MARKERS OF OXIDATIVE STRESS AFTER THREE DAYS OF NANOTIO$_2$ SUNSCREEN USE IN HUMANS: A PILOT STUDY

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SUMMARY

Objective: Recent experimental studies point to a high reactivity of nanoparticles and the potential of sunscreens to penetrate the skin. We measured 20 markers of oxidative stress and inflammation to find out whether skin exposure to nanoTiO$_2$ sunscreen may elevate the level of the markers in exhaled breath condensate (EBC) and urine of exposed subjects, as was suggested by our earlier study.

Methods: Six volunteers (3 males and 3 females), with a mean age of 48.0 ± 6.7 years, used commercial sunscreen for three days continuously. The first samples were collected before the test. The second samples were collected on day 4, before the sunscreen was washed off, and the third samples on day 11. The following biomarkers were measured: malondialdehyde, 4-hydroxy-*trans*-hexenal, 4-hydroxy-*trans*-nonenal, aldehydes C6-C12, 8-*iso*Prostaglandin F2α, *o*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine, 8-hydroxy-2-deoxyguanosine, 8-hydroxyguanosine, 5-hydroxymethyl uracil, and leukotrienes B4, C4, D4, and E4, using liquid chromatography-electrospray ionisation-tandem mass spectrometry.

Results: In the urine, 4-hydroxy-*trans*-hexenal was significantly higher in post-exposure sample 2, and the same trend was seen in all urinary markers. In EBC, no difference was seen between the mean values of 20 post-test markers as compared with pre-test samples.

Conclusion: This study suggests potential side effects of the sunscreen – borderline elevation of markers of oxidative stress/inflammation – which may relate to the absorption of the nanoTiO$_2$, and the non-significant difference may be explained by the small number of subjects. The effect was not seen in EBC, where nanoTiO$_2$ was not found. A larger study is needed, as according to our previous study, the beneficial effect of the sunscreen to suppress oxidative stress caused by UV radiation may be questioned.

Key words: sunscreen, nanoTiO$_2$, oxidative stress, inflammation, urine, exhaled breath condensate, nanoparticles absorption, nanotoxicology

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INTRODUCTION

Sunscreen use has been recommended as a cost-effective prevention of skin cancer. However, experimental studies have reported potentially unfavourable effects of sunscreens, and there are concerns regarding the safety of nanoTiO$_2$, because it can extend the duration of sunbathing and increase the risk of skin malignancies (1, 2). Other studies point to a high reactivity of nanoparticles and their potential to produce reactive oxygen species (ROS) (3–6), alter the skin structure (7), and/or penetrate the skin (8–10), similarly to organic components (11, 12). For particles 4–20 nm, the penetration happens mostly through the hair follicles, and particles smaller than 4 nm may pass based on the skin physiology and diffusion theory (13, 14).

One of the most frequently used nanoparticles in inorganic sunscreens is TiO$_2$, which is able to reflect, scatter, and absorb UV radiation (15); however, production of ROS capable of altering DNA has also been described (16, 17). In 2010, the International Agency for Research on Cancer reclassified TiO$_2$ as a group 2B carcinogen, i.e., possibly carcinogenic to humans (18). Additionally, in experimental studies, nanoTiO$_2$ toxicity was higher than that of bulk TiO$_2$ because of the higher reactivity of the nanoparticles due to a highly active surface area (hundreds m^2/g). Three main mechanisms have been suggested for nanoTiO$_2$: ROS production following the induction of electron-hole pairs; damage of the cell membranes due to lipid peroxidation by the attachment of nanoparticles to cells via electrostatic forces as a result of their large specific surface area; and TiO$_2$ nanoparticle attachment to intracellular organelles and macromolecules following cell membrane damage (15).

