

Does exercise really induce lymphocyte apoptosis?

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1. ABSTRACT

While the stress associated with acute exercise has been reported to induce significant lymphocyte apoptosis, not all investigations have confirmed this finding. Regardless of animal or human subjects, exercise-induced lymphocyte apoptosis may be induced via an external receptor-mediated pathway, or internally via the mitochondria through an oxidative-mediated pathway. On the other hand, investigators reporting no effect of acute exercise on lymphocyte apoptosis speculate that cell death may be dissociated from these pathways, and explain exercise lymphocytopenia by selective migration of the lymphocytes back into the lymphoid pools. Discrepancies may be due to sensitivity issues related to the methodology used to assess cell death. Limitations to various methods used to evaluate exercise-induced lymphocyte apoptosis are detailed, and considerations for a new technique are outlined.

2. INTRODUCTION

Leukocytes have a finite lifespan and are eliminated from the body through the process of programmed cell death. This sequence of events was identified morphologically and termed “apoptosis” by Kerr *et al.* as the Greek word used to describe the dropping off, or falling off of petals from flowers or leaves from trees (1). Unlike necrosis, apoptosis occurs in an orderly fashion with morphological events including cell shrinkage, the breakdown of DNA into characteristic lengths, orderly condensation of the nucleus, and the production of membrane “blebs” which are phagocytosed by surrounding cells. Following acute exercise, leukocytes are particularly vulnerable to deletion through programmed cell death, which may have implications for immunity. The physiological stress associated with strenuous acute exercise (75-85% of aerobic capacity, or exercise until

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Table 1. Animal studies and exercise-induced lymphocyte apoptosis

Reference	Subject	Exercise Intervention	Assessment Technique	Lymphocyte Apoptosis
3	Young male Wistar rats	To exhaustion 10% grade, 20 m·min ⁻¹ Half run	DNA fragmentation	↑ with maximal and submaximal exercise
5	8 wk old male Wistar rats	60-90 min, 13.8 m·min ⁻¹	Gel electrophoresis and ELISA	↑ with exercise
7	6 wk old female C57BL/6 mice	Voluntary wheel running	Annexin-V FITC and Propidium Iodide	↓ with voluntary exercise
15	Female C57BL/6 mice	To exhaustion 8% grade, 32 m·min ⁻¹	Lipid peroxide assay	↑ Immediate and 24h postexercise
16	Female C57BL/6 mice	90 min run: 30 min at 2% grade, 22 m·min ⁻¹ ; 30 min at 2% grade, 25 m·min ⁻¹ ; and 30 min at 2% grade, 28 m·min ⁻¹	Western blot analysis of Bcl-2, caspase 3, cytochrome c	↑ with exercise
17	Female B6D2F ₁ mice	Voluntary wheel running	Annexin-V FITC and Propidium Iodide	↔ with voluntary exercise
19	Female C57BL/6 mice	90 min: 30 min at 24 m·min ⁻¹ ; increases of 2 m·min ⁻¹ thereafter to exhaustion	Annexin-V FITC and Propidium Iodide	↑ with exercise and α-adrenergic blockade, ↓ with exercise and β-adrenergic blockade
20	Female C57BL/6	90 min at 6% grade, 35 m·min ⁻¹	Annexin-V FITC and Propidium Iodide	↓ thymocyte apoptosis with exercise

volitional fatigue) significantly increases lymphocyte apoptosis in both animals (2-7) and humans (8-13).

The aim of this manuscript is 1) to review the animal and human literature pertaining to exercise-induced lymphocyte cell death, 2) to detail inherent limitations to the evaluation techniques that have been used in the literature, and 3) to discuss considerations for the modification of apoptotic assessment for leukocytes collected from an exercising individual. Current apoptotic cell biology techniques require substantial modification to use with an exercising individual because they were designed to analyze a homogeneous population of cells that were cultured *in vitro* and not mixed population of peripheral blood lymphocytes collected *in vivo*.

3. EXERCISE-INDUCED LYMPHOCYTE APOPTOSIS

3.1. Animal research

3.1.1 Pathway-directed investigations in cell death

Two primary pathways initiate apoptotic cell death. Induction of apoptosis may be mediated externally by death receptors on the cell membrane, or internally through a mitochondrial involved pathway. Investigations in which animals performed an exercise intervention have reported increased factors associated with each of these cell death inducing pathways. In an animal model, exercise increases both external (glucocorticoids, Fas) and internal factors (oxidative stress, free radicals) involved in the apoptotic process (see Table 1).

