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Small Amounts of Zinc from Zinc Oxide Particles in Sunscreens Applied Outdoors Are Absorbed through Human Skin

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Metal oxide nanoparticles are commonly used in personal-care formulations as protective agents against exposure to ultraviolet radiation. Although previous research has concluded that nanoparticles do not penetrate healthy skin, it remains contentious whether this conclusion holds under normal conditions of sunscreen use. Humans ($n = 20$) were exposed to sunscreens containing zinc oxide (ZnO) particles to determine if Zn from the particles was absorbed through skin over five consecutive days under outdoor conditions. Two sunscreens were tested—"nano sunscreen" containing 19-nm nanoparticles and "bulk sunscreen" containing > 100-nm particles. Venous blood and urine samples were collected 8 days before exposure, twice daily during the trial, and 6 days post-exposure. As the first application in nanotechnology studies, stable isotope tracing was used where the ZnO, enriched to > 99% with the stable isotope ^{68}Zn, allowed dermally absorbed zinc to be distinguished from naturally occurring zinc. The overwhelming majority of applied ^{68}Zn was not absorbed, although blood and urine samples from all subjects exhibited small increases in levels of tracer ^{68}Zn. The amount of tracer detected in blood after the 5-day application period was ~1/1000th that of total Zn in the blood compartment. Tracer levels in blood continued to increase beyond the 5-day application phase in contrast to those in urine. Levels of ^{68}Zn in blood and urine from females receiving the nano sunscreen appeared to be higher than males receiving the same treatment and higher than all subjects receiving the bulk sunscreen. It is not known whether ^{68}Zn has been absorbed as ZnO particles or soluble Zn or both.

Key Words: **human; *in vivo*; nanoparticles; sunscreen; short-term absorption; zinc isotopes; zinc oxide.**

The incidence of skin cancer is increasing globally. In Australia, more than 1600 people died from melanoma and nonmelanoma skin cancer in 2005 (Australian Institute of Health and Welfare [AIHW], 2005), and it was predicted that more than 430,000 people would be diagnosed with nonmelanoma skin cancer in 2008 (AIHW, 2008). In the United States, it was estimated that 68,720 men and women would be diagnosed with malignant melanoma in 2009 with the overwhelming proportion among Caucasians (National Cancer Institute, 2009).

The use of sunscreens is advocated for outdoor recreational and occupational activities in order to reduce the risk of skin burn and the development of skin cancer from exposure to ultraviolet (UV) radiation. Historically, organic chemical UV absorbers have been used in sunscreens, and they have their own set of potential health concerns (Environmental Working Group, 2009). Advances in formulation using nanotechnologies have seen the incorporation of the inorganic UV filters titanium dioxide (TiO_2) and zinc oxide (ZnO) in nanoparticulate form into commercial sunscreens at amounts ranging from 4 to 30% wt/wt. Such sunscreens coat the skin as a film and work primarily by reflecting and absorbing UV light. One commercial advantage in using nanoparticulate metal oxides as opposed to larger particle sizes is that the film formed on the skin appears transparent in the visible spectrum rather than opaque. The Australian Therapeutic Goods Administration stated that there were almost 400 sunscreen products commercially available in Australia in 2006 (Therapeutic Goods Administration [TGA], Australia, 2006), with many containing nanoparticulate TiO_2 and/or ZnO.

Nanoparticles are discrete objects, which have all three Cartesian dimensions less than 100 nm (ISO, 2008). At these sizes, there is potential for nanoparticles to penetrate cell walls and the blood-brain barrier and interact with

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

biomolecules (reviewed in Osmond and McCall, 2010). Furthermore, for the metal oxides typically used in sunscreens, the greater specific surface area and chemical reactivity compared with larger particles potentially result in the generation of higher levels of free radicals and reactive oxygen species per unit mass (Nel *et al*., 2006). Free radicals resulting from the photoactivity of nanoparticles of TiO_2 and ZnO have been reported to damage DNA in human skin cells when exposed to UV light (Dunford *et al*., 1997; Nakagawa *et al*., 1997).

The potential for, and consequences of, dermal absorption or penetration of metal oxide nanoparticles from personal-care products has not been determined conclusively. Following the World Health Organization (2006) definition, dermal absorption describes the transport of chemicals from the outer surface of the skin to the systemic circulation, and dermal penetration describes the entry of a substance into a particular layer or structure (e.g., the stratum corneum). Most investigations so far have been *in vitro* using diffusion cells, with few animal studies and fewer *in vivo* human studies (reviewed in Monteiro-Riviere and Baroli, 2010 and Sadrieh *et al*., 2010). In recent trials of sunscreen formulations applied to weanling pigs, Inman *et al*. (2010) and Monteiro-Riviere *et al*. (2010) found that TiO_2 and ZnO nanoparticles were localized in the stratum corneum; the ZnO particles had a mean size of 140 nm and range 60–200 nm. Related studies to evaluate the dermal penetration of metallic or metal-bearing nanoparticles such as maghemite (Baroli *et al*., 2007), quantum dots (Ryman-Rasmussen *et al*., 2006), or silver (Larese *et al*., 2009) in excised human or porcine skin have shown limited penetration (see additional references in Baroli *et al*., 2007; Osmond and McCall, 2010; and the review of Monteiro-Riviere and Baroli, 2010).

Several reviews (Monteiro-Riviere and Baroli, 2010; Nohynek *et al*., 2007, 2010; TGA, 2009) and recent investigations (Cross *et al*., 2007; Inman *et al*., 2010; Monteiro-Riviere *et al*., 2010; Roberts *et al*., 2008; Sadrieh *et al*., 2010; Zyvagin *et al*., 2008) have concluded that metal oxide nanoparticles do not penetrate the stratum corneum, although they can lodge in hair follicles (Lademann *et al*., 1999, 2006; Nanoderm, 2007), sweat glands, or skin folds.

