Is there a morning-to-evening difference in the acute IL-6 and cortisol responses to resistance exercise?

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Running head: IL-6 and Cortisol exercise-induced surges and Time

Abstract

Exercise training is known to induce a molecular adaptation process involving inflammatory responses. However any time-of-day effect of exercise on inflammatory responses remains unknown. The aim of the present study was to investigate whether acute bouts of intense exercise performed at different times of the day would affect the release Interleukin-6 (IL-6), one of the most abundant cytokines in mammalian endocrine response to exercise. Cortisol levels were measured as a confirmation of correct timing of exercise and to determine any impact it may have on the cytokine release. Twelve healthy male participants carried out 30 minutes of intense exercise (3 sets of 8-12 repetitions for 4 resistance exercises at 70% of 1RM) in morning (08:15 – 09:00 h), and evening (18:15 – 19:00 h) sessions. An 8h fasting period was required before each exercise session. Blood samples were taken immediately pre and post each exercise sessions to determine IL-6 and cortisol levels. Our data show that whilst the training group showed no post-exercise changes in serum_IL-6 levels (P>0.05), the control group on the other hand showed significant time-of-day modifications in serum_IL-6 levels (P=0.008). Moreover, a significant interaction between intervention phase (pre-post training, AM vs. PM) and group (Exercise vs. Control) is evidenced in terms of serum_IL-6 levels (P=0.014). This interaction however was nullified when the between group differences at baseline were partialled out in a covariate analysis (P>0.05). We also found that the main effect of experimental phase on Cortisol was present in both the trained (P=0.004) and control groups (p<0.001) with no significant interaction (P>0.05). Based on the current data, we would propose that exercise and/or time-of-day would not interfere with clinical endocrine profiling of IL-6 in a population.

Key Words: Acute response; Circadian rhythm; Cortisol; Interleukin-6; Resistance exercise;

1. *Introduction*

Early studies reported that strenuous exercise is followed by an inflammatory response similar to that found in infectious disease [1, 2]. It has been shown that both eccentric and concentric exercise lead to mechanical stress inducing muscle damage [3], which is initially followed by the inflammatory response before starting the muscle repair process [4]. When muscle damage occurs, the inflammatory process is initiated and consists of a combination of two phases [5]. The first being the fluid phase reaction where vasoconstriction is followed by sustained dilation of arterioles, capillaries and venules. The second phase known as the cellular response corresponds to the emigration of leukocytes from blood vessels followed by swelling of the vascular endothelial cells, causing them to adhere to the leukocytes. MacIntyre et al. [6], found the inflammatory response to be responsible for initiating, amplifying, and/or resolving skeletal muscle injury. During the inflammatory response, damage to tissue in the local area occurs. More recently it has been proposed that during regeneration of muscle tissue after damage, an increase in the number of sarcomeres in muscle fibres can be found, leading to longer, more stable fibres within the area that underwent damage/exercise (For a Review read [7]).

Hirose et al. [8] found that after the first bout of eccentric exercises of the elbow flexors, muscle soreness increased significantly. Further bouts of exercise indicated muscle adaptation as muscle soreness decreased dramatically, and function improved. Indeed the inflammatory response to exercise shows signs of the acute-phase response where there is an orchestrated response to tissue infection and inflammation. The acute phase includes an in vivo biochemical and endocrine array of changes following infections, traumas, and the inflammatory processes in general. Leucocytosis and leucocyte activation, release of inflammatory mediators, tissue damage and cellular infiltrates, have all shown signs of increased reaction in the inflammatory response [9, 10]. Drenth et al. [11] showed that interleukin -6 (IL-6) exhibits the largest circulation of exercise-induced cytokines. However, whilst some of the previous research [12], argue that IL-6 has a negative impact on the acute phase response as it is an anti-inflammatory and immuno-suppressor. Others including Al-Shanti et al. [13], suggest that the cytokine IL-6 may have a positive relationship with the inflammatory response. The literature thereby demonstrates both pro- and anti-inflammatory IL-6 characteristics (for a review read [14]). In addition, evidence that continuously contracting skeletal muscle, rather than blood leukocytes, is responsible for the exercise-induced increase in IL-6 concentration has been shown from studies investigating active and

non-active leg muscles [15, 16]. IL-6 is thus also known as the first myokine, or a cytokine that is produced and released by skeletal muscle fibres.

