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Quantification of F_2-isoprostane isomers in cultured human lung epithelial cells after silica oxide and metal oxide nanoparticle treatment by liquid chromatography/tandem mass spectrometry

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ABSTRACT

F_2-isoprostanes are lipid peroxidation products of arachidonic acid in cell membrane and are reliable biomarkers for oxidative stress and cell membrane damage. Nanomaterials are widely used as raw materials in many industries and will have high potentials to be used in life science and medical fields. However, the human health impact of nanoparticles has caused people's great concern. Unfortunately, the mechanisms of cytotoxicity of many nanoparticles are not well defined. By measuring the levels of F_2-isoprostane isomers in cultured cells after nanoparticle exposure, the information can be used to explain whether the cytotoxicity of nanoparticles is caused by lipid peroxidation and to investigate the biological pathways. In this study, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to separate and quantify F_2-isoprostane isomers in nanoparticle-treated human lung cancer cells. Silica oxide (15 nm) and other four metal oxide nanoparticles including Fe_2O_3 (30 nm), Al_2O_3 (13 nm), TiO_2 (40 nm) and ZnO (70 nm) are chosen in this study. The isotope forms of F_2-isoprostane isomers, 8-iso-$PGF_{2\alpha}$-d_4 and $PGF_{2\alpha}$-d_4, were used as internal standard (IS). After human lung epithelial cells were exposed to different nanoparticles for 24 h, F_2-isoprostanes were extracted by a single step solid phase extraction with Oasis HLB cartridge. For the first time, six F_2-isoprostane isomers were tentatively identified and quantified in human lung epithelial cells. The levels of F_2-isoprostane isomers in the cells increased after the treatment with nanoparticles. For SiO_2, Fe_2O_3, and ZnO nanoparticles, F_2-isoprostane isomers increasing are consistent with nanoparticles' cytotoxicity data. For Al_2O_3 and TiO_2 nanoparticles, F_2-isoprostane isomers levels increased even before nanoparticles showed significant cytotoxicity at 100 μg/mL concentration in 24 h. Based on our best knowledge, this is the first study on the F_2-isoprostane isomers corresponding to nanoparticles' exposure *in vitro*. Our study demonstrates that SiO_2 (15 nm) nanoparticle showed the highest degree of lipid peroxidation and cell membrane damage among the studied nanoparticles.

1. Introduction

Nanotechnology has been developed for several decades depending on nanoparticles as its raw materials. The key features of nanoparticles are small particle size and large specific surface area [1]. Because of these special properties, nanoparticles have wide applications in catalysts, semiconductors, additives, and cosmetics [2–4]. Moreover, many researchers have used nanoparticles in biosensor, drug delivery and clinical treatment, especially for cancer treatment [5–9]. Recently, nanotoxicity, which means the toxicity was caused by nanoparticles, has caused people's great concern [1,10,11]. Many studies, including our previous studies [12–17], have shown that nanoparticles are cytotoxic and can cause oxidative stress and DNA damage *in vitro* [18–20]. Our previous study reported that nanoparticles can cause lipid peroxidation and cell membrane damage by measuring total F_2-isoprostane levels as biomarkers [21].

F_2-isoprostanes are a series of compounds formed from free radical initiated lipid peroxidation of arachidonic acid in the cell membrane. They are reliable biomarkers for oxidative stress and lipid peroxidation. The proposed mechanism is that free radicals such as hydroxyl radicals can attack C_7, C_{10} and C_{13} three different sites of arachidonic acid carboxyl chain and abstract an allylic hydrogen. This process results in a delocalized pentadienyl carbon-centered radical. Subsequently, an oxygen molecule is inserted and a peroxyl radical is formed. These peroxyl radicals undergo further cyclization, followed by the addition of another oxygen molecule

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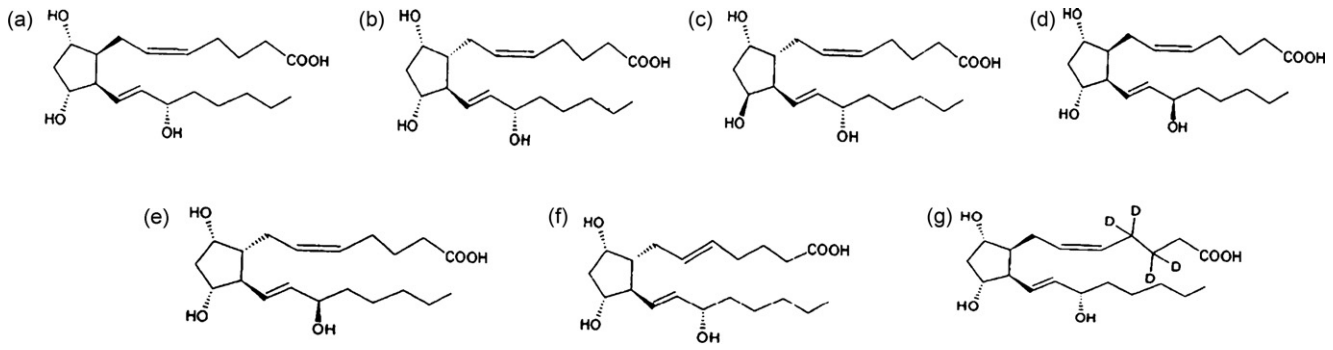

Fig. 1. Structures of F_2-isoprostane regioisomers. (a) 8-Iso-$PGF_{2\alpha}$, (b) $PGF_{2\alpha}$, (c) 11β-$PGF_{2\alpha}$, (d) 8-iso-15R-$PGF_{2\alpha}$, (e) 15R-$PGF_{2\alpha}$, (f) 5-trans-$PGF_{2\alpha}$, and (g) 8-iso-$PGF_{2\alpha}$-d_4.

to yield bicyclic endoperoxide molecules. These intermediates are then reduced to F_2-isoprostanes, named because they possess F-type prostane rings. In theory, four series (5-, 8-, 12-, 15-series) and 64 regioisomers of peroxidation products can be formed [22]. F_2-isoprostanes are formed in situ as esterified form in phospholipids of cell membrane. The enzyme named phospholipase catalyzes the esterified F_2-isoprostanes to free F_2-isoprostanes and free F_2-isoprostanes are released to biological fluids such as plasma and urine. In normal people, urinary F_2-isoprostanes concentrations were 0.2–1.5 μg/g creatinine [23]. It is important to study the isomers of F_2-isoprostanes corresponding to oxidative injury because each isomer may have different biological functions. Moreover, it can help researchers to study the pathway of F_2-isoprostanes formation in more detail. Among these isomers, 8-iso-$PGF_{2\alpha}$ has been widely studied and was found to possess some biological functions like nonspecific vasoconstriction, brochoconstriction, and modulation of platelet function [24–26]. But other isomer's biological functions are not clear yet.

