin-specific DTH reactions involve two phases: sensitization phase of T memory cells formation; challenge phase including antigen-presenting cells, T cells, neutrophils and macrophages [14, 28]. Dhabhar

and colleagues performed a series of studies implicating glucocorticoids in the enhancement of the DTH [30, 31, 36]. They showed that adrenalectomized (abolishes the corticosterone and epinephrine stress response) rats [30, 31, 36] or cyanoketone treatment (abolishes the corticosterone stress response) [31] reduced blood leukocyte redistribution and DTH responses associated with stress. On the contrary, catecholamines (epinephrine and norepinephrine) released by stress-induced sympathetic nervous system (SNS) activity is thought to increase blood leukocyte counts.[35]. Studies reported that adrenaline or noradrenaline administration increased neutrophil and natural killer (NK) cell numbers, but decreased T cells and B cells [37, 38]. Therefore, stress hormones may affect total blood leukocyte numbers and their subtypes.

Cytokines and chemokines released by stress-induced inflammatory responses are another key potential mechanism for immunoenhancement. Those cytokines and chemokines are interferon γ (IFNγ), tumor necrosis factor alpha (TNF-α), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 1β (IL-1β). IFNγ is important in both stages of DTH development including the increase of antigen presentation efficiency, increase in leukocyte recruitment, and activation of macrophages [32]. Dhabhar et al suggested that IFNγ is a local mediator of a stress-induced enhancement of skin DTH [32]. Acutely stressed wild-type mice had a higher DTH response than non-stressed mice, but IFNγ receptor-deficient mice failed to show a stress-induced DTH improvement [32]. IL-6 is another important cytokine candidate for stress-induced immunoenhancement. IL-6 is believed to be one of the first elevated cytokines after exercise and it has both

pro-inflammatory and anti-inflammatory effects [39]. Lee et at implicated that mice administered with the IL-6 gene and influenza vaccine were completely protected from a lethal dose of virus challenge [40].

In conclusion, previous studies suggest that potential mechanisms of acute stress- induced immunoenhancement involve leukocyte redistribution, increased stress hormones and cytokine gene expression.

2.2 Aging and Immunity

Aging is a global problem and will become more and more serious in the near future. The World Health Organization (WHO) reports that there are 900 million adults aged 60 or older in 2015, which constitutes 12% of the world population. What is worse, it is predicted to increase to 22% (2 billion people) of the population globally in 2050 [33].

Immunosenescence

Aging is associated with a decline in the immune system and its function, termed “immunosenescence”. Immunosenescence results in higher susceptibility to infection and depressed responses to vaccination. Infectious diseases, such as influenza, affect a large number of people every year, especially the aged population because of their depressed immunity. Vaccination has been the most effective intervention against infectious diseases. However, vaccine efficacy is remarkably reduced in the older adults compared to younger people [41]. Thus, immunosenescence is a big concern for the public health. It is of great significance to understand the mechanisms behind how immunosenescence

affects the immune system and, importantly, to find the potential exogenous and endogenous adjuvants that increase vaccine efficacy.

Aging and Innate Immunity

Aging alters both innate and adaptive immune systems. For the innate immune system, immunosenescence affects both numbers of functions of neutrophils, macrophages, natural killer cells (NK cells) and dendritic cells (DCs), as well as cytokine secretion.

The first line of defense against pathogen infection to the host is phagocytosis by neutrophils and macrophages. It is generally accepted that aging is associated with decreased function of neutrophils and macrophages, but increased or no change in the absolute numbers of these cells [42]. Reduced functional capability of neutrophils are mainly reduced phagocytosis, superoxide production, chemotaxis, signal transduction and apoptosis [43]. However, we should note that depressed phagocytic capacity of neutrophils is equivocal. Butcher et al. reported a decreased expression of CD16 (marker of neutrophil phagocytic ability) in the aged, while Plackett et al. reported aged adults had similar neutrophil phagocytosis as young adults [42, 44]. Impairment of macrophages' function are mainly reduced phagocytosis, superoxide production, chemotaxis, apoptosis, signal transduction, cytokine production and Toll-like receptor (TLR) activation [43]. Loss of TLR1/2-induced tumor necrosis factor-alpha (TNF-α) and Interlukin-6 (IL-6) production in the aged has also been shown [45].

Natural killer (NK) cells are cytotoxic effectors of the innate immune system, with characteristics of adaptive immunity, such as antigen-specific receptors and ability to produce memory cells [46]. However, unlike CD8 cytotoxic cells, NK cells do not require activation, but can respond to cytokines (IL-2, IL-12) directly and attack target cells. It is known that aging results in decrease rates of NK cell proliferation and production [47]. It is debatable whether cytotoxicity, signal transduction and response to cytokines are preserved or decreased in the aged [47]. The variability may be because of the health status of the studied population [48, 49].

Dendritic cells (DCs) are antigen presenting cells, which are significant for the development of adaptive immune responses. There are mainly two types of DCs: myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). Relatively little is known about the effects of aging on DCs. Age-associated reductions in the total numbers of DCs and mDCs in peripheral blood, thymic DCs, Langerhans' cells numbers, and DCs-induced IL-12 production have been reported [50].

Other than the changes in innate immunity cells caused by aging, inflammatory mediators are also altered in the aged. "Inflamm-aging" is a term that describes the elevated status of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, in the aged population, which may result in chronic illnesses, such as cardiovascular diseases, Alzheimer's disease, and cancers [51].

Aging and Adaptive Immunity

Aging is associated with several alterations in T cells, B cells, their subsets and cytokine production in the adaptive immune system.

Thymic involution is the most largely-studied change in the adaptive immune system in the aged. Total volume of thymus starts to decrease from the beginning of life with only a small amount left at the age of 50 [41]. As a result of the thymic involution, the number and function of naïve T cells is impaired in the aged population, which leads to a reduced immune response to primary vaccination. In the late stage of life, mainly memory T cells and effector T cells remain. Aging results in a reduction in naïve (CD45RA+CD28+) CD8+ T cells, T-cell receptor (TCR) repertoire, capacity to replicate and respond to novel antigens, and effector memory CD4+ T cells; whilst an increase in memory (CD45RA-CD28+)CD8+ T cells, effector (CD45RA+CD28-) T cells, end-stage differentiated effector T cells, IL-4 producing CD8+ T cells, and central memory CD4+ T cells [50].

Like many changes in T cells, aging also results in a decrease in naïve B cells, but an increase in effector B cells. As a result, there is an impaired antibody responses in the aged [52]. Aging also leads to a reduced class switching and somatic recombination TCR repertoire, which contributes to a lower diversity of antibody responses and a reduced immunoglobulin G (IgG) antibodies, which contributes to a lower affinity of antibody responses [53].

Cytokine production and signaling processes are also altered in the aged population, especially IL-2. It has been reported that aging results in a reduction

in synthesis of IL-2 and expression of high affinity IL-2 receptor, which is critical for effector-memory T cells formation [54].

Taken together, aging result in a series impairments in both innate and adaptive immunity, including alterations in numbers and functions of leukocytes, and different cytokine secretion. All of these contribute to the reduced immune responses to natural pathogens and vaccinations in the aged.

2.3 Aging and Exercise and Immunity

Immunosenescence refers to a cascade of dysregulation of the immune system associated with aging, which leads to an increase in incidence of infectious disease, morbidity and mortality [55]. There is a rapid growth of older adults (aged ≥ 65 years) in both industrialized and developing countries and infectious diseases are a major cause of death in the older population [56]. Vaccination against infectious diseases has been one of the most successful public health interventions [1]. Unfortunately, elderly individuals have inadequate immune responses to vaccination as compared to younger population. In order to augment vaccine responses, we need to either improve vaccines themselves or find behavioral interventions that alter host factors to improve vaccine responses. Here, I summarize the current literature for effects of both chronic and acute exercise on vaccination responses in older adults and the hypothesized mechanisms.

Aging, Exercise Training and Vaccination

Most of the studies examining the effects of exercise training on the immune responses to vaccination were conducted in the aged because of their

impaired vaccine efficacy and high risk of infectious disease associated morbidity and mortality.

Three cross-sectional studies reported that exercise training was positively correlated with either humoral or cell-mediated immunity to vaccinations in older adults [57-59]. Our group recruited older adults (60-76 year) and assigned them to high-fit, low-fit and sedentary groups based on maximal oxygen uptake test [57]. We showed that high-fit elderly participants had higher antibody responses to influenza vaccine, but no differences in cell-mediated responses among three groups [57]. Kohut et al. found that adults aged 62 years and older, who vigorously exercised three or more times per week, had higher anti-influenza IgG, IgM and greater peripheral blood mononuclear cell proliferation compared with the elderly adults who exercised with less intensity, frequency, and/or duration when compared to sedentary controls [58]. Schuler et al. also reported a positive relationship between self-reported physical activity and antibody responses to influenza vaccination in older adult [59].

In addition, there were four randomized controlled trials evaluating exercise training on vaccination responses in the elderly [11, 12, 60, 61]. Our lab has conducted two experiments comparing 10 months of aerobic moderate exercise intervention (30-60 min/session, 3 sessions/week) to flexibility/balance training in older adults [11, 12]. We found that cardiovascular exercise resulted in a longer lasting seroprotection to influenza vaccine when measured 24 weeks post-vaccination, whereas flexibility/balance training did not [12]. Similarly, aerobic exercise training induced higher primary IgG1 and IgM antibody

responses compared to the sedentary controls [11]. Kohut et al also reported that 10 months of aerobic exercise training enhanced the antibody responses and granzyme B activity to influenza vaccine [60]. Besides the commonly used aerobic exercise intervention, we examined the effects of Taiji and Qigong (a fusion of martial arts with meditation and traditional Chinese medicine) training on the antibody response to influenza vaccine in older adults [61]. Participants who practiced moderate 3 x 60 min Taiji and Qigong for 20 weeks had significantly higher antibody responses at 3 and 20 weeks post-vaccine, whereas the control group did not [61].

In conclusion, both cross-sectional studies and randomized controlled trials suggest a beneficial effect of exercise training on either antibody responses or cell-mediated responses to vaccination in older adults.

However, results of exercise training on immunity in animals are not consistent. Kohut et al. examined the effects of 8-week treadmill running exercise on the immune response to herpes simplex virus type 1(HSV-1) vaccination in both young and older mice [62]. Exercise training significantly increased IL-2 and IFN-gamma production, but not IgM antibody responses in older mice while exercise had no effect in young mice. Our lab has previous investigated a four-month moderate treadmill running on T lymphocyte profiles in young and aged mice [63]. We found exercise training improved splenic naïve to memory T cell subset ratios in CD4 and CD8 cells in the aged mice, but not young mice. Barnes et al. reported a 10-week treadmill exercise training did not alter antibody

responses to KLH vaccination in old rates when compared to the sedentary animals [64].

