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Innate immune responses to a single session of Sprint Interval Training.

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Abstract

Sprint Interval Training (SIT) is a potent stimulus for physiological and metabolic adaptations comparable with those induced by traditional 'aerobic' endurance training. There has been a great deal of recent research on SIT, which may lead to increased use of this type of training. The purpose of the present study was to determine the acute effects of SIT on aspects of innate immunity not previously researched in this context. Nine males completed one SIT and one resting control trial in a crossover design. Blood and saliva samples were obtained at pre-, post- and 30 minutes post-exercise to measure blood neutrophil oxidative burst activity (OBA) in addition to saliva secretory IgA (s-IgA) and lysozyme. SIT induced a significant depression of neutrophil fMLP-stimulated OBA (−30% 30 minutes post-exercise, $P < 0.01$), PMA-stimulated OBA (−14% post-exercise, −21% 30 minutes post-exercise, $P < 0.01$) and bacterial-stimulated degranulation (−23% post-exercise, −32% 30 minutes post-exercise, $P < 0.01$) but not fMLP-then-PMA-stimulated OBA, saliva lysozyme or s-IgA concentrations or secretion rates ($P > 0.05$). The main novel finding of the present study is that a single session of SIT causes significant exercise-induced immunodepression of some neutrophil functions but mucosal immunity was not depressed.

Key words: neutrophil; high intensity interval exercise; saliva; mucosal immunity; antioxidant; oxidative stress.

Introduction

Sprint Interval Training (SIT, also described as High Intensity Interval Training or HIT) has recently attracted a great deal of research interest. In particular, there is a growing body of research on the effects of Wingate-based SIT, which typically involves 3-6 repeated ‘all-out’ 30-second Wingate tests with short recovery intervals of between 3 and 5 minutes. There is now substantial evidence for the physiological adaptation and performance enhancing effects that can be induced by this type of exercise. It has been demonstrated that relatively short-term periods (2 weeks) of Wingate-based SIT are a potent stimulus for physiological, metabolic and molecular adaptations associated with enhanced functional capacity, exercise performance (Burgomaster et al. 2005, 2007, 2008; Gibala et al. 2006; Gibala and McGee, 2008), metabolic regulation and other health-related indices (Babraj et al. 2009; Richards et al. 2010; Whyte, Gill, and Cathcart, 2010). In many cases the magnitude of these SIT-induced physiological adaptations are comparable with or greater than those associated with traditional ‘aerobic’ endurance training (referred to hereafter as endurance training) (Gibala & Little, 2010; Gibala & McGee, 2008). Therefore, SIT has been proposed as a time-efficient exercise protocol for the enhancement of endurance performance, but also with possible implications for health and the prevention or management of chronic or metabolic diseases (such as insulin resistance and Type 2 Diabetes).

Many physiological responses to SIT have been well researched and described in the literature. However, the immune responses to such exercise have not. The immune response to physical exertion is an important aspect of the physiological responses which can have implications for training. For example, it is well known that individuals engaged in intensive training regimes, particularly endurance athletes, report a higher infection incidence,

especially of the upper respiratory tract, compared with their less active counterparts (Gleeson, 2007; Neville et al. 2008; Spence et al. 2007). It is generally accepted that this is attributable, at least in part, to exercise-induced alterations in immune function, termed exercise-induced immunodepression, and the acute response to exercise bouts are of significance. This includes changes in the distribution and functional capacity of immunocompetent cells, and effector and regulatory molecules of the immune system (Gleeson, 2007; Nagatomi, 2006; Nieman, 1997, 2007; Pedersen and Hoffman-Goetz, 2000). The magnitude and direction of exercise-induced immune perturbations are related to training volume and intensity, with the largest acute immune perturbations typically seen after prolonged exercise (Gleeson, 2007; Gleeson et al. 2004; Nieman, 1994; Spence et al. 2007). As the training load (or impulse) is influenced by the intensity and duration of exercise, it is unclear how extremely high-intensity and low volume training, such as Wingate-based SIT, fits within this paradigm. A small number of previous studies (Fahlman et al. 2001; Hall et al. 2007; MacKinnon and Jenkins, 1993) have investigated the acute effects of such exercise bouts on mucosal immunity, with observations of compromised salivary IgA concentration and secretion, but there is a distinct lack of research on other aspects of the immune system. The author is not aware of any previous study that has assessed aspects of innate immunity following this type of repeated Wingate SIT. Innate immune function plays a key role in resistance to infection. For example, neutrophils are a key first line of defence against infection and populations with sub-normal neutrophil functions are known to have an increased infection incidence compared with healthy controls (Kowalska, 2003; Kutter et al. 2000; Matsuzaka et al. 2008; Nagatomi, 2006; Pütsep et al. 2002). This demonstrates the need to study other aspects of immunity to further clarify the effects of SIT on human immune function. Hence, the purpose of the present study was to explore the acute effects of Wingate-based SIT on selected immunological parameters including key aspects of innate

immunity. A number of mechanisms have been implicated in the acute immunodepressive effects that occur following endurance exercise, including the direct and indirect effects of stress hormones, the premature release of functionally sub-mature leukocytes from the bone marrow, substrate depletion, and oxidative stress (Davison & Gleeson, 2007; Gleeson, 2007). It is likely that SIT will be associated with high physiological and oxidative stress responses but the duration of body tissue exposure will be less when compared with prolonged endurance exercise. Taken together with the fact that carbohydrate and glycogen depletion is unlikely to occur during SIT, to the extent seen with prolonged exercise, it was hypothesised that there will be little or no negative exercise-induced immunodepression following a single session of SIT.

Materials and Methods

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and in accordance with the Human Tissue Act. All procedures were approved by Aberystwyth University Research Ethics Committee for research involving human participants. Written informed consent was obtained from all subjects. Subjects also completed a pre-exercise screening questionnaire (Physical Activity Readiness Questionnaire) before participating in each test.

Subjects

Nine healthy recreationally active men (age 27 ± 5 years, body mass 74 ± 11 kg, stature 1.8 ± 0.1 m; means \pm standard deviation) participated in this study. All of the volunteers had previously experienced the Wingate test but none had undertaken repeated Wingate tests or other SIT in the prior month. All subjects took part in a resting control trial, a familiarisation

trial and a SIT trial. The familiarisation trial was conducted first and then the main experimental trials (resting control and SIT) were conducted in a counterbalanced crossover design (as $n = 9$ there was not a perfect balance: 5 subjects undertook the control first and 4 undertook the SIT first). All trials were separated by at least 1 week.

Testing protocols

The familiarisation trial was designed to familiarise subjects to the testing procedures and performing more than one Wingate test. It consisted of 2×30 -second Wingate tests separated by 4 minutes of recovery. The SIT trial consisted of 4 repeated tests (4×30 -second Wingate tests) with 4 minute recovery intervals.

