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Increased Level of α 2,6-Sialylated Glycans on HaCaT Cells Induced by Titanium Dioxide Nanoparticles under UV Radiation

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Abstract: As one of the most widely used nanomaterials, the safety of nano-TiO₂ for human beings has raised concern in recent years. Sialylation is an important glycosylation modification that plays a critical role in signal transduction, apoptosis, and tumor metastasis. The aim of this work was to investigate the cytotoxicity and phototoxicity of nano-TiO₂ with different crystalline phases for human skin keratinocytes (HaCaT cells) under ultraviolet (UV) irradiation and detect sialic acid alterations. The results showed that the mixture of crystalline P25 had the highest cytotoxicity and phototoxicity, followed by pure anatase A25, whereas pure rutile R25 had the lowest cytotoxicity and phototoxicity. A25 and R25 had no effects on the expression of sialic acids on HaCaT cells. However, HaCaT cells treated with P25 and UV showed an increased level of alterations in α 2,6-linked sialic acids, which was related to the level of reactive oxygen species (ROS) generated by nano-TiO₂ and UV. The abundance of α 2,6-linked sialic acids increased as ROS production increased, and vice versa. Antioxidant vitamin C (VC) reversed the abnormal expression of α 2,6-linked sialic acids caused by nano-TiO₂ and protected cells by eliminating ROS. These findings indicate that nano-TiO₂ can alter the sialylation status of HaCaT cells under UV irradiation in a process mediated by ROS.

Keywords: TiO₂ nanoparticles; phototoxicity; sialic acids; reactive oxygen species; vitamin C

1. Introduction

Nanoparticles and nanotechnology are being developed rapidly and are increasingly encountered during the course of daily life. Due to the unique properties of titanium dioxide nanoparticles (nano-TiO₂), including absorbing and scattering ultraviolet (UV) and photocatalysis, these nanoparticles have a variety of uses in many fields, such as additives in the cosmetics industry and whiteness enhancers in the paper industry [1,2]. However, nano-TiO₂ have a high surface-to-volume

ratio because of their nanoscale size, which may result in high surface energy and biological reactivity [3]. Studies of the potential health risk of nano-TiO₂ for humans have revealed that the toxicity of nano-TiO₂ is dependent on the size, shape, and crystalline phase of the particles, as well as their distribution in the body [4–6].

Due to the use of nano-TiO₂ in paints, wastewater treatment, food products, and cosmetics, nano-TiO₂ may come into contact with human skin and mucous membranes and thus enter the human body [7]. However, under UV irradiation, electrons in the nano-TiO₂ valence band absorb photon energy and jump to the conduction band, leaving valence band holes that extract electrons from water or hydroxyl ions and generate reactive oxygen species (ROS), which are cytotoxic and genotoxic [8,9]. The phototoxicity and degree of damage associated with nano-TiO₂ are dependent on the crystalline phase, size and concentration of the nanoparticles [4,10]. Nano-TiO₂ can inhibit the growth of HaCaT cells via ROS generation and decrease the activity levels of enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), under UV light [11]. ROS damage cells, tissues, and organisms by causing lipid peroxidation, altering the abundance of proteins, producing DNA mutations, and triggering apoptosis [12–15].

As previously reported, extensive studies have focused on how nano-TiO₂ and UV disrupt and interfere with the processes of mRNA transcription and protein translation, but few studies have examined the effects of nano-TiO₂ on post-translational modifications of proteins under UV light [16,17]. Glycosylation is an important post-translational modification. Advanced glycation end-products exist in the extracellular matrix of the dermis and the cell surface, where they influence healing of the skin, aging, elasticity and several skin disorders [18]. Keratinocytes, the main epidermal cells in the skin, were used in this study to investigate the effects of TiO₂ nanoparticles on glycosylation.

The process of apoptosis is associated with alterations in glycans, including sialic acids, mannose, and fucose [19]. In cell lines of different histological origin (colon, breast, pancreas, and bladder cancer), as well as in normal human and mouse neutrophils, apoptosis is accompanied by the exposure of sugar chains recognized by the lectin from *Sambucus nigra* agglutinin (SNA), which is specific for sialic acid structures [20]. After induction of lymphocyte apoptosis, changes in 2,6-terminal sialic acids on the surface of apoptotic membrane blebs can either directly mediate cellular engulfment or enhance phagocytosis by cooperation with further engulfment signals [21]. Sialic acids are particularly abundant in mucins and at the epithelial cell surface, and they are usually the outermost monosaccharide of the glycocalyx.

Sialic acids are nine-carbon monosaccharides at the terminal position of oligosaccharides on the cell surface that are involved in cell metabolism [22], signal transduction [23,24], and tumor proliferation, invasion, and angiogenesis [25,26]. For example, influenza A viruses infect birds by recognizing α 2,3-linked sialic acids on glycan chains, but they cannot recognize α 2,6-linked sialic acids [27]. Nevertheless, the relationship between nano-TiO₂ toxicity and alterations in sialylated glycans on the cell surface has not been studied.

In this study, the cytotoxicity and phototoxicity of different concentrations and crystalline phases of nano-TiO₂ were investigated, and changes in sialylation on the surface of HaCaT cells caused by nano-TiO₂ and UV were explored. Cells treated with UV and nano-TiO₂ showed enhanced reactivity with *Sambucus nigra* agglutinin (SNA) and increased binding with α 2,6-linked sialic acid. These changes were related to ROS generated by nano-TiO₂ and UV, which led to changes in sialic acids. These findings suggest that sialic acid expression plays an important role in the toxic effects of nano-TiO₂ on the skin. The results of this analysis offer insight into the toxicity of nano-TiO₂ to guide the development of safety guidelines and future research into nanoparticle toxicity.

2. Materials and Methods

2.1. Materials

Nano-TiO₂, an anatase-rutile mixture of nano-TiO₂ P25 (Degussa Company, Essen, Germany), anatase nano-TiO₂ (Sigma Company, St. Louis, MO, USA) and rutile nano-TiO₂ (Macklin, Shanghai, China) were obtained from commercial sources. UV light was generated by an ultraviolet lamp (ZF-5, 365 nm, 8 W, 0.6 mW/cm², Shanghai Huxi Instrument, Shanghai, China).

Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS, pH 7.4), penicillin, streptomycin, and trypsin-EDTA were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). *Sambucus nigra* lectin (SNA) labeled with fluorescein isothiocyanate (FITC) FITC and *Maackia amurensis* lectin I (MAL-I) were purchased from Vector labs (Burlingame, CA, USA).

Anhydrous dimethyl sulfoxide (DMSO) and vitamin C (VC) were purchased from Sigma Aldrich (St. Louis, MO, USA). ROSup was purchased from Beyotime (Shanghai, China).

2.2. Cell Culture

HaCaT cells, a spontaneously immortalized human keratinocyte cell line (ATCC), were cultured in 95% air and 5% CO₂ at 37 °C in c supplemented with 10% FBS and 1% penicillin/streptomycin.

2.3. Preparation and Characterization of Nano-TiO₂

The size and morphology of nano-TiO₂ were visualized using transmission electron microscopy (TEM) (HT7700, Hitachi, Tokyo, Japan). The crystalline phase of nano-TiO₂ P25 was detected by a Philips XPert PRO MPD X-ray diffractometer (Philips, Eindhoven, The Netherlands). Nano-TiO₂ powder was sterilized by an autoclave, suspended in DMEM, and sonicated for 20 min in an ultrasonic bath. Before application to cells, the suspension was freshly prepared and immediately applied.

2.4. Cell Viability

Cells were seeded in 96-well-plates (Corning, Corning, NY, USA) at a density of 1×10^5 cells per well and cultured for 24 h at 37 °C to reach 90% confluency. Cells were treated with 0, 10, 50, 100, 500, 1000 µg/mL of various crystalline phases of nano-TiO₂ for 24, 48 and 72 h. After incubation, each well was washed with PBS three times and filled with DMEM containing 0.5 mg/mL MTT. The cells were incubated for 4 h at 37 °C, the supernatant was removed, DMSO was added to each well, and the cells were allowed to sit for 15 min. The plates were assessed at 492 nm using a microplate reader (Spectrafluor Plus, Tecan US, RTP, Durham, NC, USA).

2.5. Phototoxicity

Phototoxicity was assessed using MTT. Cells were seeded in 96-well plates and cultured for 24 h. The supernatant was replaced with different concentrations of nano-TiO₂ P25 and cells were cultured for 21 h. The medium was replaced by PBS before UV radiation at 365 nm to avoid the effect of phenol red. After 1 h irradiation, fresh medium was added, and the cells were incubated for 2 h [7,28], after which cell viability was detected with the procedure described above.

2.6. Lectin Staining

HaCaT cells were cultivated in 6-well plates with sterilized coverslips to reach a density of 5×10^5 cells per well. The cells were treated with 0 or 50 µg/mL nano-TiO₂ P25 for 21 h, followed by UV irradiation for one hour. The cells were washed with cold PBS three times and fixed with 4% paraformaldehyde solution for 15 min. The fixed cells were incubated with 15 µg/mL FITC fluorescein-labeled lectins (SNA, MAL-I) for one hour in the dark at room temperature (RT) [29].

The cells were then observed under a laser confocal scanning microscope (Olympus, FV1000, Tokyo, Japan) to evaluate changes in sialylation. In order to compare sialic acid expression after various treatments, the fluorescence intensity of each pixel was quantified using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Intracellular ROS Detection

DCFH-DA, a non-fluorescent, membrane-permeable compound, was used to detect intracellular ROS. After entering cells, DCFH-DA is rapidly de-acetylated to 2',7'-dichlorodihydrofluorescein diacetate (DCFH), which can be converted by a broad range of ROS into 2,7-dichlorofluorescein (DCF), which is highly fluorescent and readily detected [30]. The fluorescence intensity was detected by flow cytometry and a fluorescence spectrophotometer at excitation and emission wavelengths of 485 and 530 nm, respectively. HaCaT cells were seeded in 12-well plates and cultured for 24 h. The cells were exposed to 0 or 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ P25 for 3 h and washed twice with PBS, followed by exposure to pre-warmed PBS containing 20 μM DCFH-DA. The plates were treated with UV irradiation or left in the dark for one hour [28]. The cells were washed with cold PBS, trypsinized, centrifuged, and resuspended in 0.5 mL of cold PBS prior to measurement and flow cytometry analysis.

2.8. Statistical Analysis

All results are shown as the mean and standard deviation from at least three independent experiments. Statistical analyses were performed with Student's two-tailed paired *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of TiO₂ Nanoparticles

The morphology of nano-TiO₂ was visualized under a transmission electron microscope (TEM) (Figure 1). The particle size was approximately 25 nm. Aggregation and large clusters were easily observed in all samples. The three tested crystalline phases of nano-TiO₂ were A25, R25 and P25. X-ray diffraction (XRD) analysis showed that the crystalline composition of P25 was approximately 14% rutile and 86% anatase. R25 was pure rutile, whereas A25 was pure anatase (Table 1). The specific surface areas of the particles were similar among the nano-TiO₂ samples.

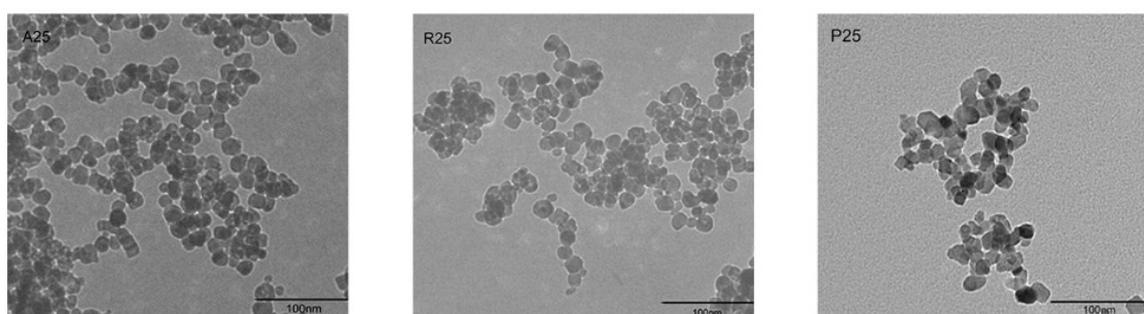


Figure 1. Morphological characterization of nano-TiO₂ A25, R25, and P25 via TEM.

Table 1. Physicochemical properties of the nano-TiO₂ used in this study.

