UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN SCIENZE DELLA PREVENZIONE

CICLO XXII

STUDI DI PERMEAZIONE CUTANEA IN VITRO DI POLVERI E NANOPARTICELLE METALLICHE

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RIASSUNTO

Il ruolo della cute è stato a lungo sottostimato, soprattutto in ambito professionale: essa era considerata una barriera impermeabile alle sostanze chimiche. Oggi è noto che molti agenti tossici sono in grado di penetrare attraverso la pelle e l'assorbimento cutaneo è un fenomeno molto studiato nell'ambito di una completa valutazione dei fattori di rischio sia in ambito professionale che ambientale. Gli studi di assorbimento percutaneo *in vitro* possono essere usati per definire le caratteristiche di diffusione dei composti chimici in differenti settori industriali, per la valutazione del rischio da esposizione cutanea a sostanze tossiche, ma anche come metodo per documentare la loro biodisponibilità.

L'assorbimento attraverso la pelle di metalli in polvere (cobalto, nichel, cromo) è stato evidenziato in lavoratori esposti e in esperimenti di laboratorio con volontari, ma in generale ci sono ancora pochi dati sull'argomento.

Questo lavoro di dottorato ha l'obiettivo di studiare l'effetto di alcune variabili, quali il pH del sudore sintetico, l'utilizzo di detergenti e la presenza di lesioni cutanee, sull'assorbimento cutaneo di questi metalli in un sistema di diffusione in-vitro.

Inoltre si è scelto di applicare l'esperienza nell'utilizzo delle celle di diffusione di Franz ai nuovi nanomateriali ed, in particolare, alle nanoparticelle metalliche dato che lo sviluppo delle nanotecnologie è molto veloce, ma l'impatto dei nanomateriali sulla salute umana è ancora da studiare.

I risultati dei nostri esperimenti hanno mostrato che, *in vitro*, ioni di cobalto, nichel e cromo rilasciati da polveri metalliche, ma anche nanoparticelle di argento e oro, sono in grado di permeare attraverso la cute, e che la presenza di lesioni cutanee, l'uso di detergenti e il pH della fase donatrice, sono in grado di volta in volta di modificare tale processo.

PAROLE CHIAVE: Assorbimento cutaneo; *in vitro*; Celle di Franz; Polveri metalliche; Nanoparticelle

ABSTRACT

The role of the skin, in occupational and public hygiene, has been underestimated for a long time: until the mid-1960s it was considered as an almost impermeable barrier for chemicals. Actually is known that many hazardous substances can permeate through the skin and dermal absorption is studied from a complete risk assessment point of view.

In vitro percutaneous permeation studies can be used to define the diffusion characteristic of chemicals in different industrial settings for the dermal exposure risk assessment, but also as one way of documenting their bioavailability.

Skin absorption of metal powders (cobalt, nickel and chromium) has been found in exposed workers and in laboratory experiments with volunteers, but in general there are few available data on this argument.

This doctoral work aims to study the skin absorption of metal powders and the effect of some variables, like the pH of the synthetic sweat, the decontamination with detergents, or the presence of lesions on the skin in an in vitro diffusion system.

Moreover, it has been decided to apply our experience with Franz cells use to the study of new nanomaterials, and, in particular, to metal nanoparticles, since nanotechnology development is very fast, but the impact of nanomaterial on human health is less clear.

Our results shown that ions of cobalt, nickel and chromium released from metal powders, but also silver and gold nanoparticles, can permeate through the skin in in vitro experiments. This process can be modified by the presence of skin lesions, by the decontamination with detergents and by the pH of the donor phase.

KEYWORDS: Dermal absorption; *in vitro*; Franz Cells; Metal powders; Nanoparticles

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1. SCOPO DELLO STUDIO

Il progetto di ricerca nel quale si inserisce questo lavoro di dottorato nasce dalla collaborazione tra il gruppo di ricerca di Chimica Analitica e Ambientale e l'Unità Clinica Operativa di Medicina del Lavoro dell'Università di Trieste e si propone di studiare la cute come barriera semipermeabile bidirezionale con l'obiettivo di comprendere il suo ruolo nell'assorbimento, nell'escrezione e nella sensibilizzazione allergica da contatto e gli aspetti molecolari a questi correlati.

In generale, nel campo della tutela della salute occupazionale e ambientale, infatti, l'assorbimento percutaneo sta riscuotendo negli ultimi anni sempre maggior interesse, al pari delle vie classiche di assorbimento quali inalazione e ingestione, nella logica di una valutazione completa dei rischi dovuti all'esposizione a sostanze tossiche.

Gli studi di assorbimento percutaneo *in vitro* possono essere usati per definire le caratteristiche di diffusione dei composti chimici in differenti settori industriali, dalla cosmetica alla farmaceutica, dalla produzione di detergenti all'agrochimica, per la valutazione del rischio da esposizione cutanea a sostanze tossiche, ma anche come metodo per verificare la loro biodisponibilità. Per questo in alcuni paesi i test *in vitro* sono inclusi tra i criteri per assegnare le skin notation ai composti chimici (Drexler, 1998).

In questo progetto di ricerca, dopo i primi lavori riguardati l'assorbimento percutaneo *in vitro* di composti organici, quali benzine, glicoleteri, testosterone, acido benzoico e caffeina, e alla partecipazione al progetto europeo EDETOX (2000) per la standardizzazione dei metodi degli studi *in vitro* (van der Sandt et al., 2004), si è passati allo studio di polveri di metalli, come cobalto, nichel e cromo (Larese et al., 2004; 2007), e di ossidi metallici, quale il biossido di piombo (Larese et al., 2006).

L'assorbimento attraverso la pelle di metalli in polvere (cobalto, nichel, cromo, rame, ecc.) è stato evidenziato in lavoratori esposti e in esperimenti di laboratorio con volontari, ma in generale ci sono ancora pochi dati sull'argomento.

Cobalto, nichel e cromo sono dei comuni sensibilizzanti e la loro presenza in oggetti metallici di uso quotidiano può aumentare il rischio di dermatiti allergiche da contatto in soggetti sensibilizzati. La valutazione dell'assorbimento attraverso la pelle di tali metalli in un sistema *in vitro,* come le celle a diffusione di Franz, può contribuire a definire un quadro più completo del rischio da esposizione cutanea, sia in ambito professionale che domestico.

Questo di dottorato si inserisce a questo livello, con l'obiettivo di studiare l'effetto sull'assorbimento cutaneo di questi metalli, di alcune variabili, quali il pH del sudore sintetico, l'utilizzo di detergenti, l'entità dell'esposizione e la presenza di lesioni cutanee, in un sistema *in vitro*.

Inoltre si è scelto di applicare l'esperienza maturata nel corso degli anni nell'utilizzo delle celle di diffusione di Franz ai nuovi nanomateriali ed, in particolare, alle nanoparticelle metalliche. La penetrazione cutanea di nanoparticelle deve essere ancora studiata e i protocolli classici di valutazione devono essere adattati e ristandardizzati per i nuovi nanocomposti.

Le colture cellulari (Bernstein and Vaughan, 1999), le celle di diffusione di Franz (Franz, 1975), il "tape stripping" (Escobar-Chávez et al, 2008) e gli impianti di cute umana su animali, rimangono strumenti importanti per studiare le interazioni tra le nanoparticelle e il tessuto cutaneo umano, ma devono anche essere sviluppati nuovi metodi e nuove applicazioni tecniche (Moger et al., 2008, Monteiro-Riviere and Inman, 2006; SCCP, 2007).

La valutazione della tossicità delle nanoparticelle metalliche richiede innanzitutto la determinazione delle loro caratteristiche quali forma, dimensioni, concentrazione, purezza e tendenza all'aggregazione. Le caratteristiche chimico-fisiche delle nanoparticelle, infatti, sono intrinsecamente collegate alla loro reattività e quindi alle loro possibili applicazioni, ma anche alla loro tossicità (Auffan et al., 2009).

Una differenza molto importante nello studio di polveri metalliche nanometriche rispetto alle micropolveri risulta essere il meccanismo con cui il metallo permea la cute. Nel caso di particelle grossolane, esse non sono in grado di penetrare la cute, ma in sudore sintetico il metallo si ossida e gli ioni possono eventualmente attraversare la barriera cutanea. Nel caso delle nanoparticelle, invece, essere potrebbero penetrare la cute anche in quanto tali; inoltre l'alto rapporto superficie/volume delle nanoparticelle fa sì che anche la ionizzazione del metallo possa avvenire in maniera più estesa rispetto alle micropolveri.

La scelta dei metodi analitici più adatti alla tipologia del composto ed alle sue concentrazioni nelle varie fasi e la ricerca di nuove applicazioni strumentali necessarie all'attuale campo di indagine, rivestono quindi un ruolo molto importante nel progetto in corso.

2. INTRODUZIONE

2.1. LA CUTE

La cute è l'organo più grande del corpo umano e, oltre alla sua primaria funzione di barriera verso l'ambiente esterno, ricopre diversi ruoli nel mantenimento dell'omeostasi fisiologica. Essa, per esempio, previene la disidratazione minimizzando la perdita di acqua, partecipa alla termoregolazione, fornisce supporto per gli organi interni, protegge il corpo dalle radiazioni UV e partecipa alla sintesi di vitamina D.