Aging, Acute Exercise and Vaccination

Fewer studies have examined the effects of acute exercise bouts on vaccination in older adults compared to the exercise training intervention. Our lab investigated the effect of acute 40-minutes moderate intensity walking on vaccine efficacy in adults between 55 and 75 years old [65]. Exercised women had a higher antibody response against the H1N1 influenza strain compared to the control women, but men did not have such an elevation [65]. These results were similar to the ones observed in previous studies regarding acute exercise intervention in young adults mentioned above. It was postulated that the sex difference was probably because of the lower pre-vaccine titers to the vaccine in women. Long et al performed a similar study, using an acute bout of brisk walk at moderate intensity for 45 minutes in young and middle-older aged adults [66]. They did not find an exercise effect to influenza or pneumonia vaccination in either age cohort. But they showed higher antibody responses to influenza vaccine in younger participants [66].

Similar to human studies, not many experiments have been investigated acute exercise on immunity in aged animals. Kapasi et al examined the effects of intense exercise on secondary antibody response to HAS in young and old mice [67]. A single bout of exercise enhanced antibody response to the extent similar to the young mice, while no effect on the young animals.

2.4 Stress and Vaccination Response

Stress consists a cascade of proceedings: starting with a stimulus (stressor), eliciting stress perception in the brain, causing physiologic stress responses in the body [68]. Several studies have shown that chronic stress is immunosuppressive and decreases both antibody and cell-mediated immune responses to vaccination; whilst acute stress can be immunoenhancing [32, 69].

Previous studies have demonstrated different forms of chronic stress suppress immune function including DTH responses and antibody responses to vaccination [70-75]. Dhabhar's group conducted a series of studies and compared the effects of different intensities and durations of restraint stress on cell-mediated immunity in animals [14, 27-32]. Acute stress ranging from 2h to 5h prior to antigenic challenge was shown to improve DTH responses. Increasing intensity of the acute stress was associated with enhancing DTH and leukocyte redeployment. On the contrary, DTH responses were reduced when increasing the stress to 6h/day for 3 weeks (before sensitization) to 5 weeks (after antigenic challenge). Increasing stress exposure was also associated with reduction of peripheral blood lymphocyte redistribution and decreased glucocorticoid responsivity [14]. Another study examined the effects of a social stressor, a colony intruder paradigm, on antibody responses to KLH in rats. Stressed rats had a significant lower serum anti-KLH IgG responses compared to the controls at 2 and 3 weeks post-vaccination [15].

Human studies have also demonstrated chronic stress dysregulates vaccine immune responses. Smith et al. examined the relationship between distress and primary immune response to KLH [76]. The more distressed

subjects had a lower DTH skin responses to KLH, while anti-KLH IgG responses were not affected by stress. In another study, suppressed cell-mediated immunity was observed in the participants with more depressive symptoms in metastatic breast cancer [77].

2.5 Exercise and Stress and Immunity

Few studies have investigated the effects of exercise on immune responses to vaccination in stressful situations, more have focused on effects of stress and disease-related immune regulation. Luo et al. examined the moderating impact of moderate exercise on chronic stress- induced intestinal barrier dysfunction [78]. Mice were subjected to repeated restraint stress for 6h per day for 7 days (or no stress), receiving 30min swimming prior to each stress session (or sedentary). Swimming before stress attenuated bacterial translocation, maintained intestinal permeability and significantly increased four antimicrobial peptides gene expression. In conclusion, they proved that brief moderate exercise attenuated chronic stress-induced intestinal barrier dysfunction and enhanced innate mucosal defenses. Some human studies also reported beneficial effect of physical activity or structured exercise on disease-related immune regulation [79, 80]. Bote et al. showed an 8-month aquatic exercise training resulted in an anti-inflammatory effect, including decreased systemic levels of IL-8 and tempered neutrophil activation (chemotaxis) in fibromyalgia syndrome patients [79].

Early evidence has suggested that moderate exercise training have the potential to alleviate stress-induced immunosuppression. Brown and Siegel

investigated the ability of physical exercise to buffer stress-induced disease incidence in adolescence [81]. The findings suggest that girls who were moderately physically active were more protected against the immunologically deleterious consequences of stress compared to the sedentary girls under high stress. In an animal study, Moraska and Fleshner reported that a four-week voluntary wheel running reduced tail shock stress-induced behavioral depression and prevented stress-induced suppression of anti-KLH IgM and IgG(2a) antibodies in rats [82].

2.6 Summary

In conclusion, acute bouts of eccentric exercise may have a potential beneficial effects on vaccination responses. However, the favorable effect was only observed in the low-immunogenic vaccine strain, or only in the population that have poor responses based on previous studies. Thus, a definitive animal study is needed to test effect of acute eccentric exercise on a primary vaccination response.

Due to the inconsistencies of the current literature regarding the efficacy of a single bout of eccentric exercise to improve vaccination responses in people, the purpose of our study was to determine if eccentric exercise could improve the primary immune response to a suboptimal vaccination dose in both young and aged mice. Examination of the effects of exercise on a primary response is important because data interpretation is not confounded by prior exposure history as is the case with influenza vaccine studies in people. The novelty of this study lies in the use of a primary vaccine and in the use of an animal model to control

for confounds affecting immune responses. If a benefit is seen using this paradigm in mice, future studies could focus on the mechanism of the beneficial effect.

CHAPTER 3

EFFECTS OF ECCENTRIC EXERCISE ON IMMUNE RESPONSES TO VACCINATION

3.1 INTRODUCTION

Vaccination against infectious diseases has been one of the most successful public health interventions. Unfortunately, some at-risk populations (e.g. aged, immunosuppressed) have inadequate immune responses to vaccination which increases their susceptibility to infectious disease. In order to augment vaccine responses, we need to either improve vaccines themselves or find behavioral interventions that alter host factors to improve vaccine responses.

Prior work in our lab has demonstrated that 10 months of cardiovascular exercise training can extend the protective antibody response to influenza vaccination in older adults [12]. While beneficial, further studies are necessary to determine whether less time consuming, equally efficacious exercise strategies can be used to augment the immune response to vaccination. Along these lines, a study has shown that a single acute bout of eccentric exercise augments the antibody response to influenza vaccination in humans [5]. Eccentric exercise causes the exercising muscle to produce continuous force while it lengthens, leading to damage of the internal structure of the muscle fibers and connective tissue [7]. The substantial increase in plasma creatine kinase indicates this damage is greater than that caused by concentric or shortening muscle contractions [8]. Muscle damage can result in a localized inflammatory response

and delayed onset muscle soreness when conducted in naive participants [9]. It is hypothesized that the influx of immune cells and the release of inflammatory mediators caused by this eccentrically-induced muscle damage creates a pro-inflammatory environment in a muscle that may activate dendritic and other cells to augment the immune response to vaccination when given intramuscularly in the damaged muscle [10]. This heightened inflammatory environment may be a particularly effective behavioral adjuvant as eccentric exercise localizes muscle damage to the specific site of intramuscular vaccine administration.

Due to the inconsistencies of the current literature regarding the efficacy of a single bout of eccentric exercise to improve vaccination responses in people, the purpose of our study was to determine if eccentric exercise could improve the primary immune response to a suboptimal vaccination dose in both young and aged mice. Examination of the effects of exercise on a primary response is important because data interpretation is not confounded by prior exposure history as is the case with influenza vaccine studies in people. Based upon prior human literature [5, 6], we hypothesized that eccentric exercise would improve both the antibody and cell-mediated immune response (i.e. delayed-type hypersensitivity response) to ovalbumin vaccination in aged mice exhibiting immunosenescence, but not in young mice. The novelty of this study lies in the use of a primary vaccine and in the use of an animal model to control for confounds affecting immune responses. If a benefit is seen using this paradigm in mice, future studies could focus on the mechanism of the beneficial effect.

3.2 METHODS

Animals

Young Mice

Six to eight-week-old male C57BL/6J (n=30) and Balb/cJ (n=13) were purchased from Jackson Laboratory (Bar Harbor, ME) and were individually housed in an AAALAC-accredited animal facility for at least 2 weeks of acclimation before experimentation. C57BL/6 and Balb/c mouse strains were both utilized in this study for the purposes of examining mice with heterogeneous immune responses (e.g. TH1 , C57BL/6 and TH2, Balb/c) [83].

Aged Mice

C57BL/6J male mice, aged 27 months (n=16), purchased from Jackson Laboratory (Bar Harbor, ME) were individually housed in an AAALAC-accredited animal facility. Retired breeder mice were purchased from Jackson Laboratory at 7 or 8 months of age and individually housed in an AAALAC-accredited animal facility till 27 months of age for experiment.

Housing and Feeding Protocols

Mice were allowed ad libitum access to water and food (Teklad 8640, Harlan Laboratories, Indianapolis, IN). All animals were maintained on a 12-hour light-dark cycle. All experiments were approved by the University of Illinois at Urbana-Champaign IACUC. Cages were changed weekly by animal care staff. Mice were allowed ad libitum access to water and food (Teklad 8640, Harlan Laboratories, Indianapolis, IN). All animals were maintained on a 12-hour light-dark cycle. All experiments were approved by the University of Illinois at Urbana-

Champaign Institutional Animal Care and Use Committee (IACUC) prior to the onset of the studies. Procedures for these studies were performed on IACUC protocols 13410, 14157 and 17026.

Eccentric Exercise Protocol in Young Mice

Eccentrically-Biased Treadmill Exercise

Young C57BL/6J mice were randomly assigned to a single bout of eccentric running (ECC1, n=5) or remained sedentary (SED, n=5). Mice were placed on a non-moving treadmill at -20º for 10 minutes for 5 consecutive days for acclimation. After acclimation period, ECC1 mice were exercised at 17 m/min speed at -20% grade for 45 minutes on a treadmill. No electrical shock was used. Lopez et al. have shown this exercise protocol elicited muscle inflammation by significantly increasing intracellular Tumor necrosis factor alpha (TNF-α), Monocyte chemotactic protein 1 (MCP-1), Interleukin-6 (IL-6), and Interleukin-10 (IL-10) [84]. Mice were vaccinated in the gastrocnemius of their right hindlimbs with ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO) immediately exercise based on the study design of Edwards et al. [5] who demonstrated an eccentric-exercise induced benefit in influenza vaccine response. The SED mice remained in their home cages during the experimental period.

In another experiment, two bouts of eccentrically-biased downhill treadmill running (ECC2) were performed on consecutive days. Peak inflammation following eccentric exercise occurs 24 h post-exercise [85]. In addition, Edwards et al. vaccinated humans with influenza vaccine immediately after eccentric exercise [6]. We rationalized that injecting immediately following a second bout of

eccentric exercise at 24 h would maximize an exercise-induced increase in vaccine response. Thus, young Balb/cJ mice were randomly assigned to eccentric running (ECC2, n=6) or remained sedentary (SED, n=7) followed by vaccination immediately after the second exercise bout. The running protocol was the same as the previous experiment except that 2 bouts of exercise were administered on consecutive days. We and others have shown that these eccentric exercise protocols induce muscle damage and local inflammation in the gastrocnemius-soleus complex of mice [86-89].