A major difficulty facing these studies is that highly sensitive methods are required to ensure the detection of very low levels (if any) of dermally absorbed nanoparticles, without altering the properties of the nanoparticles and hence their potential for dermal penetration. A concept proposed by Gulson and Wong (2006) to trace the metal is to make use of stable (non-radioactive) isotopes. Zinc has five stable isotopes, one of which, ^{68}Zn, has a natural abundance of 18.8%. If ZnO particles highly enriched with ^{68}Zn were incorporated into sunscreens and applied to skin, then increases in levels of ^{68}Zn in blood and urine samples, relative to a different naturally occurring stable isotope not included in the sunscreen (in this case, ^{64}Zn whose natural abundance is 48.6%), would indicate dermal penetration of Zn from the sunscreen even if the body were exposed to natural Zn from other sources.

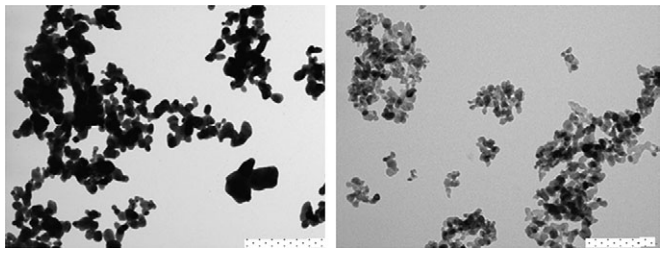

FIG. 1. Transmission electron microscopy (TEM) images of the two types of ZnO particles used in the sunscreens. For the bulk particles (image on left), the scale bar on the lower right-hand side is 1000 nm, and for the nanoparticles (image on right), the bar is 200 nm.

This paper describes the first application of stable isotopes in nanotechnology specifically for tracing absorption or penetration of Zn from ZnO nanoparticles in sunscreen applied to healthy human skin under conditions of normal use.

MATERIALS AND METHODS

^{68}ZnO particles. ZnO powder enriched to > 99% ^{68}Zn was purchased from Isoflex. Half of the stock was used to make nanoparticles with a final crystallite size of about 19 nm (± 8 nm; minimum 3 nm, maximum 60 nm) using a proprietary method based on high-energy attrition milling (Casey *et al*., 2006). The rest was used to make larger particles with an average crystallite size of 110 nm (± 46 nm; minimum 25 nm, maximum 284 nm) produced by a modified version of the same method. Primary crystallite size and phase were determined by x-ray diffractometry. Crystallite phase was determined using a Bruker ASXD8 X-Ray Diffractometer with Cu Kα radiation over a 2θ range of 5°–85° with a step size of 0.02°. The crystal structure of all particles was identical and the same as used in commercial sunscreens—single-phase hexagonal wurtzite. Crystallite size was determined by performing a Rietveld refinement of the diffraction data using Siroquant Version 2.5 software and confirmed by transmission electron microscopy (Fig. 1), which indicated that primary particles existed as single crystals, and the polydispersity of particle distributions was smaller at the smaller particle size.

Sunscreen formulation. The uncoated particles were incorporated into an oil-water formulation using a commercial process for preparing sunscreens. Both sunscreens contained ~20% wt/wt ^{68}ZnO particles. Sun protection factor (SPF) was measured. To determine the character of the ZnO nanoparticles in sunscreen when applied to skin, a tape-stripping method was used, whereby about 12 mg of sunscreen was applied to a marked area of skin on the underside of the forearm over an area ~6 cm^2 (1.5 × 4 cm), equivalent to a dose of 2 mg/cm^2 (the recommended dose to determine the SPF factor; Gabard *et al*., 2000; Lademann *et al*., 2004). The sunscreen was gently spread across the area by a gloved forefinger (disposable nitrile glove) and gently rubbed until an even coverage was obtained. A piece of tape of matching size was then applied on the marked area of skin. A total weight of 1.2 kg was placed on top of the tape for 30 s, introducing a pressure of 200 g/cm^2 prior to removal of the tape from the skin.

The stripped tape was subsequently imaged by scanning electron microscopy (SEM) in back-scattered mode. The SEM analysis showed a relatively even distribution of the nanoparticles on the skin (Fig. 2).

Treatment. The study was conducted over five consecutive days in early March 2009 (late summer in the Southern Hemisphere) using a tracer that was > 99% enriched in ^{68}Zn. Sunscreen containing nanoparticles of ^{68}ZnO was applied to a group consisting of 11 people (the "nano" group), whereas the

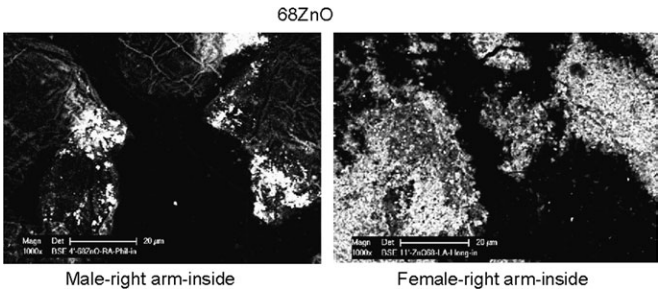

FIG. 2. Scanning electron microscope (SEM) images illustrating the character of the nanoparticle sunscreen on the skin of two subjects using a skin tape–stripping method. The scale bars are 20 μm.

other sunscreen containing non-nanoparticles of ^{68}ZnO was applied to a group of 9 people (the "bulk" group) (see Fig. 3 for appearance on the skin as applied outdoors). Information on the subjects—age, gender, skin type, and race—is given in Table 1. UV exposure was continuously monitored throughout the trial days with a UV spectrophotometer. The mean UVA and UVB measures averaged over 5 days were 26.7 (± 10.1 SD) and 1.2 (± 0.6) W/m^2, respectively. The protocol was developed with the aid of a pilot study conducted earlier with three subjects and using ZnO that was only 51% enriched in ^{68}Zn.

The first day of the study was conducted at an aquatic center to refine protocols, with the following 4 days conducted at a Sydney beach. Subjects wore UV protective upper body garments, the backs of which had a specific section cut out. Sunscreen was applied twice daily for a period of 5 days to the skin left uncovered by the garment. All sunscreen applications and removals for all subjects were performed by the same person ("X," Table 1). After each application, a period of about 30 min was allowed for the formulation to equilibrate with the skin, as recommended by manufacturers, before subjects lay on their stomachs in the sun for a minimum of 30 min.

