

Human skin penetration of cobalt nanoparticles through intact and damaged skin

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ABSTRACT

Cobalt nanoparticles (CoNPs) are produced for several industrial and biomedical applications but there is a lack of data on human cutaneous absorption. Cobalt is also a skin sensitizer that can cause allergic contact dermatitis. Co applied as NPs, due to their small size and high surface, can penetrate into the skin in higher amount than bulk material. The aim of this study was to evaluate the absorption of Co applied as NPs in both intact and damaged skin. Experiments were performed using Franz cells and 1.0 mg cm^{-2} of CoNPs was applied as donor phase for 24 h. Mean Co content of $8.5 \pm 1.2 \text{ ng cm}^{-2}$ and $1.87 \pm 0.86 \text{ } \mu\text{g cm}^{-2}$ were found in the receiving solutions of Franz cells when the CoNPs suspension was applied on intact skin and on damaged skin, respectively. Twenty-four hours Co flux permeation was $76 \pm 49 \text{ ng cm}^{-2} \text{ h}^{-1}$ in damaged skin with a lag time of $2.8 \pm 2.1 \text{ h}$. This study suggests that Co applied as NPs is able to penetrate the human skin in an in vitro diffusion cell system.

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1. Introduction

Metallic and magnetic nanoparticles with different structures, due to their small size (100 nm or less) compared to that of biological molecules, represent an enormous opportunity in the life sciences and in the medical field. The manufacture and manipulation of monodispersed metallic and magnetic nanoparticles (NPs) are increasing for their promising applications in magnetic resonance imaging, separation of different catalytic solids, fuel cells, catalyses (Kamat, 2002; Yang et al., 2006; Fernandez-Garcia et al., 2010).

Among these new nanocompounds, cobalt nanoparticles (CoNPs) are particularly attractive for their use in industry, as well as in biology and medicine where they are utilised in different forms, such as cobalt oxide, complex organic compounds or biopolymers (Wang et al., 2005). CoNPs offer powerful tools for research activity and have been proposed for a number of applications in many different fields because of their magnetic properties (Wu, 2010; Lin et al., 2009).

Cobalt is a sensitizer that can cause allergic contact dermatitis after skin contact. The contact with Co containing objects increases the risk of Co skin penetration that can cause allergic sensitization (Rui et al., 2010). CoNPs could have higher penetration potential

related to their small size and their higher surface can increase ions release compared to bulk material.

Moreover the International Agency on Research on Cancer (IARC) has classified this metal and its compounds as possibly carcinogenic to humans (2B) and Co can cause lung diseases in exposed workers as well as cardiac toxicity (Linna et al., 2004).

A study of CoNPs by Ponti et al. (2009) reported genotoxicity and morphological transformation of Balb/3T3 mouse fibroblasts with a dose-dependent statistically significant increase in morphological transforming activity. On analysing chromosomal aberrations, they observed an increase, not dose-dependent, in the frequency of micronucleated cells for CoNPs but not for Co^{2+} . The difference in the activity between the two forms of Co tends to suggest that CoNPs is a potential genotoxic carcinogen. On using the alkaline comet-assay and the micronucleus test, Colognato et al. (2008), showed that in human peripheral leukocytes there is an evident dose-dependent increase in the primary DNA damage for CoNPs but not for Co^{2+} . It has been suggested that these different effects may be attributed to the increased cellular uptake of CoNPs compared to Co-ions (Ponti et al., 2009).

On studying CoNPs aggregates compared to Co-ions, Limor et al. (2011) did not observe a different toxic effect on cell lines from lung, liver, kidney, intestine and immune system but they confirmed Co toxicity in vitro.

Skin absorption of Co has been found in occupationally exposed workers, in laboratory experiments with volunteers (Scansetti et al., 1994; Limnainmaa and Kiilunen, 1997) and in vitro experiments

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(Larese Filon et al., 2004). The penetration of nanosized Co into the skin could increase the risk of allergic sensitization or could induce a direct toxic effect on cells.

To investigate the potential CoNPs skin absorption in vitro, we carried out a series of permeation experiments with human skin using a Franz static diffusion cells (Franz, 1975; EDETOX, 2000). With this technique, we investigated the total amount of cobalt permeating through human skin during a 24-h period.

