Polyethylene glycol functionalized cerium oxide nanoparticle confer protection against UV- induced oxidative damage in skin: evidences for a new class of UV filter

Aditya Arya, Anamika Gangwar, Sushil Kumar Singh and Kalpana Bhargava

Abstract

Acute exposure to high dose of ultraviolet (UV) radiations is known to cause significant harm to skin, primarily due to the generation of free radicals and damage to DNA, which often culminate in rapid aging of the skin, or cancers. Keratinocytes being the most abundant skin’s cells are affected most by UV. Although a degree of endogenous protection is present, the vulnerability of UV-induced damaged can be minimized using protective agents. A few UV filters (organic and inorganic) have been successfully commercialized, yet, due to prevailing disadvantages such as low solubility, photostability, and aesthetic sense, suitable and more efficient UV filters continue to be explored as potential ingredients of cosmaceutical agents. A recently studied antioxidant enzyme mimetic cerium oxide nanoparticles showed emerging piece of evidence on benefits under environmental stress. However, its protective abilities as potential UV filter and therefore applicability in cosmaceutical has not yet been completely explored. This study provides a piece of evidence in support of beneficial effects of this new class of UV filters, polyethylene-glycol functionalized nanoceria (PEG-CNP) against UV - induced damage in vitro and in vivo. The nanomolar concentration of PEG-CNPs in the cell culture showed significant protection from UV exposure, by direct ROS scavenging, the rescue of cells from cell cycle arrest and DNA damage. Further, a proof of the concept study in dehaired rat skin showed that the topical application of 50 μM PEG-CNPs prevented the initial signs of UV induced damage. Unlike conventional UV filters, PEG-CNPs confer protection by internalizing the cells, and scavenging the radicals.

1. Introduction

Ultraviolet radiations represent one of the most harmful and prevalent environmental stressor among various electromagnetic radiations. UV radiations (100 nm–315 nm) are grouped into three bands, UV A (315 nm–399 nm), UV B (280–314 nm) and UV C (100 nm – 279 nm). Most of the UV C radiations are reflected back from the stratosphere while UV A and UV B from the predominant bands of UV radiation incident on the surface of earth [1], constituting 96% and 4%, respectively of total UV incident on earth surface [2]. UV B and UV Care ionizing radiation due to their high energy while UV A is a non-ionizing radiation [3]. A fraction of UV rays is able to penetrate the dermal skin layer and results in generation of reactive oxygen species (ROS) responsible for oxidative damage, alterations in gene expression, DNA damage, leading to cell inflammation, photo-ageing and carcinogenesis [4]. Due to the difference in the energies of the different UV bands their histopathological effects are also variable [5]. UV B and UV C are primarily associated with variety of skin
cancers while UV A is reportedly linked to phosensitive skin reaction, erythema and photo ageing, however some report also suggest the role of UV A in development of skin cancers [6].

The harmful effects of the ultraviolet radiations (UV), especially on the skin, are well-known for years and have been widely studied [7, 8]. Individuals exposed to high degree of UV radiation are at the risk of cutaneous melanoma [9], squamous cell carcinoma of skin [10], Basal cell carcinoma of skin [11], cortical cataract [12], pterygium [13], reactivation of herpes labialis [14]. Solar keratosis and sunburns caused by UV, have also been proposed as initiator of cancers [15]. The degree of susceptibility of an individual for aforesaid conditions primarily depend upon the UV band type, duration of exposure and the skin adaptability [5].

Mammalian skin, is a complex tissue made up of several layers primarily subdivided into outer epidermis and inner dermis. Keratinocytes are the major cells, constituting 95% of the epidermis, present at the interphase of environment and organisms. Other cells in the epidermis are melanocyte, langerhans cells and merkel cells. As keratinocytes are the immediate responders and affected cell types after UV exposure on skin, they are commonly used in vitro model [16, 17]. Abilities of skin to resist the damage caused by UV radiations primarily depend upon cellular metabolites (such as melanin) and molecular defence against radicals and ions [18, 19].