The tubes containing sunscreen were weighed by the same investigator (subject 12) before and after each application. Mean doses of sunscreen are listed in Table 1. There was no significant difference in the dose of sunscreen (miligrams per square centimeter) for the first and second applications of the day, with an overall mean of 4.3 mg/cm^2 and range 2.8–5.8 mg/cm^2. This is more than double the usual testing dose of 2 mg/cm^2. There was no significant difference between the doses for nanoparticle or bulk sunscreen, but there was a significant difference between doses for males and females with a mean of 4.6 mg/cm^2 for males versus 3.7 mg/cm^2 for females.

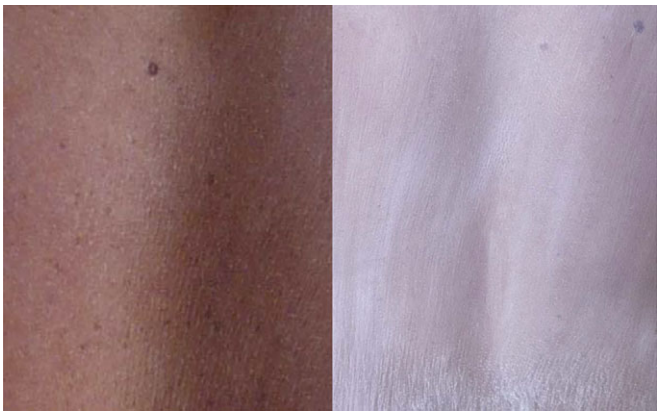

FIG. 3. Appearance on the skin of the two sunscreen formulations after the sixth application at the beach. The left-hand image is for a female subject who had nano sunscreen applied to her back, whereas the right-hand image is for a male who had sunscreen with larger particles applied. The transparency of the sunscreen on the female indicates that there is little agglomeration of the ZnO nanoparticles.

For other body areas not used in the trial, such as the face and legs, subjects were encouraged to apply a commercial sunscreen of similar formulation to the test sunscreen but where the ZnO was replaced by chemical UV absorbers. Our aim was to determine dermal absorption of Zn from sunscreens in humans undergoing normal activities at the beach. Therefore, after a 30-min UV exposure following equilibration of each sunscreen application, the subjects were free for the rest of the day to swim, surf, sunbathe, walk, or spend time sheltered from the sun in a surf life–saving club. No effort was made to control perspiration or movement (resulting in skin flexing).

Blood and urine samples were collected 8 days before the start of the trial, just prior to the first application and after the removal of the last application on each of the 5 days of the trial and also 6 days after the end of the trial. Venous blood was collected by venipuncture into low-metal Vacutainer tubes. Urine samples were collected in precleaned standard containers. Additional urine samples were provided during the day and subsequent to the trial from a subset of subjects. Collection of blood and urine samples prior to any application of these sunscreens meant that each subject acted as his or her own control. This obviated the necessity of having a control group to which we would have applied equivalent sunscreen except with naturally occurring Zn.

Because of the sensitivity of the isotope method, subjects were continuously reminded, verbally and with signage, of the need to minimize contamination from the sunscreen especially during urine collection. Beach towels and a paper towel covering (renewed each day) were supplied to assist in eliminating the potential for contamination. The towels were washed each evening by one of the organizers and UV clothing by another. Sunscreen was removed from the subjects' backs at the end of each day by using alcohol-lanoline wipes. Subjects kept a diary of UV exposure, other activities, changes of garments, any concerns over inadvertently touching their backs, or other unusual happenings.

Laboratory procedures. Zn was purified from blood (0.2 ml) and urine (2–6 ml) samples by ion exchange through macroporous resin following digestion with ultraclean nitric acid and hydrogen peroxide. Total Zn levels in blank controls processed by these procedures were routinely less than 3 ng, which is insignificant compared with the total amounts of Zn in the blood and urine samples. Changes in the isotopic abundance of ^{68}Zn of the purified samples were measured by multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) at the Research School of Earth Sciences, Australian National University, and used to evaluate the dermal absorption of Zn from the sunscreens. A Zn solution with naturally occurring (normal) isotopic ratios was measured several times during each analytical session to obtain an estimate of the precision of the isotopic ratios. These data were complemented by "control" blood and urine samples as well as the base-line (before exposure) samples obtained before any sunscreen containing ^{68}ZnO was applied. To enable comparisons between laboratories, the isotope ratios have been normalized to a ^{66}Zn/^{64}Zn value of 0.596. The total Zn concentrations in all samples analyzed for their Zn isotopic ratios were determined by ICP-MS.

Isotopic measures. The use of MC-ICP-MS technology enables the precise measurement of isotopic ratios in samples. The key measure used in this study to determine whether Zn from sunscreens is absorbed through the skin is the change, if any, of the ratio of ^{68}Zn to ^{64}Zn with sunscreen exposure. More precisely, the percentage change in ^{68}Zn with sunscreen application (denoted Δ^{68}Zn%) is defined as:

$$\Delta^{68}\mathrm{Zn}\% = [(^{68}\mathrm{Zn}/^{64}\mathrm{Zn}_{\text{exposure}} - {}^{68}\mathrm{Zn}/^{64}\mathrm{Zn}_{\text{before exposure}}) / {}^{68}\mathrm{Zn}/^{64}\mathrm{Zn}_{\text{before exposure}}] \times 100 \ldots, \quad (1)$$

where the ^{68}Zn/^{64}Zn$_{\text{exposure}}$ refers to the measurement for samples taken at the beach or post-exposure.