An endogenous circadian rhythm exists in all species that repeats approximately every 24 hours. The circadian rhythm can affect core body temperature, hormone secretion, sleep, and alertness [17]. Light, heat, activity patterns and social activities can also affect the circadian rhythm, which are all important factors to consider when deciding on the best time of day to train. Many authors have demonstrated that maximal short-term performance is increased by the end of the afternoon, coinciding with the peak of the temperature curve [18-22] but also with hormonal fluctuations. DeRijk et al. [23] studied the changes in cortisol and several cytokines 20mins immediately after strenuous exercise. They found that cortisol appeared to suppress Tumor Necrosis Factor-α, and Interleukin 1-β, but had no effect on the release of IL-6, a result which suggests that IL-6 has both pro- and anti-inflammatory properties as it is generally accepted that cortisol suppresses all pro-inflammatory cytokines [24]. Cortisol is a glucocorticoid released following physical and/or psychological stress. Cortisol contributes towards physical performance by maintaining blood glucose levels during exercise, acting upon skeletal muscle and adipose tissue to increase amino acid and lipid mobilisation. Cortisol is at its highest level in the morning and at its lowest levels in the evening, following a circadian rhythm [24]. Moreover, the levels of cortisol are generally found to increase from pre to post exercise [25] so long as the sample is taken immediately (up to 20 mins) rather than following an elongated time after cessation of the exercise (e.g. 12 hours; [26]) in which case 'stress hormones' like cortisol levels are found to not differ from baseline resting values.

From a practical point of view, short duration exercise is more likely to be complied with owing to time restraints that athletes and members of the general public, encounter. Moreover, it is evident that prolonged exercise bouts evoke a pronounced cytokine response, which is potentially inhibited by the concurrent variations in the levels of cortisol in particular. Whether the same effect would be seen with short duration exercise is however unclear, a significant oversight in current literature as such an effect could be used to advise athletes on the most beneficial time to train. Thus, the aim of the present study was to examine whether the time of day (morning vs. evening) affects the inflammatory and cortisol responses to short intense bouts of exercise. It was hypothesised that, by conducting acute bouts of exercise in the morning vs. evening, a more notable increment in IL-6 levels will occur in the former compared with the latter.

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2. *Methods*

2.1. *Participants*

The present study was carried out on a group of 12 healthy male participants matched for age and lifestyles. They were aged 20 ± 1.6 years, body mass 76 ± 6.0 kg, and stature 176 ± 6.6 cm. Participants were selected if they fulfilled the criteria (healthy, low/medium physical activity (i.e. recreationally active but not formally taking part in a sport), free from serious illness/injury for at least 3 months). A further 6 young and healthy males (21 ± 1.9 years, body mass 77 ± 4.6 kg, and stature 178 ± 7.9 cm), the controls, were also tested in the absence of an exercise intervention in order to determine changes in IL-6 and Cortisol in a time dependent manner. Each participant completed an informed consent form prior to testing. The study was approved by the local Ethics Committee. All procedures adhered to high ethical and methodological standards as described in Portaluppi et al. [27] and in accordance with the declaration of Helsinki.

2.2. *Exercise Protocol*

Exercise interventions took place at two different times of the day: between 08:15 – 09:00h and between 18:15 – 19:00h. Each participant was exercised at both time slots, and their individual blood samples were taken. A counterbalanced study design was used so that 6 of the participants were randomly assigned to testing in the evening first then in the morning and the others in reverse order.

