

DOES DOWNHILL RUNNING ALTER MONOCYTE
SUSCEPTIBILITY TO APOPTOSIS?

Kathryn A. F. Pennel

Thesis Prepared for the Degree of
MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

August 2016

APPROVED:

Brian K. McFarlin, Major Professor
Jakob L. Vingren, Committee Member
David W. Hill, Committee Member
Allen Jackson, Chair of the Department
of Kinesiology, Health
Promotion and Recreation
Bertina Hildreth Combes, Interim Dean
of the College of Education
Victor Prybutok, Vice Provost of the
Toulouse Graduate School

Pennel, Kathryn A. F. *Does Downhill Running Alter Monocyte Susceptibility to Apoptosis?* Master of Science (Kinesiology), August 2016, 30 pp., 13 figures, references, 22 titles.

Introduction/purpose: Recovery from muscle damage involves a type of programmed cell death known as apoptosis. Damage Associated Molecular Patterns (DAMPs) are released after muscle damage and may cause premature apoptosis in monocytes infiltrating the damaged site. This may alter the time course of events towards recovery. Therefore, the purpose of this study was to investigate if downhill running causes a change in the susceptibility of monocytes to apoptosis. Methods: Participants (5 male, 6 female) completed a downhill running protocol consisting of 6-5 minute bouts at a speed of 6-9mph on a -15% grade treadmill. Venous blood samples were collected immediately pre-exercise (PRE), in addition to 4 -h, 24 -h and 48 -h post-exercise. Creatine kinase (CK) was measured to give an indication of muscle damage. Monocytes were analyzed by flow cytometry for expression of multicaspase and annexin v reagent was used to detect changes in the plasma membrane. A MILLIPLEX MAP human early apoptosis magnetic bead 7-plex kit (EMD Millipore, Billerica, MA) was used to assess the relative concentration of phosphorylated protein kinase B (Akt), Bcl-2 associated death promoter (BAD), B cell lymphoma-2 (Bcl-2), active caspase-8, active caspase-9, c jun N terminal kinase (JNK) and tumor protein p53 by Luminex multiplex assay. Results: CK peaked at 24- h. Monocytes showed greater expression of multicaspase at 24 -h and 48 -h than at PRE. Bcl-2, p53 and caspase-8 were all significantly greater at 24 -h than at PRE. Conclusion: Downhill running did alter the

apoptotic response of monocytes and therefore may be important in the recovery process from muscle damage.

Copyright 2016

by

Kathryn A. F. Pennel

DOES DOWNHILL RUNNING ALTER MONOCYTE SUSCEPTIBILITY TO APOPTOSIS?

Introduction

Strenuous exercise often leads to short-term muscle damage, which can hinder subsequent activity in beginners to elite athletes. When a muscle becomes damaged the sarcolemma bursts, resulting in the release of intracellular proteins referred to as Damage Associated Molecular Patterns (DAMPs). A large number of DAMPs have been identified including heat shock proteins (HSP), High Mobility Group Box 1 (HMGB1) and nucleic acids (1). DAMPs initiate a cascade of physiological events that are collectively referred to as sterile inflammation (1, 2). Monocytes are recruited via cytokines and chemokines to the damaged area, where their function is to phagocytose debris and move the muscle towards recovery (1, 2, 3).

The role of the immune system in muscle inflammation and recovery is an established; however, despite this knowledge there is significant individual variability with respect to recovery from exercise. Variability in muscle recovery is partially explained by differences such as age, gender and fitness level (4, 5, 6). However these factors do not account for all the variation observed. Thus, it is reasonable to speculate that perhaps the individual variability may be attributed to specific differences in the efficiency of DAMPs response to damage. For example, while DAMPs initiate sterile inflammation, recent research has also demonstrated that they may stimulate premature apoptosis in infiltrating phagocytes.

Apoptosis is a form of programmed cell death that is characterized by distinct morphological changes including membrane blebbing and DNA fragmentation (7).

Apoptosis can be initiated via intrinsic or extrinsic pathways, which each consist of a

different cascade of events ultimately leading to a caspase cascade and cell death (7, 8).

Lymphocytopenia is known to occur following exercise and this may in part be due to apoptosis (9). Premature apoptosis would result in incomplete action of phagocytes and delayed resolution of sterile inflammation. The immune response would be perpetuated with the need for recruitment of a greater number of monocytes after the early responding monocytes died via apoptosis. It is not currently known which specific DAMPs cause an apoptotic response and through which pathways they act. Chemotactic agents such as Staurosporine have been shown to induce apoptosis in monocytes (7, 8). Staurosporine initiates apoptosis in a broad way, through both the extrinsic and intrinsic pathway (7, 8).

Apoptosis can be initiated extrinsically through multiple different receptors and intrinsically from many different stimuli. The most common way that apoptosis is initiated is through death receptor activation such as Toll-like Receptor-4, TRAIL and FasL (10, 11). Once activated these death receptors can activate caspase-8, which can lead to a caspase cascade involving caspase-9, caspase-3, caspase-6 and/or caspase-7, which ultimately results in the morphological changes associated with apoptosis (10, 11). Apoptosis can also be initiated intrinsically by factors such as tumor protein p53 and c-jun N terminal kinase (JNK) acting on the mitochondria to cause release of downstream signaling proteins that lead to cell death by apoptosis (10). Other proteins involved in apoptosis include protein kinase B (Akt), which can be activated by growth factor receptor. Akt can block the actions of pro-apoptotic protein B-cl2 associated death promoter (Bad), which

functions in blocking B cell lymphoma-2 (Bcl-2) (10). The anti-apoptotic protein Bcl-2 blocks the release of pro-apoptotic proteins from the mitochondria (10). It is unclear which specific proteins are involved in the increased apoptosis observed after exercise.

Previous studies have shown that both leukocytes and lymphocytes have an increased apoptotic index following strenuous aerobic exercise (12, 13, 14). The apoptotic index is defined as the ratio of apoptotic cells to non-apoptotic cells. Only one published study to our knowledge has looked at the effect of eccentric exercise on apoptosis and they reported an increase in apoptosis of lymphocytes after a bout of downhill running (15). However, no studies, to our knowledge have investigated the effect of eccentric exercise on the susceptibility of monocytes to undergo apoptosis. Eccentric exercise is known to induce greater muscle damage than aerobic exercise and therefore may cause greater release of apoptosis-inducing DAMPs (16). This study aims to determine if downhill running causes a change in the susceptibility of monocytes to apoptotic signals from DAMPs/Staurosporine

Purpose

The purpose of this study was to determine if downhill running causes a change in the susceptibility of monocytes to apoptosis.

Specific Aims and Hypothesis

Specific Aim 1: To use a flow cytometry technique to determine if a bout of downhill running changes the susceptibility of monocytes to apoptosis. The early and middle phase apoptotic response was determined through the measurement of phosphatidylserine and Caspases-3 caspase-6 and caspase-7.

Specific Aim 2: To use a multiplex protein technique to determine if a bout of downhill running changes the susceptibility of monocytes to apoptosis. The relative concentration of phosphorylated pro-apoptotic markers active Caspase- 3, Caspase-8, Bcl2- associated death promoter, and tumor protein p53 and the anti-apoptotic markers protein kinase B (Akt), c-jun N terminal kinase (JNK) and B cell lymphoma-2 (Bcl-2) were analyzed.

We hypothesize that downhill running will cause an increase in monocyte apoptosis and expect to detect greater expression of phosphatidylserine on the outer surface of the plasma membrane and greater expression of multicaspase. We also hypothesize that there will be increased relative concentration of phosphorylated caspase-8, caspase-9, Bad, Bcl-2, Akt and JNK in the post-exercise samples compared to pre-exercise.

Experimental Design and Methodology

Inclusion and Exclusion Criteria

Eleven participants (5 men and 6 women) aged between 19 and 34 were recruited for the study. Participants were required to have a body mass index between 18 and 25 kg·m⁻² and to be non-resistance trained in the 6- months prior to the study. Additionally participants needed to have a normal percentage body

fatness (24 to 35% women) or (15 to 20% men) and maximal oxygen uptake. Subjects were allowed to participate only if they were free from pre-existing musculo-skeletal problems, chronic inflammatory diseases, and autoimmune disorders, and if they appeared healthy in accordance with the standards of ACSM (17). Individuals who habitually consumed anti-inflammatory medications were excluded. Pregnancy and cigarette smoking also resulted in exclusion from the study. All participants were required to give written and verbal consent before undertaking any aspect of the study. The study gained approval from the University of North Texas Institutional Review Board before proceeding and was conducted in accordance with the declaration of Helsinki.

