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In vitro skin toxicity of CuO and ZnO nanoparticles: Application in the safety assessment of antimicrobial coated textiles

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ABSTRACT

In the context of nosocomial infections, there is an urgent need to develop efficient nanomaterials (NMs) with antibacterial properties for the prevention of infection diseases. Metal oxide nanoparticles (MeO-NPs) are promising candidates for the development of new antibacterial textiles. However, the direct exposure to MeO-NPs and MeO-coated NMs through skin contact could constitute a severe hazard for human health. In this work, the toxicity of copper and zinc oxide (CuO, ZnO) NPs antimicrobial-coated textiles was assessed on an in vitro reconstructed 3D model of epidermis. Thus, MeO-NPs and extracts from MeO-coated NMs were tested on EpiDerm™ skin model according to OECD TG 431 (Corrosion Test) and 439 (Irritation Test), respectively. Skin surface fluids composition is a crucial aspect to be considered in the development of NMs that have to encounter this tissue. So, for the irritation test, coated textiles were extracted in artificial sweat solutions at pH 4.7 and 6.5. Skin tissue viability, pro-inflammatory interleukin-8 secretion and morphological alteration of intermediate and actin filaments of keratinocytes were evaluated after 18 h exposure to extracts from CuO- and ZnO-coated textiles. Analysis of extracts at the two pH conditions indicated that released ions and not NPs are involved in promoting adverse effects on epidermis. Since Cu^{2+} and Zn^{2+} ions are known to penetrate epidermis, Balb/3 T3 cells were used as model of dermis. Fibroblasts viability was investigated after the exposure to trans-epidermis permeated ions, collected from EpiDerm™ basal supernatants, and to extracts, as representative of a direct interaction of ions with dermis cells by wounded skin. From our data we can conclude that: 1) skin surface fluids composition is a key parameter for the stability of NPs-coated textiles; 2) MeO ions released from coated textiles can deeply affect the epidermal tissue and the underlying dermal cells upon trans-epidermal permeation; 3) skin barrier integrity is a fundamental prerequisite that should be taken into account during the assessment of NMs safety by direct contact exposure.

1. Introduction

The production and application of nanoparticles (NPs) and nanomaterials (NMs) is a growing phenomenon and several consumer products, including cosmetic, electronic, textiles and medicines are enabled with NPs, constituting a new category of commercial goods called nanoparticles enabled products (NEPs).

In the context of nosocomial infections, which represent a great health issue worldwide (Ahonen et al., 2017), the increasing problem of bacterial disease determined strong need for new antibacterial agents. Indeed, the resistance of bacterial strains to the conventional antibiotics, due to their abuse or misuse, is now a public health problem of global proportions (Hawkey, 2008). Furthermore, during the COVID-19 pandemic, it has been further enlighten how the persistence of viruses and pathogens in indoor environments or on surfaces could be responsible of the spread of infectious diseases (Chia et al., 2020), posing a great attention on this topic.

The development of new antimicrobial nanomaterials and NEPs

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seems to be highly helpful in this perspective. Metal-based NPs and metal oxide NPs (MeO-NPs), have been already considered as additives in different products including medical articles, thanks to their antibacterial, antiviral and antifungal properties (Nikolova and Chavali, 2020). In particular, copper oxide (CuO) and zinc oxide (ZnO) NPs have been demonstrated to be highly effective against different strains of bacteria (Perelshtein et al., 2015a) and viruses (Aderibigbe, 2017; Borkow and Gabbay, 2004; Ghaffari et al., 2019; Tavakoli and Hashemzadeh, 2020) and therefore they are among the most eligible nanomaterials for the coating of textiles and other medical devices (Perelshtein et al., 2015b).

The antimicrobial effects of MeO-NPs is due to their ability to induce damage to cell membranes, proteins or enzymes, to the generation of reactive oxygen species (ROS) and to the imbalance of metal/metal ions homeostasis (Raghunath and Perumal, 2017). On the counterpart, most of these mechanisms responsible for the killing of pathogens, are also involved in the induced-MeO-NPs toxicity in mammalian cells (Kheiri et al., 2019; Stankic et al., 2016).

Thus, in parallel to the increased use of NEPs, also including those products modified with metal-based NPs, the issue of the potential adverse effects caused by these materials on human health and environment needs to be considered too. It is known that silver NPs (Ag-NPs), which have been used in numerous commercial products to prevent bacterial infections, induce significant adverse effects on biological systems of different levels of complexity (Vazquez-Muñoz et al., 2017). In this perspective, CuO and ZnO NPs are more promising candidates, since they have the same good antibacterial properties but with a lower toxicity compared to Ag-NPs (Perelshtein et al., 2015b). However, a certain toxicity has been demonstrated also for these MeO-NPs. In 2017, Wang Y. et al. reported several studies on the toxic effect of MeO-NPs both in vitro and in vivo, describing two mechanisms of toxicity: reactive oxygen species (ROS)-dependent and ROS-independent. In ROS-mediated toxicity, ROS activate oxidative damages including cell membrane lesions, protein denaturation, DNA damage and changes in membrane permeability. Furthermore, ROS can activate caspase 3 and caspase 9, which induce the apoptotic pathway. The non-ROS-mediated mechanism involves an excessive accumulation of NP on the cell surface, the binding of NPs with the receptors that activate the transduction of apoptotic signals and the extracellular release of ions (Wang et al., 2017). We have recently described another mechanism based on the release of CuO and ZnO NPs and ions into the cytoplasm following MeO-NP degradation in lysosomes. The toxicological effects are associated to a ROS-dependent mechanism, by the interaction of NPs and ions with mitochondria, or by a direct contact with the DNA, due to their permeation across nucleopores (Fiandra et al., 2020).

The antibacterial MeO-NPs that are used for the coating of medical fabrics come into contact with epidermidis and they furthermore may reach the dermis following the hair follicle, or by penetration through the different layers of the skin, or through little wounds. To evaluate the hazard of CuO- and ZnO-coated textiles on human skin, a reconstructed 3D model of human epidermis has been selected and standardized OECD protocols, coupled to more mechanistic studies, have been employed on this tissue to evaluate the corrosive and irritative potential of NPs and NEP extracts. Moreover, fibroblasts have been chosen as model of connective tissue cells to evaluate the effect of NEPs extracts on the dermal layer.

2. Experimental section

2.1. Synthesis of MeO-NPS and coating of textiles

The sonochemical coating was performed on a small pilot machine that can be used to coat various substrates in a roll-to-roll mode. Cotton/polyester samples (3 m × 0.1 m) were coated with CuO and ZnO nanoparticles. In a typical coating run, 8.8 g $Zn(CH_3COO)_2 \bullet 2H_2O$ (0.01 M) was dissolved in 4 L of double deionized water. The solution was heated to 55 °C using the sonotrodes, and 20 mL of ammonia (28% wt.) was added dropwise until a pH of 8 was reached. At this stage, the rolling was started and the fabric was pulled through the solution in a roll-to-roll mode. The rolling speed used was 22 cm/min. The same procedure was utilized for CuO coating, the only difference was in the precursor. For CuO coating, a copper acetate solution (0.01 M) was used.

2.2. Characterization of MeO-NPs and coated textiles

The Dynamic Light Scattering (DLS) (Malvern Zetasizer, Malvern, UK) analysis was used to characterize the hydrodynamic behavior of the CuO and ZnO NPs used for the textiles' coating, after 72 h from dissolution in AS. The samples were diluted to a final concentration of 100 ppm in artificial sweat at pH 4.7 and 6.5 (composition in paragraph 2.3).