Before each participant attended their first exercise intervention session, they were required to attend the gym for a familiarisation session, followed by exercise to ascertain 1 repetition maximum (1 RM). Following appropriate warm-up procedures consisting of 8-10 sub maximal repetitions, exercise proper included chest press (Pulse 310E class 's' 7/99, wide grip. Pulse-fitness, Congleton, England), seated leg press (Pulse S77E class 's' 8/97. Pulse-fitness, Congleton, England), shoulder press (Pulse 305E class 's' 7/99, wide grip. Pulse-fitness, Congleton, England), and front Latissimus Dorsi pull down (Pulse 380E class 's' 6/97, wide grip. Pulse-fitness, Congleton, England) exercises. Participants were instructed to perform slow controlled contractions and were monitored throughout. To determine their 1 RM, an initial weight was selected which was perceived to be within their capacity (50-70%), then raised by 2.5kg up to 20kg each set of repetitions. The testing took four sets with rests of 3-5 minutes between each. The highest and last weight to be successfully lifted was recorded as the absolute 1 RM (ACSM guidelines). During the leg press exercise, a

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number of participants could reach the maximum weight available so the Brzycki formula ([(r/30)+1]×w, where r is the number of repetitions performed and w is the amount of weight lifted; [28]) was used to evaluate their 1 RM.

The exercise session for testing proper consisted of a five minute general warm-up using a treadmill (Pulse Ascent, 260F-T Low impact treadmill. Pulse-fitness, Congleton, England), and carrying out 5 repetitions at 50% of their 1 RM on each exercise involved in the protocol. The participants were then required to carry out 3 sets of 8-12 repetitions on the chest press, lat pull down, leg press, and shoulder press. These were performed at 70% 1 RM with a rest of 1 minute between series. The controls did not partake into any exercise training between blood sampling sessions.

2.3. *Assessment of serum IL-6 and cortisol levels*

After an 8 hour fasting period, blood samples were taken from each participant from the anticubital vein of the forearm by a trained phlebotomist using a 21ml gauge needle (S-Monovette, Sarstedt, Germany). Five millilitres of blood were taken pre- and post- exercise from each participant and subsequently allowed to clot on ice for up to 1 hour. The controls had blood samples taken at similar times and intervals, though in the absence of exercise. These samples were then centrifuged (Hermle Z 380, Huddersfield) at 5ºC and at 5000 rpm for 10 minutes to separate the serum from the blood cells. Two aliquots (~900µl each) of the resulting sera samples were taken using a 500 - 2500µl pipette (Eppendorf), and where then stored at -20ºC for later analysis. IL-6 (R&D Systems inc. Minneapolis, USA. Sensitivity <0.7 pg/ml; Intra-assay variability of 2.6%) and cortisol (R&D Systems inc. Minneapolis, USA. Sensitivity <0.071 ng/ml; Intra-assay variability of 7.0%) were later analysed using the standard ELISA (enzyme linked immunosorbant assays) procedure.

2.4. Statistical Analyses

A Shapiro-Wilk test was conducted and raw data was transformed to obtain a normal distribution. The relationship between time-of-day and ligand levels was analysed using a Mixed-design repeated measures analysis of variance (rANOVA), with post-hoc Sidak adjustment for multiple pairwise comparisons. Bivariate regressions were run to highlight any influence of baseline values on post-exercise hormonal responses. Where such a regression was significant (i.e. when data was heterogeneous at baseline (i.e. before

exercise)), an ANCOVA test was used. Data are presented as mean $\pm$ standard deviation (SD), except for Fig.1 which has s.e.m. for schematic representation. Statistical significance was set at an alpha of 0.05.

3. Theory

Should a clear advantage of training at a specific time of the day be found in the current study, this would lend itself to further investigations, this time in different study populations. Of particular interest (not least owing to the age demographics of Western Society), would be the elderly, where not only is decreased muscle mass a significant impediment to daily physical function [29], but also, a background of inflamed endocrine milieu is known to prevail [30] and hence the manner in which this age group may or may not respond to a targeted training protocol is not obvious.

4. *Results*

Endocrine data sets by intervention sequence were firstly compared to ascertain the presence of a testing order effect on the results (i.e. any change in blood markers when testing AM followed by PM compared with testing so that PM was followed by AM). None was found. Consequently data of all AM and that of all PM were pooled and are described below.

