BEHAVIOURAL ADJUVANTS TO VACCINATION

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ABSTRACT

This thesis investigated the effects of acute eccentric exercise on the immune response to vaccination in young humans. Study one investigated whether the efficacy of the eccentric exercise intervention was affected by manipulating the timings of exercise prior to influenza vaccination. Three exercise groups were vaccinated immediately, 6 hr or 48 hr after exercise and antibody responses at 28 days post-vaccination were compared to those from a resting control group. All participants exhibited robust antibody responses to the vaccine and no effect of exercise was observed; therefore, it was not possible to determine the effects of exercise timing on vaccine responses. Study two investigated whether the antibody response to influenza vaccination was influenced by the intensity of eccentric exercise. Three groups exercised at an intensity eliciting 60%, 85% or 110% of one repetition maximum, and the antibody responses at 28 days post-vaccination were compared to those from a resting control group. In the exercise groups, both men and women showed enhanced antibody responses against the B/Florida strain, and men had enhanced responses against A/Uruguay, in comparison to resting controls. In both cases, the control group exhibited poorer responses against these strains, but no effect of exercise intensity was observed. Study three investigated whether the site of vaccine administration affected the efficacy of the immune response to hepatitis B vaccination following eccentric exercise. The antibody seroconversion rate to the vaccine was low (approx. 5 %), and thus, further analysis between exercise and control participants was not feasible. In sum, supporting previous research, it appears that acute eccentric exercise can enhance the immune response to poorly immunogenic strains of influenza, but research is needed to establish if exercise can enhance other poorly immunogenic vaccines, or vaccine responses in the immuno-compromised.

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LIST OF PAPERS

This thesis incorporates the following three papers, each corresponding to one of three original empirical studies:

1. Campbell J.P., Edwards K.M., Ring C., Drayson M.T., Bosch J.A., Inskip A., Long J.E., Pulsford D., Burns V.E. The effects of vaccine timing on the efficacy of an acute exercise intervention on the immune response to an influenza vaccine in young adults. *Brain, Behavior and Immunity*. In press.

2. Campbell J.P., Edwards K.M., Ring C., Drayson M.T., Bosch J.A., Downes C., Long J.E., Lumb J.A., Merry A., Paine N.J., Burns V.E. Eccentric exercise as a behavioural adjuvant to influenza vaccination: effects of exercise intensity. *Brain, Behavior and Immunity*. In submission.

3. Campbell J.P., Edwards K.M., Ring C., Drayson M.T., Bosch J.A., Carrack M., Gould L., Holmes M., Voke H., Burns V.E. The influence of vaccine administration site on the efficacy of eccentric exercise as an adjuvant to hepatitis B vaccination. *Brain, Behavior and Immunity*. In preparation.

In addition, the following presentations arose from material from this thesis:

- Campbell J.P., Edwards K.M., Ring C., Drayson M.T., Bosch J.A., Inskip A., Long J.E., Pulsford D., Burns V.E. Effects of eccentric exercise on the antibody response to a highly immunogenic influenza vaccine. *American College of Sports Medicine meeting*, 2009.

- Campbell J.P., Edwards K.M., Ring C., Drayson M.T., Bosch J.A., Downes C., Long J.E., Lumb J.A., Merry A., Paine N.J., Burns V.E. Eccentric exercise enhances the antibody response to a half-dose influenza vaccine. *International Society of Exercise Immunology meeting*, 2009.

During the period of postgraduate study at the University of Birmingham the following papers and presentations were produced:

Papers

- Campbell J.P., Guy K., Cosgrove C., Florida-James G.D., Simpson R.J. Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human peripheral blood following an acute bout of high-intensity exercise. 2008. *Brain, Behavior and Immunity*, 22 (3), 375-380.

- Campbell J.P., Riddell N.E., Burns V.E., Turner M., van Zanten J.J., Drayson M.T., Bosch J.A. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. 2009. *Brain, Behavior and Immunity*, 23 (6), 767-775.

- Burns V.E., Edwards K.M., Campbell J.P., Carroll D., Drayson MT., Ring C. Acute behavioural interventions prior to vaccination reduce the likelihood of self-reported adverse events. In preparation.

Presentations

- Campbell J.P., Drayson M.T., Burns V.E., van Zanten J.J., Bosch J.A. Selective mobilisation of functionally distinct NK subsets during moderate and high intensity exercise. *Psychoneuroimmunology Research Society meeting, 2007*

- Campbell J.P., Drayson M.T., Burns V.E., van Zanten J.J., Bosch J.A. Brief exercise mobilises cytotoxic T cells with an effector-memory phenotype *Psychoneuroimmunology Research Society meeting, 2007.*

- Campbell J.P., Edwards K.M., Burns V.E., Ring C., Carroll D., Drayson MT. Acute behavioural interventions prior to vaccination reduce the likelihood of self-reported adverse events. *Psychoneuroimmunology Research Society meeting, 2008.*

Finally, this thesis received the following media coverage:

- Stress: beneficial to health? Live interview on Australian Broadcasting Corporation Radio Darwin 23rd October 2009.

CHAPTER ONE

ECCENTRIC EXERCISE AS A BEHAVIOURAL ADJUVANT TO VACCINATION

INTRODUCTION

The introduction of vaccines against diseases has been one of the most successful public health interventions ever and, at present, is the most effective and cost-efficient method of disease prevention in humans (Nicholson et al. 2003). Despite the remarkable success of vaccination, there are variations in the efficacy of some vaccines, and many are only capable of inducing poorly immunogenic responses. For example, the effectiveness of influenza vaccination at preventing disease can be as high as 71 %, but there is research to suggest that the efficacy may be as low as 53 % in healthy adults (Villari et al. 2004), whereas other vaccines such as hepatitis B induce even poorer immune responses, and further 'booster' vaccinations are thus required (Hilleman, 2000). Given the often poor immunogenicity of some common vaccines, there is a large body of research now devoted to developing adjuvants to improve immune responses (Aguilar and Rodriguez, 2007).

The immune responses to vaccination and the role of adjuvants

The immune response to vaccination is characterised by a synergistic activation of both the innate and adaptive immune systems to execute an effective response to the antigens contained within the vaccine. Upon first exposure to antigen following intra-muscular injection, antigen presenting cells (APCs) of the innate immune system, typically the tissue-specific dendritic cells, recognise antigen, before migrating to the lymph nodes to present the processed antigen to CD4+ T cells via MHC class II signalling to T cell receptor (CD3). Following a reduction of the CD45RA receptor, and upon receipt of a secondary signal via the co-stimulatory molecule CD28 with CD80 or CD86 on the APC, these antigen specific CD4+ T cells then differentiate. Differentiation is driven by the rapid release of potent T cell growth-factor, the cytokine IL-2, that in combination with interferon-γ (IFN- γ), leads to the development of Th1 CD4+ T cells. In addition, IL-4 is released which leads to the creation of Th2 CD4+ T Cells. Th1 CD4+ T cells drive cell-mediated responses, whereas Th2 CD4+ T cells provide signalling to naïve B cells that drive humoral responses to the vaccine. The cell-mediated response is characterised by high proliferation of CD8+ T cells that are capable of releasing cytotoxic agents (e.g., perforin, granzyme A, granzyme B) that kill infected host cells. The humoral response is mostly comprised of antigen specific B cells which release antibodies. The process described above is a thymus-dependent response, in which the help of T cells is required to initiate an antibody responses from B cells. A thymus-dependent response occurs when a protein antigen is encountered, and this is the most common type of antibody response. In contrast, thymus

independent responses occur with exposure to polysaccharide vaccines; in this process, B cells are able to generate a response without T cell help.

The first class of antibody released during a primary infection are IgM antibodies; these bind antigen with high avidity, due to their pentameric formation and have the ability to activate complement. IgG antibodies are also released during a primary response, and do not typically peak until approximately 4 weeks post-vaccination. When the initial threat of infection has passed, the primary response to antigen is scaled back, and memory B and T cells remain in the event of re-infection with similar antigenic challenge. The secondary immune response is a much more rapid response that functions on the pre-existence of specific memory B and T cells. Upon re-infection with the same antigen, APCs present to antigen-specific memory cells, and they rapidly undergo clonal expansion and produce specific IgG much more quickly than during the primary response.

Adjuvants, (from *Adjuvare*: Latin, meaning to help) have been described as the immunologist's 'dirty little secret' (Janeway, Jr., 1989) and are agents that can stimulate the immune system. They can be broadly separated into two subcategories: exogenous and endogenous adjuvants. Exogenous adjuvants can be chemicals (e.g., alum, oils and detergent) or microbial components or toxins (e.g., lipopolysaccharide and Pertussis toxin) and typically function by promoting recognition by the innate immune system, which subsequently enhances the speed and magnitude of the adaptive immune response (Aguilar and Rodriguez, 2007). Despite the advances made using exogenous adjuvants, the efficacy of many vaccines remains low and the pursuit of new strategies to promote immune responses is desired. As a result, research has recently begun to

focus on the role of endogenous adjuvants to vaccination (Rock et al. 2005). Endogenous adjuvants are agents produced by various cells of the body that, like exogenous adjuvants, promote the activity of APCs which in turn increases the speed and magnitude of immune responses (Rock et al. 2005). 'Danger signals' (e.g., IL-6) are released from sites around the body in response to acute physical (Edwards et al. 2006b) or immunological stress and assist in the activation of both innate and adaptive arms of the immune system (Matzinger, 2002).

The role of behaviourally-induced endogenous adjuvants to vaccination

The acute-stress immuno-enhancement hypothesis (Edwards et al. 2007b) has been developed based on a number of studies suggesting that acute stress, experienced at close temporal proximity to vaccine administration, may induce endogenous adjuvants and thus augment the subsequent immune response. For a number of years, researchers have suggested that, from an evolutionary standpoint, acute stress is an integral component of the fight or flight response (Dhabhar and McEwen, 1999). This response initiates a series of immunological changes, presumably in anticipation of exposure to antigenic challenge, with the primary aim of maximising immune responses against the antigen. The literature surrounding this area is mainly comprised of research conducted in animals, with an increasing number of studies also conducted in humans. In order to assess the effectiveness of the immune responses to vaccination, researchers in this area have typically investigated either humoral immunity status (e.g., specific antibodies from memory B lymphocytes) or cellular responses (e.g., delayed type hypersensitivity reactions or interferon γ response to *in vitro* stimulation). In animals, a variety of behavioural stressors have been used, including restraint stress, inescapable footshock, and

exercise. Restraint stress involves placing the animal in a ventilated plastic tube. The animals are not physically squeezed or harmed; instead, it is conceived as a psychological stressor due to the feeling of confinement (Glavin et al. 1994). Inescapable footshock involves animals receiving random electric shocks delivered through a wired floor, cage or box. During exercise, the animals are typically exposed to exercise on a treadmill until a certain time elapses, or until volitional exhaustion. Human studies have typically used exercise with either dynamic whole-body exercise (e.g., cycling) or isolated muscle actions of isolated muscle groups (e.g., eccentric movements). Human studies using psychological stress have utilised a naturalistic stressor, such as an examination, or more contrived laboratory stress-tasks, such as public speaking or mental arithmetic. Regardless of the mode chosen, the hypothesis is that the stressor temporarily primes the immune system, to a similar extent, and augments the response to any proximal pathogen exposure.

ACUTE STRESS-INDUCED ENHANCEMENT OF VACCINATION RESPONSES: EVIDENCE FROM ANIMALS

Acute stress-induced enhancement of vaccination response in animals: humoral immunity

Studies conducted in animals investigating the effects of acute stress on the humoral response to vaccination support the stress-induced immuno-enhancement hypothesis. For example, Millan and colleagues investigated whether short or long duration restraint stress affected the antibody response to sheep red blood cell (SRBC) inoculation in rats. In this study, all rats were inoculated immediately prior to the stressor. Results indicated that rats exposed to short periods

of restraint stress (2 hours over 2 consecutive days) had higher primary antibody titres at 7 days post-immunisation compared with control rats. Further, no differences were observed between rats exposed to long stress (6 hours over 4 consecutive days) and control rats. This finding highlights the important distinction between acute and chronic stress, whereby acute stress appears to enhance whereas chronic stress appears to suppress immune responses (Millan et al. 1996).

In a similar study investigating acute stress and antibody responses to SRBC inoculation, it was found that 2 hours of restraint stress immediately prior to vaccination enhanced the primary IgG response 10 days post-inoculation (Silberman et al. 2003). The animals were then given another SRBC inoculation on day 11 and the secondary IgG antibody responses were measured on day 18. Animals exposed to acute stress at the time of primary inoculation also showed enhanced secondary responses compared with control animals. Another group of animals exposed to chronic stress at the time of primary inoculation showed poorer primary and secondary IgG responses to the vaccine. These results again indicate that exposure to an acute stressor may augment antibody responses, whereas prolonged periods of stress do not.

In a study conducted by Wood and colleagues, it was found that rats exposed to electrical footshock on the day of, and the day after, keyhole limpet hemocyanin (KLH) inoculation had enhanced IgG antibody responses at 17 days post-vaccination, compared with control rats. Rats exposed to electrical footshock one day, or three days, post-vaccination did not show augmented responses compared with control (Wood et al. 1993). These results seem to highlight the

importance of vaccinating at close temporal proximity to the acute stress exposure, as otherwise, it seems that the adjuvant effect is either reduced or lost.

Persoons and colleagues investigated the effects of stress exposure on the antibody responses to trinitrophenol-KLH (TNP-KLH) inoculation. In this study, 20 minutes of electrical footshock immediately prior to intracheal TNP-KLH vaccination augmented the IgA, IgE and IgG antibody responses of these stressed animals, compared with non-stressed controls (Persoons et al. 1995). Persoons and colleagues hypothesised that acute stress augmented these responses by stimulating the release of cytokines from activated macrophages in the alveoli. Although speculative, this idea supports the general contention that acute stress has the potential to augment processes involved in the early stages of immune responses, such as antigen identification and presentation.

Karp and colleagues found that mice exposed to restraint stress immediately after (KLH) vaccination showed enhanced IgM antibody responses at three time points post-inoculation (8, 14 and 21 days). The authors of this study were interested in whether restraint stress could enhance the humoral response in immuno-deficient mice, modelling the poorer immune systems seen in elderly adults. The results indicated that mice administered an immunosuppressive drug, cyclophosphamide, prior to vaccination appeared to show the largest increase in antibody responses when exposed to restraint stress (Karp et al. 2000). These findings appear to illustrate that acute stress exposure may be more beneficial at augmenting immune responses in circumstances where poorer responses would otherwise be expected. In addition, it seems that more robust responses, as seen in the control mice, are less enhanced by stress.

To our knowledge, only one study has investigated the effects of acute exercise on the humoral immune response to vaccination in animals. In this study, Kapasi and colleagues randomly allocated young and old mice previously inoculated with human serum albumin (HSA) to one of three groups. Two groups were exposed to a single bout of exhaustive intense exercise and immediately re-administered an HSA inoculation. The first group did not exercise further, but the second group continued with a more chronic regimen of daily bouts of intense exercise to exhaustion for 9 days. The third group acted as a control group and did not undergo intense exercise, but received the booster injection of HSA at the same time as the other groups. Results indicated no effects of acute or chronic exercise on the antibody response to HSA in young mice. However, older mice exposed to the acute exercise condition had elevated antibody responses in comparison to other older mice in either the control group or the chronic exercise group. Further, control young mice had higher antibody responses than control elderly mice, but elderly mice exposed to the acute exercise task had antibody responses similar to those seen in the young mice (Kapasi et al. 2000). These findings lend further support to the notion that acute stress may have the potential to help return otherwise weak immune responses to more "normal" levels (Karp et al. 2000).

In summary, the animal literature offers strong evidence to suggest that acute stress administered at close temporal proximity to vaccination can enhance both the primary and secondary antibody responses. Further, the data suggest that poorer immune responses may be particularly enhanced by acute stress, and that this augmentation appears to result from early interactions in the immune response to antigenic challenge. Despite the majority of literature supporting this line of research, it should be conceded that not all acute stress studies have found enhancements to

vaccine responses. In a study by Laudenslager and colleagues, IgG antibody responses to KLH were examined in rats following either one or three daily exposures to tail shock. Results indicated that all animals exposed to shock showed reduced levels of IgG antibodies to KLH regardless of the experimental conditions of shock exposure (Laudenslager et al. 1988). These findings were supported in a second study by this group, showing that antigen-specific total IgG, IgG1 and IgG2 antibodies were decreased in rats exposed to acute footshock. The authors suggest that antibody responses that are T-cell dependent are more susceptible to stress-induced immuno-suppression (Gazda et al. 2003). Together, these findings remain inconsistent with other findings in the field, and given that the type of stressor, duration of stressor and animals experimented on, are similar to those used in other studies showing an immune enhancement by acute stress, the reasons underpinning these inconsistencies remain unknown. Despite these contrasting results in the literature, the majority of studies investigating the effects of acute physical and psychological stress on the humoral response in animals support the acute-stress immuno-enhancement hypothesis.

Acute stress-induced enhancement of vaccination response in animals: cellular immunity

Cellular immunity in animals is the most researched area in the acute stress and vaccination literature. A large number of studies have been conducted and the majority show that acute stress can enhance the immune response to vaccination. In one of the earliest studies, Blecha and colleagues investigated the effects of various stressors on the cell-mediated responses to 2, 4-dinitro-1-1flurobenzene (DNFB) and SRBC (Blecha et al. 1982a). Cell mediated responses were determined by measuring the delayed type hypersensitivity (DTH) response. This involves an

initial sensitisation by dermal administration of antigen, followed 6 days later by antigenic challenge, which is the re-administration of the antigen to the derma of the earlobe. The extent of the response is then determined by measuring pinna thickness. Results indicated that three different types of whole-body stress (immobilisation, cold-stress, and heat-stress) administered at close temporal proximity to the DNFB challenge all enhanced the DTH responses. Subsequent to this study, a number of studies have demonstrated similar findings. Indeed, in the first of a number of studies conducted by Dhabhar, McEwen and colleagues, it was shown that rats previously exposed to DNFB showed enhanced DTH responses when administered at the same time as exposure to restraint stress (Dhabhar et al. 1996).

Mechanistically, it is hypothesised that the acute stress-induced enhancement of cell-mediated responses is associated with the rapid and robust leukocytosis observed during acute stress (Dhabhar and McEwen, 1996) and exercise (Kruger and Mooren, 2007). This leukocytosis is driven by the rapid release of adrenaline during stress; leukocytes with a high density expression of adrenergic receptors on their cell surfaces, such as natural killer cells and cytotoxic lymphocytes, are preferentially mobilised (Anane et al. 2009; Campbell et al. 2009; Kruger et al. 2008). It has been hypothesised that a greater availability of these cells may enhance cell-mediated responses upon antigenic challenge (Dhabhar, 2002). In order to explain this hypothesis, Dhabhar, McEwan and colleagues utilised a military metaphor. They suggest that the initial leukocytosis observed during stress represented the organism's 'soldiers' (blood leukocytes) leaving the 'barracks' (spleen, lymph nodes) where they are typically located, and navigate the 'boulevards' (peripheral vasculature) to patrol, ready for action. They hypothesise that the frequently observed decrease in lymphocytes to below baseline levels after acute stress

represents a recruitment of leukocytes to potential 'battle stations' (skin, lymph nodes) in preparation for immune challenge. This contention appears to be supported by previous work by that group. Indeed, Dhabhar, McEwan and colleagues have shown that the intensity of the stress was positively correlated with the DTH response to DNFB; as more intense stress induces a greater release of adrenaline, and concomitant redistribution of leukocytes (Dhabhar et al. 2000), this observation is consistent with the hypothesis that these changes may be underpinning the improved cell-mediated responses. Further, the duration of the stress did not appear to be associated with response. In this study, rats exposed to the 'New York City Subway Stress' (restraint stress and shaking for 2 hours) exhibited higher DTH responses than rats exposed to more moderate stress (restraint stress for 2 hours) (Dhabhar and McEwen, 1997). Further, more direct evidence in favour of this mechanism was presented in another study; it was shown that adrenalectomy, which eliminates the acute-stress release of adrenaline and cortisol, abolished the acute-stress enhancement of DTH responses (Dhabhar and McEwen, 1999). In a more recent study investigating the effects of long and short days of light cycles on DTH responses in hamsters, it was found that DTH responses were enhanced in males exposed to short-days compared with long-days; but not females. Furthermore, acute stress significantly augmented DTH compared with non-stressed hamsters in short-day males only. Short-day males exhibited higher levels of cortisol, and it was hypothesised that the elevated DTH responses was associated with the HPA-axis (Bilbo and Nelson, 2003).

It has also been proposed that IFN- γ could be a possible mediator of stress-induced enhancement of cellular responses to vaccination. Indeed, results indicated that both immuno-neutralisation and gene knockout of IFN-γ eliminated the enhancement by restraint stress of the DTH response

(Dhabhar et al. 2000). IFN-γ is an important cytokine involved in many aspects of the early stages of immune responses, by increasing antigen presentation to macrophages, promoting the migration of leukocytes, and activating APCs (Schroder et al. 2004). This novel finding by Dhabhar, McEwan and colleagues therefore adds support to the contention that acute stress augments the immune system at the early stages of immune responses. The idea that acute stress augments early immune interactions was also supported in a study by Saint-Mezard and colleagues, where it was found that acute restraint stress enhanced the DTH response to DNFB via direct adjuvant effects on the function of dendritic cells *in vivo*. They showed that stress induced release of noradrenaline exerted an adjuvant effect on dendritic cells by promoting enhanced migration to lymph nodes. This enhanced CD8 T cell priming, resulted in an enhancement of primary responses. Further, it was demonstrated that acute stress could also enhance the response to a secondary antigen exposure; this may be the result of enhanced recruitment of CD8 T cells in the skin upon DNFB re-challenge (Saint-Mezard et al. 2003).