Anatomicamente, la cute è costituita da due strati principali: l'epidermide, di circa 0,05-0,1 mm, e il derma di spessore 0,3-3 mm. L'epidermide è ulteriormente divisa in strati: partendo dall'interno verso la superficie, troviamo lo strato basale, lo strato spinoso, lo strato granuloso, lo strato lucido e lo strato corneo (figura 1). Questi strati di cellule hanno sia il ruolo di ancorare l'epidermide al sottostante derma, sia quello di sostituire le cellule morte che si staccano costantemente dalla superficie dello strato corneo e di formare una barriera semipermeabile che protegge l'ambiente biologico interno dall'ambiente esterno. Il derma consiste invece di una rete irregolare composta da collagene, elastina e fibre reticolari. che fornisce un supporto meccanico al tessuto. Una rete estesa di capillari, nervi e vasi linfatici sono situati nel derma per facilitare lo scambio di metaboliti fra i tessuti e il sangue, per il riparo dei tessuti, per la protezione dalle infezioni. Al di sotto del derma è presente uno strato sottocutaneo adiposo che lega la pelle ai tessuti sottostati, muscoli ed ossa (Monteiro-Riviere, 2004, 2006).

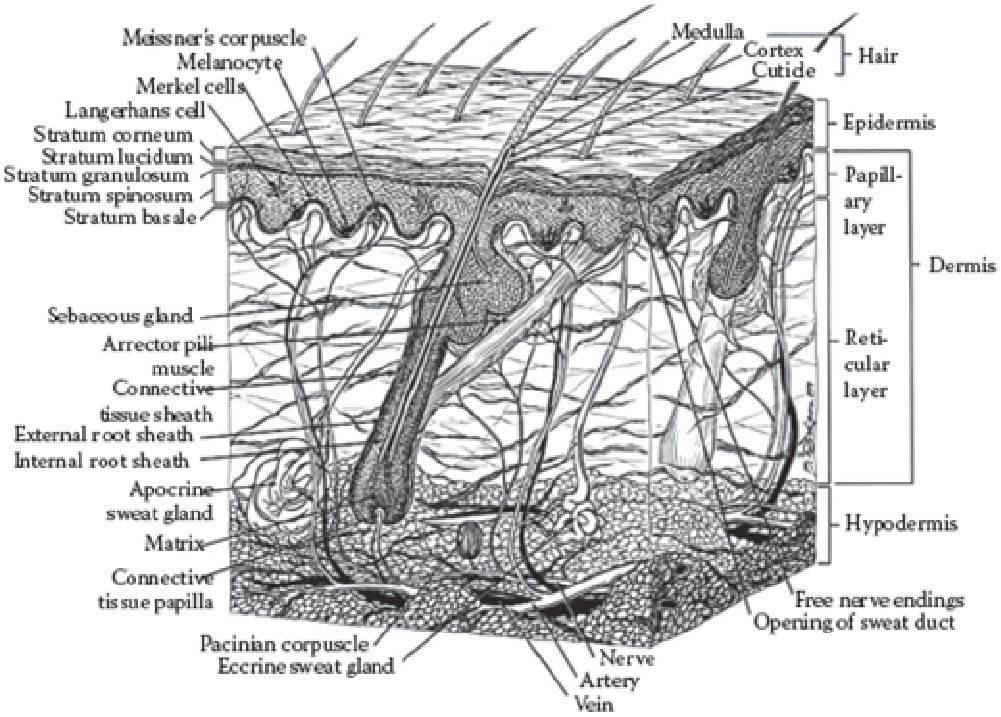

Figura 1. Rappresentazione schematica del tessuto cutaneo (Monteiro-Riviere, 2006)

2.1.1. L'epidermide

L'epidermide consiste di epitelio stratificato, squamoso e cheratinizzato. Lo spessore e il numero degli strati varia in funzione del sito anatomico. I cheratinociti sono il tipo cellulare più rappresentato costituendo circa l'80% della popolazione cellulare (Monteiro-Riviere, 2004). Queste cellule originate dallo strato basale vanno incontro ad un costante processo di proliferazione, differenziazione e cheratinizzazione durante la loro migrazione dallo strato basale, generativo verso gli strati superiori. Durante questo processo le cellule crescono in dimensioni, cambiano forma fino ad appiattirsi, arricchendosi in cheratina e andando alla fine a sostituire le cellule morte che si staccano dalla superficie dello strato corneo. Questo processo rappresenta uno dei meccanismi di difesa dell'organismo: con l'esfoliazione degli strati più superficiali se ne vanno anche batteri, particelle, composti chimici, ecc, che si accumulano costantemente sull'esterno della cute (Monteiro-Riviere, 2006). Le rimanenti cellule dell'epidermide sono costituite da melanociti, cellule di Langerhans, cellule di Merkel, che non sono coinvolte nel processo di cheratinizzazione (Smack, et al., 1994).

Lo *strato basale* è lo strato dell'epidermide posto più vicino al derma ed è costituito da un singolo strato di cellule cilindriche o cubiche che sono attaccate alle cellule del sovrastante strato spinoso e alle cellule adiacenti attraverso desmosomi e alla sottostante membrana basale dagli emidesmosomi. I desmosomi sono piccoli siti di adesione che mediano il contatto diretto cellula-cellula costituendo dei siti di ancoraggio per i filamenti intermedi del citoscheletro cellulare. Gli emidesmosomi invece fungono da siti di forte adesione fra i filamenti intermedi delle cellule e la matrice extracellulare della lamina basale.

Oltre al ruolo di sintetizzare i componenti della lamina basale, le cellule dello strato basale funzionano come cellule staminali per la continua proliferazione dei cheratinociti, che in seguito migrano verso l'esterno seguendo il processo di cheratinizzazione. I cheratinociti non maturi dello strato basale sono in grado di iniziare la sintesi di cheratina, che poi viene assemblata in filamenti chiamati tonofilamenti. Nello strato basale sono presenti anche cellule di Merkel, associate alle fibre nervose con funzione di meccanocettori, e melanociti, che producono e secernono melanina che protegge la cute dalle radiazioni ultraviolette.

Lo *strato spinoso* è situato sopra lo strato basale ed è costituito di diversi strati di cellule poliedriche di forma irregolare. "Tight junctions" e desmosomi congiungono cellule adiacenti e il sottostante strato basale. In questo strato si trovano anche cellule di Langerhans, importanti per la risposta immunitaria della cute. Qui i cheratinociti maturano, cominciano ad assemblare i tonofilamenti, crescono in dimensioni e si appiattiscono parallelamente alla

superficie. Quando i cheratinociti raggiungono la parte alta di questo strato iniziano a produrre i granuli di cheratoialina e i corpi lamellari che sono tratti distintivi delle cellule dello strato granuloso.

Lo *strato granuloso* contiene diversi strati di cellule appiattite parallelamente alla superficie della pelle. I granuli presenti nelle cellule contegono i precursori per la proteina filaggrina che è responsabile dell'aggregazione dei filamenti di cheratina presenti nelle cellule dello strato corneo. Questi granuli si fondono con la membrana cellulare e secernono per esocitosi il loro contenuto nello spazio intercellulare, fra lo strato granuloso e lo strato corneo. Il contenuto lipidico dei granuli forma la componente lipidica intercellulare della barriera dello strato corneo.

Lo *strato lucido* è una suddivisione dello strato corneo ed è presente solo in aree spesse della pelle, come i palmi delle mani o le piante dei piedi. Questo strato cutaneo è sottile, traslucido, costituito da cellule che hanno perso il nucleo e gli organelli citoplasmatici. Queste cellule sono cheratinizzate e contengono un fluido viscoso, eleidina, che è simile alla cheratina.

Lo *strato corneo*, infine, è lo strato più esterno dell'epidermide e la sua composizione e la sua organizzazione contribuiscono significativamente alle caratteristiche di semipermeabilità della cute. Lo strato corneo è costituito da cellule differenziate, organizzate in pile multicellulari perpendicolari alla superficie della pelle, che hanno perso il nucleo e gli organelli cellulari e sono quasi completamente riempite di filamenti di cheratina. Le colonne di cellule interconnesse tra loro sono immerse in una matrice lamellare che consiste di lipidi specializzati secreti dalle cellule dello strato granuloso. Questa barriera funziona riducendo la penetrazione attraverso la pelle delle sostanze idrofiliche e di grandi dimensioni e prevenendo l'eccessiva perdita di fluidi corporei (Langbein et al., 2002; Monteiro-Riviere, 2006; Smack et al., 1994).

2.1.2. Il derma

Il derma è costituito da collagene, elastina e fibre reticolari, immerse in un fondo amorfo di proteoglicani, che creano una rete di denso tessuto connettivo. I tipi cellulare predominani nel derma sono fibroblasti, mast cells e macrofagi, ma sono spesso presenti anche cellule plasmatiche, adipociti, cromatofori e leucociti stravasati. Lo strato più superficiale del derma, lo strato papillare, giace immediatamente sotto la lamina basale e contiene una rete irregolare e poco densa di molecole di collagene di tipo I e di tipo III e di fibre di elastiche. Questa regione contiene anche vasi sanguigni e linfatici, che forniscono l'epidermide senza entrarvi,

e terminazioni nervose che possono fermarsi nel derma o entrare nell'epidermide. Le protrusioni del tessuto connettivo nell'epidermide sono chiamate papille cutanee. Lo strato reticolare del derma giace sotto lo strato papillare. Questo strato è sostanzialmente più spesso di quello superficiale ed è caratterizzato da collagene di tipo I, fibre elastiche e poche cellule (Monteiro-Riviere, 2006).