Our last study proved the skin absorption of nanoTiO$_2$ by both Ti measured by inductively coupled plasma mass spectrometry and nanoTiO$_2$ particles detected by transmission and scanning electron microscopy (TEM and SEM) in the plasma and urine in all sunscreen users. Importantly, nanoTiO$_2$ particles have not been found in exhaled breath condensate (EBC) (19) – i.e. liquid, collected during tidal breathing, presumably originating from the airway lining fluid in the form of aerosolised particles generated during the re-opening of distal airways. This excludes entering by inhalation route and documents that nanoTiO$_2$ can penetrate beyond the outer layers of stratum corneum to viable cells and reach the general circulation. The amounts, however, were very small (mean 8.9 ± 2.8 ng/ml and 6.7 ± 1.6 in plasma and urine, respectively). In that study, we tested the efficiency of nanoTiO$_2$

sunscreen to prevent systemic oxidative stress/inflammation caused by ultraviolet (UV) radiation using biomarkers in subjects' plasma, urine, and EBC. Indeed, UV exposure alone in the commercial solarium significantly increased all biomarkers in the plasma, urine, and EBC of the volunteers, but rather surprisingly, the sunscreens applied before UV exposure did not supress the significant elevation of any oxidative stress/inflammatory marker due to UV radiation.

In the urine, leukotriene C4 (LTC4) was significantly higher on day 4 ($p < 0.05$). A few markers were elevated in the post-exposure EBC samples ($p < 0.05$), but there was not a clear trend. Among them, 3-chlorotyrosine (3-ClTyr) was found on day 4, and 8-*iso*-Prostaglandin F2α (8-isoprostane), LTC4, and LTD4 on day 11.

In humans, several markers of oxidation have been used to evaluate oxidative stress in biological fluids, especially blood and urine. In proteins, 3-ClTyr is a specific molecular marker of the production of chlorinating oxidants in leukocytes; 3-nitrotyrosine (3-NOTyr) is a marker for nitration stress, which may lead to functional relationship with the neutrophilic inflammation; and *o*-tyrosine (*o*-Tyr) is an amino-acid oxidation biomarker (20). They have also been found in the EBC or sputum of patients with interstitial lung diseases (21).

For the lipid oxidation, 8-isoprostane represents an *in vivo* marker produced by free-radical lipid peroxidation of arachidonic acid. Oxidative modification of lipids occurs *in vivo* during ageing and in several disease conditions (20). Lipid peroxides are unstable indicators of oxidative stress in cells that form more reactive compounds, such as aldehydes C6-C12, malondialdehyde (MDA), 4-hydroxy-*trans*-hexenal (HHE), and 4-hydroxy-*trans*-nonenal (HNE), forming covalent adducts with biomolecules including DNA and proteins, and thus are regarded as genotoxic and cytotoxic (22). Oxidative damage to nucleic acids may be measured using 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) formed by oxidation of guanine from DNA and 5-hydroxymethyl uracil (5-OHMeU) from RNA (23).

LTs are low-molecular inflammatory biomarkers. They are primarily produced by leukocytes from the arachidonic acid and are known to have powerful effects over short distances within the body (20). They play an active role in inflammatory responses and initiate tissue repair. They are involved in the pathogenesis of different respiratory disorders, especially chronic obstructive pulmonary disease (LTB4), asthma (24), and fibrosis (25).

The aim of this study was to replicate sunscreen exposure (19) under the same conditions (identical sunscreen type, length of sunscreen exposure, and length of the study with 5/6 of the same subjects) with three collected samples using non-invasive collection methods (i.e., EBC and urine) to confirm or exclude the effect.

MATERIALS AND METHODS

Six volunteers (3 males and 3 females), all non-smokers with a mean age of 48.0±6.7 years, used commercial sunscreen containing TiO_2 nanoparticles for three days. Five out of the six subjects participated in the earlier sunscreen study. The study was approved by the Ethics Committee of Charles University according to the Helsinki criteria. All participants were informed of the study aim and signed an informed consent form prior to the beginning of the study, and they filled in a standardised questionnaire that included the personal and occupational history, acute and chronic symptoms, and medications. Their urine and EBC were subsequently collected.