The morphology and size of the particles on textiles were studied using the high-resolution scanning electron microscopy Magellan (FEI microscope) at an accelerating voltage, over the range of 5–15 kV. To improve the quality of the images, the samples were coated with a carbon layer by sputtering in rarefied atmosphere of Argon at 0.1–0.2 mbar by means of an Emitech K550 Sputter Coater. The amount of coating was determined by ICP-OES (Horiba ULTIMA 2 spectrometer) analysis. A piece of coated textiles was immersed in 0.5 M HNO_3 and was heated for 15 min at 200 °C. Then, water was added and the solution was boiled for another 15 min. At the end, the total volume was adjusted to 50 mL. This solution was probed by ICP for calculation of Zn^{+2} and Cu^{+2} ions.

2.3. Preparation of textile extracts and ICP-OES analysis

The extracts from textiles sonochemically coated with CuO (0.8% wt) and ZnO (2.2% wt) NPs and from non-coated textiles (reference) were obtained according to ISO 10993-12. In detail, antibacterial textiles which could be in contact with skin for a prolonged time, were incubated in extraction solution (artificial sweat) for 72 h (maximum time of exposure from the ISO guidance) at 37 °C. Each piece of textile, 0.1 g (corresponding to 2 cm^2), was put in triplicates in borosilicate glass vials with 1 mL of AS with a different composition according to the desired pH:

1. AS pH 6.5: NaCl 0,5%; urea 0,1%; lactic acid 0,1% (BS EN 18:11:2011)
2. AS pH 4.7: 20 g/l NaCl, 17.5 g/l urea, 5 g/l acetic acid, 15 g/l lactic acid (ISO3160-2)

Vials were put on a shaker and, at the end of the incubation, the extracts (about 850 μL) were collected and stored at 4 °C until use. In Fig. S1 are represented the three pieces of textile before and after the extraction procedure.

The quantification of total Cu and Zn ions in the extraction AS was performed by ICP-OES (PerkinElmer, Optima 7000 DV Perkin Elmer), after acid digestion of the solutions and of the textiles. Extraction solutions were digested with nitric acid 5%, overnight at RT; textile digestion was performed in Aqua Regia (4 mL HNO_3 65% +12 HCl 37%) in the MILESTONE ETHOS TC microwave mineralizer (hot digestion).

To determine the amount of the only free ions in the extracts, solutions were filtered across a 10 KD cut-off membrane (Vivaspin 6, Sartorius) before processing for ICP-analysis.

2.4. In vitro 3D assays

2.4.1. The reconstructed human epidermis model EpiDerm™

The toxicity of MeO-NPs was assessed on the reconstructed human epidermis model EpiDerm™ (MatTek Corporation), where a highly differentiated 3D tissue model consisting of human-derived epidermal keratinocytes is cultured on specially prepared inserts. The tissues were reconstituted overnight in apposite culture medium (Assay medium,

MatTek Corporation), in order to remove cellular debris and other compounds used for their transport. The agar film was removed from the apical compartment of the inserts and the medium was added. Inserts were then maintained overnight in the incubator at 37 °C, 5% CO_2. After this pre-incubation, the EpiDerm™ models are ready for the exposure to NPs suspensions or textile extracts according to the Corrosion or Irritation tests, respectively.

2.4.2. Corrosion test on EpiDerm™

For the corrosion test (OECD TG 431) (OECD, 2015), EpiDerm™ were exposed to NPs suspensions (30 μL) for 3 min and 1 h at different concentrations (1, 10, 100 and 1000 ppm). PBS 1× was used as negative control (NC) and KOH 8 M as positive control (PC). After the exposure, inserts were washed twenty times in PBS 1×, dried and put in a new 24 multiwell containing a solution of culture medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. Cells were then solubilized with an extraction solution (isopropanol) overnight. After that, tissues were broken with a needle to recover all the liquid inside the well and absorbance was read by a multi-plate reader (Infinite 200 Pro, TECAN, Männedorf, Switzerland) at 570 nm. The tissue viability was calculated as % respect to the mean of the negative control (Abs sample/Abs control*100).

According to OECD TG 431 protocol, each experimental condition is analyzed in duplicate and the final result is the mean value of the two repetitions. A single testing run, composed of two tissue replicates is sufficient for testing a substance when the resulting classification is unambiguous. In our work, duplicates ($n = 2$ tissues for tested condition) of EpiDerm™ for each treatment and each time point (3 min and 1 h) were used for the in vitro classification of the tested material corrosiveness and acceptance criteria were respected (Δ tissue [%] < 30%).

The potential corrosiveness of the NPs was classified according the residual viability obtained after exposure and confronting the values with the Global Harmonized System (GSH) table adopted by the OECD (Table 1).

2.4.3. Irritation test on EpiDerm™

Irritation test (OECD TG 439) (OECD, 2019) includes the exposure of EpiDerm™ skin models to substances for 18 h to then perform the cell viability MTT test. In this test, skin models are not exposed directly to NPs suspensions, but to textile extracts, according to the ISO/TC 194/WG 8 for medical device. For skin Irritation test, EpiDerm™ models upon arrival were reconstituted overnight, as described above, and then exposed to 100 μL of the extracts. PBS 1× was used as negative control (NC); SDS 1% was used as positive control (PC), and AS solution at both pHs was used as internal control for every irritation test. After 18 h of incubation, inserts were washed 15 times with PBS 1×, moved in a solution of MTT and medium for 3 h, subjected to isopropanol extraction, and read at 570 nm as described for the Corrosion test. The irritant potential of NPs was evaluated by calculating the % of tissue viability respect to the mean of the negative control, as described above. If the obtained values are below the 50%, the substance is considered irritant of 2nd category according to GHS classification, otherwise is considered non-irritant (Table 1).

After the 18 h of incubations with the textile extracts, and before the insert incubation with MTT, the supernatants were collected, centrifuged and stored at −80 °C for ELISA analysis.

For Skin Irritation tests, textiles were extracted in AS (at different pH conditions) in triplicate and 3 different tissues ($N = 3$) were used for textiles extracted in AS at pH 6.5, while 6 different tissues ($N = 6$) for AS at pH 4.7. Since, according to OECD 439, skin irritancy potential is predicted from the mean viability determined on 3 single tissues for each tested compound, the variability of tissue replicates should be acceptably low. The assay met the acceptance criteria of the MS EXCEL spreadsheets (obtained from MatTek Corporation), since the SD calculated from individual % tissue viabilities of the 3 identically treated replicates was <20%.

Table 1
Classification of skin corrosion and irritation hazard according to the GHS System adopted by the OECD.

OECD Test	Mean Tissue viability (% of NC)	Classification
Corrosion test	3 min < 50%	Corrosive
	3 min ≥ 50% and 1 h < 15%	Corrosive
	3 min ≥ 50% and 1 h ≥ 15%	Non-Corrosive
Irritation test	≥ 50%	Irritant
	< 50%	Non-Irritant

2.4.4. Interleukin-8 quantification

The release of IL-8 from the EpiDerm™ model was evaluated in the supernatants derived from the apical part of the inserts. Supernatants were collected after 18 h of exposure, centrifuged at 1200 rpm for 6 min and then stored at −80 °C until analysis. The quantification of IL-8 was performed through IL-8 ELISA matched antibody pair kit (Invitrogen, Life Technologies, Monza, Italy) according to the manufacturer's instruction and data were shown as pg/mL.