Early studies evaluating the effect of acute exercise on lymphocyte cell death suggested that glucocorticoids, which increase with intensity, could contribute to apoptotic cell death. Ferry *et al.* studied the

effects of exhaustive physical exercise and glucocorticoids on T-cell function of trained Male Wistar rats (2). Physical exhaustion in trained control rats significantly reduced the total number of thymocytes and numbers of thymocyte subsets. Dexamethasone treatment combined with exercise resulted in a further reduction of these cells. Although apoptotic measures were not reported, it was suggested that the decrease in thymocyte numbers observed following exercise was due to cell death, and may be mediated by glucocorticoids. In a follow up study, Concordet and Ferry (1993) investigated the effect of physical stress and glucocorticoid blockade on apoptosis in male Wistar rats (3). Similar levels of apoptosis were present following two maximal bouts of running, as well as after milder exercise (half-run, and single run to exhaustion). The observed cell death was transient however, as it was not detected 24h following both the single or double exhaustive bouts of exercise. It is of interest to note that animals treated with the glucocorticoid receptor antagonist showed less apoptosis following mild exercise versus untreated controls, and it was proposed that thymocyte apoptosis in exercised rats was partly mediated by the glucocorticoid receptor. Hoffman-Goetz and Zajchowski used female C57BL/6 mice to study the effect of corticosterone incubation applied over increasing periods of time at concentrations similar to those found at submaximal levels of exercise (14). Main effects were observed for both concentration and time in thymocytes, but only time in splenocytes. Thymocytes incubated for greater time periods (120 min) were observed to have greater apoptosis compared to 0 and 90 min (although not significant). Taken together, it is apparent that in an animal model, humoral factors which are known to increase with exercise intensity also appear to induce cell death.

Another mediator of lymphocyte apoptosis is the Fas (CD95) receptor and its associated signaling pathway. Yin *et al.* carried out a study to assess the effect of chronic

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restraint stress on lymphocyte apoptosis and Fas expression (6). Mice were placed in a conical centrifuge tube that allowed for ventilation and held horizontally for 12 h. Mice stressed for two-days showed a 35-40% decrease in the number of splenic lymphocytes compared with unstressed controls. Administration of opioid antagonists before physical restraint blocked the stress-induced reduction in splenocytes. After 12 h of restraint stress, Fas (CD95) expression was increased significantly, however, Fas-ligand (CD95L) was unaltered. Splenocytes from chronically stressed mice showed signs of apoptosis, whereas only minimal cell death was observed in control cells. To assess the relationship between physical stress and apoptosis, splenocytes from stressed mice were cultured with cells transfected with CD95L cDNA in sense or anti-sense orientation. The percent of apoptotic lymphocytes increased significantly over control values when cultured with sense cDNA, while cells cultured with anti-sense cDNA was not different from control. The authors final conclusion was that stress primes lymphocytes for Fas-mediated apoptosis. Based on the information presented above concerning glucocorticoids, it is reasonable to speculate that this may partially mediate the stress response to due to their classification as “Fight or Flight” hormones.

In addition to the external receptor-mediated pathways, apoptosis may be induced internally via an oxidative pathway. Azenabor and Hoffman-Goetz investigated the oxidative stress to thymocytes and splenocytes associated with exhaustive exercise in female C57BL/6 mice (15). While apoptotic cell counts were not reported, a biochemical index of cell death, lipid peroxide, was significantly increased in thymocytes from both exercising groups. It was suggested that free radicals produced with exercise could contribute to apoptosis observed following exercise. A study by Lin *et al.* was carried out to investigate the effect antioxidants played in exercise-induced apoptosis of thymocytes (5). Olive oil alone or the antioxidant butylated hydroxyanisole (BHA) in olive oil was administered for 7 days to Male Wistar rats. Compared to sedentary controls, two days of exercise significantly induced DNA fragmentation in thymocytes but not splenocytes. BHA blocked apoptosis in thymocytes from exercised rats to levels similar of what was observed in control animals. It was concluded that the production of reactive oxygen species during exercise is involved in thymocyte apoptosis. Quadrilatero and Hoffman-Goetz designed a study to investigate the role of oxidative stress on exercise-induced apoptosis in intestinal lymphocytes of female C57BL/6 mice (16). Exercising controls displayed significantly greater proapoptotic proteins (caspase 3 and cytosolic cytochrome c) compared to the exercised animals that received antioxidant supplementation. It was suggested that oxidative stress mediated via a mitochondrial pathway could contribute to apoptosis in intestinal lymphocytes following exercise. From the previous investigations, it is apparent that the oxidative stress associated with acute exercise represents a physiological influence capable of inducing cell death in animal leukocyte populations.

3.1.2. Unequivocal reports

While acute exercise may increase cell death factors in an animal model, not all investigations have shown significant exercise-induced apoptosis. Significant apoptosis in lymphocytes can generally be observed following exhaustive bouts of exercise, whereas studies utilizing voluntary exercise (i.e. access to an exercise wheel) have not reported changes with regards to lymphocyte cell death and therefore may not be of sufficient intensity to induce a response (see Table 1). In addition, apoptosis is an attractive explanation for the reduction in lymphocyte numbers that occurs following exercise. Studies using blockades of various cell death factors have reported a dissociation with exercise while lymphocytopenia is still evident. Given this, the role of lymphocyte apoptosis in the postexercise response is one that must be more clearly defined.

