Effects of Exhaustive Exercise on Peripheral Blood Lymphocytes

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Summary:

Background: Acute exercise stress is associated with a lower lymphocyte functional response. It is suggested that exercise can enhance or reduce immunity depending on the frequency, duration, and intensity of the exercise.

Subjects and Methods: From November 2007 to January 2008, thirty healthy Volunteers were recruited from medical, paramedical staffs, and students of Baghdad College of Medicine (all males). Participants were chosen according to the following: (1)Age (between 20 and 35 years), (2) Non obese : Body mass index (BMI in kg/m^2) was used as an index of obesity, (3) Non-smoker, (4) No recent medical problem, (5) No recent medications, and (6) No musculoskeletal complaint. After a general medical checkup, the thirty subjects were subjected to the exercise protocol. Blood samples were taken before, and immediately after the end of exercise.

Results: The mean age of individuals enrolled in the study was 29 ± 4years. Exercise induced significant granulocytosis whereas lymphocytes declined to a level significantly below the baseline. Trypan blue exclusion test showed that the initial percentage of nonviable lymphocytes was 2.90% ± 0.2%. After exhaustive exercise, cell viability decreased where percentage of nonviable cells was 4.3% ± 0.3% ($p<0.005$). Early apoptotic lymphocytes showed membrane blebbing and did not take the dye, while dead cells appeared blue from dye uptake. MDA was 3.0 ± 0.23 nmol/ml. An increment in the mean value of MDA was observed in the post exercise blood sample that was withdrawn immediately after exercise ($p<0.005$).

Conclusion: The present study shows that exhaustive exercise induces apoptosis in peripheral blood lymphocytes manifested by increased level of malondialdehyde, an oxidative stress marker. This finding supports the hypothesis that apoptosis could be involved in exercise-induced lymphocytopenia and this effect depends on exercise intensity.

Keywords: Apoptosis, Exercise, MDA, lymphocyte.

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Introduction:

Apoptosis is a physiological type of cell death that occurs as a part of normal development and regulation of growth and cell number (1). After exposure to antigens, the immune system becomes activated and immune cells proliferate and secrete cytokines and antibodies. After the pathogen is eliminated, the immunological response needs to be terminated. Apoptosis of immune cells plays an important role in this regulatory process (2).

Exercise has been shown to induce inflammatory-like changes in immune cell counts (3). Acute exercise stress is associated with a lower lymphocyte functional response. It is suggested that exercise can enhance or reduce immunity depending on the frequency, duration, and intensity of the exercise (4). All leukocyte subsets increase during exhaustive exercise. When exercise ends, granulocytes remain increased for several hours after the test, whereas lymphocytes decrease rapidly to a level well below the pre-exercise value (5). Depending on the intensity and type of exercise, this decline in lymphocyte count may last from several hours up to days and may account for post-exercise immune suppression (6). Mechanisms responsible for exercise-induced lymphocytopenia remain to be elucidated. Homing to lymphoreticular structures may play a role in lymphocytopenia (7). Strenuous exercise modulates several factors, such as reactive oxygen species, DNA damage, and hormone and cytokine levels, which are involved in the regulation of apoptosis in various cell types (8).

In this study the effect of exhaustive exercise on the generation of reactive oxygen species was investigated. Exhausting exercise increases the oxygen demand 10 to 15 times compared to rest, resulting in increased oxygen consumption, increased oxygen cell uptake and increased flow in the electron transport chain, leading to an increased release of reactive oxygen species (ROS) (9).

Oxygen free radicals react with polyunsaturated fatty acids and form new radicals which initiate a chain reaction of lipid peroxidation. So malondialdehyde, the end-product of lipid peroxidation, level is considered as an indicator of free radical generation in the human body (10).

Subjects and method:

From November 2007 to January 2008, thirty healthy individuals (all males) were enrolled in this study after being informed about the nature, purpose, and potential risks, each subject signed an informed consent statement. Volunteers were recruited from medical, paramedical staffs and students of Baghdad College of Medicine. Selection criteria: (1)Age (ranging between 20 and 35 years of age), (2) Non obese : Body mass index (BMI in kg/m^2) measurement was used as an index of obesity, and is

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calculated using the following formula: BMI= Weight (kg) / [Height (m)]^2, participants were within the normal range (18.5 – 24.9) (11), (3) Non-smoker, (4) No recent medical problem, especially viral infection or lymphocyte disorders such as lymphoma and leukemia, (5) No recent medications, and (6) No musculoskeletal complaint that may prevent the performance of the exercise. After a general medical checkup, including the pulse rate, temperature, blood pressure, respiratory examination, and complete blood picture, the thirty subjects were subjected to the exercise protocol. Blood samples were taken before exercise, and immediately after the end of exercise. Oral temperature, blood pressure and pulse rate were measured for participants before and after exercise.

