COMPLEMENT PROTEINS (C1est, C4, C6), CIRCULATING IMMUNE COMPLEXES AND THE REPEATED BOUT EFFECT

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ABSTRACT

Purpose. To determine if the complement system is activated following strenuous eccentrically-biased exercise. Secondly, to determine if complement activation is attenuated (repeated bout effect) following a second bout of the same exercise.

Basic procedures. Healthy, active but untrained males performed 2 × 60 min bouts of downhill running, 14 days apart. Samples were taken pre, immediately post (IP), then every hour for twelve hours, and at 24, 48, 72, 120 and 144 h post exercise. Concentrations of C1est, C4, and circulating immune complexes (CIC’s) were determined using standardised nephelometry. C6 was determined using radial immunodifusion. The variables were analysed using a repeated measures ANOVA, with significance set at \( p < 0.05 \).

Main findings. A significant \( p < 0.01 \) run effect was observed for C1est, C4, C6 and CIC’s with the concentrations elevated after run 2 compared with run 1. C1est and C4 exhibited significant time effects \( p < 0.001 \).

Conclusions. The complement system is activated following a strenuous bout of downhill running. Complement proteins and circulating immune complexes do not exhibit the same traditional “repeated bout effect” as many other common markers of muscle damage/inflammation. The increase in complement proteins following the second bout may indicate enhanced innate immune function and/or an amplification of the immune response to tissue damage through interaction with the adaptive immune system.

Key words: inflammation, eccentric exercise, complement, muscle damage

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Introduction

Exercise that is prolonged, intense or eccentric in nature has been shown to elicit structural damage to muscle [1, 2]. It has been well documented that a subsequent bout of the same exercise that induced muscle damage, results in less damage and delayed onset of muscle soreness (DOMS) [3–5]. This is known as the repeated bout effect. The precise mechanism(s) underlying the beneficial adaptive process associated with the repeated bout effect are not well understood. There have, however, been a number of theories postulated, including neural, cellular and mechanical adaptation theories (for review, see [6, 7]). An aspect relating to cellular adaptations that has received considerable attention within the literature is that of the inflammatory response [8].

Complement proteins are found in plasma throughout the body and upon activation, react with each other through an enzymatic cascade [9, 10]. The complement system is intricately involved in inflammatory processes. More specifically, complement proteins are responsible for the manifestation of key inflammatory related actions: (a) changing vascular permeability, (b) altering vascular flow, (c) chemotaxis, and (d) extravasation of leukocytes [11]. Increase in complement proteins C1est, C4, C6 and circulating immune complexes indicates upregulation of the inflammatory response and as such provides insight into the body’s response to tissue injury; in this case benign exercise-induced muscle damage.

To the best of the authors’ knowledge, no other study has investigated the effects of repeated eccentric exercise on complement proteins and circulating immune complexes. Therefore, the aims of this study were firstly to determine if the complement system is activated following a strenuous bout of eccentrically biased exercise. Secondly, to determine if this response would be
attenuated after a second bout of the same exercise, i.e. a repeated bout effect.

**Material and methods**

**Subject selection and screening**

Healthy, active but untrained, males (n = 11) were recruited for the study (age: 19.7 ± 0.37 years; weight: 78.5 ± 30.6 kg; body fat: 14.6 ± 3.2%; VO₂max: 47.8 ± 3.6 ml/kg/min). Height and weight were recorded using a calibrated medical height gauge (stadiometer) and balance scale (Detecto, Webb City, Missouri, USA). Harpenden skinfold callipers were utilised to measure subcutaneous fat. Body composition was assessed using the Drinkwater-Ross method [12]. Selection criteria included the following: age between 18–30 years; no history of leg injury or any other medical condition that would be exacerbated by two bouts of downhill running; no current or regular usage of anti-inflammatory medication. The protocol as well as an informed consent were approved by the Tshwane University of Technology Ethics committee.

**Study method and design**

**VO₂max**

Subjects performed a VO₂max test (Bruce protocol) in the exercise-testing laboratory (ETL), a minimum of two weeks before the first downhill run. The treadmill speed that elicited 75% of the subjects VO₂max was then calculated as the speed for both downhill runs.