While many studies utilizing an animal model have found evidence of exercise-induced lymphocyte apoptosis (see previous section), other reports are unequivocal. Hoffman-Goetz *et al.* studied the interaction between 17 β -estradiol and voluntary exercise on lymphocyte apoptosis in female B6D2F₁ mice (17). Mice received low estrogen, high estrogen, or placebo over the experimental period, and were separated into cages that either had access to wheels or no wheels. The result of interest to us is that no main effect was observed for lymphocyte apoptosis when voluntary wheel running was assessed. This may indicate that when left on their own, animals do not voluntarily exercise at a high enough intensity or long enough duration (or combination) to induce cell death. Mice that were provided free access to wheels over 10 months displayed no difference in FasL or TNF-alpha expression compared to controls despite running approximately 33.7 km·week⁻¹ and gaining 43.5% less body weight over the treatment period than their sedentary counterparts (10).

In addition to the internal pathway, evidence is emerging that calls into question the receptor-mediated pathways of exercise-induced lymphocyte apoptosis. Quadrilatero and Hoffman-Goetz investigated corticosteroid administration and apoptosis in intestinal lymphocytes in female C57BL/6 mice (18). Corticosterone was injected at concentration levels similar to those found following a strenuous 90 min treadmill run. Indicators of apoptosis (phosphatidylserine externalization, mitochondrial membrane depolarization, caspase 3, and Bcl-2) were unaltered by elevated levels of corticosterone. Marra and Hoffman-Goetz studied the response of intestinal lymphocytes to exercise in female C57BL/6 mice who received β -adrenergic receptor blockade (19). All exercised mice had lower numbers of intestinal lymphocytes than sedentary mice, regardless of drug treatment. While apoptosis in the sedentary group was not reported, β -adrenergic receptor blockade decreased intestinal lymphocyte apoptosis whereas α -adrenergic receptor blockade appeared to increase cell death compared to the exercising control. It was suggested that the classic postexercise lymphocytopenia is not due to epinephrine and apoptosis, but that other factors may modulate this response.

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Table 2. Human investigations and exercise-induced lymphocyte apoptosis

Reference	Subjects	Exercise Intervention	Assessment Technique	Lymphocyte Apoptosis
8	Healthy males (N = 11)	Incremental treadmill test to exhaustion	Single cell electrophoresis assay	↑ with maximal exercise immediate, 24h, and 48h postexercise
9	Trained male runners (N = 12)	Treadmill runs for 3 consecutive days at 35%, 60%, and 85% $\text{VO}_{2\text{max}}$	TUNEL assay	35% $\text{VO}_{2\text{max}}$ = ↔, 60% $\text{VO}_{2\text{max}}$ = ↑ after d3, 85% $\text{VO}_{2\text{max}}$ = ↑ after d3
10	Healthy males and females (N = 14)	Treadmill runs at 80% and 60% $\text{VO}_{2\text{max}}$	Annexin-V FITC and Propidium Iodide	↑ with maximal exercise immediate postexercise
11	Male runners (N = 38)	Marathon run	Annexin-V FITC and Propidium Iodide	Highly trained = ↔, badly trained = ↑ 3h post marathon
11	Healthy males (N = 10)	Treadmill runs at 80% and 60% $\text{VO}_{2\text{max}}$	Annexin-V FITC and Propidium Iodide	60% $\text{VO}_{2\text{max}}$ = ↔, 80% $\text{VO}_{2\text{max}}$ = ↑
12	Endurance-trained male runners (N = 11)	2.5h treadmill run at 75% $\text{VO}_{2\text{max}}$	Annexin-fluorescein and 7-aminoactinomycin-D	↑ 2h postexercise
13	Healthy untrained males and females (N = 13)	Incremental treadmill test to exhaustion	May-Grünwald Giemsa staining	↑ with maximal exercise immediate postexercise
21	2 males and 1 female, healthy	Incremental treadmill test to exhaustion	Single cell electrophoresis assay	↑ with maximal exercise 6h and 24h postexercise
22	Untrained (N = 5), and distance trained males (N = 6)	Incremental treadmill test to exhaustion	Single cell electrophoresis assay, DNA migration	↑ with maximal exercise 24h postexercise
23	Healthy untrained males and females (N = 14)	Incremental treadmill test to exhaustion	May-Grünwald Giemsa staining	↑ with maximal exercise immediate postexercise
24	Healthy untrained females (N = 7) and males (N = 7)	Incremental treadmill test to exhaustion	May-Grünwald Giemsa staining	↑ with maximal exercise immediate postexercise
25	Healthy sedentary males (N = 18)	40 min cycle ride at 60% and 80% $\text{VO}_{2\text{max}}$	Annexin-V FITC and Propidium Iodide	↔ with 60% or 80% $\text{VO}_{2\text{max}}$
26	Endurance-trained males (N = 8)	2.5h treadmill run at 75% $\text{VO}_{2\text{max}}$	Annexin-V FITC and Propidium Iodide	↔ with exercise
27	Aerobically trained males (N = 8)	Treadmill runs at 80% and 60% $\text{VO}_{2\text{max}}$ Downhill run at 80% $\text{VO}_{2\text{max}}$	Annexin-V Fluos and Propidium Iodide HSP60 expression	↔ with exercise