2.4.5. Morphological alterations of epidermal cells

EpiDerm™, incubated to MeO-NPs or to textile extracts in experimental conditions similar to those applied for the Irritation test, were processed for the immunodecoration of a specific protein of the keratinocytes, the cytokeratin-10 (CK-10), and for the actin microfilaments of the cytoskeleton. Briefly, the epidermis models were treated for 18 h with textile extracts at the two pHs. At the end of treatment, the tissue-bearing inserts were washed 15 times with PBS 1× and fixed in paraformaldehyde for 20 min and then washed 3 times with PBS. With the help of a scalpel, the tissues on the inserts were removed from the support and put on a glass slide (Superfrost Ultra Plus™, Thermo Scientific). The skin tissues were removed from the insert under the stereomicroscope by mean of tweezers and located on the glass slide on a PBS drop. The epidermis were then incubated for 2 h at room temperature (RT) with anti-human CK-10 antibody (1:100, 0.001 mg/mL) prepared in bovine serum albumin (BSA, 5%) blocking medium, washed 3 times with PBS 1× and then incubated with the secondary antibody Alexa Fluor 488 (1:300) + DRAQ5 (nuclei, 5 μM) in BSA 5%, for 2 h at RT. After washing with PBS, tissues were stained with the actin filament probe rhodamine-phalloidin (1:350, Cytoskeleton Inc.) for 20 min, washed in PBS, dried at the air and finally mounted with Vectashield® Antifade Mounting Medium and covered with a class cover slide. Cover slides were observed at confocal microscope Leica TCS SP5 II. Rhodamine-phalloidin was excited at 561 nm and detected in the band centered at 585 nm (red), Alexa Fluor 488 was excited at 488 nm and detected in the band centered at 520 nm (green) and DRAQ5 emission wavelength was excited at 633 nm and detected in the band centered at 680 nm (blue).

2.5. In vitro 2D assays

2.5.1. Fibroblasts culture maintenance and treatments

Balb/3 T3 murine fibroblasts cell line (ATCC® CCL-163™, American Type Culture Collection, Manassas, VA, USA) was used as model of dermis cells. Cells were cultivated in DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 100 U/mL) and maintained in incubator at 37 °C and 5% CO_2. For toxicity assays, cells were seeded on a 6-well multiwell at the density of $3.2*10^5$ cells/well and, after 24 h, were treated with the basal supernatants obtained after EpiDerm™ exposure to extracts from CuO- and ZnO-textiles at pH 4.7 or 6.5. Fibroblasts that were exposed directly to AS were seeded on 6-well Transwell™ ($5*10^5$ cells/well) for 24 h and the exposed apically to the different AS for 18 h, while in the basal compartment was added fresh complete DMEM medium.

2.5.2. Viability assay on fibroblast culture

The cytotoxicity of CuO and ZnO NPs on Balb/3 T3 cells was evaluated by the MTT (Sigma Aldrich, Milano, Italy) assay. MTT test was performed according to previous works (Bengalli et al., 2019; Mosmann, 1983). Briefly, after 18 h of exposure to the samples reported in paragraph 4.5.1, cells were rinsed with phosphate buffered saline (PBS), and MTT solution was added to fresh medium (final concentration 0.3 mg/mL) for 3 h. Cells were dissolved in 1 mL Dimethyl sulfoxide (DMSO, Sigma Aldrich) and absorbance was analyzed at 570 nm by mean of a multi-plate reader (Infinite 200 Pro, TECAN, Männedorf, Switzerland). The percentage of cell viability was calculated according to the formula: Abs treated sample/Abs control sample*100.

2.6. Statistical analysis

Statistical analyses were performed using Sigma Stat 3.2 software, using unpaired Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Synthesis of MeO-NPs and coating of textiles

The synthesis and the coating of MeO-NPs was done in a one-step process. The starting materials are corresponding salts of acetates that were hydrolyzed in an alkaline environment for formation of CuO and ZnO NPs under ultrasound irradiation. Subsequently to the generation of the NPs, they are deposited onto the cotton/polyester fabrics surface due to the microjets formed in the solution after collapsing of the bubbles. The mechanism of the sonochemical coating was discussed in details in our previous publications (Perelshtein et al., 2009b). At the end of the coating process, the coated textiles were washed and dried. The NPs powder was collected by centrifugation, washed and dried. Then all substances were further subjected to various characterizations. The amount of coating was probed by Inductive Coupled Plasma (ICP), 0.8 wt% of CuO was found on the textile and 2.2 wt% of ZnO.

3.2. Characterization of MeO-coated textiles

The DLS analysis provided the hydrodynamic size and the polydispersity index (Pdl) of the NPs used for textile coating. In Table 2 are reported the DLS-derived parameters for CuO and ZnO NPs dissolved in AS for 72 h, in agreement with the extraction procedure for the samples to be applied on EpiDerm™. The *Z*-average sizes of MeO-NPs suspensions in milli-Q water are 193 ± 3 nm and 557 ± 45 nm for CuO and ZnO NPs respectively indeed (Supplementary Table S1). The hydrodynamic size for CuO and ZnO NPs in AS pH 4.7 was around 350 and 650 nm, respectively. At pH 6.5, the Z-average of CuO NPs further increased over 1000 nm, while ZnO NPs size did not vary significantly. The PdI of both CuO and ZnO NPs dissolved in AS reaches values over 0.7 at pH 4.7 and even proximate to 1 at pH 6.5. These results indicate that MeO-NPs have a tendency to aggregate in AS and forms a suspension with a broad size distribution, especially at pH values 6.5.

The morphology of the coating was studied by high-resolution scanning electron microscopy (HR-SEM) (Fig. 1). Primary ZnO NPs have an irregular rectangular shape, with tendency to agglomerate (Supplementary Fig. S2A), while CuO NPs have a leaf-shape morphology (Supplementary Fig. S2B). HR-SEM depicted images showed that CuO NPs more homogeneously and finely coat the polyester/cotton fibers (Fig. 1A), in accordance with the NPs shape and lower tendency to agglomerate. The coating with ZnO NPs, in comparison with CuO NPs coating, resulted indeed more rough and heterogeneous (Fig. 1B), with clusters of particles along the cotton fiber, likely due to the propensity of these NPs to form agglomerates.

Table 2
Hydrodynamic size (Z-average) and polydispersity index (Pdl) of 100 ppm CuO and ZnO NPs as measured by dynamic light scattering (DLS), after 72 h from dissolution in AS pH 4.7 and 6.5. Means ± SD of 3 replicates.

NPs	Medium	t72 h	
		Z-ave ± SD (nm)	PdI ± SD
CuO	AS pH 4.7	350 ± 150	0.76 ± 0.06
	AS pH 6.5	1292 ± 232	0.95 ± 0.04
ZnO	AS pH 4.7	650 ± 164	0.76 ± 0.03
	AS pH 6.5	549 ± 91	0.84 ± 0.05

3.3. Release of metal ions in the extracts from coated products

The amount of Cu and Zn ions in textile extracts were detected by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), comparing the total amount of free Cu^{2+} and Zn^{2+} released from the CuO- and ZnO-coated textiles during the extraction in Artificial Sweat (AS), with the total amount of ions (free or complexed in NPs) contained in the extraction AS and with those loaded on the textiles (Table 3). The comparable concentrations of ions measured before (total amount in extraction AS) and after (amount of free ions in extraction AS) ultrafiltration of the solutions across a 10 KD cut-off membrane, indicates that not the whole NP, but only free metal ions are released during the extraction procedure in AS. Data of table 4 also show that~17% of total ions loaded on the textiles are released at pH 4.7, while <1% of ions are released at pH 6.5.

3.4. Toxicity of MeO-NPs and NEP extracts on reconstructed human epidermis

3.4.1. Corrosion test

Skin corrosion is an irreversible damage of the epidermis that can be consequent to the direct cutaneous contact with a chemical. The evaluation of a substance corrosiveness is essential for handling, managing, transport and labelling chemicals. In order to investigate the potential corrosiveness of CuO and ZnO-NPs, EpiDerm™ skin model inserts were exposed directly to NPs water suspensions at different concentrations for 3 min and 1 h following the OECD TG 431 protocol for Skin Corrosion test. Data in Fig. 2 show that the tissue viability of the model was not affected by the exposure to the tested MeO-NPs, even at the highest concentrations (1000 ppm). These results demonstrate that CuO and ZnO NPs are not corrosive for the skin according the classification criteria of the Globally Harmonized System (GHS), since the tissue viability is >50% and > 15% at 3 min and 1 h respectively.

