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Comparative analysis of the relative potential of silver, zinc-oxide and titanium-dioxide nanoparticles against UVB-induced DNA damage for the prevention of skin carcinogenesis

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Abstract

Sunscreen formulations containing UVB filters, such as Zinc-oxide (ZnO) and titanium-dioxide (TiO₂) nanoparticles (NPs) have been developed to limit the exposure of human skin to UV-radiations. Unfortunately, these UVB protective agents have failed in controlling the skin cancer incidence. We recently demonstrated that silver nanoparticles (Ag-NPs) could serve as novel protective agents against UVB-radiations. Here our goal was to perform comparative analysis of direct and indirect UVB-protection efficacy of ZnO-, TiO₂- and Ag-NPs. Sun-protection-factor calculated based on their UVB-reflective/absorption abilities was the highest for TiO₂-NPs followed by Ag- and ZnO-NPs. This was further confirmed by studying indirect protection of UVB radiation-induced death of HaCaT cells. However, only Ag-NPs were active in protecting

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Author's contributions

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HaCaT cells against direct UVB-induced DNA-damage by repairing bulky-DNA lesions through nucleotide-excision-repair mechanism. Moreover, Ag-NPs were also effective in protecting HaCaT cells from UVB-induced oxidative DNA damage by enhancing SOD/CAT/GPx activity. In contrast, ZnO- and TiO₂-NPs not only failed in providing any direct protection from DNA-damage, but rather enhanced oxidative DNA-damage by increasing ROS production. Together, these findings raise concerns about safety of ZnO- and TiO₂-NPs and establish superior protective efficacy of Ag-NPs.

Keywords

Silver nanoparticles (AgNPs); Zinc oxide nanoparticles (ZnONPs); Titanium dioxide nanoparticles (TiO₂NPs); Reactive oxygen Species (ROS); Sun protection factor (SPF); DNA lesions (CPDs, 6-4 PPs)

1. INTRODUCTION

Skin cancer is the most commonly diagnosed malignancy in the United States [1]. Each year, there are more new cases of skin cancer than the combined incidence of cancers of the breast, prostate, lung and colon, thus underscoring the severity of this health problem. As per an estimate of Skin Cancer Foundation, ~3.5 million people are expected to be inflicted by skin cancer each year in the United States alone, which means more than 8,500 Americans diagnosed with this malignancy every day [2, 3]. This high incidence of skin cancer imposes significant economic burden, besides its morbidity and mortality to the cancer patients [4]. As a consequence, significant emphasis is placed on its prevention with a need to develop newer and more effective approaches to reduce overall incidence and/or halt its progression.

Exposure to Ultraviolet (UV) radiations (more specifically its UV-B component) is considered the most established etiological cause of skin carcinogenesis [5-7]. UVB irradiation harms skin cells in various ways, such as causing DNA damage, inflammation, cutaneous photoageing, oxidative stress and immunosuppression that all may contribute to the process of skin carcinogenesis [6, 8]. To limit the exposure of human skin to UV radiation, topical application of various commercial sunscreen creams is recommended by Skin Cancer Foundation and U.S. Food and Drug Administration [9, 10]. These sunscreen products contain ingredients that act as UVB filters (absorb UVB and/or cause its scattering and reflection) by forming a thin protective film on the skin surface [11, 12]. Zinc-oxide (ZnO) and titanium-dioxide (TiO₂) nanoparticles (NPs) are the most commonly used active ingredients in sunscreens due to their broad spectrum protection from UVB (absorption, reflection and scattering) as compared to organic compounds that merely absorb UVB radiation [13, 14]. The protection level of sunscreen products against UVB irradiation is reflected by a Sun Protection Factor (SPF), which is defined as the ratio of the least amount of ultraviolet energy required to produce a minimal erythema on sunscreen protected skin to the amount of energy required to produce the same erythema on unprotected skin [15, 16]. Although the available sunscreens in the market claim high SPF value, the incidence of UV-induced skin cancer has continued to increase at an alarming rate, thus questioning their true

efficacy in practical human use. Moreover, in certain instance, concerns have been raised regarding their safety in human applications as well [13].