The first samples of EBC and urine were taken at the beginning of the test before the sunscreen application. The sunscreen was applied to approximately 80% of the body surface twice daily and was not washed off. The volunteers wore white shirts and white trousers that remained unchanged until day 4. After the second samples were collected, the used sunscreen was thoroughly washed off. The third samples were collected on day 11 (i.e., one week after the end of sunscreen exposure).

The sunscreen type was identical to the sunscreen used in the published study (19). It had a skin protection factor (SPF) of 50. The total content of the tube was 150 g. The recommended dosage was 2 mg/cm^2 skin – i.e., approximately 6 teaspoons (30 g)/adult – to be re-applied to maintain a sufficient level typically twice a day.

EBC samples were collected using an Ecoscreen Turbo (DECCS, Jaeger, Hochberg, Germany). All subjects wore a nose clip to avoid nasal contamination and breathed tidally for approximately 15 min through a mouthpiece connected to the condenser (−20 °C), as described in our previous study (19).

An identical panel of markers of oxidative stress/inflammation was analysed in urine and EBC. These markers included MDA, HHE, HNE, aldehydes C6–C12, 8-isoprostane, *o*-Tyr, 3-ClTyr, 3-NOTyr, 8-OHdG, 8-OHG, 5-OHMeU, and LTs. The analysis was performed following solid-phase extraction (SPE) in the same laboratory using high-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (HPLC−ESI−MS/MS), using a quaternary pump, Accela 600, and Accela autosampler coupled to a triple quadrupole mass spectrometer TSQ Vantage equipped with heated electrospray ionisation (HESI) (Thermo Fisher Scientific).

Basic descriptive statistics were computed and subsequently tested for normality using the Kolmogorov-Smirnov test. For comparison of frequency counts of demographic categorical variables, Fischer's exact test was used. Differences in interval variables were tested using the Mann-Whitney U test. A paired sample t-test was used to compare pre-exposure and post-exposure values of the markers. Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using MS Excel 365, QC Expert 3.3, and SPSS version 22.0..

RESULTS

Two subjects reported allergic rhinitis without treatment, and one woman was being treated with thyroxin for hypothyroidism and with local corticosteroids for bronchial asthma; these subjects also participated in the previous study (19). No subject had symptoms of an acute viral disease, chronic bronchitis, or dyspnoea. The average total cream consumption was 131.3 ± 9.9 g, which was similar to the first study. The amylase concentration in all samples was less than 0.01% of the alpha-amylase concentration in saliva.

Mean levels of oxidative stress and inflammatory markers in the pre-exposure urine and post-exposure on day 4 (sample 2), when the sunscreen was removed, and one week later (day 11, sample 3) are shown in Figure 1 and Figure 2. Only one marker,