Molecular mechanism explaining the damaging effects of UV radiations can be subdivided into three categories [20]. One route is direct or indirect DNA damage caused by UV radiations culminating in mutation of ‘cell-cycle regulatory genes’ and therefore causing skin cancers [21]. Second route, generation of free radicals and downstream modifications of several biomolecules inducing the molecular pathways responsible for apoptosis, therefore causing skin ageing [22, 23]. A third route may be associated with activation of inflammatory responses against UV radiations leading to erythema or in severe cases immunosuppression [24]. Although the harmful effects of UV radiation are proven, but the exact correlation between the molecular changes and nature of UV radiation remains unsolved.

Protective strategies against the UV–induced damage are primarily prophylactic, usually avoidance of UV exposure and topical application of sunscreens. Most sunscreens contain UV filters as active ingredients that absorb, reflect or dissipate UV radiation are some of the most commonly used UV protective agents [25–27]. Their efficiency depends on their spectral profile and photo-stability which should then be reflected in biological protection of underlying skin. A significant number of natural or synthetic UV filters and ROS scavengers have been suggested and successfully commercialized for human use [28]. At present, both organic and inorganic absorbers of UV radiations are constituents of commercial cosmetic formulations. Among organic UV filters, more than 30 molecules have been approved by FDA. Menthyl anthranilate, ethylhexyl dimethyl para-aminobenzoate (ED-PABA) are among commonly used filters [28, 29]. However, their use biological suitability still raise multiple considerations due to their potential ability to penetrate the skin causing chronic toxicity [30, 31], capability to affect body hormone activity [32], photochemical reactions occurring in the treated skin [33, 34]. Low water solubility and narrow protection window are few other studied factors [29]. Among Inorganic UV-filters only two compounds TiO2 and ZnO, could successfully qualify for commercialization [28].

Despite the proven advantages, the use of these filters is limited by the poor aesthetic, primarily due to their larger particle size or opaque nature. In order to improve aesthetic sense and reduce opaqueness, attempts have been made to reduce the micro size particles to nano-size [35]. However, these changes, pose some adverse effects on the efficacy of the particles, such as increased, photo-catalysis and generation of reactive oxygen species. Moreover, both ZnO and TiO2 NPs have been reported to induce (photo) cyto- and genotoxicity [35]. Also they have been sporadically observed in viable skin layers especially in case of long-term exposures and ZnO [36]. Due to these existing challenges, improvements are being made and new filters are still being explored. Recently we and several others have studied the biological benefits of cerium oxide nanoparticles, owing to their exceptional antioxidant enzyme mimetic activity and surface antioxidant features [37–40]. Therefore, cerium oxide present a promising choice of alternate UV filter, which we have explored at the proof-of-the concept level in the present study. We, explored the UV protecting capacities of polyethylene functionalized cerium oxide nanoparticles (PEG-CNPs) on adult human keratinocytes as well as albino rat skin and found that PEG-CNPs confer excellent protection against simulated UV exposure by scavenging the reactive oxygen species generated by UV radiations, enhancement of cell survival and prevention of UV-induced oxidative DNA damage. Moreover, unlike other existing UV filters, PEG-CNPs were also capable of minimizing the apoptotic cell death, and non-toxic at the concentrations used in the experiment. We, therefore propose PEG-CNPs as potential inorganic filter that could be taken forward for more pre-clinical studies.

2. Materials and methods

Polyethylene glycol, cerium (III) nitrate, disodium hydrogen orthophosphate, potassium dihydrogen phosphate, potassium chloride and sodium chloride were obtained from sigma Aldrich. Cell culture media for
human epidermal keratinocytes, carboxymethyl dichlorofluoresceindiacetate (CM-DCFHDA), DiD dyes were purchased from life technologies. Sources of other kits/reagents are specified in respective methods section.

2.1. Experiment design
The UV protective efficacy of Nanoceria was initially evaluated on human adult epidermal keratinocyte (HAEK) cell line purchased from Himedia (Himedia, India) and then tested with topical application in Rattus norvegicus. For in vivo evaluation 6 week old male Spargue Dawley rats (weight 200–250 g) were used. UV exposure limit was decided on the basis of mean UV index at high altitude during high irradiance time points of a year. Reportedly these value vary from 7–8 on UV index scale set by world health organization, that equals ~200 Wm\(^{-2}\)CIE (International committee on illumination) weighted irradiance levels. Molecular imaging system fitted with UV lamp (365 nm) was used under epilluminatation mode (Ultra Lum Inc., USA) and the distance of the exposure was set such that total irradiance is no more than 200 Wm\(^{-2}\).

