

Technical Note

Acquisition Delays Affect Lymphocyte Subset Counts but not Markers of Exercise-induced Apoptosis

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ABSTRACT

International Journal of Exercise Science 6(3) : 250-255, 2013. With the emergence of shared facilities, the possibility of a processing delay is increased as time on instrumentation becomes less available. The purpose of this investigation was to evaluate the effect of a 24h time delay on lymphocyte subset concentration, as well as for the apoptotic marker annexin V. Fourteen healthy individuals completed an incremental treadmill test to exhaustion, and blood samples were obtained before and after exercise. The samples were labeled in duplicate with mixtures consisting of flow cytometry staining buffer, the biomarker for early-phase cell death (annexin V), and antibodies for specific lymphocyte phenotypes (CD4, CD8, and CD19). Samples were labeled for 30-min, centrifuged, and decanted, before the addition of RBC Lysis Buffer. Upon the completion of processing, the first set of samples were immediately analyzed using flow cytometry and the remaining duplicate samples were acquired 24 h later. Data were analyzed using a paired sample t-test with significance accepted at the $p < 0.05$ level. For blood draws obtained at rest, no differences between acquisition days were observed with regard to cell volume for any lymphocyte subfraction. However, blood samples obtained following an exhaustive exercise bout had significant decreases in CD4+ ($p=0.002$) and CD8+ ($p= 0.021$) concentration between acquisition days. Processing delays did not affect the number of apoptotic cells in any lymphocyte subset either at rest or following exercise. As the number of apoptotic cells was unaffected by processing, the reduction in cell concentration is likely due to mechanisms other than programmed cell death. It is possible that exercise makes lymphocytes more susceptible to necrosis during the post-activity period.

KEY WORDS: Programmed cell death, annexin V, exhaustive physical activity

INTRODUCTION

Intense physical activity has been shown to alter immune system variables such as subset count (1, 5), cytokine production (4,

16, 20), and proliferative response (17, 18). One common response is a biphasic modulation in overall lymphocytes, resulting in an increase during exercise, but a reduction in number through the recovery

period following exertion (1, 6). Our laboratory has investigated potential mechanisms surrounding the reduction of lymphocytes with exercise, termed exercise-induced lymphocytopenia, in the framework of elimination via apoptosis, egress from the cardiovascular system into the lymphoid pools, or contributions by both processes (2, 10). While strong evidence exists to support cellular migration as the primary influence (2, 3, 22), the contribution of lymphocyte apoptosis is less clear. Some studies have observed increased lymphocyte apoptosis with acute high-intensity exercise (7, 8, 14), however other studies do not indicate a change in cell death status of lymphocytes following an acute bout (19, 21).

We have noted that a potential explanation for the differences observed in the exercise-induced lymphocyte apoptosis literature is the lack of a standardized methodology for obtaining this measurement (11). Cell death due to exercise has been assessed morphologically (7, 14), and using various biomarkers such as annexin V (8, 9, 23), CD95 (8, 9), and alterations in mitochondrial transmembrane potential (24, 25). This has resulted in apoptotic yields following exercise that have varied between 2.5 (23) and 51% (7).

With regard to biomarker methodology, many investigators have limited access or time on equipment, particularly when utilizing a shared or "core" facility. It is possible that blood samples obtained on an exercise testing day would not be able to be processed until a day later when the investigator was able to access equipment. In a previous investigation, we found that a delay in blood processing following exercise similar to methods used in many of

the previously noted biomarker investigations, resulted in decreased overall lymphocyte apoptotic index assessed via morphology (13). As an acquisition delay has the ability to affect exercise-induced apoptosis, we wished to determine the consequence of suspending flow cytometric measures on lymphocyte subfractions following exercise while utilizing a common biomarker for cell death, annexin V. We hypothesized that evaluated measurements taken on fresh blood samples would provide a greater yield with regards to cell volume and cell death compared with readings from the same samples obtained 24h later.

METHODS

Participants

A total of 14 individuals (female=6, male=8) were recruited from Western Kentucky University's Kinesiology, Recreation and Sport Department, women's volleyball team, and swim team (see Table 1). The purpose of the study was explained to each participant and they signed a written informed consent, which was approved by the institutional Human Subject Review Board.