We recently demonstrated UVB protective effects of silver nanoparticles (Ag-NPs) in human immortalized keratinocytes (HaCaT) [17, 18]. Here our objective was to conduct comparative analysis of UVB protective efficacy of Ag-NPs against ZnO- and TiO₂-NPs. We observed comparable SPF value and indirect photoprotection of Ag-, ZnO- and TiO₂-NPs; however, the latter two failed to provide any direct protection from DNA damage. On the contrary, ZnO- and TiO₂-NPs promoted UVB-induced DNA damage likely through generation of reactive-oxygen species (ROS). Moreover, Ag-NPs were largely non-toxic and protected HaCaT cells against UVB-induced DNA damage by repairing bulky DNA lesions (CPDs and 6-4-PPs) through the nucleotide excision repair (NER) mechanism. In addition, Ag-NPs also provided protection to skin cells against ROS by enhancing SOD/CAT/GPx activity. Together, these findings highlight the superior efficacy and relative safety of Ag-NPs in protection of skin cells from UVB.

2. MATERIALS AND METHODS

2.1. Reagents

Fetal-bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were procured from Atlanta Biologicals (Lawrenceville, GA) and Thermo Scientific (Logan, UT), respectively. Penstrep (50X) and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). Silver nitrate, sodium borohydride, sodium citrate tribasic dehydrates, 2', 7'-dichlorofluorescein diacetate (DCFH-DA), Zinc-oxide nanoparticles (ZnO-NPs) and titanium-dioxide nanoparticles (TiO₂-NPs) were purchased from Sigma-Aldrich (St. Louis, MO). Silver nanoparticles (Ag-NPs) were synthesized and characterized as described earlier [18]. Countess cell counting chamber slides and trypan blue were obtained from Life technologies (Grand Island, NY) and Thermo Scientific, respectively. Tris buffered saline (TBS) and Tween 20 were from Boston Bioproducts (Ashland, MA). Cell proliferation reagent (WST-1) and DNAzol was procured from Roche Diagnostics (Mannheim, Germany) and Life Technologies, respectively. ECL plus detection kit was obtained from Thermo Scientific. Following antibodies were used: Cyclobutane pyrimidine dimer (Kamiya Biomedical Company, Seattle, WA), (6-4) photoproducts (Cosmo Bio Co. Ltd, Tokyo, Japan) and 8 hydroxyguanosine (Abcam, Cambridge, MA). All respective horseradish peroxidase-conjugated secondary antibodies were procured from Santa Cruz Biotechnology (Dallas, Texas).

2.2. Cell culture

The human immortalized keratinocytes, HaCaT cells were obtained from German Cancer Research Center (Heidelberg, Germany). Nucleotide excision repair (NER)-proficient (GM637) and defective (XPA, GM04429) human fibroblast cells were obtained from Coriell Institute for Medical Research (Camden, NJ). Cells were maintained in a humidified atmosphere of 5 % CO₂ at 37 °C as monolayer culture in DMEM medium supplemented with 10 % FBS, 2 % Penstrep solution (50X). They were continuously monitored for their

typical morphology and intermittently tested for mycoplasma contamination using MycoSensor PCR assay kit (Stratagene, La Jolla, CA) as per manufacturer's protocol.

2.3. UVB-irradiation of HaCaT cells

Cells were exposed to UVB (20 mJ/cm²) using Daavlin Research Irradiator (Bryan, OH) equipped with four UVB lamps and an electronic controller to regulate UVB dosage. Majority of UVB radiations were in the range of 290-320 nm wavelengths.

2.4. Cell viability studies

Toxicity evaluation of Ag-, ZnO- and TiO₂-NPs on HaCaT cells was performed using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl) - 2H-5-tetrazolio]-1, 3-benzene di-sulfonate) kit as per manufacturer's instructions. In brief, cells (1×10⁴ cells/well) were seeded in 96 well plates, cultured overnight, and treated with various concentration of NPs (0.5 to 40 µg/mL). Subsequently, at various time intervals (12, 24 or 48 h), supernatants were aspirated; cells washed (PBS) and WST-1 reagent (100 µL) was added, incubated for 30 minutes and absorbance was measured at 450 nm wavelength (using a Bio-Rad Benchmark microplate reader). Percent viability was calculated using the formula: [(A/B) X100], where A and B are the absorbance of treated and control cells, respectively. To mimic the actual scenario of sunscreen applications and long term toxicity, cells were incubated with NPs for 3, 6 and 12 h. After respective time interval, NPs containing medium was replaced with fresh medium and cells were further incubated for 72 h, and percent viability was examined as described above.

2.5. Trypan blue dye exclusion method

The photoprotective efficacy of Ag-, ZnO- and TiO₂-NPs on UVB-induced cell death was examined by trypan blue dye-exclusion method. After harvesting, cell were suspended in 0.4 % trypan blue solution (1:1) followed by cell counting using Countess® Automated Cell Counter (Life Technologies).