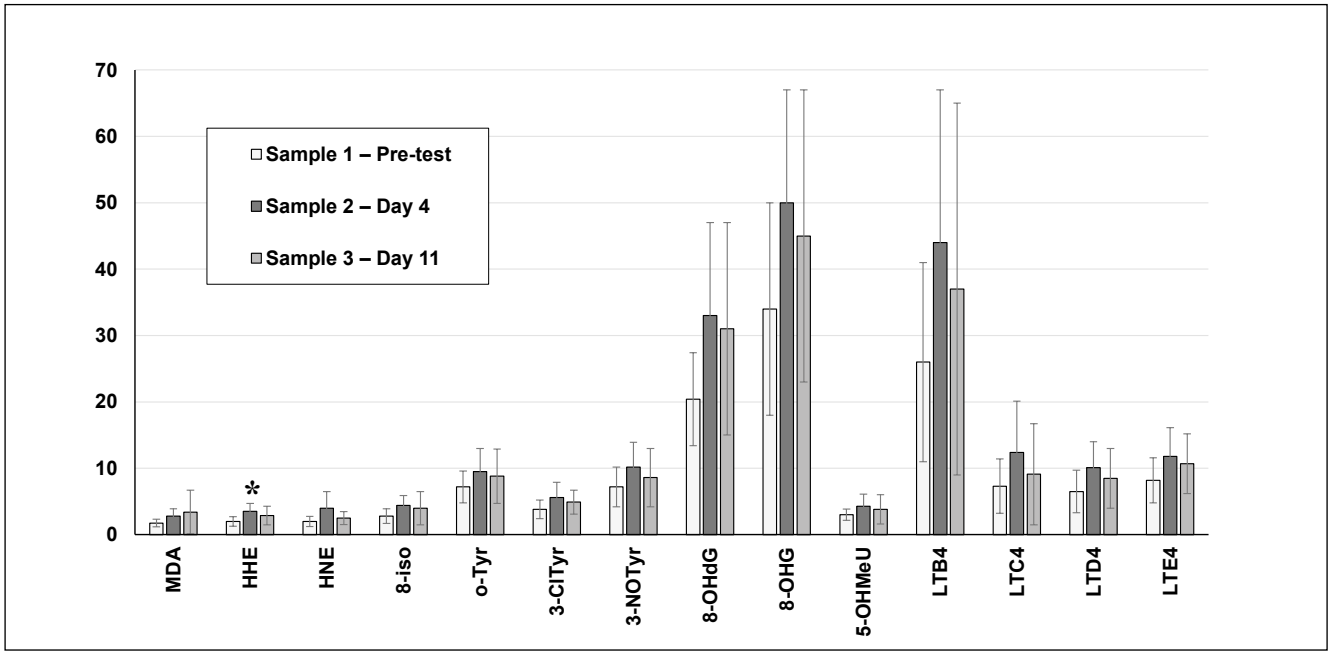

Fig. 1. *Markers of oxidative stress and inflammatory markers in urine pre-exposure (sample 1) and after sunscreen exposure (samples 2−3), mean ± confidence intervals*

MDA – malondialdehyde; HHE – 4-hydroxy-*trans*-hexenal; HNE – 4-hydroxy-*trans*-nonenal (all ng/mmol creatinine); 8-*iso* – 8-*iso*Prostaglandin F2*α*; *o*-Tyr – *o*-tyrosine; 3-ClTyr – 3-chlorotyrosine; 3-NOTyr – 3-nitrotyrosine; 8-OHdG – 8-hydroxy-2-deoxyguanosine; 8-OHG – 8-hydroxyguanosine; 5-OHMeU – 5-hydroxymethyl uracil; leukotrienes (LT) LTB4, LTC4, LTD4, LTE4 (all pg/mmol creatinine).
*p = 0.027

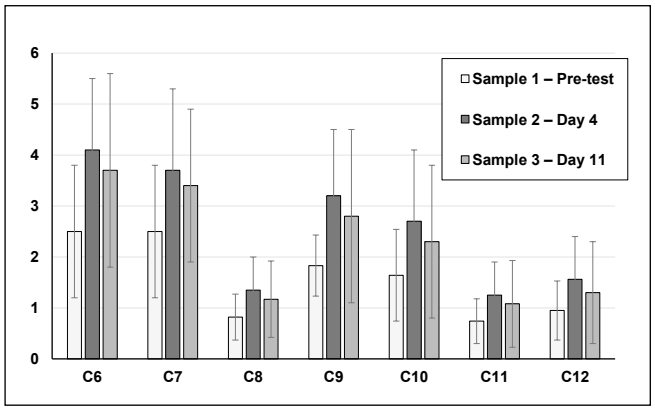

Fig. 2. *Markers of oxidative stress – aldehydes C6-C12 in urine (ng/mmol creatinine), mean ± confidence intervals*

HHE, was significantly increased in sample 2 (p = 0.027) in the urine, and a trend in the elevation of all post-exposure markers in sample 2 could be noticed in both figures in comparison with pre-exposure levels. Also, the absolute values of all markers in sample 3 were higher than in sample 1, although the difference was not significant. No statistically significant elevation of any marker of oxidative stress and/or inflammation could be seen in EBC, as presented in Figure 3 and Figure 4.

DISCUSSION

A large body of *in vivo* and *in vitro* nanotoxicology studies has shown that nanoparticles induce intracellular ROS and pro-inflammatory mediators. Much less data are available concerning studies on human subjects using mineral nano sunscreens.