Gas chromatography/mass spectrometry (GC/MS) method has been used for measuring F_2-isoprostanes. Even though the method is sensitive, the procedure is very tedious and time-consuming. It required multi-steps of solid phase extraction, thin layer chromatography, and derivatization reactions [27,28]. Enzyme immunoassay (ELISA) is commercial available. But it can cause cross-linking reaction, making the data not reliable. Moreover, each assay kit can only measure one isomer of F_2-isoprostanes, making the assay not efficient as well. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been proven to be sensitive and selective for simultaneously measurement of different isomers of F_2-isoprostanes without derivatizations in urine, plasma, and cell culture matrix [29–31]. In our previous study, total free F_2-isoprostanes were determinate by LC–MS method after the treatment of selected nanoparticles in human lung epithelial cells [21]. However, the changes of each F_2-isoprostane isomer after nanoparticle treatments were not investigated. In this study, a one-cartridge solid phase extraction method using oasis HLB cartridge was further improved to efficiently extract F_2-isoprostanes from complicated cell culture matrix, and a LC–MS/MS method was developed to separate and quantify six F_2-isoprostane isomers in human lung epithelial cells after silica oxide and metal oxide nanoparticle exposure.

In this paper, five different nanoparticles were chosen for the study because they are more abundant in the environment and widely used in the industry. These nanoparticles include SiO_2 (15 nm), Fe_2O_3 (30 nm), Al_2O_3 (13 nm), TiO_2 (40 nm) and ZnO (70 nm). A549 human lung epithelial cell line (CCL-185, ATCC number) was used to study the exposure to nanoparticles because lung is the major organ affected when nanoparticles are inhaled. Six 15-series F_2-isoprostane isomers were tentatively determined in the cell culture after cellular exposure to the nanoparticles. The molecular structures of these isomers are shown in Fig. 1. The levels of F_2-isoprostane isomers were quantified by LC–MS/MS method after exposure to each type of selected nanoparticles. The goal of this study was to explore and compare the response of different isomers and the extents of lipid peroxidation corresponding to nanoparticle treatment. This information can be very useful for revealing the mechanism of nanoparticle cytotoxicity and discover the biological functions of F_2-isoprostane isomers.

2. Experimental

2.1. Chemicals

Authentic standards of 8-iso-$PGF_{2\alpha}$, 8-iso-15R-$PGF_{2\alpha}$, 5-trans-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 15R-$PGF_{2\alpha}$, $PGF_{2\alpha}$ and internal standards of 8-iso-$PGF_{2\alpha}$-d_4, $PGF_{2\alpha}$-d_4 were purchased from Cayman Chemical (Ann Arbor, MI, USA). HPLC/MS grade acetonitrile, methanol, HPLC grade hexane, ethyl acetate, and 2-propanol were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Ham's F-12 medium with L-glutamine was purchased from Fisher Scientific (Pittsburgh, PA). Trypsin–EDTA (1×) was purchased from Invitrogen (Carlsbad, CA). HPLC/MS grade formic acid, penicillin–streptomycin, and citrate acid were obtained from Sigma–Aldrich (St. Louis, MO). Ultra pure deionized water was obtained from an Elix Milli-Q system (Millipore, Bedford, MA). All pH measurements were performed using an Accumet XL15 meter (Fisher Scientific).

2.2. Nanoparticles

The SiO_2 nanoparticle was purchased from Degussa Co. (Parsippany, NJ). The Fe_2O_3 nanoparticle was purchased from Nanophase Technologies (Romeoville, IL). The ZnO nanoparticle was purchased from Sigma–Aldrich (St. Louis, MO). The TiO_2 nanoparticle was purchased from NanoScale Corporation (Manhattan, KS). The Al_2O_3 nanoparticle was synthesized by the room temperature homogeneous nucleation method [32]. Transmission electron microscopy (TEM; Philips EM420) was used to measure particle size and distribution. The particle sizes and distributions of these nanoparticles were listed in Table 1. Characterization of nanoparticle has been studied previously from our group [18,19]. Nanoparticles tend to

Table 1
Nanoparticles size and distribution.

Nanoparticle	Size and distribution (mean ± std) (nm)
SiO_2	15 ± 5
Fe_2O_3	30 ± 5
Al_2O_3	13 ± 2
TiO_2	40 ± 8
ZnO	70 ± 13

aggregate in the solution over time. Therefore, nanoparticles were prepared freshly each time and dispersed by sonication for 15 min. Nanoparticle suspensions were applied to the cells immediately.

2.3. Assessment of nanoparticle cytotoxicity

The cells were treated with various dosages of nanoparticles. After exposure for 24–48 h, cell viability was measured by MTS assay (from Promega). The MTS assay was conducted as follows: under yellow light, 100 μL of PMS (phenazine methosulfate) solution was added to 2.0 mL of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution immediately. 20 μL of the combined MTS/PMS solution was added into each well of a 96-well plate. Each well contained 100 μL of cells medium with 4000 cells. The plate was then incubated for 2 h at 37 °C in a humidified, 5% CO_2 incubator. Absorbance at 490 nm was recorded using a plate reader (FLUOstar OPTIMA).

2.4. Cell culture and treatment with nanoparticles

The human lung epithelial cell line (A549) was purchased from ATCC (Manassas, VA). This cell line was chosen because it is a typical cell line that has widely been used for *in vitro* cytotoxicity study [18,19]. The cells were grown in T-75 cell culture flasks filled with Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37 °C in a 5% CO_2 humidified incubator, and were split every 2–3 days.

