

Original Article**Effect of regular aerobic exercise with ozone exposure on peripheral leukocyte populations in Wistar male rats**

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Abstract

BACKGROUND: The immune system in endurance athletes may be at risk for deleterious effects of gaseous pollutants such as ambient ozone. Therefore, this study was performed to assess the effect of regular aerobic exercise with ozone exposure on peripheral leukocytes populations in male Wistar rats.

METHODS: Twenty eight 8 weeks old rats were selected and randomly divided into four groups of ozone-unexposed and untrained (control or group 1, n = 6), ozone-exposed and untrained (group 2, n = 6), ozone-unexposed and trained (group 3, n = 8), ozone-exposed and trained (group 4, n = 8). All animals in groups 3 and 4 were regularly running (20 m/min, 30 min/day) on a treadmill for 7 weeks (5 day/week). After the last ozone exposure [0.3 ppm, 30 min per sessions], blood samples were obtained from the cardiac puncture and hematological parameters as well as blood lactate were measured using automatic analyzers. Data were expressed as means (\pm SD) and analyzed by ANOVA and Pearson's correlation tests at $p < 0.05$.

RESULTS: All the hematological parameters differences (except RBC and hemoglobin rate) were significantly higher in the trained groups ($p < 0.001$). However, ozone-induced leukocytosis in the trained (but not in the sedentary) rats was statistically higher than in the counterpart groups.

CONCLUSIONS: Repeated acute ozone exposure has more additive effect on peripheral leukocyte counts in active animals. But, more researches are needed to identify effects of ozone exposure on other components of the immune system in athletes and non-athletes.

KEYWORDS: Moderate Aerobic Exercise, Ozone Exposure, Leukocytosis, Wistar Rats.

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Despite control measures, urban air pollution remains a problem in many densely populated areas.¹ Tropospheric ozone (ground level O₃) is one of the most toxic and oxidizing components of the photochemical air pollution mixture that significantly contributes to increased morbidity in human populations and it is thought to be of greatest concern regarding acute effects on health.²⁻⁵ Indeed, athletes may be one of the groups at risk for deleterious pulmonary

effects in urban areas which have elevated levels of ozone.⁶ Because, athletes often exercise vigorously (with high ventilation rates) in smoggy outdoor environments in the mid- to late-afternoon when diurnal ozone levels are highest. Therefore, they may receive more exposure to ambient ozone and enhance different inflammatory responses.^{3,6-8} Although most studies have focused on immune responses in the lung, numerous investigators have provided evidence to support the hypothesis that O₃ ex-

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posure can have profound effects on systemic (Cellular and Humoral) immunity indices.^{9,10} Several indices such as peripheral leukocyte populations' count are typically elevated after O₃ exposure.^{2,11-13} Nevertheless, it is generally believed that various physical stressors such as exercise training exert profound effects on total and differential leukocyte counts (leukocytosis).¹⁴⁻¹⁶ The magnitude of exercise-induced leukocytosis appears to be directly related to exercise intensity and duration and inversely related to fitness level.¹⁷ However, a number of investigations have been conducted separately to study the effects of ozone exposure or exercise training on leukocyte count, but not combined together. Therefore, this study was conducted to determine synergistic or additive effect repeated acute exposure to ozone on total and differential leukocyte counts in inactive and trained animals.

Methods

Experimental Design

An experimental design was employed for this study and all experimental procedures were performed according to the guidelines of Helsinki declaration and approved by the Regional Research Ethics Committee of Tabriz University of Medical Sciences. Twenty eight male Wistar rats (*Rattus norvegicus*) were obtained from the Pasteur Institute in Tehran, Iran. The rats were housed in air-conditioned room maintained at 22 ± 2°C, with a relative humidity of 50 ± 10% and a 12 hours light/dark cycle (07:00 to 19:00) with free access to food (commercial rat chow: Pars Animal Feed Co, Tehran, Iran) and water. At 8 weeks old, the animals were randomly divided into the following four groups: ozone-unexposed, untrained group (control or group 1, n = 6); ozone-exposed, untrained group (group 2, n = 6); ozone-unexposed, trained group (group 3, n = 8); ozone-exposed, trained group (group 4, n = 8).

