Assessment of phototoxicity, skin irritation, and sensitization potential of polystyrene and TiO$_2$ nanoparticles

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Abstract. The human skin equivalent model (HSEM) is well known as an attractive alternative model for evaluation of dermal toxicity. However, only limited data are available on the usefulness of an HSEM for nanotoxocity testing. This study was designed to investigate cutaneous toxicity of polystyrene and TiO$_2$ nanoparticles using cultured keratinocytes, an HSEM, and an animal model. In addition, we also evaluated the skin sensitization potential of nanoparticles using a local lymph node assay with incorporation of BrdU. Findings from the present study indicate that polystyrene and TiO$_2$ nanoparticles do not induce phototoxicity, acute cutaneous irritation, or skin sensitization. Results from evaluation of the HSEMs correspond well with those from animal models. Our findings suggest that the HSEM might be a useful alternative model for evaluation of dermal nanotoxicity.

1. Introduction

Despite wide use of NPs, few studies have reported on the toxicity (safety) of manufactured NPs to humans and the environment. Therefore, studies into in vitro and in vivo toxicity of NPs are critically needed.

Phototoxicity can be defined as a skin inflammatory reaction by topical application of chemicals, drugs, and subsequent exposure to light, particularly ultraviolet A (UVA) radiation (320–400 nm) [1,2].

The human skin equivalent model (HSEM) is well known as an attractive alternative model for evaluation of dermal toxicity. However, only a limited number of papers have suggested the usefulness of the HSEM as a screening method for determination of the dermal irritation potential of NPs [3,4]. To the best of our knowledge, little data are available on the usefulness of an HSEM for phototoxicity testing of NPs.

In the present study, using cultured keratinocytes, an HSEM, and an animal model, we investigated the dermal phototoxicity and irritation potential of polystyrene and TiO$_2$ NPs. In addition, the murine local lymph node assay has been used for evaluation of the skin sensitization potential of NPs.
2. Materials and methods

2.1. Cell culture
The human immortalized HaCaT human keratinocyte cell line was grown at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), and 1% penicillin-streptomycin (10,000 unit /ml).

2.2. Nanoparticle preparation
Polystyrene latex beads (amine-modified 50 nm labeled with blue and fluorophores) and TiO₂ were purchased from Sigma-Aldrich (St. Lois, MO, USA). Polystyrene and TiO₂ were diluted in 1X concentration of PBS. In order to produce a stable, less-aggregated nanocrystalline suspension, suspended TiO₂ in PBS were dispersed by sonication for 30 min at 4°C immediately prior to preparation of treatment-dilutions in serum-free DMEM.

2.3. Nanoparticle characterization
To obtain photographs, diameter, size distribution, and morphology information, polystyrene and TiO₂ were morphologically characterized by transmission electron microscopy (TEM) using Tecnai 20 (FEI Co., Eindhoven, Netherlands) at an acceleration voltage of 200 kV.

2.4. MTT viability assays
The thiazolyl blue tetrazolium bromide (3-[4,5]dimethylthiazol-2,5 dephenyltetrazolium bromide (MTT)) assay was used as described to determine the cytotoxicity of polystyrene and TiO₂. HaCaT cells were plated in 96-well multititer plates (5,000 cells per well) and treated with 1~100 μg/ml polystyrene and 25~1000 μg/ml TiO₂ for 24 and 48 h. MTT (0.5 mg/ml) was then added to each well, and cells were further incubated for 4 h at 37°C. Barillet et al. suggested that NPs can interact with the substrate during toxicity assessment [5]. Therefore, to reduce such a potential interference of NPs in the MTT assay, NPs were allowed to sediment for 1 h and a 50 µl solution from each well was then transferred to another plate.

2.5. EpiDerm skin irritation test
3D EpiDerm™ models (EPI-200), which were used as HSEMs, were obtained from the MatTek Corporation. On arrival, the gauze pad, which covered the top of the HSEMs, was removed; HSEMs were transferred into 6-well plates containing 0.9 ml of the pre-warmed assay medium. And HSEMs were incubated for 1 h at 37°C and 5% CO₂. The medium was then replaced with pre-warmed, fresh assay medium, and models were pre-incubated overnight at 37°C in a 5% CO₂ atmosphere. HSEMs were treated with 1000 μg/ml polystyrene, 100 μg/ml TiO₂, PBS as a negative control, or 5% SDS as a positive control on top of the models for 1 h.