For in vitro study, cultured cells were divided into four different experimental groups; a. control with no UV exposure or nanoceria treatment b. PEG-CNPs which received PEGylated nanoceria, third group was UV-30 min contained the cells were exposed to 200 Wm\(^{-2}\) UV radiation for 30 min. Fourth group was PEG-CNPs + UV, this group received 50 nM PEGylated nanoceria in culture media and then exposed to similar doses of UV as in previous group.

For in vivo studies, experimental animals were also divided into four groups Control, UV (30 min), PEG-CNPs and PEG-CNPs+UV (n = 6 in each group) as described above. PEGylated nanoceria was soluble in water and homogeneous at 50 μM concentration. The doses were decided on the basis of optimized doses from previous experiments and considering that 0.1% drug is retained in vivo we used 1000 fold higher doses in vivo [41].

2.2. Synthesis and characterization of PEG-CNPs
PEGylated cerium oxide nanoparticles were synthesized from aqueous solutions of cerium (III) nitrate and Polyethylene glycol (Sigma Aldrich, USA) using micro-emulsion method as previously described [37]. In brief, 20 ml 0.0125 M Cerium (III) nitrate was added drop by drop to 200 ml 0.02 M polyethylene glycol with continuous stirring for 0.5 h. The mixture was heated at 70 °C with continuous stirring till a light yellow suspension was obtained. Nanoparticles were separated from the suspension by centrifugation at 1500 g for 30 min, washed with deionized water and acetone and dried in vacuum. The particle size and morphology were determined using phase-contrast high-resolution TEM (Philips Technai G230 transmission electron microscope) operating at an accelerating voltage of 200 kV and equipped with selected area electron diffraction (SAED) facility was used. Zeta potential and relative hydrodynamic radius was estimated using dynamic light scattering on Nanotrac™ (Microtrac, USA).

2.3. Cell culture methods
2.3.1. Culture maintenance
Frozen vials of Human Adult Epidermal Keratinocytes (HAEK) were purchased from HiMedia labs (cat# CL006, Himedia, India). The frozen cells were immediately brought to 37 °C pelleted down by spinning at 2500 rpm for 15 min and resuspended in keratinocytes serum free media (cat # 17005-042, Life Technologies, India) Supplemented with S7 supplement as per the manufacturer’s instructions and further incubated at conventional cell culture conditions (37 °C and 5% CO\(_2\), humidified). On confluence the cells were subcultured in polystyrene dishes and used for further experiments. Cells beyond passage 5 were not suitable for experiment and showed heterogenous morphology.

2.3.2. Fluorescence microscopy
In fluorescent microscopy, samples were incubated with context specific fluorophore that included, CM-DCFHDA and DiD were excited by specific radiations, thereby emitting complementary fluorescence which was viewed in the microscope. Illumination source for the fluorescence microscope is high energy arc lamps. In order to visualize the localization of the nanoceria within the cells, the nanoceria was encapsulated within a hydrophobic cyanine dye included in VibramTM multicolour cell labelling kit (cat # V22889, life technologies, CA, USA) as per the manufacturer’s instructions.

2.3.3. Cell cycle and cell death analysis (Flow cytometry)
Cell proliferation and apoptosis assay was performed using DNA damage and cell Proliferation kit (cat #562253, BD Pharmigen, USA) as per the manufacturer’s instructions. Initially, cells were cultured in animal cell culture medium till the sub confluence and BrdU was added to culture media at a final concentration of 10 μM and incubated for 4 h (for keratinocytes), further the cells were centrifuged, fixed and permeabilized with
Cytofix/Cytoperm Plus permeabilization Buffer for 10 min at 4 °C. Then, cells were treated with 100 μl of DNase (300 μg ml⁻¹) for 1 h at 37 °C. Further, the cells resuspended in 20 μl BD Perm/Wash Buffer + PerCP-Cy™5.5 Anti-BrdU (5 μl/test) + Alexa Fluor® 647 Mouse Anti-H2AX (pS139) (5 μl/test) + PE Anti-Cleaved PARP (Asp214) (5 μl/test) and incubated for 20 min at room temperature. Finally, the cells were washed and immunofluorescence measurements were performed on multicolour flow cytometer (LSR II, BD, USA).