Exercise protocol: A standardized exercise challenge test was performed on a bicycle ergometer (Lode Institute, N.V., and Holland). Exercise was commenced at 25 watts (153 kg-m/min), and the work load was raised by 25 watts each minute until 85% of maximal predicted heart rate (0.85 x [210 – 0.65 x age in years]) (11) was reached. Exercise was then sustained at that load for another four to six minutes, for total exercise duration of five to ten minutes. The subject is free to choose his own number of revolutions per minute (rpm) at a certain load, this means that the load (power output), once adjusted, cannot be, altered by the subject through changing the rate of speed, which may thus be freely chosen. The maximal work load in watts was 80% of the predicted maximum workload. The predicted maximum workload was calculated according to the method of Eggleston and Guerrant, 1976 (12) (For men, a load of maximum 2 watts per kg body weight). 1 watt=6.12 kg-m/min (McArdle et al.)(13)

Process of lymphocyte separation:

Lymphocyte separation media: Ficoll 400, (Pharmacia Fine Chemicals) lymphocyte separation medium is an aqueous solution of a high-density sucrose epichlorohydrin polymer and sodium diatrizoate. The instructions of the manufacturer of this medium stated that the concentration of the constituents is such that a density 1.077 ± 0.001 g/ml is achieved whilst maintaining a low viscosity and an osmolality of 270 - 300 mOsm/Kg. Phosphate buffered saline (PBS, pH 7.2) solution was used to washout and re-suspends separated lymphocytes for different analytical procedures. Phosphate buffer solution was freshly prepared in each experimental set. Procedure of lymphocyte separation: Two millilitres of the blood sample was put in anticoagulant (EDTA-potassium) and diluted 1:1 ratio with phosphate buffer solution. Two millilitres of this mixture was carefully layered (not mixed) on the top of 4 millilitres of Ficoll, which was dispersed in 10 millilitres siliconized glass centrifuge tube. Then, the tube was centrifuged at 400 g (2100 rpm), for 20 -25 minutes at room temperature. As a result of centrifugation different layers has been separated, the lymphocytes formed a white puffy coat at the interface of the blood plasma and the separating medium. The lymphocytes separated layer was aspirated (not disturbed) by Pasteur pipette and transferred into a 10 ml siliconized tube containing approximately three volumes of PBS. The above mixture was centrifuged at 400 g for 20 minutes and the supernatant was discarded. Then, the lymphocytes pellet was resuspended again in PBS and centrifuged at 200 g for 10 minutes. This step allowed most of blood platelets to be maintained in the supernatant, which was discarded. Finally, the lymphocyte pellet was re-suspended in 0.5 ml of PBS. This represents the lymphocyte suspension, which was examined for morphology.

Examination Methodology:

Cell viability: Trypan blue exclusion test was applied for assessment of cell viability for each sample. The principle of this test is that the viable cells exclude Trypan blue dye (i.e. not stained), while cells that accept the dye (stained blue) were regarded as dead cells (Fig 3). A known volume of lymphocyte suspension (100 microliter) was mixed with an equivalent volume of Trypan blue dye and examined by light microscope using X40 lens power. Cell count: The number of lymphocytes was determined by using haemocytometer chamber and the results were expressed as cell/mm.

Total number of viable cells = A x B x C x 10^4

Total dead cell count = A x B x D x 10^4

To give a total cell count = viable cell count + dead cell count

$$\% \text{ viability} = \frac{\text{Viable cell}}{\text{Total cell}}$$

Where A = volume of cells, B = dilution factor in trypan blue, C = mean number of unstained cells, D = is the mean number of dead/stained cells, 10^4 is the conversion of 0.1 mm^3 to ml.

Assessment of biochemical variables relevant to oxidative stress Malondialdehyde level: Malondialdehyde was measured according to the procedure described by Guidet et al. (14). Statistical analysis: The results were expressed as (mean ± standard error). Paired T test was used for comparison between two groups, before and after exercise. P value of <0.05 was considered significant.