The important advantage afforded by this measure is the sensitivity with which it can detect the presence of ^{68}Zn in blood and urine samples absorbed through the sunscreen-exposed skin. Δ^{68}Zn% will be zero if no ^{68}Zn from the sunscreens enters the blood or urine via dermal penetration, even if naturally

TABLE 1
Subject Information and Key Experimental Results

Subject	Particles	Δ^{68}Zn% beach	Δ^{68}Zn% post	Gender	Age (years)	Skin type[a]	Country	Relationship	Average dose (mg/cm^2)
1	NP	0.18	0.26	Male	60	IV	South America		5.1
2	NP		0.24	Male	20	II	Australia		5.3
3	NP	0.20	0.37	Female	23	II	Australia	Sibling of 4	3.7
4	NP	0.22	0.42	Male	20	II/III	Australia	Sibling of 3	4.7
6	Bulk	0.16	0.22	Male	24	I/II	Australia		5.1
7	NP	0.83	1.31	Female	44	II/III	South America		3.8
8	Bulk	0.30	0.43	Female	21	III	Australia		4.0
9	NP	0.27	0.52	Female	60	II/III	South America		3.9
10	Bulk	0.09	0.15	Female	34	I	UK		3.7
12	NP	0.08	0.24	Male	66	I/II	Australia		4.6
13	Bulk	0.20	0.41	Male	23	II/III	Australia		4.6
14	Bulk	0.26	0.40	Male	21	II/III	Australia/South American parents	Brother of 20	3.8
15	Bulk	0.25	0.42	Female	27	I	Germany		3.7
16	NP	0.11	0.23	Male	27	I	Australia	Son of 17	4.3
17	Bulk	0.06	0.17	Male	59	I	Australia	Father of 16	5.3
18	NP	0.45	0.69	Female	21	IV	South America		3.2
19	NP		0.80	Female	19	III	United States		4.1
20	Bulk	0.18	0.32	Male	20	IV	Australia/South American parents	Brother of 14	5.2
21	Bulk	0.10	0.22	Female	24	IV	South America	Twin of 22	3.3
22	NP	0.28	0.58	Female	24	IV	South America	Twin of 21	4.0
X	NP and Bulk	0.35	0.45	Female	64	I/II	Australia		Sunscreen applicator

Note. Subjects 5 and 11 withdrew after the first night.
[a]Rated according to the Fitzpatrick (1988) classification and assessed by Gavin Greenoak, director of the Australian Photobiology Testing Facility, Sydney. Type IV is darker than I.

occurring Zn enters the body from another source. This is because the ^{68}Zn/^{64}Zn ratio of naturally occurring Zn is essentially constant (as vindicated by the preexposure results to be discussed later) and additional amounts of natural Zn that may enter the body during the trial period will not change the (natural) ratio. Any increase, on the other hand, in Δ^{68}Zn% provides an unambiguous indication of ^{68}Zn from ^{68}ZnO in the sunscreens entering the body.

Although this is a sensitive and robust method of detecting the presence of absorbed Zn from sunscreens, for a particular score of Δ^{68}Zn%, further analysis is required before the amount of ^{68}Zn absorbed in absolute terms can be ascertained. Δ^{68}Zn% is not only proportional to the absorbed ^{68}Zn from the sunscreen but also inversely proportional to the amount of natural Zn present in the reservoir measured. For example, if knowledge was required of the absolute amount of absorbed ^{68}Zn from the sunscreen into the blood, in addition to the Δ^{68}Zn% score, it is necessary to know the amount of natural Zn present in the blood of that subject. Two subjects with the same Δ^{68}Zn% score may in fact have different amounts of absorbed ^{68}Zn from sunscreens if the amount of naturally occurring Zn in their blood is different.

Our interest was in comparing absolute amounts of absorbed ^{68}Zn from sunscreen exposure in four different groups, with gender as a factor. Two approaches are available. The first approach is to use fat-free mass as a basis for representing the natural Zn reservoir. To obtain a more reliable estimate of how much Zn an individual absorbed relative to all other subjects in the study, we used the fat-free mass based on the formula of Deurenberg *et al*. (1991) and the measured blood Zn concentration as representative of the total natural Zn for each subject to adjust the Δ^{68}Zn%; this formula is based on body mass index, gender, and age. This adjustment removed the gender bias inherent in the Δ^{68}Zn% measure, and the adjusted Δ^{68}Zn% score was used in statistical analyses to make comparisons between different groups.

The second approach for estimating the total amount of natural Zn present in the blood of each subject is to use the measured total blood Zn concentration in the before exposure blood sample and multiply this by an estimate of the individual's blood volume obtained using the formula of Nadler *et al*. (1962). These estimations provide amounts in micrograms of the ^{68}Zn tracer in blood for individuals and allow comparisons with the known amounts of naturally occurring Zn in blood and other tissues such as liver.

Statistical analyses. Changes in Δ^{68}Zn% for blood and urine samples were estimated on the last day of the 5-day sunscreen exposure phase and then 6 days later (post-exposure) relative to the initial (before exposure) values using Equation (1) above. The data come from 20 subjects split between the nano group with 6 females and 5 males and the bulk group with 4 females and 5 males.

To evaluate the overall difference between the exposure phase and post-exposure results, the Wilcoxon test (SPSS version 18.0; SPSS Inc., Chicago, IL) was used. It is important to note that comparing exposure and post-exposure results does not involve comparisons across groups but within groups and therefore does not necessitate adjusted Δ^{68}Zn%.

Because the distribution of the dependent variable, Δ^{68}Zn%, was positively skewed (skewness index = 2.1), $\log_{10}$-transformed versions of the data (skewness index = 0.02) were used in analyses. This transformation, as it compresses the scale at the higher end, also reduced the influence of the high value observed for subject 7.

Initial analysis using a mixed model showed a very strong time effect. Further analyses, also exploring male and female differences, were based on post-exposure, Deurenberg fat-free mass adjusted (and $\log_{10}$-transformed) Δ^{68}Zn% as described above, and utilized a 2 × 2 independent groups ANOVA. In addition, differences in the amount of ^{68}Zn tracer in blood (micrograms) were also evaluated with a 2 × 2 independent groups ANOVA. Effect sizes were calculated using partial eta-squared (η_p^2; Olejnik and Algina, 2003).

These studies have been approved by human ethics committees at Macquarie University and CSIRO.