2.1.3. L'ipoderma

L'ipoderma è la fascia di natura prevalentemente adiposa che giace sotto la pelle con funzioni di isolante, di riserva di calorie, di ancoraggio della pelle ai muscoli e alle ossa. Esso è costituito prevalentemente di adipociti, cellule piene di lipidi, e di tessuto connettivo contenente fibre di collagene ed elastina che danno flessibilità e libero movimento alla pelle rispetto alle strutture più profonde (Monteiro-Riviere, 2006).

2.1.4. Appendici cutanee

I follicoli piliferi, le ghiandole sebacee associate, i muscoli erettori dei peli e le ghiandole sudoripare sono le appendici comunemente associate alla cute. I peli sono prodotti dai follicoli pilieri e sono strutture cheratinizzate che derivano da invaginazioni dell'epidermide che attraversano il derma e possono estendersi fino all'ipoderma. Sebbene la permeazione cutanea attraverso i follicoli piliferi richieda comunque l'attraversamento dello strato corneo, i follicoli rappresentano le regioni di maggior area e possono quindi contribuire in maniera significativa all'aumento dell'assorbimento transcutaneo (Monteiro-Riviere, 2004). Il tessuto connettivo alla base dei follicoli piliferi costituisce un sito di ancoraggio per i muscoli erettori dei peli che, in seguito alla contrazione, non solo danno luogo all'erezione dei peli, ma aiutano anche lo svuotamento delle ghiandole sebacee che secernono il loro prodotto di secrezione, il sebo, nel canale del follicolo pilifero.

2.2. L'ASSORBIMENTO CUTANEO

Il ruolo della cute è stato a lungo sottostimato, soprattutto in ambito professionale: fino agli anni '60, essa era considerata una barriera impermeabile alle sostanze chimiche. L'igiene occupazionale si è tradizionalmente focalizzata sull'inalazione in quanto era considerata la più importate via di espozione alle sostanze tossiche e la percezione del rischio era più alta. Oggi è noto che molti agenti tossici sono in grado di penetrare anche attraverso la pelle e l'assorbimento cutaneo è ormai un fenomeno studiato nell'ambito di una completa valutazione dei fattori di rischio sia professionali che ambientali. Sono stati sviluppati diversi metodi pratici per valutare l'esposizione cutanea e si è aperto il dibattito per lo sviluppo di limiti per l'esposizione cutanea al pari di quelli per la via inalatoria. In generale, però, rispetto all'inalazione, nell'esposizione cutanea sono stati fatti meno progressi riguardo la standardizzazione di metodi di valutazione condivisi e solo negli ultimi anni si è sviluppata una terminologia di base e dei modelli teorici che consentano il confronto dei dati dei vari studi (Cherrie and Robertson, 1995).

Uno dei modelli concettuali più accettati si basa sull'osservazione che il processo di trasporto dei composti chimici attraverso la cute è spinto dal gradiente di concentrazione tra la superficie cutanea e il tessuto vascolarizzato sottostante che rimuove costantemente le sostanze che penetrano e quindi il fattore determinante risulta essere la concentrazione del composto sulla superficie cutanea (Schneider et al., 1999).

Le sostanze possono arrivare alla cute in diversi modi: possono depositarsi direttamente dall'aria, trasferirsi per contatto della cute con superfici contaminate o per immersione di una parte del corpo nella sostanza stessa. Allo stesso modo i contaminanti possono lasciare la pelle attraverso altri meccanismi: essi possono evaporare o essere rimossi tramite abrasione o lavaggio prima di venire assorbiti dal tessuto cutaneo. Infine, un ruolo molto importante è svolto dall'uso dell'equipaggiamento di protezione che può modificare la quantità di sostanza che arriva a contatto con la pelle.

Nel modello multicompartimentale di Schneider et al, (1999) vengono identificati sei compartimenti e otto processi di trasferimento del contaminante. I compartimenti sono rappresentati dalla sorgente del contaminante, dall'aria circostante, dal contaminate depositato sulle superfici, dagli strati di contaminante all'esterno e all'interno dei vestiti e allo strato di contaminante sulla superficie della cute. Tra questi compartimenti vi è un continuo scambio di contaminante per emissione, deposizione, risospensione o evaporazione, trasferimento, rimozione, ridistribuzione, decontaminazione e penetrazione.

L'entità dei vari processi di scambio fra un compartimento e l'altro determina la quantità e la concentrazione del contaminante sulla superficie della pelle e, quindi, l'eventuale assorbimento nel tessuto e nel sistema circolatorio.

Per quanto riguarda il fenomeno dell'assorbimento cutaneo, esso è un termine generale che descrive il trasporto di sostanze chimiche dalla superficie esterna della cute fino a raggiungere il sistema circolatorio (EHC 235, 2006). Questo processo viene spesso diviso in:

- *penetrazione*: l'ingresso di una sostanza all'interno di un particolare strato o struttura;
- *permeazione*: la penetrazione di un composto in un secondo strato che sia funzionalmente e strutturalmente differente dal primo strato attraversato;
- *assorbimento*: l'assorbimento del composto nel sistema vascolare locale e nel sistema linfatico della pelle. Questo generalmente porta ad un assorbimento nella circolazione sistemica (*assorbimento sistemico*).

L'assorbimento cutaneo non viene studiato solo in funzione di un'esposizione a sostanze tossiche con effettti nocivi, ma viene anche sfruttato in farmacologia per la somministrazione di alcuni farmaci che, una volta assorbiti nel circolo locale della pelle, passano alla circolazione sistemica e vengono distribuiti in tutto l'organismo.

Lo strato che controlla l'assorbimento attraverso la cute è sostanzialmente lo strato corneo che, nonostante sia di spessore molto ridotto, offre con i suoi strati di corneociti immersi nella matrice lipidica un'efficiente barriera contro la penetrazione di xenobiotici.

Sono state identificate tre vie di penetrazione dei composti attraverso la cute: (i) intercellulare, (ii) transcellulare e (iii) attraverso gli annessi cutanei, quali follicoli piliferi e ghiandole sudoripare e sebacee: tutte e tre le vie possono dare un contributo alla diffusione dei composti chimici attraverso la barriera cutanea.

L'assorbimento cutaneo può essere influenzato da una grande varietà di fattori (tabella 1), quali l'integrità della cute, il sito anatomico, la presenza di patologie (dermatiti da contatto, eczema atopico, psoriasi) la presenza di veicolanti, la flessione meccanica (Rouse et al., 2007) e l'uso di detergenti o di altri composti chimici (Larese et al., 2006).

Questi fattori possono essere raggruppati in tre classi: fattori intrinseci della cute, fattori chimico-fisici del composto e fattori dipendenti dal tipo di esposizione.

I composti chimici possono attraversare la cute per diffusione attiva, legandosi a lipidi o proteine con funzione di carrier: tuttavia il processo di assorbimento cutaneo è descrivibile fondamentalmente come una diffusone passiva.

Il flusso di un composto chimico allo steady-state può essere predetto attraverso la 1ª legge di Fick per la diffusione in condizioni stazionarie:

$$J = \frac{DK\Delta C}{h}$$

dove J è il flusso di materia attraverso la superficie (g cm^{-2} h^{-1}), D è il coefficiente di diffusione (cm^2 h^{-1}) che dipende dalla temperatura, dal tipo di soluto e di solvente, K è il coefficiente di ripartizione della sostanza fra la fase lipidica e la fase acquosa, ΔC esprime il gradiente di concentrazione attraverso la cute (g cm^{-3}) e *h* è lo spessore della membrana (cm).

Il coefficiente di diffusione di un composto è collegato al suo meccanismo di diffusione e alle sue caratteristiche chimico-fisiche come le dimensione molecolari, la struttura e la solubilità nella membrana cutanea. Il coefficiente di partizione rappresenta una misura dell'idrofilicità o idrofobicità del composto e riflette la sua capacità di diffondere in un ambiente lipofilo.

Variabilità della cute	**Caratteristiche chimico-fisiche del composto**	**Fattori di esposizione**
Spessore	Peso molecolare	Tipo di lavoro
Condizioni/Integrità	Coefficiente di ripartizione	Durata
Presenza di patologie	Irritante	Superficie di pelle esposta
cutanee	Struttura	Uso di protezioni
Parti anatomiche esposte	Presenza di altri composti	Concentrazione
Temperatura e idratazione	chimici	Igiene
Perfusione della cute	…	…
Densità dei pori e		
sudorazione		
pH del sudore		
Metabolismo della cute		
…		

Tabella 1. Riassunto variabili che possono modificare il fenomeno dell'assorbimento cutaneo.

2.3. ASSORBIMENTO CUTANEO DI COBALTO, NICHEL E CROMO

I metalli come cobalto, nichel e cromo sono particolarmente diffusi nel nostro ambiente e si trovano in molti oggetti di uso quotidiano, come monete, gioielli, chiavi, bottoni, maniglie, telefoni cellulari, pelle conciata, ecc. E' stato dimostrato che monete contenenti nichel possono rilasciare ioni metallici quando vengono in contatto ripetuto con la pelle (Liden e Carter, 2001), e allo stesso modo polveri metalliche e di ossidi metallici possono ionizzarsi in sudore sintetico e in altri fluidi biologici (Larese et al., 2007; Midander et al., 2007).