Product	Crystalline Phase	Purity	Particle Size	Particle Specific Surface Area
Degussa P25	25% rutile/75% anatase	99.5%	21 nm	50 m ² /g
Rutile 25	Rutile	99%	25 nm	51.02 m ² /g
Anatase 25	Anatase	99%	25 nm	49.5 m ² /g

3.2. Cytotoxicity and Phototoxicity of Nano-TiO₂

The viability of HaCaT cells was tested following treatment with nano-TiO₂ for 24 h (Figure 2). The low concentration of nano-TiO₂ P25 (<50 µg/mL) was non-toxic. High concentrations (100 µg/mL) of pure anatase (A25) and rutile (R25) nano-TiO₂ showed no significant cytotoxicity. However, exposure to 100 µg/mL P25 reduced cell viability by approximately 20% (Figure 2C). Exposure to 1000 µg/mL A25 reduced cell viability by approximately 30%, whereas exposure to 1000 µg/mL R25 reduced cell viability by approximately 20%. Exposure to 1000 µg/mL P25 reduced cell viability by approximately 45% ($p < 0.001$). The viability of HaCaT cells was decreased by nano-TiO₂ in a concentration-dependent manner. The toxic effects of nano-TiO₂ were related to its crystalline phase. P25, the mixture of crystalline nano-TiO₂ phases, showed more severe cytotoxicity in comparison with the pure anatase and pure rutile samples.

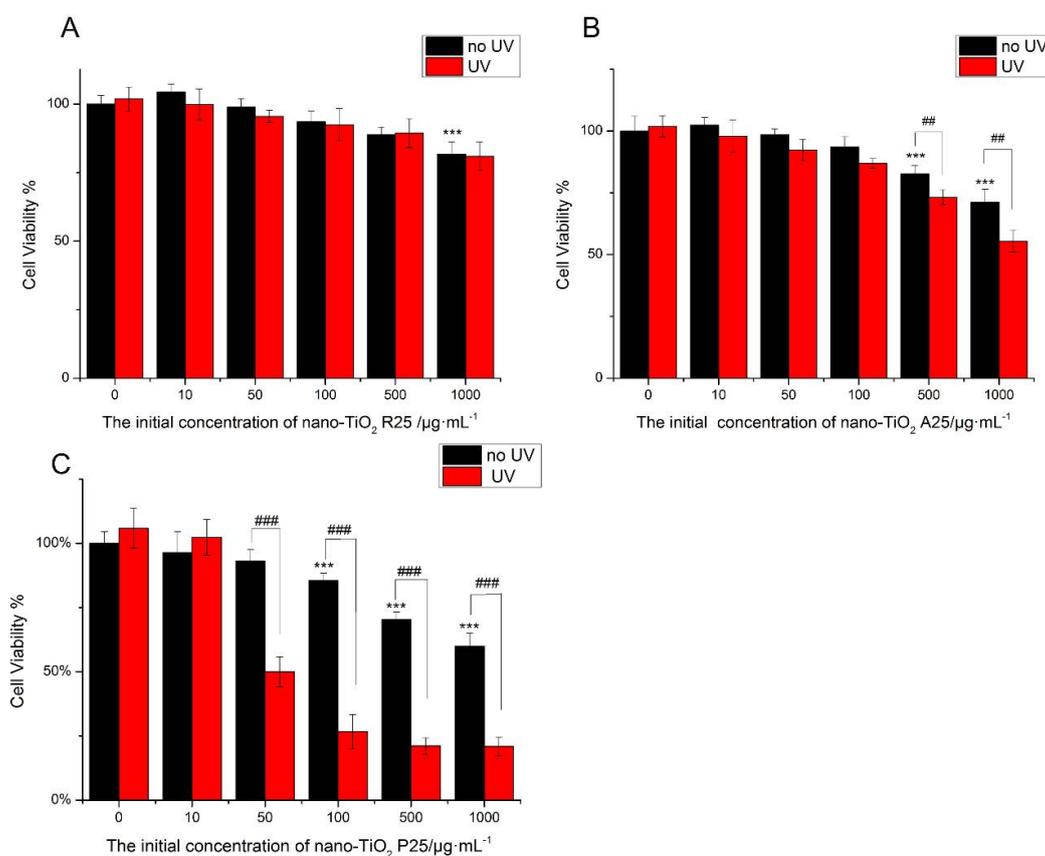


Figure 2. The effects of 24 h of exposure of human skin keratinocytes (HaCaT) cells to nano-TiO₂ (A) R25, (B) A25, and (C) P25 with or without ultraviolet (UV) irradiation. Cell viability was quantified by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Significant differences are indicated where ($n = 5$) \pm SEM, *** $p < 0.001$, ## $p < 0.01$, compared with the 0 µg/mL group. ### $p < 0.001$, compared with the same concentration of nano-TiO₂ in the presence of UV.

To investigate the phototoxicity of nano-TiO₂, cells were subjected to UV irradiation for one hour after treatment with nano-TiO₂, after which cell viability was tested. There was no decrease in viability when HaCaT cells were exposed to UV irradiation alone. R25 showed no significant phototoxicity, and cell viability was similar with or without UV irradiation. However, UV light increased the damage caused by nano-TiO₂ P25 and A25. As shown in Figure 2, P25 was more phototoxic than A25. Exposure to 50 µg/mL P25 and UV light decreased cell viability by 50% ($p < 0.001$). Exposure to 500 µg/mL A25 and UV light reduced cell viability by approximately 10% ($p < 0.01$). The mixture of

crystalline phases had the highest photo-cytotoxicity. Therefore, P25, which had severe phototoxicity and lacked cytotoxic effects, was used in subsequent experiments at a concentration of 50 $\mu\text{g}/\text{mL}$.