Eccentrically-Biased Electrical Stimulation Exercise

In a third experiment, young C57BL/6J mice were randomly assigned to an eccentric electrically-stimulated group (ECCstim, n=10) or a sham operation group (Sham, n=10). All mice were anesthetized with isoflurane (2-3% isoflurane, 0.9 L/min oxygen). The right hindlimb was shaved and aseptically prepared. The foot was placed in a miniature metal foot plate attached to the shaft of a servomotor (model 300 B-LR, Aurora Scientific, Aurora, ON, Canada) perpendicular to the tibia. Two platinum electrodes were inserted through the skin on either side of the sciatic nerve. A stimulator and stimulus unit stimulated the sciatic nerve via platinum electrodes to induce a contraction of the hindlimb crural muscles. The optimal voltage was determined by delivering 100 Hz pulses of 0.1 ms duration and measuring peak twitch force. The posterior crural muscles were injured by performing 100 eccentric contractions using the optimal voltage at 150 Hz. During stimulation, the posterior crural muscles were stretched from 19° of ankle plantarflexion to 19° of ankle dorsiflexion. Every 5 contractions were

separated by 10 second rest periods and the entire protocol lasted 10-20 minutes. Corona et al. have demonstrated that this electrical stimulation protocol causes muscle inflammation in mice [90, 91]. Sedentary sham control mice were anesthetized and subjected to electrode insertion but the muscles were not stimulated. All mice were vaccinated with OVA plus alum 6 hours post-exercise.

Eccentric Exercise in Aged Mice

Aged C57BL/6J mice were randomly assigned to a single bout of eccentric running (ECCaged, n=8) or remained sedentary (SED, n=8). ECCaged mice followed the same acute one bout of eccentric treadmill exercise protocol as ECC1 mice described above. Mice were vaccinated in the gastrocnemius of their right hindlimbs with ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO) immediately after exercise based on the study design of Edwards et al [6] who demonstrated an eccentric-exercise induced benefit in influenza vaccine response. The SED mice remained in their home cages during the experimental period.

Vaccination Protocol

In order to maximize the chance of finding an effect of eccentric exercise and because Pascoe et al. found that eccentric exercise augmented vaccine responses only when a sub-optimal dose of vaccine was used [92], we titrated our vaccine dosage in preliminary experiments across a wide range of doses (10, 50, 100, 200μg) of OVA (Sigma-Aldrich, St. Louis, MO) with 200μg of aluminum hydroxide (Sigma-Aldrich, St. Louis, MO) as adjuvant. We found that 100μg/mouse of OVA with alum yielded significantly elevated but suboptimal antibody levels when measured at four week post vaccination (data not shown).

Thus, in our experiments, all mice were intramuscularly inoculated in the gastrocnemius of their right hindlimbs with 100μg of OVA and 200μg of alum in 50μl sterile saline using a 25g needle.

Delayed-type hypersensitivity (DTH)

The DTH response is to measure the in vivo inflammatory reaction to a specific antigen. It has been shown that there is a positive relationship between the ear swelling increase and the ongoing immune response [93, 94]. Mice were injected with 100μg OVA dissolved in 10μl PBS into the dorsal side of the right ear using a Hamilton syringed fitted with a 30-gauge needle on 21 day post eccentric exercise. The left ear received 10μl PBS alone as a control for non-specific ear swelling. Both ears' thicknesses were measured immediately before, and every 24h after intradermal injection using a digital microcaliper (Tresna). The measurements were performed in triplicate by an assistant who was blinded to the treatments. Results were expressed in two ways: Both right and left ear thickness with 6 time points and the difference between two ears' swelling at 1d post intradermal injection. Maximum ear swelling occurred on day 1 post ear inoculation.

Blood Collection

All mice were bled approximately 200μl from the retro-orbital vein using a Pasteur pipette prior to vaccination and at one, two and four weeks post-vaccination. Prior to blood collection, mice were administered isoflurane with oxygen at a flow rate of 2-3 L/min until unresponsive. Blood was dispensed into heparinized tubes and centrifuged at 1100 x g at 4°C for 15 min. Plasma was

harvested and frozen at -20°C until analysis. At four week post-inoculation, blood was drawn after euthanasia from the inferior vena cava and processed as described before. In each experiment, mice were euthanized by CO2 asphyxiation followed by cervical dislocation at four weeks post-vaccination.

Plasma Antibody Measures

Plasma total anti-OVA IgG was determined using ELISA procedures. Ninety-six-well microtiter plates were coated with 50µl of 20µg/ml OVA in carbonate coating buffer and incubated overnight at 4°C. Nonspecific binding was blocked with PBS supplemented with 10% fetal bovine serum (FBS) and incubated for 1h at 37°C. After washing three times with phosphate buffered saline (PBS)-Tween 20, 50µl of plasma samples were added at a dilution of 1:20 in a diluting buffer of PBS/1% FBS and incubated for 1h at 37°C. Plates were washed again, and 50µl of horseradish peroxidase (HRP)-rabbit anti-mouse IgG (Life Technologies, Frederick, MD) diluted 1:800 in diluting buffer was added. Plates were incubated again and then washed. Plates were incubated for 20 minutes in 50µl of a 1:1 mixture of 3, 3', 5,5' tetramethylbenzidine (TMB) and hydrogen peroxide (TMB Substrate Reagent Set, BD Biosciences, San Jose, CA) and read at 405 nm on a spectrophotometric plate reader (Labsystems Multiskan, Fisher Scientific, Pittsburgh, PA). Plasma anti-OVA IgG was quantified as the difference in optical density (OD) at 405 nm from the pre-injection time point.

Statistical Analysis

Results were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL). Plasma anti-OVA IgG was assessed by repeated-measures analysis of variance (ANOVA). All analyses were followed by post-hoc Bonferroni tests in the event of a significant main effect or interaction. Ear swelling on day 1 post ear injection was assessed by independent T test. Statistical significance was set at $P \leq 0.05$ for all tests. Results are reported as mean ± SEM.

3.3 RESULTS

Effects of eccentric exercise on primary antibody responses to OVA vaccination in young mice.

We examined the effects of acute eccentric exercise on primary antibody responses to vaccination in young mice using one bout of eccentrically-biased downhill running, two bouts of downhill treadmill running, and eccentrically-biased electrical stimulation.

Effects of one bout of eccentric exercise on the immune responses to OVA vaccination in young mice.

We first addressed whether one bout of eccentric downhill running immediately prior to suboptimal OVA vaccination altered the antibody response in young mice. As we expected, we found a significant time main effect ($F_{2,34}$ = 226, $P \leq 0.001$), demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization levels (Figure 3.1). However, there was no significant time x treatment effect ($F_{2, 34} = 0.64$, $P = 0.54$) or treatment main effect ($F_{1, 17} = 4.0$, $P = 0.06$). Our results indicated that, in this

animal model, one bout of eccentric treadmill exercise immediately prior to vaccination did not improve the antibody response in young mice.

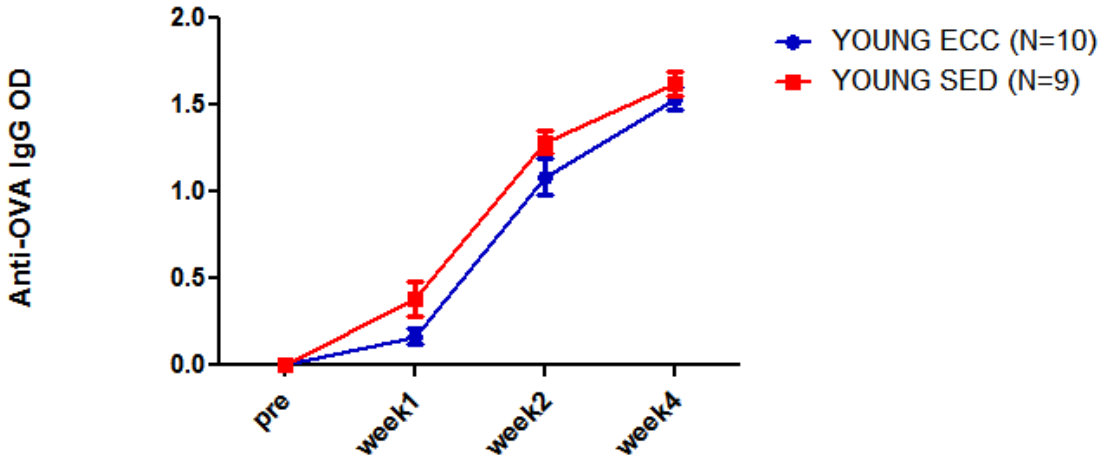

Figure 3.1: Plasma anti-OVA IgG responses to vaccination immediately after one bout of eccentric exercise in young mice. There was significant time main effect ($F_{2, 34}$=226, P < 0.001), but no significant time x treatment effect ($F_{2, 34}$=0.64, P=0.54) or treatment main effect ($F_{1, 17}$=4.0, P=0.06).

We also examined the effect of one bout of eccentric downhill running on the DTH immune response to OVA in young mice. We found there was a significant time main effect ($F_{5, 85}$ = 25.9, P < 0.001) (Figure 3.2), demonstrating that ear swelling increased significantly in both groups. However, we did not find a significant time x treatment interaction ($F_{5, 85}$ = 0.41, P = 0.89) or treatment main effect ($F_{1, 17}$ = 1.44, P = 0.25). Acute eccentric exercise did not enhance cell-mediated immune responses in young mice.

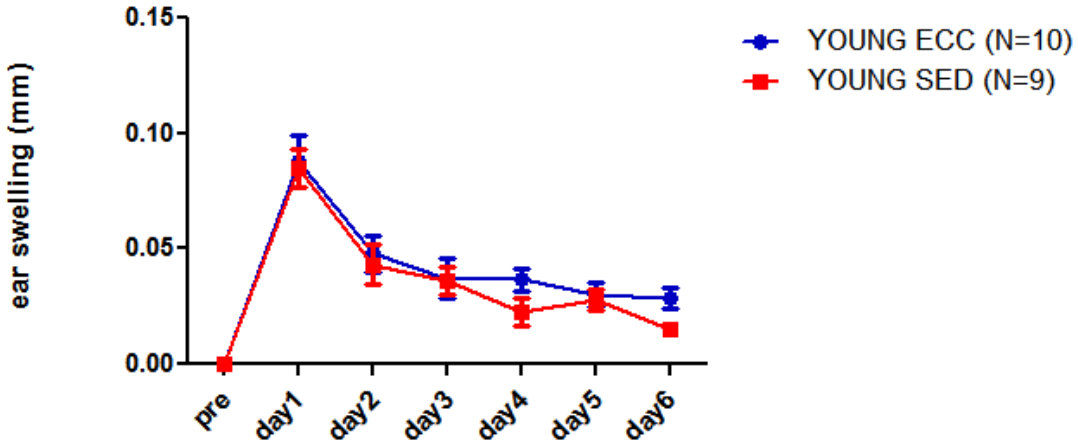

Figure 3.2: DTH responses to vaccination post one bout of eccentric exercise in young mice. There was significant time main effect ($F_{5, 85}$=25.9, P < 0.001), but no significant time x treatment effect ($F_{5, 85}$=0.41, P=0.89) or treatment main effect ($F_{1, 17}$=1.44, P=0.25).

Effects of two bouts of eccentric exercise on the antibody responses to OVA vaccination in young mice.