The Co into the skin were evaluated by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Magnesium nitrate hexahydrate, sodium hydrogenphosphate, potassium dihydrogenphosphate, and hydrogen peroxide (30% v/v) were purchased from Carlo Erba (Milan, Italy); nitric acid (>69% v/v) and hydrochloric acid (36.5–38% v/v) from Sigma Aldrich (Milan, Italy), ammonium hydroxide (25% w/v) was bought from J.T. Baker (Deventer, Holland). Water reagent grade was produced with a Millipore purification pack system (milliQ water).

2.2. Nanoparticles characterization

CoNPs were supplied by Sigma–Aldrich, Italy (20 nm < Co-nano < 500 nm; mean peak 80 nm) and characterized in our laboratory. Freshly prepared stock suspensions of Co-nano were washed by centrifugation (13 000×g for 30 min) to remove the Co²⁺ that arose from nanoparticles synthesis (10–15% of the initial concentration); they were ultrasonicated for 15 min and immediately diluted in synthetic sweat (0.5% sodium chloride, 0.1% urea and 0.1% lactic acid in milliQ water; pH 4.5 was adjusted with ammonia) to give test concentrations. The compounds were analysed for the elemental impurities (other metals) by Inductively Coupled Plasma Mass Spectrometry (Perkin-Elmer SCIEX, Ontario, Canada). The results of the analysis (data not shown) indicated a good degree of purity. Co-nano were characterized for their morphology by scanning electron microscope/energy dispersive using X-ray (SEM-EDX) technique and size distribution by nanoparticles tracking analysis (NanoSight LM20 Nanoparticles Analysis System, Salisbury, UK).

The CoNPs were visualized by Transmission Electron Microscopy (TEM) once they were dispersed in synthetic sweat and at the end of the experiments, after the 24 h exposure time.

2.3. Preparation of skin membranes

Human abdominal full thickness skin was obtained as surgical waste after the authorization of the local Ethical Committee and it was used for the absorption experiments immediately after the surgical operations. After the skin excision, subcutaneous fat was removed. From each skin specimen, 4×4 cm² pieces were cut and mounted separately on the diffusion cells, that were previously washed a first time with freshly prepared Aqua Regia, a second time with diluted nitric acid (6% v/v) and rinsed three times with milliQ water.

Skin integrity was tested before and after each experiment using electrical conductivity by means of a conductometer (Metrohm, 660, Metrohm AG Oberdorfstr. 68 CH-9100 Herisau) operating at 300 Hz and connected to two stainless steel electrodes (Fasano et al., 2009). The conductivity data in μS were converted into $\text{K}\Omega\text{ cm}^{-2}$. Cells with a resistance lower than

$3.95 \pm 0.27 \text{ K}\Omega\text{ cm}^{-2}$ were considered to be damaged and rejected as suggested by Davies et al. (2004).

2.4. In vitro diffusion system

Percutaneous absorption studies were performed using static diffusion cells following the Franz method (Franz, 1975). The receptor compartment had a mean volume of 14.0 mL and was maintained at 32 °C by means of circulation of thermostated water in the jacket surrounding the cell. This temperature value was chosen in order to reproduce the hand physiological temperature at normal conditions. The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na₂HPO₄, 0.19 g of KH₂PO₄ and 9 g of NaCl into 1 L of milliQ water (final pH 7.35). The concentration of the salt in the receptor fluid was approximately the same that can be found in the blood. The solution in each cell was continuously stirred using a Teflon coated magnetic stirrer.

Each piece of skin was clamped between the donor and the receptor compartment; the mean exposed skin area was 3.29 cm² and the average membranes thickness was 1 mm.

The experiments were carried out as follows:

2.4.1. Experiment 1

The donor phase was prepared just before the experiment: 100 mg of CoNPs were dispersed by sonication in 100 ml of synthetic sweat at pH 4.5 to reproduce in vivo condition. The total cobalt concentrations of the donor phases were confirmed in analyzed after the experiments by means of Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES).

At time 0, the exposure chambers of 4 Franz diffusion cells were filled with 3.3 mL of the donor suspension (1.0 mg cm^{-2}) to ensure an infinite dose. At selected intervals (2, 4, 8, 16, 24 h) 1.5 ml of the dermal bathing solution was removed and analyzed. Each receptor sample was immediately replaced with an equal volume of fresh physiological solution.