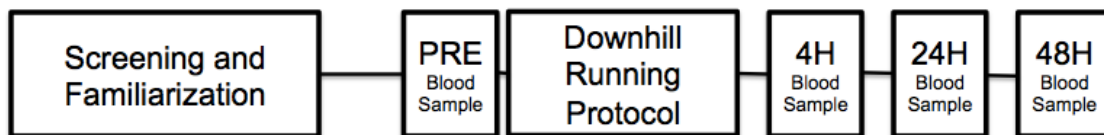


Figure 1. Timeline of events in study

Initial Testing

Participants initially attended the laboratory for familiarization and initial performance measures. Participants were then asked to complete a medical history questionnaire and to provide written consent. A whole body Dual-Energy X-ray absorptiometry (DXA) scan (Lunar Prodigy General Electric Company, Madison, WI) was used to determine percentage body fat to ensure each participant met the inclusion criteria.

Participants then performed a VO₂ max test on the treadmill using a metabolic analysis system (Ultima PF, Medical Graphics, St Paul, MN). The test consisted of a 5-min warm up at a speed that was individually selected for each participant (between 94 and 121). For the first stage of the test the speed was increased to 5.0-6.5mph for 2-min. The subsequent stages involved increasing the speed by 1mph every 2-min. Each participant was asked to continue running until they were unable to continue, at which point they were asked to signal to stop the treadmill or to jump off holding the hand rails. The oxygen uptake was recorded at the end of each 2-min stage. The Borgs Rating of Perceived Exertion scale (18) was used to gauge participants perceived rate of exertion at the end of each stage. Heart rate was monitored using a Polar monitor (Polar Electro Inc, Lake Success, NY) and recorded after each stage. If the participant passed the initial screening (average % body fatness and maximal oxygen uptake) they were accepted into the rest of the study.

Downhill Running

On the day of the downhill running bout participants arrived in the morning 10-Hours fasted. A skilled technician collected the pre-exercise venous blood sample using a butterfly needle (Greiner Bio-One). Heparin tubes were used to collect blood for the apoptosis assay and serum tubes were used to collect blood sample to be analyzed for creatine kinase (CK).

Participants then performed a downhill running protocol involving a maximum of 6, 5-minute bouts of running at a -15% grade on the treadmill. Men

began the initial 6-minute stage at 6mph and the speed was increased incrementally to 9mph. Female participants speed was increased gradually from 6mph to 8mph throughout the exercise protocol. The speed was increased by 0.5mph to 1.0mph per stage. Blood was collected 4-Hours, 24-Hours and 48-Hours after completion of the downhill running protocol and analysis occurred immediately.

Serum Creatine Kinase Measurement.

An enzymatic assay (Pointe Scientific) was used to assess CK levels in serum. CK enzyme reagent was added to CK buffer at a 1:4 ratio to make the working reagent, which was incubated at 37°C for 4 -min. The serum from each participant at each time point was added to the working reagent water at a ratio of 1:2. After mixing the cuvette for 2 -min the absorbance was read and recorded twice. The average absorbance difference was calculated and multiplied by 3376 to obtain results in U/L. This unit refers to the NADP ($\mu\text{mol/L}$) oxidized by CK in 1 minute. The purpose of the CK measure was to give confirmation that muscle damage had occurred.

Isolation of PBMCs

To assess monocyte activity, Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood using a density gradient method. Ficoll-Paque reagent (Sigma Aldrich, St Louis, MO) was added to a filter tube at room temperature and centrifuged (10 -min at 500 x g). Any liquid remaining above the

filter was decanted and 15mL of whole blood was added to the tube. An equal volume of phosphate-buffered saline (PBS) (15mL) was added to the blood and the mixture was centrifuged (30 -min at 300 x g). The supernatant was decanted into a new tube and PBS was added so that the total volume was 45mL. The mixture was centrifuged (10 -min at 500 x g). The supernatant was discarded again. The pellet containing the PBMCs was resuspended in 1mL PBS and broken up by racking.

The cells were then counted using a Millipore Sceptor™ (EMD Millipore, Billerica, MA) handheld automated cell counter. They were resuspended at a concentration of 4×10^6 /mL in sterile complete RPMI and ready for use in the flow cytometry technique and the intracellular multiplex protein technique.

Staurosporine Doses

Staurosporine is a chemotactic agent and protein kinase inhibitor known to induce apoptosis (7). There are a large variety of different DAMPs, and no research has currently looked at the pathways through which specific DAMPs act. Therefore, staurosporine was used as a less specific inducer of apoptosis. It is unknown which specific DAMPs induce apoptosis so before testing the DAMPs specifically using Staurosporine enabled us to first observe if there was a change in monocyte susceptibility following downhill running. Assays were performed after treatment with four different of staurosporine and Dimethyl Sulfoxide (DMSO) was used as a control reagent. The highest dose was 0.500 mg/mL (1D) and the middle doses were 0.250mg/mL (2D) and 0.125mg/mL (3D). The lowest

dose was 0.063mg/mL (4D). The doses were made using serial dilutions starting with the manufactures recommended dose as a middle dose (i.e. 3D). Diluting Staurosporine stock solution in PBS at a ratio of 1:10 made the 1D solution. Adding the 1D solution to PBS at a 1:2 ratio made the 2D solution. The 3D solution was made in a similar way with 2D solution being diluted in PBS at a ratio of 1:2. Finally the 3D solution was be diluted in PBS (1:2) to make the 4D solution.

Apoptosis Susceptibility Assay Conditions

The isolated cells were combined with the respective Staurosporine doses/DMSO in a 96-well plate. The plates were then incubated for 4 -H at 37°C, 5.0% CO₂.

Flow Cytometry Technique

A MILLIPLEX MAP Human Early Apoptosis Magnetic Bead 7-Plex kit (EMD Millipore, Billerica, MA) was used to assess the early and middle phase apoptosis using flow cytometry. Multicaspase working reagent was added to every library tube 3 -H into the incubation. The cells were then washed with 700µL flow cytometry staining buffer and centrifuged (15 -min at 450xg) and then washed with 700µL Annexin Binding Buffer (10 -min at 450xg). Annexin binding buffer (100µL), Annexin-V reagent (5µL), 7AAD (5µL), CD14 (1:10) (50µL) were added to each of the library tubes. Following addition of the reagents the plate was incubated in the dark on ice for 30 -min.

Intracellular Multiplex Protein Technique

Following the 4-H incubation (37°C, 5.0% CO₂) the cells were centrifuged (10 -min at 1000xg). After decanting the liquid, the cells were washed with 800µL of ice-cold PBS (10 -min at 1000xg). After decanting, 800µL of lysis buffer and protease inhibitors were added. Following incubation for 15 -min at 4°C on the shaker, the tubes were be capped and frozen at -80°C until analysis.

Flow Cytometer Acquisition

Two lasers were turned on, Blue (488nm) and Red (633nm). Using a multi-channel pipette, 150µL from each assay tube was added to each well on a 96-well plate. To ensure that a minimum of 10,000 CD14+ events (monocytes) were collected, 200,000 PBMCs were collected. Uncompensated data files were collected and analyzed offline using FC Express software (v.5; De Novo Software city).

Multiplex Protein Acquisition

The cells were removed from the freezer and thawed. MILLIPLEX MAP Assay buffer (EMD Millipore, Billerica, MA) was used to wash the cells, which were then incubated with magnetic bead provided in the kit (EMD Millipore, Billerica, MA). The kit required addition of control and stimulated cell lysates to create calibration curves for relative quantification of the analytes as opposed to creating standard curves. The plate was attached to a magnetic separation block

and washed with assay buffer. The detection antibody was added and the samples were incubated for 1 -H on ice. After removal of the detection antibody, Streptavidin-PE was added and the samples were incubated for a further 30 - min in the dark before analysis on the Luminex system.