Nel ventesimo secolo, l'industrializzazione ha portato ad un aumento dell'esposizione cutanea a questi metalli e di conseguenza anche ad un aumento delle allergie: è stato stimato che fino al 17% delle donne e il 3% degli uomini sono allergici al nichel e che circa l'1-3% della popolazione è allergica a cobalto e cromo. Fra i pazienti affetti da dermatite, la prevalenza delle allergie ai metalli è molto alta (Dotterud and Smith-Sivertsen, 2007; Thyssen and Menné, 2009); in particolare l'allergia al nichel risulta essere la causa principale dell'eczema alle mani che colpisce circa il 10% della popolazione adulta (Meding and Swanbeck, 1987). Nel 1994 l'Unione Europea ha adottato una direttiva più restrittiva sul contenuto e il rilascio di nichel dagli oggetti per proteggere i cittadini dall'allergia al nichel (1994/27/EC) e, in seguito al recepimento dei vari paesi membri, l'incidenza dell'allergia al nichel ha iniziato a diminuire (Thyssen and Menné, 2009).

L'assorbimento cutaneo di cobalto è stato riscontrato sia in lavoratori esposti che in esperimenti di laboratorio con volontari, ma i dati di letteratura su questo argomento sono ancora pochi (Kogan and Petukhova, 1986; Scansetti et al., 1994; Linnainmaa and Kiilunen, 1997). Inoltre il cobalto è un comune sensibilizzante e la sua presenza in oggetti metallici può aumentare il rischio di dermatiti allergiche da contatto in soggetti sensibilizzati. In questo caso c'è un'azione locale sulla pelle, ma le osservazioni di Scansetti et al., (1994) sulla escrezione urinaria dopo esposizione volontaria a polveri miste contenenti cobalto e i test *in vitro* con il metodo delle celle di diffusione di Franz utilizzando una dispersione di polvere di cobalto in sudore sintetico (Larese et al., 2004, 2007), sembrano confermare che questo metallo sia in grado di permeare la pelle.

L'assorbimento cutaneo del nichel è meglio conosciuto: è stato dimostrato che sali di nichel in soluzione acquosa sono in grado di attraversare lo strato corneo (Tanojo et al., 2001), mentre polveri di nichel *in vivo* possono penetrarvi in profondità (Hostynek et al., 2001). Le conclusioni di questi lavori sono in linea con i risultati ottenuti *in vitro* usando il metodo delle celle di diffusione di Franz con cute umana (Larese et al., 2007).

Per quanto riguarda la permeazione cutanea del cromo, vi sono molti dati in letteratura sull'assorbimento cutaneo di sali di cromo (Gammelgaard et al., 1992; Van Lierde et al., 2006) ed è già stato dimostrato un possibile assorbimento sistemico di cromo in seguito al contatto della cute con sali di cromo esavalente (Corbett et al., 1997), ma in generale sono ancora poche le informazioni sul rilascio di ioni di cromo dal cromo metallico e da oggetti metallici contenti cromo e quindi sul possibile assorbimento attraverso la cute.

In un precedente lavoro del nostro gruppo sono stati studiati la ionizzazione in sudore sintetico e il successivo assorbimento cutaneo *in vitro* di polveri di cobalto, nichel e cromo utilizzando il metodo delle celle di diffusione di Franz con cute umana intera (Larese et al., 2007). Come fasi donatrici in questi test di permeazione cutanea sono state usate delle dispersioni al 5% di polveri metalliche in sudore sintetico: al termine delle 24 ore di esposizione, le concentrazioni di ioni metallici nelle fasi donatrici erano rispettivamente 33,3±3,2 mg L^{-1} per il cobalto divalente e 27,1±3,2 mg L^{-1} per il nichel divalente, mentre la concentrazione degli ioni di cromo era al di sotto del limite di rivelabilità (0,1 mg L^{-1}). Dalle analisi delle concentrazioni nelle fasi riceventi è stato calcolato un flusso di permeazione di 12,3±5,4 ng cm^{-2} h^{-1} e un lag time di 1,5±0,7 h per la polvere di cobalto e un flusso di 16,5±3,6 ng cm^{-2} h^{-1} e un lag time di 14,6±0,6 h per la polvere di nichel, mentre concentrazioni inferiori a 0,1 μg L^{-1} sono state trovate in tutte le fasi riceventi delle celle allestite con polvere di cromo. Questi primi risultati pubblicati dal nostro gruppo (Larese et al., 2004, 2007) suggeriscono che il sudore sintetico sia in grado di ossidare cobalto e nichel metallici ai rispettivi ioni divalenti, mentre il cromo metallico probabilmente ha bisogno di condizioni ossidanti più forti. Questi ioni metallici possono permeare la cute ed è stato possibile misurare un flusso per il nichel ed il cobalto in un sistema *in vitro*.

2.4. ASSORBIMENTO CUTANEO DI NANOPARTICELLE METALLICHE

2.4.1. Nanoscienze, nanotecnologie e nanomateriali

La Royal Society & Royal Academy of Engineering (2004) ha definito le nanoscienze come lo studio di fenomeni e manipolazione di materiali a livello atomico, molecolare e macromolecolare, dove le proprietà differiscono significativamente da quelle a livelli più grandi, e le nanotecnologie come progettazione, caratterizzazione, produzione e applicazioni di strutture, sistemi e dispositivi, controllando forma e dimensione in scala nanometrica.

I nanomateriali sono in genere definiti come materiali che hanno almeno una dimensione inferiore a 100 nanometri (1 nm = 10^{-9} m) e possono essere distinti in due grandi gruppi: particelle ultrafini prodotte non intenzionalmente e nanoparticelle ingegnerizzate, prodotte in modo controllato (Oberdörster et al., 2005).

Le nanotecnologie sono tra le aree di ricerca in più veloce espansione con importanti applicazioni in una grande varietà di campi. Negli ultimi anni, governi e industrie in tutto il mondo hanno investito molto in ricerca e sviluppo nel campo delle nanoscience. E' stato stimato che nel 2015 prodotti commerciali per un valore di un trilione di dollari conterranno nanomateriali in componenti fondamentali. La corrispondente industria richiederà circa due milioni di lavoratori nel settore nanotecnologico e circa il triplo in attività di supporto (Roco, 2005). I nanomateriali sono stati introdotti in prodotti commerciali già disponibili, come cosmetici e creme solari, prodotti farmaceutici, abbigliamento tecnico, equipaggiamento sportivo, catalizzatori per automobili, prodotti per pulizia, bendaggi per ferite e ustioni, componenti elettronici, ma molti altri sono i settori in cui sono possibili applicazioni future di nanotecnologie come sistemi di rilascio controllato per farmaci, nanomedicine, imaging cellulare, etc.

2.4.2. Nanotossicologia

Le nanoparticelle ingegnerizzate, per il loro grande rapporto superficie-volume, mostrano proprietà chimiche, fisiche e biologiche differenti dagli stessi materiali di dimensioni maggiori, ma alcune di queste proprietà possono portare ad effetti pericolosi per la salute umana e per i sistemi ambientali. Ogni anno nuovi prodotti contenenti nanomateriali entrano in commercio (Woodrow Wilson International Center for Scholars, 2007) e nel prossimo futuro molti lavoratori e consumatori entreranno in contatto con questi nanoprodotti. Per

questo motivo vi è la necessità di capire a fondo gli effetti tossicologici e ambientali di questi prodotti, il loro ciclo vitale, le vie di esposizione per l'uomo, i rischi per i lavoratori, il comportamento delle nanoparticelle una volta che sono all'interno del corpo, ecc, in modo da poter usare questi nuovi materiali con sicurezza (EPA, 2007; NIOSH, 2007). La nanotossicologia è una disciplina emergente (Oberdörster et al., 2005) e c'è un gap tra la valutazione della sicurezza dei nanomateriali e lo sviluppo nanotecnologico che ogni giorno produce nuovi materiali, nuove vie di sintesi, nuove applicazioni e, infine, nuovi prodotti pronti per il commercio. Come suggerito nel report della Royal Society & Royal Academy of Engineering (2004), le nanoparticelle dovranno essere trattate come nuovi composti chimici dal punto di vista del rischio per la salute perché grazie alle loro dimensioni possono attraversare le normali barriere protettive del corpo (Schulte et al., 2008). Inoltre, particelle ultrafini, differenti in sorgenti e composizione sono una componente del particolato atmosferico e il loro assorbimento per via polmonare e cutanea in popolazioni che vivono in aree inquinate devono essere meglio studiate (Ayres et al., 2008).

2.4.3. Assorbimento cutaneo di nanoparticelle metalliche (Allegato III)

Ad oggi in letteratura sono disponibili pochi studi sulla permeazione e penetrazione cutanea di nanoparticelle con risultati non omogenei probabilmente dovuti a differenze nei metodi e nelle tecniche impiegati, alle condizioni di laboratorio e soprattutto all'assenza di protocolli di valutazione standardizzati. Inoltre, mentre la via di esposizione respiratoria è sempre materia di attenzione e preoccupazione per i lavoratori e per la popolazione in generale (Donaldson et al., 2006; Oberdörster et al., 2005; Rotoli et al., 2008), la cute è considerata impermeabile e la percezione del rischio è molto bassa.