3.3. The Effects of UV and Nano-TiO₂ on α 2,3- and α 2,6-Linked Sialic Acids

To study whether UV and nano-TiO₂ influence sialic acids on HaCaT cells, fluorescence-labeled lectins were detected (Figure 3). The fluorescent signal intensity of SNA (sia2-6Gal β 1-4GlcNAc) on the cells treated with UV and nano-TiO₂ P25 was significantly higher than that of the untreated cells, which indicated high expression of α 2,6-linked sialic acids. In the presence of UV and nano-TiO₂ P25 at a concentration of 50 $\mu\text{g}/\text{mL}$, the abundance of α 2,6-linked sialic acids was increased by 5.9-fold in comparison with that of the control group and significantly elevated in comparison with that of the group exposed to UV treatment only and that of the group exposed to nano-TiO₂ P25 only ($p < 0.001$). The UV group exhibited a significant increase of 2.4-fold in fluorescence intensity in comparison with that of the control group, indicating that the abundance of α 2,6-linked sialic acids was increased in the UV group ($p < 0.001$). MAL-I staining for α 2,3-linked sialic acids did not change significantly after any of the treatments. The fluorescent intensity analysis suggests that the groups had no significant differences in the abundance of α 2,3-linked sialic acids.

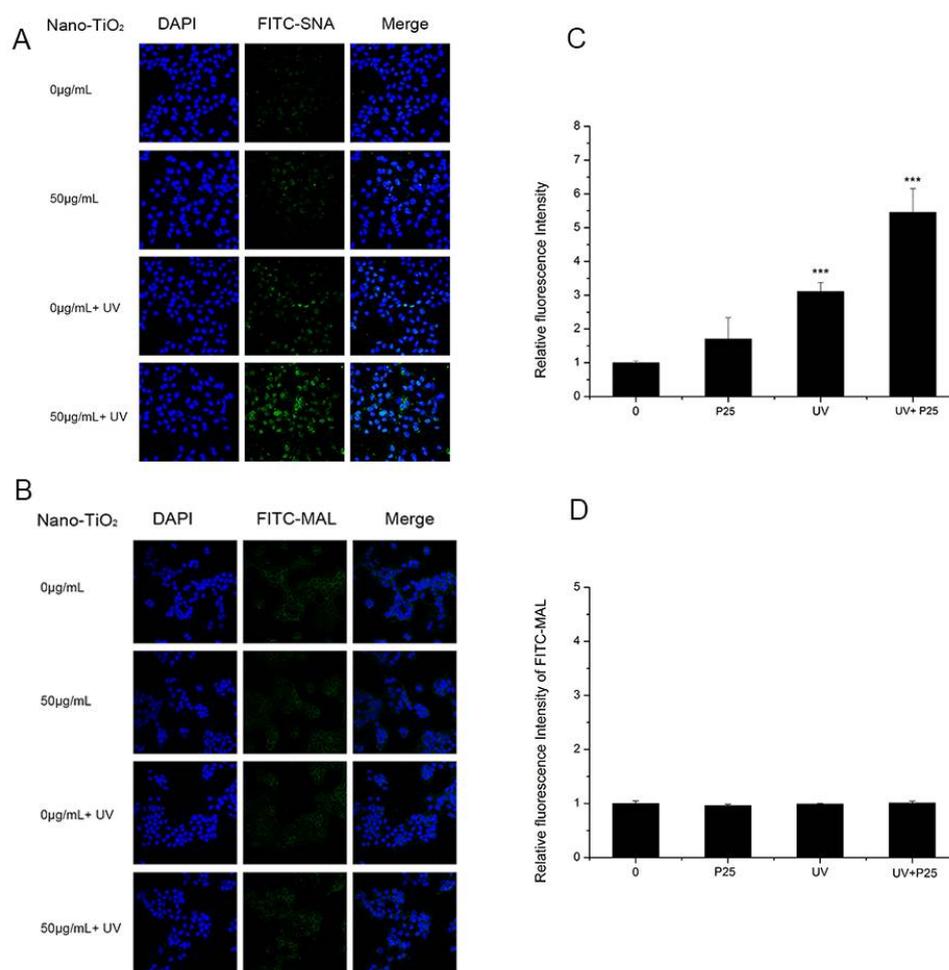


Figure 3. Assessment of changes in sialic acids by lectin staining. HaCaT cells were treated with 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ P25, UV or the combination of nano-TiO₂ P25 and UV. Staining was performed with fluorescein isothiocyanate (FITC)-labeled (A) SNA for α -2,6 sialic acid residues (green), (B) Maackia amurensis lectin (MAL) for α -2,3 sialic acid residues (green), and 4',6-diamidino-2-phenylindole (DAPI) for nuclei (blue). The bar graphs show the fluorescent intensity analysis for (C) A and (D) B (***) $p < 0.001$, error bars are standard error of the mean).

As shown in Figures 4 and 5, A25 and R25 did not alter staining for SNA or MAL-I with or without UV irradiation, which indicated that A25 and R25 did not change the abundance of sialic acids at a concentration of 50 $\mu\text{g}/\text{mL}$. These findings indicate that UV light and nano-TiO₂ P25 can affect expression of α 2,6-linked sialic acids and have no effect on expression of α 2,3-linked sialic acids.