Results:

The mean age of individuals enrolled in the study was 29 ± 4 years. Blood pressure and body temperature were measured before and after exercise. Peripheral radial pulse rate was monitored throughout the exercise. (Table 1) Results were expressed under two distinct lines of changes: Morphological Changes and Lymphocyte Count: They are related to structural changes and lymphocyte viability as assessed by trypan blue exclusion test. Biochemical Changes; that are related to lipid peroxidation activity assessed by malondialdehyde level.

Pre and Post Exercise Data:

Lymphocyte count: The exhaustive exercise induced significant granulocytosis whereas lymphocytes declined to a level significantly below the baseline (table 1 and figure 1). Lymphocyte Viability: Trypan blue exclusion test showed that the initial percentage of nonviable lymphocytes was 2.90% ± 0.2%. After exhaustive exercise, cell viability decreased where percentage of nonviable cells was 4.3% ± 0.3% ($p<0.005$). (Fig. 2). Early apoptotic lymphocytes showed membrane blebbing and did not take the dye, while dead cells appeared blue from dye uptake. (Fig. 3 and Fig. 4) Biochemical Changes (Malondialdehyde Level): MDA is measured in the serum of the freshly obtained blood sample of each individual and the mean was 3.0 ± 0.23 nmol/ml. An increment in the mean value of MDA was observed in the post exercise blood sample that was withdrawn immediately after exercise ($p<0.005$). (Table 1, Fig 5).

Table 1: Pre and Post exercise data

	Pre-exercise (n = 30) Mean ± SE	Post exercise (n = 30) Mean ± SE *	P value
Heart Rate	71 ± 6	130 ± 7	P<0.01
Blood Pressure (mmHg)	110/77 ± 12/10	140/89 ± 7/6	P<0.05
Temperature (°C)	36.9 ± 0.2	37.2 ± 0.23	P<0.05
Trypan blue exclusion test Percent of nonviable cells	2.90% ± 0.2%	4.3% ± 0.3%	P<0.005
Percent of Viable cells	97.1%	95.7%	P<0.005
Leukocytes (x 10^9)	6.120±2.90	7.080±5.20	P<0.05
Lymphocytes(x 10^9)	1.960± 1.50	1.380 ±1.30	P<0.05
MDA (nmol/ml)	2.6 ± 0.23	3.38 ± 0.17	P<0.005

* Values presented as mean ± standard error

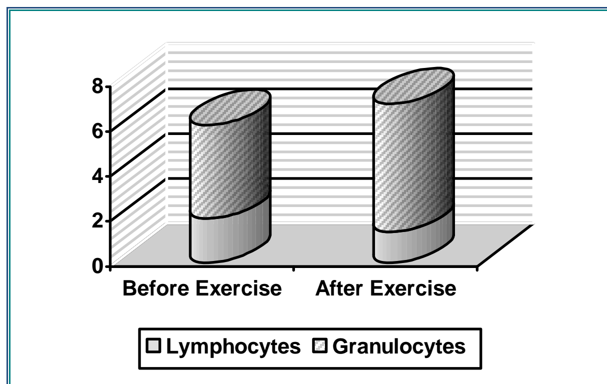

Fig. 1: Percent of white blood cells before and after exhaustive exercise.

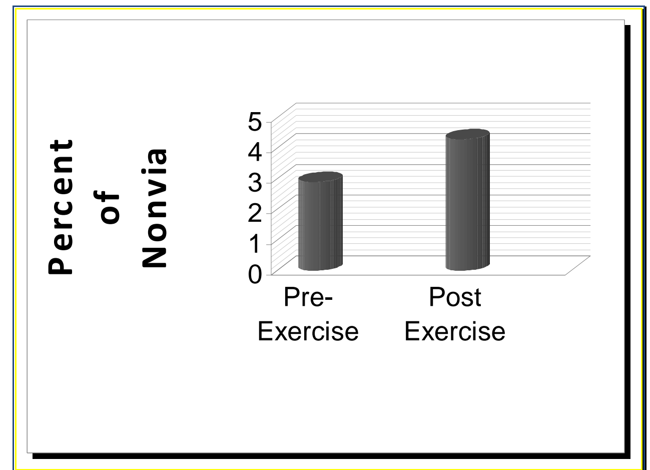

Fig. 2: Percent of nonviable cells before and after exhaustive exercise.