Training Protocol

All animals in group 3 and 4 were regularly trained by running 5 days/week for 7 weeks on a motor driven treadmill designed for rats

(ST008, the first smart treadmill designed for rats, made by University of Tabriz, Iran). Exercise started at 19:30 (initiation of active phase) in dark chamber. The intensity and duration of aerobic exercise was gradually increased during the initial 3 weeks of the program. The intensity of exercise was increased gradually by 2 m/min from 10 to 20 m/min during the first week, while the duration of exercise was increased gradually by 5 min/week from 10 to 30 min after the intensity was fixed. In the third week of program, the incline of treadmill was increased gradually by 1° degree/session from 0° to 5° degree. Work rate during treadmill running was expressed as kg.m.min⁻¹ and defined as [speed (m/min)]*[Sin(5°)]*[time (min)]*[body wt (kg)]. Oxygen consumption (VO₂) was calculated using the equation [Y (ml/kg/min) = 42.685 + 0.697 × X (speed m/min)].¹⁸

Exposure Protocol

All animals were placed on treadmill inside a 130-L exposure chamber. This chamber made it possible to expose animals to ozone [0.3 ppm (parts per million)] or clean, filtered room air during exercise for 30 minutes. Ozone was generated by a high-voltage discharge device (OREC Model O3V1-O; Ozone Research and Equip. Co., Phoenix, AZ). A small circulation fan was used for mixing chamber air. The ozone concentration in the exposure chamber was continuously measured with ozone sensor A-22 (Eco Sensors Division of KWJ Engineering Inc. 1570 Pacheco Street, Unit E-12 Santa Fe, New Mexico 87505 USA). After each exposure, the animals were returned to cages for 24 hours and exposed to filtered room air.

Blood Sampling

Immediately after the last exposure, all animals were anesthetized by inhalation of diethyl ether and they were then sacrificed. Blood samples were obtained from the cardiac puncture using syringe and drawn immediately into disposable glass tubes containing tripotassium ethylenediamine tetraacetic acid (K3EDTA) as an anticoagulant.

Hematological Methods

Hematological parameters, namely red blood cells (RBC), total and differential leukocyte (White Blood cell: WBC), and platelets counts; as well as hemoglobin (Hgb) and hematocrit (Hct) levels were determined by automatic blood analyzer (Technicon H1, Technicon, Tarrytown, NY, USA). Blood lactate was measured using the portable Lactate Scout device (EKF-diagnostic GMBH, Germany).

Statistical Analysis

Data were expressed as means (\pm SD). The characteristic differences between the four groups were analyzed by ANOVA and Tukey's multiple comparison tests. The data from group 1 was set as reference averages (controls) for each comparison. Correlations between the parameters were examined with the Pearson's correlation test (r). All statistical analyses were performed using the SPSS statistical software package (SPSS version 15.0 for Windows, SPSS Inc., Chicago, IL, USA). The significance level was set at $p < 0.05$.

Results

The results indicated that all hematological parameters differences (except RBC and hemoglobin rate) between the four groups were statistically significant ($p < 0.001$). On the other hand, total and differential leukocyte counts along with platelet counts and hematocrit level were significantly higher in the trained groups