Nella letteratura disponibile, diversi studi suggeriscono che la cute sia un'importante via d'ingresso per alcuni tipi di nanoparticelle sia in campo occupazionale che per i consumatori.

Alvarez-Roman et al. (2004) hanno usato il microscopio confocale a scansione laser per visualizzare la distribuzione di due tipi di nanoparticelle di polistirene, fluorescenti e non biodegradabili, di diametro di 20 e 200 nm, nella cute di maiale dopo 0,5, 1 e 2 ore di esposizione in celle di diffusione verticale. Le immagini della superficie hanno mostrato che le nanoparticelle di polistirene si accumulavano preferenzialmente nelle aperture dei follicoli piliferi, aumentando in modo tempo-dipendente, e la localizzazione nei follicoli era maggiore per le dimensioni particellari più piccole. Tinkle et al. (2003) hanno studiato gli effetti dei movimenti di flessione sull'assorbimento cutaneo con cute integra di particelle micrometriche fluorescenti di destrano: al termine degli esperimenti le particelle sono state osservate anche

negli strati cutanei più profondi. Kim et al. (2004) hanno scoperto che nanoparticelle introdotte nel derma migravano verso i nodi linfatici, probabilmente trasportate da macrofagi e cellule di Langerhans, sollevando preoccupazione per l'immunomodulazione.

In generale l'attenzione dei ricercatori in queste prime fasi sì è concentrata sull'assorbimento cutaneo delle più diffuse categorie di nanoparticelle (nanotubi di carbonio, fullereni, quantum dots e il biossido di titanio). Nonostante alcuni studi abbiano evidenziato uno scarso o nullo assorbimento attraverso la cute (NANODERM, 2007), molti altri lavori sollevano dei dubbi sulla loro effettiva sicurezza e molte delle variabili in gioco non sono state prese ancora in debita considerazione.

Molto meno studiate sono invece le nanoparticelle metalliche e di ossidi metallici. I ricercatori stanno sviluppando soluzioni tecniche per applicazioni industriali, farmacologiche, mediche, ecc, per nanoparticelle metalliche, leghe e ossidi metallici. E' però già stato dimostrato che polveri metalliche più grossolane di ossidi metallici poste in media biologici possono rilasciare ioni metallici (Midander et al., 2006) che eventualmente possono permeare attraverso la cute (Larese et al., 2007) e questo fenomeno sembra essere più consistente con particelle nanometriche considerata la loro grande reattività superficiale (Auffan et al., 2009).

Per quanto riguarda l'eventuale permeazione cutanea e la tossicità cellulare di nanopolveri metalliche si sa ancora molto poco: Baroli et al. (2007) hanno dimostrato, in un esperimento *in vitro*, che nanoparticelle di derivati del ferro (<10 nm) sono in grado di penetrare passivamente nella cute attraverso la matrice lipidica dello strato corneo e i follicoli piliferi, fino allo strato granuloso. In alcuni casi le nanoparticelle sono state trovate anche negli strati vitali dell'epidermide. Papageorgiou et al. (2007), invece, hanno comparato la citotossicità e la genotossicità di nanoparticelle e microparticelle di una lega di cromo e cobalto in fibroblasti umani in coltura riscontrando un maggiore danno cellulare da parte delle particelle più piccole. Berry et al. (2004) hanno investigato la tossicità di nanoparticelle di ossido di ferro di 8 e 15 nm su fibroblasti cutanei in coltura: le nanoparticelle venivano rapidamente internalizzate per endocitosi causando la distruzione del citoscheletro e diminuendo la proliferazione cellulare.

Le nanoparticelle d'argento vengono largamente usate per la loro attività antibatterica in molti prodotti commerciali come tessuti, dispositivi medici, contraccettivi, potabilizzanti (Woodrow Wilson International Center for Scholars, 2007). Inoltre il nano-argento è utilizzato nel trattamento di ferite e scottature e come rivestimento nelle protesi ortopediche. L'argento, tradizionalmente poco tossico per i mammiferi, può causare argiria o argirosi in soggetti occupazionalmente esposti in modo cronico. Quindi, a causa di un esteso impiego di

nanoparticelle d'argento in prodotti che vanno a diretto contatto con la cute, quali tessuti, abbigliamento sportivo, garze per ferite ed ustioni, l'assorbimento cutaneo deve essere valutato attentamente. Sebbene alcuni studi abbiano suggerito la biocompatibilità di questi prodotti (Leaper, 2006; Supp et al., 2005; Muangman et al., 2006; Wright et al., 2002), test tossicologici con cheratinociti e fibroblasti in coltura hanno mostrato che alcuni tipi di nanoparticelle d'argento rilasciate da tessuti commerciali risultavano tossiche per le cellule, inibendo la proliferazione e modificando la morfologia cellulare (Lam et al., 2004; Paddle-Ledinek et al., 2006; Poon and Burd, 2004). Altri Autori hanno suggerito un incremento della permeazione cutanea di argento in seguito ad esposizione *in vitro* a nano-argento in situazione di cute lesa (Larese et al., 2009) o in seguito all'uso di bendaggi al nano-argento per il trattamento di ustioni estese (Trop et al., 2006).

Il nano-oro invece riveste un particolare interesse per applicazioni nell'imaging cellulare e in medicina, ma alcuni studi hanno sollevato alcuni dubbi sulla sua sicurezza per l'uptake di cluster d'oro (1,4 nm) e di nanoparticelle d'oro/citrato (13 nm) da differenti tipi di cellule, compresi fibroblasti cutanei umani, e le conseguenti interazioni con il DNA (Connor et al., 2005; Tsoli et al., 2005) e alterazioni di attività cellulari quali adesione, crescita, sintesi proteica, formazione della matrice extracellulare (Pernodet et al., 2006). Inoltre Sonavane et al. (2008) hanno usato le celle di diffusione di Franz per dimostrare una permeazione dimensione-dipendente di nano-oro attraverso cute di ratto.

3. MATERIALI E METODI

3.1. LE CELLE DI DIFFUSIONE DI FRANZ

Il processo di assorbimento attraverso la pelle può essere studiato sia *in vivo* che *in vitro*, sia sull'uomo che sugli animali. La ricerca *in vivo* si serve di volontari, di animali vivi, di impianti di cute umana su animali, ecc, mentre negli esperimenti *in vitro* è possibile utilizzare lembi di cute animale o umana, proveniente da autopsie o da interventi di chirurgia plastica.

I metodi *in vitro* sono particolarmente usati per gli studi di assorbimento cutaneo di composti chimici in differenti settori industriali, dalla cosmetica alla farmaceutica, dalla produzione di detergenti all'agrochimica, ma anche per la valutazione del rischio da esposizione cutanea a xenobiotici e sostanze tossiche. Per questo in alcuni paesi i test *in vitro* sono inclusi tra i criteri per assegnare le "skin notation" ai composti chimici (Drexler et al, 1998).

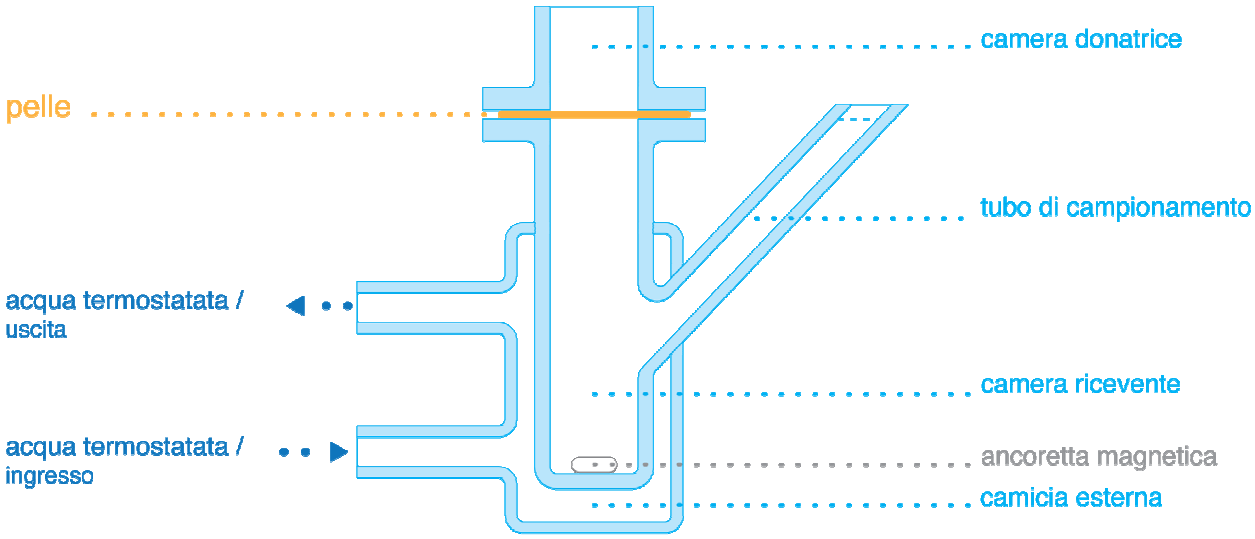

Figura 2. Rappresentazione schematica di una celle di diffusione di Franz usata negli esperimenti di permeazione cutanea.

