Controlled killing of human cervical cancer cells by combined action of blue light and C-doped TiO₂ nanoparticles

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Abstract

In this study, C-doped TiO₂ nanoparticles (C-TiO₂) were prepared and tested as a photosensitizer for visible-light-driven photodynamic therapy against cervical cancer cells (HeLa). X-ray diffraction and Transmission Electron Microscopy confirmed the anatase form of nanoparticles, spherical shape, and size distribution from 5 to 15 nm. Ultraviolet–visible light spectroscopy showed that C doping of TiO₂ enhances the optical absorption in the visible light range caused by a bandgap narrowing. The photo-cytotoxic activity of C-TiO₂ was investigated in vitro against HeLa cells. The lack of dark cytotoxicity indicates good biocompatibility of C-TiO₂. In contrast, a combination with blue light significantly reduced the survival of HeLa cells: illumination only decreased cell viability by 30% (15 min of illumination, 120 μW power), and 60% when HeLa cells were preincubated with C-TiO₂. We have also confirmed blue light-induced C-TiO₂-catalyzed generation of reactive oxygen species in vitro and intracellularly. Oxidative stress triggered by C-TiO₂/blue light was the leading cause of HeLa cell death. Fluorescent labeling of treated HeLa cells showed distinct morphological changes after the C-TiO₂/blue light treatment. Unlike blue light illumination, which caused the appearance of large necrotic cells with deformed nuclei, cytoplasm swelling, and membrane blebbing, a combination of C-TiO₂/blue light leads to controlled cell death, thus providing a better outcome of local anticancer therapy.

Graphic abstract

Keywords C-doped TiO₂ · Photodynamic therapy · Blue light · HeLa · Autophagy · Reactive oxygen species

Abbreviations

•OH	: Hydroxyl radicals
ANOVA	: Analysis of variance
AO	: Acridine orange
ATCC	: American Type Culture Collection

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AVOs	Acidic vesicular organelles
CB	Conduction band
C-TiO ₂	Carbon-doped titanium dioxide
DCF	Dichlorofluorescein
DCFH-DA	2,7-Dichlorofluorescein diacetate
DEPMPO	5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic spectroscopy
FBS	Fetal bovine serum
H ₂ DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
HeLa	Henrietta Lacks (in the text referred to the human epithelial cervical cancer cells)
HRTM	High-resolution transmission electron microscopy
LSCM	Laser scanning confocal microscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NPs	Nanoparticles
PBS	Phosphate-buffered saline
PDT	Photodynamic therapy
PI	Propidium iodide
PS	Photosensitizer
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SRB	Sulforhodamine B
TBHB	Tert-butyl hydroperoxide
TEM	Transmission electron microscopy
TiO ₂	Titanium dioxide
UV	Ultraviolet
UV-Vis	Ultraviolet-visible light
VB	Valence band
XRPD	X-ray powder diffraction

1 Introduction

TiO₂ nanoparticles (NPs) have great potential for various biological applications, such as drug delivery [1–3], cell imaging [2, 4, 5], biosensors [6–8], and photodynamic therapy (PDT) [2, 9, 10]. In recent years, PDT has been vastly explored as less damaging to normal tissues than surgery, radiation, or chemotherapy to treat various cancer types. PDT employs the photosensitizer (PS), which in the presence of molecular oxygen exhibits photo-cytotoxic activity by generating reactive oxygen species (ROS). In other words, upon light irradiation, PS triggers a set of chemical reactions that produce ROS resulting in cells' death by necrosis, apoptosis, and/or autophagy. PDT has demonstrated its advantages for treating surface tumors such as melanoma. The most important are: no scarring effect after

healing, tolerance to repeated doses, and no need for significant investments for its implementation and maintenance [11]. Nevertheless, PDT is not restricted to melanoma cancer treatment, as the optical fiber may be applied for selective illumination at the site of challenging accessible tumors. Considering that only a colposcope for the laser optical path is required to access the cervical tumor, PDT has been successfully applied in clinics for carcinoma in situ and dysplasia of the uterine cervix [12]. As it may preserve fertility and is more comfortable, this therapy is an excellent alternative to the clinically used treatments for eliminating cervical cancer, known as the third most common cause of cancer incidence and mortality in women worldwide [13]. However, clinically applied PDT treatments have two significant drawbacks: PSs internalize in normal and tumor cells unselectively and have demonstrated undesired dark cytotoxicity.

Hence, metal oxide semiconductor-based NPs have drawn much attention as possible PSs and cytostatic carriers. They exhibit unique chemical and physical properties due to the high density and limited size of corner or edge on the surface site and have a large surface-to-volume ratio, photo-responsivity, and possibility of surface modification. The most investigated nano-sized metal oxides for PDT are mesoporous SiO₂, TiO₂, and ZnO. For our investigations, we have selected TiO₂, typical n-type semiconductor material, as it is cost-effective, photo-stable, and chemically inert. Furthermore, multiple studies have confirmed that photoexcited TiO₂ NPs effectively eliminate cancer cells [14–16]. Still, this therapy might be challenging to implement in clinical applications because of the high bandgap energy (3.2 eV) and the necessity to apply UV light for photo-stimulation. In addition to being harmful, UV light cannot penetrate deeply through human tissues; thus, TiO₂-based photo-therapy is restricted to near-to-surface tumors. Therefore, modifications of TiO₂ to extend its responsiveness to visible light have become one of the most important goals to increase the applications of TiO₂. Doping of NPs, particularly with p-block elements, is one option to modify the photodynamic response of TiO₂ by shifting the bandgap to lower energies, i.e., to the visible light region [17–19]. Whereas nitrogen doping is the most studied approach [17, 20, 21], carbon(C)-doping remains less explored up to date [22, 23].

In this work, we have produced C-doped TiO₂ NPs (denoted as C-TiO₂), for which we have shown that they are biocompatible yet effective as a PS in the light-driven treatment of the human cervical cancer cells line—HeLa. Furthermore, we have tested C-TiO₂ photosensing properties in the visible region using blue light to reach the absorption efficacy of C-TiO₂. For these NPs, we determined morphology, surface properties, and optical properties. Moreover, in vitro cytotoxicity, photo-cytotoxicity, cell internalization, and morphology of HeLa cells were investigated after treatment and illumination. The combined therapeutic effect was

compared through MTT assay, SRB assay, laser scanning confocal microscopy (LSCM), and fluorescence microscopy. Furthermore, we studied ROS production by electron paramagnetic spectroscopy (EPR) and ROS cell assay as the possible involved mechanisms for the photokilling effect by C-TiO₂.