3.4.2. Irritation test

While corrosion is an irreversible damage, irritation is a reversible damage that can occur at the skin after exposure to tested compounds. The Irritation test was performed according to the OECD TG 439 and after extraction of textiles in artificial sweat according to the ISO for biomedical devices. The exposure to NEP extract is a more physiological and realistic exposure condition that mimics the possible release of NPs from the textiles which are in contact with the skin. Since the pH value of sweat can vary from person to person, depending on age, sex, different body district and health status, two different pH of AS were used: 4.7 and 6.5. Fig. 3A shows that at pH 6.5 CuO and ZnO extract are not irritants, since the tissue viability percentage is above the 50%. At lower pH (Fig. 3B), the tissue viability is drastically and significantly decreased compared to control samples, and also to AS and Reference textile. The values of tissue viability were below the 50%, confirming that at this condition the tested textile extracts are irritants. The irritative activity of textile extracts at pH 4.7, correlates with the high release of Cu^{2+} and Zn^{2+} in this acidic condition (Table 3): the large amount.

of ions leached from the textiles in extraction solution impacts the epidermis and significantly affects tissue viability.

It is well known that copper and other metals are irritants for skin,

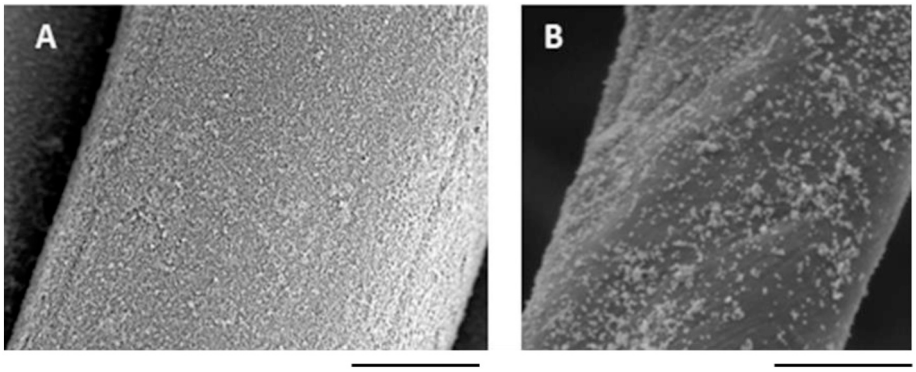

Fig. 1. HR-SEM of A) CuO coated textile (bar = 4 μm); b) ZnO coated textile (bar = 5 μm).

Table 3
ICP-OES quantification of total Cu^{2+} and Zn^{2+} on the textile and in extraction AS (about 850 μL), measured after acid digestion. Ref: Reference fabric. Means ± SE of 3 replicates.

		Amount of free ions in extraction AS (mg)		Total amount in extraction AS (mg)		Total amount on the textiles (mg/0.1 g textile)
		pH 4.7	pH 6.5	pH 4.7	pH 6.5	
Cu^{2+}		0.65 ± 0.005	0.04 ± 0.001	0.61 ± 0.017	0.02 ± 0.010	3.38 ± 0.142
Zn^{2+}		1.60 ± 0.044	0.07 ± 0.001	1.78 ± 0.047	0.07 ± 0.004	9.57 ± 0.119
Ref	Cu^{2+}	2.38×10^{-6}	8.5×10^{-7}	2.4×10^{-4}	9.0×10^{-5}	$7.5 \times 10^{-4} \pm 2.5 \times 10^{-4}$
	Zn^{2+}	5.44×10^{-5}	5.1×10^{-6}	6.1×10^{-4}	4.4×10^{-4}	$7.2 \times 10^{-3} \pm 3.0 \times 10^{-4}$

mainly when they are oxidized and thus convertible by exudates (sweat and sebum) to hydrophilic ionized salts or lipophilic soaps. In these forms, metals are able to penetrate skin and induce irritation or allergic reactions (Hostynek and Maibach, 2004). Electrophilic metal ions as Cu^{2+} and Zn^{2+} easily complex with the proteins of stratum corneum (SC), and in particular to sulfhydryl groups in cysteine, glutathione, or thioglycolic acid, leading to the formation of deposits (Hostynek and Maibach, 2004). Such depots, that in the case of essential metals as copper or zinc are useful reservoirs for the homeostatic control of these elements (Schaefer et al., 1979), can be also responsible for the toxic effect of these ions on the underlying layers. Indeed, they can continue to diffuse from SC into the viable tissues also after exposure has stopped. The Zn^{2+} absorption by skin is mainly mediated by Zn transporters (e.g ZIP and ZnT) (Kambe et al., 2015), which allow a specific localization of this metal in the epidermal layers (Inoue et al., 2014). Data on human skin penetration by copper compounds is scarce and the ability of copper of really penetrate the skin is mainly based on its induced effects. Most of the studies dealing with Cu(II) skin absorption involve the transport of as tripeptide complex (e.g. GHK-Cu and GSH-Cu), which is able to efficiently permeate the stratum corneum (Mazurowska and Mojski, 2008). However, a passive penetration of free Cu^{2+} into the skin layers has been also described (Franken et al., 2015), with a nearly uniform distribution of this ion in epidermis and dermis (Inoue et al., 2014). A recent work using human skin in a Franz static diffusion cell model demonstrated that the intact epidermis acts as a good barrier against the Cu^{2+} ions, with a negligible passage of these ions through the first layer of the skin (Zanoni et al., 2019).

It has to be mentioned that in this work the amount of ZnO NPs used for the coating of the fabrics (2.2 wt% of ZnO) is relatively high as compared to what is usually applied for getting a good antibacterial activity: the optimal coating concentration is approximatively 0.7 wt% (Perelshtein et al., 2009a). This high coating concentration might influence the toxicity effect. However, we wanted to check how different amounts of MeO-NPs for the coating of textiles, with CuO representing low concentrations and ZnO higher ones, could have influenced the skin toxicity. This evidence highlights the need to implement safe-by-design strategies for the improvement of textiles coating processes in order to

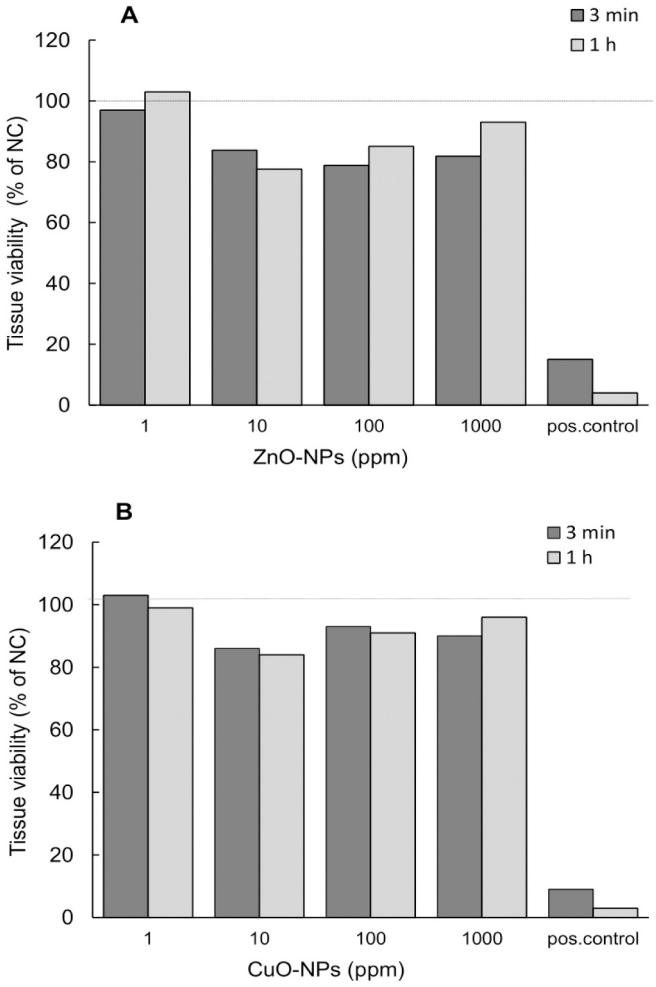

Fig. 2. Corrosion test. Tissue viability of EpiDerm™ exposed to MeO-NPs. EpiDerm™ skin model were exposed for 3 min and 1 h to the suspension of ZnO (a) and CuO (b) NPs, according to the OECD TG 431. Tissue viability was evaluated through MTT cell viability test. KOH 8 M was used as positive control. Mean values of the two repetitions ($N = 2$) for each experimental conditions according to OECD TG 431 protocol.