Given the emerging evidence from reports that are unequivocal for exercise-induced lymphocyte apoptosis in animals, other explanations for the phenomena of lymphocytopenia must be considered. Hoffman-Goetz *et al.* studied the effect of treadmill exercise on early apoptosis in female C57BL/6 mice thymus and spleen (20). The percentage of viable splenocytes was significantly greater immediately (0 min) and 120 min following acute treadmill exercise versus the control. In addition, the percentage of apoptotic thymocytes was significantly lower, and percent of viable thymocytes was greater in treadmill exercised mice at 120 min post-exercise when compared to the controls. It was proposed that exercise might alter the survival of thymocytes that would otherwise be deleted through apoptotic mechanisms, resulting in an increase of viable thymocytes after exercise. It was suggested that the higher number of viable cells and lower apoptotic thymocytes may reflect selective migration of damaged thymocytes out of the thymus. It should be noted here that this review has included animal experiments in which leucocytes have been harvested from various pools and evaluated (i.e. peripheral blood, the spleen, the thymus, etc.). Another potential explanation for the variance seen between reports and unequivocal findings is that the

apoptotic process in lymphocytes is initiated in one pool, and completed in another due to migration or extravasation into another compartment. Further investigation into this prospect may help to explain a portion of the apparent contradictory findings noted in this review.

3.2. Human investigations

3.2.1. Morphological investigations

Apoptosis was described and defined originally in terms of morphological changes that occur during the cell death process. The technique served as the gold standard to which later biochemical assays were compared. Investigations which have incorporated acute exercise and an assessment of the morphological changes induced in lymphocytes have reported significant increases in apoptosis (see Table 2). Invariably, these studies have utilized treadmill running and exhaustive exercise in the study design.

Early studies evaluating the effect of exercise on DNA damage in lymphocytes are of interest because of the commonalities shared with the apoptotic process. During biomonitoring of workers exposed to genotoxic hazards, Hartmann *et al.* observed repeatedly increased DNA

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migration versus control values, using single cell gel electrophoresis (or the comet assay) for an otherwise healthy subject (21). They hypothesized that the results were due to sports activity performed by the subject on the day before the biomonitoring test and conducted a study to control for the effect of exercise. Three healthy volunteers performed a treadmill test to maximal exhaustion, and a 45-minute run below the aerobic-anaerobic threshold. No DNA migration was observed before the test or 6-min following. DNA migration was significantly increased at 6, 24, and 48 h following the test to exhaustion. It was noted that one subject was untrained, and showed relatively greater DNA migration at 6 and 24 h after the exercise bout compared to the other two trained subjects. No increases were seen at any time point following the exercise bout below the anaerobic threshold. Niess *et al.* carried out a study to look at the effect of distance training on lymphocyte DNA damage as measured by the single cell gel electrophoresis assay (22). Five untrained and six well-trained distance runners performed a maximal exertion test. DNA migration was similar for both groups before and 15 min after exercise, with a significant increase seen in the groups at 24 h after exercise. In addition, the percent of DNA migration at the 24 h post period was significantly greater in untrained compared to trained subjects. A significant negative correlation was observed between running velocity at the individual anaerobic threshold and the change in DNA migration 24 h after exercise. Since DNA damage was delayed following exercise, it was suggested that the damage was mediated by secondary factors such as lipid peroxidation products. It was further proposed that a lower rate of lipid peroxidation at rest in trained subjects resulted in reduced DNA damage for this group.

The first study to specifically evaluate the effect of acute exercise on lymphocyte apoptosis in humans was conducted by Mars *et al.* (8). Endurance-trained males completed an incremental treadmill test to exhaustion. Blood samples were taken immediately following exercise, 24 h, and 48 h after the test, and prepared for viewing with a fluorescent microscope for four patterns of DNA lymphocyte distribution (central core, peripheral aggregation, comet tail, and nuclear condensation - patterns of DNA located in the periphery or at one end were considered characteristics of apoptosis). In the pre-exercise samples, 12% of lymphocytes were classified as apoptotic. Immediately following exercise, the percent of apoptotic cells increased to 51%, and remained significantly elevated 24 and 48 h post-exercise. It was concluded that a single bout of high intensity aerobic exercise was sufficient to significantly increase the percentage of lymphocytes that exhibit characteristics of apoptosis.