2 Materials and methods

2.1 C-TiO₂ synthesis

We prepared C-TiO₂ by the method reported by Ren et al. [24] from amorphous TiO₂ NPs, which were produced by controlled hydrolysis of titanium isopropoxide (97%, Aldrich) in ethanol. Briefly, ethanol was mixed with 0.1 M aqueous KCl solution, followed by adding 2.2 mL of titanium isopropoxide under magnetic stirring. The white precipitate was formed after stirring for 10 min and left for 24 h (aging). First, the suspension was collected by filtration and over-dried at 60 °C. Next, C-TiO₂ was synthesized by thermal treatment of the mixture of amorphous TiO₂ and glucose (22:1 mass ratio) at 160 °C for 12 h. After cooling, the product was collected, washed with water and ethanol, and dried at 60 °C.

2.2 Characterization of C-TiO₂

X-ray powder diffraction (XRPD) analysis was carried out with a Philips PW 1050 diffractometer described in [25]. The diffraction patterns were collected at 25 °C with a scanning step of 0.05° and a dwell time of 50 s. The size, shape, and appearance of the TiO₂ NPs were checked by transmission electron microscopy, TEM (JOEL JEM-2100F). The surface charge was measured by zeta potential (Zetasizer Nano ZS90, Malvern) in the manner we previously reported [10]. Finally, the optical properties of the C-TiO₂ were checked with UV–Vis spectroscopy at the Perkin Elmer Lambda spectrophotometer.

2.3 Cell culture

HeLa cell line was obtained from American Type Culture Collection (ATCC, CCL-2). HeLa cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glucose (4500 mg/L), L-glutamine, 10% fetal bovine serum (FBS), penicillin, and streptomycin (10,000 U/mL and 10 mg/mL, respectively) and humidified under 37 °C and 5% CO₂ (Heraeus, Hanau, Germany). Cells were grown until reaching 80% confluence with normal morphology for further experiments.

2.4 Cyto- and photo-cytotoxicity of C-TiO₂

For the analysis of the concentration-dependent effect of the C-TiO₂, cells (6.25×10^3 cells/cm²) were treated with three concentrations of C-TiO₂ suspension in ultrapure water in the 1:9 suspension to medium proportion (v/v), so the final treatments concentrations in the medium were 8, 80, and 160 µg/mL. Treated cells were incubated for 48 h.

To examine photo-cytotoxicity, we preincubated HeLa cells with the water suspension of C-TiO₂ (160 µg/mL) for 1 h. For comparison, HeLa cells were treated and incubated with the same concentration of colloidal TiO₂ NPs, which we had tested previously [3]. The cells were then exposed to blue light (450 nm, 120 µW, power meter NOVA II, Ophir) for 15 min. To obtain the specific wavelength of the light, we have used the shortpass 450 nm filter (ThorLabs), which transmitted the light from an incandescent light bulb (100 W) from 400 to 450 nm. Two pieces of fused silica lenses formed the concentrated parallel light beams. Photo-cytotoxicity was determined after 48 h. Three types of control were used: non-treated cells, C-TiO₂ without illumination, and illuminated cells.

For (photo-)cytotoxicity, all cell groups were assayed in triplicate in the three independently conducted experiments.

Cytotoxic effects of C-TiO₂ in HeLa cells were assessed and measured (microplate reader—Bio-TekSynergy™ HT, Bio-Tek® Instruments) by a modified MTT test by the procedure described earlier [10]. Cell viability upon light illumination was determined by a sulforhodamine B (SRB) assay described previously [26] by Wallace, VICTOR2 1420 Multilabel Counter, Perkin Elmer.

2.5 HeLa cell C-TiO₂ internalization by the imaging microscopy

HeLa cells were grown in Petri dishes and, after reaching 80% confluence, the medium was replaced by a medium containing C-TiO₂ (160 µg/mL) and incubated for 1 h. The cells were then washed three times with PBS, and the medium was replaced by a C-TiO₂ free DMEM. The uptake of C-TiO₂ by HeLa cells was observed by LSCM (Olympus, FV-300, IX71; Olympus, Tokyo, Japan) equipped with a 405 nm continuous laser excitation (Coherent, Palo Alto, USA) and a water immersion objective (×60, NA = 1.2). The images of C-TiO₂ were acquired through two channels: the reflecting signal was obtained with no filter, whereas, the cell morphology was obtained in a transmission channel by the differential interference contrast (DIC) micrographs. ImageJ software (Fiji) was applied for merging two channels and for the red-colored presentation of the NPs. The scanned area was 115 × 115 µm, with a resolution of 512 × 512 pixels.

2.6 Cells' death modalities induced by C-TiO₂ and/or blue light

To get a better insight into the cells' death modalities, we have applied AO/PI cell staining method. First, HeLa cells were stained with acridine orange (AO) and propidium iodide (PI) according to standard procedures [27, 28]. Next, stained HeLa cells were analyzed under an epifluorescent microscope Axioimager A1 (Carl Zeiss, Jena, Germany) equipped with the ISIS imaging software package (Meta-Systems, Altussheim, Germany). Afterward, treated and illuminated cells were incubated for 48 h under the standard culture conditions. After that, cells were detached by trypsin (Trypsin–EDTA solution, Gibco), blocked with a medium supplemented with 10% FBS, transferred into tubes, and centrifuged for 5 min at 600×RCF, and washed with PBS. After adding the same volumes of each fluorescent dye (10 µL, 10 µg/mL), the cell suspension was dropped onto microscopic slides, covered, and analyzed under the microscope within 15 min. At least 100 cells per treatment were counted and analyzed.

2.7 Visible light-driven ROS production of the C-TiO₂

To assess the ROS production by EPR, we added DEPMPO (5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) [29] to the C-TiO₂ (1 µg/mL) suspension in deionized water. Afterward, the mixture was transferred into the gas permeable Teflon tubes (Zeus Industries Inc., Orangeburg, USA) inserted into a special quartz tube in the EPR spectrometer (Bruker ELEXSYS-II E540, Germany) operating at X-band. EPR experimental settings were as follows: center field 3503.70 G, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 2G, microwave frequency 9.85 GHz. During measurements, one sample was continuously irradiated with the visible light source (120 µW), and the other was kept in the dark.