Main trials

Subjects were instructed to consume 500 ml of water 2 h pre-exercise before arrival at the laboratory on the morning of the main trials. All subjects were requested to arrive between 08:30 and 09:30 (each subject at precisely the same time for each trial), after an overnight fast of at least 10 h. On arrival at the laboratory, they were asked to swill and rinse their mouth with plain water before sitting on a phlebotomy chair for 10 minutes. After 10 minutes of rest, blood and saliva samples were obtained before the subjects were provided with a drink of plain water (300 ml). For the resting control trial subjects remained seated and further samples were collected at times equivalent to post-exercise and 30-minutes post-exercise in the exercise trials (see below). For the exercise (SIT) trials, subjects remained seated for a few minutes before mounting the cycle ergometer (Lode Excalibur Sport, Lode, Groningen, The Netherlands). They performed a warm-up consisting of 5 minutes at 50 W (with a 5 second 'sprint' at 3 minutes) and 5 minutes rest before beginning the repeated Wingate tests. A total of 4 Wingate tests were performed. Strong verbal encouragement was

provided during each Wingate test. Subjects remained on the ergometer during the recovery intervals and were encouraged to maintain a cadence of between 60 and 80 rpm (with the ergometer set to 0 W). After completing the last test, subjects were instructed to maintain a pedal cadence of 60-80 rpm for 2 minutes before returning to the chair for samples to be collected. In all cases the post-exercise blood samples were collected within 5 minutes of exercise and saliva samples within 8 minutes. Final blood and saliva samples were obtained at 30 minutes post-exercise so that all 3 samples (Pre-Ex, Post-Ex and 30 min Post-Ex) were approximately 30 minutes apart.

Control procedures

Subjects completed a weighed food record diary for the 24 h period before the first trial and were required to follow the same diet before the subsequent trial. Subjects were all non-smokers, not currently (or within the prior 4 weeks) taking any medication or nutritional supplements (including vitamin, mineral or 'sports' supplements). They were also required to abstain from alcohol, caffeine and strenuous activity for 48 h prior to each trial.

Blood samples

Blood samples were obtained by venepuncture, with minimal stasis, from an antecubital vein and collected into vacutainer tubes (4.5 ml into a K₃EDTA treated tube and 6 ml into a heparin treated tube). An initial resting (Pre-Ex) sample was obtained before exercise (or at the equivalent time in the resting control trial). Further venous blood samples were taken immediately after completion of the SIT exercise bout (Post-Ex) and after 30 minutes of recovery (30 min Post-Ex), or at equivalent times in the resting control trial, during which subjects remained in the laboratory and were allowed to undertake restful activities such as reading or using a computer. All blood samples were obtained while subjects were in the

seated position. Subjects were asked to sit without changes of posture and minimal movement for 10 minutes before all blood samples were drawn, except the Post-Ex sample, which was drawn as soon as possible after completing the exercise (within 5 minutes).

Saliva samples

Saliva samples were collected as previously described (Davison & Diment, 2010), at Pre-Ex, Post-Ex, and 30 min Post-Ex. Briefly, timed, unstimulated whole saliva was obtained whilst subjects were seated, with the eyes open and head tilted slightly forward and making minimal orofacial movement. They were asked to swallow to empty the mouth, before whole saliva was collected by passive dribble into a pre-weighed sterile tube for an initial period of 2 minutes. If insufficient saliva was obtained after the initial 2-minute period the process was repeated. Subjects were not allowed to drink for at least 10 minutes prior to each sample. The tube was re-weighed after collection of the sample so that saliva volume (and hence flow rate) could be estimated. Tubes were weighed to the nearest 0.1 mg (saliva density was assumed to be 1.0 g.ml^{-1}) and aliquots were frozen, at -80°C , for later analysis.

Analytical methods

All analyses were conducted in duplicate unless otherwise stated.

Blood analysis. Haematological analysis was performed on the blood samples collected into the K_3EDTA tubes using an automated haematology analyser (ABX Pentra 60C+, Horiba Medical, Montpellier, France). Blood haemoglobin concentration, haematocrit, total and differential leukocyte counts were measured. Haematocrit was also determined (in triplicate) by standard micro-centrifugation (with a Hawksley micro-centrifuge) for use, along with haemoglobin concentration, to estimate post-exercise changes in plasma volume using the

equations of Dill and Costill (1974). Blood lactate and glucose concentrations were determined in heparinised blood using an automated analyser (YSI 2300 Stat Plus, Yellow Springs, OH, USA).

One ml aliquots of whole blood were separated for the measurement of in vitro-stimulated neutrophil oxidative burst and ex vivo reactive oxygen species (ROS) (K₃EDTA samples) and degranulation (Heparin samples) as detailed below. The remaining blood was centrifuged at 1500 × g for 10 minutes at 4°C and aliquots of plasma were stored at –80°C for later analysis of plasma cortisol, Thiobarbituric acid reactive substances (TBARS) (K₃EDTA tube), elastase and antioxidant capacity (heparin tube). All samples were thawed only once prior to analysis. Plasma cortisol concentration was determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (DRG, Germany; Biomerica, Ca, USA); TBARS was assessed with a commercially available colorimetric assay kit (QuantiChrom, BioAssay Systems, CA, USA) with Malondialdehyde (MDA) as the standard, giving results in MDA equivalent units (µM); and plasma antioxidant capacity was assessed with a chemiluminescence assay (ABEL-21M2, Knight Scientific, Plymouth, UK), with ascorbate as the standard, giving results in ascorbate equivalent units (µM). All samples were thawed only once prior to analysis.

In vitro stimulated neutrophil oxidative burst. The neutrophil oxidative burst activity (OBA) response to phorbol-12-myristate-13-acetate (PMA) and formyl-leucyl-methionyl-phenylalanine (fMLP) were determined as previously described (Davison and Gleeson, 2006) using a commercially available chemiluminescence kit (ABEL-04M, Knight Scientific, Plymouth, UK). Briefly, chemiluminescence was measured in a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) reading samples in microplate

wells. Each well contained 20 μl whole blood (K_3EDTA anticoagulated) diluted 1:100 with buffer (Hank's balanced salt solution; HBSS, lacking calcium and magnesium but with 20 μM HEPES, pH 7.4), 90 μl assay buffer (HBSS with 20 μM HEPES, pH 7.4), 50 μl Pholasin, and 20 μl adjuvant K (which enhances the luminescence of Pholasin during assays with diluted whole blood). The mixture was incubated at 37°C for 1 minute (with gentle shaking for 30 seconds) in the luminometer before 20 μl of PMA (5 $\mu\text{g}\cdot\text{ml}^{-1}$), fMLP (10 μM) or assay buffer (as the unstimulated control) was injected (giving a total volume of 200 μl per well and a final concentration of 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ PMA or 1 μM fMLP for stimulated samples). When PMA was used chemiluminescence was recorded in duplicate as relative light units (RLU) per second at 20-second intervals for 30 minutes. For fMLP, chemiluminescence was measured every 2 seconds for 5 minutes. Additionally, after a further 5 minutes, 20 μl of PMA was injected into the samples that had previously been stimulated with fMLP (fMLP-then-PMA-stimulated) to activate the non-receptor dependent OBA (e.g. Figure 1B). Another 20 μl of assay buffer was also injected into the control wells to maintain the same relative concentration in stimulated and unstimulated samples. Neutrophil OBA was expressed in 2 ways: 1) as the stimulated-area under curve, in which the integral of the stimulated chemiluminescence curve was calculated and the integral of the unstimulated control curve (chemiluminescence response without stimulation) was subtracted; 2) as the stimulated-peak chemiluminescence (peak stimulated minus unstimulated chemiluminescence at the equivalent time). It was assumed that the chemiluminescence response depends almost entirely on neutrophils (Morozov et al. 2003) therefore the chemiluminescence responses were divided by the number of neutrophils per well in order to express OBA per neutrophil.