Statistical Analysis

All statistical analysis was completed using SPSS (v22; Chicago, IL) and assumptions of normality were assessed prior to formal statistical testing. For the purposes of statistical analysis, the control values were subtracted from the key outcome variables to generate a single response value for statistical testing. Prior to formal statistical analysis, data were assessed for normality, constant error variance, and outliers using the EXPLORE function in SPSS. Based on this, two of the participants showed outlier results for 10 of the variables, and so were removed from further statistical testing. Flow cytometry data was tested using a repeated measures ANOVA on time (PRE, +4, +24, and +48-H post exercise). Multiplex data were tested using a repeated measures ANOVA on time (PRE, +4 and +24 -h post exercise). The significance level was set to $p < 0.05$. LSD pairwise comparisons were used to determine the location of significant effects. Serum Creatine Kinase was tested using an ANOVA on time (PRE, +4, +24 and +48 -H) with Bonferroni post-Hoc tests and Pairwise Comparisons to find the location of significance.

After initial statistical testing using a 4 x 5 repeated measures ANOVA for time and dose, it was found that there were no significant differences between

apoptotic response to any of the doses of staurosporine. Therefore all further analysis focused on the optimum dose of 0.25mg/ml and the control samples.

Results

Creatine Kinase

Serum Creatine Kinase was found to be significantly increased at 4-Hours, 24-Hours and 48-Hours post-exercise when compared to pre-exercise. This indicates that muscle damage did occur, with the peak at 24-Hours post-exercise (Figure 2).

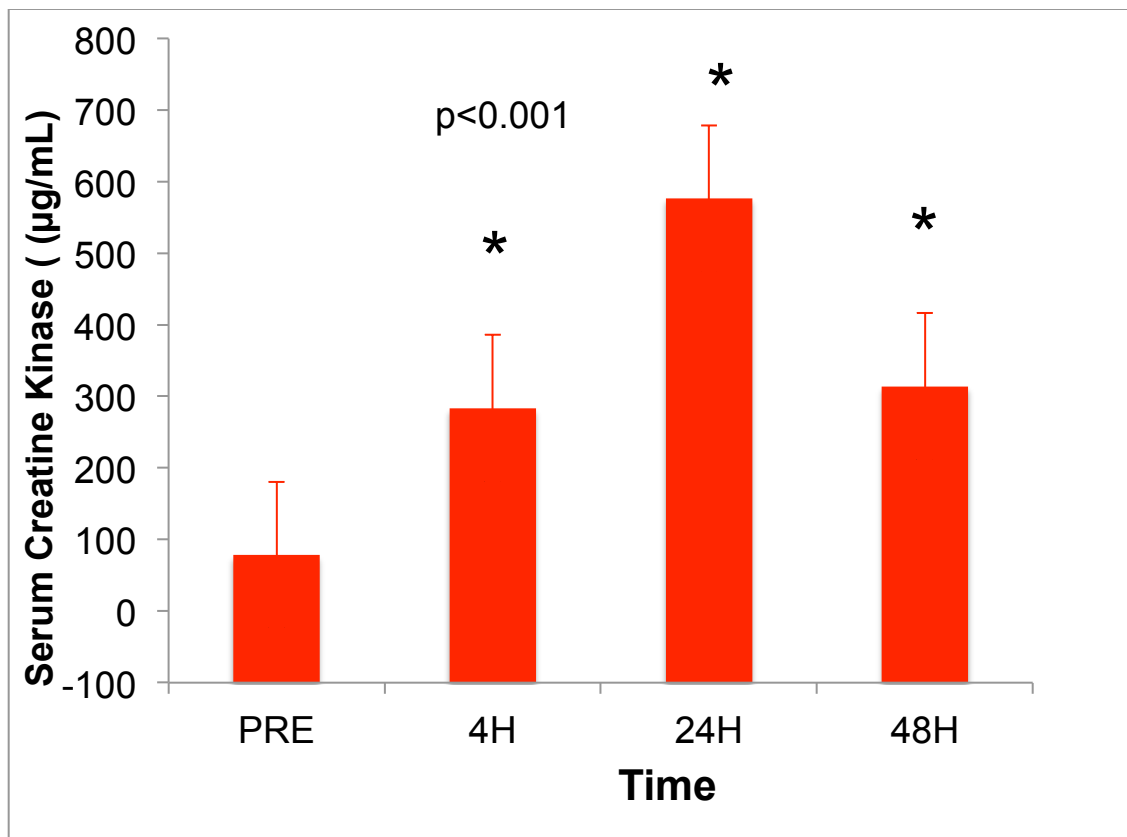


Figure 2. Serum Creatine Kinase levels at pre-exercise and 4-Hours, 24-Hours and 48-Hours post-exercise.

Monocyte Viability

A significant main effect for time was found for the percentage of dead (7AAD+) monocytes ($p=0.032$) as shown in figure 3. The percentage of dead monocytes was significantly greater ($p<0.05$) at +4 (11.17%) and +24 (19.72%) than at PRE (2.48%), and +48 (10.76%) was significantly greater than at +4. There were no significant differences between PRE and +48.

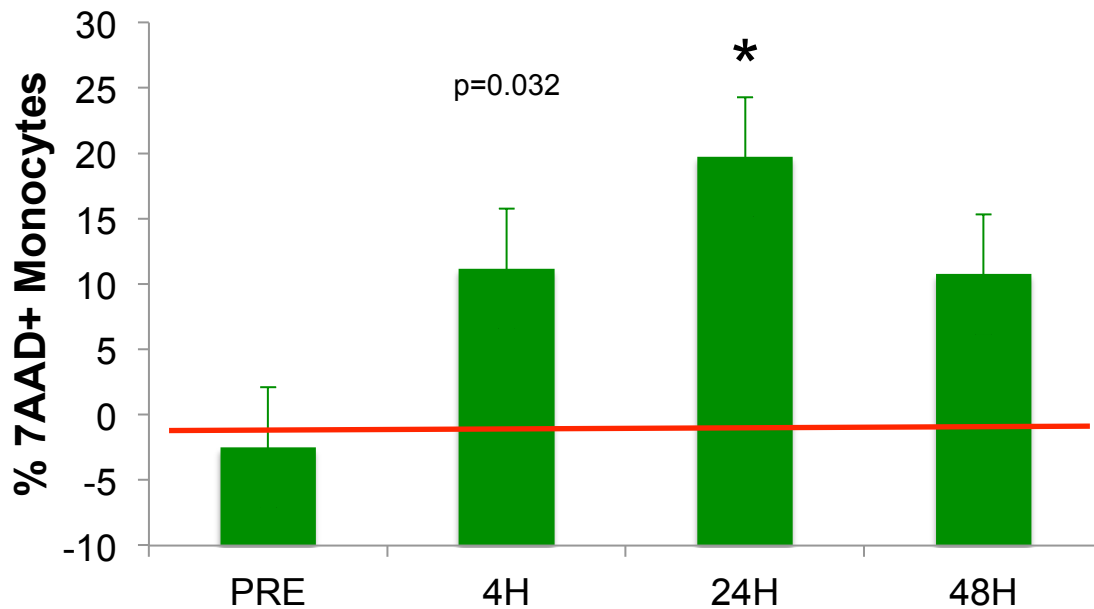


Figure 3. The percentage of dead Monocytes at time points PRE (before exercise), 4H (4 hours post-exercise), 24H (24 hours post exercise) and 48H (48 hours post exercise). * Significantly greater than PRE.

Middle Phase Apoptosis

A significant main effect of time was found for percentage of monocytes undergoing middle phase apoptosis ($p=0.041$) as shown in figure 4. The

percentage of Multicaspase+ monocytes was found to be significantly greater at +4 (2.442%) and +48 (7.99%) than at PRE (-3.587%). The staurosporine treated monocytes showed significantly increased expression of multicaspase when compared to control monocytes at +24 and +48 as shown in figure 5.