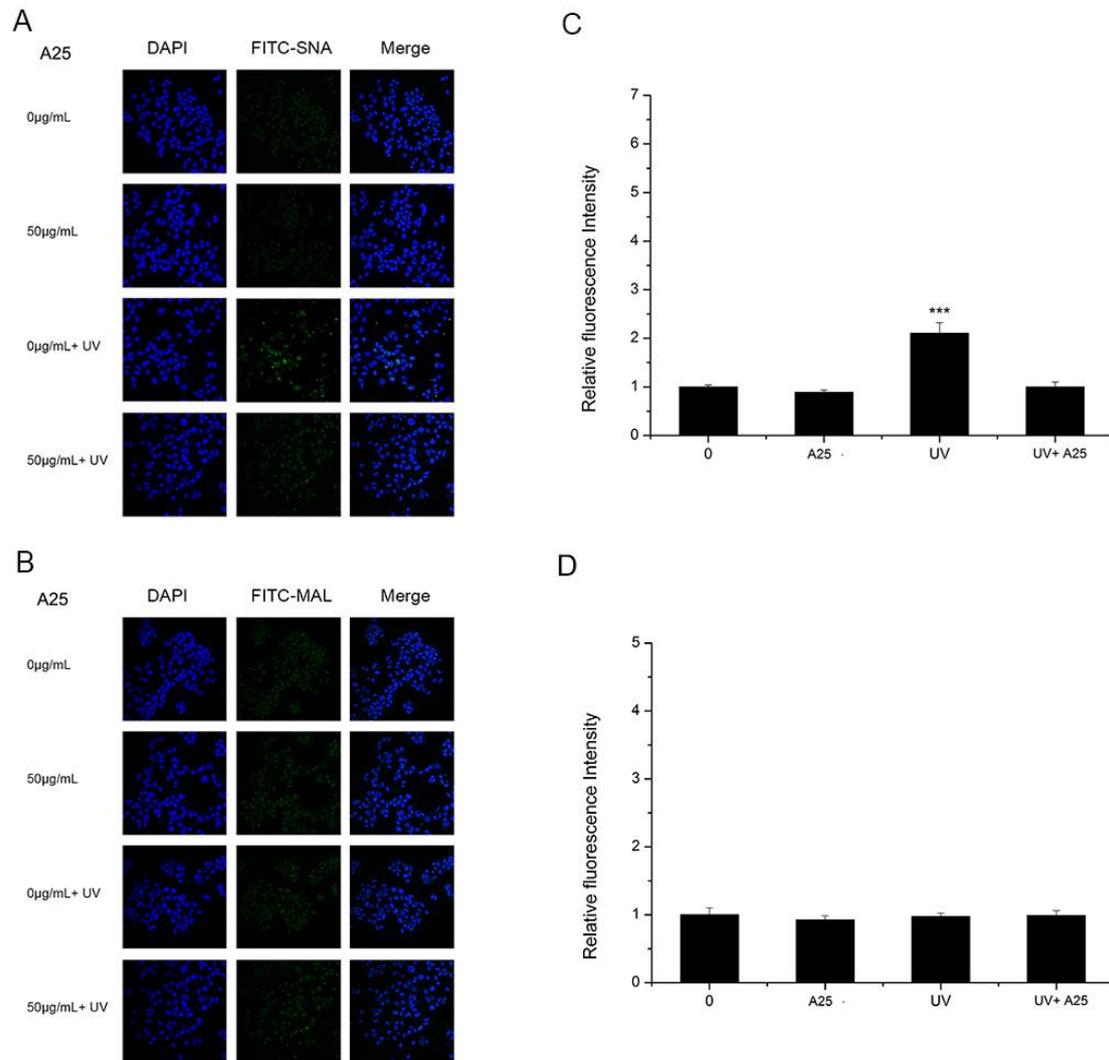


Figure 4. HaCaT cells were treated with 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ A25, UV or the combination of nano-TiO₂ A25 and UV. Staining was performed with FITC-labeled (A) SNA for α -2,6 sialic acid residues (green), (B) MAL for α -2,3 sialic acid residues (green), and DAPI for nuclei (blue). The bar graphs show the fluorescent intensity analysis for (C) A and (D) B (***) $p < 0.001$, error bars are standard error of the mean).

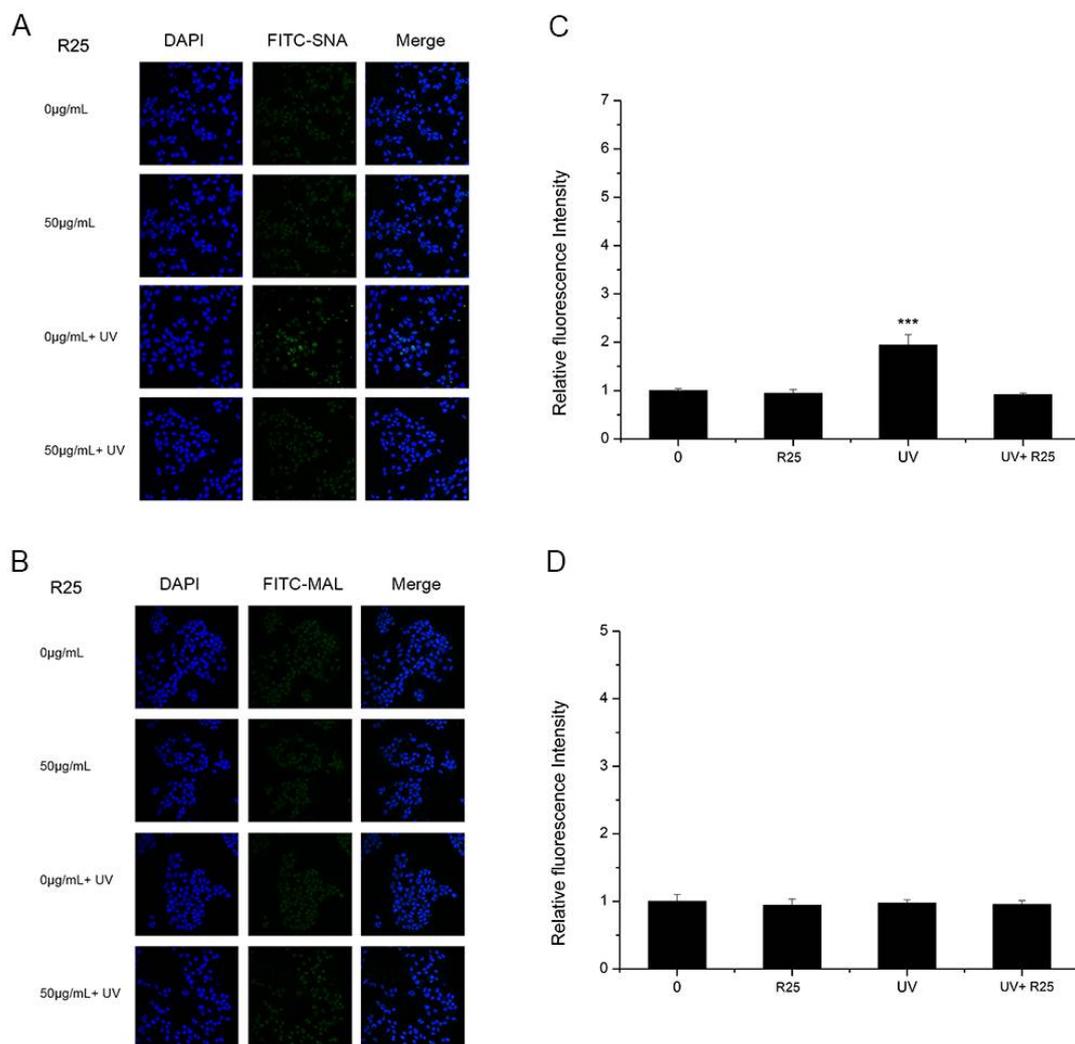


Figure 5. HaCaT cells were treated with 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ R25, UV or the combination of nano-TiO₂ R25 and UV. Staining was performed with FITC-labeled (A) Sambucus nigra agglutinin (SNA) for α -2,6 sialic acid residues (green), (B) MAL-I for α -2,3 sialic acid residues (green), and DAPI for nuclei (blue). The bar graphs show the fluorescent intensity analysis for (C) A and (D) B (***) $p < 0.001$, error bars are standard error of the mean).