2.8 Intracellular ROS production

We have used Fluorometric Assay Kit (Elabsciences, Houston, TX, USA) to evaluate intracellular ROS levels. We measured the dichlorofluorescein's fluorescence (DCF), the intracellular hydrolysis and oxidation product of 2,7-dichlorofluorescein diacetate DCFH-DA. HeLa cells (3.75×10^4 cells cm⁻², triplicate) were incubated with DMEM without phenol red (Biological Industries, Beit-Haemek) containing DCFH-DA and C-TiO₂, treated and illuminated as described. After irradiation and cells' double washing with PBS, fluorescence was determined by measuring excitation absorbance and emission absorbance at 485 nm and 535 nm wavelengths, respectively. Tert-butyl hydroperoxide (TBHP),

an analog of hydroperoxide, served as a positive control. The experiments were repeated three times independently, whereas each experiment was performed in triplicate.

2.9 Statistical analysis

Statistical differences between various treatments were assessed using one-way analysis of variance (ANOVA) with Tukey's multicomparison post-test using GraphPad Prism 8.0.1. P-values < 0.05 were considered significant.

3 Results and discussion

3.1 Size and crystalline structure of C-TiO₂

Figure 1(a) shows the TEM image of C-TiO₂, whereas, Fig. 1(b) shows the high-resolution TEM (HRTM) image of C-TiO₂. The obtained images revealed a nearly spherical shape of the C-TiO₂ (average size 5–15 nm), with the lattice spacing of 0.35 nm, corresponding to the anatase (101) plane. The anatase phase of C-TiO₂ was confirmed by XRPD, Fig. 1(c). All detected diffraction peaks represent the tetragonal anatase form of TiO₂ [25]. Results show that C-doping does not influence the crystal structure of neat TiO₂. The peaks are sharp, with (101) of anatase as the highest peak (Fig. 1(c)). Thus, we suppose that these are the most exposed facets of the materials for any photo-driven reactions.

The electrical property of C-TiO₂ was determined by zeta potential, which is the indicator of colloidal stability (at pH = 7.0). Surface charge affects various biological processes associated with NPs, such as cellular uptake and cytotoxicity. The zeta potential of C-TiO₂ was -11.0 ± 0.6 mV, which refers to negatively charged NPs with incipient stability.

3.2 Optical properties of C-TiO₂

The absorbance spectra of undoped and C-doped TiO₂ are presented in Fig. 2(a). The undoped TiO₂ displayed an absorption edge at about 400 nm. On the other hand, the C-TiO₂ showed an increase of the absorption broadband in the visible range, implying that the C-doping of TiO₂ enhanced the optical absorption, especially in the blue-light wavelength range between 380 and 500 nm. Moreover, we assume that the decrease of the energy bandgap is related to preventing electron–hole recombination and Ti–C bond formation.

Bandgap narrowing in C-TiO₂ was confirmed using the Tauc equation:

Fig. 1 Crystalline structure of C-TiO₂. TEM (a) and HRTM (b) image of C-TiO₂; (c) XRPD pattern of C-TiO₂

Fig. 2 Optical properties of pure TiO₂ and C-TiO₂. UV–Vis absorption spectra of the TiO₂ and C-TiO₂ (a) and optical bandgap calculation for non-doped and C-TiO₂ using the Tauc plot method for indirect-allowed transitions (b). A schematic representation of C-TiO₂ bandgap narrowing and production of ROS by irradiating with photons ($h\nu$) having energies $\geq E_g$. Eg stands for the bandgap energy between the valence band (VB) and the conduction band (CB) (c)

$$(\alpha h\nu)^n = A(h\nu - E_g),$$

where α is the absorption coefficient, A is the absorption constant, and n indicates indirect ($n = 1/2$) or direct ($n = 2$) allowed transitions. The bandgap energy (E_g) is determined using the optical absorption coefficient α from the experimental absorbance. The plot of the variation of $(\alpha h\nu)^{1/2}$ versus photon energy ($h\nu$) from the spectra data of pure anatase TiO₂ and C-TiO₂ are presented in Fig. 2(b). E_g values are obtained by extrapolating the curve's linear region to the abscissa ($\alpha = 0$).

The evaluated bandgap of pure anatase TiO₂ is 3.30 eV, which corresponds well to the other literature data [30, 31]. On the other hand, a direct interaction between C and Ti atoms on the surface of the NPs caused the bandgap narrowing of the C-TiO₂ as the obtained value was 2.97 eV. The literature suggests that bandgap narrowing by doping TiO₂ with p-block elements results from the formed VB by mixing the dopant p-states with the p-states of oxygen [32]. The energy bands of TiO₂ and C-TiO₂ are shown in Fig. 2(c). The VB of TiO₂ corresponds to O 2p states, and the CB corresponds to Ti 3d states, whereas, the VB of C-TiO₂ is

filled with C 2p states, and its position is above the TiO₂ VB. The bandgap narrowing decreases electron transition energy between the VB and CB, shifting the photoresponse towards the blue region (Fig. 2(c)). It has been shown that nitrogen and carbon doping could cause the narrowing of the bandgap of TiO₂ by uplifting the position of VB while preserving the position of CB [20, 33]. Hence, we assume that C-doping enhances the visible light absorption of TiO₂ due to the effect of C 2p states hybridization. This result is also consistent with the calculation data given in the literature [34, 35].