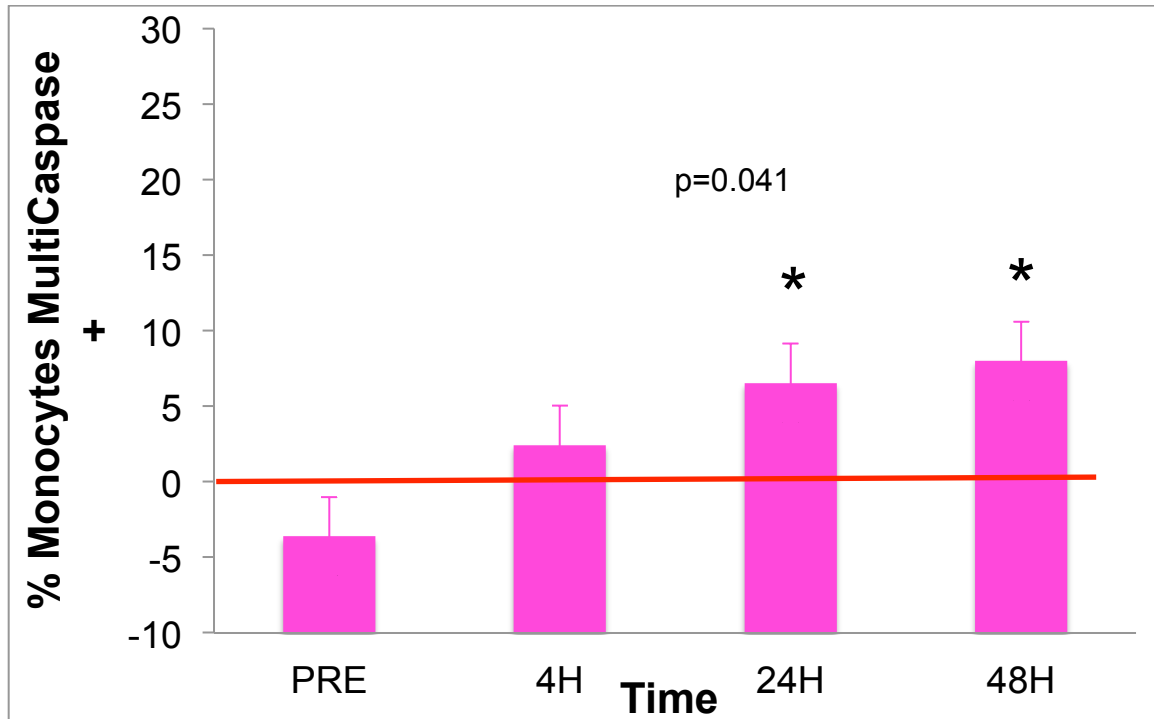


Figure 4. The percentage of monocytes undergoing middle-phase apoptosis is shown in figure 4. * Differs significantly from the pre-exercise sample.

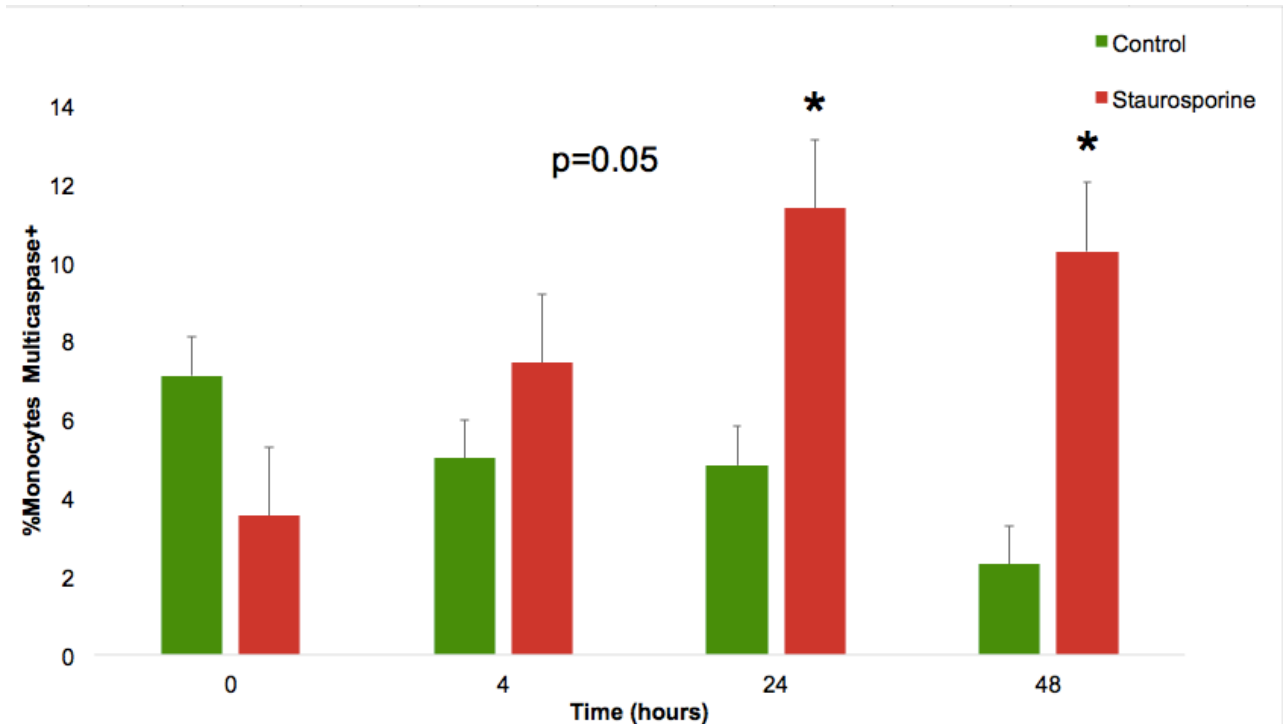


Figure 5. The percentage of monocytes positive for multicaspase in control and staurosporine treated samples.

Multiplex Analysis-Pro-apoptotic Markers

A significant main effect was found for time on the relative concentration of phosphorylated p53 ($p=0.04$) as shown in figure 6. Phosphorylation of p53 was found to be significantly greater ($p<0.05$) at +24 (1.12pg/mg total protein) than at

PRE (0.60pg/mg total protein) +4 (0.48pg/mg total protein).

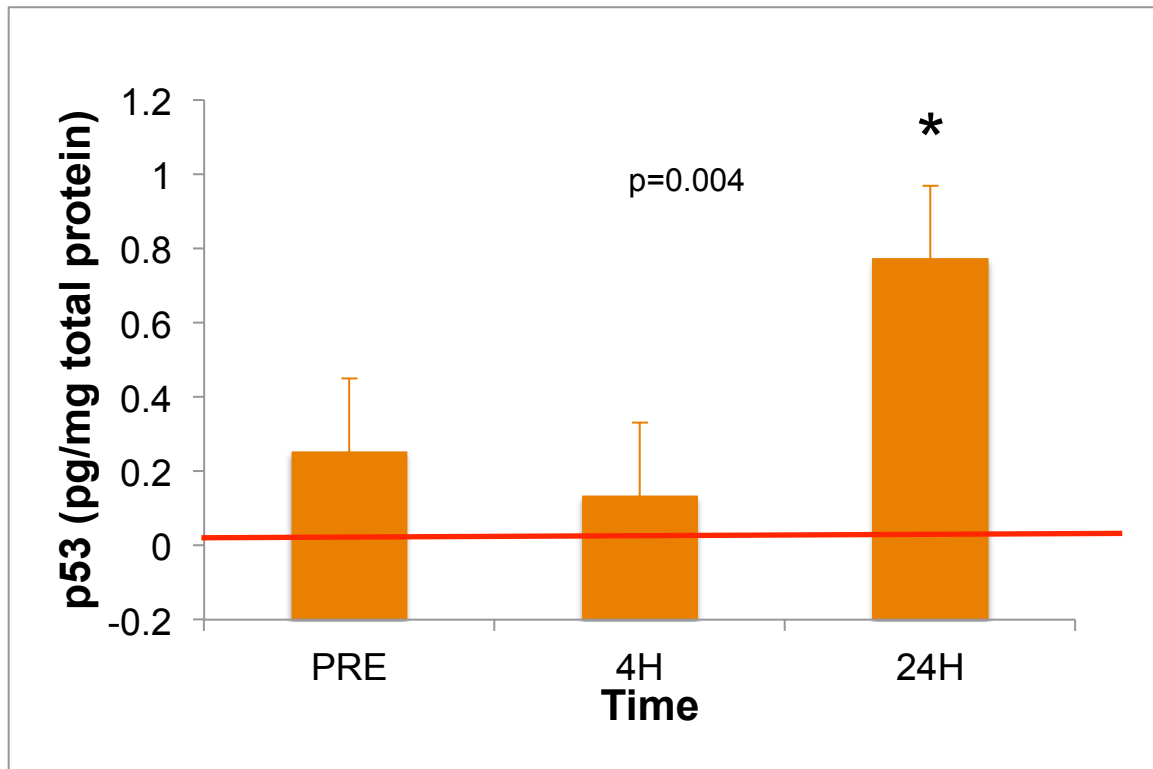


Figure 6. The change in relative concentration of phosphorylated p53 over time.

