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Can vitamin C supplementation reverse the effects of exercise training in polluted air on oxidative stress markers? A randomized controlled trial

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Original Article

Abstract

BACKGROUND: Air pollution and long-term aerobic exercise are diversely associated with cardiovascular disease (CVD) and mortality. However, the simultaneous effect of exercise in polluted air and vitamin C on oxidative stress markers is less clear. In this study, the effect of these variables on oxidative stress markers was investigated in rats.

METHODS: The study was conducted on 50 male rats. The rats were divided into 5 groups consisting of exercise, exercise with vitamin C, exercise in polluted air, exercise in polluted air with vitamin C, and control group. Animals in the exercise groups exercised on a treadmill for 12 weeks, 5 days/week, 30 minutes/day, at 50-70% of the maximum speed. Animals in the vitamin C groups received 20 mg/kg/day vitamin C orally. After 12 weeks of intervention, 2.5 ml of blood was taken from the rats' apex. Malondialdehyde (MDA) and oxidized low-density lipoprotein (OxLDL) levels were measured using NavandSalamat's Nalondi and Eastbiopharm's OxLDL ELISA kits, respectively. Two-way analysis of variance (ANOVA) was used for data analysis in SPSS software.

RESULTS: There were significant differences in MDA and OxLDL levels between all groups after 12 weeks of intervention ($P < 0.050$). The levels of MDA and OxLDL were significantly higher in the ExPo group compared to the Ex+VitC groups ($P < 0.050$). However, no significant difference was observed in MDA and OxLDL levels between the vitamin C groups ($P > 0.050$).

CONCLUSION: These findings demonstrate the oxidative stress effects of air pollution, systemically and in the respiratory tract. Moreover, polluted air significantly increased OxLDL levels in both exercise in polluted air groups. Although, vitamin C slightly decreased MDA and OxLDL levels in the ExPo groups, the difference was not significant. Different vitamin C doses could have diverse and maybe significant results.

Keywords: Aerobic Exercise; Air Pollution; Vitamin C; Malondialdehyde; Oxidized Low-Density Lipoprotein

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Introduction

Huge amounts of pollutants are produced by vehicles and released into the air every day, especially carbon monoxide (CO), sulfur dioxide (SO_2), and nitrogen dioxide (NO_2).[1] Everybody is endangered by air pollution, but some are more at risk.[2] During exercise, several physiological changes occur that could exacerbate the effects of air pollution on health. These include alterations in breathing, pollution dose, and nasal defenses. At submaximal exercise levels breathing switches from predominantly nasal to predominantly oral. This transition causes the nasal filtration system to be bypassed, potentially increasing pollutant dose, which may exacerbate the health effects of air pollution.[3]

CO is a colorless, odorless gas that is produced from the incomplete combustion of fuels containing carbon.[4] Common outdoor sources of CO include car exhaust fumes (gasoline and diesel), stationary combustion equipment such as heating

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and power-generating plants, smoke from fires, and gas-powered engines.[5,6] After reaching the lungs, CO diffuses rapidly across the alveolar and capillary membranes. It also readily crosses placental membranes. CO binds reversibly to heme proteins. Approximately 80–90% of the absorbed gas binds with hemoglobin forming carbonmonoxyhemoglobin, whose affinity for CO is 200-250 times that for oxygen. The formation of carbonmonoxyhemoglobin is reversible, but, because of the strong binding of CO, the elimination half-life while breathing room air is 2–6.5 hours depending on the initial carbonmonoxyhemoglobin level. The organs and tissues that are mostly affected include the brain, the cardiovascular system, skeletal muscle during exercising, and the developing fetus.[4]