Table 1. Subject characteristics reported as mean±SE.

	Age (years)	Height (cm)	Weight (Kg)
Male	20.0±1	170.7±2.4	84.2±2.2
Female	19.8±1	163.8±4.9	63.8±2.8

Protocol

Participants completed a single treadmill run to exhaustion. The bout began with a three minute walking warm up at 80.5 m·min⁻¹ (3.0 mph), and the first running stage was 134.1 m·min⁻¹ (5.0 mph). Speed was then increased incrementally by 26.8-52.6 m·min⁻¹ (1-2 mph) with each 3-min

stage until a comfortable running velocity was established (self-selected by the participant). Once constant running speed was attained, treadmill grade was increased 2.5% with each successive stage until volitional fatigue. Adjunct measurements obtained during the test included heart rate, and ratings of perceived exertion.

Blood Analysis: Blood samples (150 ml) were obtained via finger-stick into capillary tubes before (Pre) and immediately following the treadmill run (Post). All antibodies and reagents were obtained from eBioscience (San Diego, CA) unless otherwise noted. Whole blood (40 mL) was labeled in duplicate with mixtures consisting of flow cytometry staining buffer (250 mL), the biomarker for early-phase cell death (annexin V in a 1:900 dilution), and antibodies for specific lymphocyte phenotypes (CD4, CD8, and CD19, also in 1:900 dilutions respectively) in a microcentrifuge tube (Posi-Click, Denville Scientific Inc., South Plainfield, NJ) certified to be RNase, DNase, and pyrogen free. Blood samples were processed as described previously (12). Briefly, samples were incubated in the dark at room temperature, centrifuged, then decanted, and RBC Lysis Buffer was added and allowed to incubate for 15-min. PBS (Sigma Aldrich, St. Louis, MO) was added, then samples were centrifuged, decanted, and vortexed. The final sample of resuspended sample was 250 mL, and tubes remained capped until just prior to acquisition. Therefore, the total volume of sample was the same between acquisition days. The first set of samples were immediately analyzed using flow cytometry (C6, Accuri, Ann Arbor, MI) and the remaining duplicate samples were stored at 4°C until acquisition 24 h later.

Statistical Analysis

Data were analyzed using a paired sample t-test (SPSS Statistics 18.0, IBM Corporation, Somers, NY). Significance was accepted at the $p < 0.05$ level.

RESULTS

For baseline blood draws, no differences between acquisition days were observed with regard to cell concentration for any lymphocyte subsets (CD4+, $p = 0.23$; CD8+, $p = 0.19$; CD19+, $p = 0.45$). However, blood samples following an exhaustive exercise bout displayed significant decreases between acquisition days. CD4+ cell counts were significantly less on day 2 compared with day 1 ($P = 0.002$) (see figure 1). Similarly, CD8+ counts were significantly lower on day 2 compared with day1 ($P = 0.021$) (see figure 2).

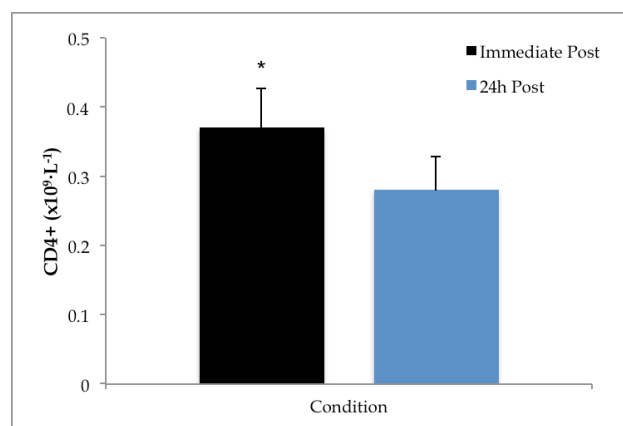


Figure 1. Postexercise helper T-cell (CD4+) volume was significantly decreased on the second day of analysis compared with volume observed on the first day of acquisition, reported as mean and SE. * denotes a significant difference between processing days ($p=0.002$).