* Differs significantly from the pre-exercise sample.

A significant main effect was found for time and relative concentration of phosphorylated caspase-8 ($p=0.019$) as shown in figure 7. The level of caspase-8 activation was significantly lower ($p<0.05$) at +4 (0.83pg/mg total protein) than PRE (1.00pg/mg total protein) and significantly greater at +24 (1.50pg/mg total protein) than at +4.

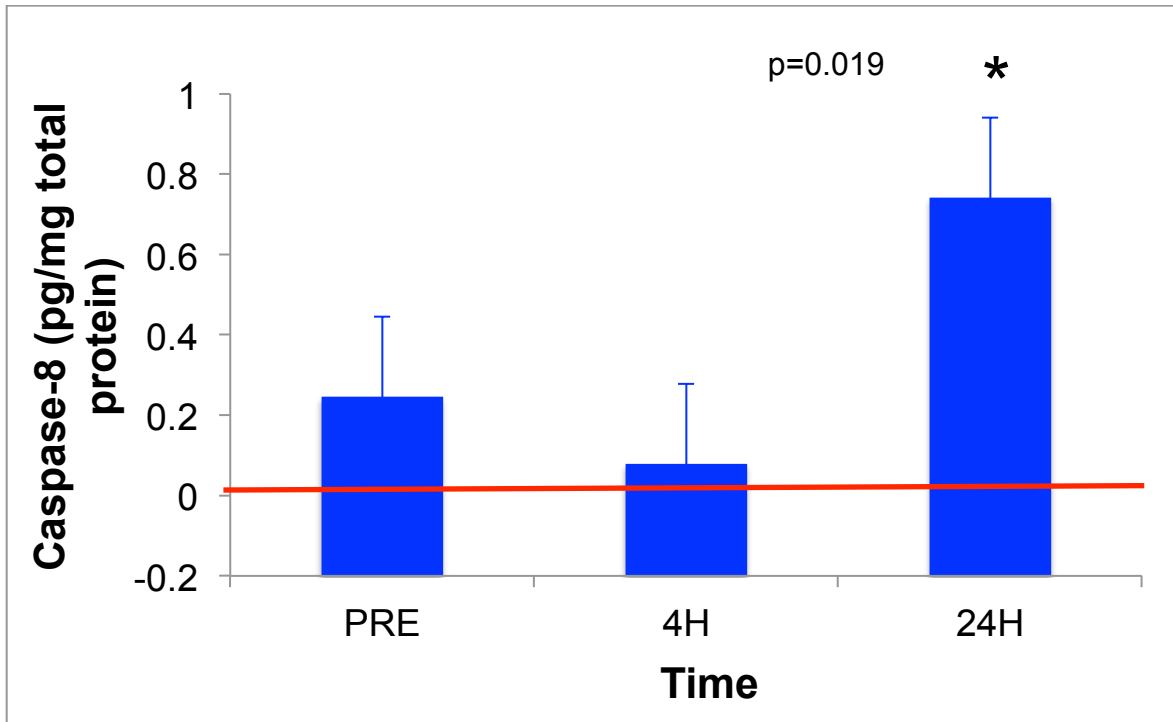


Figure 7. The change in relative concentration of phosphorylated Caspase-8 over time. * Differs significantly from the pre-exercise sample.

Multiplex Analysis- Anti-apoptotic Markers

A significant main effect for time and relative concentration of phosphorylated factor Bcl-2 was found ($p=0.012$) as shown in figure 8. The location of significance was found to be between PRE (0.84 pg/mg total protein) and +4 (0.73pg/mg total protein), and between +4 and +24 (1.39 pg/mg total protein).

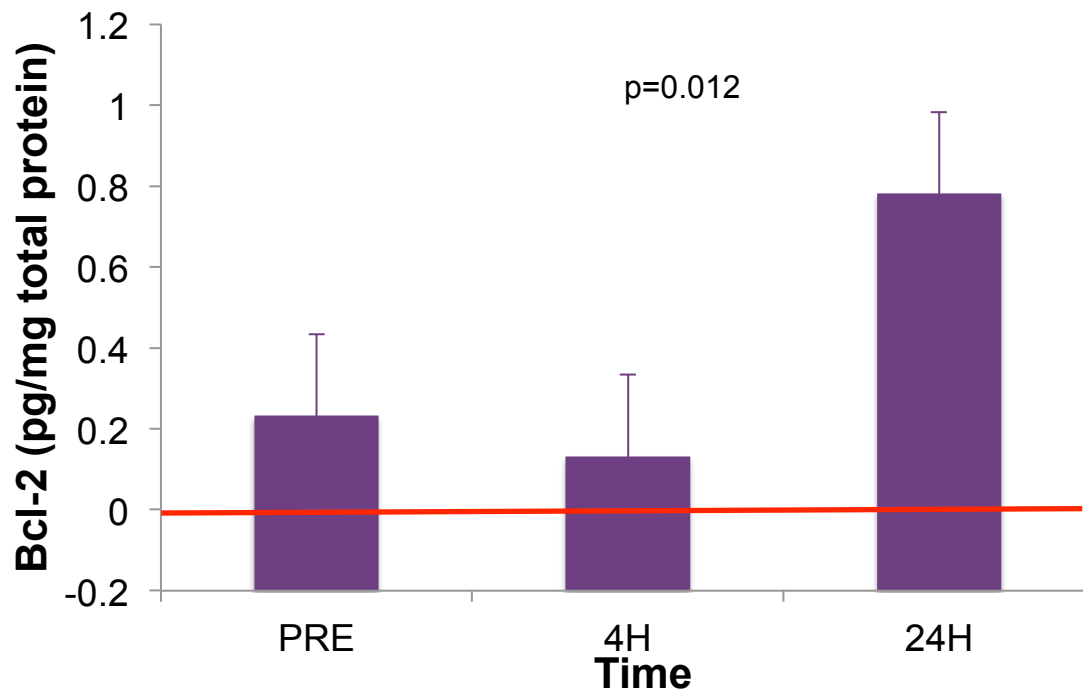


Figure 8. The change in relative concentration of phosphorylated Bcl-2 over time. * Differed significantly from the pre-exercise sample.

Annexin+ Multicaspase- Monocytes

No significant main effect was found for time and Annexin+ monocytes (figure 9). It is probable that due to the detection of middle phase apoptosis, the early phase apoptosis had already occurred by the 4-Hour time point.