SO_2 found in the atmosphere has both anthropogenic and natural sources. In addition to adverse effects on human health, SO_2 and its atmospheric products can affect the atmospheric environment at local, regional, and global scales. It is estimated that anthropogenic sources account for more than 70% of the global emission of SO_2, half of which is the result of fossil-fuel combustion.[7] The level of thiobarbituric acid reactive substances (TBARS) has been shown to be an indicator of endogenous lipid peroxidation. Superoxide dismutase (SOD), catalase (CAT), and GSH-Px are considered to be antioxidant enzymes. SO_2 inhalation (22, 56, and 112 mg/m^3 6 hours/day for 7 days) significantly increased the level of TBARS and decreased the activities of the antioxidative enzymes, SOD, GPx, and CAT in various organs in mice, including the brain, liver, lung, heart, stomach, intestine, spleen, kidney, and testicles.[8]

NO_2 is formed during the combustion of fossil fuel through the oxidation of atmospheric nitrogen and nitrogen from certain fuels such as coal and oil. In a study of air pollution near major highways in the Netherlands, NO_2 concentration was found to be positively correlated with traffic density on the nearest highway and with the percentage of time downwind from the highway, and negatively correlated with the distance from the nearest highway.[9] NO_2 is a highly reactive, nitrogen-centered free radical, poorly water-soluble gas deposited peripherally in the lungs. It is absorbed along the entire respiratory tract, but exposure studies indicate that the major target site for the action of NO_2 is the terminal bronchioles. The main mechanism of NO_2 toxicity has been suggested to involve lipid peroxidation in cell membranes and various actions of free radicals on structural and functional molecules.[10]

Moreover, it is estimated that physical inactivity is the fourth most common cause of mortality and contributes to 3.2 million deaths annually.[3] Many of the most accessible forms of exercise, such as walking, cycling, and running, are often performed outdoors. Globally, 52% of people live in urban centers, and in the developed world, this figure approaches 78%.[11] Exercising outdoors may increase exposure to urban air pollution.[3]

Furthermore, different kinds of vitamins play a vital role in a healthy lifestyle. Vitamin C (ascorbic acid), which is water-soluble and present in the cytosolic compartment of the cell, serves as an electron donor to vitamin E radicals generated in the cell membrane during oxidative stress.[12] It is a major water-soluble antioxidant that is an effective scavenger of reactive oxygen species in both intracellular and extracellular fluids. Vitamin C is also required for the regeneration of the reduced form of the lipid-soluble antioxidant, vitamin E.[13]

Air pollution can generally be a cause of oxidative stress in the body.[8] Lipid peroxidation is one of the major consequences of free radical-mediated injury to the tissue. The peroxidation of polyunsaturated fatty acids is a chain reaction that can continue until the substrate is completely consumed or termination occurs due to antioxidants. Lipid peroxidation causes structural and functional damage to membranes and has several secondary products.[14] Lipid peroxidation products are a widely accepted group of oxidative stress indices. Lipid peroxidation leads to the production of conjugated diene hydroperoxides. These unstable substances decompose into various aldehydes, such as malondialdehyde (MDA).[15] MDA interacts with proteins and is itself potentially atherogenic. MDA's reactions with lysine residues generate lysine-lysine cross-link, which has been identified in apolipoprotein B (apoB) fractions of oxidized low-density lipoprotein (OxLDL) and is postulated to impair the interaction between OxLDL and macrophages, and thereby, to promote atherosclerosis.[16,17]

The oxidation of LDL, which leads to the creation of OxLDL and phospholipids, plays a central role in the pathogenesis of atherosclerosis, with the adducts being both pro-atherogenic and pro-inflammatory.[18] OxLDL levels are higher in patients with CVD, and increasing OxLDL levels correlate with increasing severity of disease (e.g., stable angina vs. unstable angina vs. myocardial infarction).[19] OxLDL levels also appear to be predictive of future CAD in apparently healthy men.[20]

The epidemiological association between exposure to air pollution and cardiovascular morbidity and mortality has been well documented in previous studies.[21] Furthermore, long-term regular physical activity is significantly associated with a reduced risk of cardiovascular events.[22] Habitual physical exercise benefits health and longevity across the life span; however, some researchers have recently noted the possible exposure to increased risk of air contaminants during exercise since exercise amplifies respiratory uptake and deposition of air pollutants in the lung and ambient air pollution affects health.[23] Therefore, the cardiovascular health benefits of exercise may be countered to some degree by harmful actions of inhaled pollutants.[24]

This dilemma prompted the researchers of this study to examine the diverse effects of exercise in polluted air. We hypothesized that vitamin C consumption, which decreases oxidative stress, would increase the training-induced adaptation to markers of oxidative stress like MDA and OxLDL levels in polluted air.