2.6. Dot-blot assay

Genomic DNA was isolated from cells (treated or untreated) using DNAzol reagent as per manufacturer's instructions. Isolated DNA (200 ng) was transferred to a positively-charged nitrocellulose membrane, and fixed by baking the membrane for 30 minutes at 80 °C followed by blocking (5 % non-fat dry milk in TBST) for 2 h at room temperature (RT). Subsequently, membrane was incubated with primary antibody for overnight at 4 °C, washed with TBST and incubated with secondary antibody (HRP-conjugated) for 1 h. The membrane was processed with ECL plus detection kit and signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

2.7. Immunocytochemical analysis

To examine the UVB-induced DNA damage, cells were grown on UV transparent cover slips (Electron Microscopy Sciences, Hatfield, PA) and treated with NPs (2 µg/mL) for 3 h, prior to UVB-irradiation (20 mJ/cm²). Cells were washed with PBS, fixed with chilled methanol (at -20 °C; 15 minutes) and permeabilized (0.3 % Triton X-100; 30 minutes). DNA

denaturation was performed by treating the cells with 0.5 N HCl and 0.05 % pepsin at 37 °C for 30 minutes and cells were incubated with anti-CPDs or anti-6-4 PP's mouse monoclonal antibody (1:100 dilutions) for an hour at RT. Subsequently, cells were washed and incubated with anti-mouse secondary antibody. Cells were then counterstained with Hematoxylin, and stained cells were observed under optical microscope and imaged (Nikon Instruments, Melville, NY).

2.8. Sun protection factor (SPF)

The absorption spectra of samples in solution were obtained in the range of 290 to 320 nm, every 5 nm using NanoDrop 1000 UV-VIS spectrophotometer. Growth medium was used as a blank. SPF values were calculated by using the equation as shown below [16].

$$SPF_{\text{spectrophotometric}} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where CF = correction factor (10), $EE(\lambda)$ = erythemogenic effect of radiation with wavelength λ , $Abs(\lambda)$ = spectrophotometric absorbance values at wavelength λ . The values of $EE \times \lambda$ are constants.

2.9. Reactive oxygen species (ROS) measurement

HaCaT cells (1×10^6 /plate) were seeded in UVB transparent glass plates and incubated overnight. Subsequently, cells were treated with NPs (2 $\mu\text{g/mL}$) for 3 h prior to UVB-irradiation (20 mJ/cm^2). After 24 h, cells were incubated with DCFH-DA, in regular medium for 30 minutes at 37 °C. Following incubation, DCFH-DA containing medium was removed and cells were washed and resuspended in PBS. Fluorescence was recorded at an excitation/emission wavelength of 485 nm/530 nm by flow cytometry on a BD-FACS Canto Li (Becton-Dickson, San Jose).

2.10. Reverse transcription-real-time PCR

RNA isolation and RT PCR was performed as described earlier [19]. The sequences of specific primers used are given in supplementary table 1.

2.11. Anti-oxidant assay

Total anti-oxidant capacity of NPs pretreatment on HaCaT cells was measured by Cayman's anti-oxidant assay kit (Cayman Chemical Company, MI, USA). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® [2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] to ABTS®•+ by metmyoglobin. The amount of ABTS®•+ produced was measured spectrophotometrically at 750 nm. CAT activity was measured by Cayman Chemical Catalase Assay kit (Cayman Chemical Company) as per manufacturer's instructions. In this assay, CAT enzyme reacts with methanol in presence of H_2O_2 to produce formaldehyde which was measured spectrophotometrically with chromogen. GPx activity was measured using Cayman's Glutathione Peroxidase Assay Kit (Cayman Chemical Company) as per manufacturer's instructions. GPx catalyzes the reduction of hydroperoxides, including H_2O_2 , by reduced glutathione. SOD activity was

measured by Cayman's Superoxide Dismutase Assay Kit (Cayman Chemical Company) as per manufacturer's instructions. This method utilizes tetrazolium salt to detect superoxide radicals generated from xanthine oxidase and hypoxanthine.

2.12. Statistical analysis

Each experiment was performed independently and all the values were expressed as 'mean $\pm$ SD'. Wherever appropriate, the data were subjected to Student's t-test (unpaired two tailed). A value of $p < 0.05$ was considered as significant.