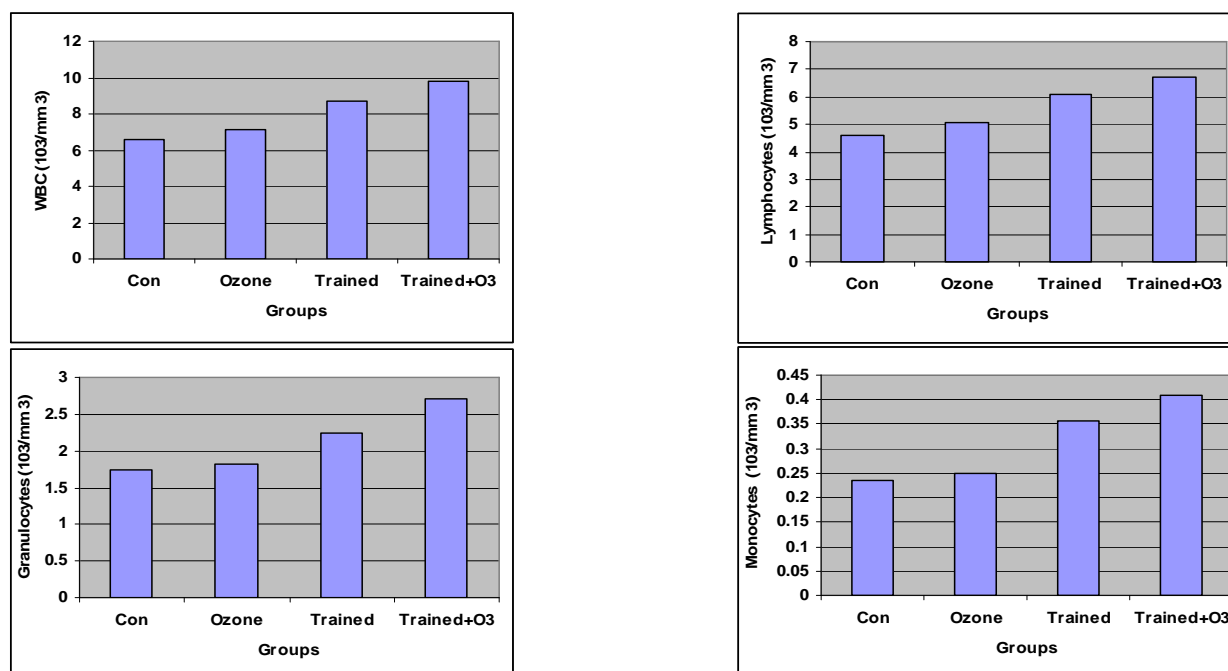
compared to the control group ($p < 0.001$). Moreover, blood lactate concentration in the ozone-exposed groups was statistically higher ($p < 0.001$) than the other groups (Table 1). Besides, body weight in the trained rats at the end study was significantly lower than in the sedentary rats ($p < 0.033$). But, body weight difference between ozone-exposed and unexposed rats was not significant ($p = 0.44$). However, ozone-induced leukocytosis in untrained rats (group 2) was not statistically higher than in the control group (effect size (ES) = 0.78). Whereas, ozone-induced leukocytosis in group 4 (ozone-exposed, trained rats) was statistically higher (ES = 2.09) than in group 3 (ozone-unexposed, trained rats). However, combined ozone-exercise stress induced significant increases of total leukocytes (53.13%), lymphocytes (46.86%), monocytes (74.79%), and granulocytes (54.95%) compared with the control group (Figure 1). On the other hand, increase in monocyte count was higher than the other leukocyte populations (Table 2). Therefore, all hematological parameters differences between the two trained groups were statistically significant (group 3 and 4). Indeed, total and differential leukocyte count in group 4 was statistically higher than other groups. Furthermore, the Pearson's test showed significant correlations between leukocytosis, platelet counts ($r = 0.617$, $p < 0.0001$) and lactate acidosis ($r = 0.880$, $p < 0.0001$) after the last exercise session.

Table 1. Variables and hematological parameters in trained and untrained rats with exposure to ozone after moderate exercise

Variables	Untrained Rats (n = 12)		Trained Rats (n = 16)	
	Without O3 Exposure (Group 1, n = 6)	With O3 Exposure (Group 2, n = 6)	Without O3 Exposure (Group 3, n = 8)	With O3 Exposure (Group 4, n = 8)
Weight (g)	300.83 \pm 39.84	288.33 \pm 29.93	266.75 \pm 24.14	262.00 \pm 17.32
Work Rate (kg-m/min)	-----	-----	0.457 \pm 0.030	0.465 \pm 0.042
VO2 (ml/kg/min)	-----	-----	14.838 \pm 0.981	15.105 \pm 1.369
Lactate (mmol/L)	1.322 \pm 0.083	1.726 \pm 0.169	4.963 \pm 0.160	5.667 \pm 0.332
WBC ($10^3/\text{mm}^3$)	6.556 \pm 0.709	7.157 \pm 0.752	8.710 \pm 0.548	9.835 \pm 0.524
Lymphocytes($10^3/\text{mm}^3$)	4.575 \pm 0.512	5.083 \pm 0.524	6.111 \pm 0.415	6.719 \pm 0.261
Monocytes ($10^3/\text{mm}^3$)	0.234 \pm 0.025	0.250 \pm 0.026	0.356 \pm 0.023	0.409 \pm 0.023
Neutrophils ($10^3/\text{mm}^3$)	1.677 \pm 0.321	1.705 \pm 0.193	2.132 \pm 0.128	2.568 \pm 0.276
Granulocytes($10^3/\text{mm}^3$)	1.747 \pm 0.304	1.823 \pm 0.204	2.2435 \pm 0.135	2.707 \pm 0.278
Platelets ($10^3/\text{mm}^3$)	494.0 \pm 15.90	581.16 \pm 18.53	639.51 \pm 73.59	753.98 \pm 129.40
Hematocrit (%)	42.042 \pm 1.16	42.00 \pm 1.25	45.13 \pm 1.49	44.23 \pm 2.41
RBC ($10^3/\text{mm}^3$)	8059.77 \pm 246.50	8500.19 \pm 442.16	8633.98 \pm 493.12	8407.08 \pm 502.22
Hemoglobin (mg/dl)	14.82 \pm 0.44	15.22 \pm 0.71	15.46 \pm 0.80	15.37 \pm 1.02

Table 2. Changes of percentages in total and differential leukocytes induced by ozone-exercise stress