Acquisition delays did not affect the number of apoptotic cells in any lymphocyte subset either at rest or following exercise ($p > 0.05$ in all cases).

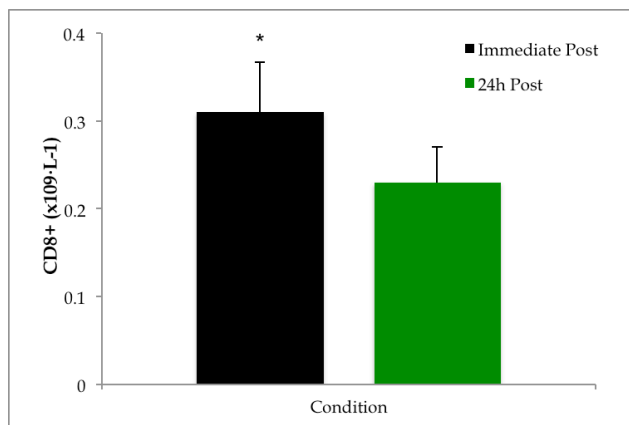


Figure 2. Cytotoxic T-lymphocyte (CD8+) volume from postexercise samples was significantly reduced with a 24h delay in processing. * represents a significant difference between days of processing ($p = 0.021$).

DISCUSSION

The purpose of this investigation was to evaluate the effect of acquisition time on lymphocyte subset count and apoptosis. We hypothesized that a delay would affect both lymphocyte cell concentration, as well as the expression of the apoptotic marker annexin V. The primary finding is that a 24-h acquisition delay significantly reduced the CD4+ and CD8+ cell concentration following exercise, while resting values were unaffected. In addition, acquisition delays of no more than 24-h appear to have little effect on early-phase apoptosis in the primary lymphocyte subsets.

While these results are not similar to our earlier investigation (13), there could be a number of explanations for the discrepancy. Previously, we reported that the apoptotic yield decreased as processing time increased (i.e. the number of observed apoptotic cells was lower as the time for processing was extended). In that study, the morphological method was utilized. Morphological techniques have generally produced much larger exercise-induced

apoptotic indexes (13-15), and it is possible that cells other than the subsets that we evaluated in the present study (CD4+, CD8+, CD19+), such as natural killer cells, could primarily account for the observed response. Additionally, the morphological method considers cells in all stages of the cell death process, whereas annexin V is a biomarker specific to early-phase apoptosis. It seems likely that the reduction of apoptotic cells in the previous investigation (13) was due to the deletion of cells in late-stage apoptosis, rather than those in the early-phase.

We found that an acquisition delay resulted in decreased cell volume for the CD4+ and CD8+ lymphocyte subsets. In one of the first investigations to evaluate the effect of physical activity on lymphocyte apoptosis, Mars et al. reported the presence of apoptotic cells up to 24 h following a bout of exhaustive exercise (7). They suggested that an apoptotic signal could persist for several hours following exercise, or that activation-induced cell death could play a more prominent role during the recovery period (7). As the absolute number of apoptotic cells in the present investigation was unaffected by processing, the reduction in cell volume is likely due to mechanisms other than programmed cell death. It is possible that exercise makes these cells more susceptible to necrosis during the post-activity period.

In conclusion, while we found that a 24h acquisition delay decreased the volume of T lymphocytes (both helper and cytotoxic) it had no effect on the number of cells expressing the early apoptotic marker annexin V. Because of this, it is likely that the reduction in lymphocyte volume was due to necrotic cell death as cells displaying

the marker for programmed cell death remained unchanged. Future investigations should employ morphological and biomarker techniques toward elucidating the contribution of these mechanisms to cell volume reductions. From a practical standpoint, processing as we have described in the current investigation should proceed to analysis by flow cytometry without delay to avoid potential error, particularly when reporting the relative percentage of apoptotic lymphocytes. Lastly, future investigations could be directed at determining modifications to the protocol that would overcome the time and workflow limitations that are associated with evaluating exercise-induced lymphocyte responses when working in a shared facility.

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