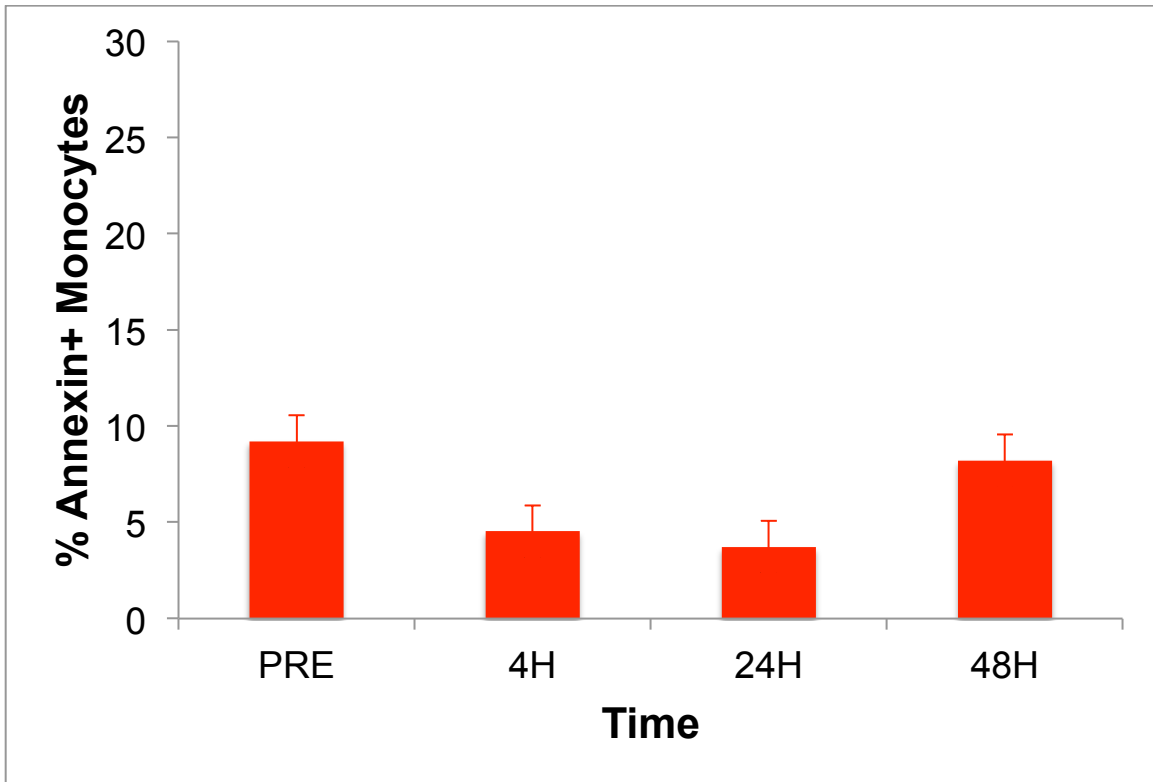


Figure 9. The percentage of monocytes undergoing early-phase apoptosis.

Pro-apoptotic markers

There was no significant effect found for time and the relative concentration of phosphorylated caspase-9 as shown in figure 10.

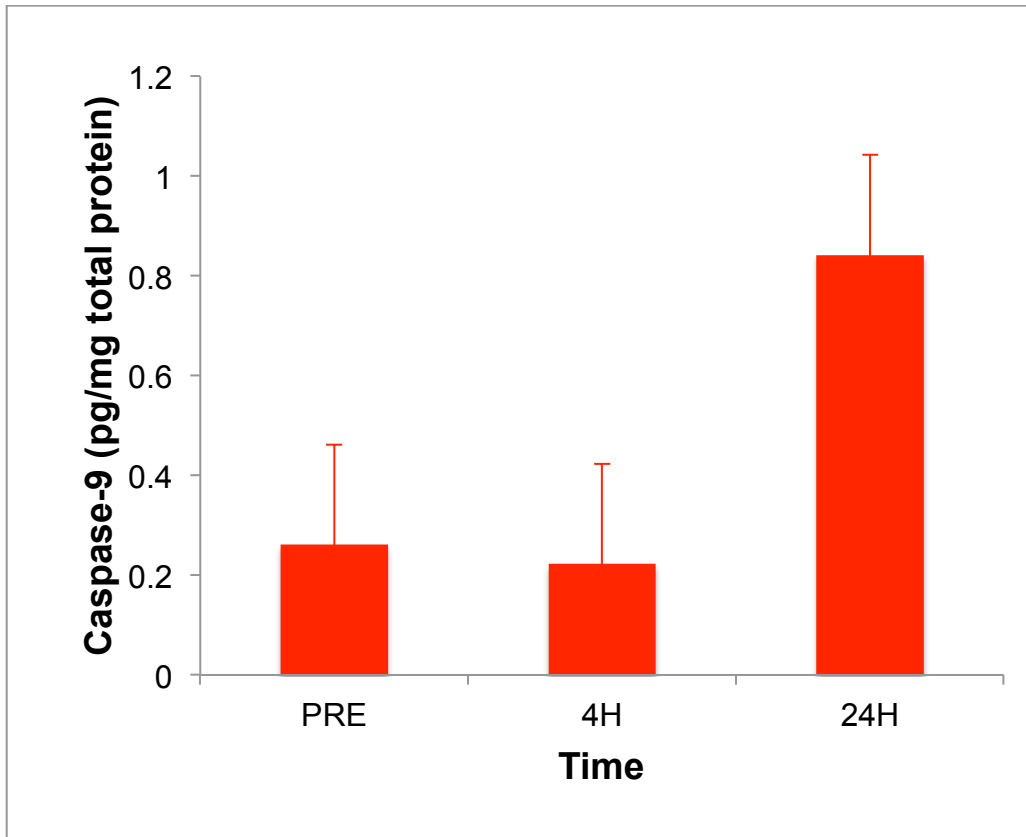


Figure 10. The change in relative concentration of phosphorylated Caspase-9.

There was no significant effect found for time and relative concentration of phosphorylated Caspase-9.

There was no significant effect found for time and relative concentration of phosphorylated BAD (Figure 11). This could be due to BAD being part of an apoptotic pathway not initiated.

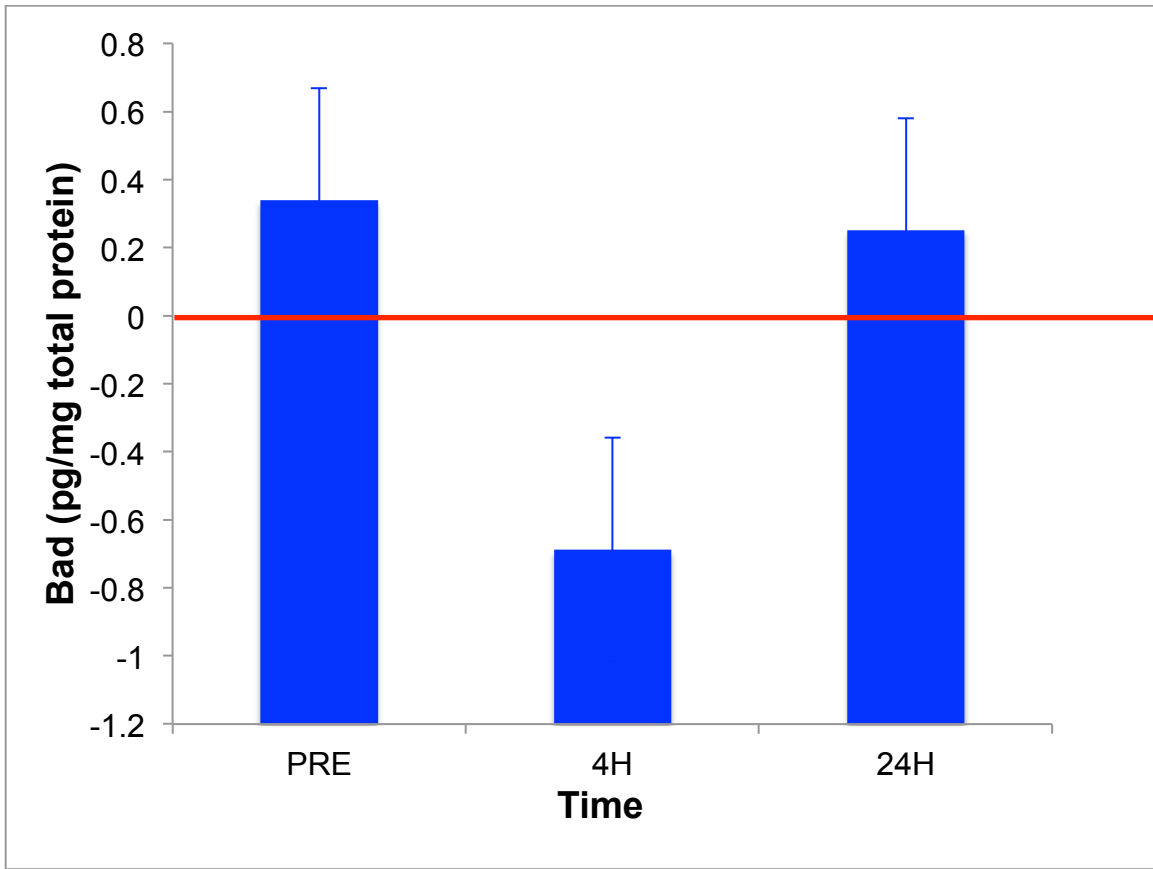


Figure 11. The change in relative concentration of phosphorylated Bad over time. * Differed significantly from the pre-exercise sample.