*** please insert Figure 1 near here ***

Ex vivo reactive oxygen species. Whole blood (predominantly from leukocytes) may contribute to exercise-induced elevations in excess ROS and oxidative stress (Nikolaidis and Jamurtas, 2009; Powers and Jackson, 2008). Therefore, the unstimulated (control) 30-minute chemiluminescence responses from the neutrophil oxidative burst assay were also expressed per μl of whole blood to give a measure of spontaneous chemiluminescence (a snapshot of the level of ROS) present in the blood at that time.

In vitro stimulated neutrophil degranulation. The neutrophil degranulation response to bacterial stimulant (840-15, Sigma, Poole, UK) was determined as previously described (Davison and Diment, 2010). Neutrophil degranulation was expressed as the amount of stimulated elastase release per neutrophil (in 1 hour, whilst incubated at 37°C). Elastase concentration was determined using a commercially available ELISA kit (Merck Calbiochem, Darmstadt, Germany).

Salivary analysis. Saliva analysis was conducted as previously described (Davison and Diment, 2010). All samples were thawed only once prior to analysis. After thawing, at room temperature, samples were centrifuged at $15000 \times g$ for 2 minutes to pellet debris, precipitate mucins and obtain a clear supernatant. Saliva osmolality was determined using a freezing point depression osmometer (Osmomat 030, Gonotec, GbBH, Berlin, Germany) calibrated with $300 \text{ mOsmol.kg}^{-1}$ saline solution, in accordance with the manufacturer's instructions. Accuracy was confirmed with a $50 \text{ mOsmol.kg}^{-1}$ reference solution. Saliva samples were screened for blood contamination by the measurement of salivary transferrin concentration using a commercially available ELISA kit (Salimetrics, PA, USA). Blood contamination was assumed if salivary transferrin concentration exceeded 1.0 mg.dl^{-1} . In the event that blood contamination was detected, all salivary data for that subject were excluded from the

analysis. Aliquots of saliva were analysed for the determination of secretory IgA (s-IgA) (Salimetrics, PA, USA) and Lysozyme (Biomedical Technologies Inc., MA, USA) using commercially available ELISA kits.

Statistical Analysis.

Statistical analyses were carried out using the statistical software package SPSS (v17.00; SPSS Inc., IL, USA). All data except saliva lysozyme secretion rate were normally distributed. Saliva lysozyme secretion rate data were normalised with log transformation before statistical analysis. To compare the responses between trials (resting control and SIT) a 2-way repeated measures ANOVA (trial \times time) was used, with post hoc Student's paired t tests (Holm-Bonferroni corrected) where appropriate. When there was evidence of an interaction the effect of time was analysed in each group independently, with 1-way repeated measures ANOVA and post hoc Student's paired t tests (Holm-Bonferroni corrected) where appropriate. The Greenhouse-Geisser correction was applied, to correct for violations of the assumption of sphericity, where necessary (indicated in the text by _{GH} following the P value if the Greenhouse-Geisser correction was applied). All results are presented as mean \pm standard deviation.

Results

*** please insert Table 1 near here ***

The average peak power output during each repetition of the Wingate test decreased progressively throughout the SIT trial: rep 1, 830 ± 78 W; rep 2, 786 ± 69 W (Student's paired t test $P = 0.04$ vs. rep 1); rep 3, 720 ± 77 W ($P = 0.003$ vs. rep 1); rep 4, 683 ± 111 W ($P = 0.002$ vs. rep 1). The average Wingate mean power output during each repetition also followed the same general pattern: rep 1, 557 ± 26 W; rep 2, 533 ± 46 W ($P = 0.021$ vs. rep

1); rep 3, 464 ± 45 W ($P = 0.002$ vs. rep 1); rep 4, 447 ± 58 W ($P = 0.002$ vs. rep 1).

Estimations of plasma volume change showed a significant trial \times time interaction (Table 1).

There were significant decreases in plasma volume at both post-exercise time points but the magnitude of the post-exercise decrease was greater on the SIT compared with resting control trials: decreases of 0.7% vs. 19.8% at Post-Ex, and 2.2% vs. 10.0% at 30 min Post-Ex in the control and SIT trials, respectively. A significant trial \times time interaction indicated that the temporal response of blood lactate concentration was different between trials. There was a significant increase in blood lactate concentration Post-Ex, which remained at 30 min Post-Ex, in the SIT trial (Table 1). There was no trial \times time interaction for blood glucose concentration but there was a slight decrease by the 30 min Post-Ex time point (Table 1). Trial \times time interaction effects ($P < 0.001$) were evident for plasma cortisol concentration and blood neutrophil count. Post hoc analysis revealed post-exercise increases in plasma cortisol concentration in the SIT trial, contrasting to the decrease over time observed in the control trial (Table 2). Post hoc analysis for blood neutrophil count revealed post-exercise increases in the SIT trial whereas there was no change in the control trial (Table 2).

*** please insert Table 2 near here ***

The OBA results, whether expressed as the chemiluminescence curve integral or peak, gave equivalent results (in terms of magnitude and direction of changes during, and comparisons between, rest or in response to exercise) so only the area under curve data are presented. There were significant trial \times time interactions for fMLP-stimulated ($P = 0.044$), and PMA-stimulated ($P < 0.001$) OBA, but not for fMLP-then-PMA-stimulated OBA ($P = 0.140$). Post hoc analyses revealed no change in the control trial for fMLP-stimulated or PMA-stimulated OBA, whereas there were significant post-SIT decreases at 30 min Post-Ex ($P = 0.008$) for

fMLP-stimulated OBA and at Post-Ex ($P = 0.008$) and 30 min Post-Ex ($P = 0.002$) for PMA-stimulated OBA (Figure 2). There was a significant trial \times time interaction for stimulated neutrophil degranulation ($P = 0.011$). Post hoc analyses revealed no change in the control trial and significant decreases at Post-Ex ($P = 0.009$) and 30 min Post-Ex ($P = 0.006$) in the SIT trial (Figure 3).

*** please insert Figure 2 near here ***

Blood contamination was detected in at least one saliva sample (rest and/or post-exercise) of 3 subjects. Data for these subjects were excluded leaving $n = 6$ for all saliva parameters.