Based on our negative finding in response to a single bout of exercise, we performed an experiment where we exercised mice on two consecutive days using our downhill running protocol. Donnelly et al. have shown that muscle inflammatory response peaks at 24 hours post-exercise (5). Therefore, in this experiment, another bout of exercise was repeated 24 hours after the first bout of downhill running. As in our first experiment, we found there was a significant time main effect ($F_{2, 10}$=31.05, P<0.001), demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization (Figure 3.3). However, there was no significant time x treatment effect ($F_{2, 10}$=0.54, P=0.52) or treatment main effect ($F_{1, 11}$=0.002, P=0.97). Thus, in contrary to published work in humans where vaccination took place immediately

after exercise (8), our results indicated that in this animal model, two bouts of eccentric treadmill exercise with vaccination immediately after the second bout did not improve the antibody response.

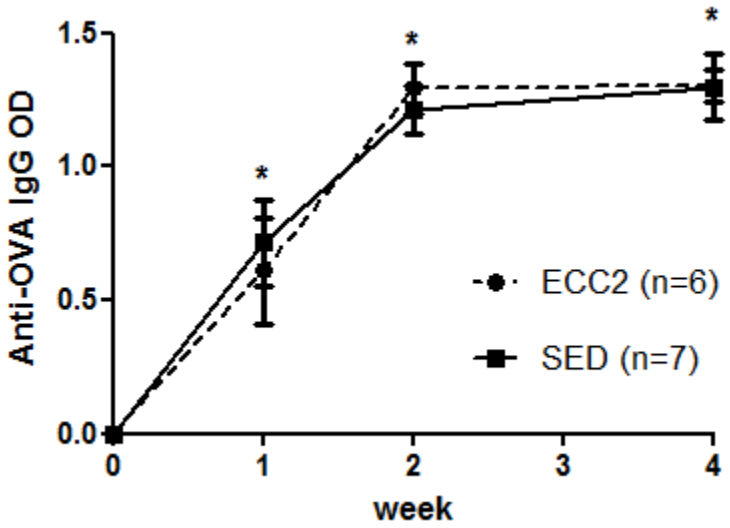

Figure 3.3: Plasma anti-OVA IgG responses to vaccination immediately the second of two bouts of eccentric exercise. There was significant time main effect ($F_{2, 10}$ =31.05, P<0.001), but no significant time x treatment effect (F2, 10=0.54, P=0.52) or treatment main effect ($F_{1, 11}$=0.002, P=0.97). *signifies statistical significance vs. pre-vaccination time point ($p < 0.05$).

Effects of electrical stimulation on the antibody responses to OVA vaccination in young mice.

Corona et al. have shown this protocol results in muscle damage and localized inflammation (4). First, we found there was a significant time main effect ($F_{2, 17}$=55.6, P<0.001), demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization (Figure 3.4).

However, there was no significant time x treatment effect ($F_{2, 17}$=0.54, P=0.59) or treatment main effect ($F_{1, 18}$=0.05, P=0.83) indicating that electrical stimulation of eccentric contraction six hours prior to vaccination did not improve the antibody response.

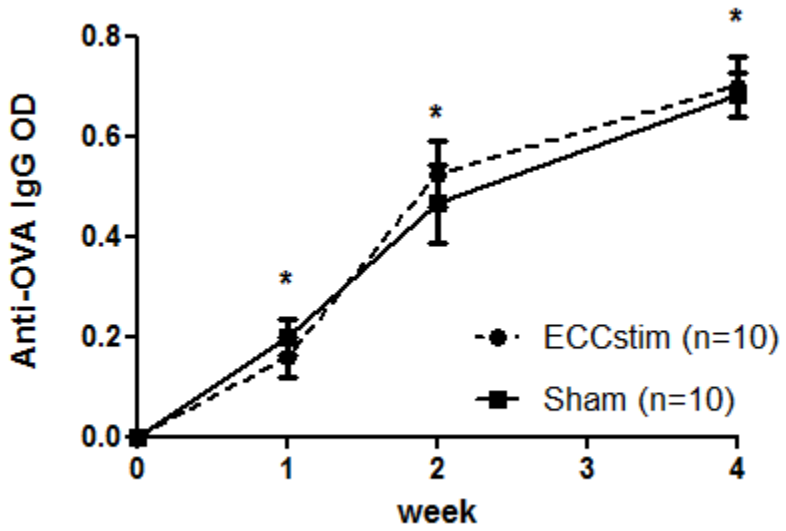

Figure 3.4: Plasma anti-OVA IgG responses to vaccination 6 h after electrically-stimulated eccentric contraction. There was a significant time main effect ($F_{2, 10}$=55.67, P<0.001), but no significant time x treatment effect ($F_{2, 10}$=0.54, P=0.59) or treatment main effect ($F_{1, 18}$=0.05, P=0.83). *signifies statistical significance vs. pre-vaccination time point ($p < 0.05$).

Effects of eccentric exercise on immune responses to OVA vaccination in aged mice.

Eccentric exercise has been previously established to be an adjuvant improving antibody responses to influenza vaccination in humans [6]. In order to replicate this finding in an animal model and understand the potential mechanisms, we first addressed whether one bout of eccentric downhill running

immediately prior to suboptimal OVA vaccination altered the antibody response. As expected, we found a significant time main effect ($F_{2, 28}$=77.045, P<0.001), demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization levels (Figure 3.5). However, there was no significant time x treatment effect ($F_{2, 28}$=0.386, P=0.683) or treatment main effect ($F_{1, 14}$=0.094, P=0.764). Thus, in contrary to published work in humans [5], our results indicated that in this animal model one bout of eccentric treadmill exercise immediately prior to vaccination did not improve the antibody response.

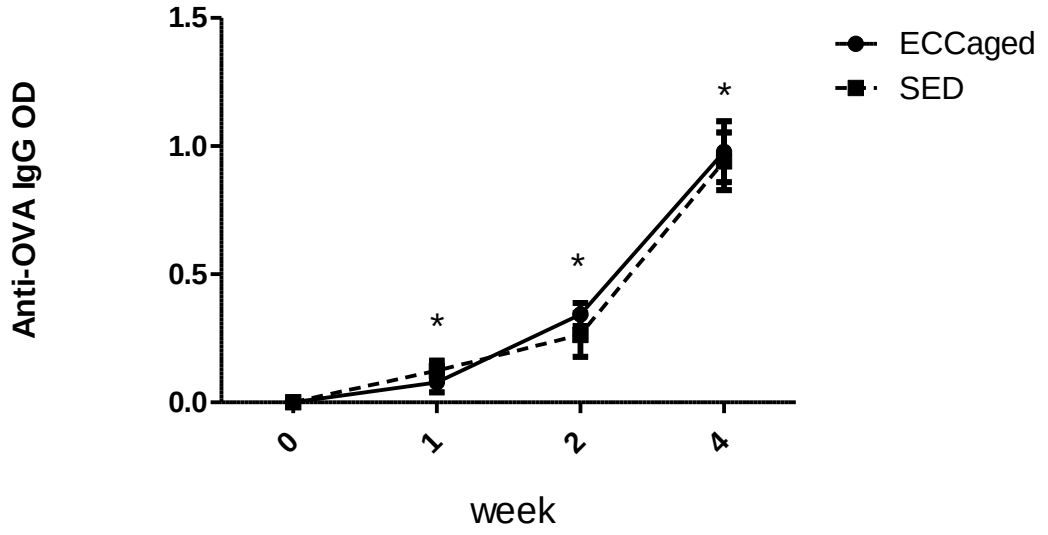

Figure 3.5: Effect of eccentric exercise on antibody response to vaccination in aged mice. * Plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization levels. No significant differences between eccentric exercise and sedentary group.

We also investigated the effect of acute eccentric exercise on the cell-mediated immune response to OVA vaccination. We inoculated OVA at three weeks post the eccentric exercise and measured the ear thickness prior to, 1d,

2d and 3d post ear injection to determine DTH responses. We found a significant time main effect ($F_{2, 30} = 33.2$, $P \leq 0.001$) as expected. Interestingly, there was a significant treatment main effect ($F_{1, 15} = 18.9$, $P = 0.001$), but not significant time x treatment interaction ($F_{2, 30} = 0.23$, $P = 0.797$), indicating that prior eccentric exercise had significantly increased the DTH responses in aged mice compared to aged sedentary controls, especially at 1 day post intradermal challenge (Figure 3.6). Thus, acute eccentric exercise improved the cell-mediated immune response to OVA vaccination in aged, but not in young, mice and failed to affect the anti-OVA antibody response in young or aged mice.

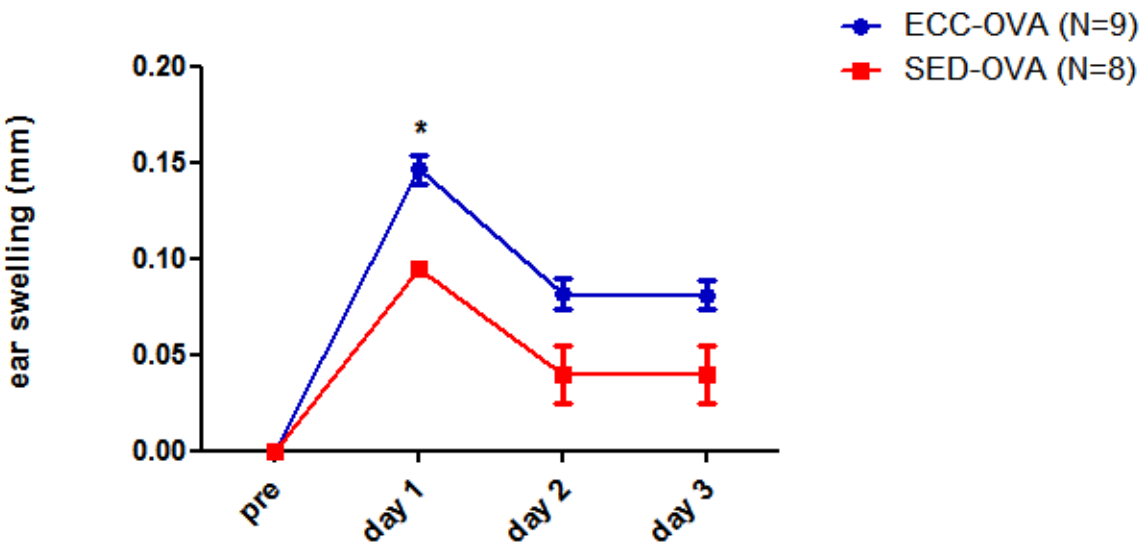

Figure 3.6: Effect of eccentric exercise on DTH responses to vaccination at 3 weeks post exercise in aged mice. There was significant time main effect ($F_{2, 30}=33.2$, $P\leq0.001$) and significant treatment main effect ($F_{1, 15}=18.9$, $P=0.001$), but no significant time x treatment effect ($F_{2, 30}=0.23$, $P=0.797$). *****signifies statistical significance between A-ECC and A-SED group at day 1. ($P < 0.05$).

Comparison of immune responses to OVA vaccination in young v.s. aged mice.