Materials and Methods

Animals and Maintenance of Conditions: Researchers used male Wistar rats (Royan Institute of Isfahan, Iran) in this study, weighing from 130 to 140 grams. The rats were stored in animal nests at Isfahan University of Medical Sciences, Isfahan, Iran. The storage conditions were stable and 12 hours of light and 12 hours of darkness were applied. The temperature was maintained at 24 ± 2 degrees Celsius. The rats were put into cages, 4 rats per cage. Food and water were always available to all rats. They spent a week in the nest to adapt to the new conditions. After the first week, a progressive test was performed to assess the maximum running speed of the rats. The test protocol was for the rats to run on the treadmill for 3 minutes at the speed of 3 meters/second and the speed was increased by 1 meter/second every 3 minutes. This protocol has been created and normalized by Leandro et al.[25]

Then, the rats were randomly divided into 5 groups of 10. Each rat was marked, then, using a random number generator, the first selected rat was placed into the first group, and the second rat into the second group, and the division went on until the last rat. The rats were divided into the following 5 groups:

1. Exercise (Ex-Cl-NoV)
2. Exercise with vitamin C (Ex-Cl-C)
3. Exercise in polluted air (Ex-Po-NoV)
4. Exercise in polluted air with vitamin C (Ex-Po-C)
5. No exercise in clean air, no vitamin supplementation (Control)

After the division of the rats into these 5 groups, they were put on the treadmill in 3 sessions for a week and ran for 10 minutes each session with 35% of the maximum speed for familiarization.

All the rats in the vitamin groups were force-fed 20 mg of vitamin C per kg of bodyweight per day, using gavage technique.[26]

Exercise and Pollution-Induction Protocol: After the first week of accommodation and the second week of familiarization, the main exercise protocol started. The main exercise protocol consisted of 3 stages. The first stage involved 5 minutes of warm-up with 40 to 50% of the maximum speed. The training stage included 30 minutes of running with 65% of the maximum speed, and the last stage of the protocol consisted of 5 minutes of cool-down with 35 to 45% of the maximum speed. To apply the overload principle after 3 weeks of exercise, the intensity of the training stage was elevated to 70% of the maximum speed. At the end of 6 weeks, another progressive test was performed on the rats to determine the new maximum speed and eliminate the effects of adaptation to the protocol. The new intensity was again 65% of the recent maximum speed measured by the test. At the end of 9 weeks, the intensity of the training stage was once more increased to 70% of the latest evaluated maximum speed, and this speed was maintained until the end of the 12 weeks of study.

The rat treadmill was placed in an isolated room. The room was 165*200*273 cm or 9.009 cubic meters or 9009 liters. The pollution induced in this study was derived from cylinders of pure CO, NO_2, and SO_2 obtained from Tarkib Gas Pars Company, Isfahan, Iran. The amount of gas to be released in the room was calculated accurately based on the volume of the room, temperature, and pressure of the air in the room, and then, released into the room using a gas syringe.

The amount of gas in the room was matched with the density of air pollution on polluted days in Isfahan city. The density of CO, NO_2, and SO_2 ranged between 10 and 15 ppm, 0.3 and 0.6 ppm, and 0.5 ± 0.1 ppm, respectively.[27] The amount of gas released into the room using the gas syringe was calculated accurately by the engineers of Tarkib Gas Pars Company using the PV = nRT formula. The amount of CO, NO_2, and SO_2 at 24 ± 2 degrees Celsius was, respectively, 95 ml, 4.3 ml, and 4.8 ml to assess the above mentioned density. Finally, the density of the released gas was measured in real-time using a portable air quality monitor (Series 200; Aeroqual, New Zealand).

An observer was present in the room during the training sessions wearing a full face gas mask for protection against the pollution. Moreover, all the pollution groups were present in the room during all of the training sessions, while the exercise groups were running on the treadmill.