At 24 h the dermal bathing solution and the donor phase of each diffusion cell were recovered for the following analysis.

2.4.2. Experiment 2

Experiment 1 was repeated using an abraded skin protocol as suggested by Bronough and Steward (1985): skin was abraded by drawing the point of a 19-gauge hypodermic needle across the surface (20 marks in one direction and 20 perpendicular).

2.4.3. Blanks

For each experiment, two cells were added as blank. The blank cells were treated as the other cells with the exception that no CoNPs were introduced to the exposure chamber.

Each experiment was repeated twice, in order to use the skin of four different donors, for a total of 8 cells with intact skin, 8 cells with damaged skin, and 8 blank cells. Donors were men and women with a range of age from 55 to 70.

2.5. Skin content evaluation

After the experiments the skin pieces were removed from the diffusion cells, washed under running water for 5 min and in distilled water for 1 min to remove CoNP on the skin. Three of them (1 blank, 1 intact and 1 damaged skin) were prepared for TEM analysis and the others were separated into epidermis and dermis by heat shock immersing them in water at 60 °C for 1 min.

All the skin fractions were collected and stored individually in freezer at −25 °C for the following digestion and analysis. At the time of the analysis, the skin membranes were dried for 2 h at room temperature, then cut into sections and put into beakers with 20 mL of HNO₃ 69% v/v for digestion (amounts of skin were

0.92 ± 0.29 g). They were agitated at room temperature overnight and then heated at the boiling point for other 8 h (after 2 h they were added, drop by drop, of 2 ml of H₂O₂ 30% v/v) till the solution volumes were approximately of 2 ml. The solutions were diluted to a final volume of 10 ml with milliQ water for the analysis with ICP-AES.

2.6. Analytical measurements

The total cobalt concentration measurements in the receiving solutions were performed by Electro-Thermal Atomic Absorption Spectrometry (ETAAS) with Zeeman background correction. A Thermo M series AA spectrometer equipped with a GF95Z Zeeman Graphite Furnace and a FS95 Furnace Autosampler (Thermo Electron Corporation, Cambridge, UK) were used for analysis. A 0.5% w/v solution of Mg(NO₃)₂ as modifying matrix. The samples were analyzed measuring against standard solutions for instrumental calibration. Co detection limit at the analytical wavelength of 240.7 nm was 0.4 µg/L and the precision of the measurements as repeatability (RSD%) for the analysis was always less than 5%.

The total cobalt concentration in the donor phases and in the solutions resulting from the skin sample mineralization were conducted by means of Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using a Spectroflame Modula E optical plasma interface (OPI) instrument (by SPECTRO, Germany). The analysis were conducted using a calibration curve obtained by dilution (range: 0–10 mg/l) of Spectrascan® Cobalt standard suspension for ICP-AES analyses (by Teknolab A/S, Norway). The limit of detection (LOD) at the operative wavelength of 228,616 was 0.05 mg/l. The precision of the measurements as repeatability (RSD%) for the analysis was always less than 5%.

2.7. Data analysis

The results of Co content in the receptor solution were converted to the total amount that penetrated in µg cm⁻² (with a correction for dilution due to sample removal) and then plotted against time. The slope of this plot gives the rate of absorption of Co through skin, simple division yielding the rate per cm². Lag time was calculated as the intercept of the curve with the X axis. We calculated flux permeation from the linear steady-state region of the above plots by dividing the rate per unit area by the concentration used. Data analysis was performed using the statistical software SPSS for Windows (version 15.0). Data are reported as mean ± standard deviation (SD). The difference between independent data was assessed by means of the Mann–Whitney and Kruskal Wallis tests. A *p* value of <0.05 was considered as the limit of statistical significance.