Aging is associated with a decline in the immune system and its function, termed “immunosenescence”. We compared both antibody responses and cell-mediated immune responses to OVA vaccination in young sedentary mice with the immune responses in aged sedentary mice.

First, we compared the anti-OVA IgG responses in young sedentary mice and aged sedentary mice (Figure 3.7). We found a significant time main effect ($F_{3,45}$=109, p<0.001) as we expected, demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization levels in both groups. We also found a significant time x age interaction ($F_{3, 45}$=17, p<0.001) and a significant age main effect ($F_{1, 15}$=26, p<0.001), demonstrating that anti-OVA IgG levels in young sedentary group were significantly higher than aged sedentary group.

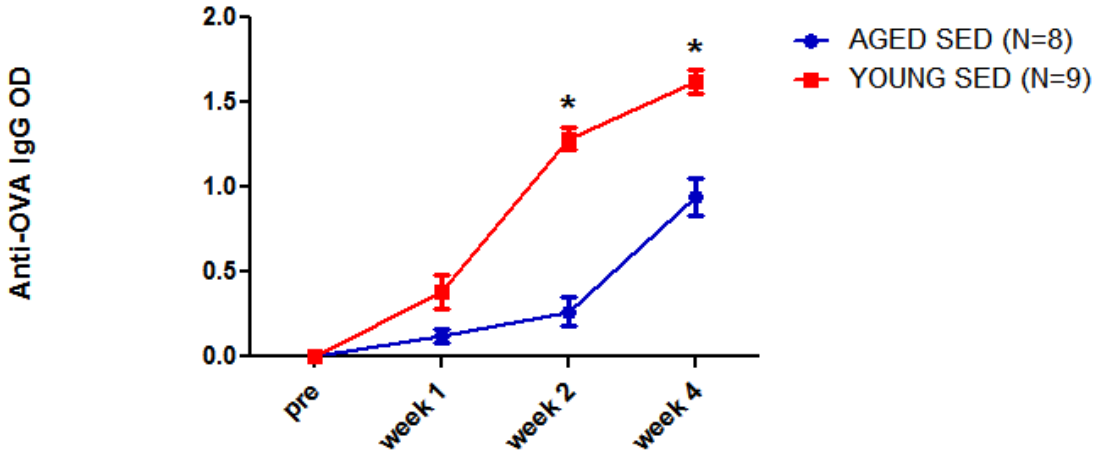

Figure 3.7: Comparison of anti-OVA IgG responses to OVA vaccination in young v.s. aged mice. There was significant time main effect ($F_{3.45}$=109, p<0.001), significant time x age interaction ($F_{3.45}$=17, p<0.001) and significant age main effect ($F_{1.15}$=26, p<0.001) *****signifies statistical significance between AGED SED and YOUNG-SED group at week 2 and week 4 post vaccination (P < 0.05).

Then we compared the DTH responses in young sedentary mice and aged sedentary mice (Figure 3.8). We found a significant time main effect ($F_{2, 30}$=22, p<0.001) as we expected, demonstrating DTH responses increased significantly relative to pre-immunization levels in both groups. However, we did not find a significant time x age interaction ($F_{2, 30}$=0.292, p=0.749) or a significant age main effect ($F_{1, 15}$=0.058, p=0.813), demonstrating that DTH responses in young sedentary group were not significantly different than aged sedentary group.

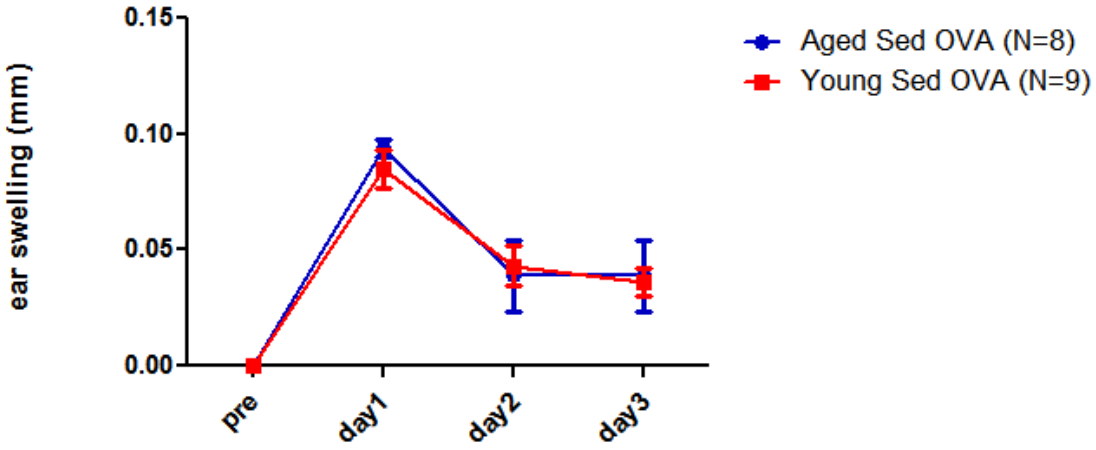

Figure 3.8: Comparison of DTH responses to OVA vaccination in young vs. aged mice. There was significant time main effect ($F_{2, 30}$=22, p<0.001), but no significant time x age interaction ($F_{2, 30}$=0.292, p=0.749) or significant age main effect ($F_{1, 15}$=0.058, p=0.813)

3.4 DISCUSSION

We investigated the effects of acute eccentric exercise on immune responses to a suboptimal dosage of ovalbumin vaccination in young and aged mice. As expected, we found that the vaccination elevated anti-IgG antibody responses at 1, 2 and 4 week post-exercise in all experiments. However,

eccentrically-biased downhill running or electrical stimulation did not further enhance these responses in young or aged mice when compared to the respective control groups. Despite the negative results of eccentric exercise on antibody responses, we found that there was beneficial effect of acute eccentric exercise on the cell-mediated immune response to vaccination in aged, but not young, mice. In conclusion, we found acute eccentric exercise enhanced cell-mediated response in aged mice, but not antibody responses in either young or aged mice.

There are several studies demonstrating acute eccentric exercise can act as an adjuvant to improve vaccination responses in humans. In the first study investigated by Edwards and colleagues, young healthy adults performed eccentric contractions of the deltoid and biceps brachii muscles and were administered influenza vaccination six hours post-exercise [5]. They found that eccentric exercise augmented antibody responses in women and interferon-γ (IFN-γ) levels in men compared to sedentary group. Similar effects were shown in another study by Edwards' group. Participants performed the same exercise task- 50 repetitions of the eccentric portion of both bicep curl and lateral raise movements, but at different intensities (light, moderate and heavy), and then received influenza vaccination immediately after exercise [6]. Eccentric exercise enhanced antibody responses specifically in men to the poorly immunogenic A/Uruguay strain compared to sedentary controls in all three intensity groups. These studies indicate that acute eccentric exercise of the muscle at the site of vaccine inoculation improves antibody responses in humans.

Based upon these findings in humans, we wanted to further explore the underlying mechanism using an animal model. Unfortunately, our null findings in antibody responses did not support the hypothesis that acute eccentric exercise prior to vaccination would enhance antibody responses in mice. However, it is consistent with some other research in humans. Campbell et al. explored the same bicep curl and lateral raise acute eccentric exercise protocol and investigated the effects of vaccine timing (immediately, 6hrs, and 48hrs post exercise) on immune response in young adults [4]. They found that eccentric exercise did not enhance antibody responses or IFN-γ production compared to the control group. However, they argued that this null finding may have been due to the fact that the influenza vaccination was strongly immunogenic in these subjects. In addition, participants completed a brisk walk at 55% heart rate maximum for 45 minutes and were injected with a full-dose pneumococcal vaccination and a half-dose influenza vaccination [66]. Four weeks post exercise, there were no significant differences in overall antibody titers between the exercise group and rest group, meaning a brisk walk prior to vaccination did not affect antibody response. Our laboratory similarly found no effect of an acute bout of walking on anti-influenza antibody responses in older adults who exhibit suboptimal responses to vaccination [65]. In another observational study, triathletes completed a half-ironman competition (3km swim, 130km cycle and 21km run) prior to tetanus toxoid, diphtheria and pneumococcal vaccination [19]. No significant differences were observed in antibody responses between exercised athletes, resting athletes and untrained sedentary controls. These

studies show either acute eccentric exercise or acute exercise in general did not improve antibody responses to vaccination in humans.

As the effects of acute exercise on vaccination responses is equivocal, chronic exercise training may be an alternative intervention method to augment antibody responses to vaccination. A previous study by our group demonstrated that cardiovascular exercise training extended influenza vaccine seroprotection in sedentary older adults [12]. Participants performing 10 months of moderate (60%-70% maximal oxygen uptake) cardiovascular exercise were compared with subjects doing flexibility and balance training. Cardiovascular exercise elicited a significant increase in the seroprotective response 24 weeks post vaccination compared to the flexibility controls. Two additional studies conducted similar moderate exercise training interventions in elderly and found that compared to sedentary group, exercise training enhanced antibody responses to influenza and KLH immunization, respectively [11, 60]. These studies above demonstrate that chronic exercise training can improve antibody responses to vaccination older adults. However, Long et al. studied a life-style physical activity intervention and the antibody response to pneumococcal vaccination in middle-aged women and found that the intervention did not increase the antibody response [13].

Our data has shown a reduction in anti-OVA IgG responses to vaccination in young sedentary mice compared to aged sedentary mice, which is consistent with previous literature [105]. Aging is associated with a decrease in naïve B cells, but an increase in effector B cells. As a result, there is an impaired antibody responses in the aged [52]. Aging also leads to a reduced class switching and

somatic recombination TCR repertoire, which contributes to a lower diversity of antibody responses and a reduced immunoglobulin G (IgG) antibodies, which contributes to a lower affinity of antibody responses [53]. Aging is also associated with a decrease in both number and function of naïve T cells. However, we did not demonstrate an aging effect in DTH responses to vaccination in our experiments. This may due to the different experiment models or different strains of mice.

In conclusion, compared to the sedentary or sham control groups, neither one bout nor two bouts of eccentric downhill treadmill exercise, nor electrical stimulation of eccentric contraction, improved the primary antibody responses to OVA vaccination in young or aged animals in our study. Despite the negative results of eccentric exercise on antibody responses, we found that there was beneficial effect of acute eccentric exercise on the cell-mediated immune response to vaccination in aged, but not young, mice. This study was the first to demonstrate the effect of acute eccentric exercise on immune responses to vaccination in an animal model. As the literature suggests that the effect of acute eccentric exercise is best found when a suboptimal dosage or poorly immunogenic strain of vaccine is utilized, in our study, we used a suboptimal dosage of OVA and yet still found no effect on the antibody response, but only beneficial effect on cell-mediated immune responses in aged animals. We also found no effect despite the fact that we utilized two different mouse strains and applied the vaccination at various times post-exercise (e.g. immediately, 6h and 24h). Future studies could utilize different forms of exercise (i.e. exercise

training) and different models of immunosuppression (for example, chronic stress).

