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Original Article

Acute exercise in ozone-polluted air induces apoptosis in rat quadriceps femoris muscle cells via mitochondrial pathway

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ABSTRACT

Ozone (O_3) pollution can decrease sport performance and induce respiratory toxicity, but relatively few studies have investigated its effects on skeletal muscles. We randomly assigned rats to the following groups based on a 2 × 4 two-factor factorial design: Air+0, Air+10, Air+15, and Air+20, O_3+0, O_3+10, O_3+15, and O_3+20. The rats in the +0 groups rested, whereas those in the +10, +15, and +20 groups ran on a treadmill (in clean air for Air groups and in air polluted with 0.14 parts per million [ppm] O_3 for O_3 groups) at speeds of 10, 15, and 20 m/min, respectively, for 1 h. Thereafter, key enzyme activities involving the tricarboxylic acid cycle, oxidative phosphorylation, adenosine triphosphate (ATP) content, histopathological changes, oxidative stress, inflammation factors, and apoptosis were assessed in the rat quadriceps femoris samples. Ozone reduced key enzyme activities and ATP contents in the quadriceps femoris regardless of whether the rats exercised. Pathological changes, inflammatory factors, oxidative stress, and mitochondria-dependent apoptosis were only evident under conditions of exercise combined with ozone and increasingly worsened as exercise intensity increased. These findings suggested that acute exercise under ozone exposure could induce damage to the quadriceps femoris, which would negatively affect sport performance. Ozone-induced disrupted energy metabolism might be an early event that becomes more critical as exercise intensity increases. Therefore, care should be taken when exercising in polluted air, even when ozone pollution is mild.

Introduction

Ambient ozone is a highly reactive gaseous pollutant. Ozone pollution is considered a worldwide health hazard, and it contributes to increasing the mortality and morbidity of many chronic diseases.[1–7] Acute ozone exposure can cause eye and nose irritation, airway hypersensitivity, lung and systemic inflammation, decreased lung function, cardiovascular effects, dyslipidemia, and enhanced fibrinolysis.[8–15] Chronic ozone exposure is associated with a higher risk of abnormal lung development in children, reduced airway function, type 2 diabetes, birth-related health outcomes, and central nervous system alterations.[16–22]

Appropriate exercise can improve cardiopulmonary function, enhance immunity and muscle strength, prevent the occurrence of chronic diseases, and delay aging.[23] However, exercising in an environment polluted with ozone has exerted deleterious effects on human health. Ozone pollution can decrease performance during training and competition and induce bronchoconstriction and airflow obstruction while exercising.[24–26] Symptoms of lung injury and early inflammatory response in trained runners exercising in an ozone-polluted, hot, and humid environment can be relieved by vitamin supplementation.[27] A study of elite cyclists exposed to low levels of ozone in a hot environment revealed impaired exercise performance and pulmonary function.[28] Intermittent exposure to various concentrations of ozone affects the lung function, lung capacity, and endurance of long-distance runners.[29] A balance between the benefits of exercise and the adverse health effects of ozone pollution is important.[30]

Most studies about ozone exposure and exercise have focused on its effects on lung function and exercise capacity. As exercise under ozone exposure can affect sport performance, we speculated whether it would also impact skeletal muscle in addition to cardiopulmonary function. The quadriceps femoris is the main component of the anterior thigh muscle

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Abbreviations

ATP	Adenosine triphosphate
ppm	parts per million
O_3	Ozone
HE	Hematoxylin and eosin
PBS	Phosphate-buffered saline
BCA	Bicinchoninic acid
MDA	Malondialdehyde
GSH-Px	Glutathione peroxidase
SOD	Superoxide dismutase
8- OHdG	8-hydroxy-2′-deoxyguanosine
IL	Interleukin
TNF	Tumor necrosis factor
ELISA	Enzyme-linked immunoassay
TCA	Tricarboxylic acid
α-KGDHC	α-ketoglutarate dehydrogenase
CS	Citrate synthase
ICD1	Isocitrate dehydrogenase 1
MRCC	Mitochondrial respiratory chain complex
COX	Cytochrome C oxidase
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
RIPA	Radioimmunoprecipitation assay
TBST	Tris-Buffered Saline-Tween 20
SD	Standard deviation
Bcl-2	B-cell lymphoma-2
Bax	Bcl-2 associated X protein
Cyt-C	Cytochrome C
ECL	Efficient chemiluminescence

group in animals and it plays an important role in maintaining knee joint stability and overall movement. However, relatively little is known about the effects of exercise under ozone exposure on skeletal muscles. Therefore, we explored the acute effects of low-level ozone pollution on the quadriceps femoris of rats after exercise at different intensities.