We have used May-Grünwald Giemsa staining to morphologically assess cell death of lymphocytes following acute exercise in humans (23-24). In one investigation, untrained subjects completed a discontinuous incremental treadmill test to exhaustion with blood samples obtained at the end of each stage. Since significant increases in lymphocyte apoptosis were reported immediately following exercise, it seemed intuitive that the apoptotic process must

begin at some point, or threshold, during the exercise bout. We found that moderate-intensity exercise ($\sim 60\%$ $\text{VO}_{2\text{max}}$) was sufficient to induce a significant rise in apoptotic lymphocytes, and that each successive increase in exercise intensity was matched by a significant rise in the apoptotic index. In another study untrained male and female subjects were tested to determine if gender differences in the exercise-induced lymphocyte apoptotic response were evident. Participants completed a maximal effort treadmill test for $\text{VO}_{2\text{max}}$ on two occasions (females, once in the follicular phase, and once in the mid-luteal phase of the menstrual cycle). While post-exercise apoptotic values were similar for men and women, and similar for women regardless of phase of the menstrual cycle, the group as a whole displayed significant increases compared to baseline measurements. It is interesting to note that all of the investigations utilizing morphological methods for evaluating lymphocyte apoptosis have observed significant increases following exercise.

3.2.2 Biochemical findings

Biochemical techniques for assessing cell death were developed to overcome the limitation of subjectivity associated with the morphological methods described in the previous section. Investigations utilizing biochemical markers to assess exercise-induced lymphocyte apoptosis are divided between results where a significant rise is detected postexercise, and those that are unequivocal (see table 2). Similar to the morphological studies, significant lymphocyte cell death has been reported following exhaustive bouts of running on a treadmill. Investigations which have found no effect of acute exercise on lymphocyte apoptosis assessed cell death following prolonged treadmill runs. The apparent conflict between these investigations may be due to methodological considerations regarding the timing of sample treatment and apoptotic evaluation.

Exercise may be modulated by manipulating intensity, frequency, and mode, and each of these factors has been shown to have a significant effect on post-exercise lymphocyte apoptosis. Mooren *et al.* characterized the effect of exhaustive and moderate intensity exercise on lymphocyte apoptosis in humans (10). Volunteers completed a run to exhaustion at 80% of $\text{VO}_{2\text{max}}$, and an equal length run at 60% of $\text{VO}_{2\text{max}}$. The exhaustive bout of exercise resulted in $\sim 50\%$ increase in apoptotic lymphocytes immediately after exercise, but values had returned to baseline levels within the hour. The moderate intensity bout resulted in no change in percentage of apoptotic cells indicating that a certain threshold level of intensity is necessary to induce the cell death process. Hsu *et al.* conducted an elegantly designed study which showed that frequency combined with intensity has an effect on leukocyte cell death (9). Trained male runners completed a $\text{VO}_{2\text{max}}$ test, and then were asked to exercise at 35% of $\text{VO}_{2\text{max}}$ for 30 min daily on three consecutive days. Four weeks following, subjects were asked to return and complete the same protocol at either 60% or 85% $\text{VO}_{2\text{max}}$, and completed the final protocol one month later. The percent of apoptotic peripheral blood mononuclear cell lymphocytes was unchanged when exercise intensity was

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low. With exercise intensities of 60 and 85% $\text{VO}_{2\text{max}}$, apoptosis did not rise significantly until after exercise on the third day, but remained elevated through seven days indicating that frequent high-intensity exercise is capable of inducing apoptosis in lymphocytes. To date, only one exercise-induced lymphocyte apoptosis study has evaluated an exercise modality other than treadmill running. Wang and Huang studied the effect of various cycling intensities on oxidative stress and lymphocyte apoptosis (25). Sedentary men completed prolonged exercise bouts at moderate and severe intensity. While caspase measurements were unaffected by moderate exercise, caspase-8 and -9 increased immediately following severe exercise. Apoptotic lymphocytes were reported to be unchanged following either moderate or severe exercise. DNA fragmentation however, was significantly increased immediately following severe exercise compared to baseline values. It was suggested that severe exercise enhances oxidative stress-induced lymphocyte apoptosis, while moderate exercise may provide resistance to cell damage by upregulating antioxidants. While it is known that intensity and frequency have an effect on lymphocyte apoptosis, other factors such as duration and different modes of exercise need to be investigated. Greater understanding of each of these components may be advantageous in designing training protocols and prescribing exercise in various populations.