3. RESULTS

3.1. Photoprotective efficacy of Zinc-oxide, titanium-dioxide and silver nanoparticles

Since ZnO and TiO₂ nanoparticles (NPs) are known to act as UV filters and thus provide indirect physical protection from UVB-induced DNA damage [13], we compared their SPF values with that of Ag-NPs. For this, we employed an *in vitro* assay that is quick, excludes human subject involvement, and now routinely used for SPF determination [15, 16]. The SPF values for three types of nanoparticles increased in a concentration-dependent manner with TiO₂-NPs exhibiting the highest curve followed by Ag-NPs and ZnO-NPs (Figure 1A). At the highest concentration tested (150 $\mu\text{g/mL}$), ZnO-, TiO₂- and Ag-NPs had ~ 3.85 , 11.68 and 7.48 SPF values, respectively (Figure 1A). To get further direct evidence of the photoprotective efficacy of these NPs by physical mechanism, we applied a uniform layer (2 mg/cm^2) of hydrophilic cream base containing 1.0 % or 10.0 % ZnO-, TiO₂- or Ag-NPs on to the backside of bottom surface of Petri-dishes growing HaCaT cells at 70 % confluence. Subsequently, cells were exposed to the UVB irradiation (20 mJ/cm^2) from the bottom surface and cell viability was examined after 48 h. As expected, UVB-irradiated HaCaT cells, with or without hydrophilic cream base, exhibited extensive cell death (44.2 and 41.2 %, respectively). The cells grown in dishes, whose bottom surface was layered with cream, containing 10 % ZnO-, TiO₂- or Ag-NPs provided significant and comparable protection (1.41 fold in ZnO-NPs; 3.05 fold in TiO₂-NPs; 2.11 fold in Ag-NPs) against UVB-induced cell death (Figure 1B). Cream formulation with 1% ZnO-, TiO₂- or Ag-NPs; however, failed to protect the skin cells from UVB-induced cell death (Figure 1B).

3.2. Only silver nanoparticles provide biological protection against UVB-induced DNA damage essentially through nucleotide excision repair (NER) mechanism

UVB radiation-induced photodamage to epidermal DNA is considered one of the most important causes for its carcinogenic impact on skin cells [20]. Common biological consequence of UVB irradiation is the formation of cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) lesions [21, 22]. We, therefore, performed immunocytochemical staining for CPDs and 6-4PPs using specific antibodies to examine the protective role of ZnO-, TiO₂- or Ag-NPs on UVB-mediated DNA damage. The result shows strong and intense staining of CPDs and 6-4PPs in the UVB-irradiated cells as compared to that in non-irradiated controls. Ag-NPs treatment prior to UVB irradiation resulted in significant reduction in the number of CPDs- as well as 6-4PPs-positive cells (Figure 2A), which was further confirmed by dot-blot assay (Figure 2B). However, we observed minimal

or no DNA damage protection in UVB-irradiated cells that were pre-treated with ZnO- or TiO₂-NPs (Figure 2A and 2B).

The nucleotide excision repair (NER) mechanism is mainly responsible for repairing UVB-induced bulky DNA lesions, in particular CPDs and 6-4 PPs [23, 24]. Therefore, we examined the expression of key NER genes (*XPA*, *XPC* and *RPA1*) by RT-PCR in UVB and NPs-treated skin cells. UVB-irradiation caused slight increase in the levels of NER genes in HaCaT cells, likely as counter-defense mechanism, however, their expression was significantly elevated in Ag-NPs pretreated and UVB-irradiated HaCaT cells (2.46 fold in *XPA*; 2.29 fold in *XPC*; 2.18 fold in *RPA1*) (Figure 3A). No significant change in NER gene expression was observed in ZnO- or TiO₂-NPs pretreated cells. To further confirm the involvement of NER mechanism in the repair of CPDs and 6-4-PPs, we examined the effect of NPs pretreatment on UVB-induced CPDs and 6-4-PPs formation in NER-proficient and -deficient cells using dot blot analysis (Figure 3B). Significant less CPDs or 6-4-PPs formation was observed in UVB-irradiated NER-proficient cells that were pretreated with Ag-NPs. However, NER-deficient cells do not show any protective effect of Ag-NPs pretreatment against UVB-induced direct DNA damage. Altogether, these results suggest that only Ag-NPs are capable of protecting the cells from UVB-induced DNA-damage by repairing bulky DNA lesions (CPDs and 6-4-PPs) through NER mechanism.