WBC	Ozone		Exercise		Ozone- Exercise
	Group 2-1	Group 4-3	Group 3-1	Group 4-2	Group 4-1
Leukocytes	9.16%	12.91%	32.84%	37.41%	53.13%
Lymphocytes	11.11%	9.94%	33.58%	32.17%	46.86%
Monocytes	6.84%	14.89%	52.14%	63.60%	74.79%
Granulocyte	4.35%	20.66%	28.42%	48.49%	54.95%

**Figure 1.** Changes in total and differential leukocytes induced by ozone-exercise stress

Discussion

Initially, a significant increase in total and differential leukocyte counts was found immediately after moderate aerobic exercise in Wistar rats (group 3). This finding confirms previous research, which indicates that leukocyte number increases immediately after the onset of endurance exercise, continues to increase throughout, and may remain elevated for several hours after prolonged exercise.¹⁹⁻²² For instance, Lim CL et al reported that the increases in concentrations of leukocyte subsets were elevated by 1.4 to 2.5 fold after endurance exercise¹⁷ whereas the present results indicated that the increase in leukocyte number (32.84%) after moderate exercise was due predominantly to increases in monocyte (52.14%) and to a lesser extent lymphocyte (33.58%) and neutrophil (27.10%) counts (Table 2). The differ-

ences between present results and previous studies may be due to differences in methodology and study designs; because in the present study an animal model was used to identify combined exercise and ozone exposure effect on leukocytosis, while, other investigators used different exercise protocols and experimental populations. For example, the percentage of lymphocytes in the total leukocyte population is much higher in rats as compared with humans (~70% in rats and 30-35% in humans).²⁰⁻²³ Nevertheless, the magnitude of leukocytosis may be influenced by exercise stress and lactate acidosis, because blood lactate concentration in the ozone-exposed groups was significantly higher ($p < 0.001$) than other groups and there was a correlation between leukocytosis, and lactate acidosis ($r = 0.880$, $p < 0.0001$) after the last exercise session. Moreover,

McCarthy et al found correlations between leukocyte, neutrophil and lymphocyte numbers and plasma lactate concentrations.²⁴ Some previous studies demonstrated that hemodynamic changes such as increased cardiac output at the onset of prolonged exercise, as well as changes in adhesion molecules were largely responsible for the rapid mobilization of cell into the circulation after any aerobic exercise. On the other hand, any stressor factors such as exercise load could lead to increase in leukocyte counts and relative proportion of subsets result from recruitment of cells (spleen and bone marrow) into the circulation, mediated at least in part by stress hormones such as corticosteroids and catecholamines.^{19,25} Nevertheless, some studies suggested that environmental stressors such as exposure to tropospheric ozone could induce increase in circulating leukocyte count.^{23,26} For example, Sakai et al reported that a change in ambient ozone may have an effect on human circulating leukocyte count.¹⁴ Likewise, in the current study, the combined ozone-exercise stress induces significant increases of total (53.13%) and differential leukocyte counts (Table 2). This may be related to loss of appetite and body weight influence by ozone exposure.⁹ Present results indicated no significant difference between body weight of ozone-exposed and unexposed rats. However, a study showed that the gaseous pollutants were not consistently associated with the circulating leukocyte counts.²⁷ The discrepancy between present results and previous studies could be related to differences in methodology and study design such as population or subjects' characteristics (species variability, genetic constitution, age, health status, nutritional factors, and sample size), immune

Conflict of Interests

Authors have no conflict of interests.

Authors' Contributions

AJ carried out the design and coordinated the study, participated in all the experiments and prepared the manuscript. MAHF provided assistance in the design of the study and participated in manuscript preparation. FA provided assistance in the design and coordinated the study. HP pro-

assay sensitivity, ozone treatments (continuous versus intermittent exposure with different concentrations), and exercise training protocol (intensity, duration and frequency of various exercises).^{9,28,29} Also, most experimental animal studies have to use O₃ exposures in excess of the ambient and spike urban O₃ concentrations to demonstrate an immunotoxic effect.⁷ And it appears that the O₃ concentrations modulate the intensity and the duration of the immune responses, as Selgrade et al found no effect on multiple immune parameters when rats were chronically exposed to O₃ in a way that simulated an urban profile.^{9,10} The present results confirm that ozone exposure has more additive effect on peripheral leukocyte counts in active animals.

Conclusions

In conclusion, repeated acute ozone exposure could deteriorate immunity response (Leukocytosis) in trained subjects. But, due to limitations of the present study, further researches are needed to identify effects of acute or chronic exposure to ozone with different dosages on other components of the immune system in athletes and non-athletes.

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vided assistance in animal training protocol and the most of experiments. All authors have read and approved the content of the manuscript.

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