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Research article

Ambient particulate matter (PM_{10})-induced injury in feline lung cells and nutritional intervention

Huasong Bai, Ying Wang, Peng Wu, Zhanzhong Wang *

Nourse Centre for Pet Nutrition, Wuhu, 241200, China

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ABSTRACT

Ambient particulate matter (APM) is extremely harmful to life's health. In this study, we investigated cellular injury in cat (*Felix catus*) lung cells (FCA-L2) exposed to organic and water-soluble extracts from APM. As well, the protective effect of vitamin E (VE), lycopene and a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (molar concentration ratio of 2:1) against this damage was evaluated. Organic and water-soluble extracts induced oxidative stress in FCA-L2 cells, as evidenced by excess reactive oxygen species production and mitochondrial damage, while treatment with VE, lycopene and EPA: DHA remarkably alleviated these indices. It was further found that treatment with EPA: DHA decreased lactate dehydrogenase and malondialdehyde, as well as increased activities of superoxide dismutase, glutathione peroxidase and catalase. Our study confirmed that nutrients mediates APM-induced oxidative stress via antioxidant proteins. Also, these findings could provide new insights into reducing APM-induced cytotoxicity by nutritional supplementation based on antioxidant compounds for animals.

1. Introduction

Ambient particulate matter (APM) is a component of air pollution which has been recognized as a serious environmental risk factor for a variety of diseases, thus determining considerable health and economic burden (Knaapen et al., 2004). APM, especially $PM_{2.5}$ and PM_{10}, can be easily inhaled and deposited in organs of the respiratory tract, such as trachea, bronchi and alveoli. APM affect the respiratory system by inducing oxidative stress as well as inflammatory and immunomodulatory responses (Zhen et al., 2013). Epidemiological studies have shown that APM is associated with human lung cancer as well as respiratory, cardiovascular and cerebrovascular diseases (Englert, 2004; Zhen et al., 2013).

Previous studies on the biological effects of APM have focused on humans, with very limited research on animals, especially cats. Animal health, especially that of companion animals, has been receiving increasing attention due to the growth of the pet industry. Since house pets, such as cats and dogs, share the same living environment as humans, these animals might also be exposed to APM. There are some reports that APM exposure increases the risk of respiratory diseases in felines and canines, such as lower respiratory tract infection, chronic bronchitis, and lymphoplasmacytic rhinitis (L et al., 2001; Lin et al., 2020; Lin et al., 2018; Montrose et al., 2015). However, to date, few studies have been reported on the mechanism and prevention strategies on the respiratory system of pets.

Several studies have shown that APM consist of a mixture of solid and liquid suspended particles with an inert carbonaceous core onto which multiple layers of chemical substances are adsorbed, and whose toxic effects are related to the presence of inorganic (sulfates, nitrates, metals, etc.) and organic components (polycyclic aromatic hydrocarbons (PAHs), etc.) (Billet et al., 2008; Deng et al., 2013). In light of this, nutritional interventions, such as dietary supplements containing antioxidants and anti-inflammatory substances, have been proposed as an effective strategy to protect against respiratory damage caused by exposure to APM (Romieu et al., 2008).

Vitamin E (VE), lycopene and omega-3 polyunsaturated fatty acids (omega-3 PUFAs) have demonstrated antioxidant activity (Bulger and Maier, 2003; Deckelbaum and Torrejon, 2012; Przybylska, 2020). VE is an essential fat-soluble vitamin which has been shown to protect against tobacco smoking-induced cardiac oxidative stress and ozone-induced lung damage (Samet et al., 2001). Additionally, lycopene is a natural carotenoid with remarkable free radical scavenging ability, which can protect endothelial cells from oxidative damage (Przybylska, 2020). Omega-3 PUFAs from natural sources (fish and linseed oils) are a mixture

* Corresponding author.
E-mail address: wzz7698@tju.edu.cn (Z. Wang).

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of short-chain and long-chain fatty acids, primarily eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Deckelbaum and Torrejon, 2012). Several in vivo and in vitro studies have shown that the mixture of EPA: DHA at a molar concentration ratio of 2:1 adequately mimics the conditions in which these substances are naturally found (Desiree et al., 2005; Simopoulos and Artemis, 2002) and which have been shown to possess excellent anti-inflammatory and antioxidative activity (Bo et al., 2016). However, the impact of these antioxidants and its underlying mechanisms on lung cells of domestic felines damaged by APM have never been reported.

In the present study, the APM standard reference material (SRM 1648a) certified by the National Institute of Standards and Technology (NIST, USA) was selected as the model sample, and its morphology was characterized. Organic and water-soluble extracts of SRM 1648a were obtained (May et al., 2000; Zeisler et al., 2020), and whose main components were certified by NIST (Zeisler et al., 2020). Furthermore, using cat (*Felix catus*) lung cells (FCA-L2) as a model, the protective effect of VE, lycopene and omega-3 PUFAs (EPA: DHA, 2:1) against damage mediated by organic and water-soluble extracts of SRM 1648a was evaluated.

2. Materials and methods

2.1. Materials and reagents

Fetal bovine serum (FBS) was purchased from Gibco (NY14072, USA). RPMI-1640 medium, penicillin/streptomycin liquid (100×), 0.25% trypsin-0.02% EDTA solution and dimethyl sulfoxide (DMSO) were purchased from Solarbio Biotechnology (Beijing, China). VE was obtained from Bide Pharmaceutical Technology Co., Ltd. (Shanghai, China). Lycopene, EPA and DHA were purchased from Acmec Biochemical Co., Ltd. (Shanghai, China). Dichloromethane was purchased from Xilong Science Co., Ltd. (Guangdong, China). Trypan blue stain and Cell Counting Kit (CCK-8) were obtained from Beyotime Biotechnology (Shanghai, China). Commercial assay kits for bicinchoninic acid (BCA) protein quantification, reactive oxygen species (ROS), lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) were all procured from Solarbio Biotechnology (Beijing, China).