2.6. Draize skin irritation test
Skin irritation potentials for polystyrene and TiO₂ were evaluated in rabbits using the Draize score test. Rabbits weighing 2.5 - 3.5 kg were acclimatized prior to the beginning of the study. On day 0 of the test period, backs were clipped free of hair. Several layers of skin were removed with adhesive tape from one half of the shaved back and areas of skin abrasion were created. Each rabbit was treated with 0.5 ml of the 1000 μg/ml polystyrene and 100 μg/ml TiO₂ solutions, which were applied over a ~6.25 cm² area of hair-free skin.

2.7. 3T3 NRU phototoxicity test
The 3T3 NRU phototoxicity test was performed as described in guidelines of the Organisation for Economic Co-operation and Development (OECD) 432 (OECD, 2004).
Absorbance was measured at 540 nm. NRU phototoxicity was assessed using photo-irritancy factor (PIF) and mean photo effect (MPE), according to a previous report [6].

2.8. EpiDerm skin phototoxicity test
HSEMs, 3D EpiDerm™ models (EPI-200), were supplied from MatTek, USA. Prior to the test, HSEMs were pre-incubated in fresh assay medium for 1 h at 37°C and 5 % CO₂. HSEMs were then transferred into 6-well plates containing 0.9 ml of the pre-warmed assay medium.

2.9. Skin phototoxicity test using an animal model
Five-week-old female, Hartley albino guinea pigs (250~300 g) were used in the study. Five female guinea pigs per group received topical application of polystyrene on the back skin, which had been clipped and shaved. Each test substance of 1000 µg/ml polystyrene and 100 µg/ml TiO₂ solutions was applied to an area measuring 1.5 cm x 1.5 cm (0.05 ml/site). Skin reactions were graded in accordance with the Draize scoring system, as follows [7,8]: Score 0: no erythema or no edema; Score 1: barely perceptible erythema or edema; Score 2: well-defined erythema or slight edema; Score 3: moderate to severe erythema or moderate edema; Score 4: severe erythema or edema. Dermal phototoxicity was tested according to the grades described in the OECD Guidelines 404 (2002).

2.10. Local lymph node assay (LLNA)
Female CBA/N mice were purchased from SLC Japan Co. Ltd (Shizuoka, Japan). Female mice were randomly allocated to four groups (four mice per group). A 25 µl volume of the concentration ranging from 10 to 1000 µg/ml polystyrene and from 10 to 1000 µg/ml TiO₂ was applied to the dorsum of both ears daily for three consecutive days. Mice were not treated with nanoparticles on day 4. A single interperitoneal injection of 0.5 ml of BrdU solution (5 mg per mouse per injection) was given to the mice on day 5. On day 6, the auricular lymph nodes from the ears of each mouse were removed, weighed, and stored at -20°C until measurement of the level of BrdU incorporation by enzyme-linked immunosorbent assay (ELISA).

2.11. Statistics
Data were analyzed using Prism 3.02 software (GraphicPad Software Inc., La Jolla, California) by one-way analysis of variance (ANOVA), followed by Dunnett’s method. P values of < 0.05 were considered statistically significant.

3. Results

3.1. MTT assay
The MTT assay was used for evaluation of the cytotoxicity of polystyrene and TiO₂. HaCaT cells were treated with medium containing various concentrations of 1~100 µg/ml polystyrene and 25~1000 µg/ml TiO₂, and cell viability was determined at 24 and 48 h. Cytotoxicity of polystyrene increased in a dose-dependent manner. However, there were no differences in cell viability for TiO₂.

3.2. EpiDerm skin irritation test
To evaluate irritation of polystyrene and TiO₂ on HSEMs, HSEMs were treated with 1000 µg/ml polystyrene and 100 µg/ml TiO₂. No significant differences in viability were observed for polystyrene and TiO₂. However, 5% SDS provided as a positive control induced a significant reduction in viability.

3.3. Draize skin irritation test
Draize skin irritation tests were conducted using polystyrene and TiO₂; 1000 µg/ml polystyrene and 100 µg/ml TiO₂ were applied to rabbit skin. However, both polystyrene and TiO₂ were found to have no irritation effect on rabbits. No observable edema or erythema was observed on the abrasion site of
rabbit skin after exposure for 24 or 72 h for any rabbit, and no skin irritation was observed after 24 or 72 h of treatment. No erythema or edema was observed on the non-abrasion site of rabbit skin.