3.3 Cellular internalization of C-TiO₂

Cellular uptake determines PSs' bioactivity, which is crucial for photodynamic tumor therapy. Thus, we have studied C-TiO₂ internalization and accumulation in cancer cells. Cell uptake of C-TiO₂ by HeLa cells after 1 h of incubation was examined by confocal fluorescence microscopy (Fig. 3(b)). Since TiO₂ is nonfluorescent and can't be detected by fluorescence imaging, we have presented the confocal micrograph as an overlay of the two channels—DIC and reflection channel in which the C-TiO₂ is shown in red for better observation. The micrograph revealed, besides cells' preserved morphology, the presence of the NPs in both extra- and intracellular space. Inside the cell, the particles seem to be mainly located around the cells' membrane, whereas, the particles' aggregates are placed in the outer cells' space. It has been reported that the internalization of TiO₂ occurred through clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis [36]. Xie et al. showed that surface charge plays an essential role in the uptake of NPs by HeLa cells by the mechanism that differs in dependence of their surface charge. They found that the clathrin-dependent pathway is related to the positively charged NPs, whereas the caveolin-dependent endocytic pathway is a dominant pathway of internalizing TiO₂-COOH with a

negative charge. For the negatively charged C-TiO₂, the last mentioned is the expected dominant pathway of the internalization. On the other hand, NPs are found at the extracellular surface of the cells despite extensive washing, as the clusters of NPs are probably remained attached to the cell membrane, being unable to be internalized.

3.4 Cyto- and photo-cytotoxicity of C-TiO₂ and blue light

We assessed the viability of HeLa cells incubated with three different concentrations of C-TiO₂ by the modified MTT test after 48 h. Figure 3(a) shows no cytotoxicity after 48 h of incubation with all tested concentrations, confirming that C-TiO₂ possess high biocompatibility. Therefore, we selected the maximal tested concentration (160 µg/mL) for further tests of the application of C-TiO₂ as the PSs.

In vitro photo-cytotoxicity was done by SRB assay after treatment with C-TiO₂/blue light and TiO₂ NPs/blue light (15 min, 120 µW) for comparison. The results are given in Fig. 4(a), and it is evident that the blue light illumination without C-TiO₂ treatment also affected the cells' viability (67.8% viable HeLa). However, the cells treated with C-TiO₂/blue light showed a statistically significant decrease in cell viability compared to illuminated control, leaving 40% viable cells. In contrast, treatment with non-doped TiO₂ NPs and blue light revealed no significant cytotoxicity compared to the blue light illumination control. Hence, HeLa cells' viability was considerably reduced by the combined treatment with C-TiO₂ and blue light illumination.

It was showed that low-intensity blue light irradiation could be effective in killing various types of cancer cells, such as lymphoma [37], skin tumors [38], colorectal cancer [39], human fibrosarcoma [40], and leukemia [41]. This treatment has several potential advantages over traditional X-rays. For example, blue LED light affects cell growth, proliferation, and apoptosis of cancer cells by regulating

Fig. 3 The effect of C-TiO₂ on HeLa cells. C-TiO₂ dose-dependent viability (8, 80, and 160 µg/mL) expressed as the percentage of the viability of control cells. Data were analyzed using one-way ANOVA with the Tukey's test and considered non-significant (ns) for $p \geq 0.05$ (a). Confocal microscopic images of HeLa cells treated with C-TiO₂ (red) for 1 h (b)

Fig. 4 The effect of the combined treatment of C-TiO₂/blue light on the HeLa cells. Photo-cytotoxicity of C-TiO₂ (160 μg/mL) induced by blue light compared to non-doped TiO₂ NPs (a). Data were analyzed using an one-way ANOVA with the Tukey's test; non-significant (ns) — $p \geq 0.05$, significant — $p < 0.05$ (*), and $p < 0.001$ (***). From (b) to (e) representative photomicrographs of AO/PI stained HeLa cells: (b) untreated cells — green, ordinary morphology, the yellow

arrows — cells with AVOs staining, (c) C-TiO₂ treated cells — normal cells are present, but also early (white arrow) and late (red arrow) apoptotic cells are observed; (d) blue light illuminated HeLa cells and (e) C-TiO₂/blue light treated HeLa cells — all types of cell death are seen as well as a large number of cells with AVOs (yellow arrows), indicating activation of autophagy. The scale bar is 30 μm

Fig. 5 Distribution of cell size (μm) in different treatments measured by ISIS imaging software package (MetaSystems, Altlussheim, Germany)

autophagy and mitochondria-mediated signaling pathways. Moreover, a combination of blue LED light with drugs/PSs was promising in treating different cancers [42, 43].

We used live/dead AO/PI cell staining to obtain better insights into the cell death modalities of HeLa cells induced by treatment with a C-TiO₂/blue light combination. The different staining pattern was revealed for cells without C-TiO₂ treatment, and blue light irradiation (Fig. 4(b)) and treated cells (C-TiO₂, blue light, and blue light/C-TiO₂; Fig. 4(c)–(e), respectively). Control HeLa cells displayed normal cell morphology and cell size (9.4 ± 0.4 μm) (Fig. 5).

Acidic vesicular organelles (AVOs) were detected at a low level, around 5% (Fig. 6.) attributed to basal autophagy described in HeLa cells [44]. After the treatment with C-TiO₂, 2.9% of early (green cells with condensed nuclei) and 6.7% of late apoptotic cells (shrunken red cells with condensed nuclei and disturbed morphology) were observed (Figs. 4(c), Fig. 6.) with an average cell size of 9.0 ± 0.5 μm, Fig. 5. Blue light-treated cells are large with deformed nuclei (32 ± 1 μm, Fig. 5.), cytoplasm swelling, and membrane blebbing (Fig. 4(d)), indicating necrosis (32.6%, Fig. 6.) with many AVOs and suggesting the activation of cytoprotective autophagy in response to increased ROS production [45, 46]. Previous research demonstrated that increased ROS production damages the subcellular compartments, triggering autophagy as a survival response. Autophagy is the recycling of damaged cellular compartments and biomolecules initiated in stressed cells to avoid apoptosis. When the cellular damage becomes too excessive, necrotic cell death is promoted [47]. Oh et al. [37] showed that blue LED irradiation induces cell death via the mitochondria-mediated pathway in conjunction with autophagy and increased intracellular Ca²⁺ levels. In our work, the combined blue light/C-TiO₂ treatment (Fig. 4(e)) induced a significant increase in the number of cells with AVOs (yellow arrows) to 36.4%, Fig. 6., which indicates activation of autophagy. Apoptosis and necrosis were also observed; 9.9%, 19.0%, and 4.9% for the early, late apoptosis and necrosis, respectively (Fig. 6). Still, the moiety of necrotic cells treated with C-TiO₂/blue light was lower

Fig. 6 Quantification of AO/PI stained HeLa cells presented as percentages of viable, early/late apoptotic, and necrotic cells after the treatments with C-TiO₂, blue light, and the combined treatment with blue light/C-TiO₂

than illumination only, suggesting a switch to controlled cell death. The diameter of C-TiO₂/blue light treated cells was also smaller ($15.9 \pm 0.4 \mu\text{m}$, Fig. 5). Considering that necrosis of malignant cells causes inflammation and promotes tumor spread and growth, a C-TiO₂/blue light combination provides a better outcome of anticancer therapy by inducing autophagy and a controlled cell death mechanism.