There was a significant trial \times time interaction for saliva flow rate (control: Pre-Ex $0.34 \pm 0.26 \text{ ml}\cdot\text{min}^{-1}$, Post-Ex $0.32 \pm 0.17 \text{ ml}\cdot\text{min}^{-1}$, 30 min Post-Ex $0.38 \pm 0.29 \text{ ml}\cdot\text{min}^{-1}$; SIT: Pre-Ex $0.48 \pm 0.18 \text{ ml}\cdot\text{min}^{-1}$, Post-Ex $0.26 \pm 0.08 \text{ ml}\cdot\text{min}^{-1}$, 30 min Post-Ex $0.38 \pm 0.18 \text{ ml}\cdot\text{min}^{-1}$, trial \times time interaction $P = 0.009$), and post hoc analysis revealed a significant Post-Ex decrease ($P = 0.018$) in the SIT trial (with no change in the control trial). There was a significant trial \times time interaction for saliva osmolality (control: Pre-Ex $53 \pm 9 \text{ mOsmol}\cdot\text{kg}^{-1}$, Post-Ex $56 \pm 7 \text{ mOsmol}\cdot\text{kg}^{-1}$, 30 min Post-Ex $58 \pm 5 \text{ mOsmol}\cdot\text{kg}^{-1}$; SIT: Pre-Ex $59 \pm 13 \text{ mOsmol}\cdot\text{kg}^{-1}$, Post-Ex $101 \pm 17 \text{ mOsmol}\cdot\text{kg}^{-1}$, 30 min Post-Ex $94 \pm 16 \text{ mOsmol}\cdot\text{kg}^{-1}$, trial \times time interaction $P = 0.008$), and post hoc analysis revealed a significant Post-Ex increase ($P < 0.001$ Post-Ex and $P = 0.012$, 30 min Post-Ex) in the SIT trial (with no change in the control trial). There was a trial \times time interaction for salivary s-IgA concentration, which increased at the post-exercise times in both trials but to a greater extent in the SIT trial (Table 2). There was no interaction for salivary s-IgA:osmolality ratio and for salivary s-IgA secretion rate. Saliva s-IgA:osmolality ratio was elevated above Pre-Ex only at the Post-Ex time (in both trials, Table 2) and salivary s-IgA secretion rate was elevated at both post-exercise time

points. There was a trial \times time interaction for salivary lysozyme concentration and post hoc analysis revealed no change in the control trial whereas there was a significant increase at the post-exercise times in the SIT trial (Table 2). There was no main interaction or time effects for salivary lysozyme:osmolality ratio and for salivary lysozyme secretion rate (Table 2).

*** please insert Figure 3 near here ***

There was a significant trial \times time interaction for plasma antioxidant capacity and post hoc analysis revealed no change in the control trial whereas there was a significant increase at the post-exercise times in the SIT trial (Table 3). There was a significant trial \times time interaction for plasma TBARS concentration and post hoc analysis revealed no change in the control trial but a significant increase in the SIT trial (Table 3). Significant time and trial \times time interaction effects were also observed for ex vivo ROS but post hoc analyses was unable to detect differences between any time points within or between trials.

*** please insert Table 3 near here ***

Discussion

The main findings of the present study are that a single session of Wingate-based SIT causes significant immunodepression of some neutrophil functions (oxidative burst and degranulation) but not mucosal immunity (salivary lysozyme and s-IgA concentration and secretion rate). The magnitude of the exercise-induced depression of some neutrophil functions (e.g. OBA) is comparable with previous studies, using the same methods, in which prolonged endurance exercise (> 2 h duration) was employed (Davison and Gleeson, 2005,

2006). However, the magnitude of the exercise-induced depression of neutrophil degranulation is considerably smaller than the changes observed in these studies (Davison and Gleeson, 2005, 2006), although similar to the changes observed following a 2 h protocol (Davison and Diment, 2010). However, such comparisons are made with caution as it was not the main aim of the present study to directly compare SIT and prolonged endurance exercise. The main aim was to determine whether SIT induced significant immunodepression, and this was evident only for some markers of neutrophil function (fMLP-stimulated and PMA-stimulated OBA and bacterial-stimulated degranulation). Additionally, the observation that SIT did not induce a significant decrease of fMLP-then-PMA-stimulated OBA, salivary s-IgA or lysozyme (whether salivary parameters were expressed as an absolute concentration, normalised to saliva osmolality or expressed as the secretion rate) is in contrast to previous studies, which have demonstrated compromised mucosal immunity (Fahlman et al. 2001; Hall et al. 2007; MacKinnon and Jenkins, 1993).

Previous research (Davison and Diment, 2010; Davison and Gleeson, 2005, 2006, 2007; Davison et al. 2007) has suggested that the decreased neutrophil functions observed following prolonged exercise are caused in part by the mobilisation of functionally immature neutrophils (Berkow and Dodson, 1986) from the bone marrow. The post-exercise increase in blood neutrophil count observed in the present study (< 2-fold) was modest by comparison with prolonged exercise of 2 to 2.5 h duration (in which there is typically an increase of between 3 and 6-fold post-exercise; Davison and Diment, 2010; Davison and Gleeson, 2005, 2006, 2007; Davison et al. 2007). Furthermore, the increase in the present study may be partly accounted for by the post-exercise change in plasma volume. Therefore, it is unlikely that this mechanism can explain the depression of neutrophil functions observed in the present study. The post-exercise increase in plasma cortisol concentration observed in the

present study is comparable with a previous study with prolonged exercise of 2 h duration (Davison and Diment, 2010) and this seems to be independent from the post-exercise change in plasma volume. However, greater cortisol responses have been observed with similar protocols, but with prolonged exercise of more than 2 h duration (Davison and Gleeson, 2005, 2006, 2007; Davison et al. 2007). Taken together, this suggests that the SIT-induced decreases of neutrophil functions are caused by more direct mechanisms, such as the direct effects of stress hormones and oxidative stress, as discussed below.

The present results for the OBA responses activated with various stimulants allows some insight into the possible mechanisms responsible for the SIT-induced depression of neutrophil OBA. For example, fMLP activates oxidative burst by binding to receptors on the neutrophil cell membrane (receptor-dependent activation of OBA). This initiates an intracellular signalling cascade, ultimately leading to the assembly of NADPH oxidase and activation of oxidative burst. PMA induces receptor-independent activation of OBA, by entering the cell (neutrophil) and directly activating protein kinase C (PKC), inducing the activation of NADPH oxidase and oxidative burst throughout the whole cell (also including the plasma membrane system). Since fMLP acts solely on the NADPH oxidase system of the plasma membrane, when PMA is used as the stimulant subsequent to stimulation with fMLP (e.g. Figure 1B), the (fMLP-then-PMA) chemiluminescence response represents the capacity for OBA remaining after the cell surface (fMLP-stimulated) system has been 'discharged'. Hence, this provides a representation of completely non-receptor-dependent OBA capacity.