In summary, the animal literature provides sound evidence to suggest that acute-stress may enhance both cell-mediated and antibody responses to vaccination. Although the mechanisms underlying these adjuvant effects remain unclear, there is preliminary evidence to suggest that augmentation of responses occurs as a result of enhanced antigenic-surveillance, antigen-detection and presentation.

ACUTE STRESS-INDUCED ENHANCEMENT OF VACCINATION RESPONSES: EVIDENCE FROM HUMANS

Acute stress-induced enhancement of vaccination response in humans: humoral immunity

There is accumulating evidence that acute stress may also enhance immunity in humans. Two early studies were conducted to investigate the apparently immuno-suppressive effects of exercise on immune function (Bruunsgaard et al. 1997b; Eskola et al. 1978). These studies were based on the 'open window hypothesis' which suggests that, after a single bout of acute strenuous and prolonged exercise, the immune system becomes suppressed and vulnerable to infections such as upper respiratory tract infections (Nieman, 1995; Peters and Bateman, 1983). The first of these studies found, contrary to their hypothesis, that the antibody response to tetanus toxoid vaccination was *higher* in the group who had completed a marathon, compared with a non-runner control group (Eskola et al. 1978). In contrast, Bruunsgaard and colleagues found no difference in the antibody responses to responses to diphtheria, tetanus toxoid and 6 pneumococcal serotypes between triathletes who had just completed a half-ironman competition prior to vaccination compared with either resting triathletes or sedentary controls (Bruunsgaard et al. 1997b). However, although these studies utilised extreme bouts of exercise to elicit an effect of exercise on vaccine responses, the sample size was relatively small and did not control the exact duration and relative intensity of the acute stress exposure in each participant. In other studies, there appears to be evidence showing that acute stress, experienced around the time of vaccination, may augment responses. A study by Burns and colleagues demonstrated that higher levels of self-reported naturalistic stress around the time of vaccination were associated with enhanced antibody responses to influenza vaccination (Burns et al. 2003). Similarly, Petry and

colleagues found that higher levels of negatively perceived stress during the induction phase of immunisation were significantly associated with higher peak anti-hepatitis B antibody titres (Petry et al. 1991). However, these findings were not replicated by Smith and colleagues who found no association between acute psychological stress and antibody responses to KLH inoculation (Smith et al. 2004).

More recently, a number of more controlled studies have demonstrated that acute stress may enhance the immune response to vaccination. A number of studies by Edwards and colleagues have demonstrated that both acute exercise and psychological stress experienced at close temporal proximity to vaccination have a beneficial effect on the subsequent antibody response. The first of these studies investigated whether humoral responses to a thymus-dependent (influenza) and thymus-independent (meningococcal) vaccine were affected by acute dynamic (cycling) exercise or psychological stress. Participants in the exercise group completed a cycling task at 55 % of Watt Max*, those in the psychological stress group completed a time-pressured mental arithmetic test, and a non-stress control group rested quietly. Following their allotted 45 min task, participants received an influenza vaccine. Results indicated that women in both the exercise group and psychological stress groups showed enhanced antibody responses to the A/Panama influenza strain at 4 and 20 weeks post-vaccination, in comparison to the resting control group (Edwards et al. 2006a). This strain elicited particularly poor antibody responses in the female control group, suggesting that the interventions successfully augmented the antibody response to an otherwise weakly-immunogenic strain. In contrast, men displayed robust responses to all strains, and no effects of exercise or stress were observed. In the same study (Edwards et al. 2008), participants were also administered a meningococcal A + C vaccine into

* Watt max = Maximal workload in Watts

the contralateral arm. Results indicated that, in men, both exercise and psychological stress improved the antibody response to meningococcal A in men, compared with control men. In agreement with the previous study, augmentation was observed where the control group response was poor; on this occasion, men, rather than women, in the control group had weaker responses to this particular strain. These results confirmed that the adjuvant effects of exercise and psychological stress are not limited to thymus-dependent (e.g., influenza) or thymus-independent (e.g., meningococcal) vaccines, and thus do not seem to be dependent on T cell interactions.

In the final study in this series by Edwards and colleagues, a resistance-based, eccentric exercise protocol was developed (Edwards et al. 2007a). An eccentric exercise task was designed to initiate a local inflammatory response in the biceps brachialis and deltoid muscles of the arm in which the influenza vaccine was administered. It was hypothesised that this inflamed local environment provides an efficient way of augmenting the antibody response to influenza vaccination. Sixty healthy young adults (31 men, 29 women) were randomly allocated to either an eccentric exercise group or a control group. The exercise group performed 50 repetitions of the eccentric portion of the bicep curl and lateral raise exercises in the non-dominant arm; the task lasted approximately 25 minutes. The control group rested quietly for the same time. Six hours after task completion, allowing for the inflammatory response to the exercise to develop (i.e., IL-6; MacIntyre et al. 2001), participants received an influenza vaccine into the non-dominant arm. Results indicated that women in the exercise group showed enhanced antibody responses to the A/Wyoming and B/Jiangsu strains of influenza at 6 and 20 weeks post-vaccination. Again, these strains had produced relatively weak antibody responses in women in the control group; again, however, men showed more robust responses against these strains and

no effects of exercise were found. In line with these findings, the other strain in the vaccine, A/New Caledonia, produced robust responses in both men and women in the control group and no effect of exercise was observed.

Acute stress-induced enhancement of vaccination response in humans: cellular immunity

Only two studies have investigated the effects of acute stress on cell-mediated responses to vaccination. The first of these studies was conducted by Smith and colleagues, who examined the extent of the DTH response to KLH inoculation in participants immediately after a viva voce examination. Results indicated that self-reported distress at the time of vaccination was negatively correlated with the likelihood of developing a DTH response (Smith et al. 2004). These findings contrast with a more recent, highly controlled study conducted by Edwards and colleagues who investigated the effects of eccentric arm exercise on the cell-mediated response to influenza vaccination. In this study, cell-mediated responses were measured indirectly by quantifying the whole blood antigen-specific interferon-γ response to stimulation with the influenza vaccine *in vitro* at 28 days post-vaccination (Edwards et al. 2007a). Results indicated that men in the exercise group exhibited higher IFN-γ responses to the vaccine at this time point, in comparison to resting controls. These findings suggest that further research investigating the role of behavioural stressors on cell-mediated immune responses in humans is warranted.

ECCENTRIC EXERCISE AS A BEHAVIOURAL ADJUVANT TO VACCINATION

During an active eccentric muscle action, the exercising muscle applies force as it lengthens, causing damage to the internal structure of the muscle fibres (Proske and Morgan, 2001). This damage is greater than that observed with concentric (shortening) muscle contractions (Sorichter et al. 1999) and, in untrained individuals when substantial damage is caused, leads to an inflammatory response in the muscle (Sorichter et al. 2006). Characterised by oedema, muscle pain, muscle weakness, leakage of muscle and cellular proteins (e.g., CK), and cellular infiltration, this response occurs early post-injury and may last anything up to 2 – 7 days (Peake et al. 2005). For example, exercise elevates the levels of inflammatory mediators released from stressed cells within the damaged muscle tissue (Peake et al. 2005). These stressed cells release a range of danger signals, such as IL-6, uric acid, HSP60, HSP70, and are thought to play a role in orchestrating inflammation and repair of the surrounding tissues (Hirose et al. 2004; Matzinger, 2002; Peake et al. 2005). Therefore, it possible that eccentric exercise, performed using the muscles located at the site of vaccine administration, improves the subsequent immune response by inducing a pro-inflammatory environment in the muscles. In support of this theory, the extent of the self-reported pain and the change in upper arm limb circumference following eccentric exercise, both indirect indicators of muscle damage, have been significantly positively correlated with the subsequent cell mediated immune response to the vaccination (Edwards et al. 2007a).

OPTIMISATION OF ECCENTRIC EXERCISE AS AN ADJUVANT TO VACCINATION

Given the relatively poor immunogenicity of many current vaccines, there is a renewed desire to identify novel, safe and easily applicable strategies to improve immune responses. Further, research now supports the contention that acute behavioural stress, in the form of eccentric exercise, may offer a realistic and efficacious means of doing this. Eccentric exercise has many advantages as an adjuvant: it is inexpensive (i.e., equipment costs are low); it is easily administered (i.e., simple training and little equipment required); it only takes a relatively short time to carry-out (i.e., 25 minutes); and most importantly, the potential health risks associated with it are low. Although rigorous investigation into the clinical use of eccentric exercise would be required, it is likely that the intervention could be implemented relatively quickly. On the other hand, exogenous adjuvants have to undergo lengthy periods of testing before they are approved for public use. For example, clinical trials can take up to 8 years to complete (World Health Organisation; http://www.who.int), and that does not include the time it takes to firstly discover, purify and test the original adjuvant in the laboratory. Given this, investigation into the use of eccentric exercise as an adjuvant to vaccination is warranted, and particular attention is needed into determining the optimal intervention protocol. In order to do this, a number of considerations have yet to be investigated: the optimal timing of vaccine administration after exercise; the minimal intensity of exercise needed to elucidate an adjuvant effect; and the ideal site of vaccine administration after eccentric exercise.

Issue of exercise timing prior to vaccination

In response to eccentric exercise-induced muscle damage, a complex inflammatory cascade is induced. Various markers of inflammation peak at different time points, with some commencing almost immediately after the onset of muscle damage, and others occurring more slowly but lasting up to 7 days post-exercise (Peake et al. 2005). Manipulating the timing of the vaccination relative to the exercise may, therefore, allow the optimization of the intervention by coinciding the vaccination with a particularly potent inflammatory response. Further, this approach also allows the indirect investigation of mechanisms, by coinciding the vaccination with post-exercise peaks in different potential mediators.

Vaccination at six hours post-exercise: role of IL-6

In a previous study, an augmented vaccine response was successfully induced using a protocol in which participants were vaccinated immediately post-cycling exercise (Edwards et al. 2006a). In this study, Edwards and colleagues examined the relationship between the IL-6 responses to acute stress and the antibody responses to vaccination. Using hierarchical linear regression analysis, which controlled for baseline antibody titres, they revealed that IL-6 concentrations at 60 minutes after stress exposure (cycling and / or arithmetic test) positively predicted the antibody response to the A/Panama strain of influenza, which was the only strain responsive to cycling exercise (Edwards et al. 2006a). In a further study conducted by Edwards and colleagues, the immune response to influenza vaccination was enhanced when the vaccine was administered 6 hr post-eccentric exercise (Edwards et al. 2007a). This timepoint was selected to

coincide with the peak blood plasma response of the cytokine interleukin-6 (IL-6) to eccentric exercise (MacIntyre et al. 2001). In response to eccentric exercise, it has been proposed that IL-6 release derived specifically from damaged myocytes in the skeletal muscle (Bruunsgaard et al. 1997a; Febbraio and Pedersen, 2002). Given that eccentric exercise induces the greatest degree of muscle damage – compared with other modes of exercise – it is interesting to consider the effects of muscle damage and concomitant IL-6 release on the immune response to vaccination. Unfortunately, IL-6 was not measured in this study, and no associations could be made between the IL-6 response to exercise and the immune response to the vaccine (Edwards et al. 2007a).

Combined, the findings from these two human studies lend support to the hypothesis that elevated plasma IL-6 may enhance vaccine responses. There is also supportive data from a number of clinical studies measuring IL-6 as an adjuvant to vaccination. Generally speaking, the IL-6 response to physical and psychological stress is thought to mimic the immunological responses observed during clinical stressors such as surgery, trauma and sepsis (Heinrich et al. 1990). High levels of IL-6 are associated with stronger antibody responses to live Francisella tularensis vaccination in humans (Krakauer 1995) and co-administration of plasmids encoding IL-6 has been shown to enhance influenza vaccine efficacy in mice (Krakauer, 1995; Lee et al. 1999). Further, there is preliminary evidence that IL-6, co-administered with vaccine, can enhance the immune response to malaria vaccination (Hui and Hashimoto, 2007), and can improve the humoral response against ovarian cancer cells (Reinartz et al. 2003). For these reasons, the IL-6 response to eccentric exercise, and its associations with the antibody response to vaccination will be assessed in Chapter Two and Chapter Three in this thesis.

From a pragmatic perspective, vaccination at six hours post-exercise is not the most ideal strategy for clinical application of eccentric exercise as this approach requires the participant to visit the laboratory or clinic twice in the same day. For this reason, it is important to consider whether vaccinating immediately after eccentric exercise enhances the immune response to vaccination.

Vaccination immediately post-exercise

Vaccination immediately post-exercise would simplify any future clinical application of this intervention; participants would only have to visit the laboratory on one occasion to complete the exercise task and to receive the vaccine. There are also sound theoretical reasons to hypothesise that this might be an effective timepoint for enhancing immune responses to vaccination. The high physical demands of physical stress result in the activation of a fight-or-flight response (Cannon, 1932), and promote survival by activating a number of defence mechanisms within the body. The acute stress response is characterised by a rapid and robust discharge of the sympathetic nervous system (SNS) which results in the release of catecholamines (e.g., adrenaline and noradrenaline) leading to a concomitant elevation in heart rate, blood pressure and respiration (Elenkov et al. 2000; Goldstein, 2003). Also activated during acute stress is the hypothalamic-pituitary-adrenal (HPA) axis, which leads to the subsequent release of cytokines causing mild inflammation (Black, 2002). As both the SNS and HPA axis are activated by exercise, and can influence immune function, they are both plausible mechanisms that may underpin the acute stress enhancement phenomenon. Studies investigating the effects of cortisol, released during acute stress, on the immune response to vaccination have produced inconsistent and often contrasting results. For example, in a study by Dhabhar, McEwan and colleagues, it

was demonstrated that DTH responses enhanced by acute-stress were impaired by adrenalectomy, and restored by exogenous corticosterone administration (Dhabhar and McEwen, 1999). These findings were not replicated however in a study by Blecha and colleagues who suggested that DTH responses were suppressed by elevated levels of corticosterone (Blecha et al. 1982b). In addition to cell-mediated responses, there appears to be mixed results regarding the effects of cortisol levels on antibody responses to vaccination. For example, it has been demonstrated that elevated glucocorticoid levels, induced by cocaine administration, were associated with an augmented antibody response to SRBC inoculation (Stanulis et al. 1997). However, Edwards and colleagues investigated the role of cortisol and the effects of acute stress on vaccination responses to influenza vaccine in humans. The extent of the cortisol response to cycling exercise and / or psychological stress was not associated with the antibody responses to any of the three viral strains of influenza (Edwards et al. 2006a). The exercise bout used in that study was performed at 55 % Watt max, and there is research to suggest that the exercise bout needs to be higher than 60% to elicit an increase in plasma cortisol (Galbo et al. 1981). However, given the results of Edwards and colleagues, it seems unlikely that cortisol is the primary mechanism underpinning the immuno-enhancement seen in response to the acute physical and psychological stress enhancement of vaccine responses in humans.

Activation of the sympathetic nervous system also leads to the release of adrenaline that has a direct effect on immune cells (Elenkov et al. 2000; Goldstein, 2003). It is thought that these effects may directly impact the immune response to vaccination (Dhabhar, 2002). Indeed, Dhabhar, McEwan and colleagues have shown that adrenaline administration resulted in a dose-dependent augmentation of DTH responses to DNFB in treated animals compared with controls.

Recent research suggests that adrenaline mobilises leukocytes exhibiting effector phenotypes and the unique capability to migrate to damaged tissues (Campbell et al. 2009; Miller, 2004). It is possible that a re-distribution of these cells to sites of muscle damage may provide a mechanism for enhanced vaccine responses.

In Chapter Two of this thesis, participants were vaccinated immediately after exercise to investigate the efficacy of vaccinating at this timepoint. If vaccination responses are improved at this timepoint, then a role of adrenaline and / or cortisol, and subsequent leukocyte infiltration, may be supported.

Vaccination at 48 hours post-exercise

Peak muscle damage, defined by pain, swelling, loss of muscle function and the release of muscle cytosolic enzymes such as creatine kinase into the circulation, is delayed and typically does not peak for days after exercise cessation (Sorichter et al. 1999). Indeed, at approximately 48 hr post-exercise, there is a peak accumulation of inflammatory infiltrate comprised of cellular (e.g. neutrophils and mononuclear cells; (Fielding et al. 1993) and secreted (e.g. cytokines, heat shock proteins, uric acid (Peake et al. 2005) factors. For example, this timepoint may coincide with a peak in number of macrophages in a pro-inflammatory state in the damaged muscle that may dually assist in antigen recognition and clearance, before reverting to an anti-inflammatory state after viral clearance (Chazaud et al. 2009). Although vaccination at this timepoint would not offer the most pragmatic approach for implementing eccentric exercise in a clinical setting, it is possible that the heightened inflammatory environment at this time may be particularly effective

at enhancing antigenic recognition and clearance at the site of muscle damage. At present, no studies have investigated this approach in humans, and for this reason, the immune response to vaccination when administered at 48 hours post-exercise will be measured in Chapter Two in this thesis.

Issue of eccentric exercise intensity

Before eccentric exercise can be implemented in a clinical setting, further research is also needed to establish the optimal intensity of eccentric exercise. In the only prior study investigating eccentric exercise on the immune response to vaccination, Edwards and colleagues found beneficial effects when eccentric arm exercise was conducted at 85 % of 1RM. This is an intensity that had been shown to elicit the desired inflammatory responses (Edwards et al. 2007a). Despite the overall success of this intervention, the intensity that was chosen has not been compared with other intensities of exercise; it is not clear whether a lesser intensity would achieve similar enhancement of vaccine responses. A lesser intensity of exercise would enable a more acceptable and safer intervention approach.

Issue of vaccination site following eccentric exercise

Before eccentric exercise as an adjuvant to vaccination can be adopted, the optimal site of vaccine administration must be identified. In a prior eccentric exercise and vaccination study, Edwards and colleagues found an adjuvant effect of exercise when the vaccine was administered into exercised muscle tissue. However, it is not clear whether this adjuvant effect would have

been achieved had the vaccine been administered into non-exercised muscle tissue, following eccentric exercise. Indeed, there are sound theoretical reasons to suggest that vaccine responses may be enhanced in both circumstances.

Locally, unaccustomed eccentric exercise causes muscle damage and induces an inflammatory response in the muscle characterised by oedema, pain and increased immunological activity (Peake et al. 2005). In particular, the release of immunological danger signals can activate dendritic cells (Gallucci et al. 1999; Shi and Rock, 2002) and animal research has shown that this can lead to enhanced antigenic transport and presentation in lymph nodes, leading to a heightened antigen-specific response from CD8+ T cells (Saint-Mezard et al. 2003). On the other hand, eccentric exercise also induces a number of systemic alterations which could lead to an augmented antibody response to vaccination. For example, during this type of exercise, it has been demonstrated that circulating levels of inflammatory mediators, such as plasma IL-6 (Peake et al. 2005) are elevated above resting levels. It is possible that increased levels of these pro-inflammatory mediators in the systemic circulation, stemming from eccentric exercise-induced muscle damage, could be a mechanism for augmented immune responses seen previously (Edwards et al. 2007a). By comparing the antibody responses of exercised participants who received their vaccine into either the exercised or the resting arm, it is possible to determine the optimal site of vaccination and to begin to elucidate some of the underlying mechanisms.

SUMMARY AND OVERVIEW OF THESIS

This thesis comprises three large experimental studies and each of these investigated the effects of eccentric exercise on the immune response to vaccination in humans. The eccentric exercise protocol was employed to induce muscle damage to the biceps brachialis and deltoid muscles of the non-dominant arm, which is known to cause a local inflammatory response, and can lead to enhanced vaccine responses (Edwards et al. 2007a).

The first study (Chapter Two) investigated the effect of exercise timing on the efficacy of the immune response to influenza vaccination. Eccentric exercise was performed either immediately, 6 hours or 48 hours prior to influenza vaccination, and the immune responses of these groups were compared with a non-exercise control group. Immune responses were measured by antibody titres and *in vitro* cell-mediated responses to the whole vaccine at 28 days post-vaccination.

The second study (Chapter Three) investigated the influence of exercise intensity on the efficacy of the immune response to influenza vaccination. Eccentric exercise was performed at 60 %, 85 % or 110 % of 1RM to determine whether the degree of exercise induced muscle damage and concomitant inflammation affected the subsequent immune response. Exercise was performed immediately prior to influenza vaccination, and antibody titres were compared with the responses of a non-exercise control group at 28 days post-vaccination.

The final study in this thesis (Chapter Four) investigated whether the site of vaccination affects the efficacy of the eccentric exercise intervention. In this study, a comparison is made between exercise participants who receive a vaccine directly into eccentrically-exercised damaged muscle tissue, or exercise participants who receive the vaccine into the non-exercised arm. Further, Chapter Four investigates whether the immune response to a relatively poor immunogenic vaccine, hepatitis B, is enhanced by eccentric exercise. Immune responses were measured by antibody titres and *in vitro* cell-mediated responses to the whole vaccine.