3.3. Zinc-oxide and titanium-dioxide nanoparticles promote, while silver nanoparticles suppress reactive oxygen species generation

UVB-induced generation of reactive oxygen species (ROS) acts as powerful oxidant resulting in oxidative DNA-damage including single- and double-strand breaks, DNA-protein cross-linking, deletion mutations, and generation of oxidized nucleotides such as 8-hydroxyl-2-deoxyguanine (8-OHdG) - a miscoding lesion causing G to T transversion [25]. Therefore, we examined the effect of ZnO-, TiO₂- or Ag-NPs on the ROS levels in HaCaT cells with or without UVB irradiation. As expected, UVB irradiation significantly enhanced ROS levels (~ 8.28 fold) in HaCaT cells whereas, those pretreated with Ag-NPs experienced only minimal induction in ROS levels (~ 2.84 fold) suggesting its anti-oxidant efficacy (Figure 4A). In contrast, the cells treated with ZnO- or TiO₂-NPs not only exhibited increase in basal intracellular ROS content (~ 2.1 and 1.8 folds, respectively), but also had greatly pronounced ROS induction in cells upon UVB irradiation (~ 13.8 and 13.0 folds, respectively). To examine the oxidative DNA damage, 8-OHdG adducts were detected by dot-blot analysis. The level of 8-OHdG adducts in DNA were higher in ZnO- or TiO₂-NPs-treated cells upon UVB irradiation as compared to UVB alone (Figure 4B). Not only this, ZnO- and TiO₂-NPs treatment also led to the formation of 8-OHdG even in the absence of UVB exposure. However, formation of 8-OHdG was significantly reduced in UVB-irradiated cells that were pretreated with Ag-NPs (Figure 4B). These findings suggest that Ag-NPs are also effective in protecting HaCaT cells from UVB-induced oxidative DNA damage, whereas ZnO- or TiO₂-NPs enhance oxidative DNA damage by increased ROS production.

3.4. Silver nanoparticles enhance SOD/CAT/GPx activity to counterbalance the oxidative stress generated by UVB irradiation in HaCaT cells

Considering the observed differences in ROS levels in HaCaT cells pretreated with Ag-, ZnO- or TiO₂-NPs, we next examined their comparative total anti-oxidant activity (TAC) which is measure of anti-oxidant activities of enzyme systems (GSH reductase, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, vitamin E, etc.) and proteins (albumin, transferrin, etc.). TAC of HaCaT cells was significantly decreased (~ 3.68 fold) upon UVB irradiation as compared to that in untreated cells (Figure 5A). Ag-NPs pretreatment successfully restored the TAC of UVB-irradiated cells. However, ZnO- and TiO₂-NPs treatment not only decreased the basal TAC (~ 1.38 and 1.34 folds, respectively) of HaCaT cells, but caused a more pronounced decrease upon UVB irradiation (~ 6.0 and 6.1 folds, respectively) (Figure 5A). Next, we determined the effect of NPs pretreatment on the expression of genes encoding for enzymes of major anti-oxidant defense system [*SOD* (superoxide dismutase), *CAT* (catalase) and *GPx* (glutathione peroxidase)]. UVB irradiation slightly enhanced the gene expression level of these anti-oxidant enzymes as compared to untreated cells. However, Ag-NPs pretreated cells exhibited slightly increased expression of *CAT* and *GPx* along with a noteworthy increase in the expression of *SOD* (p value = 0.04). Conversely, ZnO- and TiO₂-NPs pretreated cells did not show any significant effect on the expression level of these enzymes (Figure 5B). Interestingly, when we examined the enzymatic activity of SOD, CAT and GPx, it was decreased (1.37, 1.21 and 1.76 folds, respectively) in UVB-irradiated cells, whereas increased (1.85, 2.33 and 1.44 folds) in Ag-NPs pretreated cells with relative to control cells. In contrast, ZnO- and TiO₂-NPs pretreatment led to further decrease in the activity of these enzymes (Figure 5C).

3.5. Toxicity evaluation of silver, zinc-oxide and titanium-dioxide nanoparticles in HaCaT cells

Toxicity is always a major issue for human applications of metal nanoparticles. So, we next evaluated the toxicity of Ag-, ZnO- and TiO₂-NPs by WST1 assay. HaCaT cells treated with various concentrations (0.5 to 40 µg/mL) of Ag-, ZnO- or TiO₂-NPs were monitored for their viability. Ag-NPs treatment for 12 h did not cause any toxicity in HaCaT cells, whereas those treated for 24 h and 48 h exhibited some toxicity (87 % and 73 % cell viability, respectively), at the highest concentration (40 µg/mL) tested in our experiment (Figure 6A). In contrast, ZnO- and TiO₂-NPs treatment significantly reduced the cell viability and less than 45 % cells were viable after 24 h and 48 h treatment at the highest concentration (40 µg/mL). In addition, we treated HaCaT cells with these NPs for relatively short time period (3, 6 and 12 h) and toxicity was evaluated after 72 h to mimic the practical scenario of sunscreen applications. Remarkably, no toxicity was observed in Ag-NPs treated cells after 72 h, whereas TiO₂- and ZnO-NPs treatment resulted in reduced cell viability (Figure 6B). Altogether, these data suggest that AgNPs are largely non-toxic to human keratinocytes, whereas ZnO- and TiO₂-NPs were found to be significantly toxic in comparison to Ag-NPs.