Le celle di diffusione statica più usate sono le celle di Franz e consistono di due comparti, uno donatore, il superiore, ed uno ricevente, l'inferiore, tra i quali viene posta la membrana da studiare e il tutto viene fissato in modo tale che non ci possano essere perdite di soluzioni durante i test. La membrana può essere cute umana o animale, cute intera o solo l'epidermide. La determinazione della quantità di sostanza permeata viene effettuata mediante opportune tecniche analitiche previo prelievo di un'aliquota della soluzione ricevente.

Figura 3. Foto di una delle celle usate nei test di permeazione cutanea.

La sostanza da testare viene posta nel compartimento superiore (donatore), a contatto con la membrana, mentre il compartimento ricevente viene riempito di soluzione fisiologica, mantenuta in agitazione per mezzo di un'ancoretta magnetica. Il comparto ricevente è dotato di un tubo di campionamento attraverso cui possono essere effettuati i prelievi della fase ricevente ai tempi prefissati e di una camicia esterna collegata al sistema di termostatazione (figure 2 e 3). Il tutto viene posto in uno degli alloggiamenti della consolle di lavoro (figura 4) e la camicia esterna viene collegata al sistema di termostatazione.

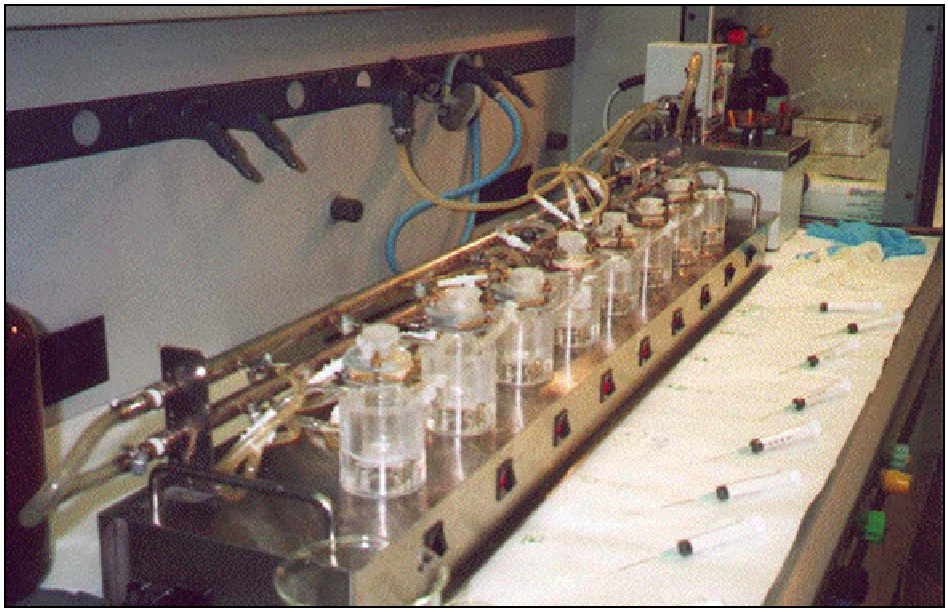

Figura 4. La consolle di lavoro per l'alloggiamento delle celle di Franz.

La concentrazione dei sali nel fluido recettore è all'incirca la stessa che può essere trovata nel sangue, cioè di circa 0,9 %.

Inoltre, collegato alla camera ricevente, vi è un tubo per i prelievi a tempi prefissati dei campioni di soluzione che vengono poi analizzati con varie tecniche analitiche.

Sono state usate 14 celle con caratteristiche leggermente diverse per diametro della cute esposta e per volume di soluzione ricevente contenuta.

Prima di iniziare gli esperimenti si sono misurati i diametri delle diverse celle con un calibro ventesimale e si è calcolato il volume: si sono pesate le singole celle, prima vuote e poi riempite d'acqua, con una bilancia di precisione monopiatto elettronica (mod. CHYO JL – 180), considerando per la densità dell'acqua un valore pari a 0,998 g ml^{-1} a temperatura ambiente di 21°C (Franz, 1975).

Infine, nella fase di organizzazione e di rielaborazione dei dati ottenuti dalle analisi delle concentrazioni nelle fasi riceventi, il passaggio del composto attraverso la pelle viene di solito espresso in termini di flusso, calcolato nella parte lineare del profilo di permeazione, di lag time, ovvero il tempo necessario affinché il flusso raggiunga un valore massimo costante (Franz, 1975; Rougier et al., 1990; Bronaugh and Franz, 1986) e di coefficiente di permeabilità K_p (cm h^{-1}) che si ottiene dividendo il flusso per la dose applicata (figura 5).

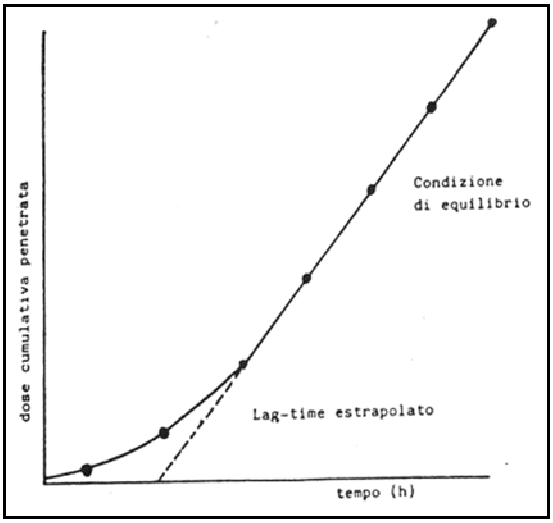

Figura 5. Esempio di una curva cumulativa di permeazione in funzione del tempo: la pendenza della parte rettilinea rappresenta il flusso, mentre l'intercetta con l'asse delle ascisse fornisce il valore di lag time.

3.2. PROCEDURA SPERIMENTALE GENERALE

3.2.1. Preparazione della cute

La cute utilizzata negli esperimenti è stata fornita dal reparto di Chirurgia Plastica degli Ospedali Riuniti di Trieste: essa costituisce scarti di interventi chirurgici ed è stata prelevata dalla regione addominale di pazienti di età compresa tra i 40 ed i 65 anni.

Dopo il prelievo, i lembi di cute vengono puliti del grasso sottocutaneo, posti in sacchetti di plastica e congelati a –25 °C per un periodo non superiore ai 4 mesi: è stato dimostrato che questo metodo di conservazione non danneggia la cute in quanto non sono state riscontrate differenze di permeabilità tra lembi freschi e congelati della stessa cute in serie separate di esperimenti (Franz, 1975).

Al momento dell'utilizzo, il tessuto cutaneo viene lasciato scongelare per circa 30 minuti a temperatura ambiente in soluzione fisiologica, viene tagliato in quadrati di circa quattro centimetri di lato e quindi si procede all'eventuale rimozione del grasso sottocutaneo residuo, in modo da ottenere dei lembi di cute di spessore costante di circa un millimetro. La misura dello spessore è eseguita con un calibro ventesimale prima di sistemarli nei siti appositi.

Gli esperimenti di permeazione sono stati eseguiti allestendo le celle sia con cute integra sia con cute lesa. La cute lesa è stata ottenuta utilizzando un protocollo di abrasione della pelle (Bronaugh e Steward, 1985), mediante strisciature effettuate con la punta di un ago di siringa su cute integra.

Nei test di permeazione, ogni lembo di cute viene posizionato tra le due camere di una cella in modo da rivolgere lo strato corneo alla soluzione donatrice e il derma alla soluzione ricevente.

In ogni esperimento vengono registrati i seguenti dati:

- Numero, età e sesso dei donatori;
- Spessore della cute;
- Provenienza della cute (operazioni di chirurgia plastica);
- Data di prelievo e di congelamento del tessuto.

Per ogni analisi è necessaria cute proveniente da più donatori diversi, in modo da minimizzare l'effetto della variabilità dovuta alle caratteristiche intrinseche del tessuto.

La soluzione fisiologica usata come fase ricevente negli esperimenti viene preparata sciogliendo 2,38 g di Na_2HPO_4, 0,19 g di KH_2PO_4 and 9 g di NaCl in 1 l di acqua milliQ con un pH finale di 7,35.

Il sudore sintetico usato per disperdere le polveri metalliche è una soluzione allo 0,5% di NaCl, 0,1% di urea e 0,1% di acido lattico. Il pH viene portato al valore finale con ammoniaca.

3.2.2. Integrità della cute

Prima e dopo ogni esperimento è stata testata l'integrità della cute attraverso misure di conducibilità (o resistenza: R = 1/C) elettrica utilizzando un conduttimetro (Metrohm, 660, Metrohm AG Oberdorfstr. 68 CH-9100 Herisau) operante a 300 Hz collegato a due elettrodi in acciaio inox (Fasano et al., 2002). Dopo aver riempito la camera ricevente della cella di soluzione fisiologica, viene posta la cute e quindi anche la camera donatrice viene riempita di soluzione fisiologica. Si attendono circa 30 minuti affinché si instauri l'equilibrio termico e a questo punto si effettua la misura di conducibilità con i due elettrodi immersi nella soluzione ricevente.

I dati di conducibilità, ottenuti in μS, sono stati convertiti in $K\Omega\ cm^{-2}$.

I lembi di pelle che avevano una resistenza inferiore a $3,95\pm0,27\ K\Omega\ cm^{-2}$ sono stati ritenuti danneggiati e scartati e le eventuali soluzioni sono state eliminate dall'esperimento (Davies et al., 2004).