In addition to the factors detailed above, the effect of training status on lymphocyte cell death must also be determined. Mooren *et al.* studied exercise-induced lymphocyte apoptosis following a marathon and treadmill bouts in individuals of various aerobic capacity (11). Male subjects who were classified as either highly trained ($\text{VO}_{2\text{max}} > 60 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) or badly trained ($\text{VO}_{2\text{max}} < 55 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) completed a marathon. It was determined that the apoptotic response is dependent on cardiovascular fitness level. In the highly trained marathon group no differences were observed for the baseline (31%), immediate-post (29%), or 3h post exercise condition (28%). In contrast, the apoptotic response of the badly trained group was similar before (24%) and immediately after the marathon (23%), then significantly increased 3h post (33%). From this data it is apparent that in individuals with lower cardiorespiratory fitness levels, exercise-induced lymphocyte apoptosis can be induced either through prolonged submaximal exercise (such as a marathon) or through short-term high intensity exercise. The response is different in highly trained individuals, and the mechanism for the apparent resistance of lymphocytes to exercise-induced apoptosis in these individuals remains to be elucidated. In addition, training and longitudinal studies in which the same cohort of untrained individuals is assessed throughout the course of a training program would offer additional insights, and such investigations are warranted.

A number of investigations have not observed any pre-to-post exercise difference in exercise-induced lymphocyte apoptosis. These studies generally include well-trained endurance athletes as participants. Steensberg *et al.* studied the effect of prolonged exercise on

lymphocyte apoptosis and other variables (12). Endurance-trained runners completed a 2.5 h treadmill bout at 75% $\text{VO}_{2\text{max}}$. The percent of apoptotic lymphocytes was similar between pre and post-exercise values (~2%), and significantly greater 2 h after exercise (~3%). Based on these results, it was suggested that the decrease in lymphocyte numbers following exercise is not related to apoptosis. Peters *et al.* investigated the effect of prolonged exercise on lymphocyte apoptosis (26). Well-trained males completed 2.5h treadmill runs at 75% $\text{VO}_{2\text{max}}$. No difference for Annexin-V positive lymphocyte was reported between any of the sampling time points. It should be noted that most subjects completed two trials, with carbohydrate supplementation once and placebo administration on the other. These trials were not separated when apoptosis was analyzed. Measurement of cortisol showed a significant increase immediately postexercise compared to both baseline and 3h postexercise values. The authors concluded that prolonged submaximal exercise in well-trained individuals does not induce lymphocyte apoptosis, and that cortisol is not a mediating factor in this response. Recently, Simpson *et al.* investigated the effect of intensive level running and downhill exercise designed to induce muscle damage on lymphocyte apoptosis (27). Aerobically trained men participated, and no changes in cell death markers were in evidence at time points up to 24-hours post exercise following all types of exercise. It is of interest to note that while downhill running increased markers of muscle damage and inflammation, these factors had no effect on lymphocyte cell death. A unique aspect of this investigation compared to previous studies, is that lymphocyte subset populations were determined. The authors concluded that the observed post exercise lymphocytopenia can most likely be explained by removal of certain lymphocyte subsets, specifically CD8^+ and CD56^+ cells, rather than through removal via a cell death program. The investigations listed above which have found no significant difference in lymphocyte cell death following exercise have all utilized well-trained aerobic participants. As Mooren *et al.* found the response to be different with cardiovascular fitness level (11), future investigations into the ability of exercise to induce cell death in lymphocytes should take this into consideration. The ability to retain viable immune cells with exercise training rather than deletion through apoptosis is likely an advantageous adaptation when presented with an immune challenge.

While some studies employing biochemical markers of apoptosis have observed significant increases following acute exercise, other investigations have not. No significant difference postexercise compared to rest has naturally led some authors to conclude that exercise-induced lymphocyte does not occur. An alternate explanation for the lymphocytopenia following exercise-induced lymphocytosis given by these authors is that the immune cells migrate back into the lymphoid pools. A study by Simpson *et al.* provides evidence that exercise increases cellular adhesion molecules which could facilitate this migration of lymphocytes (28). Given the evidence provided by the animal and morphological literature, it may be countered that exercise-induced lymphocyte apoptosis does indeed occur, but must be assessed by a technique

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both sensitive and capable of detecting it. It is also likely that postexercise lymphocytopenia is due to a combination of cell death and migration. The contribution and effect of each is an area that warrants further investigation.

4. LIMITATIONS WITH CURRENT ASSESSMENT TECHNIQUES

4.1. Wide variance between reports

With regards to exercise-induced lymphocyte apoptosis, there is a discrepancy between cell death assessment methods. Previously published studies employing morphological techniques have reported apoptotic indexes between 19.1-51.5% (8,13), whereas investigations utilizing biochemical markers report apoptotic values between 2.2-2.6% (10,12). Based on the literature, it is unclear which measurement technique provides the most accurate representation of blood lymphocyte apoptosis.