4. DISCUSSION

Skin cancer remains a major health concern, particularly in developed nations [1]. Topical sunscreens, mainly available as over the counter UVB protective agents, not only have failed

in limiting the incidence of skin cancer, but are also of concern for their unintended harmful effects. This study compared the protective efficacy of titanium-dioxide (TiO₂) and zinc-oxide (ZnO) nanoparticles (NPs) that are most common physical sun blockers in commercial sunscreens with that of Ag-NPs, the newly discovered UVB protective agents by us [17, 18].

In our study, SPF increased in a concentration-dependent manner and was observed to be the highest for TiO₂-NPs followed by Ag- and ZnO-NPs. Concentration-response curve data for the SPF of ZnO- and TiO₂-NPs is consistent with previous studies [26, 27]. ZnO- and TiO₂-NPs as well as nanodiamonds were reported to be largely ineffective at low concentrations in HaCaT and mouse embryonic fibroblasts (MEFs) cells, but exhibited enhanced protective efficiency with increasing concentrations [26]. We also observed that at very low concentration (1.0 %), the three tested metal NPs did not show photoprotective efficacy, but it was significant at higher concentration (10.0 %) suggesting dependency of SPF on the concentration of NPs. In further support, some other studies have also indicated that level of protection depends on the amount of sunscreen product applied [28, 29]. Most of the consumers actually use only 0.5–1.0 mg cm⁻² of the product instead of applying the recommended concentration (2.0 mg cm⁻²) [30], which could be one reason that even the formulation with high SPF may not be providing intended protection against UVB-induced skin damage. This also underscores the need for a better alternative having more effective active ingredients.

Our findings on the repair of CPDs and 6-4 PPs are also quite intriguing considering significant protection by Ag-NPs pretreatment on UVB-irradiated HaCaT cells, while ZnO- and TiO₂-NPs were largely inefficient. This may not be concerning as ZnO- and TiO₂-NPs do not claim direct DNA damage protection [13, 14], but it does clearly suggest an advantage for future Ag-NPs-based formulations. Our study also identified the enhanced activation of NER (as indicated by increased expression of NER genes as a possible underlying mechanism for the protective efficacy of Ag-NPs in repairing CPDs and 6-4 PPs. Nucleotide excision repair (NER) is shown to be an important mechanism in skin cells for the repair of CPDs/6-4PPs caused by UVB [23, 24].

UVB radiation is also known to cause DNA damage through ROS generation, which results from DNA oxidation [25]. Our observation on the effect of Ag-NPs on ROS levels and oxidative stress was very interesting. As per some published reports, Ag-NPs are known to cause oxidative stress by ROS generation [31]. However, we did not find any increase in ROS generation when the HaCaT cells were treated with Ag-NPs at 2 µg/mL concentration. More importantly, we observed that Ag-NPs pretreatment, in fact, protected the HaCaT cells from the oxidative DNA damage by suppressing ROS generation. Physiochemical properties of AgNPs are considered important in their pro- or anti-oxidant nature. It has been reported that Ag-NPs coated with citrate do not show any cytotoxic, genotoxic and phototoxic effects to HaCaT cells even after treatment with concentrations of upto 100 µg/mL range for 24 h [32]. Moreover, citrate-coated Ag-NPs (up to 20 µg/mL) did not induce significant ROS generation in human bronchial epithelium cells (BEAS-2B) as compared to unirradiated cells [33]. Since our synthesized Ag-NPs are coated with trisodium citrate [18], so it could be one reason for their non-toxic and non-pro-oxidant nature.

