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INTRA- AND INTERRATER RELIABILITY OF MORPHOLOGICALLY EVALUATED LYMPHOCYTE APOPTOSIS IN TRAINED AND UNTRAINED OBSERVERS

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ABSTRACT: Exercise-induced lymphocyte apoptosis has been reported using biochemical analysis and morphological assessment. Morphological evaluation is limited by the subjective nature of the technique. This investigation evaluated the intra- and interrater reliability of trained and untrained observers assessing apoptosis morphologically. Blood smears at baseline (PRE) and following cycle ergometer exercise (POST) were assessed microscopically for each condition. To obtain reliability measures, cell sets were evaluated for morphological characteristics of apoptosis on two separate occasions by trained and untrained observers using the intraclass correlation coefficient. Test-retest reliability for trained observers was higher for both conditions than untrained observers. Interrater reliability was below-average or below acceptable regardless of training status. Exercise may induce apoptotic changes in lymphocytes that are more easily discriminated by trained observers. Investigations assessing exercise-induced apoptosis should train observers in order to produce reliable results, and a single trained observer would be expected to yield the most reliable results.

KEY WORDS: Stability reliability, programmed cell death, white blood cells, methodological evaluation

INTRODUCTION

Strenuous exercise is a physiological stress that has been reported to induce lymphocyte cell death in both animals [1,5,6] and humans [7,11,14]. The sequence of this cell death process was first identified based on cellular changes (i.e. morphologically) and given the name "apoptosis" by Kerr et al. [8] from the Greek used to describe the falling off of leaves from trees or petals from flowers. Apoptosis occurs in a distinct manner and progression with characteristics including cell shrinkage, orderly condensation of the nucleus, and the production of membrane "blebbing" that eventually leads to the formation of so-called apoptotic bodies. Exercise-induced lymphocyte apoptosis has been measured through both biochemical analysis [7,12,20,22] and via morphological evaluation in the recent literature [10,15]. However, there appears to be a marked difference between the apoptotic yield reported between these two methodologies with morphological investigations observing greater incidences compared to biomarker studies (morphological = 20%, biomarker = 2.5%).

We have detailed many potential reasons and limitations associated with the discrepancy between methods [13], however the focus of the current investigation is on the morphological method of assessing apoptotic lymphocytes. Considering morphological evaluation, the main drawback is a limitation due to the subjective nature of the technique. This method requires an investigator to identify lymphocytes on a microscope slide (generally at least 100 per condition), and then make a determination about whether each cell is normal or displays characteristics of cell death (including cell shrinkage, membrane blebbing, or the formation of apoptotic bodies). It was hypothesized that individuals who were provided training with regards to cell death characteristics commonly displayed following exercise would return more reliable results when compared to individuals who were provided with literature only. Therefore, the focus of this investigation was to evaluate the intra- and interrater reliability of trained and untrained observers when assessing cell death via the morphological technique in lymphocytes before and after an exercise bout.

MATERIALS AND METHODS

Participants. Blood smears on microscope slides in which lymphocytes individually were identified, were utilized from a previous investigation [14]. The Committee for the Protection of Human

Subjects approved the study, and participants voluntarily completed the requisite informed consent documents. Descriptive data for this subject pool completing the exercise (N = 10) included an average age of 34±8 years old, and mean height and weight of 173.6±8.1 cm and 69.8±10.6 kg respectively. In the present study, two trained and two untrained individuals were used to determine reliability coefficients associated with exercise-induced lymphocyte apoptosis in resting (PRE) and postexercise conditions (POST). Trained individuals were provided with examples of apoptotic and non-apoptotic lymphocytes, and three 1h individual training sessions with an expert in identifying morphological characteristics of cell death. Untrained observers were only provided with literature [8] depicting the apoptotic process.

Protocol

The protocols for the determination of VO2peak and exercise trial are described in detail in our previous investigation [14]. Briefly, an incremental test on an electronically-braked cycle ergometer (Velotron Pro, RacerMate, Inc.; Seattle, WA) was performed to exhaustion with respiratory gases measured using an automated system (Cardio Coach Plus; Salt Lake City, UT). On a separate day, participants completed a 60-min ride at ~80% VO2peak following an overnight fast.

Whole blood smears were made at baseline following a 20 min rest (PRE) and following the prolonged cycle ergometer exercise (POST) as we have previously described [15]. Briefly, May-Grünwald stain was used to stain the slides (Sigma-Aldrich, Inc; St. Louis, MO) for 3-5 min, and then washed in phosphate buffered saline (PBS) for 3 min. A modified Giemsa stain (Sigma-Aldrich, Inc) was applied to each smear for 2 min, rinsed in deionized water, and allowed to air dry. This process was completed within 10 min of whole blood collection.