2.2. Pretreatment and characterization of urban particulate matter

SRM 1648a was obtained from NIST (USA). Certified mass fraction values for elements (Table S1), PAHs (Table S2), and polychlorinated biphenyl congeners (PCBs) (Table S3) in SRM 1648a are provided by NIST (Zeisler et al., 2020). For the organic and water-soluble extracts of SRM 1648a, the extraction processes were performed in accordance with NIST's methods (Zeisler et al., 2020). Briefly, for organic extracts, SRM 1648a was extracted using Soxhlet extraction for 20 h with dichloromethane. Subsequently, the concentrated extract was rotary evaporated, dried under nitrogen, and weighed. The stock solution was prepared with DMSO and stored at -20 °C. For water-soluble extracts, the aqueous suspension of SRM 1648a was ultrasonically extracted in an ice bath for 3 × 20 min, filtered with eight layers of gauze, lyophilized in vacuum and weighed. The stock solution was prepared using phosphate buffered saline (PBS, pH 7.2–7.4, 0.01 M) and stored at -20 °C. Before the experiment, the stock solutions of organic extracts and water-soluble extracts were diluted with RPMI-1640 medium supplemented with 10% FBS and sonicated in an ice bath for 10 min to obtain a homogeneous solution.

A suspension of SRM 1648a was dropped onto the membrane, dried and sputter-coated with gold and then observed under a scanning electron microscope (SEM) (Zeiss Supra 40, Oberkochen, Germany). The particle size distribution of SRM 1648a was obtained by measuring the particles in an aqueous suspension using a laser diffractometer (Malvern, Worchestershire, UK).

2.3. Cell culture

FCA-L2 cells were provided by C-Reagent Biotechnology Ltd. (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. At more than 90% confluency, cells were trypsinized with 0.25% trypsin-0.02% EDTA solution, collected and counted in a hemocytometer using trypan blue stain. Subsequently, cells were seeded in a 96-well plate at a density of 1×10^5 cells/mL or in a 6-well plate at a density of 5×10^5 cells/mL and cultured for 24 h to ensure cell adhesion before treatment.

2.4. Cell treatments

Cells were treated with a range of concentrations of VE (0–80 μM), lycopene (0–80 μM) and EPA: DHA (2:1; 0–80 μM) in RPMI-1640 medium for 2 h to assess the nontoxic concentration range. Likewise, cells were exposed to organic extracts (0–960 μg/mL) and water-soluble extracts (0–960 μg/mL) for 16 h to select the optimal concentration for each.

To analyze the protective effects of VE, lycopene and EPA: DHA on APM extracts against cellular damage, cells were treated with or without VE (low, medium, and high doses), lycopene (low, medium, and high doses), and EPA: DHA (2:1; low, medium and high doses) for 2 h. Subsequently, cells were challenged with organic extracts and water-soluble extracts for 16 h to induce oxidative damage.

2.5. Cell viability

Cell viability was determined using the CCK-8 which indicates mitochondrial oxidoreductase activity. After cell treatment, 10% CCK-8 solution was added followed by incubation at 37 °C for 4 h. Absorbance at 450 nm was then measured in a microplate reader (Spectra MAX 190, Molecular Devices, USA) at room temperature. Cells treated with organic or water-soluble extracts separately served as exposure controls, whereas cells without any treatment were used as negative control, and results were expressed as a percentage relative to the negative control.

2.6. ROS production

Intracellular ROS accumulation was determined using the probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). After cell treatment, the fluorescent probe DCFH-DA was added followed by incubation at 37 °C for 30 min. Cells were then washed twice with serum-free medium, and intracellular ROS accumulation was qualitatively determined in a fluorescence microscope (BDS 400, OPTEC Instruments Co., Ltd., Chongqing, China).

2.7. LDH assay

Cytoplasmic LDH released into the medium was quantified to assess cell membrane integrity. Cell culture supernatants exposed to APM extracts after pretreatment with EPA: DHA (2:1) were collected separately and measured using the LDH kit. Briefly, 10 μL of cell supernatant sample was mixed with 50 μL of lactic acid solution and 10 μL of nicotinamide adenine dinucleotide from the LDH kit, incubated at 37 °C for 15 min, followed by a reaction with 50 μL of dinitrophenylhydrazine solution for 15 min. Finally, 150 μL of potassium hydroxide solution was added to terminate the reaction. Absorbance was measured at 450 nm and normalized to LDH viability values according to the manufacturer's instructions.

2.8. MDA assay

MDA is a biomarker of cell membrane lipid peroxidation which reflects oxidative stress levels. Treated cells were collected, resuspended in cell lysate, sonicated in a SCIENTZ-IID ultrasonic homogenizer (Scientz Biotechnology Co., Ltd., Ningbo, China) in an ice bath, and then submitted to centrifugation at 8,000 × *g* at 4 °C for 10 min. After protein quantification of the cell lysis supernatants were carried out with BCA kit, MDA contents were determined by the thiobarbituric acid condensation method. Briefly, a 100 μL sample of cell lysate was incubated with the MDA detection working solution in the kit for 60 min at 100 °C, cooled in an ice bath, and then centrifuged at 10,000 × g for 10 min at room temperature. Subsequently, the absorbance of the supernatant was measured at 450 nm, 532 nm and 600 nm, and the MDA content was calculated according to the manufacturer's instructions.