3.4. 3T3 NRU phototoxicity test
The 3T3 neutral red uptake phototoxicity test (NRU PT) is designed to detect phototoxicity induced by polystyrene, TiO$_2$, and UVA light using an *in vitro* cytotoxicity assay in the Balb/c 3T3 mouse fibroblast cell line. Polystyrene on UV-irradiated cells was more cytotoxic than on non-UV-irradiated cells at concentrations higher than 75 μg/ml polystyrene. However, photo-irritancy factor (PIF) and mean photo effect (MPE) values of polystyrene were calculated to 1.149 and -0.005, respectively, indicating non-phototoxicity. No differences in viability were observed for TiO$_2$ on UV-irradiated cells and non-UV-irradiated cells. TiO$_2$ has an MPE of -0.046, indicating its non-phototoxicity and the PIF value of TiO$_2$ was not calculated. Therefore, both polystyrene and TiO$_2$ were found to be non-cytotoxic at the tested concentrations.

3.5. EpiDerm skin phototoxicity test
Phototoxicity testing in the HSEMs confirmed phototoxicity of the 3T3 NRU test for polystyrene and TiO$_2$. Polystyrene and TiO$_2$ did not exhibit phototoxicity in the HSEMs.

3.6. Skin phototoxicity test using an animal model
Polystyrene at 1000 μg/ml and TiO$_2$ at 100 μg/ml were evaluated for skin phototoxicity in guinea pigs. No visible changes with or without UV exposure were observed at any of the time points during evaluation of animals treated with polystyrene and TiO$_2$. No erythema or edema was detected. Results from evaluation of the HSEMs correspond well with those from animal models.

3.7. LLNA test
A non-RI LLNA test was performed for evaluation of the skin sensitization potential of polystyrene and TiO$_2$. The SI value of PC was in excess of 2, indicating a positive response of skin sensitization. However, SI values did not increase in polystyrene and TiO$_2$ and SI value of eugenol as a positive control was only increased.

4. Discussion
The skin is the largest organ of the body and an important route of entry for environmental toxicants, including NPs, into the body; as a result, assessment of skin penetration of NPs has attracted a great deal of attention. Our previous study has shown that quantum dot nanoparticles could actually penetrate through the stratum corneum of human skin [9]. Recent papers have also shown that, topically applied, NPs may permeate normal or damaged skin and induce a health risk to humans [10,11]. Despite the great potentialities of nanotechnology, few NPs have been evaluated for their dermal toxicity potential.

Using the MTT assay, we evaluated the cytotoxic effects of polystyrene and TiO$_2$ NPs. Cell viability decreased with increasing polystyrene NPs concentration in a dose-dependent manner. TiO$_2$ NPs had no effect on cell viability. Similar findings were reported by Tian Xia *et al.* (2006) who found that polystyrene NPs can induce cell death; however, TiO$_2$ NPs did not demonstrate cellular toxicity [12]. However, TiO$_2$ NPs induce allergic sensitization and lung inflammation [13]. And TiO$_2$ NPs have cytotoxicity and induce apoptosis [14].

Because they reflect bioavailability of phototoxic chemicals in the skin, HSEMs are regarded as a promising alternative approach designed to mimic *in vivo* models in humans as closely as possible [15,16]. HSEMs have been prevalidated in dermal studies, including phototoxicity, skin irritancy, and skin corrosivity of chemicals [16-18]. In the assessment for skin irritation and phototoxicity using HSEMs, cell viability was analyzed by MTT assay according to the protocol provided by the manufacturer.
Murine LLNA is used for measurement of proliferation in skin-draining lymph nodes following topical application of chemicals. The LLNA method should be used as an alternative to guinea pig models for the sensitization potential of chemicals [19,20]. Non-radioisotopic LLNA has recently been developed as a non-radioactive modification method based on assessment of BrdU incorporation into lymph node cells instead of radioisotopes (³H-methyl thymidine) [20,21].

In conclusion, findings from the present study demonstrate that polystyrene and TiO₂ nanoparticles induce no phototoxicity, acute cutaneous irritation, or skin sensitization. LLNA results from this study indicated that polystyrene and TiO₂ NPs were not in themselves dermal sensitizers.

Acknowledgements
This research was supported by a grant (09152KFDA696) from the Korea Food & Drug Administration in 2009.

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