In sum, this programme of research was conducted to extend our understanding of the use of eccentric exercise as an adjuvant to vaccination. The studies were designed to identify the optimal intervention strategy in healthy young adults, in order to develop an evidence-based protocol for use in other, more vulnerable, populations. Each study was also designed to allow some investigation of the mechanisms underpinning this effect.

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CHAPTER TWO

THE EFFECTS OF VACCINE TIMING ON THE EFFICACY OF AN ACUTE ECCENTRIC EXERCISE INTERVENTION ON THE IMMUNE RESPONSE TO AN INFLUENZA VACCINE IN YOUNG ADULTS

ABSTRACT

An acute bout of exercise prior to vaccination can improve the antibody and cell-mediated responses to influenza vaccination. The mechanisms underpinning this adjuvant effect remain unclear, and further investigation to determine the optimal exercise protocol is warranted. The aim of the current study was to determine whether exercise augmented the immune response to vaccination, and whether the timing of exercise relative to vaccination affected the efficacy of the intervention. One hundred and fifty six (76 men) healthy participants were randomly assigned to a control group or one of three intervention groups who exercised immediately, 6 hr or 48 hr prior to administration of a standard trivalent influenza vaccine. The exercise groups performed 50 repetitions of the eccentric portion of both the bicep curl and lateral raise movements at an intensity eliciting 85% of each participant's pre-determined concentric one repetition maximal. Antigen specific serum antibody titres were measured at baseline and 28 days post-vaccination as indicators of the humoral response. All three viral strains elicited strong antibody responses; however, eccentric exercise did not further augment any antibody responses compared with the control group. Cell-mediated immunity at 28 days post-vaccination was determined by measuring the IFN-γ response to *in vitro* stimulation of the blood with whole vaccine. There

were no differences in cell-mediated immunity among the groups. Although these null findings were unexpected, they are consistent with previous research showing that exercise-induced immuno-enhancement was only observed when the control group had relatively poor responses. In conclusion, it is likely that the robust immune responses to the vaccine observed in this study may have limited any further immune enhancement by exercise.

INTRODUCTION

Influenza is a major cause of morbidity and mortality resulting in the death of up to half a million people annually worldwide (WHO Influenza Position Paper, 2005). Vaccination is used to reduce the incidence of influenza infection but, as yet, is only effective at preventing illness in approximately two-thirds of the population (Villari et al. 2004). Low efficacy rates have prompted further research into the role of adjuvants that may enhance the immune response to vaccination (Aguilar and Rodriguez, 2007). In addition to the studies investigating exogenous adjuvants, research has begun examine the potential role of behaviourally-induced, endogenous adjuvants (Edwards et al. 2007b). For example, acute stress exposure prior to immunization has been shown to improve both humoral (Millan et al. 1996; Persoons et al. 1995; Silberman et al. 2003; Wood et al. 1993) and cell-mediated immunity in rodents (Blecha et al. 1982; Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1999; Saint-Mezard et al. 2003; Saint-Mezard et al. 2004).

Adjuvant effects of acute psychological stress and exercise have also been demonstrated in humans (Edwards et al. 2006; Edwards et al. 2007a; Edwards et al. 2008). For example, acute

stress improved the antibody response to influenza vaccination in women (Edwards et al. 2006). In order to maximise the potential adjuvant effect of the acute exercise intervention, a resistance-based, eccentric exercise protocol was developed (Edwards et al. 2007a). During an eccentric muscle action, the exercising muscle applies continuous force as it lengthens, causing damage to the internal structure of the muscle fibres (Proske and Morgan, 2001). This damage is greater than that observed with concentric (shortening) muscle contractions (Sorichter et al. 1999) and, in untrained individuals, leads to an inflammatory response in the muscle (Sorichter et al. 2006). For example, exercise elevates the levels of inflammatory mediators released from stressed cells within the damaged muscle tissue (Peake et al. 2005). Numerous cytokines are also released, and may play a role in augmenting immune interactions (Peake et al. 2005). Therefore, it possible that eccentric exercise, performed using the muscles located at the site of vaccine administration, improves the subsequent immune response by inducing a pro-inflammatory environment in the muscles. In support of this theory, the extent of the self-reported pain and the change in upper arm limb circumference following eccentric exercise, both indirect indicators of muscle damage, were significantly positively correlated with the subsequent cell mediated immune response to the vaccination (Edwards 2007a).

In light of these promising findings, it is important to improve the exercise intervention further to achieve the maximum adjuvant effect possible. One factor that can be manipulated is the timing of the exercise relative to the vaccination. Within the local muscle tissue, the complex inflammatory response to muscle damage evokes a number of changes, occurring almost immediately after the onset of muscle damage, and lasting up to 7 days post-exercise (Peake et al. 2005). Therefore, a number of different timepoints in the post-exercise recovery period may

provide the optimal environment for enhancing the immune response to vaccination. As well as having clear implications for future clinical applications of such an intervention, a timing manipulation may also help elucidate the mechanisms underlying the exercise induced augmentation of vaccine responses.

Previously, an augmented vaccine response was successfully induced using a protocol in which participants were vaccinated six hours post-exercise (Edwards et al 2007). This timepoint was selected to coincide with the peak response of the cytokine interleukin-6 (IL-6) to eccentric exercise (MacIntyre et al. 2001). High levels of IL-6 are associated with stronger antibody responses to live Francisella tularensis vaccination in humans (Krakauer 1995) and co-administration of plasmids encoding IL-6 has been shown to enhance influenza vaccine efficacy in mice (Krakauer, 1995; Lee et al. 1999). Further, the extent of the antibody response to the A/Panama strain was positively correlated with the IL-6 response to the acute stress tasks (Edwards et al 2006). Given these findings, the current study also included a group vaccinated six hours post-exercise to coincide with a predicted peak in IL-6.

It is also important to include a group vaccinated at the point of peak muscle damage, as this is a key process that could underly the eccentric exercise-induced augmentation of antibody responses. Peak muscle damage, defined by pain, swelling, loss of muscle function and the production of creatine kinase, is delayed after exercise cessation (Sorichter et al. 1999). At approximately 48 hr post-exercise, there is a peak accumulation of inflammatory infiltrate comprised of cellular (e.g. neutrophils and mononuclear cells) (Fielding et al. 1993) and secreted (e.g. cytokines, heat shock proteins, uric acid) (Peake et al. 2005) factors. We propose that this

heightened inflammatory environment may enhance antigenic recognition and clearance at the site of muscle damage. Therefore, in the current study, a second group were vaccinated 48 hr after exercise.

Finally, a group were vaccinated immediately after exercise. From a pragmatic perspective, this would simplify any future clinical application of this intervention. There were also sound theoretical reasons to hypothesise that this might be effective. It has been hypothesized that a redistribution of immune cells (Kruger et al. 2008), and subsequent demargination into sites of tissue damage immediately following stress, may be one of the principal mechanisms behind the acute-stress immunoenhancement hypothesis (Dhabhar et al. 1995). Further, there is evidence that muscular contractions are associated with a short term increase in lymphatic drainage of the exercised muscle (Havas et al. 1997) which could subsequently enhance immune cell transport to and from the site of antigen administration (Swartz et al. 2008). Therefore, a third exercise group were vaccinated immediately post-exercise in the current study.

In summary, three exercise groups (immediate, 6 hr, 48 hr post-exercise) were compared with a non-exercising control group, in terms of their humoral and cell-mediated responses to influenza vaccination. We hypothesised that the exercise groups would show augmented immune responses to the influenza vaccine in comparison to the control group, and that the timing of vaccination would influence the efficacy of the exercise intervention.

METHODS

Participants

One hundred and fifty-six healthy students (76 men, 80 women) at the University of Birmingham were recruited, and 155 completed the study (mean ± SD; age: 20.4 ± 2.4 years; body mass index: 22.8 ± 2.9 kg/m^2). None of the participants had received the influenza vaccine in the past year and none had reported influenza-like illness in the year prior to participation. Exclusion criteria included smoking, a history of immune or cardiovascular disease, current acute infection or illness, pregnancy or suspected pregnancy, and a history of vaccine-related allergies or side effects. Use of prescription medication within one month of participation was also an important exclusion criterion; but females taking the contraceptive pill were not excluded. In addition, none of the participants reported having performed any resistance training in the 6 months prior to testing. All participants were instructed to abstain from vigorous exercise and over-the-counter medication for at least 24 hr, alcohol for at least 12 hr, and food or caffeine for at least 2 hr prior to each session, and to refrain from over the-the-counter medication for up to 24 hr after the exercise / control task. All participants provided written informed consent and the study protocol was approved by the Black Country Local Research Ethics Committee. All participants were paid £30 or given research credits upon completion of the study.

Procedure

Participants were pseudo-randomised, maintaining an even sex distribution, into one of four groups: immediate exercise group (*n*=38), 6 hr exercise group (*n*=39), 48 hr exercise group (*n*=39) or a resting control group (*n*=39). Groups did not differ for stressful life events exposure, perceived stress and health behaviours (data not reported here); these factors have been associated with the extent of antibody responses (Burns et al. 2003; Cohen et al. 2001). In the first session, baseline blood samples were taken, from an antecubital vein in the dominant arm, following a 20 min rest period. Participants then had height and weight measured, and a test of maximum muscle strength was conducted. In the second session, they completed either the eccentric exercise task (exercise groups) or remained quietly resting for 25 min (control group). Participants then returned to the laboratory at varying times, depending on group allocation, for session three; the resting control group received the vaccine immediately after task completion. During this session, a nurse administered the 2007/2008 northern hemisphere influenza vaccine (0.5 ml; Inactivated Split Virion BP, Sanofi Pasteur MSD, Batch no. B9676-1) via intra-muscular injection into the deltoid muscle of the non-dominant arm. Participants returned to the laboratory at 28 days post-vaccination to provide blood samples for antibody and cell mediated immunity measurement.

Exercise Task

The exercise protocol has been described in detail elsewhere (Edwards et al. 2007a). In brief, participants performed the eccentric portions of the bicep curl and lateral raise exercises,

contracting the biceps brachii and deltoid muscles of the non-dominant arm, respectively. In both exercises, they were asked to lower the dumbbell in a controlled manner to a count of five. The weights used were 85% of each participant's concentric single repetition maximal (1RM) (1RM bicep curl: 11.68 ± 4.1 kg; 1RM lateral raise: 8.16 ± 2.9 kg); this intensity was chosen as it elicits muscle damage, whilst at the same time it is not too intensive, thus enabling all participants to complete the task. All participants performed 50 repetitions of each movement. The task lasted approximately 25 min.

Blood sampling

Blood was collected in a 10 ml vacutainer containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton-Dickinson, UK) at baseline and at the time of vaccination. Tubes were stored on ice until centrifugation (3400 g for 10 min at 1 °C) and plasma was stored at −80 °C for later assessment of IL-6 and CK. Blood serum was collected in a plain 10 ml vacutainer and allowed to clot for 1 hr at room temperature; and following centrifugation (3400 g for 5 min at 21 °C), was stored at −20 °C for later antibody titre determination. A 10ml lithium-heparin vacutainer was taken at 28 days post-vaccination for assessment of cell-mediated antigen specific immunity.

Assays

Anti-influenza antibody titres were measured using a haemagglutination inhibition test analysed by the serology laboratory of GlaxoSmithKline Beecham at Dresden, Germany. The trivalent 2007/2008 Northern Hemisphere influenza vaccine contained three viral strains: A/Solomon Islands/3/2006 (H_1N_1)-like strain; A/Wisconsin/67/2005 (H_3N_2)-like strain; and B/Malaysia/2506/2004-like strain. The antibody results are reported as titres; a titre represents the reciprocal of the highest dilution having a positive response in the haemagglutination inhibition assay.

Cell-mediated antigen-specific immunity was assessed by determining the levels of IFN-γ in cell culture supernatant following *in vitro* stimulation of blood with whole vaccine. In brief, 1 ml of fresh lithium heparinised blood was incubated for 18 hr (5% CO_2, 37 °C) with 100µL of complete vaccine. This vaccine dose was found to be optimal based on a series of titrations carried out during pilot testing (unpublished data). Supernatant was harvested by aspiration, centrifuged (200 g at 4 °C for 2 min) to remove cells, and frozen at −20 °C. IFN-γ concentrations were measured in duplicate using a sandwich ELISA (catalogue number DY285; R&D Systems, UK). All tests were performed according to the manufacturer's recommendations. A colour reaction (SIGMA*FAST*™ OPD, Sigma-Aldrich, UK) was stopped after 20 min (2N H_2SO_4, Fisher Scientific, UK) and read on a microplate reader (ELx800, BioTek©, USA) at 490 nm and 650 nm, allowing adjustment for optical imperfections in the plate. The recorded intra-assay and inter-assay variation for IFN-γ were 4.1 and 10.5 %, respectively.

Plasma IL-6 was measured using high-sensitivity ELISA (Quantikine HS Human IL-6 ELISA, R&D Systems, UK) according to manufacturer's instructions. The reported sensitivity of the assay was 0.039 pg/ml, with recorded intra-assay and inter-assay variation at 2.3 % and 9.6 %, respectively. Plasma creatine kinase (CK) activity were measured using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK) and diagnostic reagents (ABX diagnostics, UK). CK samples were analysed in triplicate, and coefficient of variations for CK were calculated as 1.9%.

Limb circumference, pain and perceived exertion

Muscle soreness was assessed using a visual analogue scale (Melzack, 1987) with anchors of 0 (no pain) and 100 (worst possible pain), to indicate an overall pain rating regarding the non-dominant arm. These measures were completed by all participants immediately after the task, and again immediately prior to vaccination. Change in upper arm circumference was used to quantify the amount of swelling in the non-dominant arm pre-task, immediately post-task and again at 6 hr post-task. Measurements were taken in an unblinded manner using tape measure at the point of flexed maximal circumference of the upper arm, and, for the forearm, 5 cm below the elbow crease in the inside of the arm. After locating the site of measurement, participants let their arm hang, relaxed, by their side for all measurements. Measurement sites were marked with a pen in two places around the arm at the first testing session to ensure consistent placement of tape measure. Exercise group participants also provided a rating of perceived exertion (Borg, 1970) immediately after the task using a 15-point scale (6= "no exertion at all", 20= "maximal exertion"), immediately after the task.

Statistical analysis

Due to the skewed distributions of the antibody titres and IFN-γ concentrations, analyses were performed on log10 transformed values. All analyses were conducted using repeated measures (antibody titres; IL-6; CK) or univariate (cell mediated immunity pain; percentage change in limb circumference) analysis of variance. We made an *a priori* decision to follow a traditional drugs trial analytic strategy. As such, it was firstly investigated whether there was any difference between treatment and control i.e. all exercise groups combined were compared with the control group. Secondly, the treatment groups were compared i.e. compared within the three exercise groups only. Finally, a series of ANOVAs compared each individual exercise group with each other and with the control group. As previous studies have found sex differences in intervention efficacy (Edwards et al. 2006; Edwards et al. 2007a), sex was entered as a between-subject factor in all parameter analyses. If no sex effects were found at this stage for a particular parameter, it was excluded from further analyses of that parameter. Effect sizes are represented by η^2, and differences in degrees of freedom reflect occasional missing data.

Results

Antibody titres

Table I displays the overall geometric mean influenza antibody titres for A/Solomon Islands, A/Wisconsin and B/Malaysia at baseline and 28 days post-vaccination. A 2 Sex (male, female) × 3 Strain (A/Solomon Islands, A/Wisconsin, B/Malaysia) × 2 Time (0, 28 days) ANOVA on log

transformed antibody titres revealed significant Strain ($F(2,151)=22.96$, $p<.001$, $\eta^2=.23$), Time ($F(1,152)=1164.91$, $p<.001$, $\eta^2=.89$), and Strain × Time effects ($F(2,151)=12.41$, $p<.001$, $\eta^2=.14$). As anticipated, separate ANOVAs (2 Time) confirmed that antibodies titres to all viral strains increased from baseline to 28 days post-vaccination ($F(1,153)=340.37–728.52$, $ps<.001$, $\eta^2=.69–.83$). Fold changes were calculated by dividing the geometric mean antibody titre at 28 days by the relevant baseline titre; results exhibited by each strain are displayed in Table I.

In order to compare differences between experimental groups, a two step analytical procedure was adopted. Firstly, the control group was compared with all exercise groups to establish if there was an overall adjuvant effect of exercise, then comparisons within exercise groups were conducted, followed by individual pairs of ANOVAs comparing individual exercise groups with each other and with control.

Exercise v control comparison

A 2 Group (combined exercise groups, control) × 2 Sex × 2 Time ANOVA revealed that there were no Group × Time interactions for antibody responses to A/Solomon Islands ($F(1,150)=0.32$, $p=.57$, $\eta^2<.01$), A/Wisconsin ($F(1,150)=2.91$, $p=.13$, $\eta^2=.02$), or B/Malaysia ($F(1,151)=2.18$, $p=.14$, $\eta^2=.01$) viral strains. Further, no main effects of Sex, and no Sex × Time interactions, were identified in the response to any of these strains.

Exercise groups comparison

A 3 Group (immediate, 6 hr, 48 hr) × 2 Sex × 2 Time ANOVA again revealed no significant Group × Time interactions for antibody responses to A/Solomon Islands ($F(2,110)=0.19$, $p=.83$, $\eta^2<.01$), A/Wisconsin ($F(2,110)=1.67$, $p=.19$, $\eta^2=.03$), or B/Malaysia ($F(2,110)=2.34$, $p=.10$, $\eta^2=.04$) viral strains. Finally, ANOVAs were conducted to establish whether any of the exercise groups differed from each other, or from control; these analyses revealed no significant differences between any of the four groups (all $p>.05$; see Figure I).

Table I. Geometric mean (95% confidence intervals) antibody titres and fold change scores for the three viral strains of influenza at baseline and 28 days post-vaccination.

Viral strain	Baseline	28 days post-vaccination	Fold change
A/Solomon Islands	33 (27 – 40)	761 (652 – 888)[a]	23.06
A/Wisconsin	36 (30 – 44)	346 (294 – 408)[a]	9.61
B/Malaysia	23 (20 – 27)	317 (262 – 383)[a]	13.78

[a] Significantly different from pre-vaccination ($p<.05$)

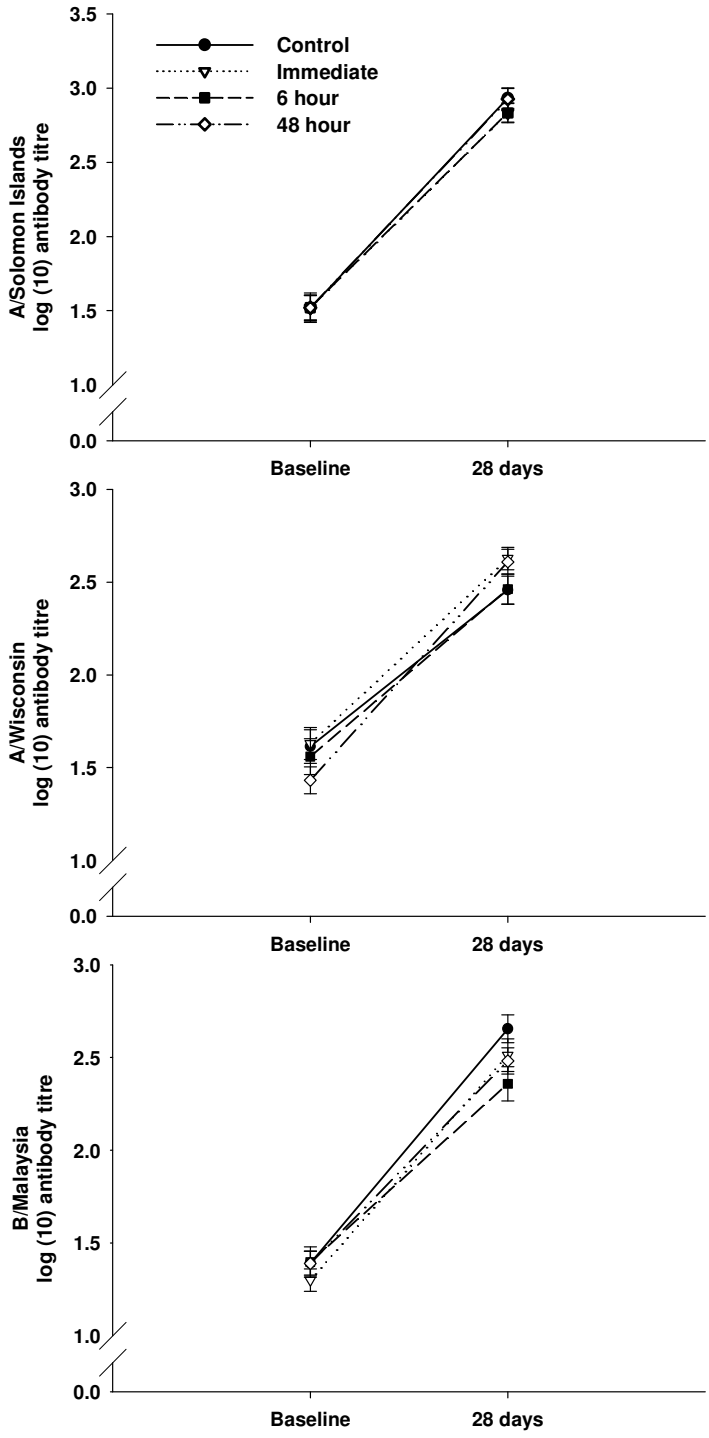

Figure I. Mean (SE) log-transformed antibody titres in each experimental group, against the three viral strains of influenza at baseline and 28 days post-vaccination.