3.5 Evaluation of photo-induced ROS generation

We studied C-TiO₂ photo-induced ROS generation since that is the leading cell injury mechanism induced by different NPs [48]. Blue LED light is reported to have phototoxic and anti-proliferative effects through the generation of intracellular ROS. Increased levels of intracellular ROS expression were found in various types of tested cancer cells (mouse preadipocytes, prechondrocytes, myoblasts, mesenchymal stromal cells, lung cancer cells, insulinoma cells, fibroblasts, human cervix adenocarcinoma cells, macrophages differentiated from lymphocytes, and rat basophilic leukemia cells) after blue laser illumination [49]. Since the optical absorption edge shifted from UV to visible region for C-TiO₂, we expected that blue light illumination would enhance the C-TiO₂ induced ROS production, which is the leading cause of induced HeLa cells' death. To explore this hypothesis, we evaluated the effect of blue light illumination on intracellular ROS production with and without preincubation of the cells with C-TiO₂.

DCFH-DA was used to estimate blue light triggered intracellular ROS production for the HeLa cells incubated with C-TiO₂. 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) penetrates the cells and is essentially nonfluorescent. Upon the acetate groups' cleavage by intracellular esterases and oxidation, H₂DCFDA converts into the highly fluorescent DCF. Figure 7(a) shows that C-TiO₂ causes the most extensive intracellular ROS production equal to an illuminated positive control (TBHP) under the blue light illumination. Without the blue light irradiation, ROS levels of HeLa cells incubated with C-TiO₂ were unaltered compared

to control (Fig. 7(a)), proving that C-TiO₂ could not stimulate intracellular ROS production in the dark. Blue light only also could cause intracellular ROS production. Still, the effect is less pronounced, indicating that the C-TiO₂/blue light combination induced the most severe responses. These results agree with the cytotoxicity and photo-cytotoxicity results and may explain the outcomes shown in Fig. 4(a).

Upon the blue light illumination, the electrons are promoted from the VB to the CB, generating highly reactive electron (e⁻)/hole (h⁺) pairs, which generated ROS in the cell culture medium. Produced ROS then randomly react with cellular lipids, proteins, and nucleic acids, damaging these macromolecules and leading to cell death. Higher ROS production ensures more efficient cell killing, Fig. 7(a) and Fig. 4(a).

The use of fluorescent probes and chemiluminescent assays is a simple and easy way of detecting intracellular ROS in cellular systems, but there are limitations and sources of artifacts [50]. In addition, ROS are short-lived and chemically distinct entities, difficult to detect in dynamic complex environments, such as biological systems. On the other hand, the EPR technique employed to study ROS generation mediated by nanomaterials has several advantages compared to fluorescent probes. Most importantly, EPR provides a more direct and chemically specific method for detecting ROS and identifying the exact type of free radical species, unlike fluorescent probes that give only information about the total amount of ROS produced by C-TiO₂ and blue light illumination. Therefore, to investigate the light-induced ROS liberation potential of C-TiO₂, EPR spectroscopy was employed. The spin-trapping method was applied to detect hydroxyl radicals (•OH) formation using DEPMPO as a spin-trap precursor. C-TiO₂ illuminated with the visible light (120 μW) for 10 min in the presence of DEPMPO led to an immediate increase in the typical eight-line signal of the spin adduct DEPMPO/OH [51], indicating the formation of •OH, Fig. 7(b). The charge compensation mechanism dominates the formation of DEPMPO/OH from the reaction between DEPMPO and water on the C-TiO₂ surface. The generated

Fig. 7 ROS generation evaluation. Intracellular ROS production assessed by the Fluorometric Assay Kit (a); ROS generation assessed by EPR (b). Fluorescence value of ROS in C-TiO₂ preincubated HeLa cells followed by the blue light illumination. Samples were treated using a DCFH-DA probe, and the intracellular ROS production was quantified. The fluorescence value in C-TiO₂/blue light treated HeLa cells was compared to the values in control, illuminated control, and

C-TiO₂-treated cells. TBHP is a positive control. Data were analyzed using an one-way ANOVA with the Tukey's test and considered non-significant (ns) — $p \geq 0.05$, significant — $p < 0.05$ (*), and $p < 0.0001$ (****) (a). EPR spectra of DEPMPO/OH adduct obtained from C-TiO₂ water solution in the dark are shown with the black line. In contrast, EPR spectra with continuous visible light illumination are red. The blue line is the EPR spectrum of DEPMPO/OH adduct (b)

•OH is the most reactive free radical with the highest 1-electron reduction potential and is primarily responsible for the aerobic organism's cytotoxic effect [52].

As already mentioned, when illuminated by blue light, photo-induced electrons and holes could be created and transferred to the surface of C-TiO₂. The photogenerated holes on the C-TiO₂ surface can further react with surrounding water to generate powerful •OH and other oxygen radicals. Multiple studies show that all forms of TiO₂ (rutile, anatase, and P25) irradiated with light can produce hydroxyl radicals. Furthermore, physicochemical factors, including the size, shape, composition, and surface coating, and doping of NPs affect ROS levels significantly [53].

Both applied methods confirmed ROS production after illumination of C-TiO₂ with visible light, leading to oxidative stress and HeLa cells death.

4 Conclusion

In the presented work, we demonstrated that carbon doping of TiO₂ NPs resulted in the higher absorbance of TiO₂ NPs in the visible region. Moreover, our study confirmed the anti-cancer effects of C-TiO₂/blue light combined treatment of HeLa cells. Furthermore, the significant photokilling effect

is associated with an increased ROS generation and the activation of autophagy as the predominant mechanism for cell death. Since autophagy is the desired cancer cell death mechanism, this system can be applied in cervical cancer therapy after fine-tuning towards better NPs uptake and short illumination times. However, further studies are needed to elucidate the effects of blue light with different power and illumination times, producing other photo-cytotoxic results. Also, in the continuation of this work, we will focus on the additional methods of modifying C-TiO₂, such as co-doping with other elements, to shift the bandgap towards the red-end of the spectrum.