Suitable single colour compensation controls and gating controls were also run parallel to experiment. Data was analysed and graphical representation was prepared using FCS Express research solutions software. Nuclii were counterstained stained with DAPI and results were analyzed with a minimum of 10,000 events. Gated G1 and S populations were estimated and represented as percentage in respective quadrant.

2.4. Topical application on skin, UV exposure and histology
An aqueous suspension of PEG-CNPs (50 μM) was applied on a dehaired region of rat skin, and rats were kept under anesthesia in the UV chamber. For performing histology, animals were perfuse according to the manufacturer instructions. Initially, tissue homogenate was prepared in cold phosphate buffer saline. 50 μl of samples, standards and blank were added with sample diluents and immediately mixed with biotinylated detection antibody to each well in duplicates. Plate was incubated at 37 °C for 45 min. The mixture was then allowed to cool at room temperature followed by centrifugation at 400 g for 5 min. About 200 μl of the supernatant was removed and the absorbance was measured at 531 nm using a spectrophotometer (Vera Max microplate reader, Molecular Devices). The values were expressed in μmol/mg protein.

2.5. Oxidative stress assays
2.5.1. ROS estimation (in tissue homogenate)
10 μl of 10 μM CM-DCHF-DA was added to the 150 μl of tissue homogenate (10% w/v in RIPA buffer) and incubated for 40 min at 37 °C in amber tubes in dark. Fluorescence was measured at 488 nm excitation and 525 nm emission (LS45 Luminescence spectrophotometer, Perkin Elmer) and converted to fluorescent units per milligram of protein.

2.5.2. MDA estimation
Lipid peroxidation was measured by direct estimation of Malondialdehyde (MDA) in tissue homogenate using method suggested by Ohkawa with slight modification [42, 43]. Initially, 750 μl of trichloroacetic acid (20% in distilled water) and 750 μl of thiobarbituric Acid (0.67% in 0.05 M NaOH) were added to 250 μl lung tissue homogenate. The mixtures were incubated in a water bath at 85 °C for 45 min. The mixture was then allowed to cool at room temperature followed by centrifugation at 400 g for 5 min. About 200 μl of the supernatant was removed and the absorbance was measured at 531 nm using a spectrophotometer (Vera Max microplate reader, Molecular Devices). The values were expressed in μmol/mg protein.

2.5.3. Protein carbonylation
Protein carbonylation assay was performed using crude protein extracts using the Protein carbonylation colorimetric assay kit (Cayman, cat # 10002050) as per the manufacturer’s instruction. Samples were quantified for protein content and equal volume of protein was dispensed and added with 800 μl DNPH, incubated at room temperature for one hour and then added with 1 ml 20% trichloroacetic acid. Tubes were then centrifuges at 10,000 g for 10 min. Supernatant was discarded and pellet was resuspended in 10% TCA and incubated on ice for 10 min. Tubes were then centrifuges at 10,000 g for 10 min and pellet was washed with 1:1 ethanol: ethyl acetate mixture. Further, pellet was resuspended in 500 μl of guanidyl hydrochloride and centrifuged at 10,000 g for 10 min to remove left over debris. Finally 220 μl sample was dispensed in duplicate in 96 well ELISA plate and optical density was measured at 370 nm using spectrophotometer.

2.5.4. 8-OHdG estimation
8-Hydroxydeoxyguanosine (8-OHdG) was estimated as a measure of DNA damage caused by reactive oxygen species using 8-OHdG competitive ELISA kit (Cat # E-EL-0028, Elabsciences) as per the manufacturer’s instructions. Initially, tissue homogenate was prepared in cold phosphate buffer saline. 50 μl of samples, standards and blank were added with sample diluents and immediately mixed with biotinylated detection antibody to each well in duplicates. Plate was incubated at 37 °C for 45 min. After 3 washed with wash buffer each well was added with 100 μl HRP conjugate and again incubated at 37 °C for 30 min. Further, plate was washed 3 times and 90 μl substrate was added and incubated at 37 °C for 15 min in dark. Finally 50 μl stop solution was added to each well and optical density was measured at 450 nm immediately. 8-OHdG concentration was measured using standard curve as described. Concentration was represented as ng/mg of protein.
2.6. Statistical analysis
All the data were statistically tested before making conclusions, central tendencies mean, geometric mean, median were calculated and data were expressed as either Mean ± standard deviation or mean ± standard error of mean. Parametric or non-parametric statistical tests were performed to determine the significance, based on the data type. Most commonly, students t-test, and Analysis of variance was used with Mann Whitney’s Post hoc analysis. A p-value of less than 0.05 or confidence interval of 95% or above was considered significant.