Cell-mediated responses (IFN-γ)

A 2 Group (combined exercise groups, control) × 2 Sex ANOVA on the IFN-γ response to whole vaccine *in-vitro* at 28 days post-vaccination revealed no significant effect of Group ($F(1,143)=1.62$, $p=.21$, $\eta^2=.01$), and no Group × Sex interaction ($F(1,143)<0.01$, $p=.99$, $\eta^2<.01$) (See Figure III). Further, 3 Group (immediate, 6 hr, 48 hr) × 2 Sex ANOVAs comparing the exercise groups revealed no significant overall Group effects ($F(2,105)=0.80$, $p=.45$, $\eta^2=.02$). Finally, ANOVAs were conducted to compare each of the separate groups with each other, and results revealed no significant differences between any of the individual groups (all $p>.05$).

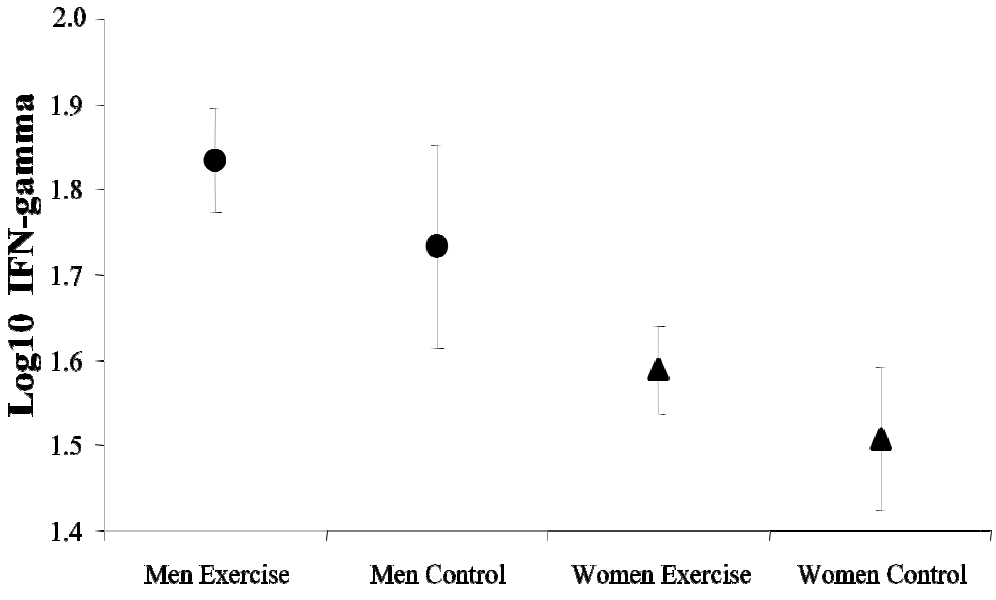

Figure III. Log 10 IFN-gamma at 28 days in the exercise groups (combined) and control group. No significant differences were observed between Sex or Group.

Limb circumference and arm pain

A 4 Group (immediate, 6 hr, 48 hr, control) ANOVA comparing limb circumference percentage changes from baseline to post-task, between all experimental groups, revealed significant Group effects for upper ($F(3,135)=4.34$, $p=.01$, $\eta^2=.09$) and forearm circumference ($F(3,136)=3.86$, $p=.01$, $\eta^2=.08$) (see Table II). Tukey post-hoc analyses revealed that each exercise group had a significantly greater percentage increase in both upper arm and forearm circumferences compared with control ($ps<.01$). None of the exercise groups differed from each other.

Similarly, a 4 Group (immediate, 6 hr, 48 hr, control) ANOVA comparing self-reported arm pain scores between all experimental groups revealed no significant Group effects immediately post-task ($F(2,109)=2.45$, $p=.09$, $\eta^2=.04$), but significant Group effects were observed immediately prior to vaccination ($F(3,145)=10.71$, $p<.01$, $\eta^2=.18$) (see Table II). Tukey post-hoc comparisons revealed that, at the time of vaccination, both the immediate ($p<.01$) and 48 hr groups ($p=.01$), but not the 6 hr group ($p=.26$), reported higher pain than the control group. Further, the pain rating of the immediate group was also significantly higher than the 6 hr group ($p<.01$); there were no other group differences.

IL-6 and CK responses to exercise

Figure II illustrates plasma IL-6 concentrations at baseline and at the time of vaccination for each of the four experimental groups. A 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (baseline, time of vaccination) ANOVA comparing the control group with the

combined exercise groups revealed a close to significant Group × Time effect ($F(1,133)=3.49$, $p=.06$; $\eta^2=.03$); suggesting that exercise groups had elevated concentrations of IL-6 at the time of vaccination, compared with the control group. Further, no main effects of Sex and no Sex × Time interactions were identified ($p>.05$). A 3 Group (immediate, 6 hr, 48 hr) × 2 Time (baseline, time of vaccination) ANOVA comparison of the three separate exercise groups revealed no significant Group × Time interaction ($F(2,99)=0.69$, $p=.51$; $\eta^2=.01$). However, comparing the control group with each of the exercise groups in turn, IL-6 appeared to be elevated in both the immediate ($F(1,59)=3.44$, $p=.07$; $\eta^2=.07$) and 48 hr ($F(1,66)=3.77$, $p=.06$; $\eta^2=.05$) exercise groups compared with the control group, but these results were non-significant. Unexpectedly, there was no significant difference between the IL-6 levels in the 6 hr and the control group ($F(1,64)=2.05$, $p=.16$; $\eta^2=.03$).

Figure II illustrates plasma CK activity s at baseline and at the time of vaccination in each of the four groups. A 2 Group × 2 Sex × 2 Time ANOVA revealed a significant main effect of Time ($F(1,118)=6.32$, $p=.01$; $\eta^2=.05$), whereby CK increased from baseline to time of vaccination. However, no Group × Time, Sex × Time, or Group × Sex × Time interactions were observed. A comparison of the three separate exercise groups using a 3 Group (immediate, 6 hr, 48 hr) × 2 Time ANOVA revealed no significant Time x Group interaction ($F(2,82)=1.4$, $p=.08$; $\eta^2=.03$). 2 Group × 2 Time ANOVAs comparing the control group with each of the exercise groups in turn revealed that the 48 hr group had, as expected, elevated CK levels compared with control ($F(1,58)=4.34$, $p=.04$; $\eta^2=.07$). No further group differences were found (all $p>.05$).

Table II. Percentage change (mean ± SD) in upper arm and forearm limb circumference from baseline to immediately post-task, and upper arm pain immediately post-task, and immediately prior to vaccination.

	Control	Immediate exercise	6 hour exercise	48 hour exercise	Group Effects
Change in upper arm limb circumference (%)	0.01 ± 0.1	1.45 ± 0.36[a]	0.86 ± 0.32[a]	1.21 ± 0.27[a]	$F(3,135)=4.34$, $p<.01$, $\eta^2=.09$
Change in forearm limb circumference (%)	0.08 ± 0.1	1.57 ± 0.26[a]	2.17 ± 0.28[a]	1.57 ± 0.36[a]	$F(3,136)=3.86$, $p=.01$, $\eta^2=.08$
Arm pain immediately post-task (1-100)	n/a	32.1 ± 4.6	29.1 ± 3.6	20.7 ± 2.8	$F(2,109)=2.45$, $p=.09$, $\eta^2=.04$
Arm pain at time of vaccination (1-100)	1.1 ± 0.1	22.1 ± 3.6[a]	8.1 ± 2.6[b]	13.0 ± 3.0[a]	$F(3,145)=10.71$, $p<.01$, $\eta^2=.18$

[a] significantly different from control group ($p\leq.01$)

[b] significantly different from immediate exercise group ($p<.01$)

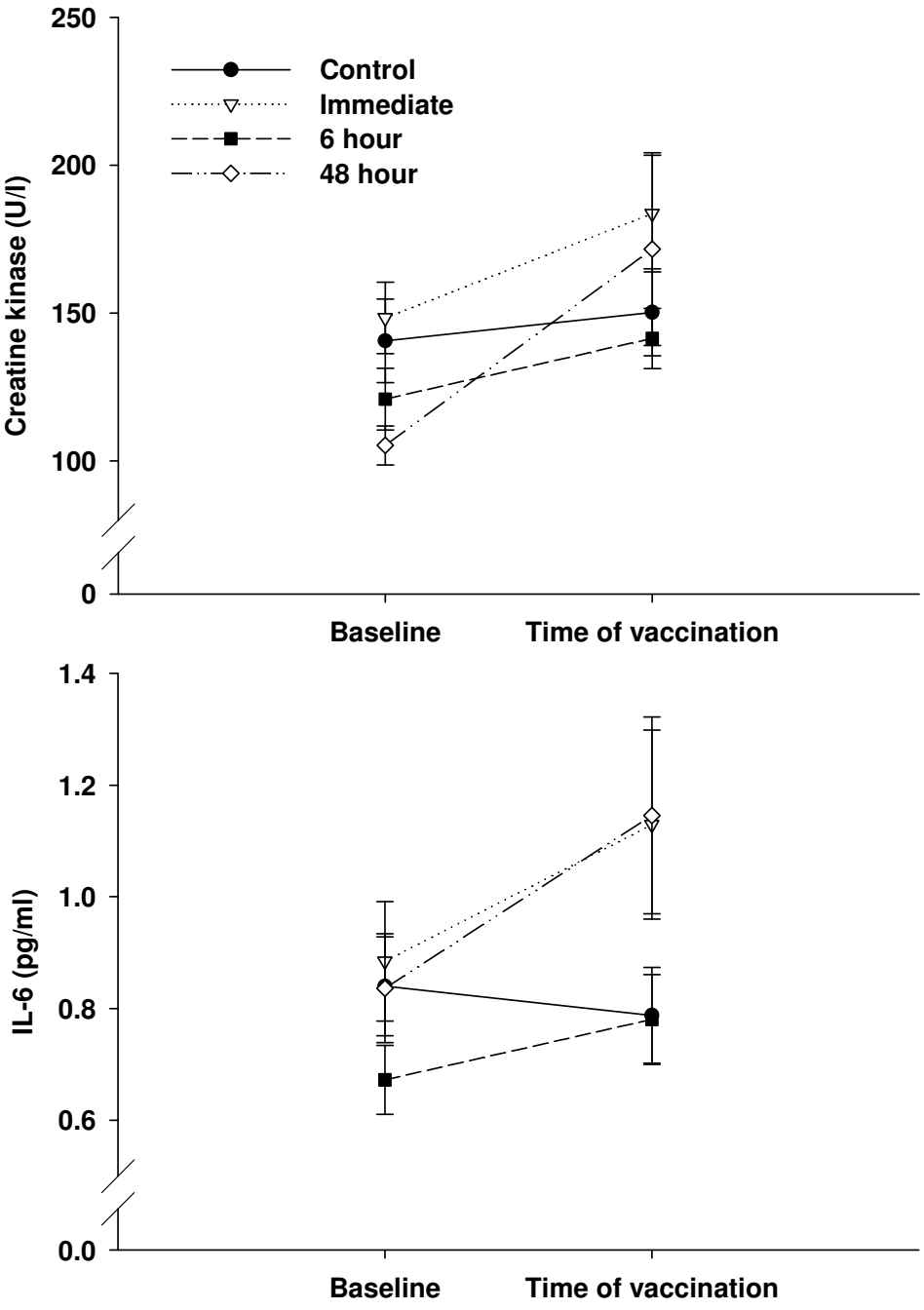

Figure II. Plasma CK (top; U/l) activity and (IL-6; pg/ml) concentrations in each experimental group at baseline and at the time of vaccination.

DISCUSSION

The current study investigated the effects of an acute bout of eccentric arm exercise on the immune response to vaccination in healthy young adults, and whether the efficacy of this intervention was modulated by the timing of the exercise relative to vaccination. All three viral strains contained in the 2007/2008 influenza vaccine elicited strong antibody responses (see Table I), and eccentric exercise did not further augment these responses, compared with the control group. There was also no difference in cell-mediated immunity between the groups. Although these null findings were unexpected, they are consistent with previous research, in which exercise-induced augmentation was only observed when the control group had relatively poor responses (Edwards et al. 2006; Edwards et al. 2007a; Edwards et al. 2008).

In a previous eccentric exercise study, antibody responses to both the A/Wyoming and B/Jiangsu strains were enhanced in female participants who completed an acute exercise task; both strains were characterised by poorer antibody responses in the control group (Edwards et al. 2007a). In contrast, the A/New Caledonia strain, which had larger antibody responses in the control group, was not further improved by exercise. A similar pattern occurred with the measure of cell mediated immunity; men in the control group had poorer IFN-γ production than women, and it was men that demonstrated an exercise-induced augmentation in this measure. Similar effects were demonstrated in another study; the relatively poor antibody responses to the A/Panama influenza strain shown by women in the control group were enhanced in those who undertook an exercise or psychological stress intervention (Edwards 2006). Again, the other two strains displayed greater immunogenicity and no effect of exercise was observed. Participants in this

study also received the meningitis A+C vaccination. Here, weak antibody responses to meningococcal A were shown by men in the control group; again, these were enhanced in both exercise and stress groups. Taken together, the data presented in this study and the findings from previous studies suggest that exercise may be an effective adjuvant where there are typically relatively poor immune responses. This may explain why an enhancement was not observed in the current study, in which the vaccine induced high levels of immunity even in the control groups.

A second aim of this study was to investigate whether the timing of vaccine administration following eccentric exercise affected the immune response to vaccination. Three specific time-points were chosen that coincide with particular physiological and immunological processes that follow eccentric exercise. As anticipated, a number of physiological differences were observed between the three exercise groups at the time of vaccination, as a result of the different timings of exercise. For example, the immediate exercise group exhibited elevated pain (Proske and Morgan, 2001) and the 48 hour group reported both elevated pain, indicative of the characteristic delayed onset muscle soreness associated with eccentric exercise, and elevated plasma CK levels (Rodenburg et al. 1993). Despite these distinct physiological group profiles, exercise timing was not associated with the immune response to vaccination. These findings were unexpected but not surprising given that no overall effect of exercise was found. Future research should investigate the effects of exercise timing on the immune response to vaccines that elicit poorer vaccine responses.

Contrary to expectations, IL-6 was not highest in the 6 hr exercise group; this cytokine was in fact more elevated in the other exercise groups. This observation suggests that the inflammatory response to acute eccentric exercise did not follow the same kinetics as has been previously reported (MacIntyre et al. 2001; Paulsen et al. 2005); this is not without precedent (Suzuki et al. 2002; Sorichter et al. 2006), and suggests that IL-6 kinetics post-exercise may be complex. Further, in contrast to our previous work, IL-6 did not predict antibody response. This may be due to the unexpected IL-6 kinetics described. Alternatively, there is evidence to suggest that plasma levels of IL-6 may not be an accurate representation of intramuscular IL-6 activity (Jonsdottir et al. 2000); it remains possible that the extent of the intramuscular IL-6 response may predict antibody response. Finally, given that all groups showed elevated IL-6 relative to control, it is unlikely that this is the source of the null findings reported in this study.

This study was the largest of its type to date and has a robust design to investigate the research question. However, in light of the null findings reported, it is important to consider possible methodological limitations. A key consideration is the choice of participants. The participants used in this study were healthy young adults with no known immune dysfunction; this population was chosen as we believe it is important to optimise the efficacy of this intervention in healthy individuals before application to more vulnerable populations. However, this choice, and the subsequent strong immune responses shown by the healthy participants, may have been responsible for the null findings. It is clear that future research would be well-directed towards investigating the impact of this intervention in populations with known immune dysfunction, such as the elderly.

In summary, there were no differences in vaccine response between the four exercise groups, despite the distinct physiological profiles observed in each of the exercise groups. We hypothesise that this result was due to the relatively robust immune responses seen to the influenza vaccine. These findings are consistent with the notion that acute exercise may only be an effective adjuvant to vaccination where the control responses are relatively poor (Edwards et al. (2007b). To explore this possibility further, future research should investigate less immunogenic vaccines, or immuno-compromised individuals, such as chronically stressed individuals (Burns et al. 2003), the elderly (Goodwin et al. 2006) or HIV patients (Kroon et al. 1994).

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CHAPTER THREE

ECCENTRIC EXERCISE AS A BEHAVIOURAL ADJUVANT TO VACCINATION: ISSUE OF EXERCISE INTENSITY

ABSTRACT

Acute exercise prior to vaccination can improve the antibody response to influenza vaccination. However, both the optimal exercise protocol and the mechanisms underpinning this adjuvant effect remain unclear. The aim of the current study was to determine whether exercise intensity influenced the efficacy of the intervention. One hundred and sixty healthy young adults were randomly assigned to a resting control group or one of three intervention groups, who exercised at an intensity of 60%, 85%, or 110% of their pre-determined concentric one-repetition maximal. The exercise groups performed 50 repetitions of the eccentric portion of both bicep curl and lateral raise movements. All participants then immediately received a reduced-dose (50 % recommended dose) influenza vaccine. Antibody titres to the three viral strains contained in the vaccine were measured at baseline and at 28 days post-vaccination. Compared with the control group, exercise enhanced the antibody response to the least immunogenic of the three strains (B/Florida). In addition, the exercise groups showed an augmented response to the A/Uruguay strain compared with control; however, this effect was observed only in men. The intervention had no effect on the antibody responses to the most immunogenic strain, A/Brisbane. Finally, antibody responses were unrelated to the intensity of the exercise bout. In conclusion, our

findings provide further evidence of exercise as an adjuvant to enhance vaccination responses. The results further show that responses to the low-immunogenic antigens are particularly responsive to augmentation by acute eccentric exercise.

INTRODUCTION

Vaccination against infectious diseases is one of the most successful public health interventions of all time (Nicholson et al. 2003). However, there is considerable inter-individual variation in the efficacy of vaccination, and additional strategies to improve immune responses are needed. Recently, behavioural interventions, such as acute exercise administered prior to vaccination, have been shown to enhance the immune response in both animals (Dhabhar, 2002) and humans (Edwards et al. 2006; 2007; 2008). For example, exposure to an acute bout of cycling exercise improved the antibody response to influenza vaccination in women (Edwards et al. 2006) and to meningococcal A vaccination in men (Edwards et al. 2007).

A recent study showed an adjuvant effect to influenza vaccination using a resistance-based, eccentric exercise protocol (Edwards et al. 2007). During an eccentric muscle action, the exercising muscle applies continuous force as it lengthens, causing damage to the internal structure of the muscle fibres (Proske and Morgan, 2001). This damage is greater than that observed with concentric (shortening) muscle actions, as indicated by a substantial increase in plasma creatine kinase (Sorichter et al. 1999). When conducted in untrained participants, eccentric exercise reliably causes muscle damage and leads to a localised inflammatory response in the muscle (Sorichter et al. 2006). It is hypothesised that the influx of immune cells and the

release of inflammatory mediators associated with this muscle damage creates a pro-inflammatory environment that may augment the immune response to vaccination by, for example, the release of 'danger signals' or by activating dendritic cells (Gallucci et al. 1999; Rock et al. 2005). Given that eccentric exercise of the arm allows the localisation of this muscle damage to the specific site of vaccine administration, it is hypothesised this heightened inflammatory environment may be a particularly effective behavioural adjuvant. A recent study showed that the antibody response to influenza vaccine was enhanced in women following an acute eccentric exercise bout (Edwards et al. 2007). Although these encouraging findings demonstrate an adjuvant effect of eccentric exercise, further investigation is needed to maximise the efficacy of this intervention.

Given that the degree of muscle damage is related to exercise intensity, it is possible that the efficacy of the adjuvant effect could be adjusted by manipulating the exercise intensity. For clinical applicability, it is important to determine the minimum intensity at which exercise causes significant improvements to vaccine responses. Further, intensity modulation may help elucidate the mechanisms underpinning the adjuvant effect of exercise. For example, an intensity-dependent augmentation may suggest that muscle damage severity, and the concomitant inflammation, are key to the immuno-enhancement effects. On the other hand, if there is no effect of intensity this will have important clinical application i.e. a more modest exercise bout can be utilised. This study therefore compared the efficacy of a relatively low (60% of concentric one repetition maximum (1RM)), medium (85% 1RM), and high (110% 1RM) intensity eccentric exercise intervention.

A key finding from the previous exercise and vaccination studies conducted by our group is that stress-induced immunoenhancement manifests only when antigens in the vaccine induce a poor immune response in the host. For example, when there are clear sex differences in the antibody response to a particular strain of the vaccine, the exercise-induced enhancements of this response are always confined to the sex who had mounted the poorer control response (Edwards et al. 2008; 2007a; 2006). For example, it has been shown before that control women showed a relatively poor response to the A/Panama strain of the influenza vaccine, compared with men; acute cycling exercise improved the antibody response to this strain in women, but not men (Edwards et al. 2006). The other strains, which induced generally robust responses and demonstrated no sex differences in their immunogenicity, showed no added benefit from the exercise intervention. Similarly, the antibody response to meningococcal A was enhanced by cycling exercise in men, who showed weaker responses than women in the control group (Edwards et al. 2008). It would thus appear that exercise augments poor responses, but does not enhance robust responses. Further support of this hypothesis emerged from a recent study, which demonstrated that the antibody responses to influenza vaccination were not enhanced by eccentric exercise when the vaccine contained viral strains that induced robust immunogenic responses (Chapter Two). In light of these findings, a reduced dose (50% recommended dose) vaccine was administered in this study in order to further investigate whether poorer responses, induced by a less immunogenic vaccine, are enhanced by exercise.