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Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

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

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References

- Abbasi, A. (2019). Chapter 7—TiO₂-based nanocarriers for drug delivery. In S. S. Mohapatra, S. Ranjan, N. Dasgupta, R. K. Mishra, & S. Thomas (Eds.), *Nanocarriers for Drug Delivery* (pp. 205–248). Amsterdam: Elsevier.
- Flak, D., Yate, L., Nowaczyk, G., & Jurga, S. (2017). Hybrid ZnPc@TiO₂ nanostructures for targeted photodynamic therapy, bioimaging and doxorubicin delivery. *Materials Science & Engineering, C: Materials for Biological Applications*, 78, 1072–1085. <https://doi.org/10.1016/j.msec.2017.04.107>
- Nešić, M., Žakula, J., Korićanac, L., Stepić, M., Radoičić, M., Popović, I., Šaponjić, Z., & Petković, M. (2017). Light controlled metallo-drug delivery system based on the TiO₂-nanoparticles and Ru-complex. *Journal of Photochemistry and Photobiology A: Chemistry*, 347, 55–66. <https://doi.org/10.1016/j.jphotochem.2017.06.045>
- Yurt, F., Ocakoglu, K., Er, O., Soylu, H. M., Ince, M., Avci, C. B., Kurt, C. C., Sari, F. A., Colak, S. G., & Gunduz, C. (2019). Evaluation of photodynamic therapy and nuclear imaging potential of subphthalocyanine integrated TiO₂ nanoparticles in mammary and cervical tumor cells. *Journal of Porphyrins and Phthalocyanines*, 23, 908–915. <https://doi.org/10.1142/S1088424619500639>
- Wei, B., Dong, F., Yang, W., Luo, C., Dong, Q., Zhou, Z., Yang, Z., & Sheng, L. (2020). Synthesis of carbon-dots@SiO₂@TiO₂ nanoplatform for photothermal imaging induced multimodal synergistic antitumor. *Journal of Advanced Research*, 23, 13–23. <https://doi.org/10.1016/j.jare.2020.01.011>
- Sakib, S., Pandey, R., Soleymani, L., & Zhitomirsky, I. (2020). Surface modification of TiO₂ for photoelectrochemical DNA biosensors. *Medical Devices & Sensors*, 3, e10066. <https://doi.org/10.1002/mds3.10066>
- Wang, J., Xu, G., Zhang, X., Lv, J., Zhang, X., Zheng, Z., & Wu, Y. (2015). Electrochemical performance and biosensor application of TiO₂ nanotube arrays with mesoporous structures constructed by chemical etching. *Dalton Transactions*, 44, 7662–7672. <https://doi.org/10.1039/C5DT00678C>
- Mavrič, T., Benčina, M., Imani, R., Junkar, I., Valant, M., Kralj-Iglič, V., & Iglič, A. (2018). Chapter three—Electrochemical biosensor based on tio₂ nanomaterials for cancer diagnostics. In A. Iglič, M. Rappolt, & A. J. García-Sáez (Eds.), *Advances in Biomembranes and Lipid Self-Assembly* (pp. 63–105). London: Academic Press. <https://doi.org/10.1016/bs.abl.2017.12.003>
- Shah, Z., Nazir, S., Mazhar, K., Abbasi, R., & Samokhvalov, I. M. (2019). PEGylated doped- and undoped-TiO₂ nanoparticles for photodynamic Therapy of cancers. *Photodiagnosis and Photodynamic Therapy*, 27, 173–183. <https://doi.org/10.1016/j.pdpdt.2019.05.019>
- Matijević, M., Nakarada, Đ, Liang, X., Korićanac, L., Rajsiglova, L., Vannucci, L., Nešić, M., Vranješ, M., Mojović, M., Mi, L., Estrela-Lopis, I., Böttner, J., Šaponjić, Z., Petković, M., & Stepić, M. (2020). Biocompatibility of TiO₂ prolate nanospheroids as a potential photosensitizer in therapy of cancer. *Journal of Nanoparticle Research*, 22, 175. <https://doi.org/10.1007/s11051-020-04899-3>
- Chizenga, E. P., Chandran, R., & Abrahamse, H. (2019). Photodynamic therapy of cervical cancer by eradication of cervical cancer cells and cervical cancer stem cells. *Oncotarget*, 10, 4380–4396. <https://doi.org/10.18632/oncotarget.27029>
- Muroya, T., Suehiro, Y., Umayahara, K., Akiya, T., Iwabuchi, H., Sakunaga, H., Sakamoto, M., Sugishita, T., & Tenjin, Y. (1996). Photodynamic therapy (PDT) for early cervical cancer, Gan to Kagaku ryoho. *Cancer & Chemotherapy*, 23, 47–56.
- ESMO, Cervical Cancer Ranked in the Top Three Cancers Affecting Women Younger Than 45 Years, (n.d.). Retrieved March 10, 2021, from <https://www.esmo.org/oncology-news/cervical-cancer-ranked-in-the-top-three-cancers-affecting-women-younger-than-45-years>.
- Kubota, Y., Shuin, T., Kawasaki, C., Hosaka, M., Kitamura, H., Cai, R., Sakai, H., Hashimoto, K., & Fujishima, A. (1994). Photokilling of T-24 human bladder cancer cells with titanium dioxide. *British Journal of Cancer*, 70, 1107–1111. <https://doi.org/10.1038/bjc.1994.456>
- Zhang, A.-P., & Sun, Y.-P. (2004). Photocatalytic killing effect of TiO₂ nanoparticles on Ls-174-t human colon carcinoma cells. *World Journal of Gastroenterology*, 10, 3191–3193. <https://doi.org/10.3748/wjg.v10.i21.3191>
- Cai, R., Hashimoto, K., Kubota, Y., & Fujishima, A. (1992). Increment of photocatalytic killing of cancer cells using TiO₂ with the aid of superoxide dismutase. *Chemistry Letters*, 21, 427–430. <https://doi.org/10.1246/cl.1992.427>
- Galata, E., Georgakopoulou, E. A., Kassalia, M.-E., Papadopoulos-Fermeli, N., & Pavlatou, E. A. (2019). Development of smart composites based on doped-tio₂ nanoparticles with visible light anticancer properties. *Materials (Basel)*. <https://doi.org/10.3390/ma12162589>
- Basavarajappa, P. S., Patil, S. B., Ganganagappa, N., Reddy, K. R., Raghu, A. V., & Reddy, Ch. V. (2020). Recent progress in metal-doped TiO₂, non-metal doped/codoped TiO₂ and TiO₂ nanostructured hybrids for enhanced photocatalysis. *International Journal of Hydrogen Energy*, 45, 7764–7778. <https://doi.org/10.1016/j.ijhydene.2019.07.241>
- Etacheri, V., Di Valentin, C., Schneider, J., Bahnemann, D., & Pillai, S. C. (2015). Visible-light activation of TiO₂ photocatalysts: Advances in theory and experiments. *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*, 25, 1–29. <https://doi.org/10.1016/j.jphotochemrev.2015.08.003>
- Ansari, S. A., Khan, M. M., Ansari, M. O., & Cho, M. H. (2016). Nitrogen-doped titanium dioxide (N-doped TiO₂) for visible light photocatalysis. *New Journal of Chemistry*, 40, 3000–3009. <https://doi.org/10.1039/C5NJ03478G>
- Li, Z., Mi, L., Wang, P.-N., & Chen, J.-Y. (2011). Study on the visible-light-induced photokilling effect of nitrogen-doped TiO₂