Materials and methods

Rat model of ozone exposure and exercise

Fifty healthy Sprague-Dawley male rats aged 7–8 weeks, weighing 240–280 g (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), were given food and water *ad libitum for* one week under a 12 h–12 h day/night cycle at a temperature of 23 ± 1 °C and 55% ± 5%

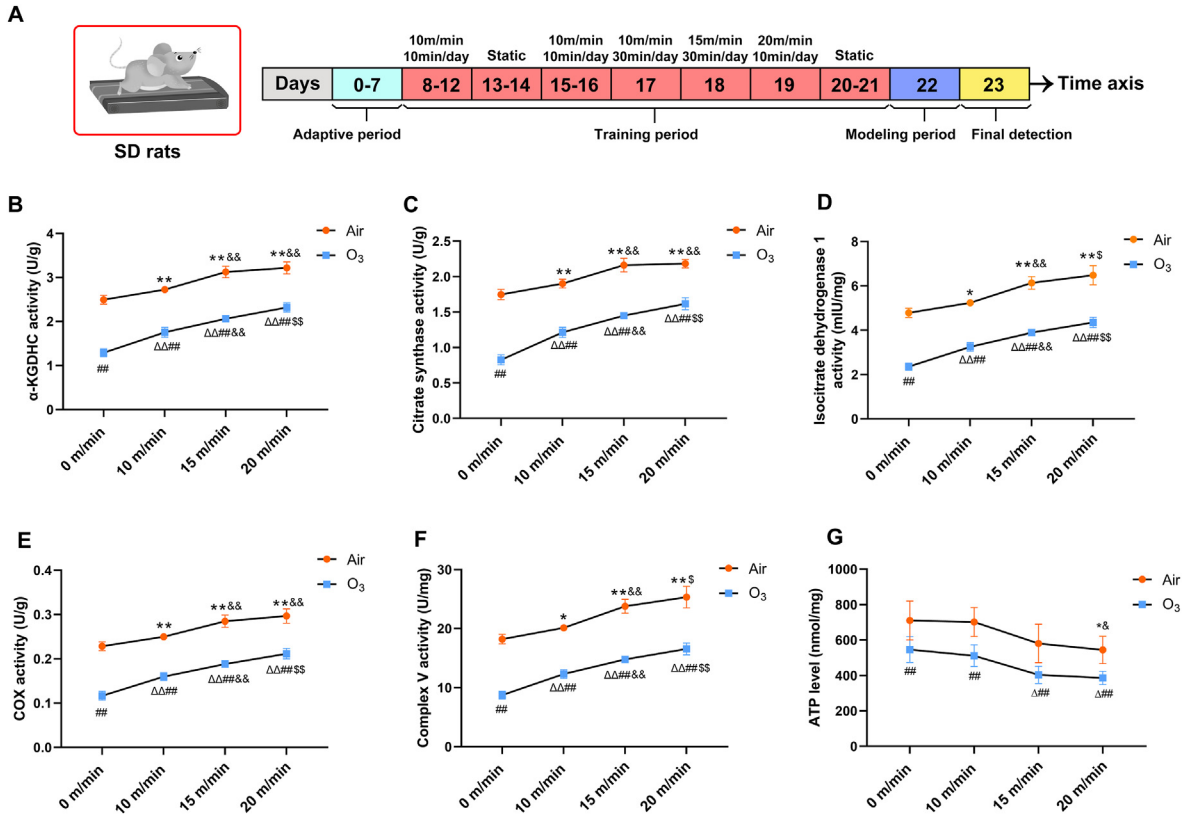

Fig. 1. Changes in the enzyme activities involving TCA and MRCC, and ATP content in quadriceps femoris of rats after acute exercise in clean air or ozone-polluted air (Mean ± *SD*, $n = 5$). A: The time axis for animal treatment; **B:** α-KGDHC; **C:** CS; **D:** ICD1; **E:** COX; **F:** Complex V; **G:** ATP content. **Air:** group of doing different speeds of exercise in clean air; **O_3**: group of doing different speeds of exercise in ozone-polluted air. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Air+0 group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs O_3+0 Group, $^{\#\#}p < 0.01$ vs the Air group with the same speed, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 10 m/min group in the same group, $^{\$}p < 0.05$, $^{\$\$}p < 0.05$ vs 15 m/min group in the same group. TCA: Tricarboxylic acid, MRCC: Mitochondrial respiratory chain complex, ATP: Adenosine triphosphate, α-KGDHC: α-ketoglutarate dehydrogenase, CS: Citrate synthase, ICD1: Isocitrate dehydrogenase 1, COX: Cytochrome C oxidase, m/min: meter/minute, min/day: minute/day, U/g: Unint/gram, U/mg: Unint/milligram, mIU/mL: million international units/milliliter, nmol/mg: nanomol/milligram.

humidity. After acclimation, the rats were familiarized with running on a treadmill (KW-PT, Nanjing Calvin, Nanjing, China) for 2 weeks. Fig. 1A shows details of the training schedule. Forty rats that learned to run on the treadmill were randomly assigned to the following groups ($n = 5$ each): Air+0 (control; no running); Air+10, Air+15, and Air+20 (running at 10, 15, and 20 m/min, respectively, in air); O_3+0 (no running, 0.14 ppm ozone); and O_3+10, O_3+15, and O_3+20 (running at 10, 15, and 20 m/min, respectively, in 0.14 ppm ozone). The animals exercised under these respective conditions for 1 h on a treadmill in an HRH-CSED-K animal gas dynamic exposure device (Beijing Huironghe, Beijing, China). Fig. 1A shows the time axis. Ozone was produced using an HMJ-CY-2 ozone generator (Beijing Himeju, Beijing, China). The ozone concentration in the gas exposure device was controlled at approximately 0.14 ppm using a XLA-BX-03 ozone monitor (Shenzhen Pulitong, Shenzhen, China). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee to minimize animal distress.