**Downhill run**

The subjects performed two bouts of downhill running spaced 14 days apart (run 1 and run 2). On both occasions the subjects arrived at the ETL at the same time of day and in a fasted state. Laboratory conditions (temperature/humidity) were the same for both run 1 and run 2. Subjects warmed up for 5 min running on a level grade. The treadmill was then lowered to –13.5% and subjects ran for 60 min at their predetermined ‘relative’ speeds. The subjects abstained from all physical activity for at least 72 h before run 1, and for the entire duration of the study.

**Blood sampling**

A venipuncture was performed to obtain the baseline blood sample. Following the 60 min downhill run a venous catheter (22 gauge, 2.2 cm) was inserted which was kept patent using saline solution. Blood was drawn at the following times: pre-exercise, immediately post exercise (IP), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h after (14 samples x 15 ml per sample = 210 ml blood over approximately 14 h). In addition, subjects were required to return to the ETL at 24, 48, 72, 96, 120 and 144 h post exercise, for additional blood draws (6 days x 15 ml = 90 ml). The same procedure was followed after 14 days.

Blood was allowed to stand at room temperature for 30 minutes (serum) and was then as spun down for 20 minutes at 1000 × g. Aliquots were frozen and stored at −70°C in .5 ml eppendorf tubes until analysed. Subjects remained in the ETL for 12 h after run 1 and run 2. They were provided with food and encouraged to drink ad libitum.

**Blood analysis**

**Validity and reliability**

All blood samples were analysed in duplicate. A coefficient variation of less than 10% between the duplicate samples was required for all measures.

**Complement proteins**

**C1est and C4**

Determination of serum complement proteins C1est and C4 was performed using specific anti­sera to C1est and C4. The immune complexes formed were measured in a Behring nephelometer (Behring Diagnostics, Germany) and the amount of C1est, and C4 was calculated by comparison with standards of known concentration.

**C6**

C6 was determined by radial immunodiffusion (The Binding Site, UK). The assay was performed by adding serum and controls of known C6 concentrations to radial immunodiffusion plates containing non-specific antibody in an agarose gel. The diameter of immunoprecipitating antigen-antibody complexes radiating out of the wells was measured and compared against a calibrated curve drawn from a range of samples of known concentration.

**Circulating immune complexes**

The levels of circulating immune complexes (CIC) were determined by particle-enhanced nephelometry. The assay utilised polystyrene particles coated with human C1q, which was added to the subjects’ sera. Light scatter due to agglutination of the C1q coated particles in the presence of CIC was measured in a Behring nephelometer (Behring Diagnostics, Germany) whereby
the concentration of CIC was determined in relation to the amount of agglutination detected.

**Data analysis**

Each dependent variable was analysed before and after the two conditions (run1/run2), using a repeated measures analysis of variance, with significance set at \( p < 0.05 \). Where significance was found for main or interaction effects, a Bonferroni *post hoc* analysis was performed.

**Results**

A significant \( (p = 0.0009) \) run effect was observed for C1esterase inhibitor, with the concentrations being elevated by 7% after run 2 compared with run 1. C1est was significantly elevated at 9 h and 10 h post exercise (time effect). No significant difference in the resting concentrations before each run was observed for C1est. By 24 h post exercise, C1est levels had returned to pre-exercise concentrations in both runs (Fig. 1).

C4 exhibited a significant \( (p < 0.0001) \) run effect, being elevated by 17% after run 2 compared with run 1. C4 was significantly elevated at 7 h and 9 h post exercise (time effect, \( p < 0.001 \)). There were no differences in the resting levels of C4 before each run. At 24 h post exercise, C4 levels had returned to baseline concentrations after both runs (Fig. 2).

A significant \( (p < 0.0001) \) run effect was observed for C6, with concentrations being significantly elevated by 4% after run 2 compared with run 1 (Fig. 3). There were no differences in the resting levels of C6 before each run.

**Figure 1.** C1est concentrations measured before and then at hourly and 24 hour intervals following two bouts of downhill running. Data are means ± SE. Significant \( (p < 0.001) \) time effect compared to baseline (pre) concentrations. The symbol “//” on the X axis signifies a change in time interval (samples no longer measured at hourly intervals). Pre = 30 min before the run; IPE = immediately post exercise

**Figure 2.** C4 concentrations measured before and then at hourly and 24 hour intervals following two bouts of downhill running. Data are means ± SE. Significant \( (p < 0.001) \) time effect compared to baseline (pre) concentrations. The symbol “//” on the X axis signifies a change in time interval (samples no longer measured at hourly intervals). Pre = 30 min before the run; IPE = immediately post exercise