3.4. Intracellular ROS Detection

Although the mechanism of nano-TiO₂ P25 toxicity was not fully deciphered, ROS was regarded as playing a critical role in the toxicity of nano-TiO₂ P25. Therefore, ROS generation was examined using a DCFH-DA probe during treatment with UV light and nano-TiO₂ P25. Images collected using a fluorescence microscope showed that the intracellular ROS generated in HaCaT cells was dependent on the treatment (Figure 6A). The cells exposed to nano-TiO₂ P25 for 3 h exhibited improved intracellular ROS. The presence of UV increased ROS production compared to the absence of UV. Moreover, the cells exposed to nano-TiO₂ P25 and UV irradiation showed the most ROS generation. Intracellular ROS were quantified by quantitative flow cytometry (Figure 6B,C). Intracellular ROS abundance was increased significantly in HaCaT cells treated with 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ P25 and UV light. Intracellular ROS abundance was increased approximately 2.5-fold in cells treated with UV light alone in comparison with that of control cells and those treated with nano-TiO₂ P25 only. A significant increase in ROS generation in HaCaT cells was detected after UV irradiation and nano-TiO₂ P25 treatment (10-fold that of the control group, $p < 0.001$).

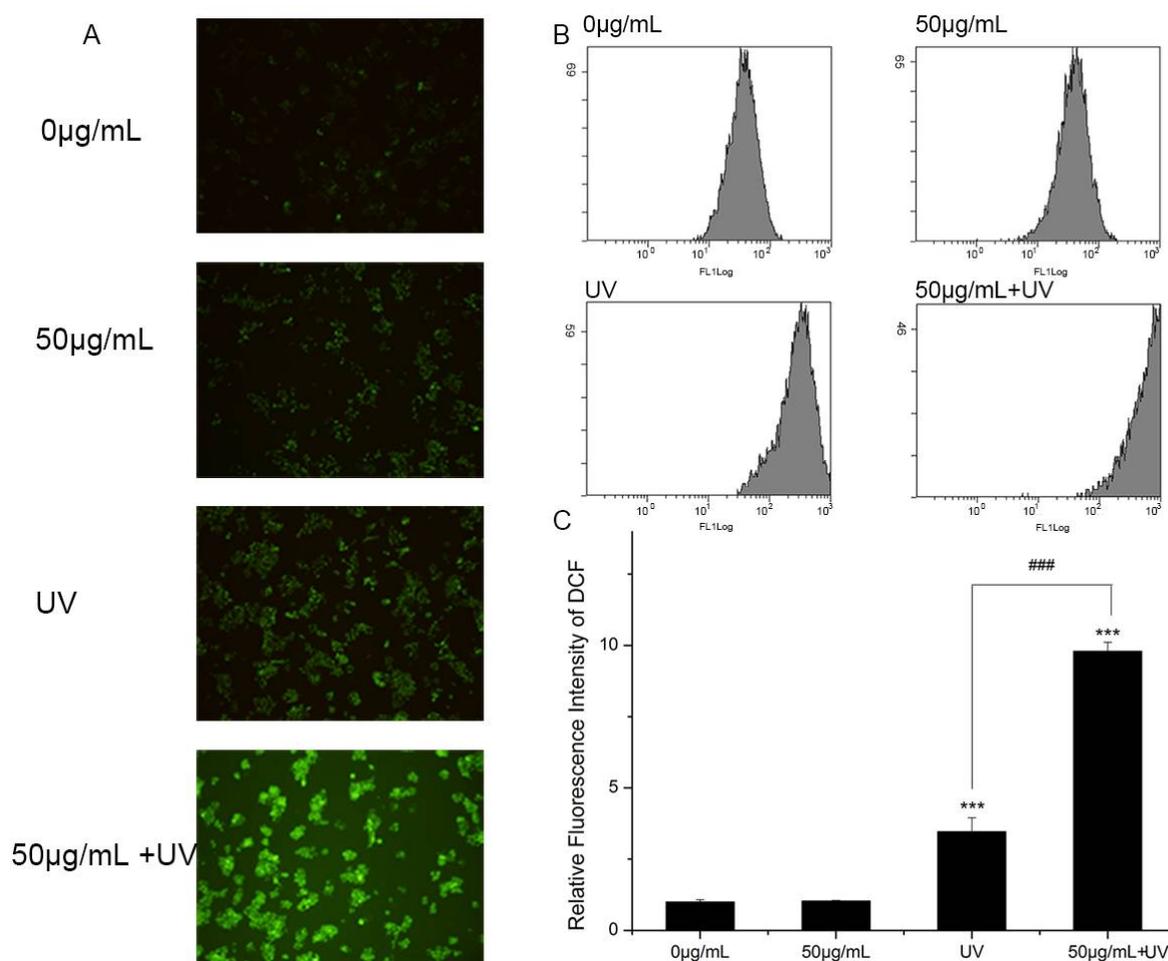


Figure 6. Reactive oxygen species (ROS) generation in HaCaT cells. Cells were treated with 0 or 50 $\mu\text{g}/\text{mL}$ nano-TiO₂ P25 with or without UV irradiation. After the treatment, 2',7'-dichlorofluorescein diacetate (DCFH-DA) was imaged by fluorescence microscopy and detected by flow cytometry. (A) A fluorescence microscopy image, (B) histograms from the flow cytometry analysis, (C) quantification of the fluorescence intensity of 2,7-dichlorofluorescein (DCF) (mean \pm SD, $n = 3$, *** $p < 0.001$, compared with the 0 $\mu\text{g}/\text{mL}$ group; ### $p < 0.001$, compared with the UV group).