2.9. Determination of antioxidant capacity

SOD, GSH-Px and CAT are important enzymes that constitute the intracellular antioxidant system and can be considered indicators of cell antioxidant capacity. After cell treatment, cell lysis supernatants were obtained and subjected to protein quantification as described in Section 2.8. SOD activity was determined by the SOD kit based on the nitroblue tetrazolium photoreduction method. Briefly, 18 μL of the lysate sample was mixed with the detection working solution containing superoxide anion and the substrate nitroblue tetrazolium, incubated at 100 °C for 60 min, and then the absorbance was measured at 560 nm. GSH-Px activity was determined by the GSH-Px kit based on the dithio-p-dinitrobenzoic acid (DTNB) method. A 20 μL lysate sample was added with 20 μL glutathione solution, 10 μL hydrogen peroxide solution, and 200 μL metaphosphoric acid precipitation solution, and preheated at 37 °C for 5 min. The mixture was centrifuged at 4000 × g for 5 min at room temperature. Subsequently, the supernatant was collected, added with 100 μL of disodium hydrogen phosphate solution and 25 μL of DTNB solution, incubated for 15 min, and finally measured the absorbance at 412 nm. CAT activity was measured using a CAT kit based on the UV absorption method. 10 μL of the lysate sample was immediately mixed with 190 μL of the working solution containing hydrogen peroxide. Subsequently, the initial absorbance and the absorbance at 10 min of reaction were measured at 240 nm. The enzymatic activities of SOD, GSH-Px and CAT per unit protein weight in cells were calculated and analyzed according to the manufacturer's method.

2.10. Statistical analysis

Data analysis was performed using SPSS software (version 26.0). Samples were compared by one-way analysis of variance (ANOVA) followed by the Least Significant Difference post hoc test. The results were obtained from at least three independent experiments and expressed as the mean ± standard deviation (SD). P values <0.05 were considered statistically significant.

3. Results

3.1. Characterization of APM

Morphology of APM SRM 1648a was determined using SEM. As shown in Figure S1A and B, SRM 1648a had irregular shapes with an approximate average diameter of 10 μm. Moreover, at higher magnification, small particles were observed adhered forming large particles (Figure S1C and D). Additionally, particle size distribution of SRM 1648a in aqueous suspension was determined by laser diffractometry. As shown in Figure S2, particle size of SRM 1648a was within the range of 0.3–100 μm with an average particle size of 10.902 μm, which is consistent with SEM observations.

3.2. Effect of nutrients and APM extracts on cell viability

As shown in Figure 1, a dose-dependent decrease in cell viability was observed in cells treated with both APM organic (Figure 1A) and water-soluble extracts (Figure 1B). Notably, compared with the control group (0 μg/mL), cells treated with APM organic extracts at 160 μg/mL and APM water-soluble extracts at 80 μg/mL for 16 h showed reduced viability—66.63 ± 1.20% ($P < 0.01$) and 61.55 ± 7.60% ($P < 0.01$), respectively. Therefore, APM organic extracts at 160 μg/mL and APM water-soluble extracts at 80 μg/mL were employed in further experiments.

The dose-response relationship of VE, lycopene and EPA: DHA (2:1) on cell viability was determined. As shown in Figure S3, cell viability increased with increasing concentrations of VE, lycopene, and EPA: DHA. Compared to the control group (0 μM), cell viability was significantly increased when the concentration of VE ($P < 0.01$), lycopene ($P < 0.01$), and EPA: DHA ($P < 0.05$) increased to 10 μM. In contrast, cell viability was significantly decreased at 40 μM VE ($P < 0.05$), 80 μM lycopene ($P < 0.01$), and 80 μM EPA: DHA ($P < 0.05$). Therefore, three concentrations (5, 10 and 20 μM) categorized as low, medium and high dose of VE, lycopene, and EPA: DHA were employed in subsequent experiments.

3.3. Effect of different nutrients on viability of APM-exposed cells

As shown in Figure 2A–C, viability of cells treated with APM organic extracts was significantly reduced when compared to untreated cells (control group) ($P < 0.05$). Interestingly, viability of cells exposed to APM organic extracts which were pretreated with low, medium or high doses of VE (Figure 2A), lycopene (Figure 2B), and EPA: DHA (Figure 2C) significantly increased ($P < 0.05$). Viability was notably increased in cells pretreated with 10 μM VE (88.86 ± 5.93%, $P < 0.01$), 20 μM lycopene (82.39 ± 4.53%, $P < 0.01$), or 20 μM EPA: DHA (89.23 ± 5.58%, $P < 0.01$).

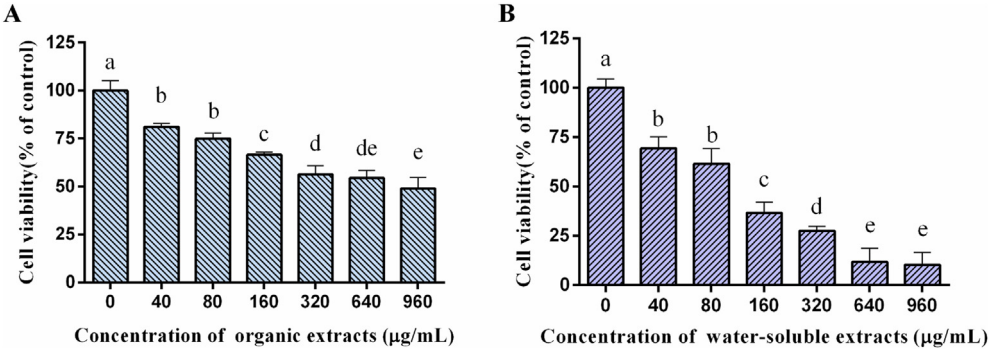

Figure 1. Effects of pretreatment with different concentrations (0–960 μg/mL) of (A) the organic extracts and (B) water-soluble extracts on the viability of FCA-L2 cells. All data are presented as the mean ± SD (n = 3). Different letters (a, b, c, d, e) indicate significant differences between each treatment group, $P < 0.05$.