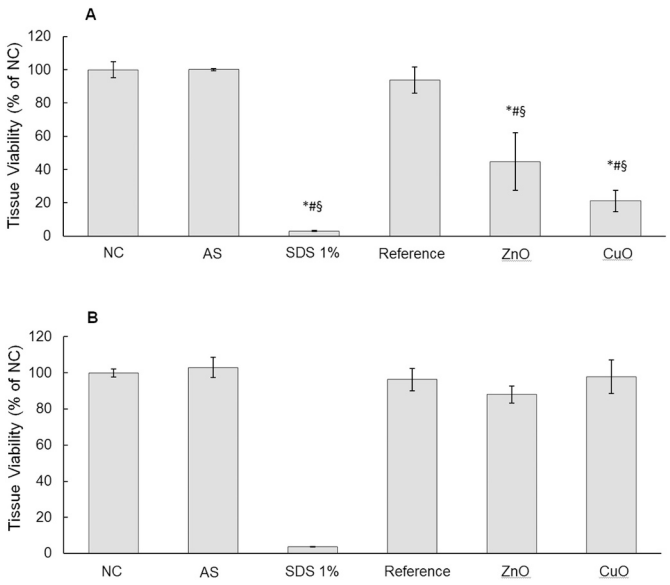

Fig. 3. Irritation test. Tissue viability of EpiDerm™ exposed to extracts from NPs-coated and reference textiles. A) Extraction in AS pH 4.7; B) extraction in AS pH 6.5. NC: negative control; SDS 1%: positive control. Data represent the mean ± SE of at least three replicates ($N = 6$ tissues for AS at pH 4.7; $N = 3$ tissues for AS at pH 6.5) according to OECD TG 439 protocol. $*p < 0.001$ vs NC; $\#p < 0.001$ vs AS; $\S p < 0.001$ vs Reference (Student's *t*-test).

obtain good antibacterial NEPs with less toxic adverse outcomes.

3.4.3. Cytoskeleton alteration

In order the investigate the adverse effects of textile extracts on the skin tissue organization, the morphological alterations of EpiDerm™ cytoskeleton were investigated upon 18 h exposure to CuO- and ZnO-coated extracts, obtained at the two AS pHs, in comparison to untreated tissues. In the samples treated with extracts in AS pH 6.5, the actin filaments maintain the same organized network observed in controls. On the other hand, a cytoskeleton disassembling is observed in the EpiDerm™ exposed to the extracts obtained at pH 4.7 (Fig. 4). The low pH itself is not responsible of actin filament alteration (Fig. S3), that is therefore associated to the high amount of Cu^{2+} and Zn^{2+} released at this pH condition.

The effect of CuO and ZnO extracts on EpiDerm™ morphology was also assessed by confocal analyses of immunodecorated cytokeratin-10 (CK-10), one of the intermediate filament proteins of the intracytoplasmic cytoskeleton of keratinocytes. Fig. 5 shows that CK-10 is strongly affected by the extracts obtained from CuO- and ZnO-textiles at both pH values. The expression of CK-10 at the periphery of cells is downregulated, and fluorescent agglomerates of condensed protein, not consistent with the cell shape, are detectable in all treated samples. Also, for CK-10, as for actin filaments, the effect seems due to the impact of the metal ions released in the extraction solutions on the epidermal cells. However, CK-10 is a more sensible target respect to actin, since the low amount of Cu^{2+} and Zn^{2+} dissolved from CuO and ZnO NPs-coated textiles at pH 6.5 (Table 3) is able to induce the same CK-10 alteration obtained with the extracts at pH 4.7. AS itself does not modify the CK-10 network at pH 6.5, while a certain CK-10 disorganization and condensation is appreciable at pH 4.7 (Fig. S4).

During adult life, the skin undergoes through periodically renewal as basal proliferative cells accomplish their terminal differentiation into squamous keratinocytes. In this transition, the epidermis homeostasis is highly regulated to maintain the dynamic balance between cell growth and differentiation (Zanoni et al., 2019). In case of the disruption of this tightly conserved mechanism, abnormal skin morphology, as well as aberrant skin functions and consequent pathological conditions may occur (Ota et al., 2014). During skin cells differentiation, spinous cells turn off cytokeratin 14 (KRT14, or CK14) expression, while turn on cytokeratin 10 (KRT10, or CK10) expression (Blanpain and Fuchs, 2009). CK10 is a fibrous protein that belongs to the family of the intermediate filaments of keratinocytes. CK10, together with keratin 1 (CK1), forms a robust cytoskeletal network connected with desmosomes (Fuchs and Green, 1980), reinforcing the cell-cell junctions and providing resistance and protection against mechanical and physical stress.

3.4.4. Interleukin-8 release

The inflammatory response induced after 18 h exposure to extracts from textiles coated with CuO and ZnO NPs was assessed by the investigation of IL-8 release from the exposed EpiDerm™. The release of metal ions from products in contact with the skin can cause inflammation and contribute to skin irritation (Fuchs and Green, 1980). A deregulated or increased cytokine release after topical exposure to toxic agents indicate an inflammatory skin condition and it is a hallmark of skin diseases such as acne, atopic, irritant and allergic dermatitis or psoriasis. Damage to skin induces the release of cytokines, such as interleukin (IL)-1α and IL-8, which is involved in skin inflammatory responses and is considered a key parameter for the classification of irritant agents (Kemény et al., 1994).

A significant increase in the production and release of IL-8 was observed after exposure to the extracts obtained in AS at pH 6.5, from the fabric coated with CuO nanoparticles and, to a lower extent, to ZnO NPs (Fig. 6). Therefore, the low percentage of Cu and Zn ions released in extraction solutions at pH 6.5 is able to exert an inflammatory response of epidermal cells, associated to release of cytokines. This result is in line with the strong impact on CK10 expression in the keratinocytes (Fig. 5). Indeed, it is known that the increase of cytokine production from reconstituted human epidermis induces dramatic changes in the biological properties of keratinocytes and, in particular, of some keratinocyte markers, including CK-10 (Rabeony et al., 2014).

Exposure of tissues to the only AS at pH 6.5 did not induce any inflammatory response, while a significant enhancement of IL-8 release was observed upon incubation with AS at pH 4.7 and no additional effects were recorded in the presence of the extracted NPs. Therefore, acidic pH itself seems to be correlated to an inflammatory response of epidermal cells which is likely responsible for CK-10 alteration (Fig. S4).