The aim of the study was to investigate whether exercise augmented the immune response to a reduced dose vaccine, and whether the intensity of exercise influenced the efficacy of the intervention. We hypothesised that the exercise groups would show augmented immune

responses to the influenza vaccine in comparison to the control group, and that higher intensity exercise would be associated with greater immune enhancement.

METHODS

Participants

One hundred and sixty healthy young students (80 men) were recruited from the University of Birmingham, of which one hundred and fifty-eight (79 men) completed the study (mean ± SD age: 21.01 ± 2.5 years; body mass index: 22.75 ± 2.8 kg/m^2). The majority of participants (N = 115) had not previously received the influenza vaccine used in the present study, and none reported influenza like-illness in the year prior to participation. Forty-five participants had received an influenza vaccine in the previous five years. As all three strains within the northern hemisphere winter 2008/2009 influenza vaccine differed from that of previous years, these participants were deemed eligible for participation. Importantly, further analysis revealed no significant differences between these groups in terms of their antibody responses to any of the three strains (data not presented). Exclusion criteria included smoking, a history of immune or cardiovascular disease, current acute infection or illness, pregnancy or suspected pregnancy, and a history of vaccine-related allergies or side effects. Use of prescription medication within one month of participation was also an important exclusion criterion; but females taking the contraceptive pill were not excluded. In addition, none of the participants reported having performed any resistance training in the 6 months prior to testing. All participants were instructed to abstain from vigorous exercise and over-the-counter medication for at least 24 h,

alcohol for at least 12 h, and food or caffeine for at least 2 hr prior to each session, and to refrain from over the-the-counter medication for up to 24 hr after the exercise / control task. All participants provided written informed consent and the study protocol was approved by the Black Country Local Research Ethics Committee. All participants were paid £30 or given research credits upon completion of the study.

Procedure

Participants were pseudo-randomised, maintaining an even sex distribution, into either one of three exercise groups, exercising at 60%, 85% or 110% of their concentric one repetition maximal (1RM) or a resting control group (all groups $N = 40$). Groups did not differ on stressful life events exposure, perceived stress, and health behaviours (data not reported here); these factors have been associated with augmentation of antibody responses (Burns et al. 2003; Cohen et al. 2001). In the first session, baseline blood samples were taken from an antecubital vein in the dominant arm, following a 20 min rest period. Participants then had height and weight measured, and a test of maximal muscle strength was conducted. Next, they completed the eccentric exercise task, with their non-dominant arm, at the relevant exercise intensity (exercise groups) or remained quietly resting for 25 min (control group). Immediately after task completion, a nurse administered a reduced dose (0.25 ml; 50% adult recommended dose) 2008/2009 northern hemisphere influenza vaccine (Fluarix, GlaxoSmithKline, Inactivated Split Virion, Lot no. AFLUA384AB) via intra-muscular injection into the deltoid muscle of the non-dominant arm. Participants returned to the laboratory after one day to provide a blood sample for

muscle damage and inflammatory marker measurement, and at 28 days post-vaccination to provide a blood sample for antibody determination.

Exercise Task

The exercise protocol has been adapted from elsewhere (Edwards et al. 2007). In brief, following the determination of maximal muscle strength (1 Repetition Maxima: Bicep Curl: 12.35 ± 2.38 kg; Lateral Raise: 8.92 ± 1.59 kg), participants performed the eccentric portions of the bicep curl and lateral raise exercises, contracting the biceps brachialis and deltoid muscles of the non-dominant arm, respectively. In both exercises, they were asked to lower the dumbbell in a controlled manner. All participants performed 50 repetitions of each muscle action (10 sets of 5 repetitions). They completed a set of lateral raises, rested 15 s, completed a set of bicep curls, and rested 30 s before the next set of lateral raises. The task lasted approximately 25 min.

Blood sampling

Blood was collected in a 10 ml vacutainer containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton-Dickinson, UK) at baseline and 24 hr post- exercise. Tubes were stored on ice until centrifugation (3400 g for 10 min at 1 °C) and plasma was stored at −80 °C for later assessment of interleukin-6 and creatine kinase. Blood serum was collected in a plain 10 ml vacutainer and allowed to clot for 1 hr at room temperature; and following centrifugation (3400 g for 5 min at 21 °C), was stored at −20 °C for later antibody titre determination.

Assays

Anti-influenza antibody titres were measured using a haemagglutination inhibition test analysed by the serology laboratory of GlaxoSmithKline Beecham at Dresden, Germany. The influenza vaccine contained three viral strains: A/Brisbane/59/2007 (H1N1)-like strain: A/Brisbane/59/2007; A/Brisbane/10/2007 (H3N2)-like strain: A/Uruguay/716/2007; B/Florida/4/2006-like strain: B/Florida/4/2006. The antibody results are reported as titres; a titre represents the reciprocal of the highest dilution having a positive response in the haemagglutination inhibition assay.

Plasma IL-6 was measured using high-sensitivity ELISA (Quantikine HS Human IL-6 ELISA, R&D Systems, UK) according to the manufacturer's instructions. The reported sensitivity of the assay was 0.039 pg/mL, with recorded intra-assay and inter-assay variation 3.9%, and 8.4%, respectively. Plasma creatine kinase (CK) activity s were measured using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK) and diagnostic reagents (ABX diagnostics, UK). CK samples were analysed in triplicate, and coefficient of variations for CK were calculated as 1.18%.

Limb circumference, pain and perceived exertion

Muscle soreness was assessed using a visual analogue scale with anchors of 0 (no pain) and 100 (worst possible pain) to indicate pain in the exercised arm (Melzack, 1987). Participants rated their pain twice immediately after task completion; one rating was giving with reference to the

arm resting by their side and the other while they replicated the movements of the exercise task without the weights. Change in upper and forearm limb circumferences were used to gauge the amount of swelling in the exercised arm. Measurements were taken in an unblinded manner using tape measure at the point of flexed maximal circumference of the biceps (upper arm) and 5 cm below the elbow crease in the inside of the arm (forearm), with the arm relaxed by the participants' side. Arm circumference was recorded at pre-task, immediately post-task, and at 24 hr post-task; measurement sites were marked with pen in two places to ensure consistent placement of tape measure. Participants also provided a rating of perceived exertion , using Borg's (1970) 15-point scale (6= "no exertion at all", 20= "maximal exertion"), immediately after the exercise task.

Statistical analysis

Due to the skewed distributions of the antibody titres analyses were performed on log10 transformed values. All analyses were conducted using repeated measures (antibody titres; IL-6; CK) or univariate (cell mediated immunity pain; percentage change in limb circumference) analysis of variance. In each case, the following analytical strategy was adopted. For example, it was first investigated whether there was any difference between treatment and control i.e. all exercise groups combined were compared with the control group. Secondly, the three treatment groups were compared with each other. Finally, a series of ANOVAs compared individual exercise groups with each other and with the control group. As previous studies have found sex differences in intervention efficacy (Edwards et al. 2006; Edwards et al. 2007), sex was entered as a between-subject factor in all parameter analyses. If no sex effects were found at this stage

for a particular parameter, it was excluded from further analyses of that parameter. Univariate post-hoc analyses were conducted using Tukey. Effect sizes are represented by η^2, and differences in degrees of freedom reflect occasional missing data. Pearson correlations were used to examine the associations between antibody titre change scores with responses to the exercise task (change in CK, IL-6, pain ratings, limb circumference). These latter analyses were performed only within the exercise group.

RESULTS

Antibody titres

Table I displays the overall geometric mean antibody titres specific to each viral strain of influenza, at baseline and 28 days post-vaccination, for the overall cohort and each individual group. A 2 Sex (male, female) × 3 Strain (A/Brisbane, A/Uruguay, B/Florida) × 2 Time (baseline, 28 days) ANOVA on log transformed antibody titres revealed significant Strain ($F(2,149)=4.82$, $p<.01$, $\eta^2=.87$), Time ($F(1,150)=8.92$, $p<.01$, $\eta^2=.86$), and Strain × Time ($F(2,149)=60.04$, $p<.01$, $\eta^2=.45$) effects. As anticipated, separate ANOVAs (2 Time) confirmed that antibodies titres to all viral strains increased from baseline to 28 days: A/Brisbane ($F(1,151)=716.64$, $p<.01$, $\eta^2=.83$); A/Uruguay ($F(1,155)=299.73$, $p<.01$, $\eta^2=.66$); B/Florida ($F(1,157)=283.3$, $p<.01$, $\eta^2=.64$). A series of ANOVAs (2 Strain × 2 Time) comparing each strain response revealed that the vaccine induced different response levels for each strain ($F(1,150)>72.59$, $p<.01$, $\eta^2>.33$). The largest fold increase was exhibited by A/Brisbane (19.25), whereas A/Uruguay (6.25) displayed greater increases than B/Florida (4.68) which displayed the

smallest change; fold increases were calculated by dividing the geometric mean antibody titre at 28 days by the baseline titre.

Effects of exercise on antibody responses

The first analytic step was to compare the control group to the combined exercise group, in order to establish if there was an adjuvant effect of exercise. A series of 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (0, 28 days) ANOVAs revealed a significant Group × Time interaction for B/Florida ($F(1,154)=7.17$, $p=.01$, $\eta^2=.04$). There were no Group × Time interactions for A/Brisbane ($F(1,148)<0.01$, $p=.98$, $\eta^2<.01$) and no Sex × Time, or Group × Sex × Time interactions were observed for either A/Brisbane or B/Florida strains. Similarly, there was no Group × Time interaction for A/Uruguay ($F(1,152)=0.14$, $p=.71$, $\eta^2<.01$), however there was however a significant Sex × Time interaction ($F(1,152)=3.94$, $p=.049$, $\eta^2=.03$); demonstrating that women had better antibody responses to this strain than men (see Figure I). There was also a significant Group × Sex × Time interaction for A/Uruguay ($F(1,152)=5.70$, $p=.02$, $\eta^2=.04$). This effect was further investigated by performing a 2 Group × 2 Time ANOVA for men and women separately. Men showed a significant Group × Time interaction for A/Uruguay ($F(1,76)=4.51$, $p=.04$, $\eta^2=.06$), whereby exercise men had higher responses than men in the control group. No Group × Time effects were found in women for this strain ($F(1,76)=1.75$, $p=.19$, $\eta^2=.02$).

Table I. Geometric mean (95% confidence intervals) antibody titres against the three viral strains of influenza at baseline and 28 days post-vaccination for all participants combined, and for each group.

	A/Brisbane		A/Uruguay		B/Florida	
	Baseline	28 days	Baseline	28 days	Baseline	28 days
Overall	20 (17 – 23)	385 (320 – 463)[a]	16 (14 – 18)	100 (79– 126)[a]	246 (206 – 294)	1152 (1028 – 1290)[a]
Control	16 (13 – 19)	333 (232 – 476)[a]	17 (13 – 23)	104 (64 – 169)[a]	388 (300 – 501)	1220 (1046 – 1423)[a]
60 %	22 (16 – 32)	425 (287 – 632)[a]	17 (14 – 21)	116 (76 – 177)[a]	197 (128 – 303)	1433 (1127 – 1821)[a]
85 %	19 (14 – 27)	323 (221 – 473)[a]	15 (12 – 18)	129 (79 – 212)[a]	180 (122 – 264)	813 (627 – 1056)[a]
110 %	15 (12 – 19)	440 (286 – 675)[a]	14 (11 – 17)	61 (38 – 99)[a]	270 (200 – 370)	1220 (951 – 1565)[a]

[a] Significantly different from baseline ($p < .05$)

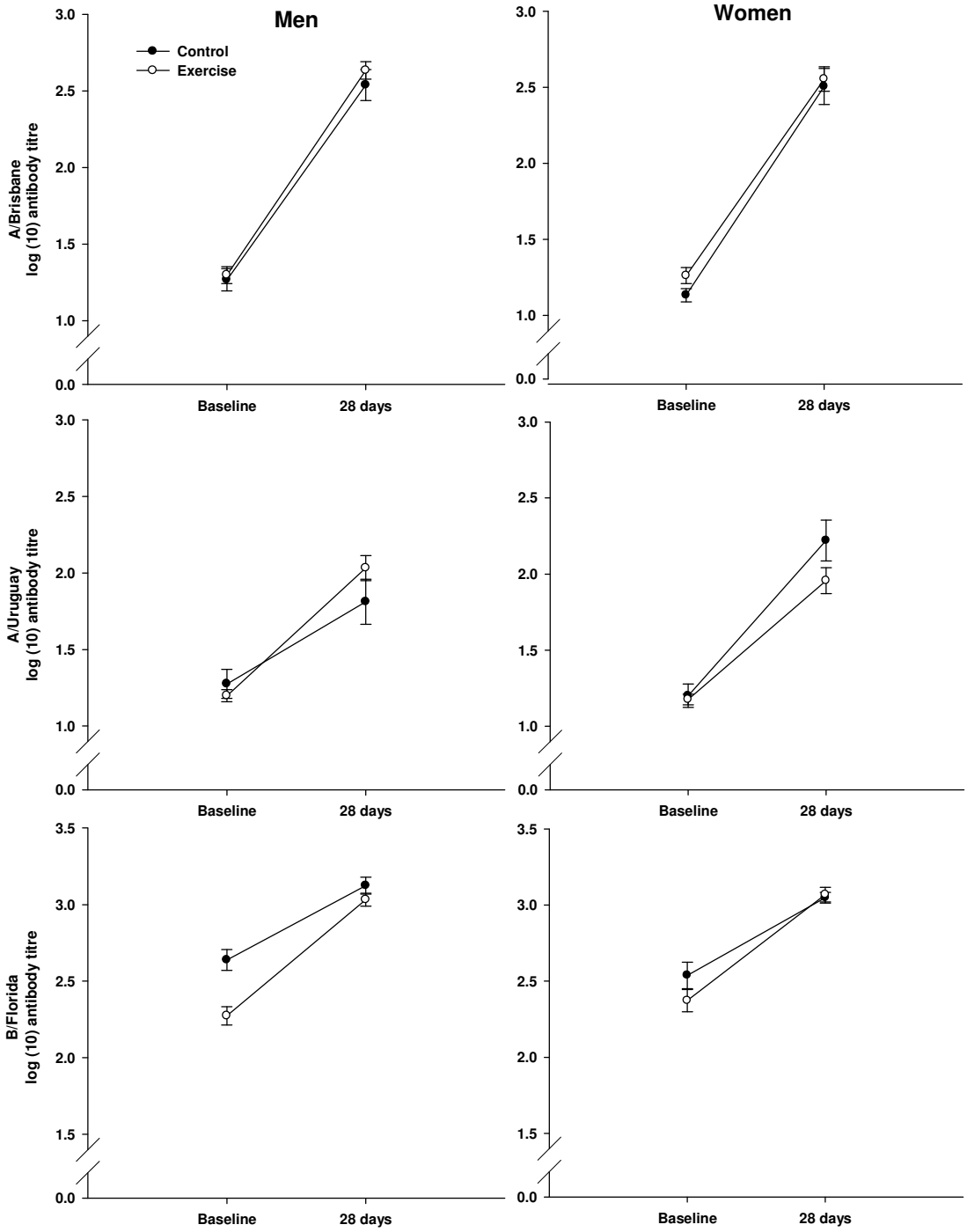

Figure I. Mean (SE) log-transformed antibody titres in men and women in both the exercise groups (combined) and control group. Titres are displayed for each of the three viral strains of influenza at baseline and 28 days post-vaccination.

Effects of exercise intensity on antibody responses

The next stage of the analysis compared the three exercise groups in terms of their efficacy in augmenting the antibody response to the influenza vaccination. A series of 3 Group (60%, 85%, 110%) × 2 Time (baseline, 28 days) ANOVAs on individual strains revealed a close to significant Group × Time effect for A/Uruguay ($F(2,111)=2.93$, $p=.058$, $\eta^2=.05$). Subsequently, a series of ANOVAs comparing each of the exercise groups revealed that the 85% group had greater elevations in antibody titre than the 110% group ($F(3,150)=3.92$, $p=.01$, $\eta^2=.07$); there were no other significant differences between the three groups (all ps < .16).

Limb circumference and arm pain

A 2 Group (combined exercise groups, control) ANOVA comparing limb circumference percentage changes from baseline to post-task revealed significant Group effects for upper ($F(1,152)=30.78$, $p<.01$, $\eta^2=.17$) and forearm ($F(1,151)=10.96$, $p<.01$, $\eta^2=.07$) circumference. These results indicated that all exercise groups had significantly greater percentage increases in upper and forearm limb circumference in comparison to control. A series of 3 Group (60%, 85%, 110%) ANOVAs revealed a Group effect for the upper ($F(2,115)=3.13$, $p=.05$, $\eta^2=.05$) and forearm ($F(2,113)=4.58$, $p=.012$, $\eta^2=.08$) circumference; Tukey post-hoc analyses confirmed a dose-dependent response with the higher intensity group exhibiting greater changes than the other groups (see Table II).

Similarly, a 2 Group (combined exercise groups, control) ANOVA comparing self-reported arm pain scores revealed significant Group effects for resting ($F(1,152)=12.02$, $p<.01$, $\eta^2=.07$) and movement ($F(1,152)=35.26$, $p<.01$, $\eta^2=.19$) arm pain immediately post-task. A series of 3 Group (60%, 85%, 110%) ANOVAs revealed a Group effect for movement ($F(2,116)=4.14$, $p=.02$, $\eta^2=.07$) but not resting ($F(2,116)=1.21$, $p=.30$, $\eta^2=.02$) arm pain. Tukey post-hoc analyses confirmed a dose-dependent response for movement arm pain with the 85% and 110% groups exhibiting greater changes than the 60% group (see Table II).

CK and IL-6 responses to exercise

Figure II illustrates plasma CK activity s at baseline and at 24 hr post-exercise in each of the four groups. A 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (baseline, 24 hr) ANOVA revealed a Group × Time effect ($F(1,140)=4.89$, $p=.03$; $\eta^2=.03$); exercise groups had elevated concentrations of IL-6 at the time of vaccination, compared with the control group. A 3 Group (60%, 85%, 110%,) × 2 Time (baseline, 24 hr post-exercise) ANOVA, comparing changes within the three exercise groups, revealed a significant Group × Time interaction ($F(2,100)=7.66$, $p<.01$; $\eta^2=.13$). A series of ANOVAs comparing the changes between the separate exercise groups showed that the 110% exercise group had a greater increase than the 60% ($p<.01$) and 85% groups ($p<.01$); there was no significant difference between the 60% and 85% groups ($p=.07$). No main effects of Sex and no Sex × Time interactions were identified for CK in any of the above analyses ($p>.05$).

Figure II illustrates plasma IL-6 concentrations at baseline and 24 hr post-exercise. A 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (baseline, 24 hr post-exercise) ANOVA revealed a significant main effect of Time ($F(1,141)=14.19$, $p<.01$; $\eta^2=.09$), but no Group × Time interaction ($F(1,141)=1.96$, $p=.16$; $\eta^2=.04$). A 3 Group (60%, 85%, 110%,) × 2 Time (baseline, 24 hr post-exercise) ANOVA comparing the three separate exercise groups again revealed no significant Group × Time interactions ($F(2,106)=0.95$, $p=.39$; $\eta^2=.09$). No main effects of Sex and no Sex × Time interactions were identified for any of the IL-6 analyses ($p>.05$).

Correlations

To explore the association between the acute response to eccentric exercise and the response to vaccination, a series of Pearson correlations were conducted. Analyses were performed only within the exercise group as participants in the control group showed, as expected, minimal changes in arm circumference and pain scores. As expected, Borg scores obtained immediately after the exercise bout were associated with CK change from baseline to 24 hr post-exercise ($p=.01$), forearm percentage change ($p = .019$), arm pain at rest ($p< .01$) and arm movement pain ($p< .01$). However, no other significant correlations were observed and, notably, no relations were observed between antibody change scores and either CK change, IL-6 change, limb circumference, pain (resting and movement) or Borg scores.

Table II. Percentage change (mean ± SD) in upper arm and forearm limb circumference from baseline to immediately post-task; and upper arm pain immediately post-task either at rest or during a movement replicating the action of the exercise tasks.