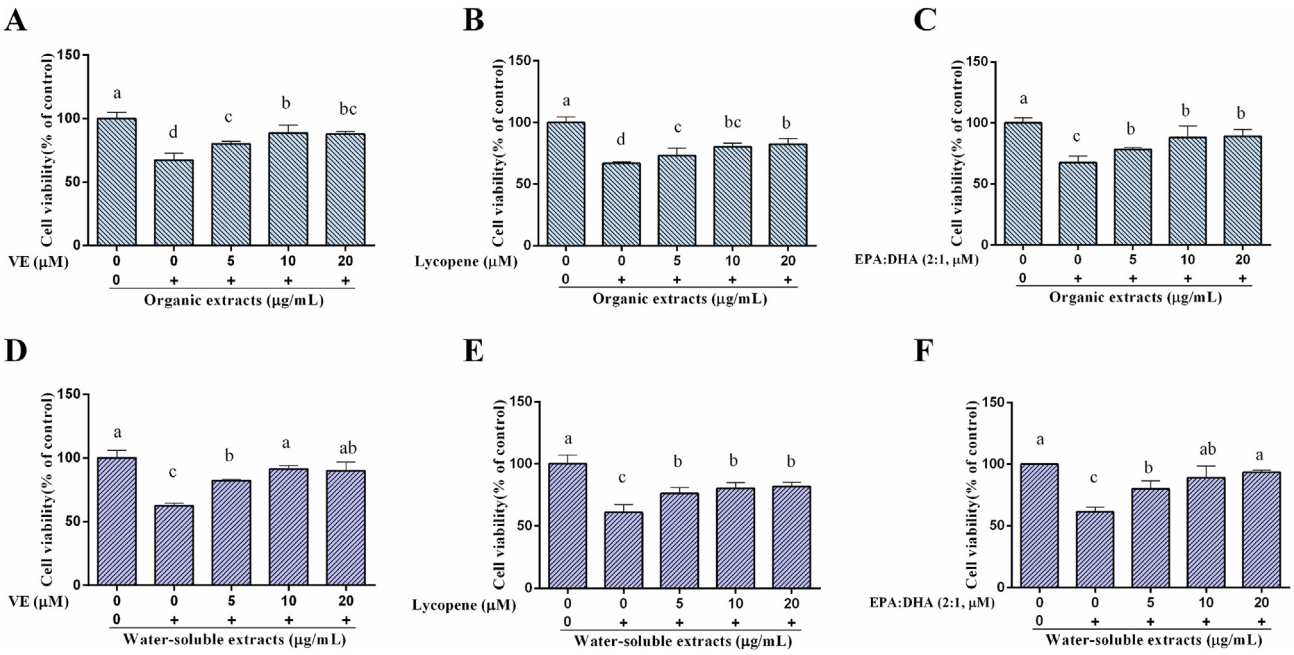

Figure 2. Effects of different nutrient pretreatments on the viability of FCA-L2 cells infected with different APM extracts. (A) VE (0, 5, 10, 20 μM), (B) lycopene (0, 5, 10, 20 μM) and (C) EPA: DHA (2:1; 0, 5, 10, 20 μM) pretreated cells were stressed with organic extracts (0 or 160 μg/mL). (D) VE (0, 5, 10, 20 μM), (E) lycopene (0, 5, 10, 20 μM) and (F) EPA: DHA (2:1; 0, 5, 10, 20 μM) pretreated cells were stressed with water-soluble extracts (0 or 80 μg/mL). All data are presented as the mean ± SD (n = 3). Different letters (a, b, c, d) indicate significant differences between each treatment group, $P < 0.05$.

A similar protective trend was observed in cells exposed to APM water-soluble extracts which were pretreated with VE (Figure 2D), lycopene (Figure 2E), and EPA: DHA (Figure 2F). Cell viability was the highest when cells were pretreated with 10 μM VE (91.49 ± 2.59%, $P < 0.01$), 20 μM lycopene (81.88 ± 3.49%, $P < 0.05$), or 20 μM EPA: DHA (93.62 ± 1.80%, $P < 0.01$) compared with cells exposed to APM water-soluble extracts.

As shown in Figure S4, under the microscope, cells in the negative control group were growth well and tightly connected with spindle shape. The nucleus and cell membrane were clear. In contrast, in the organic extract or water-soluble extract exposed groups, cells had shrunk and had distinct blister-like features on the cell membrane. When cells were pretreated with 10 μM VE, 20 μM lycopene, or 20 μM EPA, cell damage was improved in both the organic and water-soluble extract-exposed groups, as demonstrated by reduced cell shrinkage and adherent growth.

Thus, the above results indicated that pretreatment of FCA-L2 cells with VE, lycopene, and EPA: DHA can effectively alleviate damage induced by exposure to APM organic and water-soluble extracts. EPA: DHA pretreated cells were selected for further evaluation.

3.4. Nutrients attenuated APM-induced oxidative stress

Oxidative stress is generally considered as the main mechanism of cellular damage caused by external APM pollutants. Excessive ROS production is often used as a proxy for measuring oxidative stress levels. Fluorescence intensity of cells exposed to organic (Figure 3A1) and water-soluble extracts (Figure 3B1) was higher compared to that of cells in the negative control group (Figure 3A5 and B5), thus indicating that exposure to APM extracts led to ROS accumulation in cells. Interestingly, fluorescence intensity was significantly reduced in cells pretreated with 10 μM VE (Figure 3A2 and B2), 20 μM lycopene (Figure 3A3 and B3), or 20 μM EPA: DHA (Figure 3A4 and B4). These preliminary results suggest that VE, lycopene, and EPA: DHA could effectively alleviate oxidative stress induced by APM extracts as indicated by excessive ROS production in FCA-L2 cells.