3.5. Toxicity of MeO-NEP extracts on fibroblasts

Epidermis is considered an efficient barrier to limit the entry of exogenous substances, both accidentally encountered as well as deliberately applied. In particular, the stratum corneum and the tight junctions in the stratum granulosum represent a tight barrier, known to be impermeable to most drug molecules above 500 kDa (Bos and Meinardi, 2000). Topically applied NPs can penetrate beyond the epidermis by the appendageal route, the intracellular route, or the intercellular route (Palmer and DeLouise, 2016), and ions derived from NPs dissolution can also permeate to dermis through the intact epidermal layer by diffusion or active transport (Franken et al., 2015). Moreover, very often skin can present some micro lesions and loss of the integrity of the stratum corneum increases the spaces between keratinocytes, leading to the leakage of NPs that could reach the dermis. This condition could be even more relevant if antibacterial textiles are used for bandages, bed sheet or gowns which are in contact with wounded skin. For this reason, since no NPs have been seen leaching from coated textiles, we evaluated the effect of the Cu^{2+} and Zn^{2+} released at the different pH conditions into the NEP extracts and permeated across EpiDerm™ model on fibroblasts. Moreover, we considered the impact of MeO-NEP extracts on fibroblasts, as representative of a direct interaction of ions with dermis cells by wounded skin.

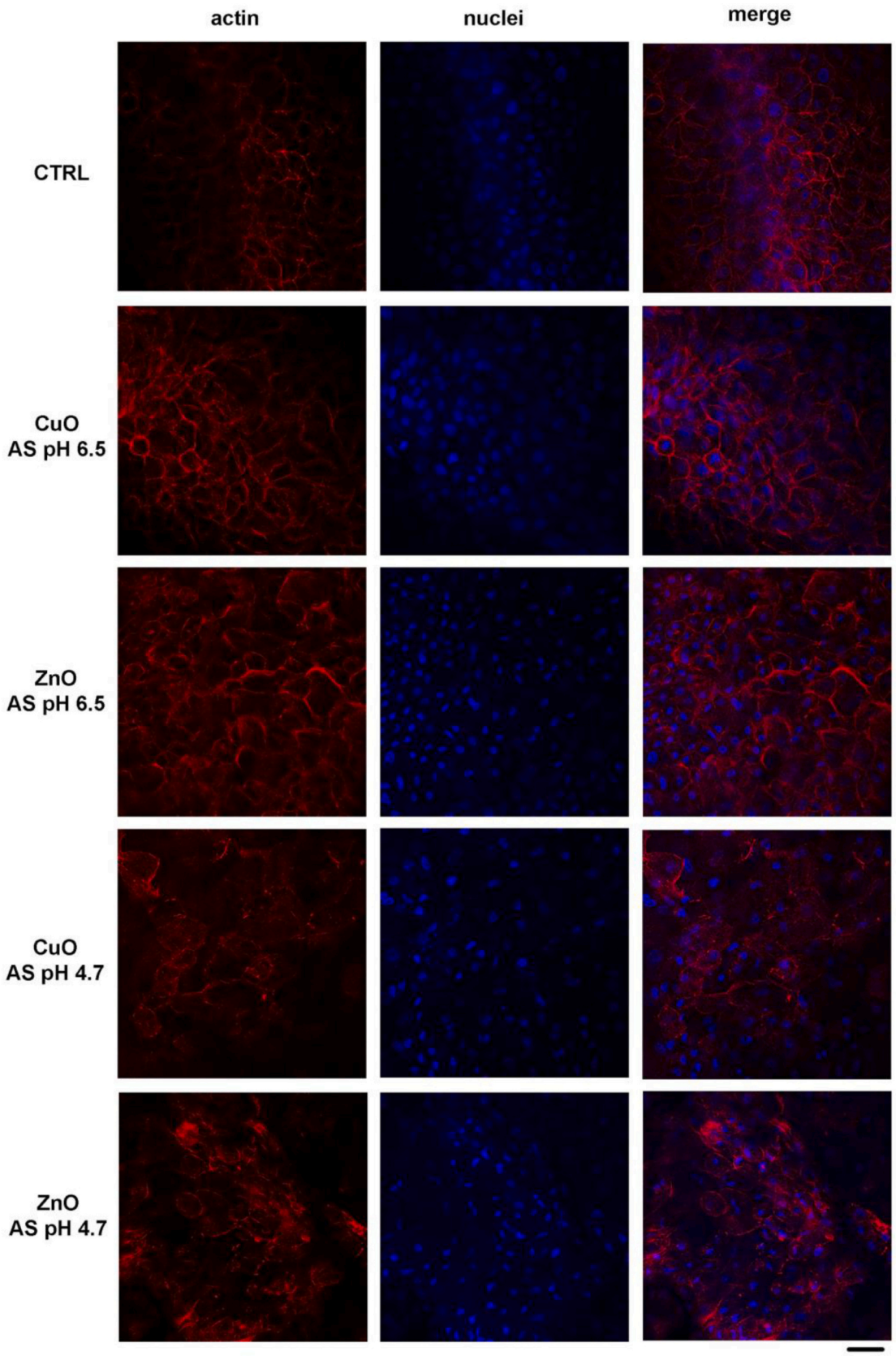

Fig. 4. Confocal microscopy of EpiDerm™ after 18 h exposure to extracts obtained in AS 6.5 and 4.7 pH from CuO- and ZnO-coated textiles. Tissues were stained for nuclei (DRAQ5, blue) and actin (rhodamine-phalloidin, red). CTRL: control tissue. Bar = 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