Digital pictures of lymphocytes from each condition were obtained using a camera (MA88 Microscope Digital Camera, C&A Scientific Co., Inc.; Manassas, VA) mounted to a light microscope (CXL Plus, Labomed, India). At least 100 cells from each condition (PRE N = 100 cells, POST N = 100 cells) were used for the evaluation of apoptotic characteristics and the determination of rater reliability.

Statistical Analysis

To obtain reliability measures, cell sets in each condition were evaluated for morphological characteristics of apoptosis on two separate occasions by two trained and two untrained observers. Evaluation sessions occurred at least one week apart. Images were presented in a randomized order on each occasion to prevent recall or an order effect. Reliability was assessed using the intraclass correlation coefficient (R) in SPSS (SPSS Inc., Chicago, IL), and acceptability categorized according to the classification standards noted by Baumgartner et al. [2]. The Fisher r-to-z transformation was utilized to determine significant differences between correlation coefficients.

RESULTS

Test-retest Reliability. Untrained observers displayed stability reliability coefficients that were considered below-average acceptability under resting conditions (R = 0.74) and following exercise (R = 0.75). No statistical difference was noted between these stability reliability coefficients (z = 0.16, p = 0.4364). In contrast, the test-retest reliability for trained observers was significantly higher for both conditions than untrained observers (rest z = 2.96, p = 0.0015; postexercise z = 5.22, p < 0.0001). At rest, an average acceptability was noted (R = 0.88), and an above average acceptability was observed when lymphocytes in a postexercise condition were evaluated (R = 0.94).

Interrater Reliability

The interrater reliability was determined between the two trained and untrained observers. We found that there was a difference between conditions, such that the coefficient was higher for trained observers in the baseline resting condition (R = 0.76) compared to untrained (R = 0.65). However, when comparing similarly trained raters evaluating lymphocytes from the postexercise condition, interrater reliability coefficients were actually lower between trained observers (R = 0.56) than untrained (R = 0.69).

Apoptotic Index

The apoptotic index was calculated as the number of apoptotic cells divided by the total number of cells, and expressed as a percentage. While untrained observers detected greater numbers of apoptotic cells for each condition compared to trained observers (see figure 1), unpaired t-tests revealed no significant differences (Pre p = 0.158, Post p = 0.07), likely due to the small number of data points for this type of analysis (N = 4).

DISCUSSION

The purpose of this investigation was to assess reliability measures associated with the morphological method of evaluating exercise-

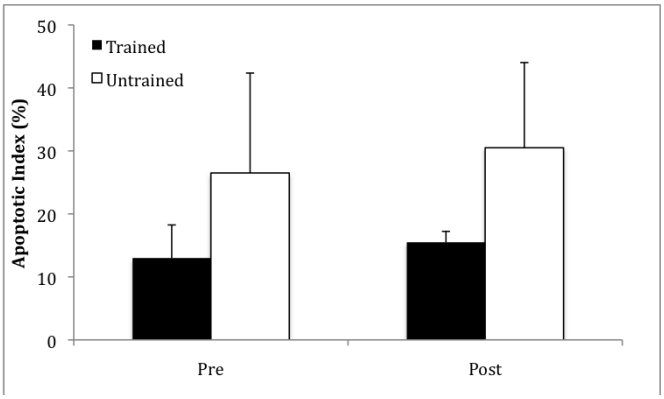

FIG. 1. MEAN AND STANDARD DEVIATION (BARS) OF APOPTOTIC LYMPHOCYTES FROM RESTING (PRE) AND EXERCISING CONDITIONS (POST) AS EVALUATED BY TRAINED AND UNTRAINED OBSERVERS.

induced lymphocyte apoptosis. Specifically, we wished to determine the effect of training sessions to enhance the ability of observers to recognize characteristics of cell death, compared to observers who were presented with literature alone (and considered untrained for our purposes). Our hypothesis was that training sessions would increase reliability measures, both stability as well as intrarater reliability, when observers rated lymphocytes from resting and postexercise conditions.

Lymphocyte apoptosis attributed to exercise was first described by Mars et. al. [10] using a morphological technique. Various authors have used single biomarkers indicative of cell death to determine that the oxidative stress from heavy exercise influences lymphocyte apoptosis [22], that consecutive days of high-intensity treadmill exercise has an impact on measurements [7], and that endurance training status affects the lymphocyte apoptotic response [12]. It appears that this methodology may be problematic as other investigators, also using a single biomarker to indicate apoptosis, have found no effect of exercise on lymphocyte cell death [18,20]. Our research group has used the classic morphological method, and found that the apoptotic index in lymphocytes increases with exercise intensity [16], that there is no difference in response based on gender or menstrual cycle phase [17], and that cycle exercise is capable of inducing increases in the cell death yield [14]. However, this technique does present various drawbacks as well, including the subjective differentiation of lymphocytes into the categories of normal or apoptotic by observers. Thus, a determination of the test-retest reliability as well as intrarater reliability of observers is important as we contribute the research in this particular area of interest.