Sample collection

After completing the task, the rats were injected intraperitoneally with 40 mg/kg body weight of 3% pentobarbital sodium, then blood was collected from the abdominal aorta to assess related indexes. Samples of quadriceps tissues from the right hind limb were fixed in 10% formalin, and the remaining tissues were frozen in liquid nitrogen.

Histopathological examination of quadriceps femoris muscle

Formalin-fixed quadriceps femoris tissues were dehydrated using a series of graded ethanol, rendered transparent with xylene, and embedded in paraffin. The tissues were sectioned (3-μm thick) and stained with hematoxylin and eosin (HE). The tissue structure and morphological changes of the quadriceps femoris were visualized and photographed using a BX51 light microscope (Olympus Corp., Tokyo, Japan). Then, 5 fields for each sample were selected and analyzed using Image Pro Plus 6.0 software. The mean area of the muscle cell and the mean muscle intercellular space in different groups were counted.

Detection of oxidative stress and inflammation factors in quadriceps femoris muscle

Fresh quadriceps femoris tissues (0.1 g) were lysed in phosphate-buffered saline (PBS) using a TJS-325 high-throughput tissue grinder (Techin, Tianjin, China). The supernatant was collected by centrifugation, and total protein was quantified using bicinchoninic acid (BCA) assays (Solarbio Co., Ltd., Beijing, China). The malondialdehyde (MDA) content and activity of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in the quadriceps femoris tissues were detected using colorimetric kits (Nanjing Jiancheng Biological Company, Nanjing, China). The oxidative damage index, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and the inflammatory indexes interleukin (IL)-1, IL-2, IL-6, IL-8, and tumor necrosis factor (TNF)-α were detected using enzyme-linked immunoassay (ELISA) kits (Jiangsu Meimian Industrial Co., Ltd, Yancheng, China). Colorimetry and ELISA results were determined using a SpectraMax® M5 plate reader (Molecular Devices LLC., San Jose, CA, USA). All assays proceeded as described by the respective manufacturers.

Detection of enzyme activities related to energy metabolism and ATP content in quadriceps femoris

Frozen quadriceps femoris tissues were disrupted in 0.01 M PBS (pH 7.0–7.4) using a high-throughput TJG-215 homogenizer (Techin, Tianjin, China). The homogenate was centrifuged at 1 690 × g for 15 min; then, the activities of enzymes involved in the tricarboxylic acid (TCA) cycle (α-ketoglutarate dehydrogenase [α-KGDHC], citrate synthase [CS], and isocitrate dehydrogenase 1 [ICD1]) and of those in the mitochondrial respiratory chain complex (MRCC) (cytochrome C oxidase [COX] and Complex V) were quantified in supernatants using corresponding ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) as described by the manufacturer. The ATP content in quadriceps femoris lysates was determined using enhanced ATP detection kits (Beyotime Biotechnology, Shanghai, China) as described by the manufacturer.

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL)

Apoptosis in rat quadriceps femoris tissue cells was assessed using TUNEL kits (Roche Holdings AG., Basel, Switzerland) as described by the manufacturer; then, stained sections were assessed using a BX51 fluorescence microscope. Blue and yellow-green fluorescence indicated normal and apoptotic nuclei, respectively. Apoptotic cells were counted three times per section using IPP6.0 software and averaged to determine the final apoptotic rate.

Western blotting

Fresh quadriceps femoris tissues (0.1 g) were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Solarbio) and centrifuged at 12 000 ×g for 30 min at 4 °C. The amount of total protein in the supernatant was quantified using BCA Protein Assay kits (Solarbio). Total protein (50 μg) was mixed with an appropriate amount of 5 × loading buffer and then boiled in a water bath for 10 min. The proteins were resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany) under a constant current of 200 mA for 1 h. Non-specific protein binding on the membranes was blocked with 5% skim milk at room temperature for 2 h and then the membranes were incubated overnight with the following rabbit primary antibodies (Bioss Antibodies, Woburn, MA, USA): Bcl-2 associated X protein (Bax) (BS-0127R, 1:800), B-cell lymphoma-2 (Bcl-2) (BS-4563R, 1:1 000), Caspase-3 (BS-0081R, 1:800), Caspase-9 (BS-0049R, 1:1 000), Cytochrome C (Cyt-C) (BS-0013R, 1:1 000), and GAPDH (BS-2188R, 1:2 000). The membranes were washed with Tris-Buffered Saline-Tween 20 (TBST) buffer 3 times for 10 min each and then incubated at room temperature for 1 h with secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (BS-0295R, 1:2 000; Bioss). The membranes were washed, after which proteins were visualized at room temperature using efficient chemiluminescence (ECL) kits (Solarbio) for 5 min. Proteins were detected and photographed using an automated chemiluminescence image analysis system (Tanon Science and Technology Co., Shanghai, China); then, gray values of the proteins were analyzed using Gel-Pro Analyzer software (Tanon Science and Technology Co.).

Statistical analysis

All data are presented as the mean ± standard deviation (*SD*). Statistical analysis of data was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). A 2 × 4 two-factor factorial design was adopted, and data were analyzed using analysis of variance. Non-normally-distributed data were analyzed using Scheirer–Rayleigh tests. Interactions between two factors or the dominant effects in each index were assessed.