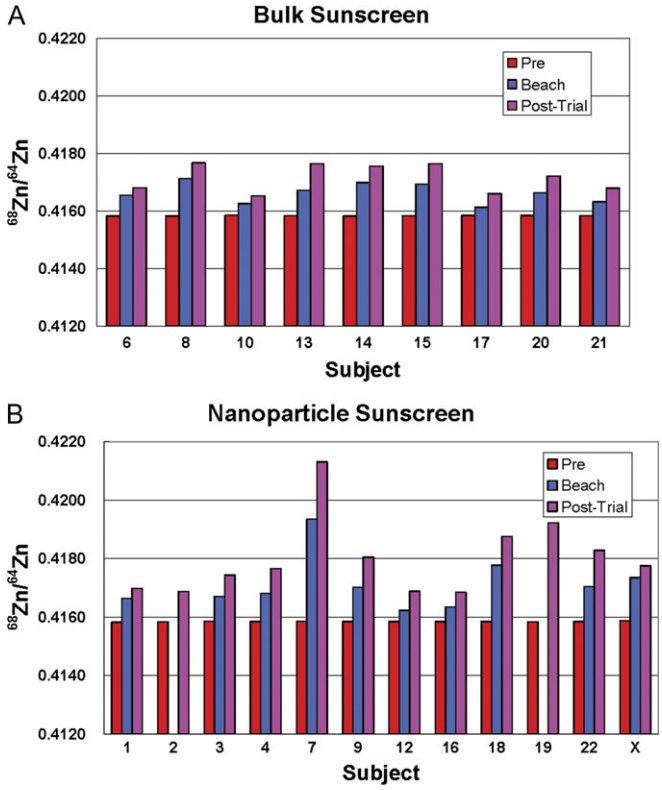

FIG. 4. (A) and (B) Bar graphs showing the ratio $^{68}Zn/^{64}Zn$ in blood from subjects on whose backs bulk or nano sunscreen was applied. The before exposure data (red bars) illustrate the uniformity in $^{68}Zn/^{64}Zn$ ratios prior to sunscreen application, reflecting the isotopic composition of naturally occurring Zn. Increases in the ratio evident in all subjects at end of the beach exposure phase (blue bars) and 6 days post-exposure (pink) are due to skin penetration of ^{68}Zn from the sunscreens. ''X'' represents the person who applied both sunscreens; her data were not included in the statistical analyses.

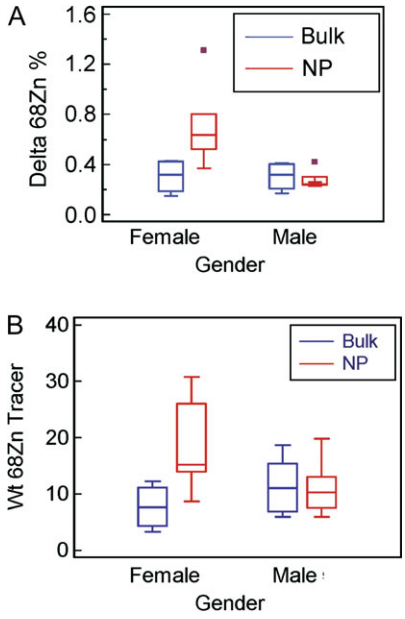

FIG. 5. (A) and (B) Box plots for $\Delta^{68}Zn\%$ and amount of ^{68}Zn tracer (micrograms) in blood samples for subjects, showing significantly higher $\Delta^{68}Zn\%$ and amounts of ^{68}Zn tracer for females in the nano group (NP) compared with females in the bulk group but no difference between the nano and bulk groups for males. Data for subject 7 (the outlier) are included in the analyses. The data are for the post-exposure sampling.

RESULTS

Seventeen of the 20 subjects completed the full trial. Subject 2 had an unforseen commitment on day 5. Subject 7 had an adverse reaction to the sunscreen, and application was discontinued on day 4; however, she continued to provide blood and urine samples. Subject 19 was unable to provide blood samples at the beach but provided a blood sample before exposure and post-exposure and urine samples throughout. Results are presented in Table 1 and in Figures 4–8.

Blood Samples

Because of constraints on access time to the MC-ICP-MS, Zn isotopic compositions have been measured for only critical samples (before exposure, end of day 5 at the beach, and post-exposure) from all subjects, whereas more complete data have been obtained for four of the subjects. The precision of the analytical method is provided by isotopic data obtained for the before exposure blood and urine samples from all 20 subjects, as well as for the person who applied the sunscreen. The before exposure $^{68}Zn/^{64}Zn$ ratios of blood data shown by the red bars in Figures 4A and 4B have a mean of 0.41584 ± 0.00002 (± 0.006% [2σ, n = 21]). The variation of ± 0.022% (2σ, n = 21) for the before exposure urine samples is not as good as that of the blood (0.006% [2σ, n = 21]) probably due to lower concentrations of Zn in urine (~1/10th) compared with blood. The extremely small variation in Zn isotopic composition of the before exposure blood and urine samples is consistent with the few other studies of very limited numbers of biological samples (Cloquet *et al.*, 2008; Steenberg *et al.*, 2005).

In contrast to the uniformity of before exposure $^{68}Zn/^{64}Zn$ ratios shown in Figures 4A and 4B, this ratio in blood increases for all subjects with sunscreen exposure and it continues to increase post-exposure. Although there are small variations in these data for the male bulk, male nano, and female bulk groups, the increases in ^{68}Zn in blood for the female nano group are greater (Fig. 5A). In the female nano group, subject 7 shows an exceptionally large increase. The sunscreen applicator (X, Table 1, Fig. 4B) was not part of the trial (and not included in statistical analyses), but increases in ^{68}Zn in her blood are comparable with the female nano group, consistent with her handling the nano sunscreen and indicating absorption through the skin of her hand.