For the nanoparticle treatment experiment, cells were seeded in a 65 × 15 mm cell culture Petri dish at a density of 6×10^5 cells/mL cell medium. Cells were allowed to attach to the Petri dish for 24 h. Nanoparticle suspensions in the cell medium were freshly prepared each time. Appropriate nanoparticles were weighed and added to the cell medium to make the stock solution. The suspensions were vortex and sonicated for 15 min. The stock solution was diluted for several steps to get the working solution; after each dilution the suspensions were vortex and sonicated for 15 min. Therefore the nanoparticles were evenly dispersed in the cell medium. The old cell medium was removed from the Petri dish, and the freshly prepared cell medium with nanoparticles was added to the cells. Cells filled with the culture medium without nanoparticles were served as the control. Cells were treated with nanoparticles for 24–48 h. After that, cell medium was collected. Antioxidant 1% 1N citrate acid was added to the cell medium. The samples were stored in −80 °C freezer immediately until analysis.

2.5. Solid phase extraction of F_2-isoprostanes

Free F_2-isoprostanes in the cell medium were extracted by Oasis HLB extraction cartridge (Waters, Milford, MA). 2 ng of internal standard 8-iso-$PGF_{2\alpha}$-d_4, $PGF_{2\alpha}$-d_4 was added to each sample. Then 3 mL of 2% formic acid was added to the samples to adjust the pH to 3. The cartridges were conditioned and equilibrated with methanol and Milli Q water. The samples were applied to the cartridges. The cartridges were washed with 5% methanol. Then, the cartridges were washed with 5% methanol and 2% ammonia hydroxide. The cartridges were further washed with 5% methanol and 2% formic acid. Finally, the cartridges were washed with 15% methanol and 2% formic acid. F_2-isoprostanes were eluted with hexane/ethyl acetate/2-propanol (30/65/5) solution. The solvent was evaporated to dry by a stream of nitrogen gas. The residue was dissolved in 200 μL of 0.1% formic acid in 50/50 Milli Q water/methanol, filtered through a 0.22 μm filter and was ready for LC–MS/MS analysis.

2.6. Instrumentation

The HPLC system was Agilent 1100 series HPLC which included G1322A vacuum degasser, G1311A binary pump, G1329A autosampler and G1330B thermostatic column compartment. The column was Synergi Hydro-RP 80 Å, 250 × 2.0 mm, 4 μm column with C-18 guard column (Phenomenex, Torrance, CA). The column temperature was controlled at 25 °C. The mobile phase A was 0.1% formic acid in Milli Q water, mobile phase B was methanol. The flow rate was 150 μL/min and the injection volume was 40 μL. The gradient was 0–14 min 70% methanol; 14–19 min 70–95% methanol; 19–21 min 95–70% methanol; then the column was re-equilibrated to the starting condition.

The mass spectrometer was 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA). The ion source was TurboIon Spray (TIS). The scan mode was multiple reaction monitoring (MRM) and the polarity was negative. Source-dependent parameters: Gas 1, Gas 2, Source temperature, Curtain gas, and Ion spray voltage were set at 40 psi, 60 psi, 450 °C, 10 psi, and −4500 V, respectively. Compound-dependent parameters: declustering potential (DP), entrance potential (EP), collision cell exit potential (CXP), collision energy (CE) were set at −86, −15, −11, and −36 V, respectively. Deprotonated molecular ion $[M-H]^-$ was selected at the first quadrupole (Q1) for standard 8-iso-$PGF_{2\alpha}$ (m/z = 353) and internal standard (IS) 8-iso-$PGF_{2\alpha}$-d_4 (m/z = 357). MRM transition ion pair 353/193 was chosen for quantification. Ion pair 353/309 was the second abundant product ion, but it was not a specific ion pair for F_2-isoprostanes. For IS 8-iso-$PGF_{2\alpha}$-d_4, ion pair 357/197 was chosen for quantification. The quantification was based on the peak area ratio of the standard 8-iso-$PGF_{2\alpha}$ MRM transition ion pair (353/193) to IS 8-iso-$PGF_{2\alpha}$-d_4 MRM transition ion pair (357/197). The calibration curve linear range was 0.1–50 ng/mL ($y = 0.6008x - 0.0966$, $r^2 = 0.9997$). The limit of detection (LOD) was 0.05 ng/mL and the LOQ the limit of quantification (LOQ) was 0.1 ng/mL. Analyst 1.4.2 software (Applied Biosystems) was used to control the LC–MS/MS instrument and process the data.

2.7. Statistics

Data were expressed as the mean ± SD of three experiments. Student's *t*-test was used for significance testing, using a p value of 0.05.

3. Results and discussion

3.1. Nanoparticle cytotoxicity study

A549 cells were exposed to each of silica oxide and metal oxide nanoparticles for 24–48 h. Cells were treated with SiO_2 (15 nm) nanoparticle for 48 h. The dosage levels were 5, 10, 25, 50, 75, and 100 μg/mL, with cell viability of 99.0%, 98.3%, 90.4%, 88.2%, 83.9%, and 78.3%, respectively, compared with the control cells. Among these, cell viability decreased significantly with dosage levels of 75 and 100 μg/mL, respectively, compared with the control group ($p < 0.05$) [21]. After cells were exposed to Fe_2O_3 (30 nm) nanoparticle for 48 h at the dosage levels of 30, 40, 50, 80 and 100 μg/mL, cell viability decreased to 93.3%, 88.9%, 83.5%, 80.5%, and 77.2%, respectively, compared with the control cells. At dosage levels of 80 and 100 μg/mL, cell viability decreased significantly. ($p < 0.05$) [21]. ZnO nanoparticle was the most toxic among the five nanoparticles. After cells were exposed to ZnO (70 nm) nanoparticle for only 24 h, the cell viability decreased to 73.4%, 34.1% and 25.9%, respectively, at the dosage levels of 12, 14, and 16 μg/mL ($p < 0.05$) [20]. Al_2O_3 (13 nm) nanoparticle did not show significant cytotoxicity after 48 h exposure at the dosage levels of 5, 10, 25, 50, 75, and

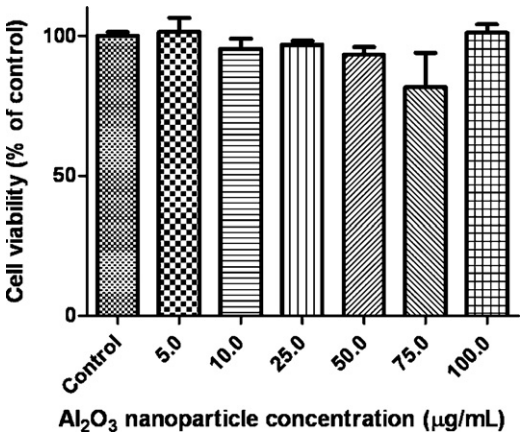

Fig. 2. A549 cell viability after 48 h exposure to 5, 10, 25, 50, 75, and 100 μg/mL of 13 nm Al_2O_3 nanoparticle. Values are mean ± SD ($n = 3$). $p > 0.05$.