2.8. Skin fixation protocol for TEM analysis

After removal some skin samples were cut into smaller sections and fixed for 3 h in a solution of 3% glutaraldehyde (Serva, Heidelberg, Germany) in 0.1 M cacodylate buffer (pH 7.3). The fixed sections were washed twice (10 min each) with 0.1 M cacodylate buffer and post fixed with 1% (w/v) osmium tetroxide for 1 h at 4 °C. Post-fixed samples were dehydrated with an ascending ethanol series ending to 100% (v/v) ethanol and then embedded in Down epoxy resin (DER332; Unione Chimica Europea, Milan, Italy) and DER732 (Serva). The last resin embedding was made under vacuum. Semi-fine and ultra thin sections were prepared with an ultra microtome Leica Ultracut UCT (Leica Microsystems, Milan, Italy) equipped with a diamond blade Drukker 3 mm (Emme3, Milan, Italy). Semi-fine sections were observed with an optical microscope Leitz Dialux 20 EB (Leica Microsystems, Milan, Italy) instead ultra thin sections were double stained with lead citrate and uranyl

acetate and observed with TEM (EM208; Philips, Heidehoven, The Netherlands) with an high definition acquisition system SIS Morada and a digital image acquisition system iTEM (FEI Italia, Milan, Italy).

3. Results

CoNPs showed a size distribution ranging between 20 and 500 nm with a peak at 80 nm. The TEM visualization confirmed a primary size of around 80 nm with the tendency to form aggregates: the presence of aggregates was much more evident in the donor solutions at the end of the 24 h exposure time as reported in Fig. 1.

Permeation profiles of Co are reported in Figs. 2 and 3: Co passed through the intact skin in small amount reaching 8.5 ± 1.2 ng cm⁻² at 24 h (Fig. 2). In damaged skin the Co permeation was significantly higher (Kruskal-Wallis test *p* = 0.001) reaching 1.87 ± 0.86 µg cm⁻² at 24 h (Fig. 3 and Table 1). In this latter condition Co flux through the skin was 0.076 ± 0.049 µg cm⁻² h⁻¹ with a lag time of 2.8 ± 2.1 h.

The cobalt concentration into the damage skin was significantly greater than into the intact skin with values of 12.0 ± 3.8 µg cm⁻² and 4.35 ± 1.36 µg cm⁻², respectively (*p* < 0.02; Fig. 4). In intact skin, epidermis contained a greater amount of Co compared with derma (3.53 vs 0.82 µg cm⁻²; *p* < 0.02), conversely, a similar concentration of Co was found in epidermis and dermis in damaged skin (6.38 vs 5.59 µg cm⁻²).

TEM investigation revealed the presence of CoNPs in the skin, from the stratum corneum to the epidermis, in both the intact and the damaged skin (Figs. 5 and 6). TEM allowed to observe, at least qualitatively, the presence of images that for size and shape can be attributable to CoNPs. Chemical analysis of the skin confirmed the presence of Co into the different skin layers.

4. Discussion

This study investigated the skin absorption of Co applied as NPs using full thickness human skin (epidermis and dermis): experiments were conducted with both intact and damaged skin in an in vitro diffusion cell system. The findings suggest that human skin absorption of CoNPs is possible in intact and damaged skin and that skin lesions increase both the penetration into the skin and the permeation in the receiving solution. The chemical analysis by means of ICP-AES confirmed the presence of Co in both the epidermis and the derma.

Different types of NPs have been tested with respect to skin absorption (Crosera et al., 2009; Baroli, 2010): TiO₂ (Kiss et al., 2008; NANODERM, 2007), Ag (Larese Filon et al., 2009a; Samberg et al., 2010), Au (Larese et al., 2011; Sonavane et al., 2008), iron-based NPs (Baroli et al., 2007), quantum dots and carbon based NPs (Monteiro-Riviere and Inman, 2006). However, there is evidence of discrepancies in nanoparticle transcutaneous penetration between studies.

NPs can permeate through pilosebaceous pores and sweat gland pores (Schaefer et al., 1990; Meidan et al., 2005). Vogt et al. (2006) showed that 40 nm nanoparticles are able to penetrate the perifollicular dermis through the hair follicles. Baroli et al. (2007) showed that iron nanoparticles can penetrate the skin through the stratum corneum lipidic matrix and hair follicle orifices, reaching the deepest layers of the stratum corneum and less often the uppermost strata of the viable epidermis, but these nanoparticles do not permeate the skin.