2.7. Ethics statement
Male Sprague-Dawley rats (Rattus norvegicus) weighing 180 to 200 g (age 6 weeks) were used in this study. Rats were housed in the experimental animal facility of the institute and received standard diet and reverse osmosis water ad libitum. Constant 25 °C temperature with 12 h automatic light and dark cycles was maintained. All the animal care and experimental protocols were approved by the institutional animal ethical committee under supervision of committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

3. Results and discussion

Among several UV filters (organic and inorganic) available in market, inorganic filters are relatively newer. Klimova et al., described some of the limitations of organic filters as their low solubility in water and hence, low perfusion into the skin [44]. Inorganic filters are relatively more soluble in aqueous solvents. Despite, the advantages of inorganic filters only two compounds TiO2 and ZnO have reached the cosmetic industry [33]. The nanoformulations of TiO2 and ZnO are excellent UV filters in UVB region of UV spectra. The primary mechanism of UV absorption by these nanoparticles is attributed to their high band gap energy and capacity to reflect most of the UV radiations incident on skin [45]. Reducing the particle size of TiO2 increases the surface and permeability into the skin, but reported to cause DNA damage and generate reactive oxygen species. Other inorganic filters such as iron oxide has also been found to be UV protective [28] but, due to its non-white texture, it has been excluded from cosmaceutical applications. Nanoceria, offers and excellent choice as UV filter as it is devoid of anomalies seen in other known UV filters. Furthermore, unlike TiO2 and ZnO, nanoceria is capable of undergoing surface modification and thereby possess tunable redox chemistry, which contributes ROS scavenging capacity [40], in particular the cycling of 3+ and 4+ oxidation states of CNPs results in superoxide and catalase mimetic activities which contribute most towards their antioxidant behavior. Like TiO2, nanoceria also possess high band gap energy and direct UV absorption [46]. These advantages advocate the potentials of nanoceria as an emerging inorganic UV filter. A comparison of existing inorganic filters and CeO2 recently performed by Jung et al, suggest the importance of cerium oxide as an emerging UV-filter [47].

PEGylation of nanoparticles is known to improve biocompatibility, minimize interference with other biomolecules and increase circulation time i.e. bio persistence, therefore promoting the efficacy of nanoceria [48, 49]. PEGylated nanoceria used in this study contained mean particle size of ~7 nm, easily permeable to skin epidermis and dermis, which was observed with the help of fluorescent microscopy.

3.1. PEG-CNPs were internalized in keratinocytes and cleared rapidly
PEG-CNPs were conjugated to fluorescent dye DiD to visualize particle internalization into the skin cells. The synthesized particles were well-characterized and evaluated for toxicity and optimal dose for biological use in our previous studies [37].

The particles used in this study were predominantly in the size range of 7–10 nm as determined using TEM (figure 1(a)). Furthermore, to understand the hydrodynamic properties, relative charge and possible agglomeration, we performed dynamic light scattering measurements (DLS) which showed that average hydrodynamic diameter of the particles ~10 nm with zeta potential of ~42.4 mV (figure 1(b)) at physiological temperature, and was found to be stable for 25 days. Fluorescence microscopy of cells pretreated with PEGylated nanoceria-DiD revealed intracellular presence of nanoparticles predominantly in cytosol (figure 1(c)). Untreated cells stained with DAPI, and fluorescent particles alone were also observed under microscope as control (figure 1(d)). Moreover, we also performed the microscopy post- 48 h of the PEG-CNPs and change of media, we observed no significant appearance of particles in the cells (figure 1(d)). Based on the previous toxicological effects and safety of CNPs at the given dose [50], and the proven benefits of cerium oxide in various cell and animal models [38, 40] internalization is suggested to be useful. The internalization was also an important reason for the direct radical scavenging abilities in cytoplasm and the benefits presented in this study, which also makes CNPs different from other classes of UV filters. However, the significant rapid clearance of the particles, from the cells was observed within 48 h after the application. This observation was consistent with our previous studies of bio distribution clearance of CNPs which we tested using ICP-MS and radiolabeled imaging.
techniques [37, 51]. However some studies point out the persistence of small fraction of particles in tissues [41], which need to be answered completely before any pre-clinical claims are made.