In the present study, there were significant SIT-induced decreases of receptor-dependent stimulated neutrophil functions (OBA and degranulation) but not the fMLP-then-PMA-stimulated OBA. Taken together, these findings suggest that the SIT-induced effects on

neutrophil dysfunction are associated with direct mechanisms acting at the cell surface, although this requires further research. Support for this idea has been previously suggested, for prolonged exercise (Davison and Gleeson, 2007; Robson et al. 2003), in which a plausible 'direct' mechanism is the exercise-induced production of excess free radicals and ROS, which may lead to oxidative stress or damage to neutrophils, since the membrane receptors are more exposed to extracellular attack by ROS. These studies (Davison and Gleeson, 2007; Robson et al. 2003) provide strong evidence for the ROS-mediated theory as they also demonstrated that the exercise-induced alterations in neutrophil function and oxidative stress markers were blunted by the administration of antioxidants. The present findings, that the receptor-dependent activation was affected by SIT whereas the receptor-independent activation was not, provide preliminary support for this but further work is required, including studies with antioxidant administration and studies of in vitro exposure of neutrophils to ROS, to determine whether these theorised mechanisms are correct. However, the observed post-exercise increase in plasma TBARS and antioxidant mobilization add further weight to the argument that SIT-induced neutrophil dysfunction is mediated, at least in part, by oxidative stress to these cells.

Salivary s-IgA and lysozyme concentrations were significantly increased following SIT. This may be largely due to the pseudo-concentrating effects induced by strenuous exercise and high ventilation rates (such as changes in hydration status, mucosal drying and evaporative loss of water). For example, when these analytes were normalised, by expressing them relative to saliva osmolality or as the secretion rate, the exercise-induced increases were markedly reduced or absent (Table 2). The present results for salivary lysozyme are in agreement with previous studies (Allgrove et al. 2008; West et al. 2010) in which continuous strenuous exercise (of up to ~30 minutes) has been shown to increase the salivary

concentration or secretion of antimicrobial proteins. While this may be viewed as a potentially beneficial response, it may actually be a result of physical damage to epithelial cells in the upper airways, and subsequent inflammatory responses, meaning that the immunological and host defence implications are unclear at present (Gleeson, 2007; West et al. 2010). In addition, it is possible that repeated stimulation of such responses during periods of intensive training are involved in the chronic lowering that has been observed for the salivary concentration and secretion rate of these proteins of innate immunity during prolonged training periods (West et al. 2010). However, such a link between the acute salivary antimicrobial proteins responses to strenuous exercise and 'chronic' training effects requires further investigation.

The discrepancy between the findings of the present study and previous research (Fahlman et al. 2001; Hall et al. 2007; MacKinnon and Jenkins, 1993) for salivary s-IgA responses to SIT may be due to differences in methodology and the subject population. For example, it has been shown (although with a maximal treadmill protocol) that males exhibited an acute increase in salivary s-IgA post-exercise whereas females exhibited a decrease (Schouten et al. 1988). Hence, the difference between the present findings, with all male subjects, and the exercise-induced decrease observed by Fahlman et al. (2001) may be explained by the fact that the subjects in the latter study were all female. Likewise, the sex of the subjects was not reported in the study by Hall et al. (2007) so it is possible that there was a mixture of males and females. Finally, the study by MacKinnon and Jenkins (1993) employed a protocol with a higher total volume of exercise (5×60 -second Wingate tests), which may explain why a post-exercise decrease was observed.

Conclusions and future directions

Research evidence showing the many potential benefits of SIT could lead to increased use of this type of training, both as a research tool and practically in sporting and athletic populations. The present study demonstrated that a single session of SIT caused significant exercise-induced immunodepression of key aspects of innate immunity (blood neutrophil OBA and degranulation capacity), which persisted for at least 30 minutes post-exercise. However, some of the measured parameters were not negatively affected (fMLP-then-PMA-stimulated OBA, salivary s-IgA and lysozyme). These findings may have implications for those considering the incorporation of SIT into a training programme. However, it must be acknowledged that the SIT protocol employed in the present study (a single session of 4 × 30-second Wingate tests) was based on the first day of the 2-week protocol from the McMaster group (i.e. Burgomaster et al. 2006, 2007, 2008; Gibala et al. 2006) in subjects with limited prior experience of Wingate-based SIT. Therefore, future research in this area should determine the responses to repeated SIT sessions (e.g. over a typical 2-week SIT period), also in subjects with prior experience of SIT, and additionally determine the chronic responses when SIT is incorporated into a long-term training programme.

References

- Allgrove, J.E., Gomes, E., Hough, J. and Gleeson M. 2008. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. *J Sports Sci*, **26**: 653-661.
- Babraj, J.A., Vollaard, N.B., Keast, C., Guppy, F.M., Cottrell, G. and Timmons, J.A. 2009. Extremely short duration high intensity interval training substantially improves insulin action in young healthy males. *BMC Endocr Disord*, **9**: 3.

- Berkow, R.L. and Dodson, R.W. 1986. Purification and functional evaluation of mature neutrophils from human bone marrow. *Blood*, **68**: 853-860.
- Burgomaster, K.A., Cermak, N.M., Phillips, S.M., Benton, C.R., Bonen, A. and Gibala, M.J. 2007. Divergent response of metabolite transport proteins in human skeletal muscle after sprint interval training and detraining. *Am J Physiol Regul Integr Comp Physiol*, **292**: R1970-R1976.
- Burgomaster, K.A., Heigenhauser, G.J. and Gibala, M.J. 2006. Effect of short-term sprint interval training on human skeletal muscle carbohydrate metabolism during exercise and time-trial performance. *J Appl Physiol*, **100**: 2041-2047.
- Burgomaster, K.A., Howarth, K.R., Phillips, S.M., Rakobowchuk, M., Macdonald, M.J., McGee, S.L. and Gibala, M.J. 2008. Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol*, **586**: 151-160.
- Burgomaster, K.A., Hughes, S.C., Heigenhauser, G.J., Bradwell, S.N. and Gibala, M.J. 2005. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol*, **98**: 1985-1990.
- Davison, G. and Diment, B.C. 2010. Bovine colostrum supplementation attenuates the decrease of salivary lysozyme and enhances the recovery of neutrophil function after prolonged exercise. *Br J Nutr*, **103**: 1425-1432.

- Davison, G. and Gleeson, M. 2005. Influence of acute vitamin C and/or carbohydrate ingestion on hormonal, cytokine, and immune responses to prolonged exercise. *Int J Sport Nutr Exerc Metab*, **15**: 465-479.
- Davison, G. and Gleeson, M. 2006. The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man. *Eur J Appl Physiol*, **97**: 454-461.
- Davison, G. and Gleeson, M. 2007. The effects of acute vitamin C supplementation on cortisol, interleukin-6, and neutrophil responses to prolonged cycling exercise. *Eur J Sports Sci*, **7**: 15-25.
- Davison, G., Gleeson, M. and Phillips, S. 2007. Antioxidant supplementation and immunoendocrine responses to prolonged exercise. *Med Sci Sports Exerc*, **39**: 645-652.
- Dill, D.B. and Costill, D.L. 1974. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol*, **37**: 247-248.
- Fahlman, M.M., Engels, H.J., Morgan, A.L. and Kolokouri, I. 2001. Mucosal IgA response to repeated wingate tests in females. *Int J Sports Med*, **22**: 127-131.
- Gibala, M.J. and Little, J.P. 2010. Just HIT it! A time-efficient exercise strategy to improve muscle insulin sensitivity. *J Physiol*, **588**: 3341-3342.