**Figure 3.** C6 concentrations measured before and then at hourly and 24 hour intervals following two bouts of downhill running. Data are means ± SE. The symbol “//” on the X axis signifies a change in time interval (samples no longer measured at hourly intervals). Pre = 30 min before the run; IPE = immediately post exercise

**Figure 4.** Concentrations of circulating immune complexes measured before and then at hourly and 24 hour intervals following two bouts of downhill running. Data are means ± SE. The symbol “//” on the X axis signifies a change in time interval (samples no longer measured at hourly intervals). Pre = 30 min before the run; IPE = immediately post exercise
Circulating immune complexes were significantly increased by 42% after run 2 compared with run 1. Peak concentrations after run 1 and run 2 were observed at 12 h and 10 h post exercise, respectively (Fig. 4). Differences in the resting concentrations before run 1 and run 2 were statistically non-significant.

Discussion

The aim of this study was to determine (a) if the complement system is activated by eccentrically-biased exercise (downhill running), and (b) if the manifestation of this response would be attenuated after a second bout of the same exercise, i.e. a repeated bout effect.

Complement proteins C1 est, C4 and C6 as well as circulating immune complexes (CIC) all exhibited a significant elevation after the second run when compared to the first run (bout effect). Increases in complement proteins and immune complexes would imply the manifestation of a more pronounced pro-inflammatory response following the second bout. It is arguable that the increased complement could in part be attributed to the elevated immune complexes, since complement may facilitate the removal of these complexes from circulation [11]. The increase in C4 implies that the classical pathway of complement was activated while the ‘simulation' [11]. The increase in C4 implies that the classical pathway was activated and the increased complement could in part be attributed to the elevated immune complexes, since complement may facilitate the removal of these complexes from circulation [11]. The increase in C4 implies that the classical pathway was activated while the ‘simulation' [11]. The increase in C4 implies that the classical pathway was activated while the 'simulation' [11]. The increase in C4 implies that the classical pathway was activated while the ‘simulation' [11].

Despite the classical repeated bout effect being produced for creatine kinase (reported elsewhere [14]) the complement proteins failed to show the same phenomenon. Recently, the study by Hubal et al. [15] produced similar observations. In their study a number of inflammatory genes were upregulated following a repeated bout of 300 lower limb eccentric-concentric actions.

The question still remains as to why a pro-inflammatory response was seemingly exacerbated after run 2 compared to run 1? Although speculative, an explanation for this observation may be related to an enhancement of the immune/inflammatory response to the second bout of tissue damaging exercise via an innate:adaptive immunity interaction or collaboration. Recent research provides support for this hypothesis. Specifically, Chan et al. [16] have proposed that injured tissue (ischemic) expresses neoepitopes that are recognised by natural antibodies. The subsequent binding of the natural antibodies to these novel antigens results in the formation of immune complexes (antigen:antibody) which in turn activate complement. This interaction may help explain the results of the present study. Following the first bout of downhill running there may have been the 'liberation' of novel antigens (neoepitopes) as a result of the muscle damage. Weiser et al. [17] have postulated that these antigenic determinants may be exposed as a result of 'subtle alterations in plasma membranes'. Natural antibodies, specifically natural IgM, would then bind to these epitopes and activate the classical pathway of complement [9]. Additional synthesis of IgM following exposure to these antigens would result in an elevation of natural antibodies (i.e development of immune memory). On exposure to the second bout of downhill running the elevated IgM (memory) would facilitate a more pronounced inflammatory response through an enhanced activation of the classical pathway of complement. In support of this, C4, which is indicative of classical pathway activation, was significantly elevated after the second run.

Conclusion

Based on our findings, the premise that inflammation as a whole is down regulated or suppressed following a repeated bout of muscle damaging exercise is questionable.

Significant elevations after run 2 compared with run 1 were observed for C4, C6, C1 est and CIC. Thus, 60 min of downhill running followed by an identical bout two weeks later, fails to ‘dampen' the systemic inflammatory response. This is in contrast to what has been observed for creatine kinase and other indirect markers of muscle damage. Overall, the surprisingly minor changes in complement proteins, in response to a relatively severe bout of eccentric exercise suggests that either (a) little muscle damage was incurred (which based on CK responses [14] did not seem to be the case), or (b) changes in systemic complement protein concentration may not be sensitive markers of what is occurring in skeletal muscle. These results warrant further investigation as this is the first study to report such an effect and participant numbers were limited.

References


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