It is already known that metallic cobalt can permeate the skin in volunteers (Scansetti et al., 1994; Limnainmaa and Kiilunen, 1997) and in in vitro experiments using Franz cells (Larese Filon et al.,

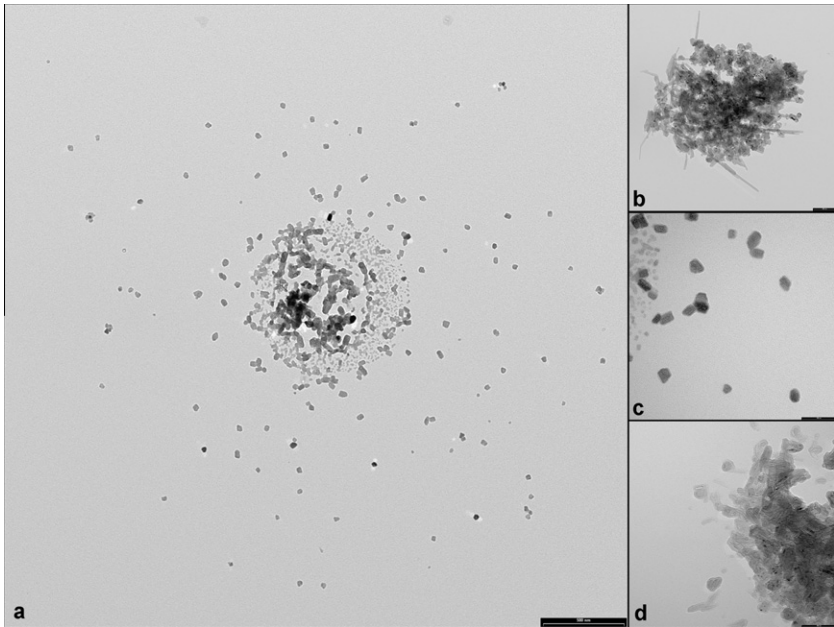


Fig. 1. CoNPs visualized by TEM in the donor suspension after the experiment: (a) bar 500 nm; (b) bar 200 nm; (c) 100 nm, (d) 100 nm.

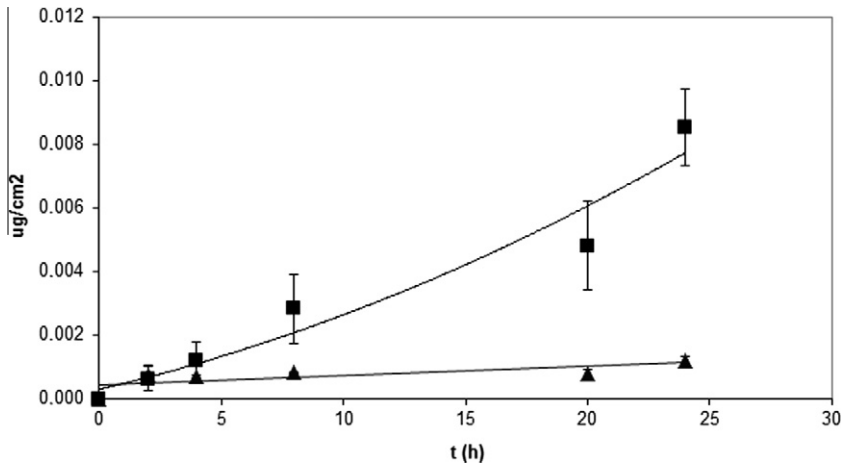


Fig. 2. Co in receiving phase in 8 intact (square) and 8 blank (triangle) cells. Human skin from 4 donors. Two replicates.