Blood Sample Collection: After 12 weeks of training, the rats were anesthetized using an intraperitoneal injection of Ketamine-Xylazine,[28] the combination of 100 mg.kg^{-1} Ketamine and 10 mg.kg^{-1} Xylazine,[29] then, the blood samples were drawn from the apex of the rats' hearts. Next, the blood specimens were centrifuged at 2000 × g for 10 minutes within 30 minutes after collection. The second round of centrifuge was performed right away at 2500 × g for 15 minutes in room temperature to obtain platelet-poor plasma (PPP).[30] All the samples were kept at -80°C until the preparation of all the enzyme-linked immunosorbent assay (ELISA) kits.

ELISA kits: The ELISA kit used to assess the amounts of MDA was named Nalondipurchased from NavandSalamat Company, Urmiah, Iran. OxLDL kit used was purchased from Eastbiopharm Company, Hangzhou, China. The kits were specially made for rats.

All the ELISA tests were performed by laboratory professionals at Doctor Baradaran Medical Laboratory, Isfahan, Iran.

Statistical Analysis: Data were reported as mean ± SD. Normality of all variables were tested using the Shapiro-Wilk test.[31] Two-way analysis of variance (ANOVA) was performed to compare the study groups. When a significant intergroup effect was detected at a significance level of $P < 0.050$, the Least Square Difference (LSD) correction was used for post hoc comparison. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) (version 23.0; IBM Corp., Armonk, NY, USA). All graphs were made using Prism software (Version 8.0.2; GraphPad Software Inc., California, USA).

Results

Differences in MDA and OxLDL levels, as biomarkers of oxidative stress in each group, are presented in. After the 12 weeks of intervention, significant differences were observed between the groups in terms of the MDA levels ($P < 0.001$) and OxLDL levels ($P < 0.010$).

MDA: Mean MDA levels were significantly higher in the ExPoNoV group compared to the exercise in clean air and control groups. No significant difference was detected in MDA levels between the exercise in clean air and control groups (Table 1 and Figure 1).

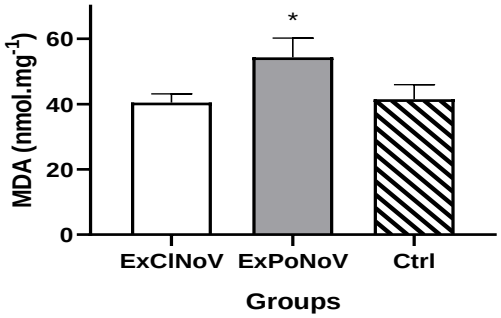

Figure 1. The effect of exercise in polluted and clean air on malondialdehyde (MDA) levels
*Significant difference with other groups ($P < 0.050$); ExClNoV: Exercise in clean air; ExPoNoV: Exercise in polluted air; Ctrl: Control group

Nevertheless, after the 12 weeks of intervention, MDA levels were significantly higher in the ExPoC air group than the control group ($P < 0.050$). However, these results did not show any significant differences between the ExPoC and Exercise in clean air with vitamin C (ExClC) groups (Table 1 and Figure 2).

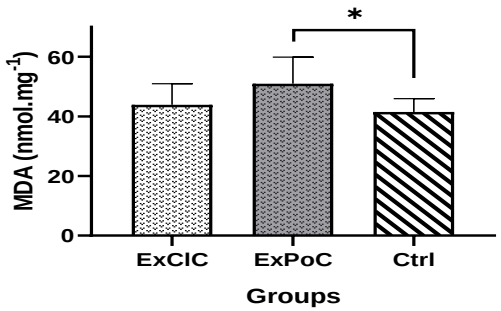

Figure 2. The effect of exercise in polluted and clean air on malondialdehyde (MDA) levels
* Significant difference between groups ($P < 0.050$); ExClC: Exercise in clean air with vitamin C; ExPoC: Exercise in polluted air with vitamin C; Ctrl: Control group