It was shown that the skin absorption of nanoTiO_2 already occurs after the first 6 hours of exposure, although in a small quantity (19). This agrees with the histological proof of nanoTiO_2 particles in the viable cells of both the epidermis and dermis in the study by Tan et al. (26). This may explain the borderline elevation of the markers of oxidative stress and inflammation in the biological samples collected in this study with the limited number of volunteers using nanoTiO_2 sunscreen. Here, only one significantly elevated biomarker in the urine, HHE (p = 0.027), was found in sample 2 after three days of exposure, and although it decreased on day 11, its absolute level was still (from the statistical point of view non-significantly, p = 0.13) higher than in sample 1. All remaining urine markers showed the same trend.

Differently, no EBC markers were elevated in this study, and no positive trend was noted. Some elevations in our previous study concerned especially LTs, inflammation markers that may reflect subclinical inflammation in the respiratory system, as they are involved in the response to injury, infection, or allergy (27–29). Therefore, markers in EBC usually reflect occupational exposures of workers to nanomaterials by inhalation (30–34), including office employees exposed to nanoTiO_2 (35). Due to the potential of sunscreen in the spray form to be absorbed by inhalation, such products have been classified as potentially hazardous and are no longer allowed (36). Differently, elevated markers of oxidative stress in the plasma and urine were associated with ageing, metabolic diseases (20, 37), or systemic intoxications (38). Under the conditions of skin sunscreen exposure, the inhalational way of exposure was excluded in our last study (19), which did not find nanoTiO_2 particles in EBC,