a)

b)

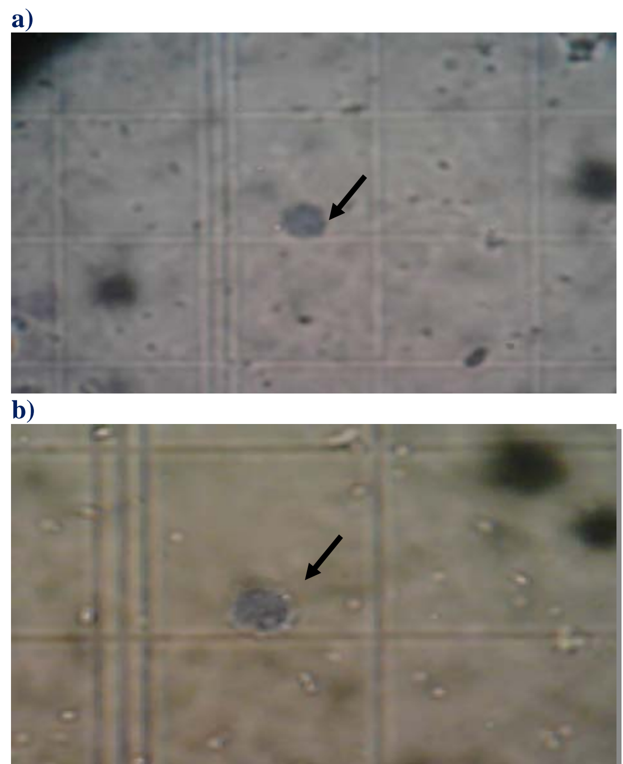

Fig. 3 (a) and (b): Dead Lymphocyte that took up trypan blue dyes (x40).

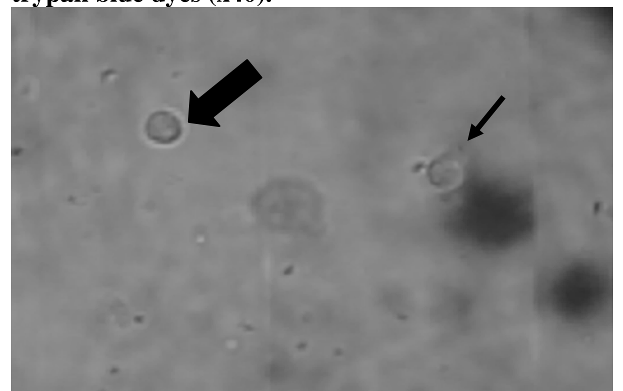

Fig 4:- Viable lymphocyte not taking up the dye (thick arrow)
-Early apoptotic lymphocyte showing bleb formation (thin arrow) (x40).

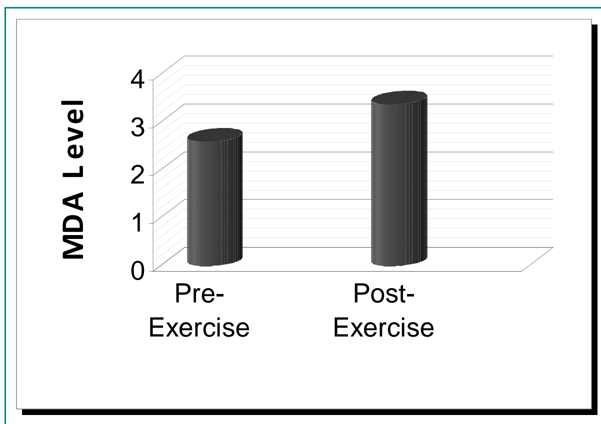

Fig.5: MDA level pre- and post-exercise.

Discussion:

Exercise has been shown to induce inflammatory-like changes in immune cell counts and acute-phase protein release (2, 3). It is well documented that strenuous exercise recruits both neutrophils and lymphocytes into the circulation. However, during recovery lymphocyte concentrations rapidly decrease, resulting in lymphocytopenia if exercise intensity and duration is of sufficient magnitude (15).Maximal exercise stimulates a variety of signals, such as increases in glucocorticoid secretion, intracellular calcium levels, and reactive oxygen species, which can potentially induce apoptosis. In addition, glutathione depletion, thiol oxidation, DNA damage, and hypoxia have been reported during exercise and may likely contribute to programmed cell death(16).Several studies in humans have confirmed that exercise is able to induce DNA damage. Niess et al. (17) found DNA damage in leukocytes after a half-marathon race. After maximal exercise the percentage of apoptotic lymphocytes increases immediately after the test. Likewise, Mars M. et al. study investigating human subjects exercising on a treadmill until exhaustion found DNA strand breaks in lymphocytes immediately after exercise. These events could possibly explain the greater incidence of upper respiratory infections in highly trained athletes following maximal training periods. (18) Hoffmann-Goetz and co-workers investigated whether exercise-induced changes in glucocorticoid levels were responsible for the apoptosis induction. Their data suggest that in vitro exposure to corticosterone at the physiological concentrations observed after moderate exercise were already effective in inducing apoptosis in thymocytes. Likewise, catecholamines were found to induce apoptosis in peripheral blood lymphocytes in a time- and concentration-dependent manner (19). Although we did not determine either catecholamines or cortisol levels in the present investigation, their levels are known to correlate to exercise intensity. Another major trigger of apoptosis is changes in the intracellular free calcium concentration buffer. Mooren et al study has demonstrated [(20)] that maximal exercise is associated with alterations in intracellular calcium signaling of lymphocytes, and this would support the hypothesis that apoptosis could be involved in exercise-induced lymphocytopenia and this effect depends on exercise intensity. Finally, changes in the cellular redox status may be another important apoptosis trigger. The generation of free radicals and ROS is markedly enhanced during exercise. ROS are well known to induce damage of cellular structures and DNA. Therefore, in a number of cell types, ROS are involved as initiators and mediators of apoptosis (21). In the present study, the changes in neutrophil and lymphocyte concentrations after exhaustive exercise followed the patterns of cell trafficking described above. The percentage of lymphocytes undergoing apoptosis following maximal exercise was significantly higher than the pre-exercise level (P value < 0.001). In a previous study using the Tunel method, Mars et al. (22) found that lymphocyte apoptosis occurs in about 63% of lymphocytes immediately after high-intensity exercise. At 24 hours after exercise, 86% of cells still showed an apoptotic pattern of DNA distribution. In this study, the percentage of apoptotic cells was considerably lower. This discrepancy between the two results can probably be attributed to the different exercise protocol, different methods used and different of numbers of subjects.

Another important factor which affects the percentage of lymphocytes exhibiting apoptotic pattern is the time factor. Because apoptosis is a rapid process, it is likely that any significant delay reduces the percentage of cells that may be considered apoptotic. Sedlock A (23) study on exercise-induced lymphocyte apoptosis in human indicates that the amount of the time between post-exercise blood sampling and the assessment of apoptosis is critical for the determination of exercise-induced lymphocyte apoptosis. Significantly less apoptosis was observed in samples, which were allowed to sit before centrifugation (time-treated), than whole blood samples. These alterations may be of clinical importance to elite athletes assuming they represent a temporary immune impairment during the immediate recovery period. Exercise training results in a variety of adaptations that may be beneficial in attenuating apoptosis. For example, it has been shown that several critical antioxidants and antioxidant enzymes are upregulated with exercise training, thus providing additional free radical protection (24). Malondialdehyde (MDA) is a natural product formed in all mammalian cells either as a product of lipid peroxidation, or as a by-product of prostaglandin and thromboxane biosynthesis. Although MDA can be broken down by aldehyde dehydrogenases, its production is accelerated by oxidative stress and when its concentrations reach critical levels, it may escape this detoxification process. It is highly reactive and known to bind covalently with primary amino groups of proteins, phospholipids, or DNA. This covalence modification of cellular molecules may cause structural

modifications, which results in dysfunction or inactivation. MDA is toxic and has been implicated in aging, mutagenesis, carcinogenesis, radiation damage and a number of other pathological processes (25). In this study, MDA level before and after exercise was measured. Maximal exercise yielded higher readings of MDA than the pre-exercise results. This indicates that high intensity exercise causes oxidative stress with resultant lipid peroxidation as a result of free oxygen radical high levels, manifested by increasing concentration of MDA, an oxidative stress marker.

Conclusion:

The present study shows that exhaustive exercise induces apoptosis in peripheral blood lymphocytes, which could be attributed to oxidative stress assessed by increased level of malondialdehyde. This effect depends on exercise intensity. Further studies will have to elucidate the molecular mechanisms of exercise-induced apoptosis in lymphocytes and their subsets.

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