CHAPTER 4

EXERCISE AS A MEANS TO ATTENUATE STRESS-INDUCED REDUCTIONS IN VACCINATION RESPONSES

4.1 INTRODUCTION

Chronic stress has been shown to suppress immune responses, leading to morbidity and mortality due to infectious diseases. Vaccines have been one of the most successful interventions to protect against infectious diseases and improve human health. Unfortunately, people undergoing chronic stress have impaired vaccine efficacy and a weakened capacity to protect against infectious disease. If our hypotheses are verified, that acute eccentric exercise or exercise training enhances immune response to vaccination in chronically stressed mice, results from our study would be potentially useful clinically to prescribe acute exercise or exercise training as a behavioral adjuvant to augment vaccine efficacy in people undergoing chronic stress. We hypothesized that chronic restraint stress would suppress both antibody and DTH responses to OVA vaccination compared to unstressed control mice. Moreover, chronic restraint stress would also decrease body weight and spleen weight, but would cause adrenal hypertrophy compared to controls. Importantly, we hypothesized that both acute eccentrically-biased downhill running exercise and voluntary wheel exercise training would attenuate chronic restraint stress-induced reductions in antibody and cell-mediated immune response to vaccination.

4.2 METHODS

Animals

C57BL/6J male mice aged 6-8 week (n=35) were purchased from Jackson Laboratory (Bar Harbor, ME) and individually housed in our AAALAC-accredited animal facility. Mice were allowed ad libitum access to water and food (Teklad 8640, Harlan Laboratories, Indianapolis, IN). All animals were maintained on a 12-hour light-dark cycle. All experiments were approved by the University of Illinois at Urbana-Champaign IACUC.

Restraint Stress

Restraint stress was applied by placing mice in adequately ventilated 60-ml syringes, while ensuring that they were capable of moving laterally, but not vertically. It has been shown that restraint stress is widely used as a psychological stressor including activation of the autonomic nervous system, hypothalamic-pituitary-adrenal axis and adrenal steroid receptors [95]. Mice in the three stress groups were exposed to the stressor 5 days/week for six hours per day for 3 weeks. The stress sessions were conducted from 9am to 3pm Monday through Fridayd during the dark cycle (dark cycle: 3am to 3pm).

Study design

Mice were randomized into four groups: mice that did not receive restraint stress and were sedentary (No stress, n=9), mice that received chronic restraint stress and performed a single acute eccentric exercise bout (Stress + ECC, n=9), mice that received chronic restraint stress and performed voluntary wheel running (Stress + VWR, n=10), and mice received chronic restraint stress and

remained sedentary (Stress-SED, n=7). All three stressed groups received chronic restraint stress for 3 weeks. Mice in the ECC group performed a single acute bout of eccentrically-biased downhill running (as described before) 42h after the last stress session (Figure 4.1). Mice in the VWR group were housed in cages with free access to telemetered running wheels (manufacturer?) starting from the first day of restraint stress, continuing throughout the entire experiment (Figure 4.2). Mice in the SED group were handled similarly and housed in the same room with the treadmill and wheel cages to control for incidental stress (Figure 4.3). Mice in the “No stress” group stayed in their home cage for the entire experiment. All mice received 100μg/mouse of OVA with 200μg alum intramuscularly at 43h after the first week’s stress session (immediately after eccentric exercise for ECC group). Two weeks after the sensitization, all mice were challenged with 100μg OVA to assess DTH responses. Body weight was measured immediate after stress daily. Mice were euthanizeded for tissue collection at 4wk post initial OVA sensitization.

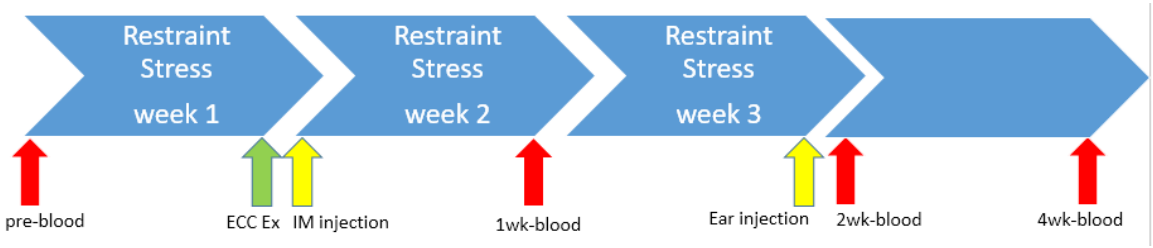

Figure 4.1: Study design: effects of acute eccentric exercise on immune response to vaccination in chronically stressed mice.

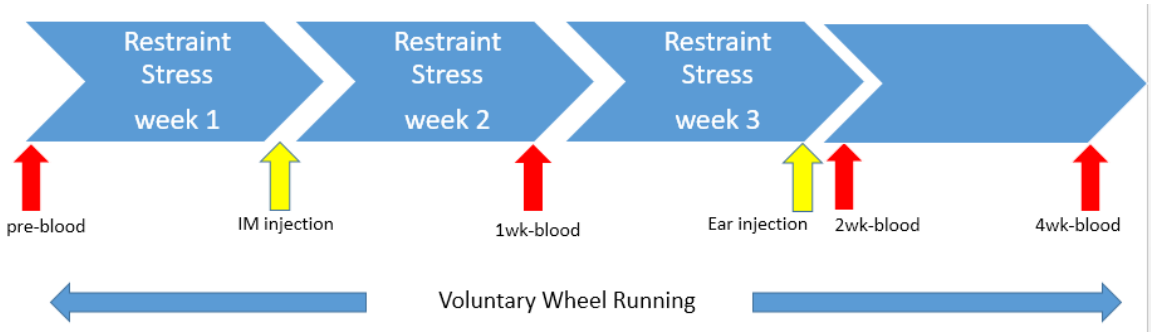

Figure 4.2: Study design: effects of voluntary wheel exercise training on immune response to vaccination in chronically stressed mice.

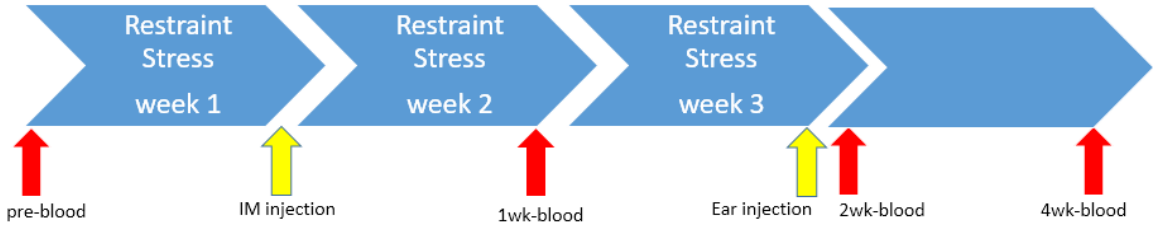

Figure 4.3: Study design: effects of chronic restraint stress on immune response to vaccination.

Eccentric Exercise

Eccentrically-biased downhill running protocol was the same as described before. Briefly, Stress+ ECC mice were exercised at 17 m/min speed at -20% grade for 45 minutes on a treadmill. No electrical shock was used.

Voluntary Wheel Running

Stress-VWR mice were given constant access to a telemetered running wheel (Respironics, Bend, OR) during the entire experiment (other than restraint stress period). Mice in the other groups were housed in similar cages lacking the running wheel and were subjected to similar handling throughout the experiment.

Vaccination Protocol

Intramuscular injections were the same as described before. Briefly, at 43h after the first weeks stress session (immediately after eccentric exercise for ECC group) all mice were intramuscularly inoculated in the gastrocnemius of their right hind limb with 100µg of OVA and 200µg of alum in 50µl sterile saline using a 25-gauge needle.

Delayed-type hypersensitivity (DTH)

The DTH response protocol was the same as described above. Briefly, two weeks after the sensitization, all mice were injected with 100µg OVA dissolved in 10µl PBS into the dorsal side of the right ear using a Hamilton syringed fitted with a 30-gauge needle. The left ear received 10µl PBS as a control for non-specific ear swelling due to injection. Both ears' thicknesses were measured immediately before, and every 24h after intradermal injection using a digital microcaliper (Tresna). The measurements were performed in triplicate by a single assistant who was blinded to the treatments.

Blood Collection

All mice were bled approximately 200µl from the retro-orbital vein using a Pasteur pipette prior to vaccination and at one, two and four weeks post-vaccination. Prior to blood collection, mice were administered isoflurane with oxygen at a flow rate of 2-3 L/min until unresponsive. Blood was dispensed into heparinized tubes and centrifuged at 1100 x g at 4°C for 15 min. Plasma was harvested and frozen at -20°C until analysis. At four week post-inoculation, blood was drawn after euthanasia from the inferior vena cava and processed as

described before. In each experiment, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation at four weeks post-vaccination.

Plasma Antibody Measures

Plasma total anti-OVA IgG and anti-OVA IgM were determined using ELISA procedures. Ninety-six-well microtiter plates were coated with 50µl of 20µg/ml OVA in carbonate coating buffer and incubated overnight at 4°C. Nonspecific binding was blocked with PBS supplemented with 10% fetal bovine serum (FBS) and incubated for 1h at 37°C. After washing three times with phosphate buffered saline (PBS)-Tween 20, 50µl of plasma was added at a dilution of 1:20 (IgG) or 1:200 (IgM) in a diluting buffer of PBS/1% FBS and incubated for 1h at 37°C. Plates were washed again, and 50µl of horseradish peroxidase (HRP)-rabbit anti-mouse IgG or HRP-goat anti-mouse IgM (Life Technologies, Frederick, MD), diluted 1:800 or 1:400 respectively in diluting buffer, was added. Plates were incubated again and then washed. Plates were incubated for 20 minutes in 50µl of a 1:1 mixture of 3, 3', 5,5' tetramethylbenzidine (TMB) and hydrogen peroxide (TMB Substrate Reagent Set, BD Biosciences, San Jose, CA). Lastly, 25µl of stop solution (sulfuric acid) was added and read at 450 nm on a spectrophotometric plate reader (Labsystems Multiskan, Fisher Scientific, Pittsburgh, PA). Plasma anti-OVA IgG or IgM was quantified as the difference in optical density (OD) at 450 nm from the pre-injection time point.

Statistical Analysis

Results were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL). Plasma anti-OVA IgG was assessed by repeated-measures analysis of variance

(ANOVA). All analyses were followed by post-hoc Bonferroni tests in the event of a significant main effect or interaction. Ear swelling on day 1 post ear injection was assessed by independent T test. Statistical significance was set at $P \leq 0.05$ for all tests. Results are reported as mean ± SEM.

4.3 RESULTS

Chronic restraint stress reduced body weights and induced adrenal hypertrophy.