	Control	60% exercise	85% exercise	110% exercise	Group Effects
Change in upper arm limb circumference (%)	−0.15 ± 0.45	0.64 ± 1.49	0.92 ± 2.56	1.85 ± 2.49[a b]	$F(3,145)=6.40$, $p<.01$, $\eta^2=.12$
Change in forearm limb circumference (%)	−0.20 ± 1.12	1.37 ± 1.61[a]	2.52 ± 2.06[a]	3.17 ± 3.85[a b]	$F(3,146)=14.86$, $p<.01$, $\eta^2=.23$
Resting arm pain immediately post-task (1-100)	1.37 ± 1.24	8.33 ± 15.55[a]	9.84 ± 13.80[a]	13.64 ± 17.37[a]	$F(3,146)=5.09$, $p<.01$, $\eta^2=.10$
Movement arm pain immediately post-task (1-100)	2.66 ± 2.91	15.08 ± 15.62[a]	25.25 ± 18.47[a b]	25.74 ± 21.52[a b]	$F(3,146)=15.53$, $p<.01$, $\eta^2=.24$

[a] significantly different from control group ($p<.05$)

[b] significantly different from 60% exercise group ($p<.05$)

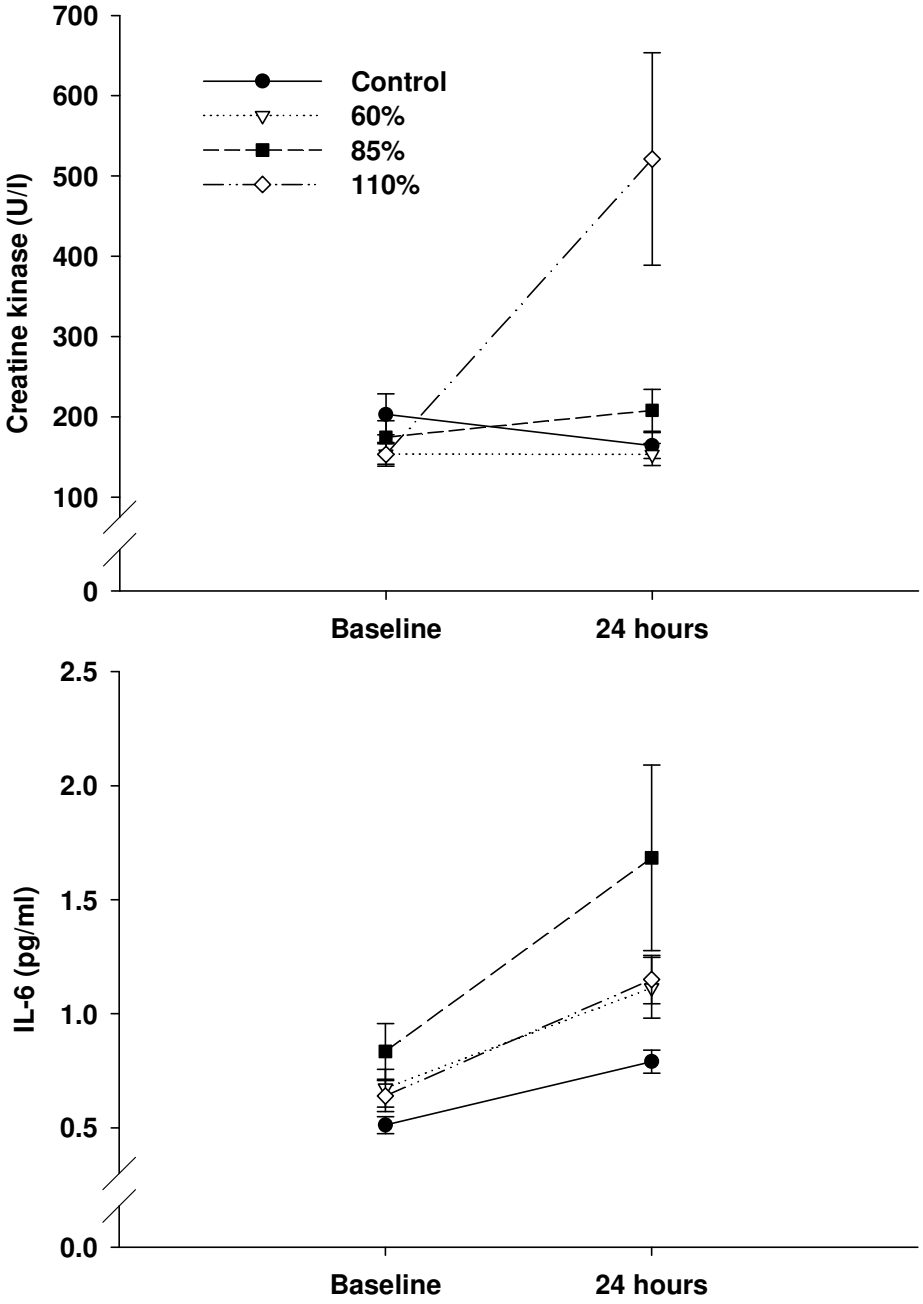

Figure II. Plasma CK (top; U/l) activity and IL-6 (bottom; pg/ml) concentrations in each experimental group at baseline and at 24 hr post-exercise.

DISCUSSION

This study evaluated the adjuvant effect of a single bout of eccentric exercise on the antibody response to influenza vaccination, and whether the efficacy of this intervention was modulated by the intensity of the exercise bout. Replicating prior research, eccentric exercise enhanced the antibody responses to the least immunogenic of the three influenza strains (B/Florida) in both men and women. In addition, exercise also augmented the response to the A/Uruguay strain, although this was apparent only in men. Antibody responses to the most immunogenic strain, A/Brisbane, showed no effect of the intervention. Varying the eccentric exercise intensity did not influence intervention efficacy.

These results provide further evidence that eccentric exercise is particularly effective at augmenting the immune response when the antibody response to the vaccination is weak. By using a reduced dose influenza vaccine, a poorer immune response was elicited in a healthy population, particularly to two of the three vaccination viral strains (A/Uruguay, B/Florida). As anticipated, it was these strains that demonstrated immuno-enhancement by the exercise intervention. The third influenza strain (A/Brisbane) induced higher antibody responses and there was no effect of exercise found. These findings are consistent with previous research showing that exercise-induced augmentation of antibody responses was only observed when the control group had relatively poor responses (Edwards et al. 2006; Edwards et al. 2007; Edwards et al. 2008). Taken together, the findings from these studies suggest that exercise is effective in augmenting otherwise relatively poor immune responses.

Another key aim of this study was to evaluate whether the intensity of the exercise bout modulated the efficacy of the eccentric exercise intervention. Edwards et al (2007) showed that eccentric exercise at 85% 1RM enhanced immune responses to influenza vaccination; the present study compared a lower (60%) and higher (110%) exercise intensity to evaluate the effect of this modulation on adjuvant efficacy. As anticipated, a number of physiological differences were observed between the three exercise groups after the exercise bout. For example, self-reported arm pain and change in limb circumference exhibited dose-dependent responses, where the greatest increases were observed in participants exposed to the highest intensity exercise. Indeed, CK also showed the highest increase in the high-intensity group. However, despite these physiological differences, there was no association between the extent of these changes and the antibody response to vaccination. For example, contrary to previous research (Edwards et al. 2006), there was no association between the antibody response to vaccination and the IL-6 response to exercise, measured at 24 hr post-exercise. It may be that plasma levels of these markers did not accurately represent actual intramuscular damage and inflammatory status following eccentric exercise, as has been shown previously (Jonsdottir et al. 2000). More likely, however, is the possibility that the changes in plasma IL-6 reflect the nature of the exercise bout being conducted. In this study, eccentric exercise induced relatively small changes in IL-6, whereas in the previous study a concentric based (cycling) protocol was used (Edwards et al. 2007), and changes in plasma IL-6 are typically higher following this type of exercise. Importantly, it should also be noted that the timings of IL-6 measurement also differed between these two studies; in this study IL-6 was measured post-vaccination, whereas in the previous study it was measured before vaccine administration (Edwards et al. 2007). Further, it is also possible that the IL-6 response to exercise was confounded by an additional IL-6 response to

vaccination (Tsai et al. 2005); the observed increase in IL-6 even in the control group suggests that this is a plausible explanation. Finally, it is possible that the physiological measures taken may not accurately represent the *peak* response to the exercise. For pragmatic reasons, they were assessed either immediately post-exercise at the time of vaccination (pain, limb circumference), or 24 hours later (IL-6, CK), whereas it is well established that greatest inflammatory responses are observed in the damaged skeletal muscle tissue up to 48 hr after muscle-damaging exercise (Proske and Morgan, 2001). It remains possible that the magnitude of the later peak could relate more accurately to the antibody response which is developing during this time (Cox et al. 1994). This could be addressed in future studies by conducting a more detailed kinetic profile of the inflammatory response to the exercise.

Given that enhancement of antibody responses to vaccination occurred independently of exercise intensity, it is important to consider potential mechanisms. There is evidence that muscular contractions are associated with a temporary increase in lymph drainage around the site of exercised muscle tissue (Havas et al. 1997) which could subsequently enhance immune cell transport to and from the site of antigen administration (Swartz et al. 2008). It is possible, therefore, that simply performing the eccentric exercise is sufficient to enhance this process. Alternatively, a threshold effect of exercise may exist whereby some degree of exercise enhances antibody responses but higher intensities do not cause further augmentation; it may be that this intensity threshold may be lower than the exercise intensities used in this study. Future research should investigate the mechanisms underpinning the adjuvant potential of exercise to help clarify this issue.

In summary, eccentric exercise improved the antibody response to a reduced dose influenza vaccine. The reduced dose vaccine produced weak antibody responses to the B/Florida and A/Uruguay strains, and the intervention was effective in both cases, increasing the response in both men and women for B/Florida and in men only for A/Uruguay. The A/Brisbane strain produced the most robust immunogenic response and no adjuvant effects were found. These findings add to previous research, and support the hypothesis that acute behavioural adjuvants enhance weak immune responses to vaccination. Given this, future research should consider whether less immunogenic vaccines which currently require multiple doses can be enhanced by acute behavioural stress, and whether this intervention could augment immune responses in the immuno-comprised, such as the chronically stressed (Burns et al. 2003), the elderly (Goodwin et al. 2006) and HIV patients (Kroon et al. 1994).

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CHAPTER FOUR

THE INFLUENCE OF VACCINE ADMINISTRATION SITE ON THE EFFICACY OF ECCENTRIC EXERCISE AS AN ADJUVANT TO HEPATITIS B VACCINATION IN YOUNG ADULTS

ABSTRACT

Behavioural interventions, such as acute exercise, have been shown to improve responses to poorly immunogenic vaccines. It has been speculated that eccentric exercise involving muscles close to the site of vaccine administration induces a pro-inflammatory local environment that is beneficial to the specific antibody response. However, eccentric exercise also induces immunological changes at a systemic level, which raises the possibility that the adjuvant effect could be more widespread. The aim of the current study was to determine whether exercise induced changes in the antibody response are mediated via local or systemic effects of exercise. Seventy-eight healthy participants (39 men) were randomly assigned to an exercise group (n = 59) or a resting control group (n = 19). The exercise group performed 50 repetitions of the eccentric portion of both the bicep curl and lateral raise movements, in the non-dominant arm, at an intensity eliciting 85% of the participant's concentric one repetition maxima. All participants then received a hepatitis B vaccination; this vaccine was chosen because a single dose induces a relatively poor antibody response in humans. Thirty-one exercise participants received the hepatitis B vaccine into the non-dominant (exercised) arm and twenty-nine exercise participants

received the vaccine into the dominant (resting) arm. Anti-hepatitis antibody levels were measured at baseline and 28 days post-vaccination as indicators of the humoral response. Anti-hepatitis B antibodies increased from baseline to 28 days, but exercise had no effect on these responses, regardless of the site of administration. These findings may indicate that eccentric exercise has a limited adjuvant effect on the primary antibody response to vaccination.

INTRODUCTION

Acute behavioural interventions, such as exercise and psychological stress, can improve the immune response to a variety of vaccinations (Edwards et al. 2006; Edwards et al. 2007a; Edwards et al. 2008). For example, a bout of eccentric arm exercise performed prior to influenza vaccination improved the antibody response to influenza vaccination in women. Specifically, exercised women showed enhanced responses against the A/Wyoming and B/Jiangsu strains of influenza compared with women in the control group, who showed poor responses to these strains. The other strain, A/Fujian, induced relatively robust responses in the control group and no further enhancement by exercise was found. These results suggest that exercise may enhance poorer antibody responses, but have no effect on normal robust immune responses.

This account is supported by two large-scale randomised control trials. The first study revealed that exercise had no effect on the immune response to influenza vaccination when the vaccine contained relatively immunogenic strains (Chapter Two). In the second study, a half-dose influenza vaccine was used to reduce the immunogenicity of the vaccine (Chapter Three). Results indicated that exercise enhanced the antibody response to the B/Florida strain in men and

women, and to A/Uruguay in men only. In these cases, relatively poor responses were exhibited by the relevant control group suggesting that exercise augmented these otherwise weakened responses. Again, no effect of exercise was observed in the most immunogenic strain, A/Brisbane. Taken together, we hypothesise that eccentric exercise may enhance antibody responses to poorly immunogenic vaccine strains. To investigate this hypothesis further, this study investigated the effects of exercise on the immune response to a single hepatitis B vaccine.

Hepatitis B virus (HBV) is a highly infectious DNA virus responsible for infecting approximately two billion people worldwide and killing up to 600,000 individuals each year (http://www.who.int). Currently, vaccination is the primary method of preventing HBV infection; however, a single vaccine induces poor immunogenic responses and, as a consequence, a primary vaccination, followed by at least two 'booster' secondary doses, is recommended. This lengthy and uneconomical schedule has led to widespread interest in the development of adjuvants used to enhance immune responses (Sanyal and Shi, 2009).

Although there is now considerable evidence of the utility of acute-behavioural stress as an adjuvant to vaccination in the literature (Edwards et al. 2007b); Campbell et al. *in submission*), the underlying mechanisms underpinning these adjuvant effects remain unclear. Given this, one important factor to consider is whether the adjuvant effects of exercise are driven by mediators within the local damaged muscle tissue or from systemic inflammatory mediators released in response to exercise.

Unaccustomed eccentric exercise causes local muscle damage to skeletal muscle by disrupting internal structures in the muscle fibres (Proske and Morgan, 2001). This physical damage induces an inflammatory response in the muscle characterised by oedema, pain and increased immunological signalling and leukocyte infiltration (Peake et al. 2005). This pro-inflammatory environment may facilitate antigenic identification and clearance. For example, there is evidence to suggest that stressed skeletal muscle cells release danger signals that activate dendritic cells (Gallucci et al. 1999; Shi and Rock, 2002). Further, muscular contractions from exercise are associated with a temporary increase in lymph drainage around the site of exercised muscle tissue (Havas et al. 1997) which could subsequently enhance immune cell transport to and from the site of antigen administration (Swartz et al. 2008). Together, it appears that localised muscle damage and increased lymph drainage arising from eccentric exercise may offer an environment which can enhance the immune response to vaccination. For this reason, one group in this study were administered a single hepatitis B vaccine into eccentrically-exercised muscle tissue.

However, eccentric exercise also induces a number of systemic alterations which could lead to an augmented antibody response to vaccination. For example, during this type of exercise, it has been demonstrated that circulating levels of inflammatory mediators, such as plasma IL-6 (Campbell et al. *in press*) and a host of other pro-inflammatory cytokines (Peake et al. 2005), are elevated above resting levels. Elevated concentrations of cytokines, such as IL-6, have previously been implicated in enhancing vaccine responses (Krakauer, 1995; Lee et al. 1999). It is, therefore, possible that increased levels of these pro-inflammatory mediators in the systemic circulation, stemming from eccentric exercise-induced muscle damage, could be a mechanism for augmented immune responses seen previously (Edwards et al. 2007a; Campbell et al. *in press*).

A recent study has shown that completion of concentric (cycling) exercise and / or acute psychological stress (pressurised mental arithmetic test) leads to both an increase in systemic IL-6 and an enhanced antibody response to vaccination (Edwards et al. 2006); the extent of the IL-6 response was also associated with the antibody response at six weeks post-vaccination . This suggests that elevations of inflammatory mediators in the systemic circulation may be a crucial component in the adjuvant effect of acute behavioural stress. Therefore, in this study, a second exercise group received the vaccine in the non-exercising arm. If both exercised groups were to demonstrate an augmented response to the vaccination, this would support a "systemic" adjuvant mechanism. In contrast, if any enhancements were limited to the group receiving their vaccination directly into the exercised arm, a more local mechanism would be indicated.

In summary, the primary aim of this study was to investigated the effects of eccentric exercise on the IgG antibody responses to a single dose hepatitis B vaccine. IgG antibodies were measured at baseline and at 28 days post-vaccination as this represents the peak IgG response to primary vaccination (Odinsen et al. 2007). A further aim of this study was to investigate whether immune augmentation was mediated via local or systemic mechanisms, by comparing the antibody responses of exercised participants who were vaccinated in either their exercised or non-exercised arm.

METHODS

Participants

Seventy-eight healthy, physically active students (39 men) were recruited from the University of Birmingham, and seventy-six completed this study (mean ± SD age: 21.08 ± 1.6 years; body mass index: 22.72 ± 2.3 kg/m^2). None of the participants had received the Hepatitis B vaccine previously, or had reported symptoms indicative of the condition in the past. Exclusion criteria included smoking, a history of immune or cardiovascular disease, current acute infection or illness, pregnancy or suspected pregnancy, a history of vaccine-related allergies or side effects, and use of prescription medication (excluding the contraceptive pill) within one month of participation. In addition, none of the participants reported having performed any resistance training in the 6 months prior to testing. All participants were instructed to abstain from vigorous exercise and over-the-counter medication for at least 24 hr, alcohol for at least 12 hr, and food or caffeine for at least 2 hr prior to each session, and to refrain from over the-the-counter medication for up to 24 hr after the exercise / control task. All participants gave written informed consent, and the study was approved by the South Birmingham Local Research Ethics Committee. They were paid ten pounds or given student research hours upon completion of the study.

Procedure

Participants were pseudo-randomised, maintaining an even sex distribution, into either exercise group A (*n*=31), exercise group B (*n*=28), or a non-exercise control group (*n*=19). Groups did

not differ for stressful life events exposure, perceived stress and health behaviours (data not reported here); these factors have been associated with augmentation of antibody responses (Burns et al. 2003; Cohen et al. 2001). In the first session, height and weight were determined, and a test of maximal muscle strength was conducted. After 7 days or more, participants returned to the laboratory for session 2, between 1000 hr and 1200 hr, for baseline blood sampling. Blood samples were taken from an antecubital vein in the dominant arm following a 20 min rest period. Next, participants completed either the eccentric exercise task with their non-dominant arm (exercise groups) or remained resting quietly for 25min (control group). Participants then returned to the laboratory 6 hr after completion of the morning session, between 1600 hr and 1800 hr; this delayed administration allows development of the response to the eccentric exercise task (Edwards et al. 2007a; MacIntyre et al. 2001). A nurse administered a Hepatitis B (Engerix B, GlaxoSmithKline, Batch number AHBVB253AB) and a Meningococcal ACWY (ACWY Vax, GlaxoSmithKline, Batch number A73FA135A) vaccine via intra-muscular injection into opposite deltoid muscles (only data from the hepatitis B vaccine is presented here due to assay problems associated with the meningococcal assay). One group received the Hepatitis B vaccine into the non-dominant (exercised arm group) arm and the Meningococcal ACWY vaccine into the dominant (rested) arm, whereas the opposite group (non-exercise arm group) received the alternate configuration. The control group were also randomly matched for arm of vaccination; 9 participants received the Hepatitis B vaccine into the non-dominant arm and the Meningococcal ACWY vaccine into the dominant arm, whereas the other 10 participants in the control group received the vaccines into the opposite arms. Participants returned to the laboratory after one day to provide a blood sample for indirect measurement of muscle damage (plasma CK), and at 28 days post-vaccination for IgG antibody determination.

Exercise Task

The exercise protocol was adapted from a previous study protocol (Edwards et al. 2007a). In brief, following the determination of maximal muscle strength (1 Repetition Maxima: Bicep Curl: 12.08 ± 4.4 kg; Lateral Raise: 8.61 ± 3.0 kg), participants performed the eccentric portions of the bicep curl and lateral raise exercises, contracting the biceps brachialis and deltoid muscles of the non-dominant arm, respectively. In both exercises, they were asked to lower the dumbbell in a controlled manner. All participants performed 50 repetitions of each muscle action at an intensity eliciting 85% of their individual 1RM (6 sets of 7 repetitions followed by 2 sets of 4 repetitions). They completed a set of lateral raises, rested 30 s, completed a set of bicep curls, and rested 60 s before the next set of lateral raises. The task lasted approximately 25 min.

Blood sampling

Blood was collected in a 10 ml vacutainer containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton-Dickinson, UK) at baseline and 24 hr post-vaccination. Tubes were stored on ice until centrifugation (3400 g for 10 min at 1 °C) and plasma was stored at −80 °C for later assessment of creatine kinase. Blood serum was collected in a plain 10 ml vacutainer and allowed to clot for 1 hr at room temperature; and following centrifugation (3400 g for 5 min at 21 °C), was stored at −20 °C for later antibody titre determination.