The Wilcoxon test of the difference in $\Delta^{68}Zn\%$ between exposure and post-exposure results is highly significant ($p < 0.0001$). The mean increase in $\Delta^{68}Zn\%$ is 0.42 for post-exposure results compared with the mean increase of 0.23 on

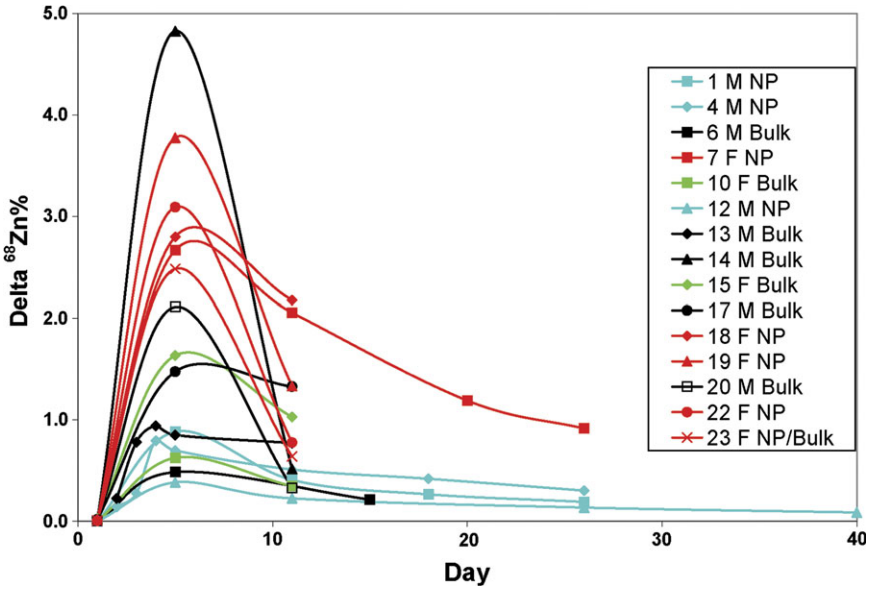

FIG. 6. Changes in Δ^{68}Zn% for urine samples. All values peak at the end of the beach exposure phase (day 5) and thereafter show a decrease over time. The red lines are for female subjects administered nano sunscreen. The Δ^{68}Zn% values for five subjects (2, 3, 8, 9, and 16) at day 5 range up to 35, and for subject 21, the value was 330. Urine samples for all 20 subjects were collected up to 6 days post-exposure; samples from six subjects were collected at various times out to 40 days post-exposure.

the last day of the exposure phase, relative to the before exposure mean (Fig. 5A).

The ANOVA results for the fat-free adjusted Δ^{68}Zn% failed to show any significant effects at the 0.05 level. However, the interaction of gender and particle type was marginally significant, $F(1, 16) = 4.34$, $p = 0.053$, and because of the relatively large effect size ($\eta_p^2 = 0.21$; i.e., the interaction of particle type and gender explained 21% of the variance in the adjusted Δ^{68}Zn%), the simple effects of particle type were examined. Although effect of particle type was clearly insignificant for males, $t(16) = 0.22$, $p = 0.83$, $\eta_p^2 = 0.003$, it was significant for females, $t(16) = 2.71$, $p = 0.016$, $\eta_p^2 = 0.314$; i.e., 31% of the variance in adjusted Δ^{68}Zn% for females can be accounted for by particle type.

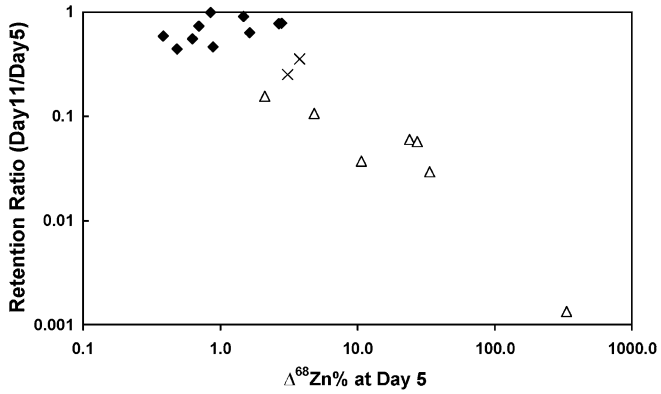

FIG. 7. Plot of log(retention ratios) versus log(peak Δ^{68}Zn%) to evaluate possible urine contamination with ^{68}Zn from the sunscreen. Data for samples with minimal contamination (♦) lie on a line with a slope of approximately zero. For urine samples significantly affected by contamination (Δ), the data define a linear function with a negative slope of around −1. The two data points (marked X) in between the above groups do not affect the statistical outcomes.

The equivalent ANOVA on the data from the alternative method (to that of Deurenberg *et al.* fat-free adjusted Δ^{68}Zn%) for estimating absolute amounts of absorbed Zn from sunscreens, based on measured Zn concentrations and estimated blood volumes, produced essentially identical results. The interaction of gender and particle was marginally significant, $F(1, 16) = 4.44$, $p = 0.051$. The effect of particle type was only significant for females ($p = 0.012$). Figure 5B shows that data for absolute amounts of absorbed ^{68}Zn are consistent with Δ^{68}Zn%.

Before accepting this result, it is necessary to consider whether any individual had an undue influence on it, in light of the small sample. To this end, standardized residuals and Cook's distance (Cook, 1977) were calculated for each case. None of the values obtained was large: the highest absolute residual was 1.47 and the mean Cook's distance was 0.20. Particular attention was paid to subject 7, who had the highest value of Δ^{68}Zn%. Partly as a result of the logarithmic transformation, neither the standardized residual (1.21) nor Cook's distance (0.09) for this subject suggested that she had a misleading influence on the overall results.

In further analyses of the fat-free data, we considered possible confounding effects, which might modify the conclusion that particle type affected the female subjects but not the males. Analyses were carried out in which age, average dose of sunscreen, skin type (treated as a numeric variable), and country (three categories), respectively, were added to the

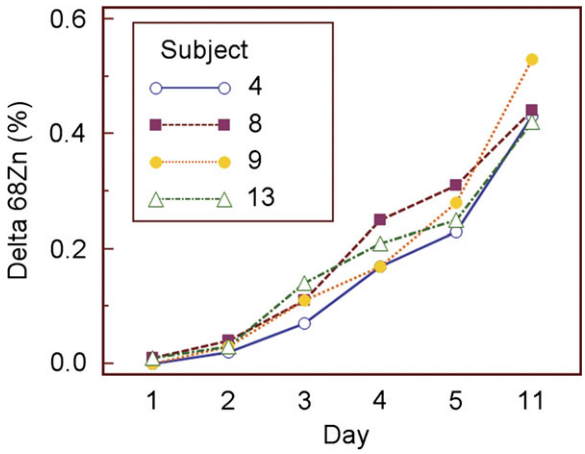

FIG. 8. Δ^{68}Zn% results for daily blood sampling collected at about 3 P.M. showing a linear increase with dose over the 5 days of sunscreen application for subjects 4, 8, 9, and 13. Note the nonlinear scale for the x-axis. Individual lines of best fit to the data from day 1 to day 5 have squared Pearson correlation coefficients (R^2) of 0.95–0.96. Detection of the ^{68}Zn tracer from the sunscreen was found from day 2.

original 2 × 2 ANOVA as covariates. The simple effect of particle type for females was significant with each covariate held constant: with age, $t(15) = 2.95$, $p = 0.010$, $\eta_p^2 = 0.366$; with skin type, $t(15) = 2.35$, $p = 0.032$, $\eta_p^2 = 0.271$; and with country, $t(14) = 2.43$, $p = 0.029$, $\eta_p^2 = 0.297$. For males, the simple effect of particle type remained nonsignificant in each case ($p > 0.8$).