Our results show that regarding test-retest reliability, training sessions appear to increase the stability coefficient. When evaluating lymphocytes from blood smears taken at rest, trained evaluators displayed average acceptability (R = 0.88), while we found a below average acceptability for untrained observers (R = 0.74). In the postexercise condition following exhaustive exercise the difference becomes more dramatic, as trained observers exhibit an above average acceptability (R = 0.94), whereas untrained evaluators maintain a below average acceptability (R = 0.75). It appears from these results, that individualized training increases the stability reliability measures above the ability for an individual to make determinations based on the literature alone. This is particular note when cells from the postexercise period are evaluated (i.e. exercise-induced lymphocyte apoptosis). While we are unable to make a definite determination behind this phenomenon, it is possible that exercise may induce apoptotic changes in lymphocytes that are more easily discriminated morphologically by trained observers.

To our knowledge, this is the only investigation to report stability reliability for exercise-induced immune parameters. Regarding plasma values of factors associated with health, one previous study measured the reliability and stability of biomarkers for lipids in the blood from a cohort of the Shanghai Men's Health Study [9]. The intraclass correlation coefficient for LDL-cholesterol was reported to be 0.58, 0.65 for triglycerides, 0.75 for total cholesterol, and 0.83 for HDL-cholesterol [9]. In a different investigation, the stability reliability of a progressive endurance run to predict maximal oxygen consumption was reported to be 0.96 [19] for different laps of a submaximal test. In a test of a novel repetitive jumping device, the stability intraclass correlation coefficient between measurements taken seven days apart yielded values of 0.95 for oxygen uptake, 0.89 for heart rate, and 0.75 for ratings of perceived exertion [21]. Thus, the stability reliability coefficients reported in the present study are similar to values from tangentially related literature.

In addition to stability measures, we wished to determine the interrater reliability between individuals of the same trained status. Regardless of condition, untrained observers displayed coefficients that were not considered to be of an acceptable value (Pre R = 0.65, Post R = 0.69). Trained observers returned an acceptable interrater reliability in the resting condition only (Pre R = 0.76, Post R = 0.56). It appears that while three training sessions are sufficient to develop acceptable stability measures, interrater coefficients may require more instruction and training to develop. This is particularly true with the evaluation of lymphocytes from a postexercise condition. In trained observers, although initially receiving a similar training protocol, it is possible that the one became more adept at identifying the difference between apoptotic and non-apoptotic cells from session to session. It is possible that this effect contributed to differences between the scores of the trained observers and lead to the unacceptable interrater reliability reported in the postexercise condition. This finding underscores the variability inherent with the subjective nature of the visual morphological assessment method, and the importance of trained investigators when utilizing this technique.

While this is the first study to report interrater reliability measures for exercise-induced lymphocyte apoptosis, previous investigations have evaluated the ability of multiple observers to rate various exercises [23], as well as measures associated with cell death [3,4]. Weir et al. asked six observers to assess participant performance on various core stability exercises, and then again five weeks later [23]. Intrarater reliability ranged between 0.09 and 0.55 while utilizing a four point visual scoring system. An intrarater reliability coefficient of 0.89 was reported between independent observers who evaluated apoptosis in colonic cells induced by bile acids [3]. In the investigation by Brugnon et al., two observers analyzed fluorescein labeled apoptotic spermatozoa via fluorescence microscopy from two different samples [4]. Using caspase expression as the indicator for impending apoptosis, the intraclass correlation coefficient between observers for this biomarker was 0.939. When compared to the previous investigations evaluating apoptosis in various tissues, the intrarater reliability coefficients in the present study were somewhat lower. Potential explanations for this difference are likely in the methodology used to assess apoptosis (May-Grunwald Geimsa in the present study, compared to specific antibodies), or differences in tissues observed (lymphocytes in the current investigation).

CONCLUSIONS

In summary, we designed this investigation to determine the effect of using trained observers when assessing exercise-induced lymphocyte apoptosis using the morphological method. We found that stability reliability was increased with a series of training sessions in the evaluation of both baseline and postexercise lymphocytes when compared to untrained raters. On the other hand, interrater reliability was below-average or below acceptable regardless of training status. Based on these results it is recommended that investigations assessing exercise-induced lymphocyte apoptosis via the morphological method train observers in order to produce reliable results. Because of low interrater reliability, a single trained observer with the greatest amount of experience would be expected to yield the most reliable results.

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