3.2. PEG-CNPs confers protection against UV exposure in cultured keratinocytes

We, then evaluated the levels of reactive oxygen species in the cells using standard florescent dye based methods. A semi quantitative analysis of CM-DCFH-DA stained cells using flow cytometry showed that, percentage of DCF positive cells in UV exposed group was 35.88%. PEGylated nanoceria pretreatment before 30 min UV exposure however, limited the fraction of DCF positive cells to 18.64% showing a significant decrease in UV induced ROS in keratinocytes as compared to PEG-CNPs group (Figure 2(a)). Moreover, the direct visualization of cells under phase contrast and fluorescence microscope was also performed, which showed similar differences in the reactive oxygen species. A high fluorescence was observed in UV exposed cells, while PEG-CNP pre-treated cells showed reduced fluorescence as an indicative or ROS scavenging at source (Figure 2(b)). Lipid peroxidation that results from reaction of lipids with reactive oxygen species was also elevated by 2.4 fold after UV exposure, PEG-CNPs pre-treatment significantly reduced the levels of lipid peroxides (Figure 2(c)). Oxidation of protein by reactive oxygen species leads to the formation of protein carbonylation. The

Figure 1. PEG-CNPs microscopic observation and evaluation of internalization in keratinocytes. (a). Transmission electron micrograph showing PEG-CNPs with an average size distribution pattern (scale bar—100 nm) (b). Dynamic light scattering based particle size distribution (c). Fluorescent micrographs showing the internalization of PEG-CNPs labeled with DiD fluorescent dye (scale bar—50 μm). (d). Control cells with no PEG-CNPs and cells after 48 h of PEG-CNPs treatment with no red fluorescence.
concentration of protein carbonyls was elevated by 5 fold in UV exposed cells while PEG-CNP pretreatment prevented such abrupt increment in protein carbonyls \((\text{figure } 2)\).

Generation of reactive oxygen species is the primary cause of UV-induced damage in skin cells, where superoxide anion, hydrogen peroxide and the hydroxyl radicals are being primary radical generated from UV and responsible for damage \([52]\). Reactive oxygen species (ROS) are associated with premature skin aging (photo-aging), local and systemic immunosuppression, many cutaneous inflammatory disorders, and photo carcinogenesis \([53]\). Among several downstream effects, protein oxidation and lipid peroxidation are most
common damage to biomolecules [54]. Besides this, activation prostaglandin pathway and generation of cytokines has also been found to be involved in UV induced inflammatory events in skin cells [24]. PEG-CNPs being SOD and catalase mimetic augments the weakening antioxidant defence and therefore apparently curbed the ROS generation.

3.3. PEG-CNPs prevented UV induced DNA damage and apoptotic death in keratinocytes

UV insult is known to induce the DNA damage and therefore directly influences the cell cycle [54]. Cell death of keratinocytes was studied after UV exposure, utilizing BrdU incorporation followed by flow cytometry analysis (PerCPCy5.5 labelled anti BrdU antibody). (figure 3(a)). Control group showed 40.5% of cells in S-phase while this was reduced to 20.8% in UV exposed cells, showing a suppression of replication by nearly 50 percent. PEG-CNPs pretreatment rescued cells from this damage and S-phase cells showed and increase to 28.9%, an eight percent increase compared to UV exposed cells. Furthermore, on comparing the cell viability using propidium iodide (PI), it was observed that PEG-CNPs also prevented cells from UV induced cell death (figure 3(b)). Additionally flow cytometry of cells labelled with antibodies against cleaved poly (ADP) ribose polymerase (PARP), was used to determine overall degree of cell damage caused by UV that lead the cells to death. Elevated cleaved PARP is associated with rapid ATP depletion from the cell leading to cell death. We observed that fraction of PARP positive cells was 8.6% in control group, which increased to 13.7% in UV exposed cells, but in cells pretreated with PEG-CNPs was 7.3% indicating significant protection against the cellular damage (figure 3(c)). The phototoxic effects of UV radiations are caused direct absorption of incident photons within by DNA base pairs within a narrow wavelength range of UV radiations.