There was no significant effect found for time and relative concentration of phosphorylated JNK as shown in figure 12. However the pattern of JNK concentration was similar to that of p53 and caspase-8, with the numerical peak at 24 –H post exercise.

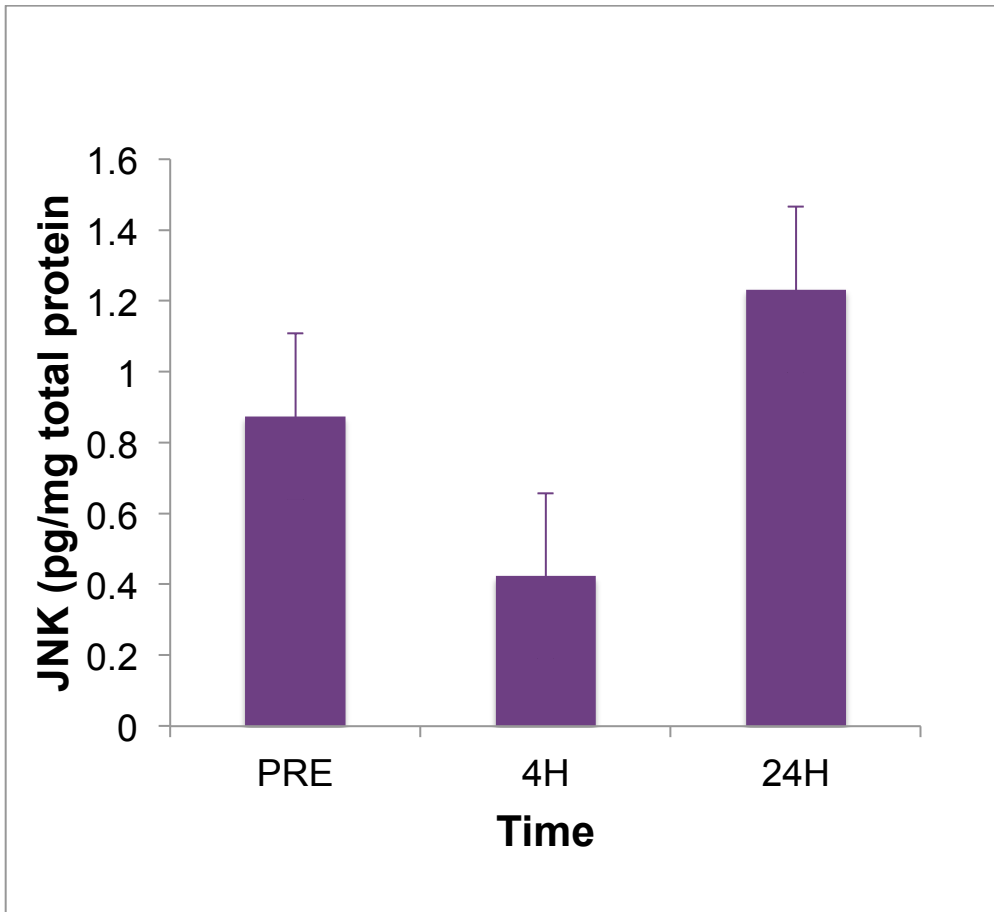


Figure 12. The change in relative concentration of phosphorylated JNK over time.

Anti-apoptotic Markers

There was no significant main effect found for time and the relative concentration of phosphorylated Akt as shown in figure 13.

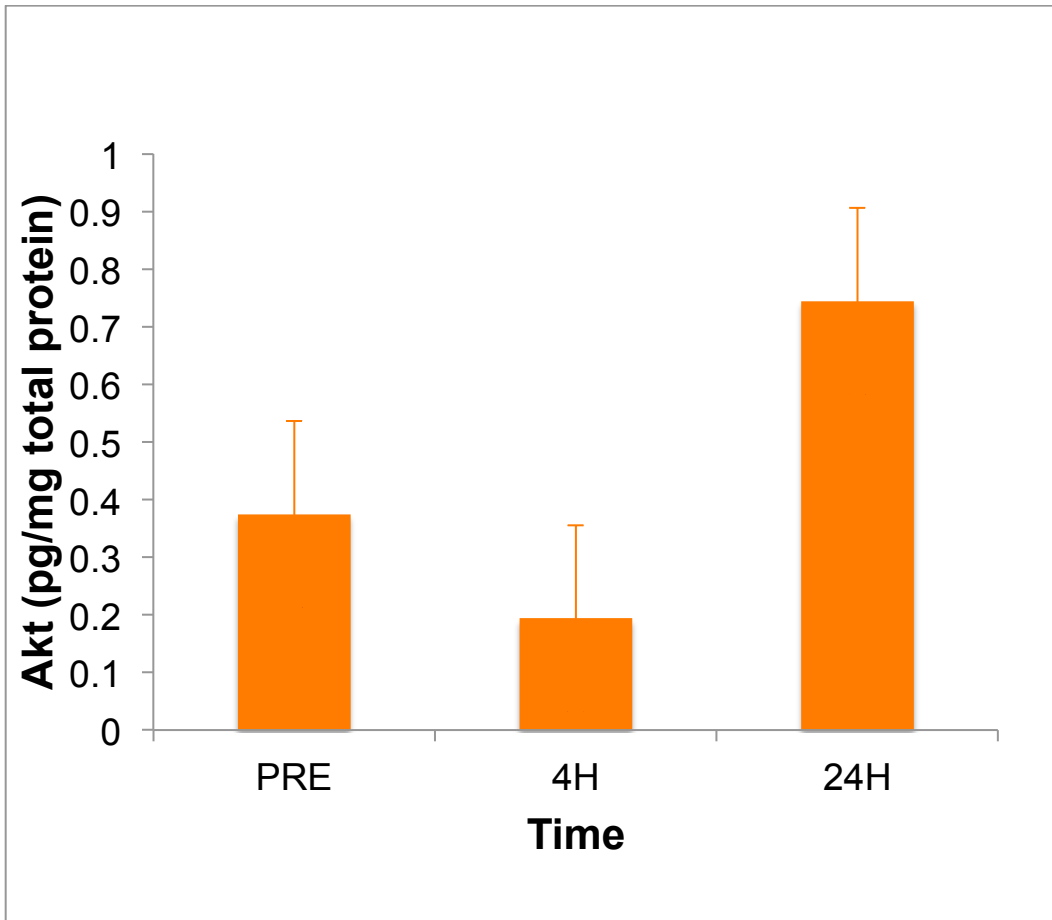


Figure 13. The change in relative concentration of phosphorylated Akt over time.

Discussion

Previous research has demonstrated that lymphocytopenia occurs after exercise (9). The effect of exercise on monocytes is less established, however it is reported that monocytes follow a similar decline in number following a bout of exercise (9). This reduction in the number of monocytes is thought to be a result of two independent factors; redistribution to tissue from the blood and death by apoptosis (9).

Apoptosis is a form of controlled cell death, which is involved in balancing the maturation of optimal functioning new cells and aging or damaged cells (10). Studies have demonstrated that lymphocytes and leukocytes exhibit decreased resistance to apoptosis following bouts of intense exercise (11, 12, 13, 14). The degree of increased apoptosis is related to the intensity of the exercise performed, the type of exercise performed and the training status of participants (11, 12, 13, 14).

Previous research has reported that the percentage of Annexin-V positive cells significantly increased 50% from before to immediately after exhaustive exercise (11). Levels returned to normal after 1 –h (11).

In the same study, cells treated with the chemotactic agent Camptothecin have shown a 200% increase in Annexin-V positive lymphocytes when compared to unstimulated cells (11). The dose of Camptothecin (chemotactic agent similar to Staurosporine) in this previous study was determined based on preliminary testing on pre-exercise samples (11). This presents a potential flaw in the methods as the dose used may not have been optimal for use on post-exercise samples. In the present study the dose of Staurosporine was determined after preliminary testing on post-exercise samples and so should have been optimal for the study.