Gibala, M.J. and McGee, S.L. 2008. Metabolic adaptations to short-term high-intensity interval training: a little pain for a lot of gain? *Exerc Sport Sci Rev*, **36**: 58-63.

Gibala, M.J., Little, J.P., van Essen, M., Wilkin, G.P., Burgomaster, K.A., Safdar, A., Raha, S. and Tarnopolsky, M.A. 2006. Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol*, **575**: 901-911.

Gleeson, M. 2007. Immune function in sport and exercise. *J Appl Physiol*, **103**: 693-699.

Gleeson, M., Nieman, D. and Pedersen, B. 2004. Exercise, nutrition and immune function. *J Sports Sci*, **22**: 115-125.

Hall, H., Fahlman, M.M. and Engels, H.J. 2007. Echinacea purpurea and mucosal immunity. *Int J Sports Med*, **28**: 792-797.

Kowalska, M. 2003. Dysfunction of peripheral blood granulocyte oxidative metabolism in children with recurrent upper respiratory tract infections. *Int J Pediatr Otorhinolaryngol*, **67**: 365-371.

Kutter, D., Devaquet, P., Vanderstocken, G., Paulus, J.M., Marchal, V. and Gothot A. 2000. Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit ? *Acta Haematol*, **104**: 10-15.

MacKinnon, L.T. and Jenkins, D.G. 1993. Decreased salivary immunoglobulins after intense interval exercise before and after training. *Med Sci Sports Exerc*, **25**: 678-683.

Matsuzaka, M., Fukuda, S., Yamai, K., Tsuya, R., Fukuoka, Y., Takahashi, I., Yaegaki, M., Shimoyama, T., Sakamoto, J., Umeda, T. and Nakaji, S. 2008. Are individuals with lower neutrophil oxidative burst activity more prone to *Helicobacter pylori* infection? *Luminescence*, **23**: 132-138.

Morozov, V.I., Pryatkin, S.A., Kalinski, M.I. and Rogozkin, V.A. 2003. Effect of exercise to exhaustion on myeloperoxidase and lysozyme release from blood neutrophils. *Eur J Appl Physiol*, **89**: 257-262.

Nagatomi, R. 2006. The implication of alterations in leukocyte subset counts on immune function. *Exerc Immunol Rev*, **12**: 54-71.

Neville, V., Gleeson, M. and Folland, J.P. 2008. Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes. *Med Sci Sports Exerc*, **40**: 1228-1236.

Nieman, D.C. 1997. Immune response to heavy exertion. *J Appl Physiol*, **82**: 1385-94.

Nieman, D.C. 1994. Exercise, upper respiratory tract infection, and the immune system. *Med Sci Sports Exerc*, **26**: 128-139.

Nieman, D.C. 2007. Marathon training and immune function. *Sports Med*, **37**: 412-415.

- Nikolaidis, M.G. and Jamurtas, A.Z. 2009. Blood as a reactive species generator and redox status regulator during exercise. *Arch Biochem Biophys*, **490**: 77-84.
- Pedersen, B.K. and Hoffman-Goetz, L. 2000. Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev*, **80**: 1055-1081.
- Powers, S.K. and Jackson, M.J. 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev*, **88**: 1243-1276.
- Pütsep, K., Carlsson, G., Boman, H.G. and Andersson, M. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet*, **360**: 1144-1149.
- Richards, J.C., Johnson, T.K., Kuzma, J.N., Lonac, M.C., Schweder, M.M., Voyles, W.F. and Bell, C. 2010. Short-term sprint interval training increases insulin sensitivity in healthy adults but does not affect the thermogenic response to beta-adrenergic stimulation. *J Physiol*, **588**: 2961-2972.
- Robson, P.J., Bouic, P.J. and Myburgh, K.H. 2003. Antioxidant supplementation enhances neutrophil oxidative burst in trained runners following prolonged exercise. *Int J Sport Nutr Exerc Metab*, **13**: 369-381.
- Schouten, W.J., Verschuur, R. and Kemper, H.C. 1988. Habitual physical activity, strenuous exercise, and salivary immunoglobulin A levels in young adults: the Amsterdam Growth and Health Study. *Int J Sports Med*, **9**: 289-293.

Spence, L., Brown, W.J., Pyne, D.B., Nissen, M.D., Sloots, T.P., McCormack, J.G., Locke, A.S. and Fricker, P.A. 2007. Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes. *Med Sci Sports Exerc*, **39**: 577-86.

West, N.P., Pyne, D.B., Kyd, J.M., Renshaw, G.M., Fricker, P.A. and Cripps, A.W. 2010. The effect of exercise on innate mucosal immunity. *Br J Sports Med*, **44**: 227-231.

Whyte, L.J., Gill, J.M. and Cathcart, A.J. 2010. Effect of 2 weeks of sprint interval training on health-related outcomes in sedentary overweight/obese men. *Metabolism*, **59**: 1421-1428.

Table Headings and Legends

Table 1: Physiological responses

	Pre-Ex	Post-Ex	30 min Post-Ex	2-way ANOVA Main Effects, P = (trial; time; interaction)
Plasma volume change (% decrease)				0.001 ; < 0.001 _{GH} ; < 0.001 _{GH}
Control	-	0.7 (± 0.1) [‡]	2.2 (± 1.9) [†]	
SIT	-	19.8 (± 4.9) ^{‡aa}	10.0 (± 7.0) ^{‡a}	
Blood [lactate] (mM)				< 0.001 ; < 0.001 ; < 0.001
Control	0.8 (± 0.3)	0.9 (± 0.3)	0.9 (± 0.4)	
SIT	0.8 (± 0.3)	10.9 (± 2.1) ^{‡aa}	5.2 (± 1.4) ^{‡aa}	
Blood [glucose] (mM)			*	0.946 ; 0.003 ; 0.085
Control	4.7 (± 0.6)	4.4 (± 0.4)	4.2 (± 0.4)	
SIT	4.7 (± 0.5)	4.7 (± 0.7)	3.9 (± 0.4)	

Values are mean (± standard deviation). Post hoc analysis for main effect of time: signifies a significant difference from Pre-Ex (either pooled data from both trials due to no interaction effect: *P < 0.05, **P < 0.01, or each trial independently when an interaction exists: [†] P < 0.05; [‡] P < 0.01). Post hoc analysis for trial × time interaction: signifies a significant difference, between trials, in the change from Pre-Ex (^{aa}P < 0.01, ^aP < 0.05).