Results

Acute exercise in air containing ozone (0.14 ppm) significantly reduced enzyme activity in TCA and MRCC and ATP content in rat quadriceps femoris

The three important rate-limiting enzymes in the TCA cycle are α-KGDHC, CS, and ICD1. They regulate important activities such as energy metabolism and biosynthesis that directly affect the speed of aerobic

oxidation and energy metabolism. Fig. 1B–D shows that α-KGDHC, CS, and ICD1 activities gradually increased with increasing exercise intensity in quadriceps femoris from rats in the Air group. In contrast, these indices were significantly lower in the rats that exercised under ozone exposure compared with those that exercised in the air ($p < 0.01$). The trends in the activities of the important mitochondrial respiratory chain complexes, COX and Complex V were the same (Fig. 1E and F, respectively). These results suggested that exercise in air increased, whereas that in 0.14 ppm ozone significantly reduced the activity of metabolism-related enzymes in the quadriceps femoris. The ATP content did not significantly differ among the Air groups, except for the 20 m/min group. However, the ATP content in the quadriceps femoris significantly declined in rats that exercised under 0.14 ppm ozone compared with those that exercised in the air ($p < 0.01$; Fig. 1G).

Histopathological changes in quadriceps femoris of exercised rats after acute exposure to ozone (0.14 ppm)

Pathological changes in the quadriceps femoris of rats were assessed via HE staining. The overall structures of the quadriceps femoris were normal, without hypertrophy, edema, or inflammatory cell infiltration in the groups exposed to air (Fig. 2A–D). However, muscle structures were slightly abnormal and intermuscular edema was evident in the O_3+0 and O_3+10 groups (Fig. 2E and F, respectively), and hypertrophic muscle cells and more intermuscular edema were evident in the O_3+15 and O_3+20 groups (Fig. 2G and H, respectively). The quantified data of the mean area of muscle cell (Fig. 2I) and the mean muscle intercellular space (Fig. 2J) in different groups also proved the observations from HE staining. These results suggested that ozone induces histopathological changes in the quadriceps femoris, which worsened as exercise intensity increased.

Acute exercise with moderate intensity in air containing ozone (0.14 ppm) induced significant inflammation in rat quadriceps femoris muscles

Increased levels of IL-1, IL-2, IL-6, IL-8, and TNF-α in the rat quadriceps femoris indicated inflammatory responses. Fig. 3 shows that levels of IL-1, IL-2, IL-6, IL-8, and TNF-α did not significantly differ in the Air groups, O_3+0 group, and O_3+10 group. In contrast, levels of IL-1, IL-2, IL-6, IL-8, and TNF-α significantly increased in the quadriceps femoris at treadmill speeds of 15 and 20 m/min under ozone exposure (Fig. 3A–E). These results suggested that moderate-intensity exercise under ozone exposure led to inflammatory reactions in quadriceps femoris muscles. In addition, exercise under ozone exposure exerted synergistic effects while facilitating an increase in the levels of inflammatory cytokines IL-2 and IL-6 (Fig. 3F and G). The relevant mechanisms require further investigation.

Moderate-intensity exercise under ozone (0.14 ppm) exposure led to oxidative stress in rat quadriceps femoris muscles

The indexes GSH-Px, MDA, SOD, and 8-OHdG indicate the status of the balance between oxidants and antioxidants, in addition to the level of oxidative stress. Fig. 4 shows that exercise at different intensities in the air did not change the oxidative stress indices, suggesting that oxidative stress was not induced. In contrast, these indices significantly changed in the O_3 groups. The activities of GSH-Px and SOD significantly decreased in the O_3 groups compared with the Air groups at the same exercise intensity ($p < 0.01$; Fig. 4A and C), whereas the content of MDA and 8-OHdG significantly increased ($p < 0.01$, Fig. 4B and D). However, ozone-induced oxidative stress became increasingly obvious as the treadmill speed increased (Fig. 4A–D). These results suggested that exercise under ozone exposure induces oxidative stress in the rat quadriceps femoris muscle. Exercise and ozone synergistically induced changes in 8-OHdG (Fig. 4E).