4.2. Lack of an adequate method

One of the major limitations for assessment of exercise-induced leukocyte apoptosis is the lack of an adequate method, since most techniques for assessing apoptosis were developed and designed for use with *in vitro* cell cultures or tissue sections (29-32). Bedner *et al.* reported that flow cytometry measurements of apoptosis typically only measure a single cell-surface antigen (33). This method may under estimate the actual apoptotic response because sample collection time effects the stage of apoptosis that the cells will be in (i.e. the cell may be at a different point in the apoptotic process which may be before or after what a particular biomarker is designed to detect). In support of this conclusion, Barrett *et al.* found that annexin V failed to label approximately 66% of human carcinoma cells which displayed morphological evidence of apoptosis (34). Because a consistent biochemical parameter has yet to be identified, Tessitore *et al.* has reported that the apoptotic process is still best described in morphological terms (35). Thus, current techniques may capture only a very small portion of the overall apoptotic process, particularly in blood obtained following the rapidly changing conditions associated with exercise.

We have examined the effects of blood handling and immune cell isolation on apoptotic yield in subjects who completed one exhaustive exercise bout (13). Our key findings were that the exercise-induced apoptotic yield significantly decreased when blood samples were subjected to an isolation process following an intense bout of exercise. This is consistent considering that cell isolation techniques selectively separate viable cells (generally reported >95%) from those that are non-viable (and may be undergoing cell death through apoptosis). In the report we discussed various drawbacks to current biomarker techniques. In addition to relying only on a single molecular change, flow cytometry has been reported to be poorly reproducible due to cell suspensions that may contain between 3-30% erythrocytes, affecting measures since both mononuclear cells and erythrocytes appear in the same region of forward scatter and side scatter (36). To overcome erythrocyte contamination, pure leukocyte

fractions may be obtained through ammonium chloride lysing, however this procedure strongly induces apoptosis in lymphocytes (37) and neutrophils (38).

Cell death through apoptosis is a process that occurs with relative speed (39-41). The time from onset of apoptosis to the formation of apoptotic bodies has been estimated at only minutes by the researchers who are credited with naming the process (42). In studies that accidentally captured the apoptotic process in immune cells, direct measurement of the time from budding to the complete breakup of human leukocytes via time-lapse cinematography was reported to be 34 minutes (43-45). Studies on the developmental process of the nematode *Caenorhabditis elegans* have determined that specific cells undergo apoptosis at characteristic points in time and are normal and necessary part of initial growth (46-47). Measurement of the time course of apoptotic cells deleted during nematode development has determined that the entire cell death process is completed within one hour of the cell's birth (48). Under the controlled conditions associated with cell culture methodology, cells can be prepared (isolated and subjected to multiple washes) prior to the addition of apoptotic stimuli without affecting yield. However, if the stimulating event occurs *in vivo*, it is clear from the literature that much of the apoptotic yield will be lost merely in the time taken to process a sample for the assessment of apoptosis. Thus, due to the dynamic and rapid progression of apoptosis and the body's internal environment during exercise, an appropriate methodology must be chosen, else the researcher may find incomplete results regarding the process of apoptosis.

Based on the current literature, existing methods for assessing apoptosis may not provide an accurate measurement in the blood of an exercise individual because of the time necessary to prepare samples for biochemical assessment. Previously we have employed a whole-blood morphological technique described in the exercise science literature because it has been speculated to more closely represent the *in vivo* exercising condition (13,24-25). However, this morphological method has numerous limitations as well. Chief among these is the subjective identification of apoptotic cells, in which an investigator must use their own judgment to determine whether a particular cell is displaying characteristics of initiated cell death. It is desirable that the investigator evaluate all conditions in a blinded fashion; however the significant increase in absolute numbers of lymphocytes with exercise should make samples from an exercised condition fairly obvious. It is possible that this knowledge, even at a subconscious level, introduces observer bias in which an investigator may expect to see a greater number of apoptotic cells with exercise and identify cells accordingly. A second limitation is that one can not separate total leukocyte into their various sub-fractions. Finally, in addition to being time consuming the morphological method only evaluates relatively few cells per sample. For example, if 100 cells are counted and five lymphocytes are identified as being apoptotic, an apoptotic index of 5% is reported, which may not truly represent the actual concentration of cell death in the blood. The advantage of cytometry methods in this regard is that many more events

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can be assessed toward identifying a representative measure of the cellular population. It is apparent that this field of study may benefit from the development of a new method which addresses the limitations of current morphological and biochemical techniques.