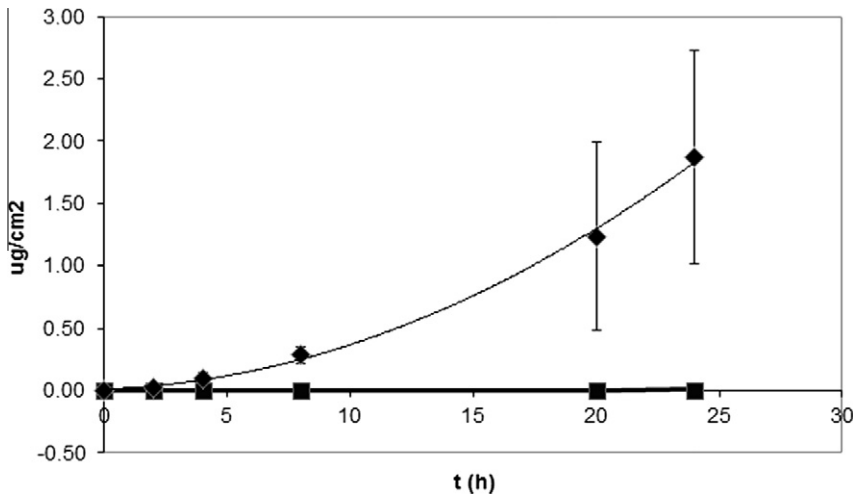


Fig. 3. Permeation profile of Co in receiving phases in 8 intact (square) and 8 damaged skin (diamond). Human skin from 4 donors. Two replicates.

Table 1Cobalt (ng cm^{-2}) in receiving phases in intact, damaged and blanks cells.

Time (h)	Intact skin		Damaged skin		Blanks	
	Mean $\pm$ SD		Mean $\pm$ SD		Mean $\pm$ SD	
2 ^a	0.67	0.5	24.6	28.0	0.8	0.3
4 ^b	1.2	0.4	94.3	53.5	0.75	0.07
8 ^c	3.0	1.1	289.6	65.8	0.85	0.07
20 ^d	5.0	1.4	1235.9	750.5	0.80	0.14
24 ^e	8.5	1.3	1870.6	860.4	1.2	0.14
Flux (ng cm^{-2})	0.55 $\pm$ 0.33		76 $\pm$ 49.3		–	
Lag time (h)	7.2 $\pm$ 7.0		2.8 $\pm$ 2.1		–	

Kruskal-Wallis test for the differences between blanks, intact and damaged skin:

^a $p = 0.045$; ^b $p = 0.0023$; ^c $p = 0.0027$; ^d $p = 0.0029$; ^e $p = 0.0028$.Contrasts: at 2 h: damaged vs intact $p = 0.011$ at 4 h, 8 h, 20 h, 24 h: damaged vs blanks $p = 0.0017$; damaged vs intact $p = 0.0045$.

2004, 2007, 2009a,b). Our findings demonstrated that Co applied as NPs can permeate the skin. The higher surface/volume ratio of the NPs compared to the bulk materials can lead up to an increase of the metal ion release (Larese Filon et al., 2004; Ponti et al., 2009), and the consequent dermal permeation. Another contribution to the total absorption rate may derive from the direct penetration into the tissue of the metal NPs. To compare results with previous studies performed using Co powder (Larese Filon et al., 2004, 2009,b) we standardized results considering different concentration of cobalt in donor solutions (Table 2). Co in receiving phases is 10 times more in cells exposed to CoNP than to Co Powder considering damaged or intact skin. Skin Co content is 3 times more in CoNP vs Co Powder exposed cells. On the contrary Co flux through the skin was similar between CoNP and Co Powder and no differences were found between receiving solutions in intact skin comparing experiments performed in 2004.

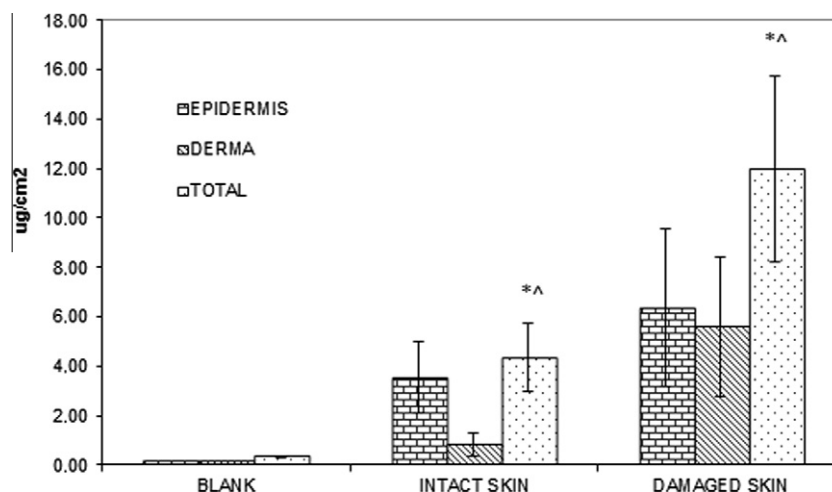


Fig. 4. Cobalt into the skin (mean values and standard deviation on 8 cells each, 2 replicates, 4 donors). *Kruskal-Wallis test between blank, intact and damaged skin ($p = 0.002$). ^Mann-Whitney test between blank and intact and between intact and damaged ($p < 0.002$).