Table 1. Changes in the malondialdehyde and oxidized low-density lipoprotein levels [Mean ± standard deviation (SD)] in the five groups after 12 weeks of intervention

Variables	ExClNoV	ExClC	ExPoNoV	ExPoC	C	F	P
MDA	40.44 ± 2.68	43.85 ± 7.12	54.32 ± 5.91	50.97 ± 8.90	41.48 ± 4.47	7.95	< 0.001
OxLDL	0.47 ± 0.09	0.39 ± 0.12	0.74 ± 0.26	0.64 ± 0.16	0.49 ± 0.15	6.02	0.001

Data are presented as mean ± standard deviation (SD).
ExClNoV: Exercise in clean air; ExClC: Exercise in clean air with vitamin C; ExPoNoV: Exercise in polluted air; ExPoC: Exercise in polluted air with vitamin C; C: Control group; MDA: Malondialdehyde; OxLDL: oxidized low-density lipoprotein

The comparison of the effects of exercise training with and without vitamin C consumption in polluted and clean air on the levels of MDA are shown in figure 3. The level of MDA was significantly increased in both ExPoNoV and ExPoC groups compared to the ExClNoV, ExClC, and control groups.

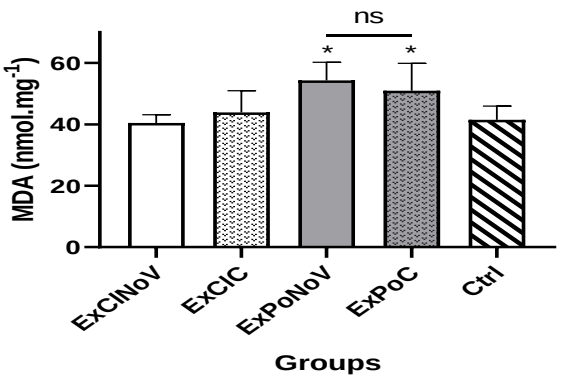

Figure 3. The comparison of effects of exercise training with and without vitamin C consumption in polluted and clean air on malondialdehyde (MDA) levels
*Significant difference with other groups (P < 0.050)
ns No significant difference between specified groups (P > 0.050); ExClNoV: Exercise in clean air; ExClC: Exercise in clean air with vitamin C; ExPoNoV: Exercise in polluted air; ExPoC: Exercise in polluted air with vitamin C; Ctrl: Control group

OxLDL: There was a significant difference between the ExPoNoV group and other groups. There was no significant difference between the ExClNoV group and the control group (Figure 4).

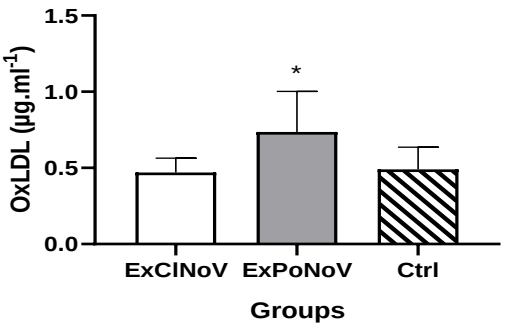

Figure 4. The effect of exercise in polluted and clean air on oxidized low-density lipoprotein (OxLDL) levels
* Significant difference with other groups (P < 0.050); ExClNoV: Exercise in clean air; ExPoNoV: Exercise in polluted air; Ctrl: Control group

The ExPoC group showed significantly higher levels of OxLDL than the ExClC group. However, neither of the mentioned groups showed a significant difference with the control group (Figure 5).

Figure 6 demonstrates the effects of exercise with and without vitamin C consumption in polluted and clean air.