3.2.3. Test di permeazione

Prima di ogni esperimento le celle di Franz vengono lavate con acido nitrico diluito (6% v/v) e risciacquate con acqua milliQ: una volta asciutte, esse vengono sistemate nella consolle di lavoro e collegate al sistema di termostatazione. Al termine dei test invece le celle di Franz vengono lavate con acqua e detersivo, quindi con acqua regia, poi con acido nitrico diluito (6% v/v) ed infine risciacquate più volte con acqua milliQ.

Ogni compartimento inferiore viene completamente riempito di soluzione ricevente e lasciato termostatare per circa mezz'ora, fino al raggiungimento dell'equilibrio termico: in generale i nostri esperimenti sono stati condotti ad una temperatura di 32°C.

Il campione di cute viene poi posizionato con il derma a contatto con la soluzione ricevente, facendo attenzione che all'interfaccia non rimangano bolle d'aria che ridurrebbero la superficie di contatto. Al di sopra della cute è posta la camera donatrice e tutto il sistema viene fissato con pinze di polietilene, in modo che non ci siano perdite delle varie fasi. La camera donatrice infine viene riempita con un volume di sostanza da testare sufficiente a ricoprire omogeneamente tutta la superficie della pelle. Una volta riempita la camera donatrice, le aperture della cella, apertura superiore e tubo laterale, vengono chiuse con tappi

in plastica o parafilm per evitare fenomeni di evaporazione delle soluzioni (Franz, 1975).

Il tempo di esposizione è stato di 24 ore in tutti i test; a intervalli prefissati vengono eseguiti i prelievi per il monitoraggio e la definizione dei profili di permeazione. Ad ogni campionamento vengono prelevati 1,5 ml di soluzione ricevente da ogni cella attraverso il tubo laterale utilizzando una siringa in polietilene da 2,5 ml. Dopo il prelievo, viene ripristinato il volume iniziale all'interno della cella aggiungendo soluzione fisiologica fresca sempre facendo attenzione alle bolle d'aria che si possono formare.

I campioni di fase ricevente prelevati durante le 24 ore, vengono posti in provette da 1,5 ml e conservati in congelatore ad una temperatura di –25 °C fino al momento dell'analisi.

Al termine delle 24 ore la fase donatrice delle varie celle viene recuperata e posta in provette da 15 ml. La camera donatrice e l'epidermide esposta vengono lavate con alcuni ml di acqua milliQ che vengono poi addizionati alla soluzione già recuperata.

La pinza di plastica viene infine rimossa e il lembo di cute e le varie fasi vengono congelate per le successive analisi.

3.2.4. Mineralizzazione della cute dopo l'esperimento

Al momento delle analisi, i lembi di cute vengono scongelati ed asciugati a temperatura ambiente per due ore, quindi le aree esposte sono tagliate, pesate e messe in becher con 10 ml di HNO_3 al 69 % v/v per la mineralizzazione (le quantità di pelle in generale sono comprese tra 0,6 e 1,2 g). Le soluzioni vengono riscaldate a 150°C per 10 ore. Dopo circa due ore, quando il tessuto è completamente disgregato, viene aggiunta, goccia a goccia, H_2O_2 al 30% v/v fino ad un totale di 2 ml; quindi si continua ad evaporare fino ad ottenere circa 2 ml di soluzione.

Infine le soluzioni vengono portate ad un volume di 10 ml con acqua milliQ e analizzate.

3.2.5. Preparazione dei campioni di cute per le analisi di microscopia elettronica

In alcuni test di permeazione cutanea di nanoparticelle metalliche, alcuni lembi di cute esposta sono stati analizzati al microscopio elettronico a trasmissione (TEM) al fine di visualizzare le nanoparticelle eventualmente penetrate nei vari strati della pelle.

I campioni scelti vengono tagliati in sezioni più piccole e fissati per 3 ore in una soluzione al 3 % di glutaraldeide in tampone di cacodilato allo 0,1 M (pH 7,3). Le sezioni di pelle fissate vengono lavate due volte (per 10 minuti ciascuna) con il tampone allo 0,1 M di cacodilato e sono state trattate con tetrossido di osmio all'1 % per 1 ora a 4 °C.

A questo punto i campioni vengono disidratati con una serie di aggiunte crescenti di

etanolo e poi immersi in una resina epossidica Dow (DER332; Unione Chimica Europea, Milan, Italy): l'ultima inclusione in resina è stata fatta sotto vuoto.

Le sezioni semi-fini e le ultra sottili vengono tagliate con un ultramicrotomo (Ultracut UCT - Leica Microsystems, Milan, Italy) dotato di una lama di diamante lunga 3 mm.

In seguito, le sezioni semi-fini vengono osservate con un microscopio ottico Leitz Dialux 20 EB (Leica Microsystems, Milan, Italy), mentre le sezioni ultra sottili vengono colorate due volte con citrato di piombo e acetato di uranile e vengono osservate con un microscopio elettronico a trasmissione (EM208; Philips, Eindhoven, The Netherlands) con un sistema di acquisizione ad alta definizione SIS Morada e un sistema di acquisizione dell'immagine digitale iTEM (FEI Italia, Milan, Italy).

3.2.6. Misure analitiche strumentali

Per le misurazioni analitiche delle concentrazioni dei metalli studiati in questo progetto, sono state utilizzate le seguenti tecniche analitiche strumentali:

- Spettroscopia di Assorbimento Atomico Elettro-Termica con Fornetto di Grafite (GF-AAS);
- Spettroscopia di Emissione Atomica con sorgente al Plasma Induttivamente Accoppiato (ICP-AES);
- Spettrometria di Massa con sorgente al Plasma Induttivamente Accoppiato (ICP-MS).

La scelta della tecnica analitica più opportuna è stata fatta in funzione delle concentrazioni attese nelle varie soluzioni da analizzare e quindi dei limiti di rilevabilità degli strumenti per i vari elementi studiati.

3.2.6.1. Spettroscopia di Assorbimento Atomico Elettro-Termica con Fornetto di Grafite

Lo strumento utilizzato per le analisi di cobalto, cromo e nichel è uno spettrometro Thermo M series GF95Z (UK) dotato di fornetto di grafite e di autocampionatore FS95, presso il Dipartimento di Scienze Chimiche, Laboratorio di Chimica Analitica Ambientale e Strumentale, Università di Trieste.

Lo strumento utilizzato per le analisi dell'argento è uno spettrometro Perkin Elmer 4100 ZL (USA) equipaggiato con fornetto di grafite HGA e autocampionatore AS/71, presso il Dipartimento di Traumatologia, Ortopedia e Medicina del Lavoro, Laboratorio di Tossicologia Industriale, Università di Torino.

3.2.6.2. Spettroscopia di Emissione Atomica con sorgente al Plasma Induttivamente Accoppiato

Lo strumento utilizzato per le analisi di cobalto, cromo e nichel è uno Spettrometro ottico al Plasma assiale Spectroflame Modula-E (SPECTRO, Germany) presso il Dipartimento di Scienze Chimiche, Laboratorio di Chimica Analitica Ambientale e Strumentale, Università di Trieste.

3.2.6.3. Spettrometria di Massa con sorgente al Plasma Induttivamente Accoppiato

Lo strumento utilizzato per le analisi dell'argento è uno spettrometro ICP-MS Agilent 7500ce (USA) equipaggiato con una cella di collisione per l'abbattimento delle interferenze, presso il Dipartimento di Traumatologia, Ortopedia e Medicina del Lavoro, Laboratorio di Tossicologia Industriale, Università di Torino.

3.3. PROCEDURE SPERIMENTALI SPECIFICHE

3.3.1. Assorbimento cutaneo *in vitro* di polvere di cromo ed effetti della detersione (Allegato I)

In questo lavoro è stato studiato l'assorbimento percutaneo del cromo metallico *in vitro*, utilizzando sudore sintetico a pH 4,5, e l'effetto di un comune detergente (contenente sodio laurilsolfato e sodio lauriletere solfato) sul processo di assorbimento del metallo stesso. Negli ambienti di lavoro la pulizia con il detergente della cute contaminata è una pratica abituale: essa dovrebbe rimuovere la maggior parte delle sostanze tossiche depositatesi, ma numerosi studi hanno dimostrato che essa può determinare un aumento dell'assorbimento percutaneo delle stesse. In particolare, il sodio laurilsolfato può facilitare la penetrazione di alcuni agenti tossici, ad esempio nichel e piombo, alterando il mantello idrolipidico e la normale funzione "barriera" della cute (Frankild et al., 1995, Larese et al., 2006).

Prima degli esperimenti di permeazione cutanea, sono stati svolti alcuni test propedeutici per studiare gli effetti del pH del sudore sintetico e della concentrazione della polvere sulla ionizzazione della metallo, processo necessario per una eventuale permeazione cutanea (Larese et al., 2007).

I test di permeazione sono stati svolti col metodo delle celle di diffusione di Franz utilizzando cute umana integra. Come fase donatrice è stato usato 1 ml di una sospensione al 5 % w/v di polvere di cromo, con una dimensione media delle particelle (APS) inferiore ai 10 µm, in sudore sintetico fresco a pH 4,5. Inoltre in 4 celle, a 30 minuti dall'inizio dell'esperimento, è stato eseguito il lavaggio della cute con un comune detergente contenente sodio laurilsolfato.