5. CONSIDERATIONS FOR DEVELOPING A NEW TECHNIQUE

5.1. Describe the process

Based on the available literature, it appears that the best method of assessing apoptosis is one which looks at the entire process and not just a snapshot at single point in time. The “snapshot” approach may under or over estimate apoptosis because a portion of the cells that are undergoing apoptosis may not fully express specific biochemical markers. The corollary is akin to viewing a sporting or cultural event, and trying to understand what has happened by viewing a single picture of the occasion. Even if a single picture is taken of many different players, it is nearly impossible to assess what has happened unless the entire event is viewed. A new method would allow for the quantitative assessment of the entire apoptotic process based on both biochemical markers and classic morphological changes associated with programmed cell death.

5.2. Objectivity

The primary drawback of the morphological technique is the lack of objectivity associated with the evaluation of apoptotic cells. A technique that describes the full range of the process would ideally incorporate both biochemical and morphological components, and overcoming the subjectivity of the morphological method would be of paramount importance. An automated system that was based on known biochemical events as well as the classic morphological characteristics of cell death (i.e. cell shrinkage, breakdown of DNA into characteristic lengths, orderly condensation of the nucleus, and the production of membrane blebs) would remove subjective limitations.

5.3. Discriminate subfractions

Another limitation with the morphological method is the inability to distinguish between various subfractions of lymphocytes. A joint biochemical and morphological technique could use current CD specific monoclonal antibodies currently available (CD20 to mark B-cells, CD4 to mark T-cells, a combination of CD3-negative and CD56 to mark NK cells, and CD14 to mark monocytes). This would allow investigators the ability to evaluate whether exercise induces apoptosis differently in various subfractions.

5.4. Timing of sampling and processing

As detailed previously, one of the primary drawbacks associated with biochemical methods is the amount of time required for cell isolation, incubation, and staining. It is probable that cells induced to die via the apoptotic process are deleted even before marking and biochemical analysis takes place. Because of this, standardized sampling procedures should be established which minimizes this possibility.

5.5. Time and cost effective

As described above, a technique that is time effective would be ideal. Another shortcoming of the morphological method is the time investment required to detect the requisite number of lymphocytes microscopically on a slide (generally at least 100 lymphocytes per slide) and evaluate them for the characteristics of apoptosis. As the timing of blood sampling is also an issue with the biochemical technique, a process that is time efficient with regards to processing and evaluation would be ideal. In addition, to maximize the possible widespread application of the technique, the cost for each sample analysis should be taken into consideration. A methodology that is not cost prohibitive would be optimal in this regard.

5.6. *In vivo* measures

To date, techniques utilized to evaluate exercise-induced lymphocyte apoptosis are not reflective real-time measures. In order to determine the effect of exercise or physical activity on lymphocyte cell death, we feel that an *in vivo* technique would be preferred. This would overcome the effect of a time-delay (i.e. either death induced during the cell preparation period following sampling, or a reduction in yield as apoptotic cells are deleted prior to evaluation for apoptosis). In addition, assessing cells in the internal milieu would remove the potential that cell death could be induced by factors other than the inducing event. Ideally, in combination with an *in vivo* measure, a standardized technique for exercise-induced lymphocyte apoptosis would also include the considerations previously listed.

6. CONCLUSION

In conclusion, our answer to the title query is yes, we feel that taken together the literature to date provides enough evidence to say that exercise really does induce lymphocyte apoptosis. The phenomenon of exercise-induced lymphocyte apoptosis has been observed in both the animal and human subject literature. Are we saying that directed cell death is responsible for the entire decrease in lymphocytes following the cessation of exercise? No, but it could explain at least a portion of the lymphocytopenia observed postexercise, although it is likely that the contribution changes according to cardiovascular fitness level. Exercise is a physiological stimulus that significantly alters the internal milieu both in terms of circulating factors (increasing catecholamines, cortisol, and Fas ligand) as well as inducing oxidative stress within the cell. While the exact mechanism of exercise-induced lymphocyte apoptosis has yet to be determined, it is likely that a combination of external and internal signals contribute to the response that has been reported in most, but not all, investigations. The studies that have not reported increased in lymphocyte cell death following acute exercise have proposed that the cells migrate back into the lymphoid pools following activity. We agree that there is compelling evidence that cells extravasate from the peripheral blood compartment into other tissues and suggest that it is likely that both the processes of migration as well as apoptosis are active to some extent in the lymphocytopenia observed following exercise. Determining the contributions of each

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mechanism is an area that requires further investigation as understanding postexercise lymphocytopenia develops. The reported discrepancies between morphological and biochemical techniques for evaluating cell death postexercise is likely due to an inadequate and unstandardized methodology for assessment. In truth, it is very difficult to make comparisons from one investigation to the next due to the wide array of techniques employed for the assessment of exercise-induced lymphocyte apoptosis. We feel that an ideal technique is one that combines both the biochemical and morphological method to assess apoptosis in lymphocytes over the entire process *in vivo*, is automated and objective, discriminates between subfractions, and is both time and cost effective. As we move forward in this area of research, a standardized and accepted methodology would allow for valid comparisons between studies and deepen our understanding of this process.

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