3.5. Effects of EPA: DHA on membrane integrity of APM-exposed cells

LDH assay was used to assess membrane integrity of FCA-L2 cells. As shown in Figure 4A, LDH levels in culture supernatant of cells exposed to APM organic extracts were significantly increased compared to those of cells in the negative control group ($P < 0.001$). Interestingly, a dose-dependent reduction in LDH levels in the supernatant of EPA: DHA-treated cells was observed (5 μM, $P < 0.05$; 10 μM, $P < 0.01$; 20 μM, $P < 0.01$) when compared to cells exposed to APM organic extracts. As shown in Figure 4B, compared with cells in the negative control group, the release of LDH was significantly increased in cells exposed to APM water-soluble extracts ($P < 0.01$). However, LDH release was reduced in cells pretreated with low-, medium- and high-dose of EPA: DHA compared to cells exposed to APM water-soluble extracts ($P < 0.01$). Notably, no significant difference was observed in released LDH levels between cells pretreated with 20 μM EPA: DHA and cells in the negative control group ($P > 0.05$).

MDA, as a typical metabolite of ROS-induced membrane lipid peroxidation, is an effective biomarker for assessing cell membrane integrity and oxidative stress. As shown in Figure 4C, compared with cells in the negative control group, exposure to APM organic extracts increased MDA content ($P < 0.01$). However, pretreatment with 10 μM and 20 μM EPA: DHA led to a significant decrease in MDA production in cells exposed to APM organic extracts (10 μM, $P < 0.01$; 20 μM, $P < 0.01$). Similar results were observed for cells exposed to APM water-soluble extracts, which had significantly higher MDA contents compared to cells in the negative control group ($P < 0.01$) (Figure 4D). MDA levels were significantly reduced in cells pretreated with 5 μM EPA: DHA ($P < 0.05$), and further decreased with treatment at increasing concentrations of EPA: DHA (10 μM, $P < 0.01$; 20 μM, $P < 0.01$) in cells exposed to APM water-soluble extracts.