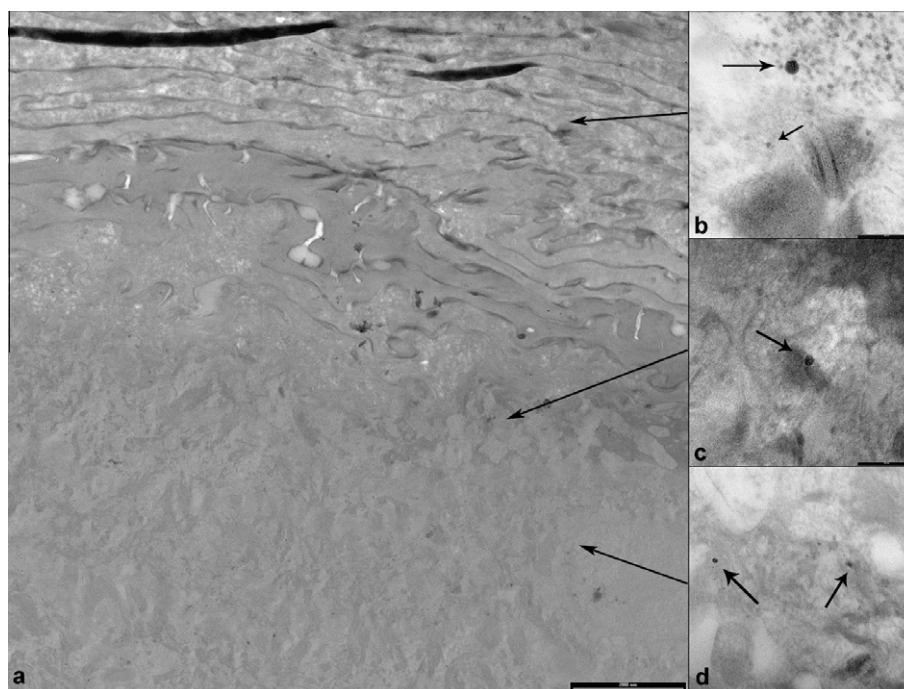


Fig. 5. Intact skin a. TEM micrograph of CoNPs-treated skin sample (Bar = 10 μm). (b–d) Some details of the epidermis (b), and of the dermis (c, d) Black arrows show Co nanoparticles in the tissue. (Bar = 200 nm).