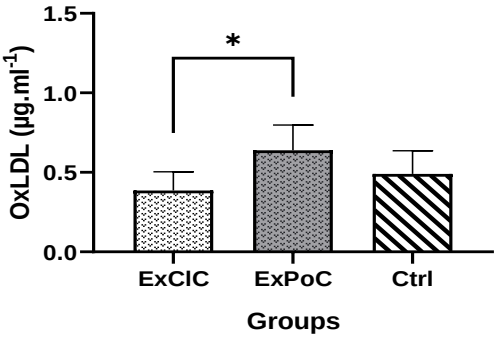

Figure 5. The effect of exercise and vitamin C in polluted and clean air on oxidized low-density lipoprotein (OxLDL) levels
*Significant difference between groups (P < 0.050); ExClC: Exercise in clean air with vitamin C; ExPoC: Exercise in polluted air with vitamin C; Ctrl: Control group

The highest amount of OxLDL was seen in the ExPoNoV group; it had a significant difference with both exercise in clean air groups. OxLDL level in the ExPoC group is slightly less than that in the ExPoNoV group, but this difference was not significant. Moreover, the ExPoC group did not have a significant difference with the control group and the ExClNoV group in terms of OxLDL level. The least amount of OxLDL was observed in the ExClC group, and it had a significant difference with the exercise in polluted air groups (Figure 6).

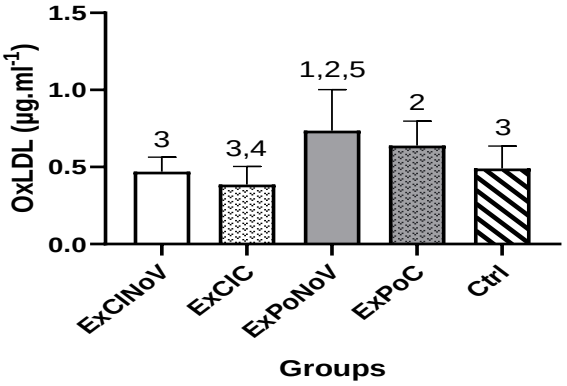

Figure 6. The comparison of effects of exercise training with and without vitamin C consumption in polluted and clean air on the levels of oxidized low-density lipoprotein (OxLDL)
Significant difference with group (P < 0.050):
1-ExClNoV2-ExClC3-ExPoNoV 4-ExPoC5-Control; ExClNoV: Exercise in clean air; ExClC: Exercise in clean air with vitamin C; ExPoNoV: Exercise in polluted air; ExPoC: Exercise in polluted air with vitamin C; Ctrl: Control group

Discussion

The present study investigated the effects of 12 weeks of vitamin C intervention and exercise training in polluted air on oxidative stress markers in rats. To our knowledge, this is the first study to investigate the simultaneous effect of antioxidant supplements and exercise in polluted air. Therefore, the novel findings of this study are that vitamin C supplementation intake with exercise training attenuates the severe effects of air pollution. Although, there was a difference between the groups that used vitamin C and those that did not, the findings around vitamin C were not significant, which nullifies our hypothesis.

Malondialdehyde: MDA levels in the ExPo groups are significantly higher than that in the ExCl groups. The ExPoC group had lower levels of MDA compared to the ExPoNoV group, but the difference was not significant. Furthermore, higher levels of MDA were observed in the ExClC group compared to the ExClNoV group. This is in accordance with the findings of Laumbach and Kipen,[32] Costa et al.,[33] and Romieu et al.[34] However, these results are in conflict with the results of the studies by Kini et al.,[35] and Ghate et al.[36]

Laumbach and Kipen showed that acute exposure to motor vehicle traffic-related air pollution increases traces of oxidative stress biomarkers like nitric oxide (NO) in the breath and nitrate, MDA, and F2-isoprostanes in exhaled breath condensate (EBC).[32] They stated that oxidative stress is a common mechanism through which diesel exhaust and other air pollutants cause adverse effects. In addition to diesel exhaust, diverse air pollutants, ranging from ozone to cigarette smoke, cause acute neutrophilic inflammation in the airways of animals and humans. Increases in proinflammatory cytokines and chemokines, such as interleukin-8 (IL-8), appear to be instrumental in this initial inflammatory process. Moreover, oxidative stress is known to be an important regulator of IL-8 gene expression, which leads to the recruitment of neutrophils,[22] upregulation of IL-8 and other proinflammatory cytokines, and increase in oxidative stress biomarkers levels.[32]