In summary, in spite of the small numbers of subjects, the simple effect of particle type is substantial, whereby there are larger amounts of the tracer ^{68}Zn in blood of females who received the nanoparticle sunscreen. This result cannot be easily dismissed.

Zinc Blood Levels

The mean total Zn content in whole blood was measured as 3.55 mg/l, ranging from 2.36 to 4.62 mg/l. There were significantly lower amounts of Zn in blood samples taken before exposure for females (mean 3.05 mg/l) compared with males (3.83 mg/l; $p = 0.01$), and differences were also present in post-exposure samples (3.21 vs. 3.63 mg/l; $p = 0.04$). Changes over time in Zn content vary for individuals but there do not appear to be any systematic trends and certainly no systematic increases associated with increases in isotopic ^{68}Zn levels.

Urine Samples

Urine samples showed large increases in Δ^{68}Zn% with sunscreen exposure when compared with the blood samples. All subjects showed the same temporal characteristic with Δ^{68}Zn% values peaking around the last sunscreen application and decaying from then onwards (Fig. 6). Positive Δ^{68}Zn% values were still detectable 6 days after the last sunscreen application and for the four subjects tested 25–40 days after application. There was no simple relationship between Δ^{68}Zn% and total Zn concentration in urine.

The large variation in the peak Δ^{68}Zn% values across subjects may appear to make it difficult to detect a relationship between urine and blood findings. Although the bulk of the subjects had peak Δ^{68}Zn% values ranging between 0 and 4, six subjects had peak values ranging from 5 to 330. If the results are taken at face value and subjected to statistical analysis, there are no significant differences between the four groups.

One factor, however, that can account for some of the high peak values is the potential for urine samples taken at the beach to be contaminated with ZnO from sunscreens, especially for females. Strictly speaking, there is no certainty that a particular result is due to contamination, even if it is atypically high. What we can do is suppose that contamination did happen, infer what the effects would be and investigate the results for the effects. Assuming that there is contamination, we would expect to see for samples that are contaminated as outcomes: (1) larger peak Δ^{68}Zn% values than if there were no contamination, given that peak Δ^{68}Zn% values coincide with samples taken at the beach; (2) smaller retention ratios of Zn following the end of sunscreen application (retention ratio being defined as Δ^{68}Zn% on day 11/Δ^{68}Zn% on day 5), and (3) the retention ratio reducing as a function of increasing peak values reached by day 5 (which would produce an asymptotic slope of −1 on a log plot; see Supplementary notes). In contrast, for those samples that are contamination free, retention ratios will be higher and they will not be a function of peak Δ^{68}Zn% values achieved by day 5.

The extent of urine contamination is evaluated in detail in the Supplementary notes. Figure 7 is a scatter log plot of retention ratio versus peak Δ^{68}Zn% for all subjects. The first two expectations complement one another; the six samples with the highest peak Δ^{68}Zn% values (day 5) also have the lowest retention ratios. The total pool of samples separate into two clusters, one (♦) being scattered around a flat straight line suggestive of no correlation between retention rate and peak Δ^{68}Zn% and hence negligible contamination, and the other (Δ) showing a linear relationship with a slope of around −1, indicative of overwhelming contamination (see Supplementary notes).

If the second cluster is omitted and a 2 by 2 factorial ANOVA is performed on the rest of the day 5 samples with particle and gender as factors, there is a significant particle effect, a gender effect, and a second-order gender-particle interaction. *Post hoc* comparison of the four groups (nano females, nano males, bulk females, and bulk males) corrected for multiplicity using the Bonferroni method show that nano females show higher Δ^{68}Zn% values than the other three groups (with p values of 0.0007, 0.009, and 0.002 for nano males, bulk females, and bulk males, respectively), between which there are no significant differences. This is consistent with the results for the blood data. Furthermore, this finding is robust with respect to the samples that fall between the two

clusters (crosses in Fig. 7). The statistical significance of the results does not change whether or not we include either one or both of these samples.

Performing a 2 by 2 factorial ANOVA on the whole set of day 11 samples (without any exclusions) serves the double purpose of testing independently the contamination model for day 5 samples (see Supplementary notes), as well as confirming the statistical finding that nano females show higher Δ^{68}Zn% values than the other three groups. The analysis of day 11 data gives essentially the same result—nano females have higher Δ^{68}Zn% than the other three groups (p values of 0.002, 0.041, and 0.009 for nano males, bulk females, and bulk males, respectively), with no other significant differences.

Tracer Changes with Dose

To evaluate if there was a positive relationship between applied dose and penetration, blood and urine samples collected at the end of each day for one male and one female from each group were analyzed. These four subjects (4, 8, 9, and 13) were selected for more detailed analysis because increases in Δ^{68}Zn% in their blood, as shown in Figures 4A and 4B, were close to the mean increase over all subjects. Figure 8 shows that increases in their Δ^{68}Zn% in blood are linear over 4 days, detectable from day 2 after the fourth application of sunscreen, and continue to increase when sunscreen is no longer applied (tested 6 days after the last application). The profiles for changes in Δ^{68}Zn% over time for most subjects in this trial are similar to those observed in a smaller pilot trial of three subjects conducted earlier to test and refine the design of this larger and more expensive trial.