Some of the chemical and structural perturbations in DNA after absorption of UV radiations formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand and double strand breaks. The major class of lesions produced is cis / syn cyclobutane pyrimidine dimers (CPDs). Pyrimidine / pyrimidone photoproducts are the second most prevalent adducts formed in DNA by UVB radiation [55].

UV induced DNA damage response (DDR) is seen as induction of γH2AX in S and G1- phase cells [56]. Moreover, the quantitative estimation of γH2AX, was also performed to understand the reason behind the cytotoxic effects of nanoceria under UV exposure. Phosphorylation of γH2AX is an initial event in the DNA damage repair culminating in early apoptotic events. It was observed that UV exposure caused an increase of 34.4% in γH2AX positive cells. Control showed 4.29%. Interestingly, UV + PEG-CNPs group showed 8.6% and PEG-CNPs group showed 8.19 γ H2AX positive cells (figure 3(d)). Besides this radical induced DNA damage can be primarily indicated by formation of 8-hydroxydeoxyguanosine (8-OHdG) [54]. Although the frequency of mutations in DNA caused by UV is high, but an extensive repair mechanism counteracts the effects. Primary nucleotide excision repair mechanism that operate in mammals are transcription coupled repair [57], global genome repair [58, 59], base excision repair [60]. Estimation of 8-OHdG using ELISA showed that UV exposure for 30 min caused a 5 fold increase in the 8-OHdG content, which was significantly reduced by PEGylated nanoceria pretreatment in keratinocytes (figure 3(e)).

3.4. Topical application of PEG-CNPs confers protection against UV exposure in rat skin

In vitro data suggested a significant prevention of UV induced radical generation and subsequent cell death, we further chose to develop a proof-of-the-concept for pre-clinical studies by extending the experiment to animal skin. Unlike conventional UV filters, which provide protection by direct filtering of UV and preventing the permeation of UV into skin, PEG-CNPs showed an alternative mechanism by reversing the immediate ill effects of UV and destruction of free radicals at source, perhaps due to well-proven superoxide dismutase activity.

Therefore, the protective effect of PEG-CNPs against UV exposure was also considered and at skin experiments were performed on rat skin. De-haired rats were exposed to UV radiations for 30 min (equivalent to UV index 7). Rat skin was excised and used for biochemical and histological examinations. Skin from the animals with topical application of PEG-CNPs (labelled with DiD) were subjected to fluorescence microscopy to observe the internalization and we observed that within 30 min of topical application nanoceria permeabilized into the rat dermis (figure 4(a)). We observed that, UV exposure for 30 min equivalent to UV index of 8 caused erythema and mild skin inflammation in de-haired skin region of the control rat. Interestingly, topical application of PEG-CNPs showed no erythema suggesting powerful anti-inflammatory activity of PEGylated nanoceria (figure 4(b)).

Furthermore, we estimated the markers of oxidative stress and inflammation in the skin and compared the PEG-CNPs skin with control in each case. We observed that, level of reactive oxygen species was elevated by 1.5 fold in UV exposed skin while, part of skin that was treated with PEG-CNPs showed no significant difference from control (figure 4(c)). Furthermore, it was observed that increased ROS due to UV was also reflected in the downstream effectors of ROS. Malondialdehyde (oxidation product of lipids) was also increased by 1.5 fold in UV exposed skin (figure 4(d)). Protein carbonyl content in the skin was found to be 2.5 fold in UV exposed skin.
compared to control. PEGylated nanoceria reduced protein carbonylation significantly (figure 4(e)). We also, estimated 8-OHdG concentration, a marker of DNA damage, and found that PEG-CNPs showed significant reduction in 8-OHdG concentration when compared to UV exposed skin of control (figure 4(f)). Interestingly, PEGylated nanoceria treatment in control animals also showed a significant reduction in ROS and MDA concentrations. Several recent studies support our hypothesis of cerium oxide nanoparticles as UV filter for cosmetics. Redox-active cerium oxide nanoparticles were shown to protect human dermal fibroblasts from PQ-induced damage [61]. CeO2 nanoparticles-based topical formulations for the skin protection against organophosphates have also been studied [62] and also Cerium oxide for sunscreen cosmetics have been.