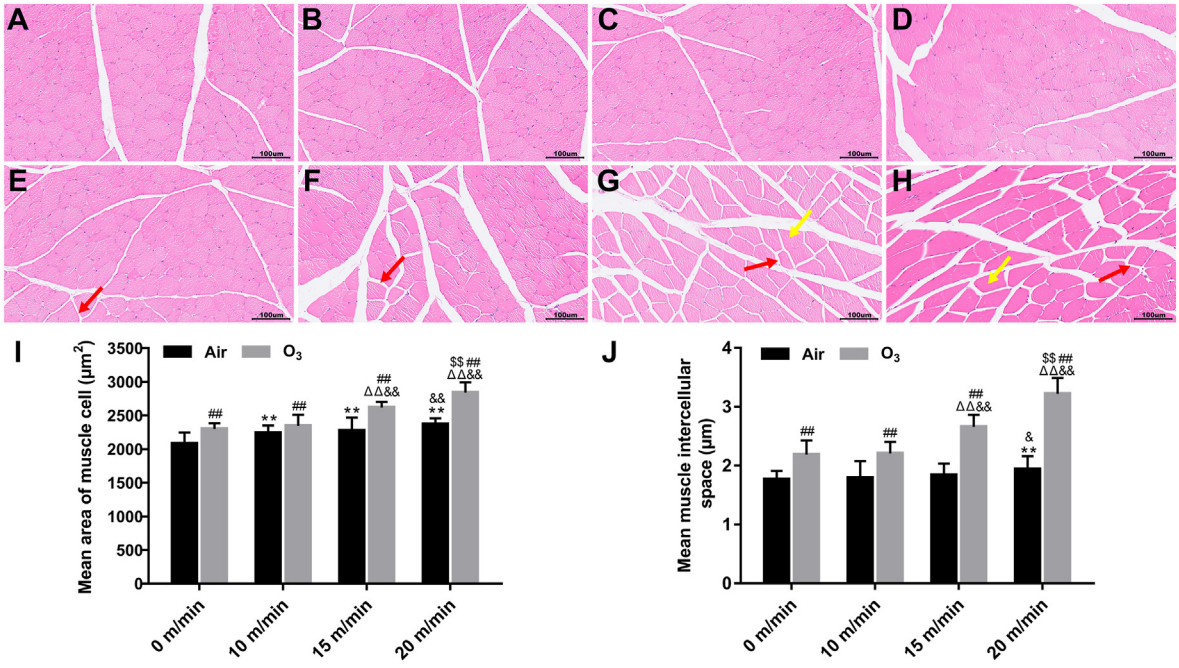

Fig. 2. The histopathological changes of the quadriceps femoral muscle of rats after acute exercise in clean air or ozone-polluted air ($n = 5$). A–H: the results of HE staining for Air+0 group (**A**), Air+10 group (**B**), Air+15 group (**C**), Air+20 group (**D**), O_3+0 group (**E**), O_3+10 group (**F**), O_3+15 group (**G**) and O_3+20 group (**H**). The yellow arrows represent muscle cell hypertrophy and the red arrows represent inter-muscular edema. Scale bar = 100 μm; **I:** the mean area of muscle cell in different groups; **J:** the mean muscle intercellular space in different groups. **Air:** group of doing different speeds of exercise in clean air; **O_3**: group of doing different speeds of exercise in ozone-polluted air. $^{**}p < 0.01$ vs Air+0 group, $^{\Delta\Delta}p < 0.01$ vs O_3+0 Group, $^{\#\#}p < 0.01$ vs the Air group with the same speed, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 10 m/min group in the same group, $^{\$\$}p < 0.05$ vs 15 m/min group in the same group. HE: Hematoxylin and eosin.

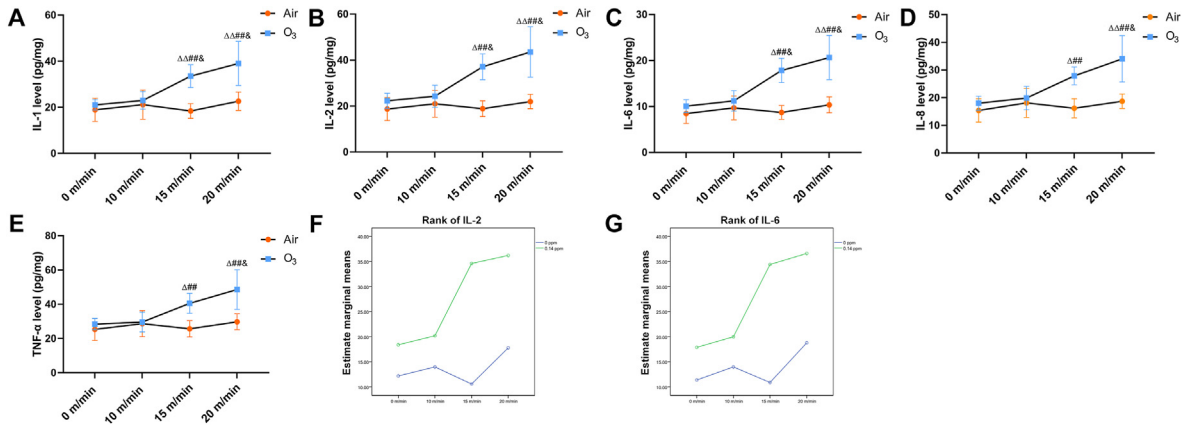

Fig. 3. Levels of inflammatory cytokines in the quadriceps femoris muscle of rats after acute exercise in clean air or ozone-polluted air (Mean ± *SD*, $n = 5$). A: IL-1; **B:** IL-2; **C:** IL-6; **D:** IL-8; **E:** TNF-α; **F:** the interaction diagrams for IL-2; **G:** the interaction diagrams for IL-6. **Air:** group of doing different speeds of exercise in clean air; **O_3:** group of doing different speeds of exercise in ozone-polluted air. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Air+0 group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs O_3+0 Group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs the Air group with the same speed, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 10 m/min group in the same group. IL: Interleukin, TNF: Tumor necrosis factor, pg/mg: picogram/milligram.