Table 2: Physiological stress and immunological responses

	Pre-Ex	Post-Ex	30 min Post-Ex	2-way ANOVA Main Effects, P = (trial; time; interaction)
Plasma [cortisol] (nM)				0.002 ; < 0.001; < 0.001
Control	330 (± 97)	276 (± 94) ‡	246 (± 97) ‡	
SIT	367 (± 123)	507 (± 120) ^{‡aa}	641 (± 150) ^{‡aa}	
Blood neutrophil count (× 10⁹ cells·l⁻¹)				0.039 ; < 0.001 _{GH} ; < 0.001
Control	2.3 (± 0.9)	2.4 (± 0.9)	2.5 (± 1.0)	
SIT	2.3 (± 0.7)	4.0 (± 1.5) ‡aa	3.1 (± 1.0) ‡a	
Saliva [s-IgA] (mg·l⁻¹)				0.003 ; < 0.001 ; 0.005
Control	148 (± 26)	190 (± 31) †	182 (± 19) ‡	
SIT	164 (± 47)	376 (± 49) ^{‡aa}	308 (± 89) ^{†aa}	
Saliva s-IgA:osmolality ratio		*		0.640 ; 0.007 ; 0.499
Control	2.8 (± 0.4)	3.4 (± 0.7)	3.2 (± 0.5)	
SIT	2.7 (± 0.3)	3.8 (± 0.7)	3.2 (± 0.6)	
Saliva s-IgA secretion rate (µg·min⁻¹)		*	*	0.033 ; 0.002 ; 0.696
Control	47 (± 18)	60 (± 32)	66 (± 43)	
SIT	76 (± 23)	99 (± 33)	106 (± 31)	
Saliva [Lysozyme] (mg·l⁻¹)				0.040 ; 0.003 ; 0.035
Control	10.5 (± 2.3)	11.6 (± 3.7)	13.9 (± 6.7)	
SIT	10.8 (± 7.5)	25.9 (± 8.6) ^{†a}	24.8 (± 12.2) [†]	
Saliva Lysozyme:osmolality ratio				0.704 ; 0.128 ; 0.498
Control	0.21 (± 0.07)	0.21 (± 0.09)	0.24 (± 0.12)	
SIT	0.19 (± 0.13)	0.26 (± 0.08)	0.27 (± 0.14)	
Saliva Lysozyme secretion rate (µg·min⁻¹)				0.341 ; 0.085 ; 0.446
Control	3.6 (± 2.3)	3.8 (± 2.9)	6.1 (± 6.7)	
SIT	6.1 (± 5.9)	6.8 (± 3.7)	9.7 (± 7.3)	

Values are mean (± standard deviation). Post hoc analysis for main effect of time: signifies a significant difference from Pre-Ex (either pooled data from both trials due to no interaction effect: *P < 0.05, **P < 0.01, or each trial independently when an interaction exists: † P < 0.05; ‡ P < 0.01). Post hoc analysis for trial × time interaction: signifies a significant difference, between trials, in the change from Pre-Ex (^{aa}P < 0.01, ^aP < 0.05).

Table 3: Oxidative stress and antioxidant capacity biomarkers.

	Pre-Ex	Post-Ex	30 min Post-Ex	2-way ANOVA Main Effects, P = (trial; time; interaction)
Plasma antioxidant capacity (μM ascorbate equivalent)				0.023 ; < 0.001; < 0.001 _{GH}
Control	348 (\pm 55)	359 (\pm 53)	377 (\pm 52)	
SIT	321 (\pm 61)	515 (\pm 44) ^{‡aa}	424 (\pm 60) ^{‡ a}	
Plasma TBARS (μM)				0.083 ; 0.022 ; 0.007
Control	1.61 (\pm 0.64)	1.60 (\pm 0.62)	1.53 (\pm 0.63)	
SIT	1.55 (\pm 0.54)	1.81 (\pm 0.48) [†]	2.06 (\pm 0.43) ^{†a}	
Blood ex vivo ROS (% of Pre-Ex)				0.124 ; 0.003 ; 0.026
Control	100 (-)	95 (\pm 9)	89 (\pm 13)	
SIT	100 (-)	117 (\pm 23)	94 (\pm 18)	

Values are mean (\pm standard deviation). Post hoc analysis for main effect of time: signifies a significant difference from Pre-Ex (each trial independently due to interaction: [†] P < 0.05; [‡] P < 0.01). Post hoc analysis for trial \times time interaction: signifies a significant difference, between trials, in the change from Pre-Ex (^{aa}P < 0.01, ^aP < 0.05).

Figure Legends

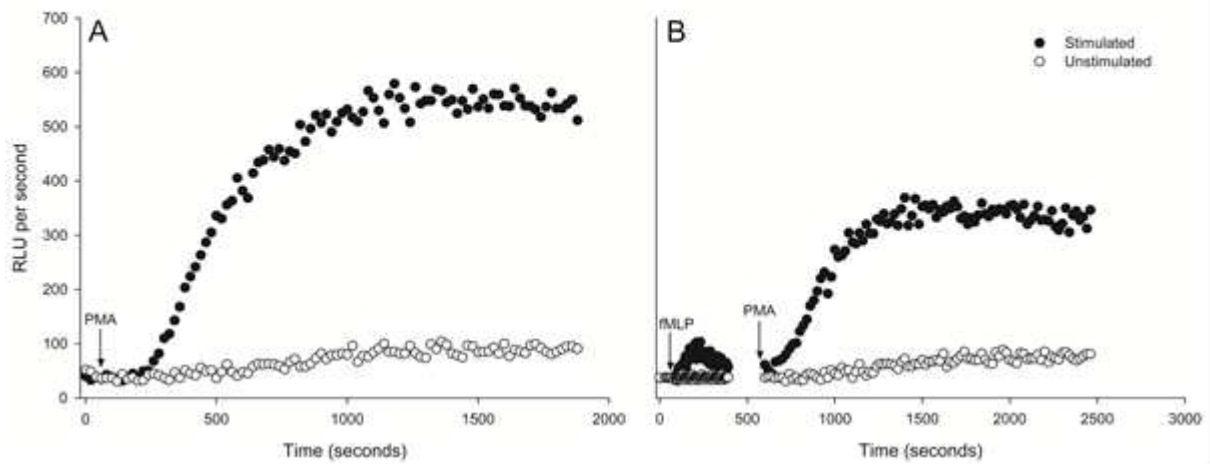


Figure 1: Example of typical chemiluminescence (OBA) response with different stimulants.

Panel A (left) shows a typical response after stimulation with PMA (and no stimulation control) and Panel B (right) shows a typical response after stimulation with fMLP and PMA subsequent to fMLP (fMLP-then-PMA).

Downward arrows indicate the point at which the stimulants were added.