- nanoparticles on cancer cells. *Nanoscale Research Letters*, 6, 356. <https://doi.org/10.1186/1556-276X-6-356>
22. Yang, C.-C., Wang, C.-X., Kuan, C.-Y., Chi, C.-Y., Chen, C.-Y., Lin, Y.-Y., Chen, G.-S., Hou, C.-H., & Lin, F.-H. (2020). Using C-doped TiO₂ nanoparticles as a novel sonosensitizer for cancer treatment. *Antioxidants (Basel)*. <https://doi.org/10.3390/antiox9090880>
 23. Dong, F., Guo, S., Wang, H., Li, X., & Wu, Z. (2011). Enhancement of the visible light photocatalytic activity of C-Doped TiO₂ nanomaterials prepared by a green synthetic approach. *Journal of Physical Chemistry C*, 115, 13285–13292. <https://doi.org/10.1021/jp111916q>
 24. Ren, W., Ai, Z., Jia, F., Zhang, L., Fan, X., & Zou, Z. (2007). Low temperature preparation and visible light photocatalytic activity of mesoporous carbon-doped crystalline TiO₂. *Applied Catalysis B: Environmental*, 69, 138–144. <https://doi.org/10.1016/j.apcatb.2006.06.015>
 25. Vranješ, M., Kuljanin Jakovljević, J., Konstantinović, Z., Pomar, A., Stojiljković, M., Mitrić, M., Radetić, T., & Šaponjić, Z. (2017). Shaped Co²⁺ doped TiO₂ nanocrystals synthesized from nanotubular precursor: Structure and ferromagnetic behavior. *Journal of Advanced Ceramics*, 6, 220–229. <https://doi.org/10.1007/s40145-017-0233-5>
 26. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., & Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI Journal of the National Cancer Institute*, 82, 1107–1112. <https://doi.org/10.1093/jnci/82.13.1107>
 27. Abdel Wahab, S. I., Abdul, A. B., Alzubairi, A. S., Mohamed Elhassan, M., & Mohan, S. (2009). In vitro ultramorphological assessment of apoptosis induced by zerumbone on (HeLa). *Journal of Biomedicine and Biotechnology*, 2009, 769568. <https://doi.org/10.1155/2009/769568>
 28. Li, T.-H., & Yan, H.-X. (2018). Antitumor- and apoptosis-inducing effects of pomolic acid against SK-MEL-2 human malignant melanoma cells are mediated via inhibition of cell migration and sub-G1 cell cycle arrest. *Molecular Medicine Reports*, 17, 1035–1040. <https://doi.org/10.3892/mmr.2017.7977>
 29. Jackson, S. K., Liu, K. J., Liu, M., & Timmins, G. S. (2002). Detection and removal of contaminating hydroxylamines from the spin trap DEPMPO, and re-evaluation of its use to indicate nitron radical cation formation and S(N)1 reactions. *Free Radical Biology & Medicine*, 32, 228–232. [https://doi.org/10.1016/S0891-5849\(01\)00795-x](https://doi.org/10.1016/S0891-5849(01)00795-x)
 30. Dubey, R. S., & Singh, S. (2017). Investigation of structural and optical properties of pure and chromium doped TiO₂ nanoparticles prepared by solvothermal method. *Results in Physics*, 7, 1283–1288. <https://doi.org/10.1016/j.rinp.2017.03.014>
 31. Singh Surah, S., Vishwakarma, M., Kumar, R., Nain, R., Sirohi, S., & Kumar, G. (2019). Tuning the electronic band alignment properties of TiO₂ nanotubes by boron doping. *Results in Physics*, 12, 1725–1731. <https://doi.org/10.1016/j.rinp.2019.01.081>
 32. Huang, F., Yan, A., & Zhao, H. (2016). Influences of doping on photocatalytic properties of TiO₂ photocatalyst, semiconductor photocatalysis in materials. *Mechanisms and Applications*. <https://doi.org/10.5772/63234>
 33. Yu, J., Dai, G., Xiang, Q., & Jaroniec, M. (2011). Fabrication and enhanced visible-light photocatalytic activity of carbon self-doped TiO₂ sheets with exposed 001 facets. *Journal of Materials Chemistry*, 21, 1049–1057. <https://doi.org/10.1039/C0JM02217A>
 34. Guo, M., & Du, J. (2013). Electronic and optical properties of C-N-codoped TiO₂: A first-principles GGA+U investigation. *International Journal of Modern Physics B*, 27, 1350123. <https://doi.org/10.1142/S0217979213501233>
 35. Zhao, C., Huang, D., & Chen, J. (2018). DFT study for combined influence of C-doping and external electric field on electronic structure and optical properties of TiO₂ (001) surface. *Journal of Materiomics*, 4, 247–255. <https://doi.org/10.1016/j.jmat.2018.05.003>
 36. Xie, J., Pan, X., Wang, M., Ma, J., Fei, Y., Wang, P.-N., & Mi, L. (2016). The role of surface modification for TiO₂ nanoparticles in cancer cells. *Colloids and Surfaces. B, Biointerfaces*, 143, 148–155. <https://doi.org/10.1016/j.colsurfb.2016.03.029>