100 μg/mL (Fig. 2). TiO_2 (40 nm) nanoparticle also did not show significant cytotoxicity at the dosage levels of 1, 10, 50, and 100 μg/mL after 48 h exposure (Fig. 3). The cell viability data demonstrated different degrees of cytotoxicity for SiO_2 (15 nm), Fe_2O_3 (30 nm) and ZnO (70 nm) nanoparticles, while Al_2O_3 (13 nm) and TiO_2 (40 nm) did not show significant cytotoxicity to the cells after 24–48 h exposure.

3.2. F_2-isoprostane isomers separation and quantification by LC–MS/MS

Six isomers were chosen in this study because of the commercial availability of these standards. The separation of F_2-isoprostane isomers using LC is challenging. These regioisomers have very close polarity and they are difficult to be separated on a LC column. To achieve the separation, different columns have been tested, and Synergi Hydro-RP 250 × 2 mm, 4 μm column (from Phenomenex) provided the best separation. The six isomers were separated between 8 and 13 min (as shown in Fig. 4).

Different mobile phases have also been studied and optimized. Since F_2-isoprostanes were easily deprotonated and formed negative ions, 5 mM ammonium formate in water and 5 mM ammonium formate in methanol was used at the beginning. The F_2-isoprostane isomers can be well separated, but the sensitivity was very low (data not shown). When the mobile phases were changed to 0.1% formic acid in water and methanol, the compounds gained 10 times

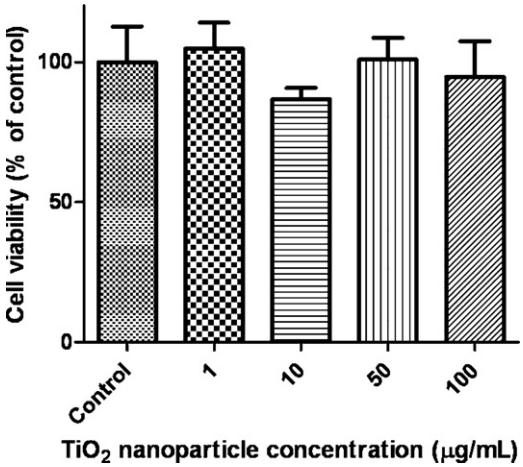

Fig. 3. A549 cell viability after 48 h exposure to 1, 10, 50, and 100 μg/mL of 40 nm TiO_2 nanoparticle. Values are mean ± SD ($n = 3$). $p > 0.05$.

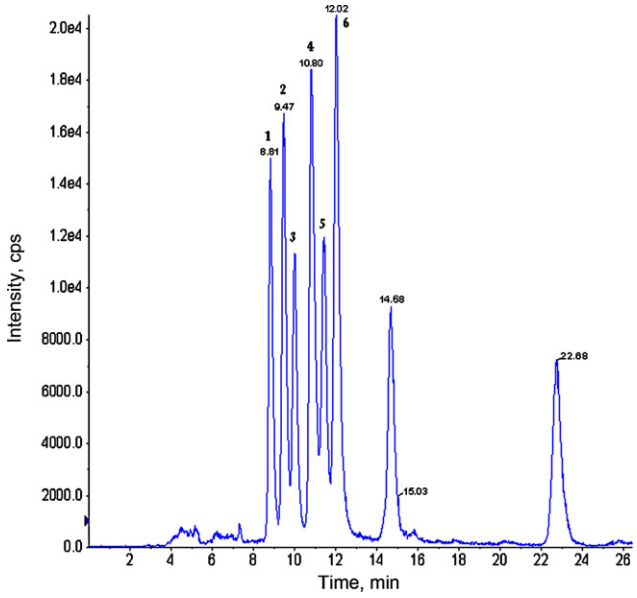

Fig. 4. The separation of F_2-isoprostane isomers. (1) 8-iso-15R-$PGF_{2\alpha}$, (2) 8-iso-$PGF_{2\alpha}$, (3) 11β-$PGF_{2\alpha}$, (4) 15R-$PGF_{2\alpha}$, and (5) 5-trans-$PGF_{2\alpha}$, (6) $PGF_{2\alpha}$.

sensitivity compared with 5 mM ammonium formate as buffer. It was also discovered that different organic solvents affected the F_2-isoprostanes detection sensitivity significantly. Methanol provided higher sensitivity than acetonitrile. Therefore, 0.1% formic acid in water and methanol were chosen for the mobile phase through the entire study.

MRM transition ion pair 353/193 was monitored for the isomers and ion pair 357/197 was monitored for the IS. These are consistent with the literature reports [22,23]. Extracted ion chromatograms of standard 8-iso-$PGF_{2\alpha}$ and IS were shown in Fig. 5. The retention times for both the standard 8-iso-$PGF_{2\alpha}$ and IS were 9.12 min.