Hepatitis B Antibody Assay

Serum was assayed for levels of immunoglobulin (IgG) antibodies against Hepatitis B surface antigen using a specially developed in-house Luminex assay. The protocol was based on assays developed by others (Lal et al. 2004; Odinsen et al. 2007). The first stage of the assay involved adhering the specific antigen to identifiable microspheres. Recombinant Hepatitis B surface antigen (HepBsAg) (IBT Systems, Germany) was separated from tris buffered saline (TBS) using a buffer exchange technique (SuperSpin™ Desalter, BioToolomics, UK) and re-suspended in storage buffer (phosphate buffered saline (PBS) with 1 % bovine serum albumin (BSA), 0.05 % NaN_3 and 0.05 % Tween 20; all Sigma Alrich). HepBsAg was then coupled to activated carboxylated microspheres (region 43). The microspheres were then activated using activation buffer (NaH_2PO_4) containing 5 mg / ml EDC (Sigma-Aldrich) and 5 mg / ml Sulpho-NHS (Sigma-Aldrich). The solution was incubated on a rotator for 20 min at room temperature in the dark. Microspheres were washed with PBS by centrifugation and re-suspended in 125 µl of HepBsAg solution, and incubated for 2 hr at RT in the dark. Following this, the microspheres were washed twice with wash buffer (PBS, 0.05 % Tween 20) and stored in 100 µl storage buffer at 4 °C in the dark.

The second part of the assay involved measuring the concentration of specific antibody using the coated microspheres. Hep B IgG standard (HepBQuin, Sanquin, NL; 100 IU / ml) was diluted at 1:3000 in diluent buffer (PBS, 1 % BSA and 0.05 % NaN_3) and a 7 point curve serially diluted 1:3 was generated. Serum samples were diluted 1:100 in diluent buffer. Samples and standards

(50 µl) were incubated with 2500 beads per well for 1 hr at room temperature on a plate shaker. After 5 washes (PBS with 1 % BSA, 0.05 % NaN_3 and 0.05 % Tween 20), contents were incubated with IgG-PE mouse anti-human secondary antibody (Invitrogen Corp, CA, USA) (100 µl, 1:200) for 40 minutes on a plate shaker at room temperature. Microspheres were washed and resuspended in 125 µl of wash buffer and read on a Luminex machine (Luminex Corp, TX, USA), programmed to collect data for 100 microspheres per well. Acquisition software (BioPlex Software Manager (version 4), BioRad Labs, CA, USA) was used to generate serotype antibody concentrations from a five parameter logistic curve fit. Serum IgG levels are reported as IU / l.

Creatine kinase assay

Plasma creatine kinase (CK), an indirect marker of muscle-damage, was measured using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK) and diagnostic reagents (ABX diagnostics, UK). CK samples were analysed in triplicate, and coefficient of variations for CK were calculated as <2%.

Limb circumference, pain and perceived exertion

Muscle soreness was assessed using a visual analogue scale (Melzack, 1987) with anchors of 0 (no pain) and 100 (worst possible pain), to indicate an overall pain rating regarding the non-dominant arm. These measures were completed by all participants immediately, 6 hr (immediately prior to vaccination), and 24 hr after the task. Change in upper arm circumference was used to quantify the amount of swelling in the non-dominant arm from pre-task baseline to

immediately post-task, and at 6 hr post-task. Measurements were taken in an unblinded manner using tape measure at the point of flexed maximal circumference of the upper arm and, 5 cm below the elbow crease in the inside of the forearm. After locating the site of measurement, participants let their arm hang, relaxed, by their side for all measurements. Measurement sites were marked in two places at the first testing session to ensure consistent placement of tape measure. Exercise group participants also provided a rating of perceived exertion (Borg, 1970) immediately after the task using a 15-point scale (6= "no exertion at all", 20= "maximal exertion"), immediately after the task.

Statistical analysis

Due to the skewed distributions of the antibody titres, analyses were performed on LOG10 transformed values. Previous studies have found sex differences in intervention efficacy (Edwards et al. 2006; Edwards et al. 2007a; Edwards et al. 2008a) and, as a result, sex was entered as a between-subject factor in all initial analyses. If no sex effects were found at this stage, it was excluded from further analyses. All analyses were conducted using ANOVA (antibody titres; CK; pain; percentage change in limb circumference). In each analysis, a combined exercise group, with both exercise sub-groups collapsed, was initially compared with the control group. Individual exercise groups were then compared with control, followed by a comparison between exercise groups. Effect sizes are represented by eta-squared (η^2). Differences in degrees of freedom reflect occasional missing data.

RESULTS

Antibodies

Figure I displays the Log transformed mean IgG Hepatitis B antibody levels at baseline and at 28 days post-vaccination for the exercise groups combined and the control group. A 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (baseline, 28 days post-vaccination) ANOVA revealed a main effect of Time ($F(1,67)=4.78$, $p=.03$, $\eta^2=.07$), indicating that antibodies against Hepatitis B increased from baseline to 28 days. However, no Group × Time effect ($F(1,67)=0.20$, $p=.66$, $\eta^2<.01$) was observed. Similarly, no Sex × Time ($F(1,67)=0.86$, $p=.77$, $\eta^2<.01$), and no Group × Sex × Time ($F(1,67)=0.31$, $p=.86$, $\eta^2<.01$) effects emerged. Similarly, separate analyses comparing each exercise group to the control group revealed no significant Sex × Time, Group × Time, or Group × Sex × Time interactions. Finally, there were no significant differences in the responses between the two exercise groups (*p*s all >.2). Further analysis revealed that 6 participants, split equally (3 vs 3) between exercise and control groups, showed the WHO recommended response (>100 IU / L) to acquire immunity to the vaccine.

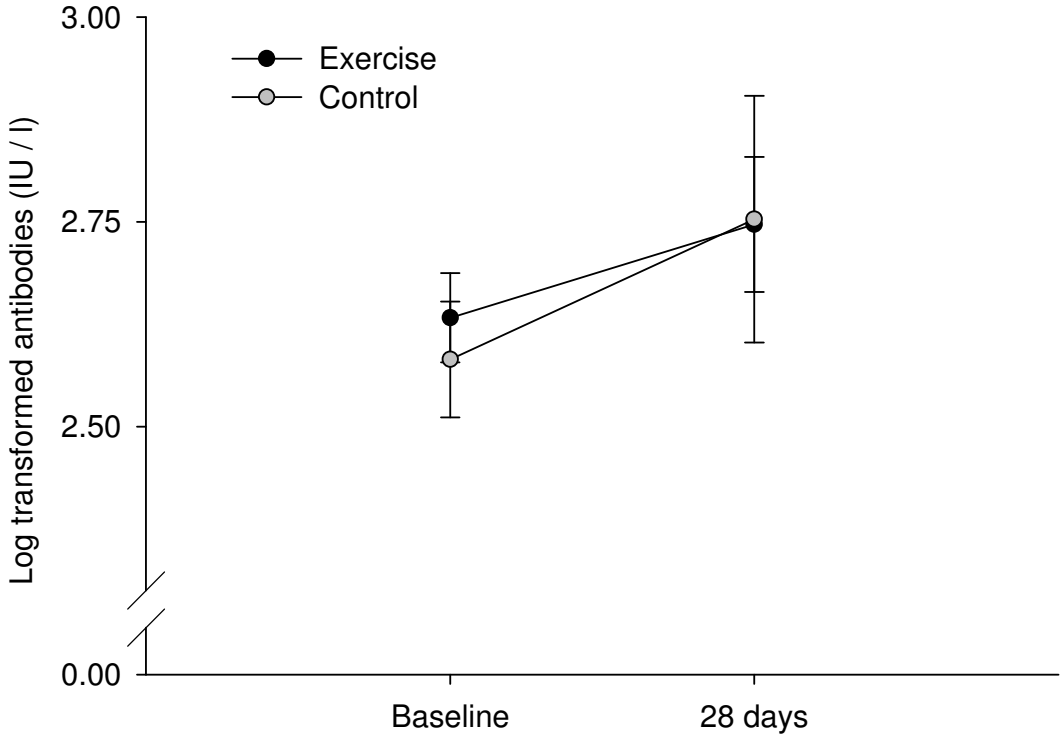

Figure I. Mean (± SEM) hepatitis B antibody levels pre-vaccination and at 28 days post-vaccination for participants in the control group and exercise groups (combined).

Limb circumference and arm pain

A 2 Group (exercise groups combined, control) × 2 Sex (male, female) ANOVA comparing percentage change limb scores from baseline to post-task showed, as anticipated, an overall effect of Group for forearm ($F(1,71)=19.90$, $p<.01$, $\eta^2=.22$) and upper-arm ($F(1,71)=17.13$, $p<.01$, $\eta^2=.19$) circumferences; (Figure II, top). ANOVAs showed that both exercise groups manifested higher bicep and forearm circumference percentage changes in comparison to control (all *p*s <.05). No differences were observed between the two exercise groups, and no Sex effects were observed (all *ps*>.05).

Similarly, a 2 Group (combined exercise groups, control) × 2 Sex (male, female) ANOVA comparing self-reported arm pain scores immediately post-task revealed significant Group effects ($F(1,72)=41.16$, $p<.01$, $\eta^2=.36$) (Figure II, bottom). ANOVAs showed that pain was elevated in both exercise groups compared with control (all *p*s <.05); no differences were observed between exercise groups and no Sex effects were observed (*p*>.05).

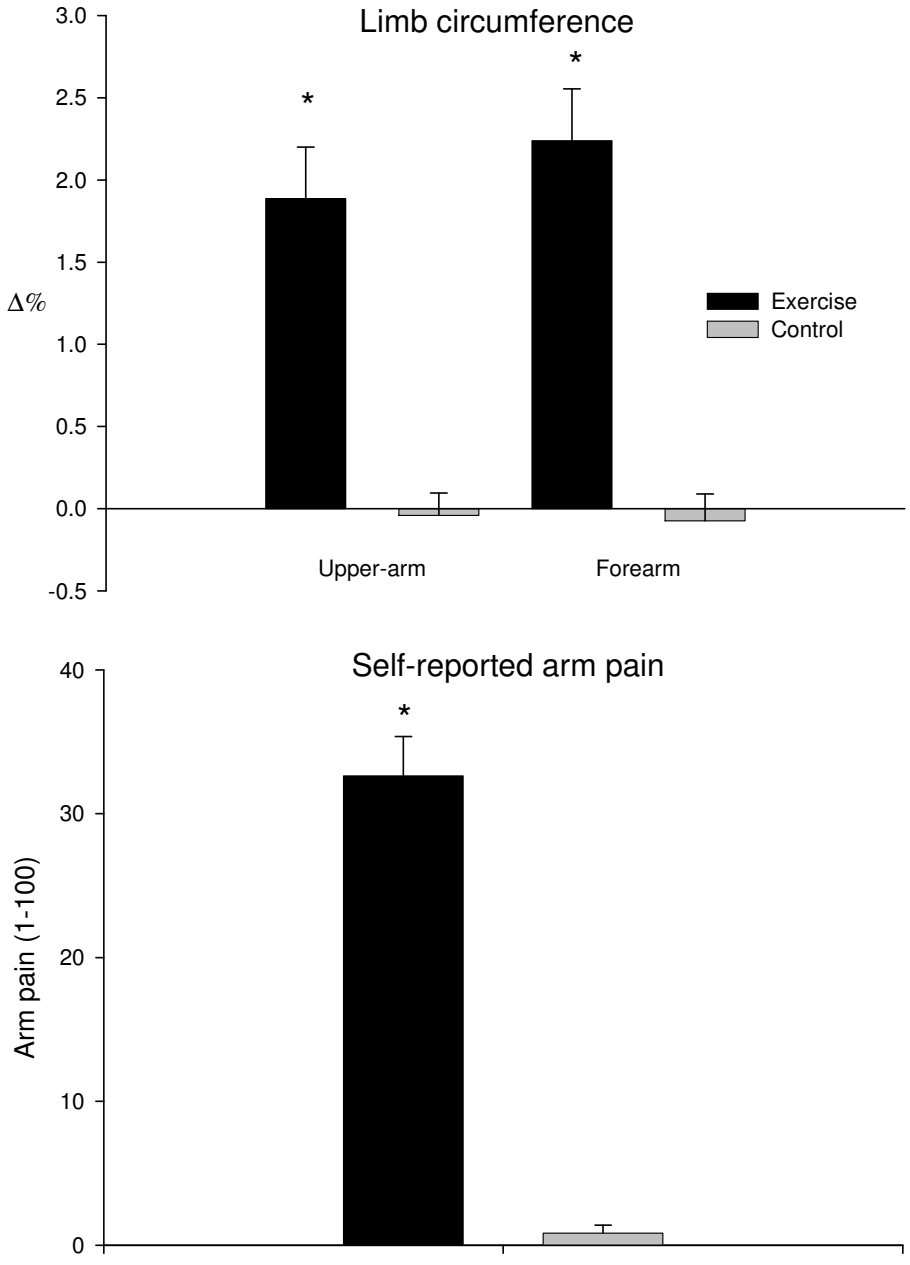

Figure II. Mean (± SEM) percentage change in upper-arm and forearm limb circumference from baseline to immediately post-task (top), and self-reported arm pain immediately post-exercise for both the exercise and control groups (bottom). * Indicates significant difference in comparison to control ($P < .05$).

Creatine kinase

A 2 Group (combined exercise groups, control) × 2 Sex (male, female) × 2 Time (baseline, 24 hr post-task) ANOVA revealed significant Group × Time effects ($F(1,60)=3.90$, $p=.05$, $\eta^2=.06$). These results indicate that exercise caused CK to increase from baseline to 24 hr post-task (Figure III). A series of ANOVAs revealed that the exercise arm group (hepatitis B vaccine into the exercised arm) had significantly higher CK than control ($F(1,36)=3.94$, $p=.05$, $\eta^2=.10$), whereas the other group (non-exercised arm group) showed a close to significant elevation in CK compared with control ($F(1,35)=3.56$, $p=.07$, $\eta^2=.09$); no differences were observed between exercise groups ($p>.05$).

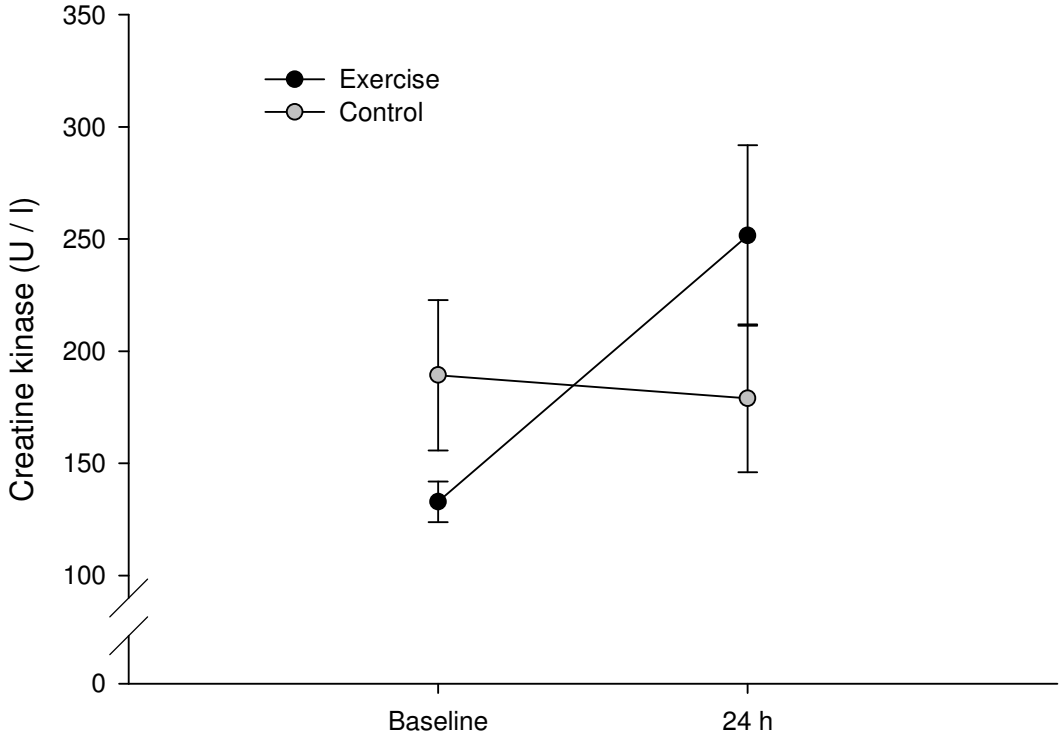

Figure III. Mean (± SEM) plasma CK (U / l) activity s in the control and exercise groups (combined) at baseline and at 24 hr post-exercise.

DISCUSSION

The primary aim of this study was to investigate whether an acute bout of eccentric arm exercise could enhance the immune response to hepatitis B vaccination in healthy young adults. As previous studies have demonstrated that eccentric exercise improved the immune response to poorly immunogenic vaccines (Edwards et al. 2007a; Chapter Two; Chapter Three), it was hypothesised that the antibody response to a single hepatitis B vaccination would be augmented by exercise. However, although the hepatitis B vaccine induced a modest increase in anti-HBV antibodies, the exercise group antibody response to vaccination was not different to control. As a consequence, it was not possible to investigate the secondary aim of this study, namely, to determine whether any adjuvant effects of exercise are mediated by vaccinating into localised damaged muscle or by systemic changes produced from the exercise bout.

Manipulation checks confirmed that eccentric exercise induced considerable physiological alterations. Participants in the exercise groups exhibited increases in their upper and forearm limb circumference, as well as greater arm pain, in comparison to control participants. Further, CK, an indirect marker of muscle damage, increased in exercised participants compared with controls. These later findings are consistent with previous studies by our group (Edwards et al. 2007a; Chapter Two, Chapter Three), and confirm that exercise induced the desired effect in terms of muscle damage and inflammation.

Given this, it is important to consider alternative reasons why eccentric exercise had no effect on antibody responses. Firstly, it should be noted that there was very low variability in the antibody response to the vaccine. The overall mean increase in anti-HBV antibodies at 28 days post-vaccination was driven mainly by a small number of participants (spread equally among experimental groups), who responded reasonably well to the vaccine; the majority showed no antibody response. This is broadly in line with the results of another study showing a low number of sero-converters after a single vaccination (Glaser et al. 1992). In our data, the dichotomous distribution somewhat disguised the otherwise low variability in the antibody responses; this lack of variability may explain, to some extent, the null findings.

This aside, other mechanisms should be investigated. For example, in this study, hepatitis B was chosen to investigate whether exercise enhances poorer antibody responses, whereas most previous studies have used seasonal influenza vaccines (Edwards et al. 2007a; Chapter Two; Chapter Three). These vaccines differ in terms of the probability of previous exposure to the antigen. Hepatitis B is likely to be a novel antigen to young, healthy adults with no previous history of vaccination or infection. In contrast, most individuals have some level of baseline immunity against H1N1 and H3N1 influenza viruses, due to cross-reactivity after previous exposure against similar seasonal strains of the virus. Given that eccentric exercise enhanced responses to influenza, but not hepatitis B, vaccines, it is possible that eccentric exercise more effectively enhances secondary immune responses. Indeed, it has been shown in animals that a single bout of intense exercise prior to human serum albumin (HSA) vaccination did not affect the primary antibody response, but did enhance the subsequent secondary response to a booster HSA vaccine in mice, compared with non-exercise controls (Kapasi et al. 2000). The possibility

that behavioural adjuvants are beneficial to secondary, but not primary, antibody responses warrants further attention in future research. This could be addressed by measuring the effects of exercise on the immune response to a full course of hepatitis vaccines.

Another possible explanation for this unexpected finding is that individual responsiveness to hepatitis B vaccination is limited by factors that are not impacted by our intervention. There is considerable evidence that the response to hepatitis B vaccination is particularly influenced by inherited genetic factors, notably by the ability of T cells to recognise antigen via human leukocyte antigen (Egea et al. 1991). As a consequence of large variation in HLA type, the variability in the number of people responding to the vaccine is high. It is plausible, although speculative, that where genetic factors are paramount in determining responsiveness, a behavioural adjuvant may be less effective.

In summary, eccentric exercise did not improve the immune responses to a single dose hepatitis B vaccine. These findings contrast with previous findings using the influenza vaccination, and reveal some limits of the eccentric exercise enhancement effect. The results raise the possibility that the adjuvant effects of eccentric exercise may be limited to secondary antibody responses. Finally, future studies should readdress whether immune augmentation is mediated via local or systemic mechanisms, using a vaccine, such as influenza, in which the exercise intervention has demonstrated more consistent benefits for antibody responses.

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CHAPTER FIVE

GENERAL DISCUSSION

SUMMARY

The aim of this thesis was to investigate the effects of eccentric exercise on the immune response to vaccination. Following prior research in humans proposing eccentric exercise as a potential adjuvant to vaccination (Edwards et al. 2007), this thesis further investigated the efficacy of this intervention through the completion of three large randomised control studies. The first study (Chapter Two) investigated the effects of eccentric exercise on the antibody and cell-mediated responses to influenza vaccination in healthy young adults, and whether the timing of the exercise affected the efficacy of the intervention. As anticipated, all 155 participants exhibited robust antibody responses to each of the trivalent strains contained within the vaccine. Exercised participants exhibited increases in limb circumference, self-reported muscle pain and CK, in comparison to a resting control group. However, results indicated that exercise had no effect on the antibody or cell-mediated responses to the vaccine. The exercise timing manipulation revealed that, despite the distinct physiological differences observed between the exercise groups, exercising immediately, 6 hr or 48 hr prior to vaccination had no effect on the antibody or cell mediated responses to the vaccine.