First, we examined the effects of three weeks of chronic restraint stress on body weight. Data were presented as percentage change compared to baseline. As expected, we found there was a significant time main effect, ($F_{33, 1023} = 261$, $p<0.001$), significant time x treatment interaction ($F_{99, 1023} = 21$, $p<0.001$), and a significant treatment main effect ($F_{3, 31} = 13$, $p<0.001$) demonstrating that chronic restraint stress significantly reduced body weight relative to non-stressed controls (Figure 4.4). We also found a significant treatment effect ($F_{3, 31} = 4.34$, $p=0.011$) for adrenal, (Figure 4.5) but not spleen, weight ($F_{3, 31} = 0.530$, $p=0.665$) (Figure 4.6) indicating that chronic restraint stress resulted in significant adrenal hypertrophy compared to non-stress controls.

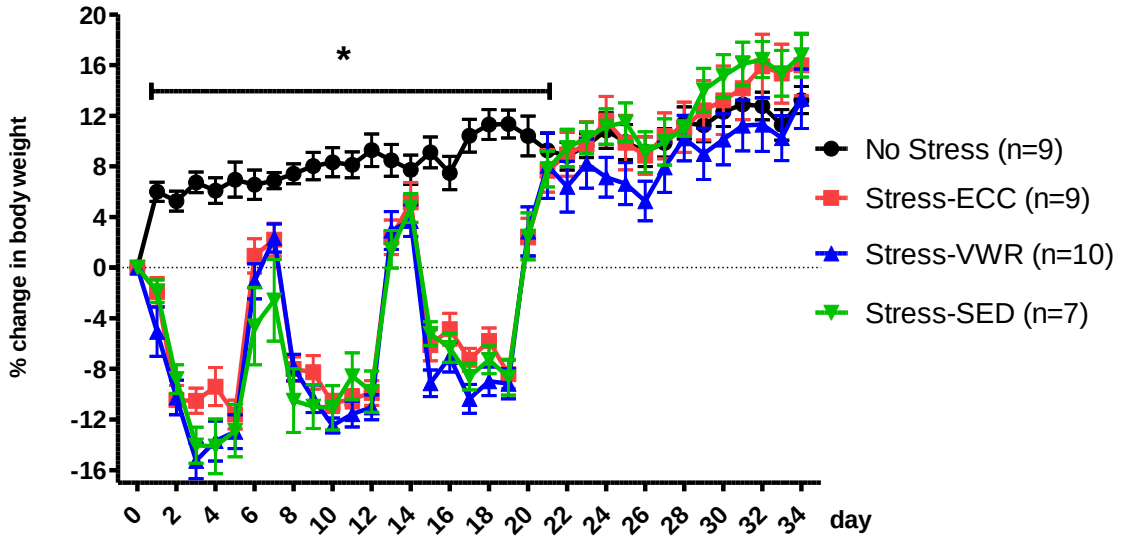

Figure 4.4: Chronic restraint stress reduces body weight. There was a significant time main effect, ($F_{33, 1023}$ = 261, p<0.001), significant time x treatment interaction ($F_{99, 1023}$ = 21, p<0.001), and a significant treatment main effect ($F_{3, 31}$ = 13, p<0.001). *signifies “No stress” group statistical significant higher than three stress groups from day 1 to day 21 (p < 0.05).

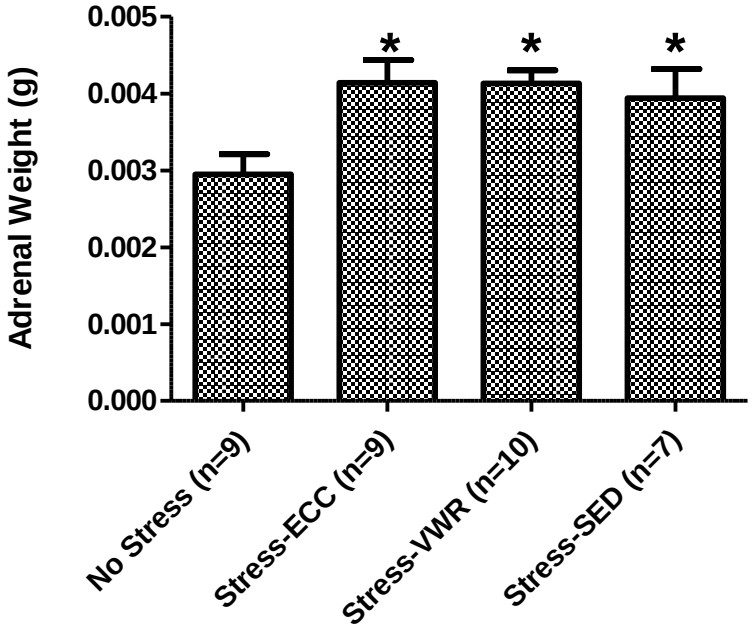

Figure 4.5: Chronic restraint stress induced adrenal hypertrophy. There was a significant treatment effect ($F_{3, 31} = 4.34$, p=0.011). *signifies "No stress" group statistical significant lower than stress groups ($p < 0.05$).

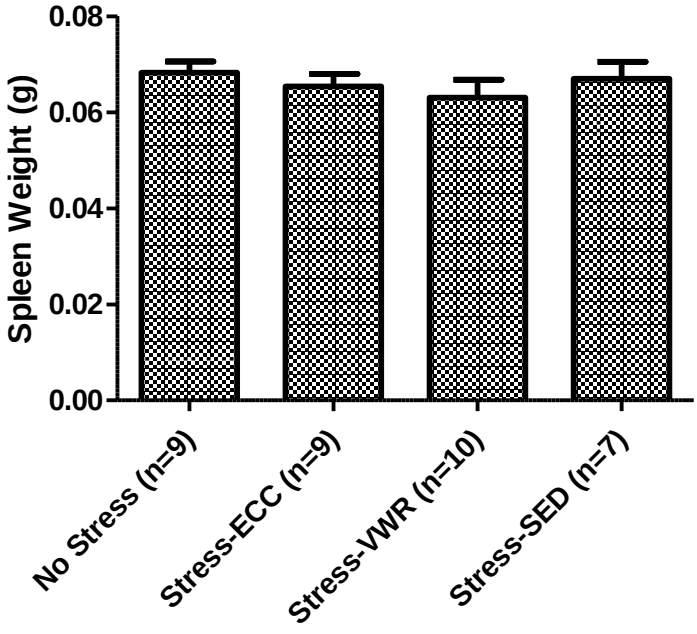

Figure 4.6: Chronic restraint stress did not affect spleen weight. There was no significant treatment effect ($F_{3, 31} = 0.530$, p=0.665).

Effects of exercise on antibody responses to vaccination in chronically-stressed mice.

Additionally, we investigated whether acute eccentrically-biased downhill running and voluntary wheel exercise training could attenuate stress-induced reductions in antibody responses to vaccination. We examined both anti-OVA IgM and anti-OVA IgG responses to vaccination. When we examined the anti-OVA IgM responses, we found that there was a significant time main effect ($F_{3, 93}$ = 16, $p<0.001$) as expected, demonstrating plasma anti-OVA IgM increased significantly at one, two and four weeks relative to pre-immunization levels. We did not find significant time x treatment ($F_{9, 93}$ = 1.1, p=0.375), but there was a trend towards significance in treatment main effect ($F_{3, 31}$ = 2.4, p=0.085), especially at two week post vaccination ($F_{3, 31}$ = 4.3, p=0.012, when analyzed separately) (Figure 4.7). When we examined the anti-OVA IgG responses, we found a significant time main effect ($F_{3, 93}$ = 257, $p<0.001$), demonstrating plasma anti-OVA IgG increased significantly at one, two and four weeks relative to pre-immunization levels. We did not find significant time x treatment ($F_{9, 93}$ = 0.87, p=0.555), but there was a trend towards significance in treatment main effect ($F_{3, 31}$ = 1.8, p=0.16). (Figure 4.8). In summary, we report that both acute eccentric exercise and voluntary wheel exercise training tend to attenuated chronic restraint stress-induced suppressions in anti-OVA IgM and anti-OVA IgG responses to vaccination.

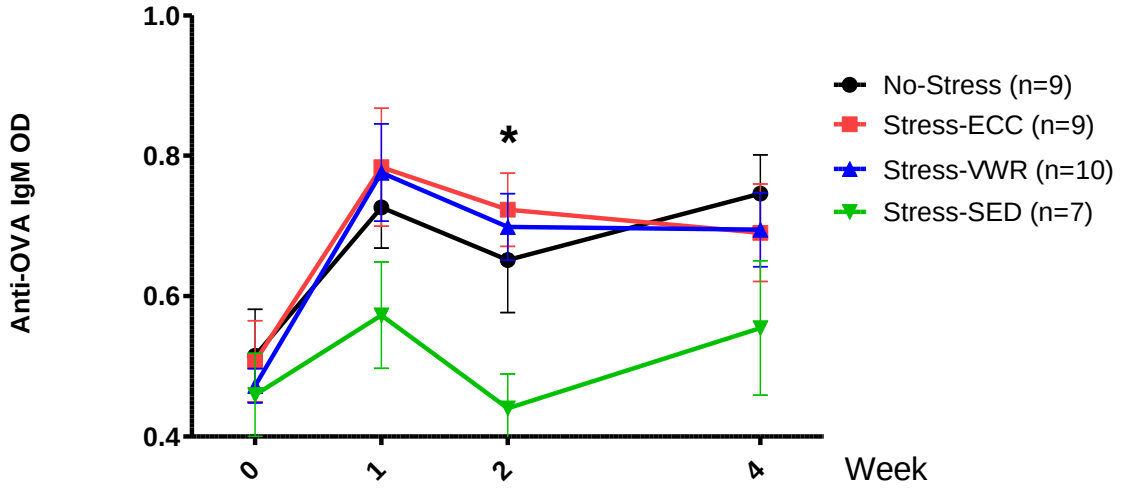

Figure 4.7: Effects of exercise on anti-OVA IgM responses in chronically-stressed mice. There was a significant time main effect ($F_{3, 93} = 16$, $p<0.001$), no significant time x treatment ($F_{9, 93} = 1.1$, $p=0.375$), a trend towards significance in treatment main effect ($F_{3, 31} = 2.4$, $p=0.085$). Univariate analysis revealed a significant treatment effect at two week post vaccination ($F_{3, 31} = 4.3$, $p=0.012$). *signifies “Stress-SED” groups statistical significant lower than other groups ($p < 0.05$).