DISCUSSION

There are four key findings from the present study. The most notable one is that, contrary to the dominant view (Baroli *et al.*, 2007; Inman *et al.*, 2010; Monteiro-Riviere *et al.*, 2010; Ryman-Rasmussen *et al.*, 2006) and for the first time, the study reveals unequivocal evidence that Zn from ZnO particles in sunscreens is absorbed through healthy human skin exposed to sunlight and is detectable in blood and urine. The Zn may have been absorbed by (intercellular or intracellular) diffusion, via hair follicles, skin folds, or sweat glands or a combination of these. The sunscreen formulation may have assisted the absorption. The commercially based formulation for the production of sunscreens used in this study contained isopropyl myristate, a known chemical enhancer of skin penetration (Chan, 2005), and EDTA, a chelating agent which is highly effective for Zn. Molecules from the formulation physically adsorbed onto the ZnO could also affect dissolution, dissolution rates, and/or skin penetration.

The second finding pertains to the absolute amounts of Zn that are absorbed from the sunscreens. The total amounts absorbed as detected in blood and urine were small when compared with the amounts of natural Zn normally present in the human body. For example, for the nano female group, which appeared to show the largest levels of absorption, the average Δ^{68}Zn% in blood post-exposure is 0.71% (Table 1, Fig. 5A) and the amounts of ^{68}Zn tracer from sunscreen circulating in whole blood range from 8.6 to 30.8 μg (mean 15.8 μg; Fig. 5B). These small amounts contrast with an average amount of Zn in whole blood, post-exposure for these females, of about 12 mg. In addition, these amounts are minute when compared with the dietary intake of Zn, recommended daily values for which are 8 mg Zn for females and 11 mg for males (National Institutes of Health, 2009). The amount of ^{68}Zn tracer detected in blood posttrial represents less than 0.001% of the applied dose. Nevertheless, there are grounds which suggest that the amount of Zn absorbed from sunscreens may be somewhat larger than indicated by the blood results. A strong empirical support provided within the confines of this study for the possibility of additional Zn being deposited elsewhere in the body is that the amount of ^{68}Zn detected in blood continued to increase 6 days after the last sunscreen application. ^{68}Zn may have concentrated in particular tissues (e.g., epidermis, liver, muscle) with subsequent slow rerelease into the blood. Though, if the absorbed Zn is in ionic form, it is reasonable to expect that the amounts of ^{68}Zn tracer would once again be overwhelmed by the naturally occurring Zn in these tissues, we cannot make any assumptions if the absorbed Zn is in form of ZnO particles. The binding and retention of residues in the lower epidermis or dermis potentially acting as a long-term chemical reservoir is termed substantivity (Ngo and Maibach, 2010). The continuing increase in blood after the peak of ^{68}Zn in urine is likely to be due to the uptake of Zn by the red blood cells; urine is "fed" from the plasma compartment which turns over rapidly, whereas Zn in the red blood cell turns over slowly (Wastney, written communication, 2010). It should also be kept in mind that sunscreens are recommended for lifetime use. In countries with a sunny climate such as Australia, this translates into many days of actual application, in contrast to the 5-day exposure of our trial.

The third key finding is the interaction between ZnO particle size and gender in determining the levels of absorption (as reflected by blood and urine measures), which may well be an expression of an underlying skin thickness and particle size interaction. Females on average have thinner skin than males. The small sample size notwithstanding, more ^{68}Zn absorption was detected in blood samples for females in the nano group compared with females in the bulk group, whereas there is no significant difference between the male nano and male bulk groups. Similarly, in urine, both exposure and post-exposure data revealed that the female nano group had higher levels of absorbed ^{68}Zn than female bulk, male nano, and male bulk groups with no significant differences within the latter three groups. Other, less obvious, gender-related factors which may

contribute to the interaction with particle size are differences in skin pH, lower surface lipid content (e.g., ceramide) in specific areas and, with aging, significant changes in ceramide ratios (reviewed in Dao and Kazin, 2007 and Tagami, 2010).

The fourth finding is that there is a time lag between the first sunscreen application and the first detection of tracer ^{68}Zn in samples. Tracer ^{68}Zn was first detected in blood after the fourth sunscreen application and on the afternoon of the second day of the exposure period. This has implications for the conclusions derived from studies, which involved fewer applications, shorter time periods, or less sensitive methods to detect absorption.

It should be noted both as a limitation and to avoid any ambiguity that Zn detected in the blood and urine from sunscreens is not necessarily in the form of ZnO (nano)-particles. Tracer ^{68}Zn may have been absorbed as either ZnO (nano)particles or soluble Zn or both. Nano-sized particles potentially release more ionic Zn due to their larger surface area. The pH of the outer layer of the skin, the stratum corneum, ranges from 5.4 to 5.9 (Schmid-Wendtner and Korting, 2006), and in such an environment, ZnO particles could partially dissolve. For example, Ågren (1990) found a 10-fold increase in Zn extracted from a ZnO dressing at a pH of 5.4 compared with 7.4. Although currently undergoing investigation by confocal microscopy in urine samples from subjects that contained significant ^{68}Zn, ZnO particles may not be detectable because of the limited amount of absorption over the relatively short time of the trial. We have been unable to find any studies investigating dermal penetration of soluble Zn in humans. Three earlier studies of human subjects used larger particles of ZnO in ointments (Ågren, 1990; Derry *et al*., 1983; Morgan *et al*., 1980). In contrast, several *in vitro* studies using diffusion cells have shown that small amounts (0.3–0.4%) of Zn salts such as ZnO and $ZnSO_4$ can be absorbed through human excised skin (e.g., Pirot *et al*., 1996).

The amount of individual UV exposure appears unrelated to the amount of dermal absorption. Some of our subjects experienced several hours of UV exposure and their results were no different to others who had only the required minimal exposure of 1 h per day. Sweating and increased skin temperature associated with sun exposure and activity may enhance skin permeability (Benech-Kieffer *et al*., 2003), but these variables are difficult to quantify.

In conclusion, using highly sensitive stable Zn isotopes as tracers, this study has demonstrated that small amounts of Zn from ZnO particles in sunscreens can pass through the protective layers of skin exposed to the sun in a real-life environment and be detected in blood and urine.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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