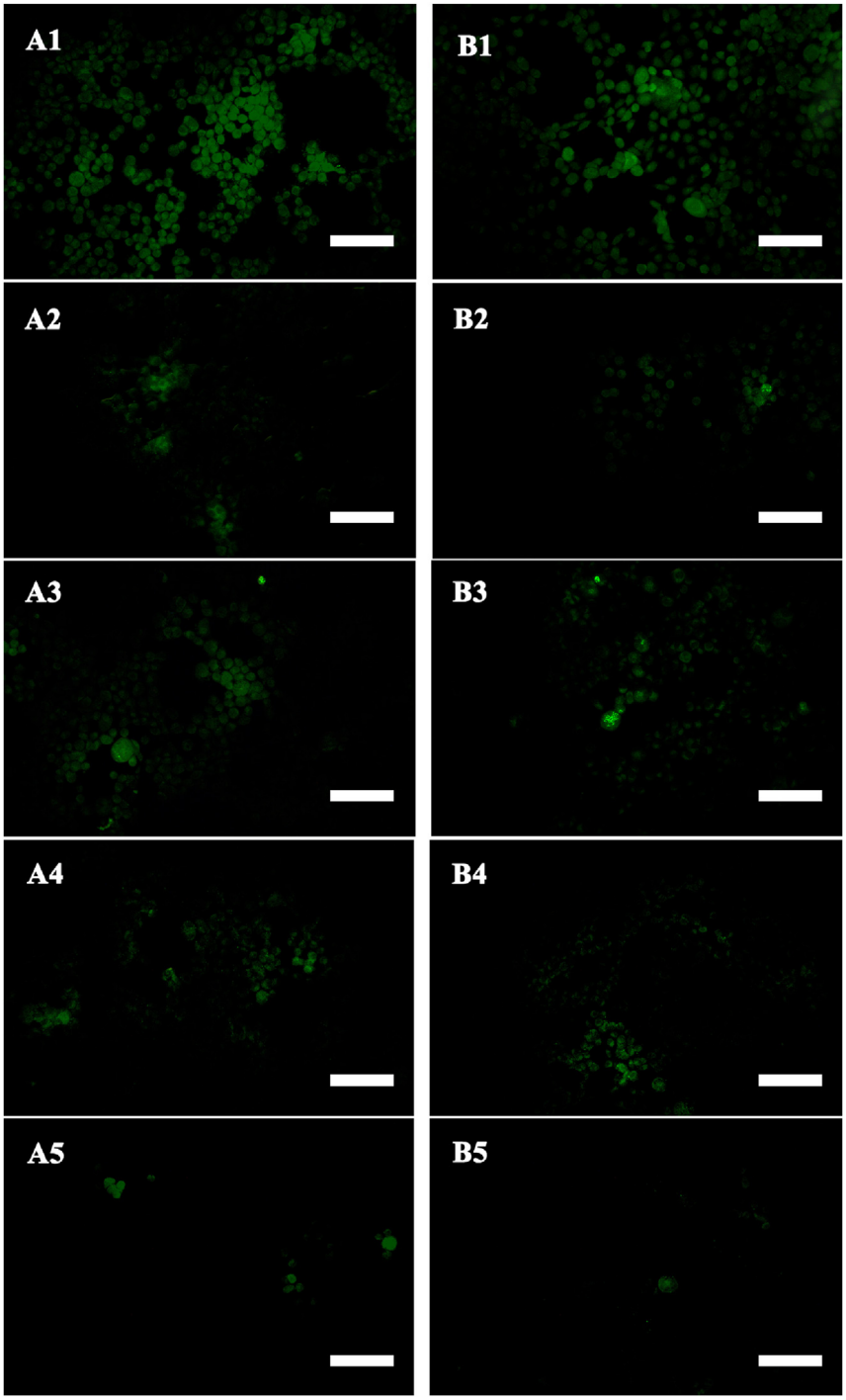

Figure 3. ROS levels in FCA-L2 cells treated with organic extracts or water-soluble extracts with VE, lycopene and EPA: DHA under fluorescence microscopy. A1-A5 represent organic extract treatment combinations: (A1) positive control, cells treated with organic extracts alone. (A2) VE (10 μM) + organic extracts (160 μg/mL). (A3) Lycopene (20 μM) + organic extracts (160 μg/mL). (A4) EPA: DHA (2:1; 20 μM) + organic extracts (160 μg/mL). (A5) Negative control, cells without any treatment. B1–B5 represent water-soluble treatment combinations: (B1) positive control, cells treated with water-soluble extracts alone. (B2) VE (10 μM) + water-soluble extracts (80 μg/mL). (B3) Lycopene (20 μM) + water-soluble extracts (80 μg/mL). (B4) EPA: DHA (2:1; 20 μM) + water-soluble extracts (80 μg/mL). (B5) Negative control, cells without any treatment. The scale bar for all groups is 100 μm.

3.6. EPA: DHA pretreatment increased antioxidant capacity of APM-exposed cells

The activity of antioxidant enzymes, including SOD, CAT, and GPH-Px, was measured to assess the protective effect of EPA: DHA pretreatment on cells exposed to APM extracts. The activity of SOD (Figure 5A; $P < 0.01$), CAT (Figure 5B; $P < 0.01$), and GPH-Px (Figure 5C; $P < 0.01$) was significantly reduced in cells exposed to APM organic extracts compared to cells in the negative control group. However, the activity of SOD (Figure 5A; 5 μM, $P < 0.01$; 10 μM, $P < 0.01$; 20 μM, $P < 0.01$), CAT (Figure 5B; 5 μM, $P < 0.01$; 10 μM, $P < 0.01$; 20 μM, $P < 0.01$), and GPH-Px (Figure 5C; 5 μM, $P < 0.01$; 10 μM, $P < 0.01$; 20 μM, $P < 0.01$) was significantly increased in cells pretreated with EPA: DHA compared to cells exposed to APM organic extracts alone. Similarly, exposure to APM water-soluble extracts remarkably led to reduced activity of SOD (Figure 6A; $P < 0.01$), CAT (Figure 6B; $P < 0.01$), and GPH-Px (Figure 6C; $P < 0.01$) compared to cells in the negative control; in contrast, pretreatment with EPA: DHA significantly increased the activity of these antioxidant enzymes in FCA-L2 cells ($P < 0.05$). Collectively, these results suggest that EPA: DHA increases the activity of antioxidant enzymes to respond to APM-induced oxidative stress.

4. Discussion

APM, which adsorbs large amounts of toxic chemicals, is a significant risk factor for developing respiratory disease (Jalava et al., 2015). Upon

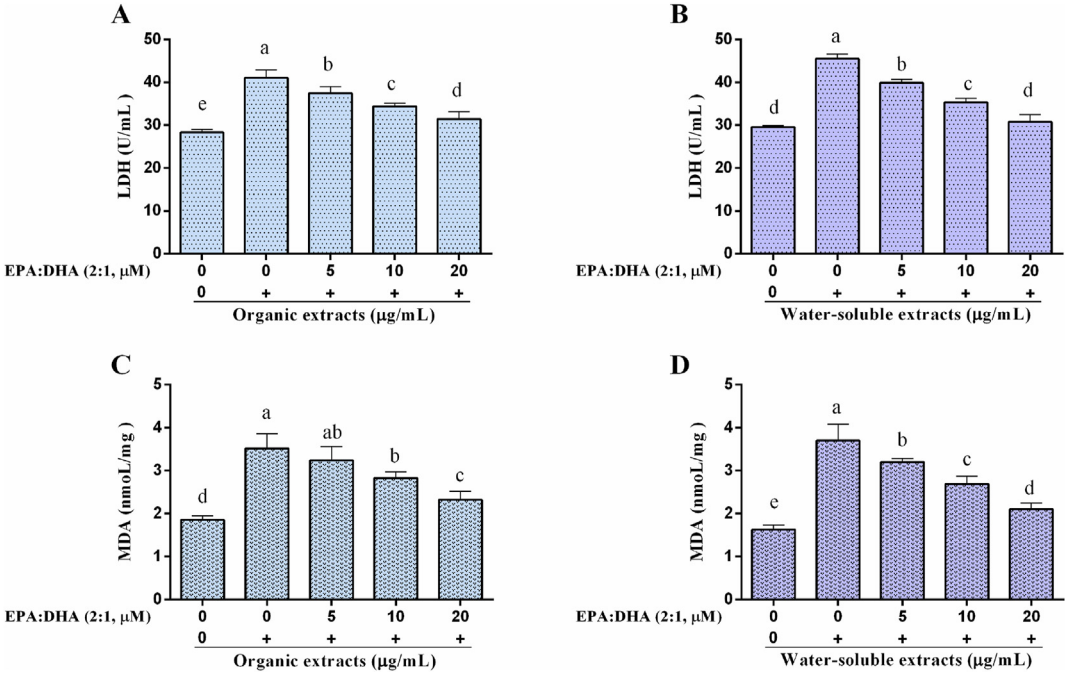

Figure 4. LDH release from culture supernatants of FCA-L2 cells pretreated with different concentrations of DHA: EPA (1:2; 0, 5, 10, 20 μM) followed by exposure to (A) organic extracts and (B) water-soluble extracts. MDA content of FCA-L2 cells pretreated with different concentrations of DHA: EPA (1:2; 0, 5, 10, 20 μM) and subsequently exposed to (C) organic extracts and (D) water-soluble extracts. All data are presented as the mean ± SD (n = 3). Different letters (a, b, c, d) indicate significant differences between each treatment group, $P < 0.05$.