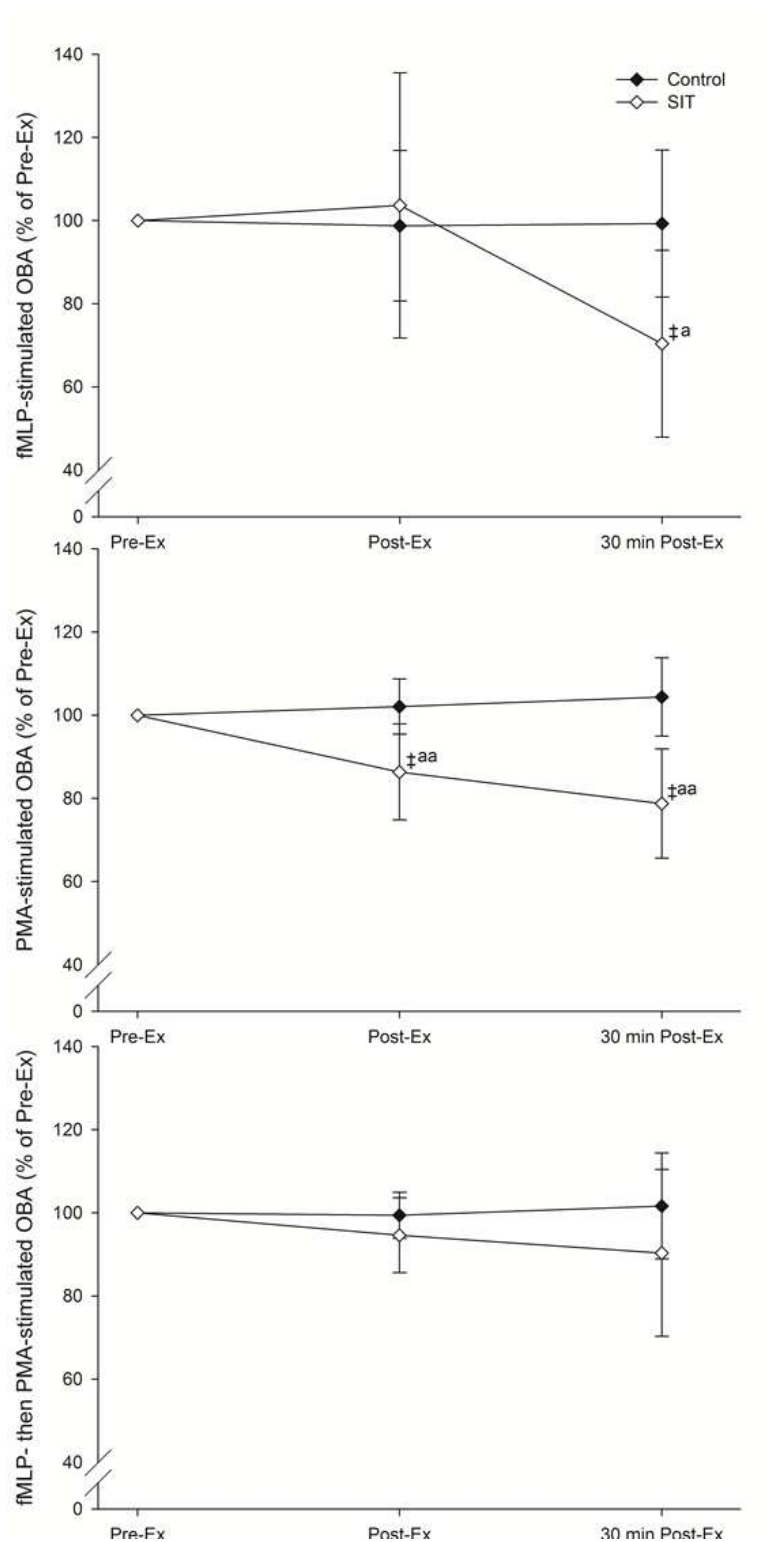


Figure 2: Stimulated neutrophil OBA responses to SIT and Control trials.

Top panel, fMLP-stimulated OBA; middle panel, PMA-stimulated OBA; bottom panel, fMLP-then-PMA-stimulated OBA. Post hoc analysis for main effect of time: signifies a significant difference from Pre-Ex (for each trial independently when an interaction exists: [†] $P < 0.05$; [‡] $P < 0.01$). Post hoc analysis for trial \times time interaction: signifies a significant difference, between trials, in the change from Pre-Ex (^{aa} $P < 0.01$, ^a $P < 0.05$).

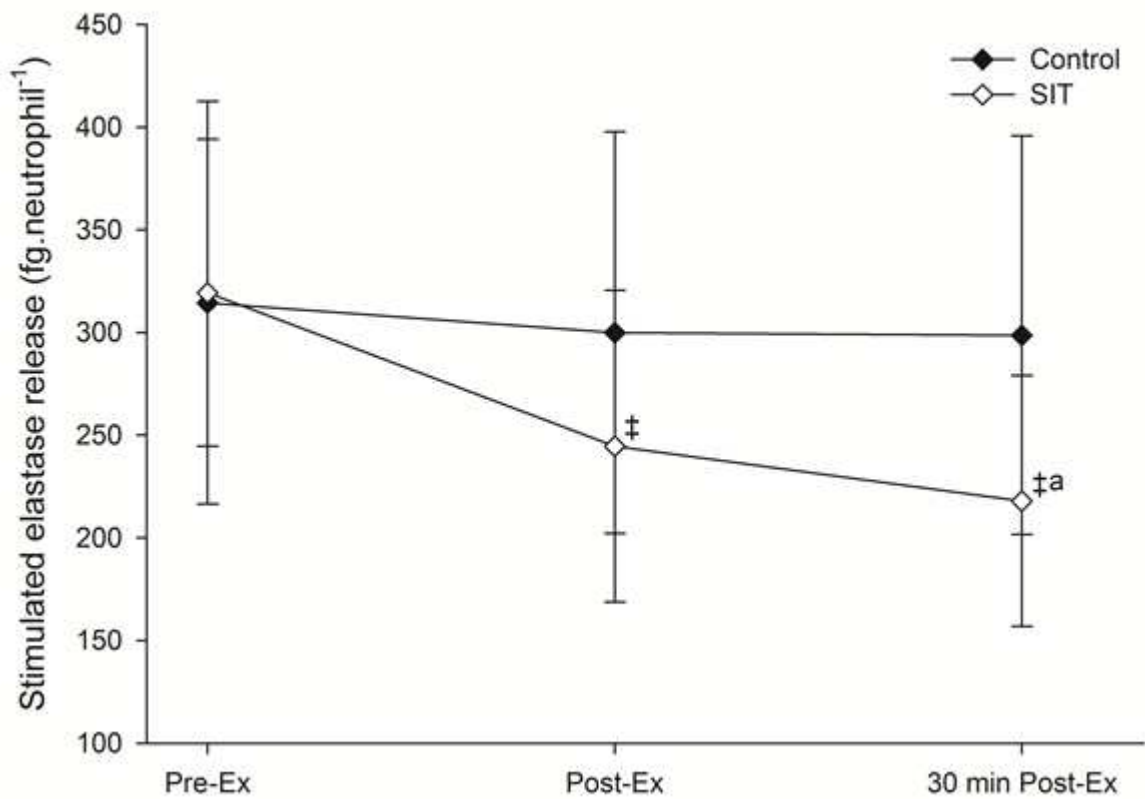


Figure 3: Stimulated neutrophil degranulation responses to SIT and Control trials.

Post hoc analysis for main effect of time: signifies a significant difference from Pre-Ex (for each trial independently when an interaction exists: [†] P < 0.05; [‡] P < 0.01). Post hoc analysis for trial × time interaction: signifies a significant difference, between trials, in the change from Pre-Ex (^{aa}P < 0.01, ^aP < 0.05).