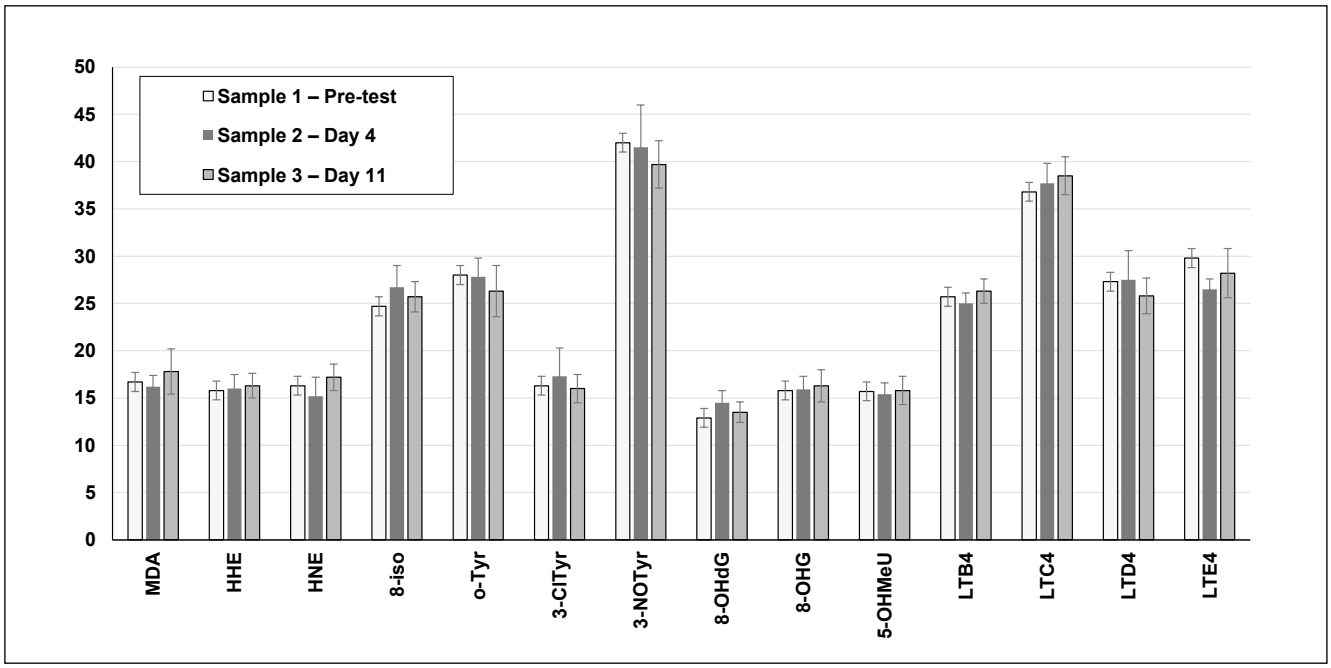

Fig. 3. *Markers of oxidative stress and inflammatory markers in exhaled breath condensate pre-exposure (sample 1) and after sunscreen exposure (samples 2−3), mean ± confidence intervals*

MDA – malondialdehyde; HHE – 4-hydroxy-*trans*-hexenal; HNE – 4-hydroxy-*trans*-nonenal (all ng/ml); 8-*iso* – 8-*iso*Prostaglandin F2*α*; *o*-Tyr – *o*-tyrosine; 3-ClTyr – 3-chlorotyrosine; 3-NOTyr – 3-nitrotyrosine; 8-OHdG – 8-hydroxy-2-deoxyguanosine; 8-OHG – 8-hydroxyguanosine; 5-OHMeU – 5-hydroxymethyl uracil; leukotrienes (LT) LTB4, LTC4, LTD4, LTE4 (all pg/ml)

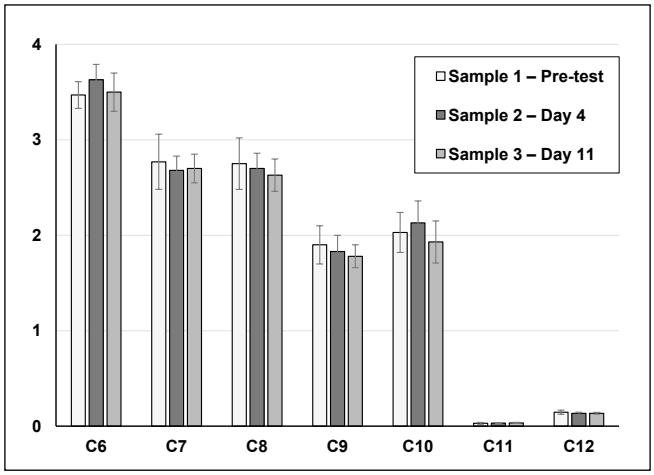

Fig. 4. *Markers of oxidative stress – aldehydes C6-C12 in exhaled breath condensate (ng/ml), mean ± confidence intervals*

but they were present in nanoTiO_2 workers at both post-shift and pre-shift (39).

Potential contamination between skin-hands-mouth cannot be completely excluded, however, the absorption from the gastrointestinal tract is very limited (40), therefore, we suppose it could bring unmeasurable plasma and urine levels. In addition, there is no explanation why it would be more pronounced in females. In addition, it would be expected, that the nanoparticles, the origin of which would be the skin-hands-mouth contamination, would keep the original size, approximately 43 nm, as oral absorption enables absorption of larger particles, with average much higher than in our plasma and urine samples. In a study in human volunteers, their oral exposure of 100-mg dose of TiO_2 particles with diameter 50–260 nm led to their appearance in the blood (41).

The main limitation of this study is the low number of exposed subjects. Based on these results, both the safety and effect of nanoTiO_2 may be questioned and should be further studied. It was shown in our previous study that they only reduced skin redness induced by UV radiation in our previous test; however, they did not lower systemic oxidative stress caused by UV radiation.

CONCLUSIONS

Since absorption of nanoTiO_2 from sunscreen occurs, systemic oxidative stress and inflammation cannot be excluded. In this study, a statistically significant increase of HHE in post-exposure sample 2 was detected. The same trend, although statistically non-significant, was seen in all remaining 19 urine markers. The inhalation route of entering the body under this condition was excluded, which agrees with the normal EBC finding in this study. A larger study is urgently needed to prove both the safety and usefulness of nanoTiO_2 sunscreens.

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Conflict of Interests

None declared

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