Video Article

Finger-stick Blood Sampling Methodology for the Determination of Exercise-induced Lymphocyte Apoptosis

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URL: http://www.jove.com/details.php?id=2595
DOI: 10.3791/2595
Citation: Navalta J., McFarlin B., Simpson R., Fedor E., Kell H., Lyons S., Arnett S., Schafer M. (2011). Finger-stick Blood Sampling Methodology for the Determination of Exercise-induced Lymphocyte Apoptosis. JoVE. 48. http://www.jove.com/details.php?id=2595, doi: 10.3791/2595

Abstract

Exercise is a physiological stimulus capable of inducing apoptosis in immune cells. To date, various limitations have been identified with the measurement of this phenomenon, particularly relating to the amount of time required to isolate and treat a blood sample prior to the assessment of cell death. Because of this, it is difficult to determine whether reported increases in immune cell apoptosis can be contributed to the actual effect of exercise on the system, or are a reflection of the time and processing necessary to eventually obtain this measurement. In this article we demonstrate a rapid and minimally invasive procedure for the analysis of exercise-induced lymphocyte apoptosis. Unlike other techniques, whole blood is added to an antibody panel immediately upon obtaining a sample. Following the incubation period, red blood cells are lysed and samples are ready to be analyzed. The use of a finger-stick sampling procedure reduces the volume of blood required, and minimizes the discomfort to subjects.

Protocol

1. Finger-Stick Blood Sampling

1. Collection of whole blood is typically taken at rest for a baseline measurement (usually following 10-15 min seated rest), during or immediately following an exercise bout, and throughout the post-exercise period (generally from 1-3 hours following cessation of the exercise).
2. Using Universal Precautions, first sterilize the fingertip with 70% isopropyl alcohol.
3. Next, puncture the site using an automated lancet or similar device. Although we use a 30-gauge lancet to provide for subject comfort, a larger gauge needle could be utilized.
4. Wipe away the first drop of blood, and then collect blood into capillary tubes or microvettes treated with lithium heparin to prevent clotting.

2. Cell Phenotyping and Apoptosis Staining

1. Add 10 µL heparinized whole blood to titred antibody panel in 250 µL binding buffer (see table 1 schematic). We have used a 1:20 dilution factor with success, but each laboratory should titre their reagents to determine the best working solution.
   Table 1. Antibody panel for the determination of exercise-induced apoptosis in leukocyte subsets. Early apoptosis was defined by the expression of Annexin V, while late apoptosis was defined by double positive labeling with Annexin V and 7-AAD. Necrotic cells were Annexin V- and 7-AAD+ cells only.

Tube 1	Tube 2	Tube 3	Tube 4
Cells only tube	Annexin V - FITC	Annexin V - FITC	Annexin V - FITC
	Anti Human CD4 - PE	Anti Human CD8 - PE	Anti Human CD19 - PE
	7-AAD	7-AAD	7-AAD
	Anti Human CD45RA - APC	Anti Human CD45RA - APC	

   7-AAD = 7-Amino-actinomycin D, APC = Allophycocyanin, CD4 = Helper T lymphocytes, CD8 = Suppressor/cytotoxic T lymphocytes, CD19 = B lymphocytes, CD45RA = naíve cell subsets, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin.
2. Incubate at room temperature in the dark for 30 minutes.
3. Centrifuge at 1075 x g for 5-10 minutes.
4. Decant, add 300 µL Red Blood Cell Lysis Buffer, and thoroughly vortex.
5. Incubate at room temperature for 15 minutes.
6. Add 300 µL PBS to stop RBC lysis reaction.
7. Centrifuge at 1075 x g for 5-10 minutes.
8. Decant, rack, and add 50 µL binding buffer.
9. Analyze by flow cytometry.

It is recommended that at a minimum, the following controls be utilized: a cells only tube to serve as a negative control in detecting background autofluorescence, and tubes containing each individual fluorochrome to set compensation during initial set up of the flow cytometer. In addition, we have used compensation standard beads successfully to set up our experiments.

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Discussion

A minimally invasive sample collection procedure and subsequent analysis is demonstrated for the analysis of both early and late phases of exercise-induced lymphocyte apoptosis. It is also possible to clearly exclude those cells, which are necrotic, and not apoptotic. This procedure overcomes the drawbacks and discomfort associated with invasive venipuncture from the antecubital region at multiple time points prior to, during, and immediately after exercise, or securing a catheter for the duration of the exercise bout. In terms of the investigators, such procedures typically require specialized training in phlebotomy, which not all individuals have. In addition, subject recruitment and retention is expected to be enhanced using this procedure, thus increasing the potential statistical power of finding a significant effect if one is present.

Previous investigations employing venipuncture blood sampling have reported resting and post-exercise values for overall lymphocytes. Utilizing annexin V, Simpson *et al.* reported approximately 1.2% apoptotic lymphocytes at rest, and ~1.3% following treadmill running [1]. Similarly, Steensberg *et al.* reported ~1.8% total apoptotic lymphocytes at rest, and approximately 2.1% after 2.5 hours of running [2]. Our results for apoptotic lymphocytes using the finger-stick procedure described here are similar, with 0.7±0.2% observed at rest, and 1.1±0.4% post-exercise in five subjects who completed a graded exercise test (unpublished results).

One of the limitations of previous methodology used to determine exercise-induced immune cell death was the amount of time required to process a blood sample. In previous investigations, samples obtained immediately postexercise by venipuncture were subjected to a lengthy isolation and preparation process (as long as 2 h) before eventually being assessed for cell death [3-5]. It is reasonable to speculate that such processing delays may artificially elevate reported post-exercise lymphocyte apoptosis values. In some cases, annexin V positive lymphocytes have been reported to be as high as ~25% [6]. Further interpretation of this methodological confounder raises the question of whether the reported values for lymphocyte apoptosis were due to exercise or blood processing delays. We suggested that previous methodology did not accurately reflect the effect that exercise has on inducing cell death in immune cells [7].

Utilizing the methodology demonstrated here decreases the amount of blood required for each collection time point. Also, our approach decreases sample-processing time, which may reduce the incidence of false positives for lymphocyte apoptosis. In this protocol, whole blood is added to the antibody panel within 30-seconds after blood collection, thus reducing the potential that lymphocytes progress through the apoptotic process and are eliminated prior to antibody staining. This procedure overcomes the measurement limitations previously discussed, and allows for the quantification of apoptotic lymphocytes induced by exercise as a physiological stimulus. Future study with regards to this methodology should evaluate the potential differences with larger gauge lancets.

In addition, the methodology described in this paper allows for the determination of both early and late stage exercise-induced apoptosis in lymphocyte subsets (see Table 1). Cells stained with Annexin V only are in the early phase of apoptosis, whereas double-staining with Annexin V and 7-AAD indicate progression through the cell death process to the latter stages. Cells marked with 7-AAD alone are indicative of undergoing cell death through necrosis. Phenotyping of lymphocytes is accomplished through cluster of differentiation markers (CD4 = helper T-lymphocytes, CD8 = cytotoxic/suppressor T-lymphocytes, CD45RA = naive subfractions, CD19 = B-lymphocytes).

Disclosures

No conflicts of interest declared.

Acknowledgements

This research was supported by a New Faculty Grant from the Faculty Scholarship Council at Western Kentucky University.

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