Figure 1A shows the raw IL-6 data in both control and exercised populations. In the exercise population, at AM IL-6 level in the serum was 4.42 $\pm$0.10 pg/mL pre-exercise and 4.51 $\pm$ 0.18pg/mL post-exercise. PM the cytokine levels were 4.63 $\pm$ 0.31 pg/mL pre-exercise and 4.64 $\pm$ 0.44 pg/mL post-exercise. In fact, the repeated measures ANOVA revealed no main effect of time frame ($p > 0.05$). In the control population, at AM IL-6 level in the serum was 4.14 $\pm$0.34 pg/mL pre-exercise and 4.03 $\pm$ 0.35pg/mL about 30 mins later. PM the cytokine levels were 4.50 $\pm$ 0.73 pg/mL pre-exercise and 4.91 $\pm$ 0.77 pg/mL about 30 mins later. Interestingly, the repeated measures ANOVA revealed a significant main effect of time frame ($p = 0.008$) with post-hoc differences evident between pre-AM and post-PM ($p = 0.049$), post-AM and post-PM ($p = 0.030$), pre-PM and post-PM ($p = 0.002$). In addition to this, a Mixed-Design analysis with Greenhouse-Geisser adjustments showed a significant interaction ($p=0.014$) between intervention phase (pre-post training, AM vs PM) and group (Exercise vs. Control).

Figure 1B shows the raw cortisol data. In the exercise population, at AM cortisol level in the serum were 109 $\pm$ 53 ng/mL pre-exercise and 93 $\pm$ 39 ng/mL post-exercise. PM cortisol levels were 54 $\pm$ 21 ng/mL pre-exercise

and 55 ± 27 ng/mL post-exercise. Interestingly, the repeated measures ANOVA revealed a significant main effect of time frame (p = 0.004) with post-hoc differences evident between AM-pre and PM-pre (p = 0.010), AM-pre and PM-post (p = 0.023), and finally AM-post and PM-post (p = 0.019). In the control population, at AM cortisol level in the serum were 85 ± 21 ng/mL pre-exercise and 110 ± 28 ng/mL about 30 mins later. PM cortisol levels were 42 ± 14 ng/mL pre-exercise and 42 ± 15 ng/mL about 30 mins later. Interestingly, the repeated measures ANOVA revealed a significant main effect of time frame (p < 0.001) with post-hoc differences evident between all pairs (p < 0.007), except for the PM-pre and PM-post comparison which was not significant (P>0.05). Nevertheless, a Mixed-Design analysis with Greenhouse-Geisser adjustments showed no significant interaction (P>0.05) between intervention phase (pre-post training, AM vs PM) and group (Exercise vs. Control).

→ [Figure 1 near here]

Further analyses were carried out in other to tease out the presence of any confounding factors in the analysis of changes in the levels of IL-6 and/or cortisol with exercise. The anslyses revealed not only that the pre-exercise levels of IL-6 differed from AM to PM (for instance in the trained group, ~4.6% lower at AM, p = 0.05), but also a bivariate analysis (see figure 2) revealed a significant association between cytokine levels pre- to cytokine levels post exercise (r = 0.85, p<0.001). Similarly a bivariate analysis also revealed a significant association between cortisol levels pre-exercise and cortisol levels post-exercise (r = 0.51, p < 0.01) as well as a significant association between cortisol levels pre-exercise and exercise-induced cortisol level changes (r = -0.41, p < 0.05).

Thus, ANCOVAs were run in order to differentiate between true acute exercise response differences and artefacts of differing baseline values. The ANCOVA on IL-6 data revealed that after accounting for pre-exercise differences, there was in fact no difference between AM and PM flux of IL-6 with exercise (p >0.05). Similarly, an ANCOVA on cortisol changes after correcting for baseline levels also revealed no effect of Time-of-day on the flux of this blood ligand (p >0.05).

→ [Figure 2 near here]

Finally, IL-6 and cortisol were neither associated in terms of absolute levels pre-exercise (r = -0.15, P>0.05) nor in terms of exercise- (or time-) induced changed (r = -0.03, P>0.05).