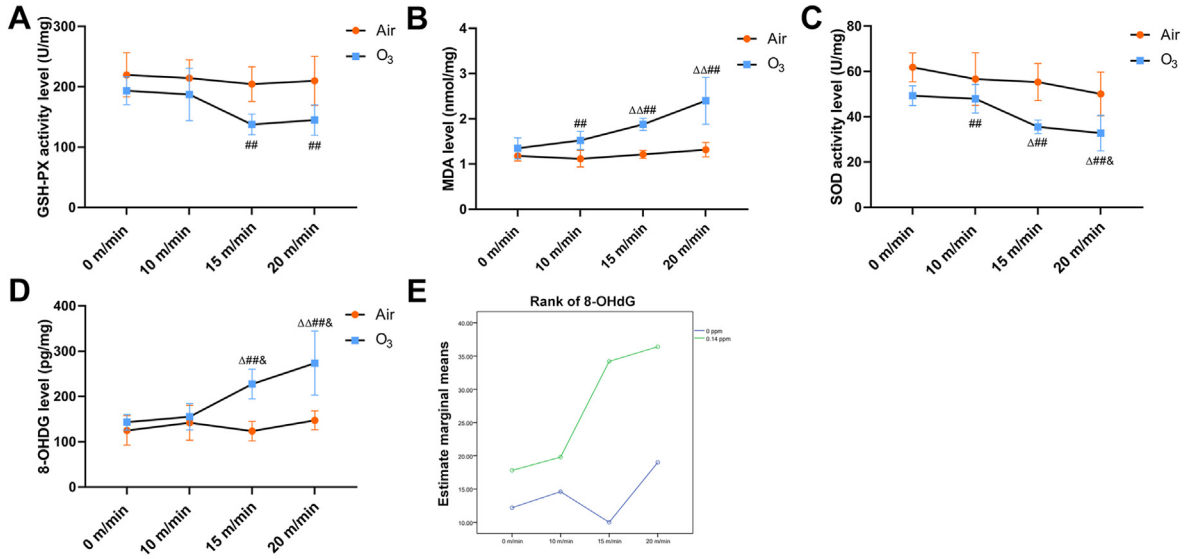

Fig. 4. Levels of the oxidative stress indexes in the quadriceps femoris muscle of rats after acute exercise in clean air or ozone-polluted air (Mean ± *SD*, $n = 5$). A: GSH-Px; **B:** MDA; **C:** SOD; **D:** 8-OHdG; **E:** The interaction diagrams for 8-OHdG. **Air:** group of doing different speeds of exercise in clean air; **O_3:** group of doing different speeds of exercise in ozone-polluted air. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs O_3+0 Group, $^{\#\#}p < 0.01$ vs the Air group with the same speed, $^{\&}p < 0.05$ vs 10 m/min group in the same group. GSH-Px: Glutathione peroxidase, MDA: Malondialdehyde, SOD: Superoxide dismutase, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, U/mg: Unint/milligram, nmol/mg: nanomol/milligram, pg/mg: picogram/milligram.

Acute exercise in air containing ozone (0.14 ppm) induced apoptosis in rat quadriceps femoris muscle cells via mitochondria-dependent pathway

Apoptosis of the quadriceps femoris was detected using TUNEL (Fig. 5A). Fig. 5B shows the apoptosis rates. Apoptotic cells were scant in all the Air groups and in the O_3+0 group. These results indicated that exercise for 1 h at different intensity in air and rest under 0.14 ppm ozone for 1 h do not induce apoptosis in quadriceps femoris muscle cells. However, cell apoptosis obviously increased after 1 h of exercise in air containing ozone (0.14 ppm) and further increased as exercise intensity increased. Therefore, exercise under ozone exposure plays an important role in inducing apoptosis.

The expression of Bax, Bcl-2, Caspase-3, Caspase-9, and Cyt-c in the mitochondrial signaling pathway of apoptosis was quantified in quadriceps femoris tissues via western blotting (Fig. 5C). Exercise in air did not significantly affect the expression of Caspase-3, Caspase-9, and Cyt-C, except in the Air+20 group, whereas exercise under ozone exposure decreased their expression regardless of exercise (Fig. 5D, E, and G). Exercise reduced Bcl-2/Bax ratio, depending on the exercise intensity in the Air and O_3 groups, but the decline was more significant in the O_3 groups (Fig. 5C and F). These results suggest that exercise under ozone activates the mitochondrial apoptosis pathway and that exercise and ozone both play important roles in this biological process.

Discussion

Globally, people engage in outdoor exercise on a frequent basis, during which they face air pollution, which is an unavoidable challenge. Ambient ozone is the main pollutant during summer in developing countries such as China and India.[31–33] Therefore, the impact of exercise in ozone-polluted air on health has become a matter of public concern. Adverse effects on physical exercise capacity and the respiratory system have been confirmed in schoolchildren,[34] male and female adults,[28,35] competitive athletes,[36,37] and well-trained individuals[35,38] who engage