3.5. The Effect of ROS on Sialic Acid Expression

The results of the analysis of ROS generation were in accordance with the results of the analysis of $\alpha 2,6$ -linked sialic acids. HaCaT cells treated with nano-TiO₂ P25 and UV showed enhanced ROS production and increased abundance of $\alpha 2,6$ -linked sialic acids. These findings suggested ROS altered $\alpha 2,6$ -linked sialic acids on the HaCaT cell surface. Vitamin C (VC), a natural antioxidant, can eliminate ROS and protect cells from oxidative damage [31]. Moreover, VC can significantly reduce intercellular ROS abundance [32]. HaCaT cells incubated with VC and nano-TiO₂ P25 under UV irradiation showed significantly decreased binding with SNA. ROSup was used as a reagent to generate a positive control group with relatively high ROS abundance. After treatment with 50 $\mu\text{g}/\text{mL}$ ROSup, a dramatic increase in SNA binding was observed. VC decreased SNA binding by approximately 5-fold in comparison with that of the group treated with UV and nano-TiO₂ P25 ($p < 0.001$). The fluorescent intensity of the group treated with VC was similar to that of the control group. The fluorescent intensity of the ROSup group was increased by approximately 6.5-fold in comparison with that of the control group (Figure 7A,C). However, VC treatment and ROSup treatment did not alter the abundance of $\alpha 2,3$ -linked sialic acids (Figure 7B,D). These findings indicate that P25 could lead to abnormal

expression of α 2,6-linked sialic acids via ROS generation. In addition, an abnormally high abundance of α 2,6-linked sialic acids can be reversed by treatment with antioxidants.

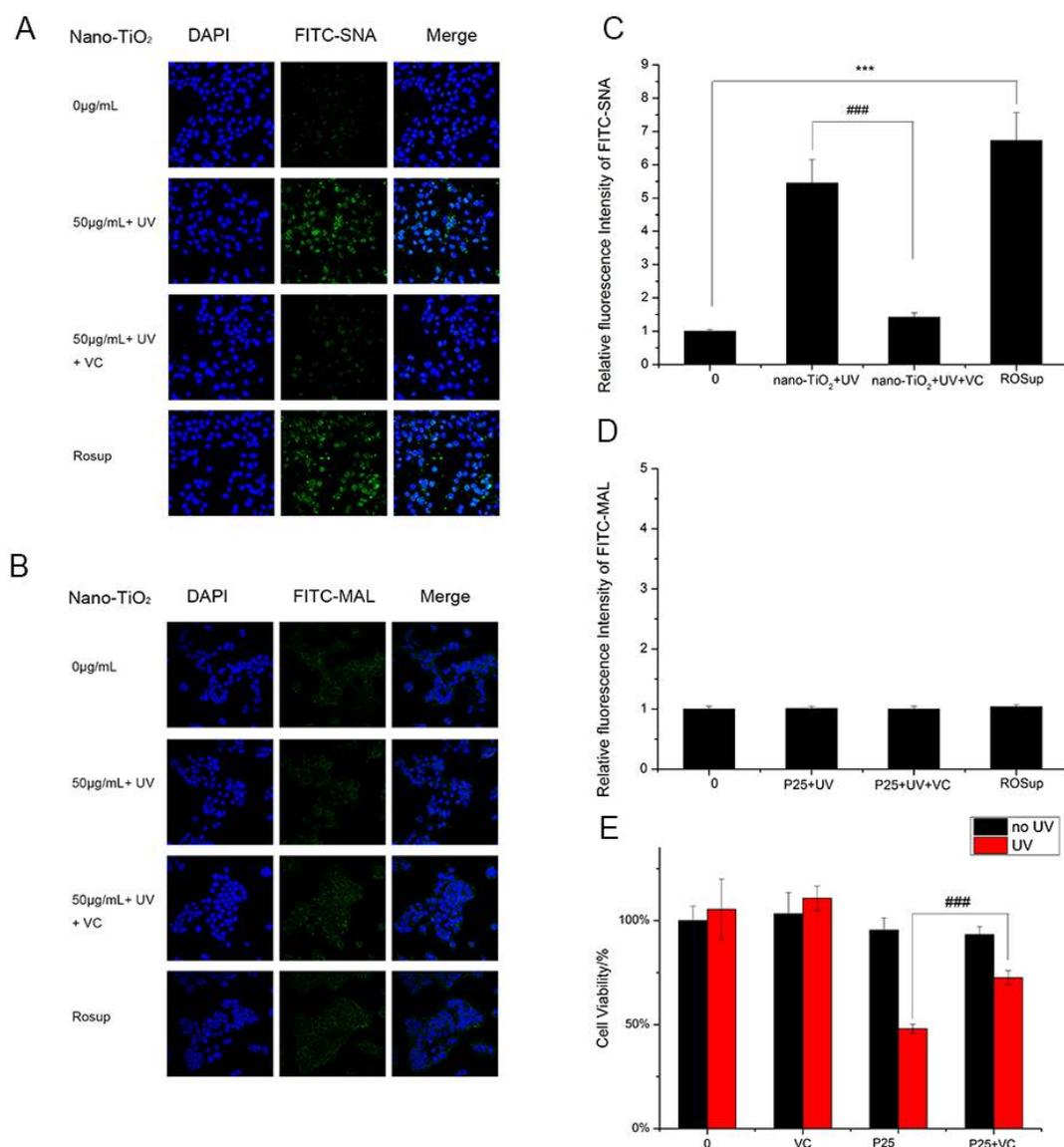


Figure 7. Detection of changes in sialic acids by lectin staining. HaCaT cells were treated with 50 μ g/mL nano-TiO₂ P25 and UV, the combination of 1 mM VC, nano-TiO₂ P25 and UV, or 50 μ g/mL ROSUp. Staining was performed with FITC-labeled (A) SNA for α -2,6 sialic acid residues (green), (B) MAL for α -2,3 sialic acid residues (green), and DAPI for nuclei (blue). The bar graphs show the fluorescence intensity analysis for (C) A and (D) B (error bars are standard error of the mean). (E) The effects of vitamin C (VC) on cell viability under treatment with nano-TiO₂ P25 and UV irradiation. Significant differences are indicated where ($n = 5$) \pm SEM, *** $p < 0.001$, ### $p < 0.001$, compared with the same concentration of nano-TiO₂ in the presence of UV without VC treatment.