 37. Oh, P.-S., Hwang, H., Jeong, H.-S., Kwon, J., Kim, H.-S., Kim, M., Lim, S., Sohn, M.-H., & Jeong, H.-J. (2016). Blue light emitting diode induces apoptosis in lymphoid cells by stimulating autophagy. *International Journal of Biochemistry & Cell Biology*, 70, 13–22. <https://doi.org/10.1016/j.biocel.2015.11.004>
 38. Oh, P.-S., Na, K. S., Hwang, H., Jeong, H.-S., Lim, S., Sohn, M.-H., & Jeong, H.-J. (2015). Effect of blue light emitting diodes on melanoma cells: Involvement of apoptotic signaling. *Journal of Photochemistry and Photobiology B: Biology*, 142, 197–203. <https://doi.org/10.1016/j.jphotobiol.2014.12.006>
 39. Yan, G., Zhang, L., Feng, C., Gong, R., Idiatullina, E., Huang, Q., He, M., Guo, S., Yang, F., Li, Y., Ding, F., Ma, W., Pavlov, V., Han, Z., Wang, Z., Xu, C., Cai, B., Yuan, Y., & Yang, L. (2018). Blue light emitting diodes irradiation causes cell death in colorectal cancer by inducing ROS production and DNA damage. *The International Journal of Biochemistry & Cell Biology*, 103, 81–88. <https://doi.org/10.1016/j.biocel.2018.08.006>
 40. Oh, P.-S., Kim, H.-S., Kim, E.-M., Hwang, H., Ryu, H. H., Lim, S., Sohn, M.-H., & Jeong, H.-J. (2017). Inhibitory effect of blue light emitting diode on migration and invasion of cancer cells. *Journal of Cellular Physiology*, 232, 3444–3453. <https://doi.org/10.1002/jcp.25805>
 41. Zhuang, J., Liu, Y., Yuan, Q., Liu, J., Liu, Y., Li, H., & Wang, D. (2018). Blue light-induced apoptosis of human promyelocytic leukemia cells via the mitochondrial-mediated signaling pathway. *Oncology Letters*, 15, 6291–6296. <https://doi.org/10.3892/ol.2018.8162>
 42. Yang, M.-Y., Chang, C.-J., & Chen, L.-Y. (2017). Blue light induced reactive oxygen species from flavin mononucleotide and flavin adenine dinucleotide on lethality of HeLa cells. *Journal of Photochemistry and Photobiology B: Biology*, 173, 325–332. <https://doi.org/10.1016/j.jphotobiol.2017.06.014>
 43. Waer, C. N., Kaur, P., Tumor, Z., Hui, D. D., Le, B., Guerra, C., et al. (2020). Rosmarinic Acid/ Blue Light Combination Treatment Inhibits Head and Neck Squamous Cell Carcinoma In Vitro. *Anticancer Res*, 40, 751–758. <https://doi.org/10.21873/anticancer.14006>
 44. Tuloup-Minguez, V., Greffard, A., Codogno, P., & Botti, J. (2011). Regulation of autophagy by extracellular matrix glycoproteins in HeLa cells. *Autophagy*, 7, 27–39. <https://doi.org/10.4161/auto.7.1.13851>
 45. Fang, C., Gu, L., Smerin, D., Mao, S., & Xiong, X. (2017). The Interrelation between Reactive Oxygen Species and Autophagy in Neurological Disorders. *Oxidative Medicine and Cellular Longevity*, 2017, e8495160. <https://doi.org/10.1155/2017/8495160>
 46. Yoboue, E. D., Sitia, R., & Simmen, T. (2018). Redox crosstalk at endoplasmic reticulum (ER) membrane contact sites (MCS) uses toxic waste to deliver messages. *Cell Death & Disease*, 9, 331. <https://doi.org/10.1038/s41419-017-0033-4>
 47. Ullman, E., Fan, Y., Stawowczyk, M., Chen, H.-M., Yue, Z., & Zong, W.-X. (2008). Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress. *Cell Death and Differentiation*, 15, 422–425. <https://doi.org/10.1038/sj.cdd.4402234>
 48. Z. Yu, Q. Li, J. Wang, Y. Yu, Y. Wang, Q. Zhou, P. Li. (2020). Reactive oxygen species-related nanoparticle toxicity in the biomedical field. *Nanoscale Res Lett*, 15. <https://doi.org/10.1186/s11671-020-03344-7>
 49. Kushibiki, T., Hirasawa, T., Okawa, S., & Ishihara, M. (2013). Blue laser irradiation generates intracellular reactive oxygen

- species in various types of cells. *Photomedicine and Laser Surgery*, 31, 95–104. <https://doi.org/10.1089/pho.2012.3361>
50. He, W., Liu, Y., Wamer, W. G., & Yin, J.-J. (2014). Electron spin resonance spectroscopy for the study of nanomaterial-mediated generation of reactive oxygen species. *Journal of Food and Drug Analysis*, 22, 49–63. <https://doi.org/10.1016/j.jfda.2014.01.004>
 51. Dvoranová, D., Barbieriková, Z., & Brezová, V. (2014). Radical Intermediates in Photo-induced Reactions on TiO₂ (An EPR Spin Trapping Study). *Molecules*, 19, 17279–17304. <https://doi.org/10.3390/molecules191117279>
 52. Krumova, K., & Cosa, G. (2016). Chapter 1, Overview of Reactive Oxygen Species 1–21. <https://doi.org/10.1039/9781782622208-00001>
 53. Shi, H., Magaye, R., Castranova, V., & Zhao, J. (2013). Titanium dioxide nanoparticles: A review of current toxicological data. *Particle and Fibre Toxicology*, 10, 15. <https://doi.org/10.1186/1743-8977-10-15>

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