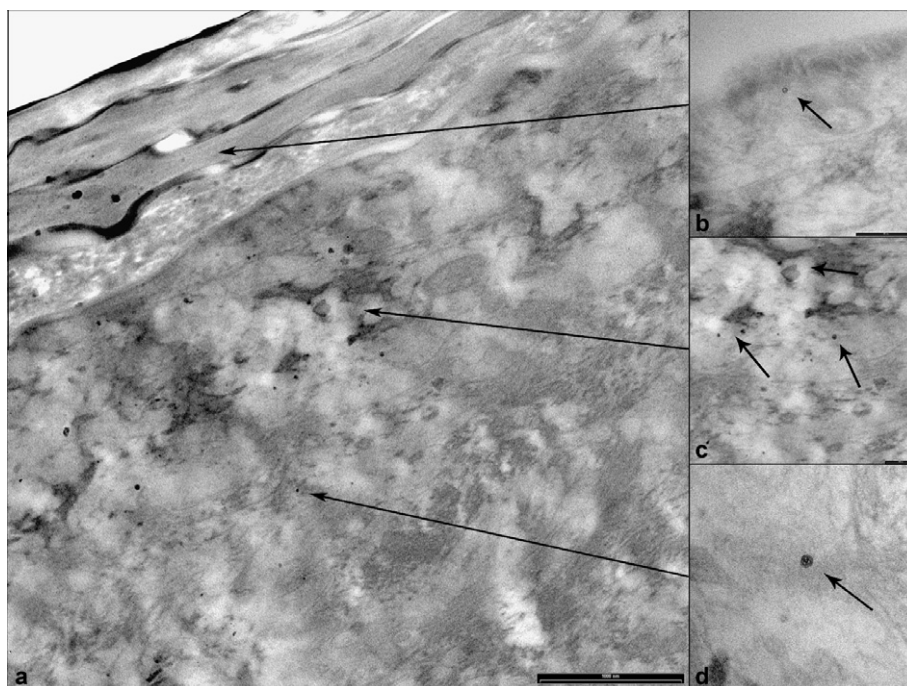


Fig. 6. Damaged skin (a) TEM micrograph of CoNPs-treated skin sample (Bar = 10 μm). (b–d) Some details of the stratum corneum (b) and of the dermis (c, d) Black arrows show Co nanoparticles in the tissue. (Bar = 200 nm).

Table 2
Cobalt concentration ($\mu\text{g cm}^{-2}$) into the skin and in receiving solution at 24 h in cobalt nano vs cobalt powder considering 2 previous studies. To compare values we standardized results considering the different concentration of Co in donor solution.

Donor suspension	Co-nano (peak 80 nm) 1.0 mg cm^{-2}		Co-powder (peak 2 μ) ^a 15.9 mg cm^{-2}		Standardized values 1.0 mg cm^{-2}	Co-powder (peak 2 μ) ^b 23.9 mg cm^{-2}		Standardized values 1.0 mg cm^{-2}
	Mean	SD	Mean	SD		Mean	SD	
<i>Skin</i>								
Intact	4.35 [*]	1.36	–	–	–	32.02	8.86	1.33
Damaged	12.0 [*]	3.8	–	–	–	79.5	65.9	3.2
<i>Receiving solution</i>								
Intact	0.0085 [*]	0.0012	0.26	0.22	0.02	0.02	0.017	0.0008
Damaged	1.87 [*]	0.86	–	–	–	3.46	2.25	0.14
<i>Flux $\text{ng cm}^{-2} \text{h}^{-1}$</i>								
Intact	0.55	0.33	12.3	5.4	0.77	–	–	–
Damaged	76	49.3						

^{*} Mann–Whitney test between Co-nano and Co-powder $p < 0.05$.

^a Data from Larese Filon et al. (2004).

^b Data from Larese Filon et al. (2009b) reported ad mean and SD.

From a toxicological viewpoint, our findings suggest that Co applied as NPs can permeate the human skin reaching the epidermis and dermis where these NPs can represent a long-term source of ions which can be involved in the sensitization process, or can be available for systemic diffusion. It has been already shown that NPs administered in the dermis migrated to regional lymph nodes, potentially via skin macrophages and Langerhans cells, raising potential concern for sensitization (Kim et al., 2004). These possible effects, together with an increased risk of Co absorption through inhalation route, suggest the need of preventive measures for workers who either produce or manipulate CoNPs.

Nevertheless the aggregation of CoNPs reduces skin penetration in a significant way and the comparison between our data with previous experiments performed using Co powder demonstrated an increase of CoNP permeation compared to Co Powder but failed to found a “dramatic” increase related to the higher surface area.

There are some limitations of this study that deserve attention. A limitation of our in vitro experiments is that the stratum cor-

neum was exposed over 24 h. Excessive hydration is known to increase the penetration of many compounds. Nevertheless, people have often mechanically damaged skin such as cuts and abrasions, which would make penetration substantially higher. Another limitation is that the experiments were carried out with Franz cells in which skin was static: human skin flexion has been suggested to lead to an increased translocation of small particles into the cell membrane (Rouse et al., 2007; Tinkle et al., 2003); in this case, the in vitro results can underestimate the risk at the workplace.

In conclusion, this experimental study showed for the first time that Co applied as NPs can permeate the skin in an in vitro diffusion system. These findings suggest, anyway, the implementation of measures which can minimize the potential contact with CoNPs on occupationally exposed workers.

Conflict of interest

The authors declare that there are no conflicts of interest.

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