3.3. Sample preparation

Sample preparation is a crucial step in the determination of F_2-isoprostanes because cell medium is a complicated biological matrix and F_2-isoprostane isomers are at trace levels in the cell. Free

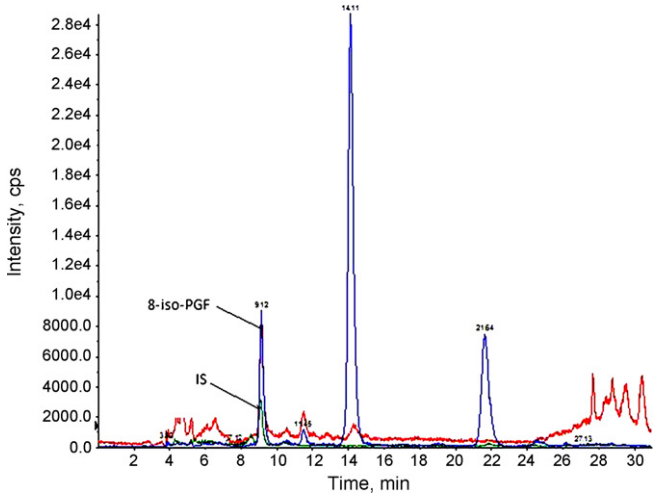

Fig. 5. Extracted ion chromatogram of standard 8-iso-$PGF_{2\alpha}$ and IS 8-iso-$PGF_{2\alpha}$-d_4. The retention time for the standard 8-iso-$PGF_{2\alpha}$ and IS is 9.12 min. The blue trace is MRM ion pair 353/193. The green trace is MRM ion pair 357/197. The red trace is MRM ion pair 353/309. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

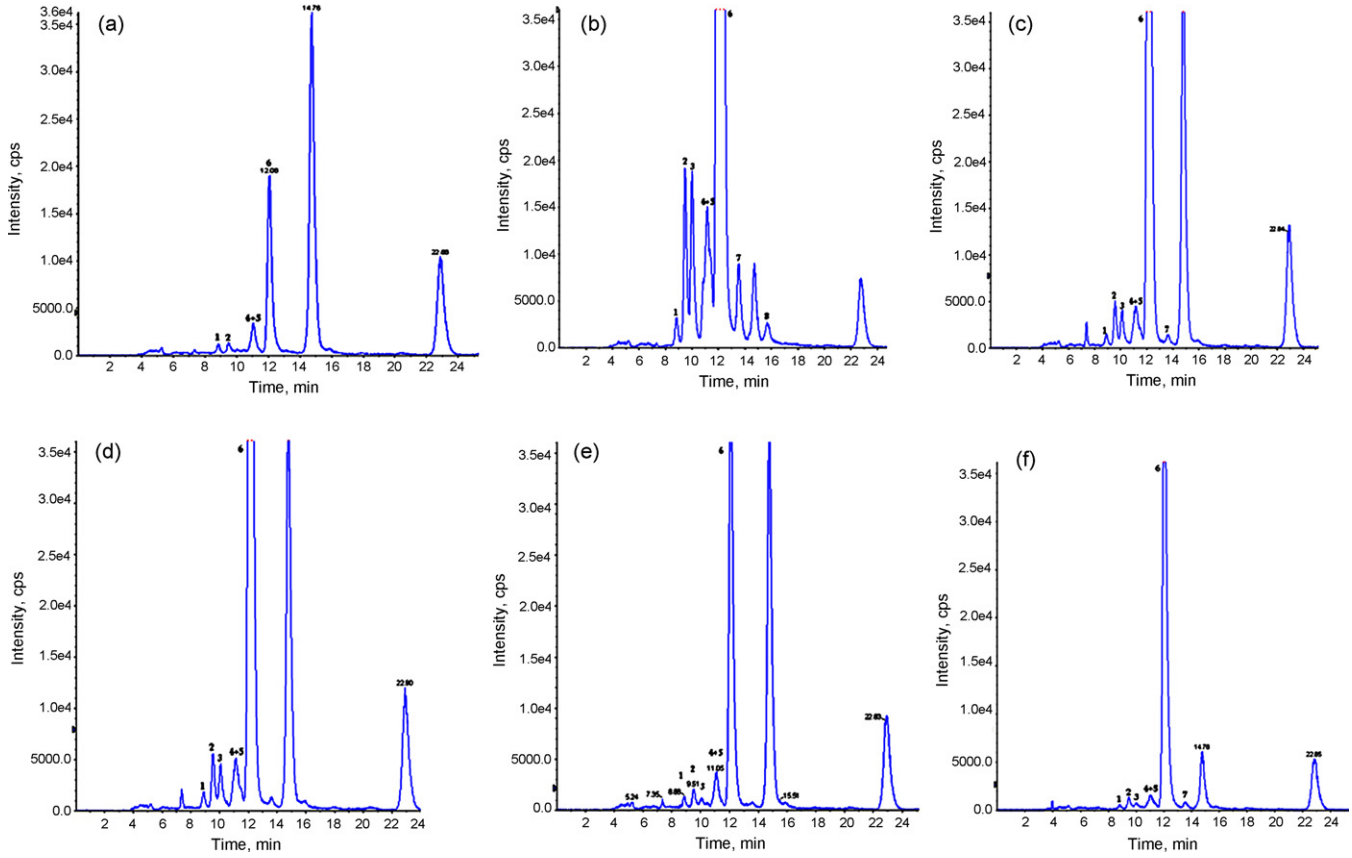

Fig. 6. The extracted ion chromatograms of F_2-isoprostane isomers in (a) the control, (b) SiO_2 (50 μg/mL), (c) Al_2O_3 (100 μg/mL), (d) TiO_2 (100 μg/mL), (e) Fe_2O_3 (100 μg/mL) and (f) ZnO (15 μg/mL) nanoparticle-treated cells. Peaks (1) 8-iso-15R-$PGF_{2\alpha}$, (2) 8-iso-$PGF_{2\alpha}$, (3) 11β-$PGF_{2\alpha}$, (4) + (5) 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$, (6) $PGF_{2\alpha}$, (7) Unknown 1, and (8) Unknown 2.

F_2-isoprostanes are released into the cell medium by the enzyme named as phospholipase. F_2-isoprostanes are extracted by solid phase extraction from the cell culture medium. At first, C18 cartridge (Waters, Milford, MA) was used to extract F_2-isoprostanes, but the recovery was only at about 20%. Oasis HLB cartridge was then used for sample extraction. After sample was loaded on the cartridge, consecutive washing with methanol, acidified water and basic water cleaned up any salted, acidic and basic compounds in the sample. At low concentration (2ng/mL), the recoveries of 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$-d_4 standards were 77.6 ± 2.4 and 77.1 ± 4.9 ($n = 3$) respectively. At high concentration (10 ng/mL), the recoveries of 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$-d_4 were 80.7 ± 2.1 and 80.5 ± 4.1 ($n = 3$) respectively. From the mass spectrum, we did not find any major peak to interfere with the F_2-isoprostane analytes. Different elution solvents have also been studied. Methanol can elute F_2-isoprostanes, but it caused ion suppression (date not shown). Ethyl acetate can effectively elute F_2-isoprostanes and no ion suppression was found. This solid phase extraction procedure is only one cartridge procedure and is easier, faster and cheaper than the previous procedures requiring both HLB cartridge and μElution plate [30].