HaCaT cells exposed to VC in the presence of nano-TiO₂ P25 and UV irradiation showed increased cell viability in comparison with that of the group treated with UV and P25 (Figure 7E). In addition, VC-treated cells showed no discernible difference in viability in comparison with that of the control group. Therefore, these results demonstrate that ROS affect the level of α 2,6-sialylation on the cell surface; greater ROS abundance is associated with enhanced α 2,6-sialylation. Finally, exposure to VC can protect cells from nano-TiO₂ and UV irradiation via ROS clearance.

4. Discussion

Nano-TiO₂ is one of the most used nanomaterials and has thus been the subject of significant research regarding toxicity and safety. Humans can be exposed to nano-TiO₂ during manufacturing [33]. Nano-TiO₂ can be found in the forms of aerosols, suspensions or emulsions, which can cause toxic effects via inhalation and dermal exposure [34]. The small size and high surface-to-volume ratio of nano-TiO₂ increase the surface energy of the particles and enhance biological reactivity [35]. ROS generated by nanoparticles can lead to cytotoxicity and genotoxicity. The skin is regularly exposed to irradiation from sunlight simultaneously with nano-TiO₂ contained in sunscreen. Therefore, the toxicity of nano-TiO₂ in the context of the skin is of great significance.

In this work, the cytotoxicity of nano-TiO₂ was related to its crystalline phase, concentration, and exposure time (Figure 2). There were no significant differences in nano-TiO₂ cytotoxicity at low concentrations of 10 µg/mL or 50 µg/mL, while nano-TiO₂ showed cytotoxicity at concentrations of 100–1000 µg/mL. The toxic effects of nano-TiO₂ increased in a dose-dependent manner. P25 at a low concentration of 50 µg/mL with UV light caused a 50% decrease in cell viability in comparison with that of cells exposed to nano-TiO₂ P25 only. The cytotoxicity and phototoxicity of nano-TiO₂ were related to its crystalline phase; P25 had greater photo-cytotoxicity in comparison with that of A25 or R25. These results are in accordance with those of previous studies, which also showed that phototoxicity was mediated by ROS generation during UVA irradiation [1,9,36]. The low concentration (less than 50 µg/mL) used for all nano-TiO₂ treatments did not affect proliferation after 24 h of treatment, but P25 caused severe phototoxicity in the presence of UV light. Therefore, 50 µg/mL nano-TiO₂ P25 was used for subsequent experiments.

Although the principle of nano-TiO₂ phototoxicity is still debated, ROS are considered to be a potential mechanism for nano-TiO₂ phototoxicity. In this study, DCFH-DA was used to estimate ROS generation. Significantly increased ROS abundance was observed in HaCaT cells after treatment with nano-TiO₂ P25 under UV irradiation (Figure 6). Treatment with UV and nano-TiO₂ together enhanced the level of ROS production in HaCaT cells by approximately 10-fold in comparison with that of the control group. Excessive ROS and reactive nitrogen species (RNS) have been reported as fundamental mechanisms underlying nanomaterial toxicity; they induced apoptotic signaling and various pathologies by disrupting intracellular redox homeostasis and inducing irreversible oxidative modifications of lipids, proteins or DNA [12]. However, during apoptosis, glycosylation changes occur with cell shrinkage, nuclear condensation and DNA disruption. Cells from different histological origins undergoing apoptosis and primary necrosis induced by TPEN and heat treatment had an α2,6-sialylated lactosaminic structure [20]. However, few studies have paid attention to the toxic effects of nanomaterials on glycans. Glycans perform functional and regulatory roles in various physiological processes. Glycan structure is defined by the expression of nucleotides in the corresponding gene and environmental factors [37]. Sialylation is an important glycosylation modification that plays an important role in cell signaling [38], cell adhesion [39], cell recognition [21], ageing, and senescence [40]. Considering the importance of sialylation, we investigated changes in sialylation on HaCaT cells treated with nano-TiO₂ and UV irradiation. Cells treated with UV or nano-TiO₂ P25 showed altered sialylation levels, while the groups treated with A25 or R25 showed no significant alterations in sialylation. However, only the abundance of α2,6-linked sialic acids was changed; no differences in α2,3-linked sialic acids were observed in the groups treated with P25 and UV (Figure 3). The group treated with UV and nano-TiO₂ P25 showed a higher level of sialylation in comparison with those of the other groups, whereas the UV group and nano-TiO₂ P25 group each showed a slight increase. The changes in sialic acid abundance on HaCaT cells followed a pattern similar to that of the changes in ROS generated by UV and nano-TiO₂ P25, which indicated that the changes in sialic acids may be related to ROS generation.

To confirm the hypothesis that ROS are involved in changes in sialylation induced by UV and nano-TiO₂ P25, we used vitamin C and ROSup to treat HaCaT cells, followed by detection of changes in α2,6-linked sialic acids and α2,3-linked sialic acids. The positive control ROSup group showed

a significant 6-fold increase in sialylation in comparison with the control group. Vitamin C, as an antioxidant and free radical scavenger, is widely used to protect bio-membranes from peroxidative damage [31]. VC can decrease ROS abundance to inhibit cellular damage [32,41]. As shown in Figure 7, VC protected cells by reducing the abundance of intracellular ROS and decreasing the abundance of α 2,6-linked sialic acids by approximately 4-fold in comparison with that of the group that was not treated with VC. However, VC did not produce a similar effect on α 2,3-linked sialic acids. VC inhibited apoptosis caused by nano-TiO₂ phototoxicity and increased cell viability by 25%. These results demonstrate that ROS generation affected the expression of α 2,6-linked sialic acids, but had no effect on the expression of α 2,3-linked sialic acids. In addition, VC reversed the abnormal expression of α 2,6-linked sialic acids caused by nano-TiO₂ and inhibited apoptosis. Changes in sialylation may be mediated by sialyltransferase in a manner regulated by ROS production. Overexpression of sialylated antigens has been reported to cause cancer, and our results suggest that clearing ROS with an antioxidant like VC can reduce sialic acid expression and thus reduced the risk of cancer. This study demonstrated that the effects of ROS generated by nanomaterials and UV irradiation on sialic acids may be a new mechanism of nanomaterial toxicity and provide insight into methods of enhancing the safety of nanomaterials by utilizing antioxidant agents to reduce ROS abundance. Moreover, change in sialic acids could be a marker of nanomaterial toxicity. Our study is the first to assess the influence of nano-TiO₂ P25 on oligosaccharides and provides a foundation for research into the impact of nanomaterials and the development of new strategies for mitigating their toxicity.

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