Previous research that looked specifically at monocytes found that the percentage of Annexin-V positive monocytes increased from immediately post-exercise to +24 and +72 (13). Similarly in three subsets of B-lymphocytes, Annexin-V positive cells were shown to significantly increase immediately post

exercise when compared to PRE following bouts of exhaustive interval running (13). The previous research therefore indicates that the propensity of lymphocytes to undergo apoptosis increases following exercise.

In the present study the percentage of Annexin-V positive monocytes tended to be increased at +4 but decreased at +24 and +48. There was no significant main effect found for time and Annexin-V positive cells in the present study. Annexin-V reagent detects changes in the permeability of the plasma membrane due to relocation of phosphatidyl serine and is a marker of early phase apoptosis (19). It could be speculated that the time points of the current study meant that early apoptosis had already occurred by +4 and was therefore undetectable.

Multicaspase reagent is used to identify cells in the middle stages of apoptosis.

Given that the percentage of Multicaspase+ monocytes was significantly increased between PRE and post-exercise samples, in the present study an increase in the susceptibility of monocytes to apoptotic signals following downhill running was observed. The differences between previous research and the present study may be attributed to the different exercise modes and protocols.

The only published study to our knowledge, which has investigated apoptosis following downhill running found that apoptotic index was increased immediately post, +2 and +24 in leukocytes (15). They also reported a significant difference in apoptotic index between participants who completed a running protocol at a negative grade and those who ran on a level surface (15). They reported no significant difference for the anti apoptotic protein Bcl-2 activation between PRE

and post samples (15). In the current study a significant effect was found for Bcl-2 between PRE and +48 suggesting further investigation is required.

To our knowledge there are currently no published studies that investigate the middle or late-phase apoptotic markers (p53, Caspase-8, Caspase-9, Akt and multicaspase) in response to exercise. Apoptosis is an 'all or nothing' cascade of events so if a cell has begun the early phases of apoptosis it is destined to die.

The results from the multiplex protein technique in the present study suggest that monocytes are less resistant to apoptosis following a bout of exercise. The pro-apoptotic proteins that were significantly increased in post exercise samples included p53, Caspase 8 and Caspase 9 (10, 20). Akt and Bcl-2 are anti-apoptotic proteins involved in regulation of the mitochondrial apoptosis pathway (21, 22). The increase in relative concentration of Bcl-2 in post exercise samples may reflect an increased need for resistance to apoptotic signals following exercise. If Bcl-2 was not increased it would be possible to speculate that the pro-apoptotic proteins may have shown an even greater increase in relative concentration. To our knowledge, no other studies have investigated the effect of downhill running on these markers of apoptosis, so further research is needed to establish if the findings are reproducible.

There are currently no published studies to our knowledge, which specifically investigate the effect of monocyte susceptibility to apoptosis following a bout of downhill running. Although preliminary, this study has shown that there are differences in some aspects of the apoptotic response of monocytes

following strenuous eccentric exercise. Apoptosis was increased in monocytes following a bout of downhill running.

Conclusion

The overall findings of the present study suggest that a downhill running protocol (6 x 5-minutes at -15% grade) increases the expression and concentration of specific apoptotic markers in monocytes. Downhill running caused an increase in the number of Multicaspase+ monocytes from PRE to post-exercise, indicative of increased middle phase apoptosis. The findings from the multiplex technique, specifically the increased activation of pro-apoptotic factors p53, caspase-8 and Bad, support the conclusion from the flow cytometry data that downhill running increases the susceptibility of monocytes to undergo apoptosis. Future research should investigate the importance of an increase in monocytes susceptibility to apoptosis. Along with previous research the present study used a sample size of 12, future research should use a greater number of participants to increase the generalizability due to individual variability. The present study suggests that there is a change in apoptosis following exercise; further research should include determination of which specific DAMPs are involved in initiating the apoptotic response. A greater understanding of monocyte apoptosis following exercise has the potential to improve recovery time and increase knowledge in the field of muscle damage.

REFERENCES

1. Chen, Y. G., and Nunez, G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*, 10(12), 826-837, 2010
2. Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology*, 81(1), 1-5, 2007
3. Shen, H., Kreisel, D., & Goldstein, D. R. Processes of sterile inflammation. *The Journal of Immunology*, 191(6), 2857-2863, 2013
4. Stupka, N., et al. "Gender differences in muscle inflammation after eccentric exercise." *Journal of Applied Physiology* 89.6 (2000): 2325-2332.
5. Fell, J., & Williams, A. D. The effect of aging on skeletal-muscle recovery from exercise: possible implications for aging athletes. *Journal of Aging and Physical Activity*, 16(1), 97, 2008
6. 13. Tomlin, D. L., & Wenger, H. A. The relationship between aerobic fitness and recovery from high intensity intermittent exercise. *Sports Medicine*, 31(1), 1-11, 2001
7. Manns, J., Daubrawa, M., Driessen, S., Paasch, F., Hoffmann, N., Löffler, A., & Wesselborg, S. Triggering of a novel intrinsic apoptosis pathway by the kinase inhibitor staurosporine: activation of caspase-9 in the absence of Apaf-1. *The FASEB Journal*, 25(9), 3250-3261, 2011
8. Bertrand, R., Solary, E., O'Connor, P., Kohn, K. W., & Pommier, Y. Induction of a common pathway of apoptosis by staurosporine. *Experimental cell research*, 211(2), 314-321.---- staurosporine broad initiator, 1994

9. Timmerman, K. L., Flynn, M. G., Coen, P. M., Markofski, M. M., & Pence, B. D. Exercise training-induced lowering of inflammatory (CD14⁺ CD16⁺) monocytes: a role in the anti-inflammatory influence of exercise?. *Journal of leukocyte biology*, 84(5), 1271-1278, 2008
10. Zimmermann, Katja C., and Douglas R. Green. "How cells die: apoptosis pathways." *Journal of allergy and clinical immunology* 108.4 (2001): S99-S103.
11. Krüger, K., & Mooren, F. C. Exercise-induced leukocyte apoptosis. *Exerc Immunol Rev*, 20(20), 117-134, 2014
12. Mooren, F. C., Blöming, D., Lechtermann, A., Lerch, M. M., & Völker, K. (2002). Lymphocyte apoptosis after exhaustive and moderate exercise. *Journal of applied physiology*, 93(1), 147-153, 2002
13. Navalta, J. W., Tibana, R. A., Fedor, E. A., Vieira, A., & Prestes, J. Three consecutive days of interval runs to exhaustion affects lymphocyte subset apoptosis and migration. *BioMed research international*, 2014
14. Tuan, T. C., Hsu, T. G., Fong, M. C., Hsu, C. F., Tsai, K. K., Lee, C. Y., & Kong, C. W. Deleterious effects of short-term, high-intensity exercise on immune function: evidence from leucocyte mitochondrial alterations and apoptosis. *British journal of sports medicine*, 42(1), 11-15, 2008
15. Park, K. S., Sedlock, D. A., Navalta, J. W., Lee, M. G., & Kim, S. H. Leukocyte apoptosis and pro-/anti-apoptotic proteins following downhill running. *European journal of applied physiology*, 111(9), 2349-2357, 2011

16. Eston, R. G., Mickleborough, J., & Baltzopoulos, V. Eccentric activation and muscle damage: biomechanical and physiological considerations during downhill running. *British Journal of Sports Medicine*, 29(2), 89-94, 1995
17. American College of Sports Medicine. *ACSM's guidelines for exercise testing and prescription*. Lippincott Williams & Wilkins, 2013
18. Borg, G. *Borg's perceived exertion and pain scales*. Human kinetics, 1998
19. Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutellingsperger, C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *Journal of immunological methods*, 184(1), 39-51, 1995
20. Kruidering, M., & Evan, G. I. Caspase-8 in Apoptosis: The Beginning of "The End"? *IUBMB life*, 50(2), 85-90, 2000
21. Tsujimoto, Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?. *Genes to Cells*, 3(11), 697-707, 1998
22. Haupt, S., Berger, M., Goldberg, Z., & Haupt, Y. Apoptosis-the p53 network. *Journal of cell science*, 116(20), 4077-4085, 2003