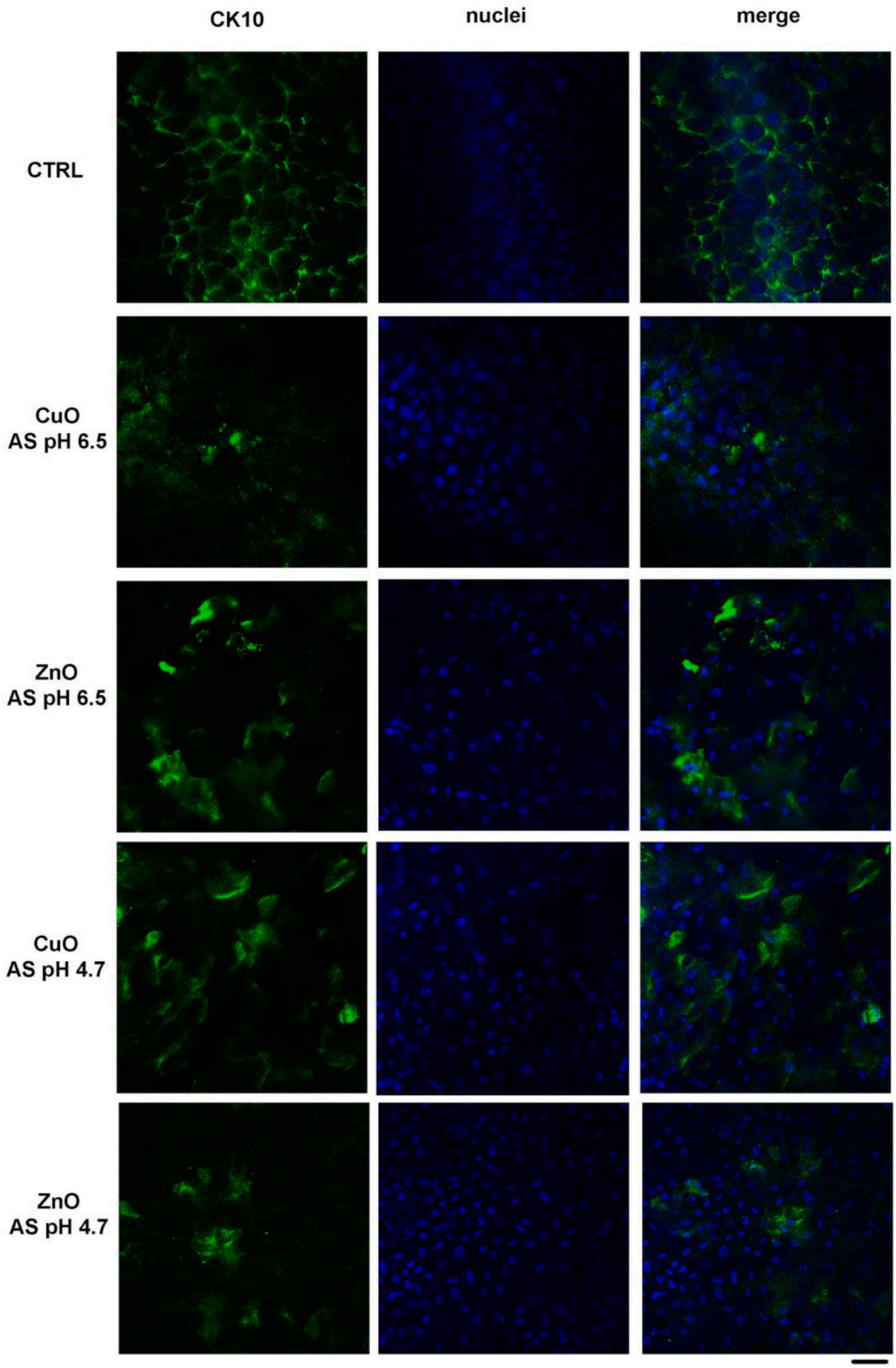

Fig. 5. Confocal microscopy of EpiDerm™ after 18 h exposure to extracts obtained in AS 6.5 and 4.7 pH from CuO- and ZnO-coated textiles. Tissues were stained for nuclei (DRAQ5, blue) and cytokeratin-10 (anti-CK10, green). CTRL: control tissue. Bar = 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5.1. Effects of trans-epidermis permeated ions on fibroblasts viability

In order to simulate the permeation of ions through the skin, fibroblasts were exposed for 18 h to the supernatants collected from the basal compartment of the EpiDerm™ models after treatment with textile extracts in AS at different pH, and cell viability was assessed. To reduce the effect of acidic pH and better appreciate the impact on cells of the released ions, we chose to buffer the solutions obtained from EpiDerm™ exposed to extracts at pH 4.7 with Hepes. Indeed, a significant cytotoxicity on fibroblasts was observed with the supernatants obtained after epidermis exposure to extracts from CuO- and ZnO-textiles, but also with the reference textile extracts and AS at acidic pH (Fig. S5A). Cell viability data obtained with buffered samples clearly showed that the high amount of Cu^{2+} and Zn^{2+} ions released in the extraction solution at pH 4.7 are able to permeate massively from the upper solution of

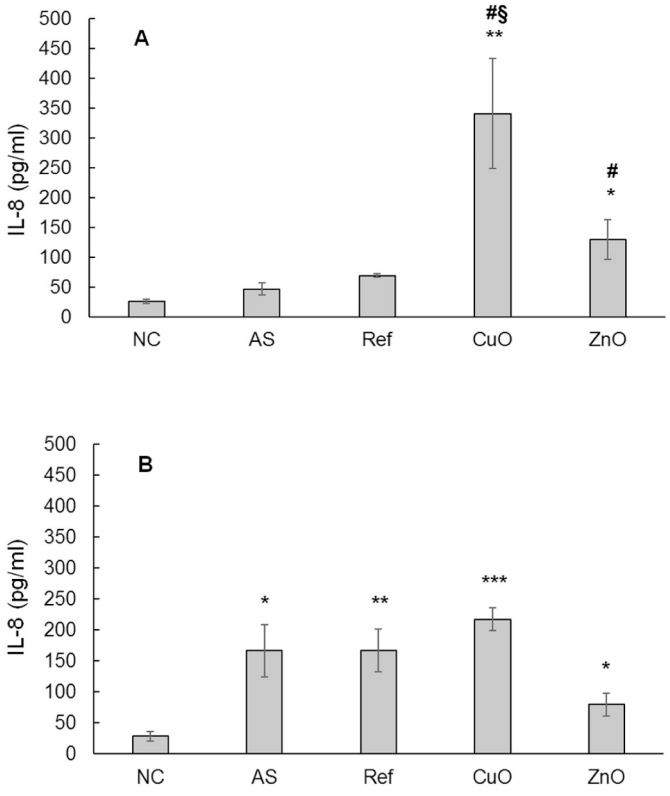

Fig. 6. Release of IL-8 after 18 h of exposure of EpiDerm ™ to tissue extracts obtained in AS at pH 6.5 (A) and 4.7 (B). NC: negative control; AS: artificial sweat; Ref: Reference fabric. Data represent the mean ± SE of at least three independent experiments ($N = 3$). * $p < 0.05$; **$p < 0.01$; $p < 0.001$ with respect to NC; # $p < 0.05$ with respect to AS; § $p < 0.05$ with respect to the Reference (Student's *t*-test).

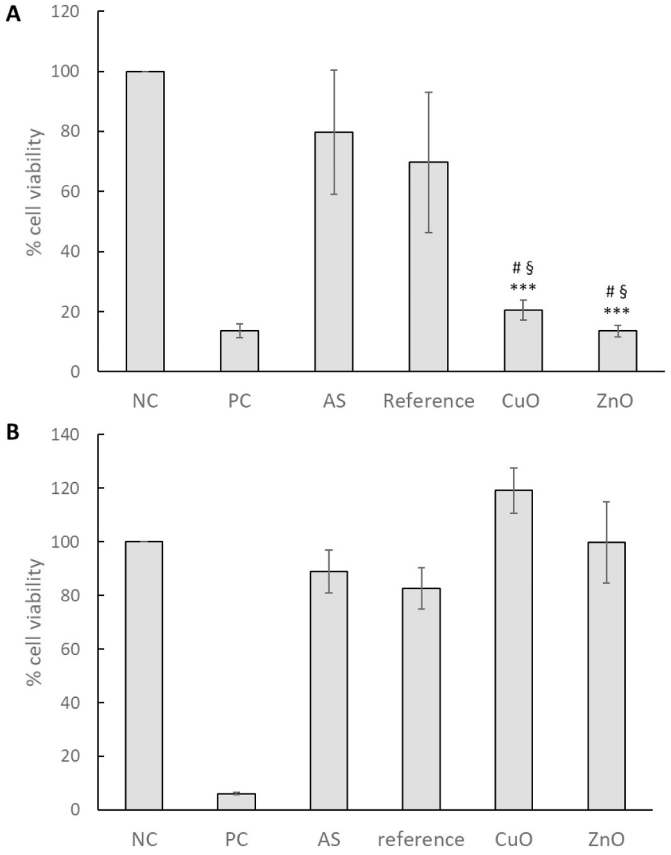

Fig. 7. Cell viability (MTT) of Balb/3 T3 cells treated for 18 h with the supernatants collected from the basal compartment of the EpiDerm™, after exposure to extracts from NPs-coated and reference textiles. A) Extraction in AS pH 4.7 buffered with Hepes, or B) pH 6.5. NC: negative control; PC (SDS 1%): positive control. Data represent the mean ± SE of at least three independent experiments ($N = 3$). ***$p < 0.001$ with respect to NC; # $p < 0.05$ with respect to AS; § $p < 0.05$ with respect to the Reference (Student's *t*-test).

EpiDerm™ to the basal compartment, to exert their toxicological activity on underlying dermis cells (Fig. 7A). As expected, exposure to the supernatants collected from basal compartment after EpiDerm™ incubation with the textile extracts at pH 6.5, did not induce a significant decrease of fibroblast viability (Fig. 7B). To the low amount of Cu^{2+} and Zn^{2+} released from the textile in this experimental condition (< 0.1 mg; Table 3), is further reduced by permeation across the tissue and ions, that reach the basal environment in very small quantities, are therefore ineffective against dermal cells. However, the contribution from other molecules released at the basal compartment, such as cytokines or other biological mediators on fibroblasts viability cannot be excluded.