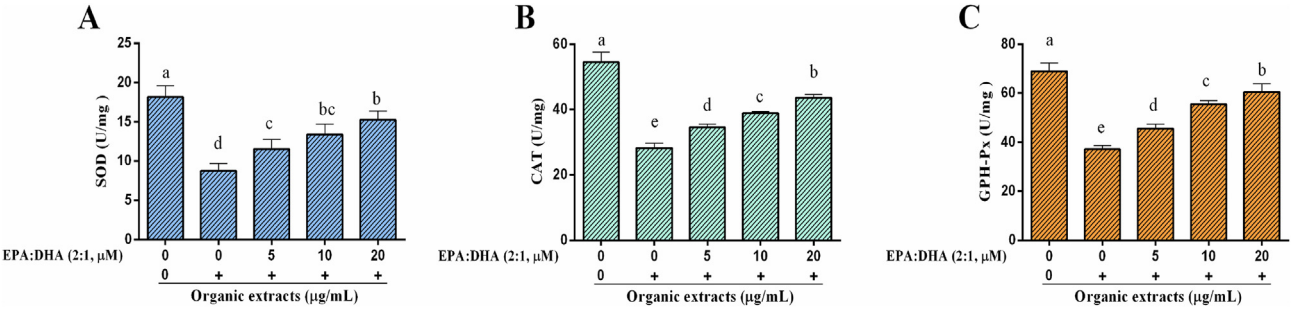

Figure 5. Activity of (A) SOD, (B) CAT and (C) GPH-Px in FCA-L2 cells pretreated with different concentrations of DHA: EPA (1:2; 0, 5, 10, 20 μM) followed by exposure to organic extracts. All data are presented as the mean ± SD (n = 3). Different letters (a, b, c, d, e) indicate significant differences between each treatment group, $P < 0.05$.

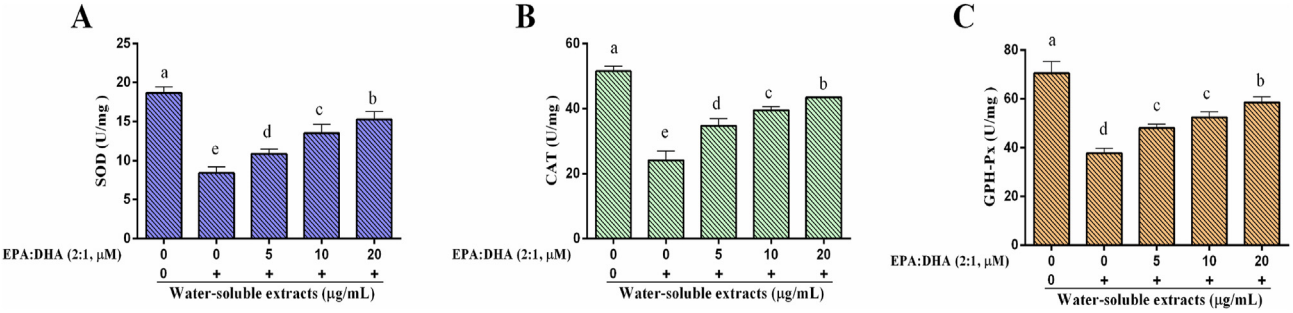

Figure 6. Activity of (A) SOD, (B) CAT and (C) GPH-Px in FCA-L2 cells pretreated with different concentrations of DHA: EPA (1:2; 0, 5, 10, 20 μM) followed by exposure to water-soluble extracts. All data are presented as the mean ± SD (n = 3). Different letters (a, b, c, d, e) indicate significant differences between each treatment group, $P < 0.05$.

inhalation, most of the water-soluble components of APM are rapidly released into lung tissue, with the remaining water-insoluble components subsequently triggering an intracellular signaling cascade (Cachon et al., 2014; Deng et al., 2013; Gualtieri et al., 2009).

In this study, organic and water-soluble extracts of APM induced oxidative stress injury in feline FCA-L2 cells. VE, lycopene and EPA: DHA effectively ameliorated the cytotoxicity induced by APM extracts, as evidenced by improved cell morphology and enhanced cell viability.