The second study (Chapter Three) investigated the effects of eccentric exercise on the immune response to a half-dose influenza vaccine, and whether the efficacy of this intervention was

modulated by the intensity of the exercise bout. Compared with the control group, exercise enhanced the antibody response to the least immunogenic of the three strains (B/Florida). In addition, the exercise groups showed an augmented response to the A/Uruguay strain compared with control; however, this effect was observed in men only, who showed poorer responses than women. The intervention had no effect on the antibody responses to the most immunogenic strain, A/Brisbane. Exercising at 60 %, 85 % and 110 % of IRM induced distinct physiological changes in each of the exercise groups, with the most intensive exercise group exhibiting higher levels of CK, self-reported arm pain, and percentage change in limb circumference. However, despite these distinctive group differences, no association between exercise intensity and antibody responses was observed.

The final study (Chapter Four) investigated the effects of eccentric exercise on the immune response to a single hepatitis B vaccination, and whether the efficacy of this intervention was influenced by the site of vaccine administration. As anticipated, anti-hepatitis B antibodies showed a small increase from baseline to 28 days, but contrary to our hypothesis, it was found that exercise had no effect on these responses.

CLINICAL APPLICATION OF ECCENTRIC EXERCISE

A key aim of the studies compiled in this thesis was to identify the optimal strategy for the implementation of eccentric exercise as an adjuvant to vaccination in a clinical setting. Prior to my research, only one study had investigated the role of eccentric exercise in improving vaccine responses (Edwards et al. 2007). In that study, a beneficial effect of exercise, at an intensity of

85% of 1RM, was found when the vaccine was administered at 6 hr post-exercise. This timing and intensity was selected on the basis of sound theoretical hypotheses, but it was important to also now test them empirically by comparing different protocols.

Issue of eccentric exercise timing prior to vaccination

In terms of intervention timing, the model used by Edwards and colleagues did not provide the most practically applicable intervention due the fact participants needed to visit the laboratory on two occasions. Chapter Two was designed to investigate this issue systematically by comparing the efficacy of the intervention when completed at differing times pre-vaccination. However, as there was no overall effect of the intervention in this study, it was not possible to gain any further understanding of the influence of intervention timing through this study. However, Chapter Three illustrated that it is possible to augment the immune response to eccentric exercise when the vaccine is administered immediately after the exercise bout. This is the first time that this has been shown, and this finding has clear and important clinical significance. Patients would only require a single visit the clinic to complete the exercise task and receive the vaccine. This allows much easier practical application of the intervention and, therefore, a more efficient strategy for patients and practitioners by saving patient effort, increasing adherence, and reducing the burden of the intervention on clinic time and resources.

Issue of eccentric exercise intensity

Exercise intensity is also an important issue in developing this intervention. Despite the overall success of using 85% of 1RM in the previous study, a lesser intensity of exercise would, if effective, enable a more acceptable and safer intervention approach. Chapter Three demonstrated that completion of an eccentric exercise task, regardless of the intensity of the bout, resulted in augmentation of the least immunogenic strains contained within the vaccine. These findings illustrate that the lowest intensity used in that study (60 % of 1RM) appears to be just as efficacious in enhancing responses as higher intensity bouts of eccentric exercise (85% and 110 % of 1RM). Clearly, this has important practical implications and means that participants may not have to participate in overly-demanding exercise, resulting in increased safety and greater ease of use, particularly for special populations such as the elderly and the disabled.

Immuno-enhancement of poorly immunogenic vaccine strains by eccentric exercise

Previous research has suggested that eccentric exercise is particularly effective at enhancing the immune response to poorly immunogenic vaccine strains. For example, it has been shown that mice administered an immunosuppressive drug, cyclophosphamide, prior to vaccination showed larger antibody responses following restraint stress in comparison to control mice. This highly controlled experiment provides strong evidence that acute stress can augment immune responses when it would otherwise be expected to be poor (Karp et al. 2000). This has been supported by a number of recent studies in humans (Edwards et al. 2006; Edwards et al. 2007; Edwards et al. 2008). In the first of these studies, psychological stress and / or cycling exercise only enhanced

the antibody response to the A/Panama strain of influenza in women; this was the least immunogenic of the trivalent strains in the 2003/2004 northern hemisphere vaccine (Edwards et al. 2006). Further, the other two strains produced relatively robust immunogenic responses and no effect of behavioural stress was found. In the same study but reported elsewhere (Edwards et al. 2008), the antibody response to meningococcal A vaccination was enhanced by stress in men; once more this strain was poorly immunogenic and induced weaker responses in men in the control group. Such specificity is also observed with the eccentric exercise intervention. For example, eccentric exercise had no effect on the antibody response to the A/New Caledonia strain of influenza, which exhibited relatively robust responses in the control group. These findings are in agreement with Chapter Two in this thesis, which showed that robust antibody responses against all three strains in the 2007/2008 northern hemisphere vaccine were not enhanced by exercise. In the same study by Edwards and colleagues, it was also shown that the antibody responses to two of the trivalent strains, A/Wyoming and B/Jiangsu, induced relatively weak responses in participants in women the control group (Edwards et al. 2007); women in the exercise group showed enhanced antibody responses to these strains at 6 and 20 weeks post-vaccination. These findings are supported in Chapter Three, which showed that the poorer immune responses to a half-dose influenza vaccine were enhanced by eccentric exercise in men and women. The final study in this thesis investigated whether the immune response to a less immunogenic vaccine, hepatitis B, was enhanced by completing a bout of eccentric exercise prior to vaccination. As anticipated, the administration of a single hepatitis B vaccine induced very weak antibody responses but, contrary to our hypothesis, these poor responses were not enhanced by eccentric exercise. It is possible that the low number of sero-responders to the vaccine limited the possibility of any effect being observed. It remains possible that the immune response to

hepatitis B vaccination is dictated by factors, such as genetics (Egea et al. 1991), that are outside the influence of this intervention.

Taken together, the findings from this thesis suggest that, when an individual exhibits a poor vaccine response, behavioural stress may be an effective intervention. Future research should investigate whether this protocol is effective in immunologically compromised individuals, such as the elderly (Goodwin et al. 2006), the chronically stressed (Burns et al. 2003), and HIV patients (Kroon et al. 1994).

Issue of site of vaccination

Chapter Four investigated whether the site of vaccine administration influenced the immune response to hepatitis B vaccination, following eccentric exercise. Unfortunately, the results indicated that there were no measurable effects of exercise on the antibody response to the vaccine, meaning it was not possible therefore to investigate the differences between vaccination sites. However, Chapter Three shows that vaccination directly into exercised muscle tissue can enhance the immune response to vaccination; from a clinical, pragmatic perspective, it may not be critical to understand whether this adjuvant effect is derived from changes within the local intra-muscular tissues or from the systemic circulation. As such, it is suggested that future clinical application of this intervention should continue to vaccinate into the exercised muscle tissue, as this provides the advantage of a heightened inflammatory environment within the intra-muscular tissues, as well as the pro-inflammatory benefits obtained from the systemic vasculature (Edwards et al. 2007). Future research should investigate whether the efficacy of exercise as an

adjuvant to vaccination is altered by completing concentric or eccentric muscle type contractions. From a more mechanistic viewpoint, it will be important for future research to readdress this issue with a vaccine, such as influenza, that has been consistently demonstrated to be modulated by behavioural intervention.

ELUCIDATING POTENTIAL MECHANISMS

In addition to designing the optimal eccentric exercise intervention, each of the studies in this thesis provided an opportunity to elucidate some of the possible mechanisms underpinning the adjuvant effects of exercise. Chapter Two investigated whether the timing of exercise (immediately, 6 hr or 48 hr) prior to vaccination influenced the efficacy of the immune response to the vaccine. Given that many of the physiological (e.g. CK) and inflammatory processes (e.g. IL-6) occur at varying timescales following eccentric exercise (Peake et al. 2005), it may have been possible to identify a key process mediating the augmentation of immune responses. Unfortunately, no overall effects of eccentric exercise were found and thus, no conclusions could be drawn on potential mechanisms in this study. However, given that a previous study found beneficial effects of eccentric exercise on the immune response to influenza vaccination, administered at 6 hr post-exercise, and the study contained in Chapter Three found that eccentric exercise enhanced the antibody response to influenza vaccination administered immediately post-exercise, it would seem that the timing of vaccination is not key in mediating these responses. This may be because antibody responses to vaccination do not occur immediately, but instead develop over the hours and days following vaccination. As such, there may be a considerable window of opportunity for exercise to have a beneficial effect on antibody response to

vaccination. More direct mechanistic studies, measuring the extent of the responses of the different potential mediators, will therefore be required to address this issue further.

Chapter Three also investigated whether the intensity of exercise (at 60%, 85% or 110% IRM) influenced the efficacy of the antibody response to a half-dose influenza vaccine. As there is greater muscle damage with more intense exercise, an effect of intensity on the intervention efficacy may indicate that the degree of muscle damage, and therefore inflammation, is key. Although distinct physiological differences were observed between the groups, in factors such as plasma CK, there was no difference between the groups in terms of antibody response. Further, no association was found between the individual extent of the observed physiological damage and the antibody response to the vaccine within the combined exercise groups. It is possible that a threshold effect exists, whereby some degree of exercise causes an enhancement of immune responses, but more intense exercise does not further augment these responses. Alternatively, it is possible that the degree of inflammation is not the main mechanism underlying this effect. For example, there is evidence to suggest that muscular contractions are associated with a temporary increase in lymph drainage around the site of exercised muscle tissue (Havas et al. 1997). In this study, radioactively labelled HSA, injected into the muscle, was cleared more quickly following exercise, compared with rest. It is possible, therefore, that exercise could subsequently lead to enhanced immune cell transport from the site of antigen administration via this altered lymph fluid dynamics. In this case, it would be hypothesised that performing *some* degree of eccentric exercise is sufficient to enhance antigenic transport to the lymph nodes where recognition by lymphocytes takes place. It would be interesting to measure this more directly in future studies.

The role of IL-6

Chapters Two and Three in this thesis investigated whether the concentration of plasma IL-6 following exercise was associated with the immune response to vaccination. IL-6 was chosen as it has been shown in a previous study that completion of concentric exercise or acute psychological stress leads to both an increase in systemic IL-6 and an enhanced antibody response to vaccination. Indeed, the extent of the IL-6 response was also associated with the antibody response at six weeks post-vaccination (Edwards et al. 2006). Elevated concentrations of pro-inflammatory cytokines, such as IL-6, have been implicated in enhancing vaccine responses (Krakauer, 1995; Lee et al. 1999). For example, higher mean levels of circulating IL-6 were found in the sera of vaccinees with good antibody responses than in the sera from non-responders, from 7 days prior to vaccination through to 63 days post-vaccination. In addition, pre-immunization levels of IL-6 in serum were also higher in responders than in non-responders (Krakauer, 1995). In another study, the co-administration of IL-6 gene with vaccine completely protected mice from a lethal challenge with influenza virus; co-administration of other cytokine genes was less successful (Lee et al. 1999). Given these findings, it has been hypothesised that increased levels of IL-6 in the systemic circulation, stemming from eccentric exercise-induced muscle damage, may play a role in augmenting the immune response to vaccination (Edwards et al. 2007).

No associations were found between the IL-6 response to exercise at 24 hr post-exercise and the antibody response to vaccination in either of the studies in this thesis. It is possible that this discrepancy is due to the mode of exercise used. The previous study used a concentric cycling

exercise, rather than an eccentric exercise intervention which is known to induce more localised muscle damage. In whole body exercise, the circulating levels of IL-6 are likely to be crucial to any adjuvant effect. As such, this may be why a positive association was observed in the previous study (Edwards et al. 2006). In contrast, in the more localised exercise used in the current study, we hypothesise that it is the tissue, rather than circulating, levels of IL-6 that are key. There is evidence that plasma levels of IL-6 do not always accurately represent actual intramuscular damage and inflammatory status following eccentric exercise (Tomiya et al. 2004). Tomiya and colleagues showed that the exercise induced increase in plasma IL-6 was not directly correlated with myofibre expression of IL-6, which peaked much later. For this reason, it remains possible that IL-6 levels in the muscular tissue, rather than plasma, are more closely related to extent of the antibody response to the vaccination. It may be possible to examine this in a future study by conducting muscle biopsies on participants after a bout of eccentric exercise, and establish whether the subsequent vaccine response is associated with myofibre expression of IL-6, extracellular IL-6 levels in the muscle, or intracellular / homogenate expression of IL-6 in cellular infiltrate.

A second difference between the two studies is the timing of IL-6 assessment. In this study IL-6 was measured 24 hr post-exercise, whereas in contrast, in the previous study, it was measured 6 hr after exercise, immediately *before* vaccine administration (Edwards et al. 2007). It is possible, therefore, that in Chapter Three the IL-6 response to exercise was confounded by an additional IL-6 response to vaccination (Tsai et al. 2005); the observed increase in IL-6 in the control group suggests that this is a plausible explanation. In sum, the findings from this thesis do not support, but cannot exclude, the contention that IL-6 is a key mediator in the acute-stress enhancement of

vaccine responses. Further research should establish whether intra-muscular levels of IL-6 are associated with improved vaccine responses.

Enhancement of secondary responses to vaccination

As discussed, Chapter Four found no effects of exercise on the antibody response to a single hepatitis B vaccine. An alternative hypothesis for these null-findings relates to the choice of vaccine. Other studies that have previously found adjuvant effects of acute concentric and eccentric exercise on vaccine responses have used either seasonal influenza (Edwards et al. 2006; Edwards et al. 2007) or meningococcal A+C vaccines (Edwards et al. 2008). These vaccines differ from hepatitis B in terms of the probability of previous exposure to the antigen. For example, influenza vaccine strains are specifically selected due to their high prevalence in the community in a particular season. For this reason, naturalistic exposure, prior to vaccination, is relatively likely. Further, seasonal influenza strains are fairly cross-reactive due to very limited mutations in the virus structure from season to season, a phenomenon known as antigenic drift (Nicholson et al. 2003). It is likely, therefore, that many individuals will have been infected by, and have antibodies against, the same or similar viruses contained within the influenza vaccines. Similarly, individuals are likely to have some degree of immunity against both meningococcal A+C as a result of meningococcal C vaccine administered in childhood and the relatively high naturalistic exposure to these bacteria. In contrast, hepatitis B is a blood-borne virus, usually transmitted via unsterile needles, unprotected sex, and direct blood-to-blood contact. There is likely to be extremely low levels of exposure in the unvaccinated, young, healthy participants used in these studies; this was confirmed by the very low baseline antibody levels observed. As

such, influenza and meningitis A+C are likely to induce secondary immune responses, whereas hepatitis B stimulates a primary immune response. It is possible, therefore, that the effects of exercise are specific to secondary immune responses.

This issue has been addressed directly in animals; Kapasi and colleagues compared the effects of acute exercise on both the primary and secondary immune responses to a novel antigen. A single bout of intense exercise prior to HSA vaccination did not affect the primary antibody response, but did enhance the subsequent secondary response to a booster HSA vaccine in mice, compared with non-exercise controls (Kapasi et al. 2000). These findings are in keeping with our hypothesis that secondary responses are more affected by acute behavioural intervention. The mechanisms underpinning this effect are, as yet, undetermined, but Kapasi hypothesised that elevated noradrenaline during exercise may enhance specific antibody synthesis in response to an antigen. Noradrenaline has been shown to promote antibody synthesis by increasing the number of antigen-specific B cells that differentiate into antibody-secreting plasma cells (Sanders and Powell-Oliver, 1992); such an effect may be particularly beneficial in a secondary immune response where this differentiation of existing antigen-specific B cells is a key process.

Alternatively, it is possible to speculate that lymphocyte mobilization, observed with exercise (Bruunsgaard et al. 1997), might be particularly beneficial for secondary immune responses. Recent research has demonstrated that exercise causes a preferential mobilisation of effector lymphocytes into the bloodstream (Campbell et al. 2008; Campbell et al. 2009), and following eccentric exercise, are re-distributed to sites of muscle damage in the tissues (Miller, 2004). These effector lymphocytes exhibit pro-inflammatory capabilities, and upon antigen exposure can

release high levels of cytokines such as IFN-γ and TNF-α (Lanzavecchia and Sallusto, 2005). Crucially, using these cytokines, these cells are able to select the most suitable cells of the innate system, such as dendritic cells, and this priming leads to stronger adaptive immune responses that follow (Moretta, 2002; Moretta et al. 2005). It is possible, therefore, that a mobilisation of effector lymphocytes during - and a subsequent re-distribution after exercise results in a more rapid, and more intensive, secondary response to antigenic challenge.

FUTURE STUDIES

It is clear from the issues raised in this thesis that further investigation into the exercise-induced augmentation of vaccine responses is warranted. For example, it appears that exercise has an adjuvant effect on vaccines containing poorly immunogenic strains. Given this, it would be interesting to test this hypothesis directly by measuring the efficacy of an exercise intervention on the antibody responses to different doses of a vaccine. If the intervention could increase the response to a reduced dose vaccine to the levels observed with a standard dose, then this would be beneficial in circumstances where vaccine availability is reduced. Alternatively, the adjuvant effect of exercise on poor immune response could be investigated by measuring the effects of exercise on the immune response to vaccination in immunologically compromised individuals. There are a host of different populations that may benefit from this approach. For example, chronically stressed individuals have poorer responses to vaccination (Burns et al. 2003) and, as such, may benefit from immuno-enhancement at the time of vaccination. Similarly, HIV patients are known to respond poorly to vaccination (Kroon et al. 1994). Finally, there is now a wide body of research implicating immunological senescence (ageing) as an important factor in the

efficacy of vaccination (Effros, 2007). Immunological senescence is characterised by an increase in cells with reduced telomere lengths that, upon antigen exposure, prevents the differentiation of antigen specific T and B cells, leading to a restriction of memory cells and an increased risk of infection (Effros, 2007). An increase in senescent cells is thought to be responsible for the poor responses seen in the older adults to vaccination (Goodwin et al. 2006). Indeed, the effectiveness of influenza vaccination in this population may be as low as 53 % (Villari et al. 2003). For these reasons, it would be of pertinent interest to investigate whether these poorer responses can be enhanced by acute exercise, as has been shown in elderly mice (Kapasi et al. 2000). It should be noted, however, that there is evidence that older adults also have reduced inflammatory responses to exercise (Przybyla et al. 2006), and this may impact upon the efficacy of this intervention. It is conceivable that similar deficiencies may be observed in the responses of HIV patients, although to our knowledge this has never been directly tested.

It would also be interesting to explore further the hypothesis that exercise has an adjuvant effect on the secondary, but not primary, immune responses to vaccination. This has been investigated using an animal model (Kapasi et al. 2000), which found that exercise enhanced the secondary but not primary response to vaccination. Clearly, it should be investigated more directly whether these findings are transferable to humans. This study could be conducted by measuring the effects of exercise on the antibody response to a series of hepatitis B vaccinations. The vaccine is poorly immunogenic and typically requires a series of booster vaccine shots. The relative effects of exercise on primary and secondary immune responses could be investigated by measuring the antibody response to the first, and then subsequent, vaccinations. Similarly, different groups could be exposed to exercise at each of the different vaccine administrations, to see if this

influenced the efficacy of the intervention. Alternatively, a novel antigen known to induce a more robust primary antibody response, such as KLH, could be used, though the clinical relevance of this approach could be questioned due to the non-pathogenic nature of this protein. Finally, it would be desirable to measure both IgM and IgG responses, as this would enable a fuller investigation into primary responses than those measured in this thesis.

Finally, future mechanistic studies should investigate whether the critical mediator of this response is a result of systemic changes in the inflammatory milieu, or instead, due to smaller changes within the damaged muscle tissue. This issue has particular relevance given the recent findings of Prymula and colleagues. This study investigated the effect of prophylactic paracetamol on fever and antibody responses to vaccination; they found that there was a substantial reduction in the primary antibody responses to each of ten pneumococcal conjugate vaccine serotypes and to Hib polysaccharide, diphtheria, tetanus, and pertactin antigens, in participants taking prophylactic paracetamol. Importantly, this reduction was not associated with a reduction in systemic symptoms, such as fever. They instead speculated prophylactic paracetamol interfered with the early interactions between APCs (e.g., dendritic cells and macrophages) and B cells and T cells, possibly through a reduction of inflammatory signals (e.g., prostaglandins) at the site of injection (Prymula et al. 2009). It may be possible that acute exercise induces the opposite effect by upregulating the early interactions of between innate and adaptive immunity. Future research could incorporate muscle biopsies to measure intra-muscular levels of cytokines and cellular infiltrate. This methodological strategy, although invasive, is likely to offer the best approach for identifying potential mechanisms underpinning

the adjuvant of exercise, as other indirect measures of muscle damage and inflammation may not provide a reliable indication of the actual intra-muscular environment.

CONCLUSION

The findings from this thesis support the acute-stress immuno-enhancement hypothesis, whereby acute eccentric exercise can, under certain circumstances, enhance the immune response to vaccination. Results indicate that timing of vaccination after eccentric exercise does not affect the efficacy of the vaccine response. In addition, it seems that altering the intensity of exercise does not affect the efficacy of the intervention, whereby exercise at 60 % IRM is just as efficacious as 110 % 1RM: it is likely that a threshold effect of exercise exists. In addition to previous exercise and vaccination studies in humans, my findings support the hypothesis that poorly immunogenic strains are enhanced by acute exercise, whereas robust responses are not. Finally, it is speculated from this thesis that exercise enhances the responses to secondary, as opposed to primary, immune responses to vaccination. Taken together, these results support further investigation of this intervention in more clinical settings, in order to realise its potential benefits for immuno-compromised populations.

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