Moreover, we observed that the activity of anti-oxidant enzymes was increased in Ag-NPs-pretreated UVB-irradiated HaCaT cells, while it was decreased in cells irradiated to UVB only. Furthermore, this scenario became worst in ZnO- and TiO₂-NPs pretreated groups. The reduced activity of anti-oxidant enzymes in UVB-irradiated HaCaT cells might be due to the damaging effects of UVB. It is known that UVB can damage the proteins/enzymes by direct absorption i.e. photo-oxidation or indirectly by free radicals generation [22]. The decreased anti-oxidant activities of SOD/CAT/GPx enzymes in UVB-irradiated HaCaT cells may thus be attributed to direct UVB absorption by heme group of CAT or inhibition of the enzymatic activity by interaction with ROS (CAT and SOD) [22, 34, 35]. Further, we are not the only ones to identify the ROS-inducing effect of ZnO- and TiO₂-NPs, but others have also reported similar findings. Jaeger et al (2012) showed that TiO₂-NPs generated ~ 7.5 fold more ROS as compared to untreated human HaCaT keratinocytes [36]. Similarly, Chan et al (2011) reported that TiO₂-NPs produced significant amount of ROS and were cytotoxic [37]. Another study revealed that nano-TiO₂ induced ROS generation and caused potential damage to HaCaT cells under UVA irradiation [38]. Higher ROS generation with decrease enzymatic activity of anti-oxidant enzymes by ZnO- and TiO₂-NPs in presence of UVB might be due to their synergistic effect of phototoxicity. It has been shown that ZnO-NPs increased the phototoxicity of UVA and visible light irradiation and resulted in oxidative DNA damage in HaCaT cells [39]. TiO₂-NPs generates most harmful hydroxyl radicals (OH[•]) upon UV-irradiations, which may overtake the anti-oxidant system of cells by oxidizing proteins of the defense system [40]. Ag-NPs-induced promotion of activity of anti-oxidant enzymes could be due to their nature to act as scaffolds for various cytosolic proteins and thus controlling their cellular responses [41, 42]. Therefore, Ag-NPs might have caused an increase in activity of anti-oxidant enzymes by immobilizing them on their surface. This assumption is supported by previous observations demonstrating drastically increased efficiency of enzymes after immobilization on solid surface as compared to their free forms [43]. Indeed, immobilization of amylase on the surface of Ag-NPs increased its activity by 60-90 % [44]. The enzymatic activity of glucose oxidase, cholinesterase, monoamine oxidase, etc. is also known to be enhanced by Ag-NPs after immobilization on their surface [45, 46].

In summary, our findings have suggested that Ag-NPs are superior to TiO₂- and ZnO-NPs in protecting human skin cells from the deleterious effects of UVB. Mechanistically (Figure 7), Ag-NPs protect HaCaT cells from UVB induced direct DNA damage by NER mechanism and oxidative DNA damage by activating the anti-oxidant enzymes i.e. SOD/CAT/GPx. However, ZnO- and TiO₂-NPs not only failed in protecting the cells from the harmful effects of UVB but also induced the oxidant environment by increasing the level of ROS as well as decreasing the activities of anti-oxidant enzymes. Further, these effects were more pronounced upon UVB-irradiation, supporting the phototoxic nature of ZnO- and TiO₂-NPs. Thus, these results suggest that Ag-NPs can serve well as replacement for current major active ingredients i.e. ZnO- and TiO₂-NPs of commercially available sunscreen creams. However, future studies are necessary to examination the real potential efficacy and more clearly defined underlying mechanisms of these nanoparticles in preclinical models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- ❖ Ag-, ZnO- and TiO₂-NPs provide significant photoprotection against UVB in HaCaT cells.
- ❖ AgNPs provide biological protection against UVB-DNA damage through NER mechanism.
- ❖ ZnO- and TiO₂-NPs promote, while AgNPs suppresses reactive oxygen species generation.
- ❖ AgNPs enhance SOD/CAT/GPx activity to counterbalance UVB-induced oxidative stress.

Figure 1. Photoprotective efficacy of Ag-, ZnO- and TiO₂-NPs

(A) The absorption spectrum of NPs suspension (1 to 150 µg/ml) were scanned in the range of 290 to 320 nm for every 5 nm and SPF values were calculated as described in materials and methods. (B) Photoprotective efficacy of NPs was monitored by applying 2 mg/cm² of NPs formulation with a hydrophilic cream base on the backside of petri-dishes prior to UVB irradiation, and cell viability was examined after 48 h using trypan blue dye exclusion assay.

Figure 2. Effect of Ag-, ZnO- and TiO₂-NPs against UVB-induced direct DNA damage
(A) HaCaT cells (1×10^4) were grown on UV transparent cover slips and treated with Ag-, ZnO- or TiO₂-NPs (2 μ g/mL) for 3 h, prior to UVB-irradiation (20 mJ/cm²). After 24 h, cells were washed, fixed (chilled methanol; 15 min) and permeabilized (0.3 % Triton X-100; 30 min). The cells were incubated with anti-CPDs or anti-6-4 PP mouse monoclonal antibody (1:100 dilutions) followed by anti-mouse secondary antibodies. Immunoreactivity was visualized by using DAB Chromogen followed by hematoxylin counterstain and stained cells were observed under optical microscope and imaged (magnification X100). **(B)** HaCaT cells (1×10^6 /plate) were grown in 6 well plates and treated with Ag-, ZnO- or TiO₂-NPs (2 μ g/ml) for 3 h prior to UVB-exposure. After 24 h, Genomic DNA was isolated, immobilized on nitrocellulose membrane and fixed by baking the membrane at 80 °C for 30 min. Thereafter, membrane was blocked for non-specific binding sites and subjected to dot-blot analysis using an antibody specific to CPDs and 6-4 PPs as described in materials and methods.