The main trigger mechanism of oxidative stress damage in APM extracts-induced FCA-L2 cells is the overproduction of ROS. Our results also demonstrated that APM extract treatment of cells resulted in high expression of ROS. ROS mainly include free radicals (e.g. superoxide anion radical, hydroxyl radical) and reactive non-radicals (e.g. singlet oxygen and hydrogen peroxide). The production of ROS disrupts the balance of pro-oxidant-antioxidant reactions, which leads to oxidative stress (Cga et al., 2022). The excessive production of ROS was attributed to both exogenous and endogenous components. On the one hand, the organic and water-soluble components of APM are responsible for the excessive production of exogenous ROS. In the present study, a variety of PAHs were found in the organic extracts of APM, including fluoranthene, chrysene, pyrene, benzo[*ghi*] perylene, phenanthrene, and benzo[*e*] pyrene at high concentrations (Zeisler et al., 2020). The inorganic extract of APM contained relatively high levels of inorganic elements (Fe, Pb, Zn, Mn and Cu) produced by anthropogenic activities, in addition to crustal elements such as Ca, Al, K, Mg, Na and Ti (Zeisler et al., 2020). These compounds and heavy metals may be the main factors for the high toxicity and induction of ROS in APM. These results are consistent with previous reports (Billet et al., 2008; Deng et al., 2013). Numerous studies have found that PAHs and metals (such as As, Pb, Fe, and Al) in APM are directly related to ROS generation capacity (Deng et al., 2013; Gualtieri et al., 2009). These pro-oxidant compounds are the main factors leading to oxidative stress in APM-exposed cells (Deng et al., 2013). Soluble chemical constituents in APM water-soluble extracts are found as smaller ions, which can rapidly be absorbed into the cells and trigger early ROS generation (Bolling et al., 2009; Gualtieri et al., 2009). Zou et al. (2016) showed that water-soluble components of APM triggered ROS production earlier (at 2, 4 and 6 h), whereas water-insoluble fractions triggered late ROS production. On the other hand, the excessive production of ROS is attributed to an imbalance in endogenous ROS metabolism, as evidenced by mitochondrial damage, elevated MDA and reduced antioxidant enzyme activity. Endogenous ROS production occurs mainly in mitochondria, microsomes, peroxisomes, or cytochrome P450 (Joanna and Květoslava, 2014). Excessive ROS production leads to DNA mutations, lipid peroxidation, protein damage and cell cycle dysregulation (Feng et al., 2016). Lipids are the main components of cell membranes, therefore lipid peroxidation can lead to increased MDA levels and cell membrane damage.

Direct antioxidants are small molecules with redox activity, such as glutathione and vitamin C. They are depleted or modified during the ROS scavenging process and need to be resupplied or restored. Indirect antioxidants act by inducing regulation of endogenous direct antioxidant synthesis and cytoprotective genes, and by influencing the metabolism and elimination of exogenous substances (Tebay et al., 2015). In the present study, VE, lycopene and EPA: DHA had a protective effect against APM-induced oxidative stress damage in FCA-L2 cells. The possible mechanism is partly attributed to the scavenging effect of their own antioxidant activity on ROS. VE is considered to be a highly effective antioxidant in hydrophobic environments (Ricciarelli et al., 2001), blocking free radical-mediated reactions by scavenging lipid peroxyl radicals and thus inhibiting lipid peroxidation (Ham and Liebler, 1995). Lycopene is a known effective scavenger of monoclinic oxygen and peroxyl radicals with anti-inflammatory, anti-autophagic and anti-apoptotic activities (Joanna and Květoslava, 2014; Krinsky and Johnson, 2006). In addition, omega-3 PUFAs, a component of cell membranes, has been shown to exert antioxidant and anti-inflammatory effects through direct modulation of ion channels or G protein-coupled receptors (GPCR) (Mollace et al., 2013; Sundaram et al., 2020). Second, its possible protective mechanism is the enhancement of antioxidant enzyme activity to reduce oxidative stress. This may be related to the regulation of Nrf2 pathway. Studies have shown that the transcription factor Nrf2 pathway controls the induced expression of genes during redox perturbations and is an important cellular defense mechanism against oxidative stress and inflammation (Tebay et al., 2015). Genes related to certain redox enzymes (e.g. CAT and GPH-Px2) have been shown to be regulated by the Nrf2 pathway (Tebay et al., 2015). As previously reported, EPA and DHA increased the expression of Nrf2 and the activity of various key enzymes regulated by Nrf2 in vascular endothelial cells (Anderson et al., 2012). In the present study, EPA: DHA was shown to enhance cellular CAT, GPH-Px and SOD antioxidant enzyme activities, although we had no direct evidence of Nrf2 relevance. Third, the protective mechanism of VE, lycopene and EPA: DHA may also be related to the inhibition of NF-κB pathway-related inflammatory responses and the regulation of apoptosis (Li et al., 2019; Przybylska, 2020; Sun et al., 2019). For example, VE and omega-3 PUFAs significantly reduced $PM_{2.5}$-induced inflammatory factor expression and oxidative stress in vascular endothelial cells through the NF-κB signaling pathway (Bo et al., 2016). Lycopene has been reported to significantly increase manganese SOD expression and decrease ROS in human mesenchymal stem cells by inhibiting the apoptosis-related pathway (PI3K-Akt pathway) (Ji et al., 2015). In addition, we speculate that the protective effects of nutrients may also be related to cellular autophagy. Excess ROS has been reported to trigger cellular autophagy in $PM_{2.5}$-induced human lung epithelial cells via Atg4, catalase, and mitochondrial electron transport chain pathways (Azad et al., 2009; Deng et al., 2013), and nutrients may directly or indirectly affect this cellular autophagic response.

Overall, this study demonstrates that nutritional intervention based on antioxidant substances such as VE, lycopene, and omega-3 PUFAs can effectively alleviate cellular oxidative stress by enhancing antioxidant enzyme activity pathway in APM extract-induced lung cell damage in cats. This study provides ideas and opportunities for further research on the nature of nutrient activity and molecular mechanism of action.

5. Conclusions

In conclusion, APM extracts had significant cytotoxic effects on the feline lung FCA-L2 cells, which is likely to trigger respiratory disease in felines. The cytotoxicity of the water-soluble extracts was more significant compared to the organic extracts in APM. We demonstrate that treatment with VE, lycopene, and omega-3 PUFAs alleviated damage caused by oxidative stress in a cat lung cell model following exposure to APM. This study provides the basis for further studies on the mechanism underlying the protective effect of VE, lycopene and omega-3 PUFAs against APM damage. Furthermore, this study offers new insights into the development of antioxidants-based nutritional interventions for preventing and/or neutralizing the adverse effects of environmental APM pollution.

Declarations

Author contribution statement

Huasong Bai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ying Wang: Performed the experiments.

Peng Wu: Contributed reagents, materials, analysis tools or data.

Zhanzhong Wang: Conceived and designed the experiments; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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