5. *Discussion*

The Principal hypothesis of the current study was that an acute bout of heavy resistance exercise would produce differing concentrations of post-exercise circulating IL-6 in the morning compared to the evening. Our findings partly support this hypothesis. Indeed we show here for the first time, that there is an interaction between time-of-day and cytokine response to resistance exercise. Morning sessions showed a significant change in IL-6 production from pre to post exercise, whereas the evening sessions exhibited no change in IL-6 form pre- to post-exercise. However, when the significant pre-exercise differences in IL-6 concentrations were accounted for in an ANCOVA, any time-of-day difference in cytokine flux was annulled. Therefore, the differences between the AM and PM interventions are merely due to the diurnal variations in the cytokine. Interestingly however, though cortisol was not found to be significantly associated with either 'absolute' or 'changes' in IL-6 levels, there was a tendency for the IL-6 levels to be low when cortisol was high and vice-versa.

The majority of the literature on IL-6 production has focused on long bouts of exercise, rather than intense short bouts. Based on the results of the present study which was conducted with a relative short bout of exercise, the suggestion may be that the production and release of this cytokine can only be seen with longer bouts of exercise. Ostrowski et al. [10], who studied marathon runners found a significant increase of IL-6 after the event. Others, such as Sprenger et al. [31] support the idea that an increased duration of exercise contributes to a greater increase in IL-6. They demonstrated that after a 20km run, IL-6 levels increased and remained elevated for a further 3 hours, while after 1 hour of cycling at 75% VO_2 max, IL-6 levels remained high for 2 hours post exercise. Similarly, Drenth et al. [11] found that IL-6 levels after a 6 hour run displayed a 3-fold increase. However, the lack of definitive stance on the topic comes with other studies including that of Langberg et al. [32] who demonstrated in their work that levels of IL-6 start to decrease after approximately 10 minutes post exercise. Smith et al. [33] found that after short bouts of eccentric exercise, cytokine levels do change, but to a lesser magnitude and at a later time period (in the case of IL-6, this is anywhere between 12-72 hours, and for other cytokines it can as late as 72-144 hours) post exercise compared to endurance exercise. Therefore, although the training-induced cytokine levels changes in the current study were found to be non-significant, it is plausible that a definitive answer could have been had if samples had been taken at later time points post exercise. It is clear from the majority of research that increased exercise duration

contributes to a more significant circulation of IL-6. Longer duration exercise however, is often of a moderate intensity which questions the theory that IL-6 production is due to muscle damage alone.

Based on the current study, the issue of whether IL-6 is solely produced by damaged muscle fibres is raised. On the one hand the baseline levels of the cytokines increased at PM relative to AM, but on the other hand the post-exercise changes in IL-6 were unexpected. They marginally increased in the morning but did not change in the afternoon. Keller et al. [15] and Steensberg [16] suggested that IL-6 production was due to skeletal muscle damage, particularly when said damage occurred due to low intensity long duration exercise, suggesting that as glycogen stores deplete, the liver is notified in order to speed up glucose production. Nonetheless, there is data (e.g. [34]) demonstrating that IL-6 flux can also be related to muscle damage caused by strenuous exercise. They found that the expression of IL-6 was increased as the myofibres were disrupted following eccentric exercise. Research contradicting such findings includes Nosaka & Clarkson [35] who investigated muscle damage after eccentric exercise. They reported pain, swelling, and loss of function after exercise, all classic signs of the inflammatory response, yet found no increase of IL-6 after exercise. Their study however, only tested for cytokine differences immediately, and 24 hours post exercise, so could have missed the fluctuations of cytokine levels. Langberg et al. [32] not only studied the release of IL-6 through muscle, but also found that the circulation of the cytokine is produced in connective tissue and released into blood plasma during prolonged exercise. Overall, it would appear that release of IL-6 is elevated when muscle glycogen is low. In partial agreement to this statement, Nieman et al. [36] found that carbohydrate supplementation during exercise inhibits the production of IL-6. This factor could have affected the results on previous studies as the majority failed to mention whether they included fasting criteria or any details on the meal composition they allowed within their protocols. What's more, since Nieman et al. [36] also demonstrated that glycogen status did not affect the carbohydrate impact on exercise-induced IL-6 production, the fact that our present study included a fasting criteria of 8-10 hours pre-exercise, therefore could not be a factor in the low cytokine levels/limited changes we found.