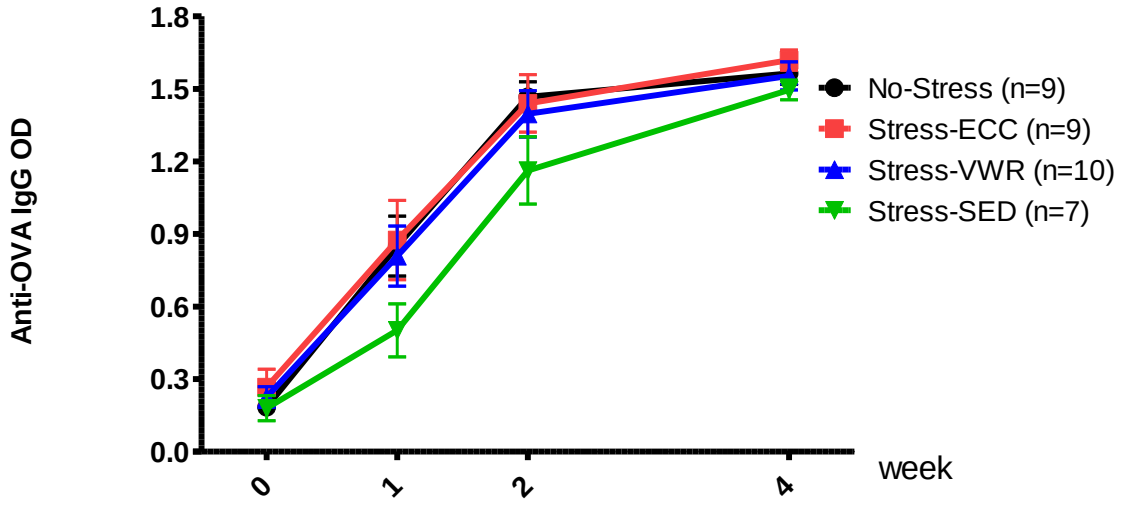

Figure 4.8: Effects of exercise on anti-OVA IgG responses in chronically-stressed mice. There was a significant time main effect ($F_{3, 93} = 257$, $p<0.001$), no significant time x treatment ($F_{9, 93} = 0.87$, $p=0.555$), but a trend towards significance in treatment main effect ($F_{3, 31} = 1.8$, $p=0.16$).

Effects of exercise on cell-mediated responses to vaccination on chronically-stressed mice.

In addition, we examined whether acute eccentrically-biased downhill running and voluntary wheel exercise training could attenuate stress-induced reductions in cell-mediated immune response to vaccination. We found there was significant time main effect ($F_{6, 186} = 9.1$, $p<0.001$), demonstrating that ear swelling increased significantly in all groups (Figure 4.9). However we did not find a significant time x treatment interaction ($F_{18, 186} = 0.559$, $p=0.925$). Mice in "No stress" group had significant higher DTH responses than all three stressed groups ($F_{3, 31} = 4.11$, $p=0.014$), but Bonferroni analysis revealed that there were no significant differences among the three stressed groups. So, chronic restraint stressed reduced DTH responses compared to non-stressed groups, but neither

acute eccentrically-biased downhill running nor voluntary wheel exercise training affected the CMI reductions to vaccination.

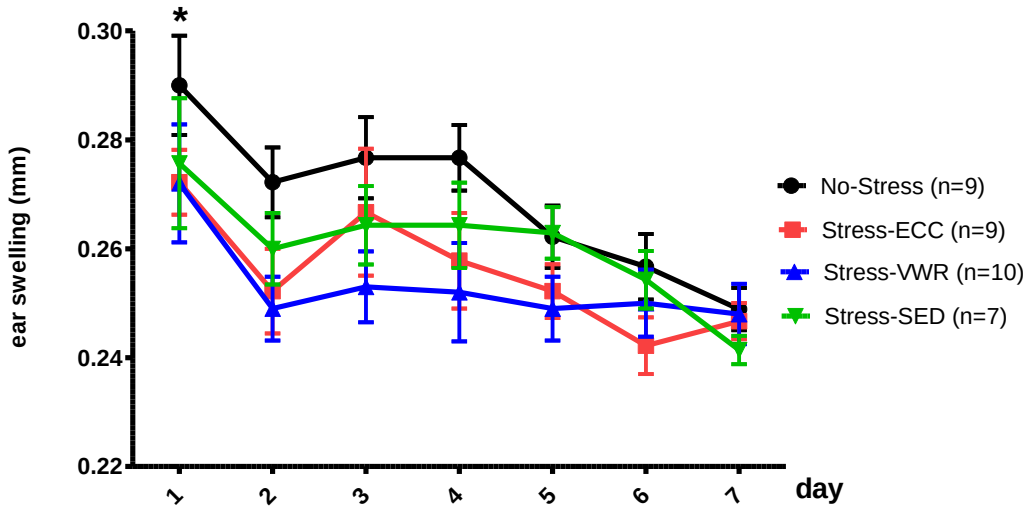

Figure 4.9: Effects of exercise on DTH responses in chronically-stressed mice. There was a significant time main effect ($F_{6, 186} = 9.1$, $p<0.001$), no significant time x treatment ($F_{18, 186} = 0.559$, $p=0.925$), significant treatment main effect ($F_{3, 31} = 4.11$, $p=0.014$). *signifies "No Stress" group statistical significant higher than other groups ($p < 0.05$).

4.4 Discussion

This study examined the effects of acute eccentrically-biased downhill running and voluntary wheel exercise training on immune responses to vaccination in chronically stressed mice. We conclude that both acute eccentric exercise and voluntary wheel running tends to attenuate chronic restraint stress-induced reductions in antibody, but not cell-mediated immune, responses to vaccination.

The mechanisms responsible for the beneficial effect of exercise on antibody responses in chronically stressed mice remain unclear. However, there may be several potential mechanisms. It has been well established that one of the main characteristics of the stress response is activation of hypothalamic pituitary adrenal (HPA) axis. After the perception of a stress, the hypothalamus is activated to release corticotrophin-releasing hormone (CRH). CRH will activate the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH will then activate adrenal cortex to release glucocorticoids (GC) into the general circulation. After acute stimulation of the HPA axis, GC will have a negative feedback to the pituitary and hypothalamus and inhibit the further secretion of ACTH and CRH. This will prevent excessive release of these hormones and terminate the stress- induced HPA axis activation [96]. Chronic stressors enhance HPA axis responses, but the sympathetic-adrenomedullary system remains unchanged, which leads to adrenal hypertrophy; consistent with our data above. Chronically elevated GC levels reduce the sensitivity of HPA negative feedback and altered plasma GC concentrations. So, one mechanism for chronic stress to suppress vaccination response is through elevated GC levels [97]. Acute exercise modulates the HPA axis by an increase in GC [98], while exercise training leads to reduced GC by an increase of GC metabolism and a decrease in adrenal responsiveness to ACTH [99].

It has been suggested that physical exercise facilitates habituation of HPA-axis activation under stress conditions [100]. Sasse et al. examined the effects of chronic voluntary wheel running on stress hormone habituation to

repeated audiogenic stress exposure in rats [100]. Rats were given access to running wheel for 6 weeks or remained sedentary. Then, all rats were exposed to 11 days of noise stress for 30 min/day. They reported that exercised animals had significant lower corticosterone, but not ACTH, levels compared to sedentary animals after repeated stress. They conclude that voluntary physical exercise facilitates habituation of HPA-axis activation to repeated stress, a phenomena known as cross-stress resistance [101]. Similarly, exercise has also been shown to modulate the HPA-axis responses to stress in humans. Wittert et al. reported that ultramarathon athletes have adaptive changes in basal HPA function in response to stress, including a phase shift and increased pituitary ACTH secretion, but a blunted adrenal cortisol response [102]. However, previous literature regarding the effect of exercise on HPA axis responses to stress are inconsistent. Moraska et al. reported four-week of voluntary wheel running did not affect corticosterone responses to inescapable tail-shock stress compared to sedentary group [82].

Another potential mechanism responsible for the beneficial effect of exercise on antibody responses in chronically stressful situations may involve the sympathetic nervous system (SNS) and norepinephrine (NE). Sympathetic nerve terminals release the neurotransmitter NE in the proximity of immune cells that express the β2 adrenergic receptor (β2AR), helping to maintain immune homeostasis [103]. For example, sympathetic nerve fibers penetrate into primary and secondary lymphoid organs. The neurotransmitter NE is released from nerve terminals located within the direct vicinity of CD4 T cells and B cells, which

express the β2AR. Th1 cells that develop from naïve CD4 T cells, which are activated by NE, produce more INF-γ per cell. NE stimulates the β2AR on B cells to increase the rate of antibody production [103]. Wang et al. investigated exercise-induced NE in counteracting stress-induced hippocampal damage [104]. They compared voluntary wheel running, three weeks of restraint stress, exercise and stress, and control groups. They reported that the exercise-alone group had the highest NE levels, while the exercise-stressed group had significant higher NE levels than the stressed-alone group. So catecholamine, specifically NE, released by the SNS may play a role in exercise attenuating stress-induced reductions in antibody responses.

Acute exercise acting as an adjuvant in increasing muscle inflammation may also play an important role in augmenting the vaccine responses. Eccentric exercise causes the exercising muscle to produce continuous force while it lengthens, leading to damage of the internal structure of the muscle fibers and connective tissue [7]. The substantial increase in plasma creatine kinase indicates this damage is greater than that caused by concentric or shortening muscle contractions [8]. Muscle damage can result in a localized inflammatory response and delayed onset muscle soreness when conducted in naive participants [9]. It is hypothesized that the influx of immune cells and the release of inflammatory mediators caused by this eccentrically-induced muscle damage creates a pro-inflammatory environment in a muscle that may activate dendritic and other cells to augment the immune response to vaccination when given intramuscularly in the damaged muscle [10]. This heightened inflammatory

environment may be a particularly effective behavioral adjuvant as eccentric exercise localizes muscle damage to the specific site of intramuscular vaccine administration.

In conclusion, our data suggested that both eccentrically-biased downhill running and voluntary wheel exercise training tends to attenuate chronically restraint stress-induced suppression in antibody, but not cell-mediated responses in mice.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

We examined the effects of exercise on age- and stress-related attenuation of vaccination responses in mice. Firstly, we investigated the effects of acute eccentric exercise on immune responses to a suboptimal dosage of ovalbumin vaccination in young and aged mice. Secondly, we examined the effects of acute eccentrically-biased downhill running and voluntary wheel exercise training on immune responses to vaccination in chronically stressed mice. We conclude that acute eccentric exercise enhanced cell-mediated response in aged mice, but not antibody responses in either young or aged mice and that both acute eccentric exercise and voluntary wheel running tends to attenuate chronic restraint stress-induced reductions in antibody, but not cell-mediated immune responses to vaccination.

There are some potential limitations to this study. First, we had a relative small sample size and only male mice were used in our experiments. Use of males only is consistent with previous rodent studies, which investigate the effects of exercise or stress in general, on immune responses to vaccination [14, 27, 28], most likely to minimize the confound of female reproductive cycle on study outcomes. However, human studies have reported different antibody or cell-mediated responses to vaccination in men and women [5]. Pascoe et al. proposed that exercise beneficial effect only observed in men or women was because of their differences in the control responses [92]. Beneficial effects were

only observed in the weaker control responses group. That being said, we used aged mice and chronically-stressed model to further investigate the mechanisms. In addition, our sample size may be smaller than other animal studies [67].

In our second set of experiments, we can only conclude that there was a tendency for acute eccentric exercise and voluntary wheel running to attenuate the stress-induced reduction in antibody responses. We did not have enough statistic power to draw a definitive conclusion because of our small sample size and relative large variability. In future studies, we would add more animals to this study in order to increase the statistical power to detect an effect and to draw a definitive conclusion. If upon finding a definitive conclusion, future experiments will need to be performed to determine the effect of exercise on stress hormones (i.e. ACTH, corticosterone, norepinephrine) in stressed conditions to further understand the mechanism underlying the beneficial effect of exercise on immune response to vaccination in chronically restraint stressed mice.

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