3.4. Levels of F_2-isoprostane isomers in cell media after nanoparticle treatment

In this study, the A549 cells were exposed to each of five nanoparticles for 24 h. F_2-isoprostane isomers were tentatively identified by retention time and MRM transition 353/193. The levels of these isomers were then quantified by the developed LC–MS/MS method. In the control cells, the 11β-$PGF_{2\alpha}$ isomer's concentration was below the detection limit. Other five isomers including 8-iso-15R-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 15R-$PGF_{2\alpha}$, 5-trans-$PGF_{2\alpha}$, and $PGF_{2\alpha}$ were detectable. 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomers were coeluted. The extracted ion chromatogram of F_2-isoprostane isomers of the control is shown in Fig. 6a.

3.4.1. F_2-isoprostane isomers in SiO_2 nanoparticle-treated cell media

After cells were exposed to SiO_2 nanoparticles (15 nm) at the dosage levels of 50 and 100 μg/mL, the levels of six F_2-isoprostane isomers increased significantly compared with the control ($p < 0.05$) and the concentration increase was dose dependent. Moreover, two new peaks (Unknown 1 and Unknown 2) were detected next to the $PGF_{2\alpha}$ isomer after SiO_2 nanoparticle exposure. It is possible that they are new isomers of F_2-isoprostanes which have the same mass to charge ratio. Due to the lack of standards, we cannot identify them at this time. The extracted ion chromatogram of F_2-isoprostane isomers after SiO_2 nanoparticle (50 μg/mL) treatment is shown in Fig. 6b.

3.4.2. F_2-isoprostane isomers in Al_2O_3 nanoparticle-treated cell media

After A549 cells were exposed to Al_2O_3 nanoparticles (13 nm) at the dosage level of 100 μg/mL, the levels of 8-iso-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$, and $PGF_{2\alpha}$ isomers increased significantly compared with the control ($p < 0.05$). The level of 8-iso-15R-$PGF_{2\alpha}$ isomer also increased compared with the control, but was not significant ($p > 0.05$). Even though the results were similar to those of SiO_2 nanoparticle treatment, only Unknown 1 was detected in Al_2O_3 nanoparticle-treated cells. The extracted ion

Table 2
11β-$PGF_{2\alpha}$ isomer and two unknowns concentrations before and after nanoparticle treatment (mean ± SD), $n = 3$.

Isomers	11β-$PGF_{2\alpha}$ (ng/million cells)	Unknown 1 (ng/million cells)	Unknown 2 (ng/million cells)
Control	<0.0065	<0.0065	<0.0065
SiO_2 (50 μg/mL)	0.700 ± 0.215*	0.773 ± 0.527*	0.118 ± 0.033*
SiO_2 (100 μg/mL)	1.87 ± 0.496*	3.32 ± 0.136*	0.481 ± 0.009*
Al_2O_3 (100 μg/mL)	0.156 ± 0.067*	0.082 ± 0.054*	<0.0065
TiO_2 (100 μg/mL)	0.151 ± 0.069*	<0.0065	<0.0065
Fe_2O_3 (100 μg/mL)	0.012 ± 0.008*	<0.0065	<0.0065
ZnO (15 μg/mL)	0.014 ± 0.005*	0.061 ± 0.031*	<0.0065

* The concentration was significantly different from that of control.

chromatogram of F_2-isoprostane isomers after Al_2O_3 nanoparticle (100 μg/mL) treatment is shown in Fig. 6c.

3.4.3. F_2-isoprostane isomers in TiO_2 nanoparticle-treated cell media

Similarly, after A549 cells were exposed to TiO_2 (40 nm) nanoparticles at the dosage level of 100 μg/mL, the levels of 8-iso-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$, and $PGF_{2\alpha}$ isomers increased significantly compared with the control ($p < 0.05$). The level of 8-iso-15R-$PGF_{2\alpha}$ isomer also increased compared with the control, but the increase was not significant ($p > 0.05$). The extracted ion chromatogram of F_2-isoprostane isomers of TiO_2 nanoparticle (100 μg/mL) treated cells is shown in Fig. 6d.

3.4.4. F_2-isoprostane isomers in Fe_2O_3 nanoparticle-treated cell media

After A549 cells were exposed to Fe_2O_3 (30 nm) nanoparticles at the dosage level of 100 μg/mL, the levels of 11β-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ isomers increased significantly compared with the control ($p < 0.05$). The levels of 8-iso-$PGF_{2\alpha}$, 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomers also increased compared with the control, but were not significant ($p > 0.05$). The level of 8-iso-15R-$PGF_{2\alpha}$ isomer did not increase compared with the control. The extracted ion chromatogram of F_2-isoprostane isomers of Fe_2O_3 nanoparticle (100 μg/mL) treated cells was shown in Fig. 6e.

3.4.5. F_2-isoprostane isomers in ZnO nanoparticle-treated cell media

After A549 cells were exposed to ZnO (70 nm) nanoparticle at the dosage level of 15 μg/mL, the levels of 8-iso-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ isomers increased significantly compared with the control ($p < 0.05$). The level of 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomer also increased compared with the control, but were not significant ($p > 0.05$). No change was observed on the level of 8-iso-15R-$PGF_{2\alpha}$ isomer. The phenomenon was very similar to those of SiO_2 (15 nm) and Al_2O_3 (13 nm) nanoparticles, and the Unknown 1 peak was detected in ZnO (70 nm) nanoparticle-treated cells. The extracted ion chromatogram of F_2-isoprostane isomers of ZnO nanoparticle (15 μg/mL) treated cells is shown in Fig. 6f.