Costa et al. showed that the mice exposed to diesel exhaust (DE) had significantly higher levels of lipid peroxidation and pro-inflammatory cytokine TNF-a (tumor necrosis factor-a) compared to mice exposed to filtered air.[33] DE exposure also caused microglia activation, as assessed by measuring ionized calcium binding adaptor molecule 1 (Iba1) levels using Western blot and immunocytochemistry, which leads to elevated MDA levels.[33]

Romieu et al. reported an increase in MDA levels in children exposed to air pollution.[34] They stated that traffic-related pollution is a complex mixture of air toxicants including particulate matter, products of incomplete fuel combustion, and secondary pollutants such as ozone, which induce oxidative stress in the lungs, especially when the antioxidant defenses have been overwhelmed. Oxidative stress in the lungs results in an influx of inflammatory cells in the lungs with the subsequent generation and release of large quantities of free radicals and reactive oxygen species (ROS). In addition, these increased levels of MDA are associated with lower lung function and increased levels of inflammatory cytokine IL-8 in nasal lavage.[34]

Lower amounts of MDA were observed in the ExPoC group compared to the ExPoNoV group; the opposite has happened in ExCl groups. This means that vitamin C had no significant effect on exercise groups, which is in accordance with the results of the study conducted by Harrison et al.[37] One possible explanation for higher levels of MDA in the ExPo groups could be oxidative stress and ROS developed by both exercise and polluted air, which exceeded the subject's antioxidant capacity. This can lead to lipid peroxidation and increased MDA levels.[38]

The comparison between polluted air groups and clean air groups has shown that pollution has an observable effect on OxLDL values. The highest amounts of OxLDL were observed in both exercise in polluted air groups. This is in accordance with the findings of Campen et al.,[39] and Soares et al.[40] LDL can be oxidized by metal ions, lipoxygenases, myeloperoxidase, and reactive nitrogen species (RNS). In vitro oxidation of LDL by metal ions (e.g., Cu21) occurs in 3 phases: an initial lag phase (consumption of endogenous antioxidants), a propagation phase (rapid oxidation of unsaturated fatty acids to lipid hydroperoxides), and a decomposition phase (hydroperoxides are converted to reactive aldehydes, e.g., MDA, and 4-hydroxynonenal). Activated phagocytes secrete myeloperoxidase that generates reactive species including hypochlorous acid (HOCl), chloramines, tyrosyl radicals, and NO_2. These reactive species oxidize antioxidants, lipids, and protein of LDL. NO is a free radical released by various vascular cells. It inhibits copper-mediated oxidation as well as cell-mediated oxidation of LDL.[41]

Studied characteristics amongst patients suggested that in vivo oxidized LDL does not originate from extensive metal ion-induced

oxidation of LDL, but that it is most likely generated by cell-associated oxidative enzymatic activity in the arterial wall.[42]

The other mechanism behind this increase could be LDL oxidation in the presence of the mixture of increased adrenaline during exercise in polluted air and ferric chloride.[43] The lowest levels of OxLDL belonged to the ExClC group, this is in conflict with the findings of Mitsui et al.[44] Yet, the ExClC group had no significant difference with the control group or other exercise in clean air groups in this regard and this is in accordance with the findings of Van Hoydonck et al.[45] Vitamin C decreased OxLDL levels in both exercise in clean air and polluted air, but this drop was not significant. This phenomenon could be because of increased amounts of MDA in polluted air groups, and lipid peroxidation in those groups.[18]

Conclusion

The findings of this study showed the oxidative stress effects of air pollution in healthy individuals. Air pollution increased MDA levels significantly, in other words, training in polluted air could have adverse effects on health. Further researches are necessary. Furthermore, polluted air amplified OxLDL levels significantly in the ExPoCl and ExPoC groups. Nevertheless, vitamin C faintly lessened MDA and OxLDL levels in the ExPo groups; this reduction was not significant. Various amounts of vitamin C consumption could end in different and maybe significant results. Thus, further studies in this regard are required. It is possible that vitamin C can help reduce the oxidative stress effects of air pollution.

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Conflict of Interests

Authors have no conflict of interests.

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