Our data show that there is no significant difference in the levels of IL-6 between morning and evening post-exercise, when resting values are corrected for. This would tend to support the findings of Derijk et al. [23], who found that IL-6 was immune to cortisol. Brownlee et al. [25] found that the glucocorticoid cortisol is at its highest levels in the morning. Nevertheless as figure 1 in our current paper shows, there was a trend for IL-6

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to be at its lowest when cortisol was high and vice versa, IL-6 tended to be high when cortisol was at its lowest level.

Do we have a definitive answer about IL-6, its diurnal fluctuations and/or release with exercise? The picture is not a simple one. Despite a paucity of literature on whether IL-6 is affected by the circadian rhythm, the research that does exist in this area suggests increases of IL-6 levels may coincide with the circadian rhythm though, it is unclear if these elevated levels of IL-6 are simply linked to the clinical conditions in which they have been monitored (i.e. arthritis [37]) and which show in any case, a diurnal rhythm in the severity of the disease. To add to which, based on our results it remains difficult to ascertain a definitive answer as to whether there is an optimal time-of-day to train. Previous work (e.g. [20, 21]) found that time-of-day impacts significantly on maximal resistance performance, so that in the evening greater muscular torques can be elicited. Training in the evening compared to the morning was also previously suggested to be a matter for consideration [18, 19, 22] owing to the likelihood that skeletal muscles are expected to be more effective in their contractile activities as a result of circadian rhythm factors including body temperature and alertness. In the present study, the amount of work done during exercise was controlled so as to remove this possible source of variation. Whilst resting levels of IL-6 seemed in phase with the expected changes in the levels of cortisol, the levels of the cytokine did not change following exercise in the evening. Future studies should investigate the possibility that an increased muscular efficiency in the evening renders the requirement for IL-6 signalling of muscle damage less necessary in the evening compared with the morning. These studies would also determine not only whether a time course of IL-6 response exits, but also whether there may be a role for IL-6 to allow greater force productions in the evening through decreased muscular sensitivity to elevated exercise activities.

Finally, it should be highlighted here that, whilst admittedly, the serum concentrations of the molecules monitored in the current study may not necessarily have been the same as seen in the tissues of interest i.e. the muscle-tendon complex, (e.g. IL-6 would have been up to 100-fold lower in the serum compared with peritendinous concentrations [32]), in fact serum patterns of changes in IL-6 are found to directly mirror those of the muscle-tendon complex [32]. In other words, the known 'linear relationship' between serum and muscle-tendon complex in situ levels allows the findings from the present study to be generalised to the events at the level of the exercising muscle-tendon complex.

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6. Conclusion

In conclusion, this study has identified that in this population of young, healthy males, there appeared to be no circadian rhythm in the exercise-induced release of IL-6. Cortisol may play a role but this is yet to be confirmed. In fact, the current data would suggest that either a short bout of intense exercise has no effect on the immediate IL-6 and cortisol release, or the timing of blood sampling, whilst optimal for assessing cortisol levels changes may have been too early to observe impact on IL-6. Future work on the possible impact of the circadian rhythm on the two ligands response to exercise, should therefore manipulate the duration of the exercise, as well as carry out multiple blood sampling in order to maximise the chance of observing any time-of-day-dependent protein responsiveness. Nevertheless, based on the current data, we would propose that exercise and/or time-of-day would not interfere with clinical endocrine profiling of a population.

Figure Legends

Fig. 1. Mean percentage change of IL-6 (in A) and cortisol levels (in B) within morning sessions (AM pre to AM post), evening sessions (PM pre to PM post) and the difference between the 2 sessions (AM vs PM). * Denotes significant pairwise comparisons (P<0.05) where the solid brackets are comparisons in the control population and the dashed lines are the comparisons in the exercised population. For IL-6, an interaction between phase and group is present (P=0.014), but this effect disappears when baseline levels are accounted for in ANCOVA analyses. For cortisol (N.B. For simplicity, as all except one pairwise comparisons were significant in the control population, only the non-significant (n.s.) comparison is shown), no interaction between phase and group is present (P>0.05). Data are mean ± SE.

Fig. 2. The impact of pre-exercise levels on protein flux post-resitance exercise. A) IL-6 and B) Cortisol. Data from both AM and PM exercise sessions are pooled.

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