3.5.2. Effect of extracts from MeO-NPs coated textiles on fibroblasts viability

Fibroblasts were also directly exposed to the extracts of CuO- and ZnO-coated textiles at pH 4.7 and 6.5, to evaluate the effects of released Cu^{2+} and Zn^{2+} on dermis in case of wounded skin. Also in this case, we buffered all the solutions at pH 4.7 with Hepes, in order to minimize the effect of acidic pH (Fig. S5B) and better appreciate the impact of the metal ions on cells. A strong impact on cell viability was observed with the extracts at pH 4.7 (Fig. 8A), in line with the high quantity of ions released at this pH value (Table 3). At pH 6.5, a small decrease of cell viability was obtained upon exposure to CuO-textiles extracts, but surprisingly we found a reduction of cell viability down to 5% with ZnO sample (Fig. 8B). This result can be explained if we consider that, although the quantity of Zn^{2+} in the extracts is relatively small, it is almost twice that of Cu^{2+} (0.07 vs. 0.04 mg, Table 3), that corresponds to a zinc concentration about three-fold higher than that of copper (1300 vs. 370 μM). In addition, a higher responsiveness to zinc respect to copper has been previously demonstrated on another model of fibroblasts (human lung fibroblasts, MRC-5) at concentration over 100 μM (Kioumourtzi, 2015).

4. Conclusions

MeO-NPs have a great potential in the medical textile coating but, in the perspective of a safe-by-design approach for the synthesis of new antibacterials, their ability to induce toxic responses on medicated skin remains a main concern. In the present study, the toxicological impact of CuO- and ZnO-NPs coated textiles on epidermis and dermis representative cells has been investigated, by using 3D human reconstructed Epiderm™ and fibroblasts monolayers, respectively. The toxicological behavior of these MeOs-NMs was assessed at different artificial sweat pHs, to simulate conditions as close as possible to the physiological ones, and according to well-standardized OECD protocols. We demonstrated that antibacterial CuO- and ZnO-NMs are overall safe on intact epidermis, unless NPs dissolution in acid sweat takes place. In particular, the high release of both Cu^{2+} and Zn^{2+} ions from textiles, obtained after the ISO guidelines-based extraction procedure in acidic environment, significantly affects keratinocytes viability during Irritation test. A strong impact on keratinocytes cytoskeleton was also observed upon exposure to extracted Cu^{2+} and Zn^{2+}, but the intermediate filament protein CK10 has proven to be a more susceptible marker of toxicity respect to actin, since able to be altered also in response to the low number of ions released at pH 6.5 or in the presence of the only acidic sweat. A strong sensitivity of epidermal tissue toward metal ions was also observed by measuring cytokine: a higher IL-8 release respect to

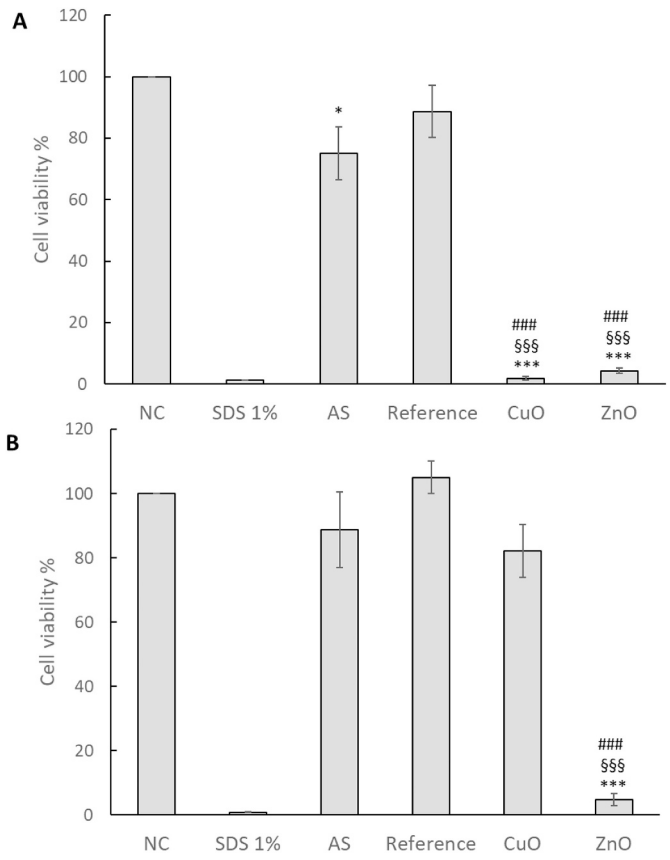

Fig. 8. Cell viability (MTT) of Balb/3 T3 cells exposed for 18 h to extracts from NPs-coated and reference textiles. A) Extraction in AS pH 4.7 buffered with Hepes, or B) pH 6.5. NC: negative control; PC (SDS 1%): positive control. Data represent the mean ± SE of at least three independent experiments (N = 3). *$p < 0.05$, ***$p < 0.001$ with respect to NC; ###$p < 0.001$ with respect to AS; §§§$p < 0.0041$ with respect to the Reference (Student's t-test).

controls occurred in the presence of ZnO- and, even more, of CuO-textiles extracts at pH 6.5. On the other hand, the inflammatory response of EpiDerm™ upon exposure to sweat at pH 4.7 did not allow appreciating the effect on this parameter of the large number of ions released from MeOs-coated textiles in this pH condition.

Analysis of fibroblasts treated with basal supernatants of EpiDerm™ exposed for 18 h to textiles' extracts indicated that released zinc and copper ions are able to permeate the epidermal tissue and reach the lower side of inserts. Only the ions produced in AS at pH 4.7 crossed epidermis in a sufficient amount to exert a toxicological effect on underlayer dermal cells, while the low number of ions released at pH 6.5 was ineffective on fibroblast viability.

In the nosocomial applications of MeOs-antibacterial textiles, the issue of skin integrity remains a critical point, since these materials could come into contact with injured skins. That's why we retained also important to assess the direct impact of textile-released Cu^{2+} and Zn^{2+}on fibroblasts. As expected, both ions released in AS pH 4.7 exerted a stronger reduction of cell viability respect to those filtrated by trans-epidermal permeation. Moreover, the much higher release of zinc versus copper ions resulted in a significant effect on dermal cells only of ZnO-textile extracts at pH 6.5.

So, we can conclude that: 1) although sonochemical coating of textiles with CuO and ZnO NPs is substantially stable, skin surface fluids composition can affect the stability of the NPs themselves, promoting the release of MeO ions; 2) sweat pH has a key role in the release of MeO ions by antibacterial textiles which can impact significantly on the epidermal tissue; 3) epidermis is not a so tight barrier to MeO ions, that can permeate through this tissue and exert adverse effects on dermal cells according to their final concentration; 4) skin barrier integrity is a fundamental parameter that needs to be taken into account when assessing the hazard potential of NPs-coated textiles, and it is therefore pivotal to investigate the toxicity on fibroblasts of a direct exposure to textiles extracts; 5) in addition to the standard cytotoxicity tests, the investigation on more sensible biomarkers is extremely important to guarantee improved safety; 6) when designing antimicrobial coatings for skin contact applications using nano CuO and ZnO, which are very powerful bactericidal and antiviral agents, it would be desirable to adopt stringent safe-by-design strategies oriented toward the limitation of NP release and metal dissolution, achievable by varying the NP surface properties and/or introducing dedicated textile finishing.

Author contributions

Conceptualization, R.B., L.F. and P.M.; Data curation, R.B., A.C., M. C. and L.F.; Funding acquisition, P.M., I.P. and A.G.; Investigation, R.B., A.C., I.P., P.M. and L.F.; Methodology, R.B., A.C., I.P., M.C. and L.F.; Supervision, L.F. and P.M; Roles/Writing - original draft, R.B., A.C. and L.F.; Writing - review & editing, R.B., A.C., I.P., A.G., M.C., P.M. and L.F. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2020.100282.

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