Total of six isomers were quantified by LC-MS/MS method in this study. In the control cells, the concentration of 8-iso-15R-$PGF_{2\alpha}$ isomer was 0.0360 ± 0.0023 ng/million cells ($n = 3$). SiO_2 (at dosage levels of 50, 100 μg/mL), Al_2O_3, and TiO_2 nanoparticles (at the dosage levels of 100 μg/mL) caused significant increase of 8-iso-15R-$PGF_{2\alpha}$ isomer concentration compared with the control. However, Fe_2O_3 (at the dosage levels of 100 μg/mL) and ZnO nanoparticles (at the dosage level of 15 μg/mL) did not cause significant increasing of 8-iso-15R-$PGF_{2\alpha}$ isomer concentration. The data of 8-iso-15R-$PGF_{2\alpha}$ isomer after nanoparticle treatment are shown in Fig. 7.

The 8-iso-$PGF_{2\alpha}$ isomer concentration in the control cell media was 0.0294 ± 0.0007 ng/million cells ($n = 3$). After cellular exposure to SiO_2, Al_2O_3, TiO_2, Fe_2O_3, and ZnO at the dosage levels described

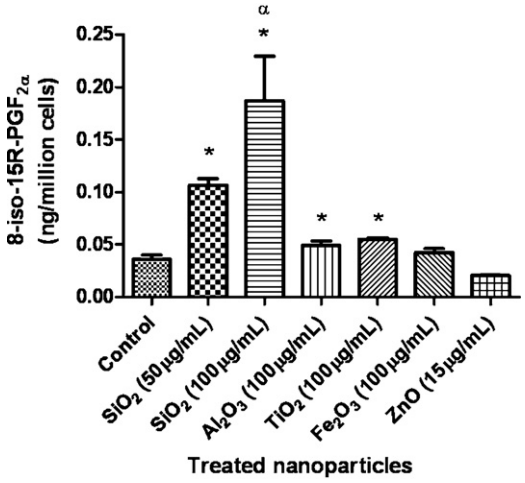

Fig. 7. 8-iso-15R-$PGF_{2\alpha}$ isomer concentrations in A549 cells after SiO_2 (15 nm), Al_2O_3 (13 nm), TiO_2 (40 nm), Fe_2O_3 (30 nm) and ZnO (70 nm) nanoparticle treatment. Data with asterisk means $p < 0.05$ compared with Control. Data with α means $p < 0.05$ compared with SiO_2 nanoparticle at the concentration 50 μg/mL.

in the last paragraph, the 8-iso-$PGF_{2\alpha}$ isomer concentration in cell media increased significantly compared with the control. The detailed data of 8-iso-$PGF_{2\alpha}$ isomer are shown in Fig. 8.

The 11β-$PGF_{2\alpha}$ isomer was below the detection limit in the A549 control cells. After nanoparticle exposure, the level of this isomer was detectable. All of the five nanoparticles caused a significant increase of 11β-$PGF_{2\alpha}$ isomer concentration compared with the control. The data of 11β-$PGF_{2\alpha}$ isomer after nanoparticle exposure

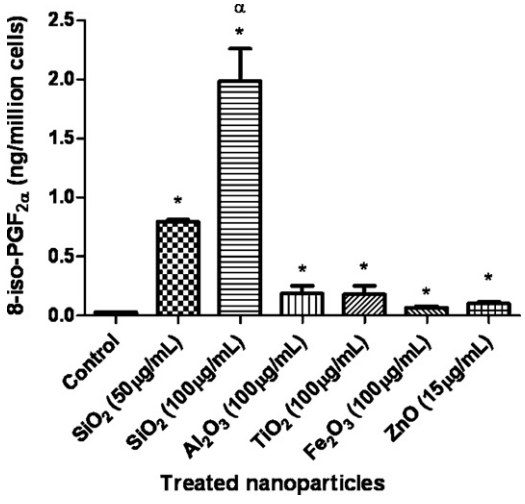

Fig. 8. 8-iso-$PGF_{2\alpha}$ isomer concentrations in A549 cells after SiO_2 (15 nm), Al_2O_3 (13 nm), TiO_2 (40 nm), Fe_2O_3 (30 nm) and ZnO (70 nm) nanoparticle treatment. Data with asterisk means $p < 0.05$ compared with Control. Data with α means $p < 0.05$ compared with SiO_2 nanoparticle at the concentration 50 μg/mL.

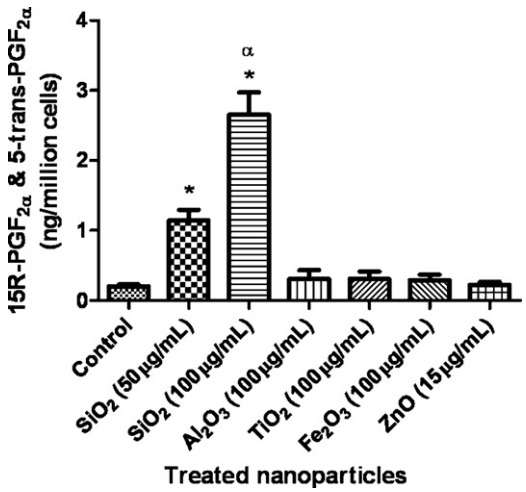

Fig. 9. 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomer concentrations in A549 cells after SiO_2 (15 nm), Al_2O_3 (13 nm), TiO_2 (40 nm), Fe_2O_3 (30 nm) and ZnO (70 nm) nanoparticle treatment. Data with asterisk means $p < 0.05$ compared with Control. Data with α means $p < 0.05$ compared with SiO_2 nanoparticle at the concentration 50 μg/mL.

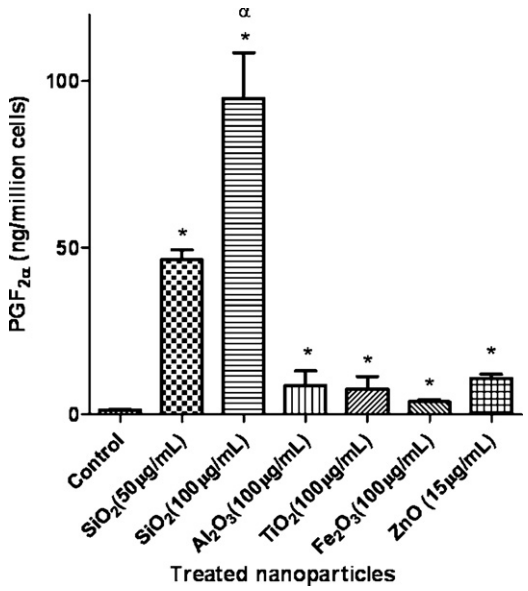

Fig. 10. $PGF_{2\alpha}$ isomer concentrations in A549 cells after SiO_2 (15 nm), Al_2O_3 (13 nm), TiO_2 (40 nm), Fe_2O_3 (30 nm) and ZnO (70 nm) nanoparticle treatment. Data with asterisk means $p < 0.05$ compared with Control. Data with α means $p < 0.05$ compared with SiO_2 nanoparticle at the concentration 50 μg/mL.

were listed in Table 2. Since 11β-$PGF_{2\alpha}$ has two *trans* ring hydroxyl groups and two *trans* alkyl chains, it is not a favorite product of free radical reaction. In addition, 5-series of isoprostanes can also form 193 product ion [31]. This deserves further study.