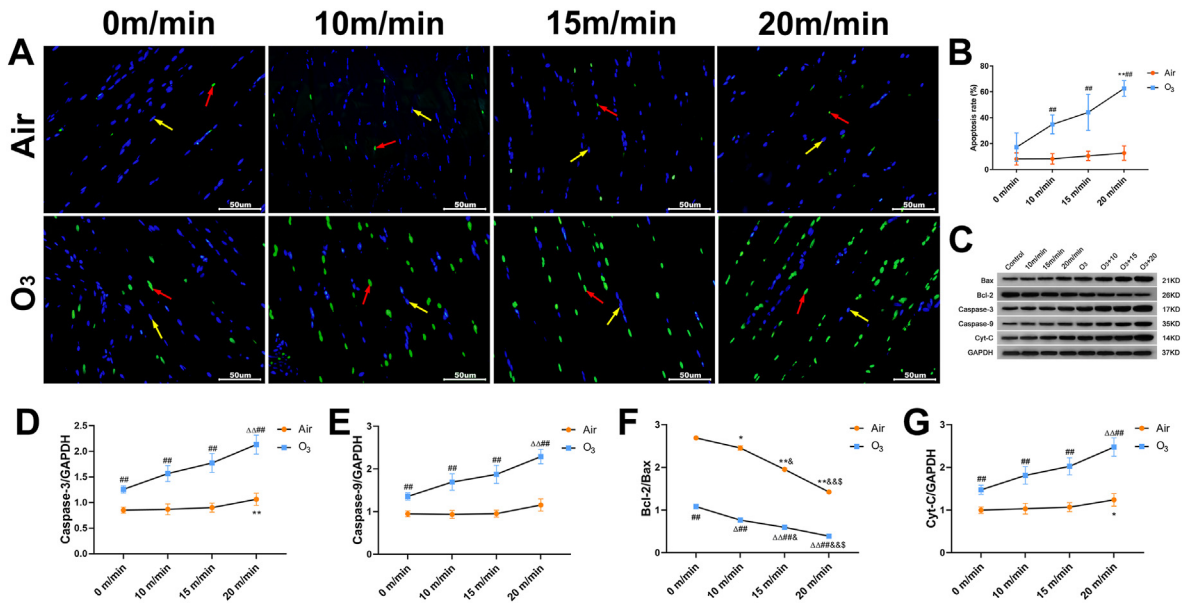

Fig. 5. Alterations in cell apoptosis and the expression of major proteins in the mitochondrial apoptosis signaling pathway in the quadriceps femoris muscle of rats after acute exercise in clean air or ozone-polluted air ($n = 5$). A: Results of TUNEL staining of quadriceps femoris muscle, scale bar = 50 μm. The yellow arrow represents normal nuclei stained with DAPI, the red arrow represents apoptotic cells stained with TUNEL; **B:** The apoptosis rate of apoptotic positive cells counted by IPP6.0 software (Mean ± *SD*); **C:** Western blot results of Bcl-2, Bax, Caspase-3, Caspase-9, Cyt-C protein expression in the quadriceps femoral muscle of rats; **D-G:** Caspase-3, Caspase-9, Bcl-2/Bax, Cyt-C protein expression histograms (Mean ± *SD*). **Air:** group of doing different speeds of exercise in clean air; **O_3:** group of doing different speeds of exercise in ozone-polluted air. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Air+0 group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs O_3+0 Group, $^{\#\#}p < 0.01$ vs the Air group with the same speed, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 10 m/min group in the same group, $^{\$}p < 0.05$ vs 15 m/min group in the same group. TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling, DAPI: 4,6-diamino-2-phenyl indole, Bcl-2: B-cell lymphoma-2, Bax: Bcl-2 associated X protein, Cyt-C: Cytochrome C, m/min: meter/minute.

in physical activities in ozone-polluted air. The effect of ozone on lung function and ventilation is considered as a major reason for the decline in physical exercise capacity. Although exercise in ozone-polluted air affects sport performance, whether it also impacts skeletal muscle is unclear. Therefore, we explored the effects of acute exercise, at different intensities under air containing a low level of ozone, on the rat quadriceps femoris.

First, treadmill speeds of 10, 15, and 20 m/min were selected to simulate the exercise intensity of rats at approximately 45%–55%, 55%–65%, and 65%–75% of the maximal oxygen consumption, respectively.[39,40] Therefore, treadmill speeds of 10, 15, and 20 m/min represented low-, moderate-, and moderate-to-high-intensity exercise, respectively. Mild (0.14 ppm) ozone pollution accounts for a high proportion of the total number of days with air pollution in China. Therefore, we considered that an average of 0.09–0.19 ppm (0.2–0.4 mg/m^3) was representative of actual exposure in this study.[41] Therefore, our model mainly focused on the acute effects of low-, moderate-, and moderate-to-high-intensity exercise under mild ozone pollution on the quadriceps femoris.

We evaluated the combined effects of exercise and ozone on the quadriceps femoris in terms of energy production. The supply of ATP is essential for skeletal muscle contraction during exercise. However, because small amounts of ATP are stored in skeletal muscle, metabolic pathways involving energy production must be activated to generate enough ATP.[42] Oxidative phosphorylation and the TCA cycle are two important energy metabolic pathways.[43] Our data showed that exercise alone increases the capacity of the TCA cycle and oxidative phosphorylation in the quadriceps femoris and maintains balanced levels of ATP in cells. However, when exposed to ozone (regardless of exercise), the activities of important enzymes involved in these two pathways and the ATP content significantly declined. These results suggested that ozone inhibited the TCA cycle and oxidative phosphorylation and disrupted energy production during exercise. Our histopathological findings showed that exercise alone did not significantly affect quadriceps femoris structures, whereas exercise under ozone exposure resulted in hypertrophy and edema of the quadriceps femoris, which gradually worsened with increasing exercise intensity. Therefore, we believe that ozone plays an important role in muscle damage.