Figure 3. Involvement of nucleotide excision repair mechanism in repair of CPDs and 6-4-PPs
 HaCaT cells (1×10^6 /plate) were grown in 6 well plates and treated with Ag-, ZnO- or TiO₂-NPs (2 μ g/ml) for 3 h prior to UVB-irradiation. **(A)** To examine the involvement of NER mechanism in the repair of UVB induced DNA damage, cells were harvested 24 h after UVB-irradiation and cellular RNA was extracted. The relative mRNA expression level of key NER genes (*XPA*, *XPC* and *RPA1*) was analyzed by RT-PCR. Bars represent mean $\pm$ S.D.; “*” ($p < 0.05$). **(B)** Effect of NPs pretreatment on UVB-induced CPDs and 6-4-PPs formation in NER-proficient and NER-deficient cells was examined using dot blot analysis.

Figure 4. Effect of Ag, ZnO, and TiO₂ nanoparticles on the reactive oxygen species generation

To examine the effect of NPs on ROS generation, HaCaT cells (1×10^6 /plate) were seeded in glass plates and treated with NPs (2 μ g/mL) for 3 h prior to UVB-irradiation. After 24 h (A), cells were stained with 2', 7'-dichloro-fluorescein diacetate (DCFH-DA; 20 μ M) for 30 min at 37 °C, and flow cytometry was performed. Bars represent mean $\pm$ S.D.; "*" ($p < 0.05$) (B) Dot-blot analysis was performed to examine the oxidative DNA damage by monitoring 8-OHdG.

Figure 5. Effect of NPs pretreatment on the oxidative stress generated by UVB in HaCaT cells
 To examine the comparative effect of Ag-, ZnO-, and TiO₂-NPs pretreatment on the anti-oxidant activity of cells, HaCaT cells (1×10^6 /plate) were seeded in glass plates and treated with NPs (2 μ g/mL) for 3 h prior to UVB-irradiation. (A) After 24 h, cells were lysed and the total anti-oxidant capacity was measured by Cayman's Total Anti-Oxidant Capacity Kit as per manufacturer's instructions. (B) To examine the effect of NPs treatment on gene expression level of anti-oxidant enzymes, cells were harvested 24 h after UVB-irradiation and cellular RNA was extracted. The relative mRNA expression level of *SOD*, *CAT* and *GPx* was analyzed by RT-PCR. Bars represent mean $\pm$ S.D.; “*” ($p < 0.05$). (C) The enzymatic activity of SOD, CAT and GPx was measured as per instructions by Cayman's superoxide dismutase, catalase and glutathione peroxidase assay kits, respectively.

Figure 6. Examining the cellular toxicity of nanoparticles

(A) For toxicity evaluation, HaCaT cells (1×10^6) were grown on UV transparent petri dishes and treated with different concentrations (0.5 to 40 $\mu\text{g/mL}$) of Ag-, ZnO- and TiO₂-NPs and effect on the viability of HaCaT cells was monitored by WST-1 assay after 12, 24 and 48 h. Data are expressed as mean $\pm$ SD; (n=3; p<0.05). (B) To mimic the actual scenario of sunscreen application, HaCaT cells were treated with NPs for 3, 6 and 12 h, and cell viability was observed after 72 h. Data are expressed as mean $\pm$ SD; (n=3; p<0.05).

Figure 7. Graphical representation of the UVB-protection efficacy of Silver, Zinc oxide and Titanium-dioxide nanoparticles

UVB irradiation cause DNA damage by direct absorption i.e. photo-oxidation or indirectly by free radicals generation (ROS) (A). Although, ZnO- and TiO2-NPs pretreatment provide physical protection (by absorbing or reflecting UVB) but failed to provide any protection from DNA damage albeit enhanced the oxidative DNA damage by increased ROS production (B). However, Ag-NPs protected HaCaT cells against UVB-induced DNA damage by repairing bulky DNA lesions (CPDs and 6-4-PPs) through the nucleotide excision repair (NER) mechanism. Moreover, Ag-NPs are also effective in protecting HaCaT cells from UVB-induced oxidative DNA damage by enhancing SOD/CAT/GPx activity (C).