In the control cells, the concentrations of 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomers were 0.207 ± 0.015 ng/million cells ($n = 3$). After SiO_2 nanoparticle exposure at the dosage levels of 50, 100 μg/mL, the concentrations of 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomers increased significantly compared with the control. However, Al_2O_3, TiO_2, Fe_2O_3, and ZnO nanoparticle at the same dosage levels described above did not caused significant increase of 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomer concentrations. The data of 15R-$PGF_{2\alpha}$ and 5-trans-$PGF_{2\alpha}$ isomers after nanoparticle treatment are shown in Fig. 9.

Among these six isomers, $PGF_{2\alpha}$ was the most abundant isomer and its concentration in the control cells was 1.032 ± 0.106 ng/million cells ($n = 3$). All five nanoparticles increased its concentration significantly compared with the control. The data of $PGF_{2\alpha}$ isomer after nanoparticle treatment are shown in Fig. 10. $PGF_{2\alpha}$ is both a cyclooxygenase (COX) enzyme and free radical product, which may explain its greater abundance in the cells. Whether nanoparticles increased COX activity deserves further study.

In the SiO_2 nanoparticle exposure study, two new peaks (Unknown 1 and Unknown 2) were found compared with the control. However, in Al_2O_3 and ZnO nanoparticle exposure study, only one new peak (Unknown 1) was detected. Their data were listed in Table 2. The data implied that even though SiO_2, Al_2O_3, and ZnO can cause oxidative stress and cellular membrane damage, the detailed mechanism may not be totally the same. Moreover, 5-series of F_2-isoprostanes also can produce 193 product ion. It is possible that Unknown 1 and Unknown 2 are 5-series of F_2-isoprostanes. This phenomenon deserves further study.

Among these five types of studied nanoparticles, SiO_2 nanoparticles induced the highest increase of F_2-isoprostane isomers compared with other four metal oxide nanoparticles. Comparing with the cytotoxicity data of SiO_2, the cell viabilities were 88.2% and 78.3%, respectively, at the dosage levels of 50 and 100 μg/mL after 48 h exposure. For ZnO (70 nm) nanoparticles, the phenomenon was very different. Our previous study showed that ZnO nanoparticle was the most cytotoxic and the cell viability was 34.1% at the dosage level of 14 μg/mL after 24 h exposure. However, F_2-isoprostane isomers concentrations were lower in ZnO nanoparticle-treated cells than those of SiO_2 nanoparticle-treated cells. These data revealed that SiO_2 nanoparticles induced higher degree of lipid peroxidation and cell membrane damage than ZnO, Al_2O_3, TiO_2, and Fe_2O_3 metal oxide nanoparticles. Here, the data showed slightly different patterns of 15-series of F_2-isoprostane isomers corresponding to six nanoparticles exposure. What caused this difference deserves further study. Our previous study showed that ZnO nanoparticle can cause DNA damage [20]. Therefore, these nanoparticles may involve different mechanisms in causing oxidative stress. Our data showed that TiO_2 nanoparticles increased F_2-isoprostane isomers and cause cell membrane damage which was consistent with previous cytotoxicity study on TiO_2 nanoparticle [14]. A separate study in our research group using imaging technique showed that Al_2O_3 (13 nm) nanoparticles can cause cell membrane depolarization in A549 cells [33], which also supported our data in this study.

F_2-isoprostane isomers have been widely studied in the plasma and urine to serve as reliable biomarkers for oxidative stress [34,35]. Urinary F_2-isoprostane metabolites including 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-8-iso-$PGF_{2\alpha}$ have greater abundance than F_2-isoprostanes and also can serve as biomarkers for oxidative stress [36]. It has been reported that cigarette smokers had higher concentrations of urinary F_2-isoprostanes and their metabolites [23]. Based on our best knowledge, our study is the first report of increased F_2-isoprostane isomers in A549 cells after nanoparticle exposure. 8-Iso-$PGF_{2\alpha}$ isomer is considered as the most important isomers of F_2-isoprostanes because its structure is favorite to be formed by free radicals and has higher abundant than other isomers [37]. Some studies have shown that it possesses some biological functions [24–26]. In our study, $PGF_{2\alpha}$ was also the most abundant isomer and increased dramatically after nanoparticle treatment. Five types of studied nanoparticles can all induce significant increase of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ isomer concentrations.

4. Conclusions

For the first time, a LC–MS/MS method has been developed to determine F_2-isoprostane isomers in human lung epithelial

cells after exposure to SiO_2 (15 nm), Al_2O_3 (13 nm), TiO_2 (40 nm), Fe_2O_3 (30 nm), and ZnO (70 nm) nanoparticles. After 24 h exposure, the concentrations of F_2-isoprostane isomers increased at different levels compared with the control cells. Data showed that these nanoparticles can induce lipid peroxidation and cell membrane damage at different degrees. SiO_2 nanoparticles induced significantly higher concentrations of F_2-isoprostane isomers than other four metal oxide nanoparticles in A549 cells. A simplified solid phase extraction procedure was also developed without ion suppression in mass spectrometer. The time response to the concentrations of biomarker F_2-isoprostane isomers and the size effects of nanoparticles on levels of the F_2-isoprostane isomers will be further studied. At the same time, the identification of two detected unknown peaks and screen for other isomers and metabolites of F_2-isoprostanes due to nanoparticle stimulation will be performed. Our data in this study will help researchers understanding the mechanism of nanotoxicity and protecting people's health when we work with nanoparticles.

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