The levels of IL-1, IL-2, IL-6, IL-8, and TNF-α in the quadriceps femoris significantly increased only in the O_3+15 and O_3+20 groups with noticeable differences among the groups with exercise alone, ozone alone, and O_3+10. Ozone and exercise exerted synergistic effects on the induction of increased IL-2 and IL-6 in quadriceps muscles. These results indicated that ozone and exercise were each important in inducing an inflammatory response, as well as that levels of inflammatory cytokines in the quadriceps femoris under 0.14 ppm ozone exposure significantly increased only when the intensity of exercise was moderate or above. Furthermore, the inflammatory response worsened as exercise intensity increased, and both ozone and exercise synergistically enhanced the levels of some inflammatory cytokines in the quadriceps femoris tissue. Although the effects of ozone with or without exercise on the skeletal muscle have remained unknown to date, ozone is known to induce inflammatory responses in the airways, lungs, and heart.[13,44,45]

We assessed oxidative stress in the rat quadriceps femoris muscle. Ozone is a powerful oxidant that can increase cellular levels of reactive oxygen species (ROS) and oxidative stress in tissues, resulting in increased MDA contents and decreased GSH-Px activity.[46–48] Our results showed that exercise alone and exposure to 0.14 ppm of O_3 alone did not induce oxidative stress in the quadriceps femoris. However, exercise under 0.14 ppm of O_3 disrupted the balance between oxidant and antioxidant levels and led to intracellular oxidative damage. Ozone and exercise both induced oxidative stress in the quadriceps femoris and synergistically increased 8-OHdG levels, suggesting that they can aggravate DNA oxidative damage.

The mechanisms underlying changes in the structure and function of the quadriceps femoris were explored. Mitochondria produce approximately 90% of physiological ATP, and thus, play important roles in maintaining exercise capacity and regulating the ROS balance.[49] Based

on our findings, we concluded that mitochondrial functions are impaired by exercising under 0.14 ppm of ozone. Valdez et al. showed exercise on a low level of O_3 decreased mitochondrial complex II activity and gene expression of critical subunits of complex I in rat hypothalamus.[50] O_3 exposure can also cause mitochondrial mtDNA damage in vascular cells,[51] and decrease mitochondrial complex I, II and IV activity in mice[52] and rats.[53] Mitochondria play an important role in inducing cell apoptosis.[54] Our analysis of the mitochondria-dependent apoptosis pathway confirmed that exercise under 0.14 ppm of ozone-induced apparent cell apoptosis via a mitochondria-dependent pathway in the quadriceps femoris, which was consistent with findings of oxidative stress and a damaged histopathological structure.

This study has some limitations. We investigated only the acute effects of exercise under ozone exposure on the rat quadriceps femoris; long-term exposure might have a more profound impact. Effects on the lungs, heart, and other organs or tissues are also topics worthy of further investigation. Moreover, interactions between tissues or organs and biological mechanisms require further investigation, in addition to determining whether the results of the current study are applicable to humans.

Conclusion

Ozone inhibited the activities of key enzymes involved in TCA and oxidative phosphorylation and decreased ATP production in the rat quadriceps femoris regardless of whether it was coupled with exercise. Exercise in air containing 0.14 ppm of ozone led to pathological changes, inflammatory responses, oxidative stress, and mitochondria-dependent apoptosis in the rat quadriceps femoris, and these became more obvious as the exercise intensity increased. Therefore, damage to the rat quadriceps femoris induced by acute exercise under ozone might have relevance to human sport performance. Exercising in air polluted even with only low concentrations of ozone should be carefully considered.

Submission statement

We submit our manuscript entitled “Acute exercise in ozone-polluted air induces apoptosis in rat quadriceps femoris muscle cells via mitochondrial pathway” for publication in Sports Medicine and Health Science. We confirm that this article is original, neither the entire manuscript nor any part of its content has been published or has been accepted elsewhere. This manuscript is not being considered for publication by any other journals, and we will not re-submit it to another journal or a conference during the reviewing procedure. All authors have seen the manuscript and approved to submit to your journal. This manuscript has no competing financial interest.

Ethical approval statement

All animals were housed under a 12 h–12 h day/night cycle at a temperature of 23 °C ± 1 °C and 55% ± 5% humidity. All procedures were reviewed and approved by the Experimental Animal Administration Committee of Tianjin Institute of Environmental and Operational Medicine.

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Authors' contribution

Xiaohua Liu and Zhuge Xi conceived the idea and designed the experiments. Ziyi Liu and Fuxu Gong established animal models and detected important indicators. Zhiyuan Liu and Pengfei Xu performed the analysis of mitochondrial related indexes. Lei Tian, Jun Yan, Kang Li, Wei Zhang and Bencheng Lin performed the other experiments. The manuscript was written by Ziyi Liu and Fuxu Gong, and reviewed and approved by all authors.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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