Figure 3. Analysis of UV induced cell death and DNA damage. (a) Cell cycle analysis using BrdU incorporation, cells in the G1 phase and S phase are represented in respective gated areas. (b) Histogram (right most) showing the degree of PI uptake by cells, each histogram was obtained from forward and side scatter plots of PI stained cells, gated for excluding dead cells and fraction of cells represented (left). (c) Contour plots representing the estimation of cleaved PARP levels in the cells, with respective fraction of cells represented in each quadrant. (d) Contour plots representing the estimation of γH2AX as a measure of DNA damage e. bar graph representing the quantitative estimation of 8-OHdG (Mean ± SEM) * p < 0.05, ** p < 0.01.
proposed with ultrafine particles doped with Mn^+ ceria [63]. Ragini et al studied that redox-sensitive cerium oxide nanoparticle confer protection to human keratinocytes from oxidative stress induced by glutathione depletion [64]. As indicated in our previous studies, CNPs are known to enhance the antioxidant capacity, we also checked the protein levels of catalase and superoxide dismutase using immunoblotting and observed that PEG-CNPs during the UV exposure also augmented the production of these enzymes (figure 4).

A number of studies in recent past strongly support the conclusions drawn by us in these experiments. Lu Z et al recently explored the protective role of silk-modified with CeO_2 against UV damage as well as for antimicrobial purposes [65]. Zholobak et al, suggested a combinatorial application of cerium oxide and panthenol for enhanced UV protective abilities [66]. Caputo et al demonstrated the UV-protective abilities of cerium oxide nanoparticles in Jurkat cells (human tumor T lymphocyte), HaCat cell lines (keratinocytes) and also provided a contrast with TiO_2 in terms of mutagenesis and associated UV-induced damage [67]. Yaxi Li in 2019, reported photoprotection of cerium oxide nanoparticles against UVA radiation-induced senescence of human skin fibroblasts due to their antioxidant properties [68]. These studies corroborate well with our finding, however we provide here additional in vivo conclusion on UV protective role and also the enhanced penetrance and efficacy with pegylation of cerium oxide nanoparticles, indicating a strong commercial prospect of PEG-CNPs as novel inorganic UV filter of the next generation cosmetics.

Despite these meaningful and significant conclusions, the present study had a few limitations which need to be answered in future. A comparison and contrast with existing UV filters at is required at later point of time to justify the advantage of PEG-CNPs. Nevertheless, a future work on development of skin compatible formulations with other conventional ingredients of cosmetics can add to the pre-clinical testing.

Figure 4. Analysis of UV induced damage in vivo (a). Section of detailed rat skin exposed to UV with and without pre-treatment of PEG-CNPs. Skin section representing H&E stained skin, and presence of DiD labelled PEG-CNPs. (d) Bar graph representing the relative quantity of ROS as a measure of CM-DCF measurement. (e) Bar graph representing the level of lipid peroxidation in cell lysate. (f) Bar graph representing the concentration of protein carbonyl levels in the cell lysate. (g) Representative immunoblots representing the levels of catalase and Superoxide dismutase (SOD) in the skin homogenates.
4. Conclusion

In conclusion, present study reports the benefits of PEG-CNPs against UV induced damage in skin cells as well as a preliminary analysis in rat skin. The benefits were observed at extremely low concentrations, 50 nM \textit{in vitro} and 50 \( \mu \text{M} \) \textit{in vivo}, due to redox cycling of nanoparticles and direct radical scavenging abilities. Besides this a significant protection from UV induced DNA damage, prevention of cell death was also observed in presence of PEG-CNPs (figure 5). Our study provides a new insight into the UV filtering abilities and a new direction for the development of nano-suspension based topical formulations for cosmaceutical applications, which can safely internalize into the skin cells, and harmlessly protect the skin cells from UV induced damaged.

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ORCID iDs

Aditya Arya @ https://orcid.org/0000-0002-9629-2532
Sushil Kumar Singh @ https://orcid.org/0000-0003-1418-3218
Kalpana Bhargava @ https://orcid.org/0000-0003-3629-084X

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