La permeazione del cromo è stata monitorata nelle 24 ore attraverso il prelievo dopo 1, 2, 4, 8, 16, 18, 20, 24 ore di 1,5 ml di fase ricevente rimpiazzata con soluzione fisiologica fresca. Alla fine degli esperimenti la cute è stata mineralizzata con acido nitrico concentrato e acqua ossigenata a caldo, per l'analisi del cromo rimasto all'interno della cute.

I valori di concentrazione del cromo nelle varie soluzioni sono stati determinati tramite spettroscopia di emissione atomica al plasma induttivamente accoppiato (ICP-AES) e spettroscopia di assorbimento atomico con fornetto di grafite (GF-AAS) con sistema di correzione del fondo Zeeman.

Il limite di rilevabilità per il cromo in ICP-AES era di 10 µg L^{-1} alla lunghezza d'onda di 267,716 nm. La precisione espressa come deviazione standard relativa percentuale (RSD%)

delle misure è stata sempre inferiore al 5%.

Il limite di rilevabilità per il cromo in GF-AAS era di 0,2 μg L^{-1} alla lunghezza d'onda di 357,9 nm. Come modificante di matrice è stata usata una soluzione di $Mg(NO_3)_2$ allo 0,5% w/v. La precisione espressa come deviazione standard relativa percentuale (RSD%) delle misure è stata sempre inferiore al 10%.

3.3.2. Assorbimento cutaneo *in vitro* di polveri di cobalto, nichel e cromo attraverso cute umana integra e lesa (Allegato II)

In questo studio è stata valutata l'influenza che le lesioni cutanee possono avere sulla permeazione cutanea *in vitro* di polveri metalliche di cobalto, nichel e cromo, utilizzando il metodo delle celle di diffusione di Franz e un protocollo di abrasione cutanea proposto da Bronaugh e Steward (1985).

Come fasi donatrici sono stati utilizzati 1,5 ml di dispersioni al 5% w/v di polveri di cobalto (APS < 2 μm), di nichel (APS =2,2 μm) e di cromo (APS < 10 μm) in sudore sintetico a pH 4,5, mentre come fase ricevente è stata usata soluzione fisiologica a pH 7,3.

Dato che i profili di permeazione nelle 24 ore di esposizione sono già stati ricavati in precedenti lavori del nostro gruppo (Larese et al., 2004, 2007), è stata studiata solo la permeazione totale alle 24 ore, senza campionamenti intermedi. Per ognuno dei tre metalli sono state allestite 2 celle come bianco, 6 celle con cute integra e 6 celle con cute lesa.

Alla fine degli esperimenti la cute è stata mineralizzata con acido nitrico concentrato e acqua ossigenata a caldo, per l'analisi del contenuto dei metalli all'interno della cute.

Le fasi donatrici sono state centrifugate per 15 minuti a 3000 giri/minuto e il surnatante è stato filtrato due volte con filtri per siringa da 0,45 μm prima delle analisi per determinare la percentuale di metallo presente in forma ionizzata.

I valori di concentrazione del cromo nelle varie soluzioni sono stati determinati tramite spettroscopia di assorbimento atomico con fornetto di grafite (GF-AAS) con sistema di correzione del fondo Zeeman. Il limite di rilevabilità per il cobalto era di 0,4 μg L^{-1} alla lunghezza d'onda di 240,7 nm; il limite di rilevabilità per il nichel era di 0,2 μg L^{-1} alla lunghezza d'onda di 232,0; il limite di rilevabilità per il cromo era di 0,2 μg L^{-1} alla lunghezza d'onda di 357,9 nm. Una soluzione di $Mg(NO_3)_2$ allo 0,5% w/v è stata usata come modificante di matrice per le analisi di cobalto e cromo. La precisione espressa come deviazione standard relativa percentuale (RSD%) delle misure è stata sempre inferiore al 10%.

3.3.3. Permeazione cutanea *in vitro* di nanoparticelle d'argento attraverso cute umana integra e lesa (Allegato IV)

In questo studio abbiamo valutato con il metodo *in vitro* delle Franz cells il passaggio di nanoparticelle di argento, rivestite con polivinilpirrolidone, attraverso la cute sia integra che lesa.

I test sono stati ripetuti due volte. Nel primo esperimento, sono state allestite 8 celle con cute integra e 8 celle con cute lesa, secondo il protocollo proposto da Bronaugh e Steward (1985). Nel compartimento donatore delle celle è stata collocata una quantità complessiva pari a 70 $\mu g\ cm^{-2}$ di nanoparticelle di argento, rivestite in polivinilpirrolidone con un diametro medio di 25 nm misurato al TEM (transmission electron microscopy), disperse in etanolo 0.14 % w/v ed infine diluite 1:10 con sudore sintetico a pH 4,5. Come fase ricevente è stata usata soluzione fisiologica a pH 7,3. Al termine delle 24 ore le soluzioni riceventi sono state recuperate per le successive analisi.

I test sono stati ripetuti una seconda volta utilizzando la stessa quantità di fase donatrice, ma allestendo 5 celle con cute integra e 5 con cute lesa. Durante le 24 ore di esposizione sono stati eseguiti i prelievi alle 4, 8, 20, 24 ore. Per ogni cella sono stati prelevati 1,5 ml di soluzione ricevente e sostituiti con soluzione fisiologica fresca.

Nei due test, 4 celle sono state allestite come bianco, quindi trattate allo stesso modo delle altre, ma come fase donatrice è stato utilizzato solo sudore sintetico, senza nanoparticelle.

Al termine delle 24 ore alcuni campioni di cute esposta sono stati utilizzati per le investigazioni al TEM.

I valori di concentrazione del cromo nelle varie soluzioni sono stati determinati tramite spettroscopia di assorbimento atomico con fornetto di grafite (GF-AAS) con sistema di correzione del fondo Zeeman. Il limite di rilevabilità per l'argento era di 0,1 $\mu g\ L^{-1}$ alla lunghezza d'onda di 328,1 nm. Come modificanti di matrice sono state usate una soluzione di $Mg(NO_3)_2$ allo 0,1% w/v e una di $Pd(NO_3)_2$ allo 0,1% w/v. La precisione espressa come deviazione standard relativa percentuale (RSD%) delle misure è stata sempre inferiore al 5%.

3.3.4. Permeazione cutanea *in vitro* di nanoparticelle d'oro attraverso cute umana integra e lesa (allegato V)

In questo studio è stata studiata la permeazione *in vitro* di nanoparticelle d'oro, con un diametro medio misurato al TEM di 12,6±0,9 nm, attraverso cute umana integra e lesa, con il metodo delle celle di diffusione di Franz.

I test sono stati ripetuti due volte. Nel primo test propedeutico, sono state allestite 8 celle con cute integra e 8 celle con cut lesa, secondo il protocollo proposto da Bronaugh e Steward (1985). Come fase donatrice sono stati usati 0,5 ml di una soluzione contenente 100 mg L^{-1} di nanoparticelle d'oro, diluiti 1:3 con sudore sintetico a pH 4,5 per un volume totale di 1,5 ml e una quantità media di 15 μg cm^{-2}. Come fase ricevente è stata usata soluzione fisiologica a pH 7,3. Al termine delle 24 ore di esposizione le fasi riceventi sono state recuperate per le successive analisi delle concentrazione.

Nel secondo test, sono state allestite 8 celle con cute integra e 8 celle con cute lesa. Come fase donatrice sono stati usati 1,5 ml di una soluzione contenente 100 mg L^{-1} di nanoparticelle d'oro, diluiti 1:1 con acqua milliQ per un volume totale di 3 ml e una quantità media di 45 μg cm^{-2}. Agli intervalli prestabiliti (4, 8, 16, 24 ore) 1,5 ml di soluzione ricevente sono stati prelevati da ogni cella per le analisi delle concentrazioni e sostituiti con lo stesso volume di soluzione fisiologica fresca. Al termine delle 24 ore di esposizione le varie fasi sono state recuperate per le successive analisi.

Per ogni esperimento, 4 celle sono state trattate come bianchi, cioè trattate come le altre celle, ma senza nanoparticelle d'oro nella fase donatrice.

I campioni di cute integra, dopo essere stati rimossi dalle celle di Franz, sono stati divisi in epidermide e derma tramite shock termico in acqua a 60°C per un minuto; per tre campioni lo strato corneo è stato rimosso tramite stripping con un nastro adesivo di VC. I vari strati sono stati mineralizzati separatamente con acido nitrico e acqua ossigenata a caldo. Alcuni campioni di cute, sia integra che lesa, sono invece stati utilizzati per le analisi al TEM.

I valori di concentrazione del cromo nelle varie soluzioni sono stati determinati tramite spettroscopia massa al plasma induttivamente accoppiato (ICP-MS). Il limite di rilevabilità per l'oro era di 0,001 μg L^{-1}. La precisione espressa come deviazione standard relativa percentuale (RSD%) delle misure è stata sempre inferiore al 3%.

4. RISULTATI E DISCUSSIONI

4.1. ASSORBIMENTO CUTANEO *IN VITRO* DI POLVERE DI CROMO ED EFFETTI DELLA DETERSIONE

(Allegato I)

4.1.1. Risultati

Il nostro studio ha evidenziato un incremento progressivo nel passaggio del metallo. Alle 24 ore, la quantità di cromo permeata nella fase ricevente delle celle non trattate con il detergente è pari a 0,016±0,005 μg cm^{-2} con un flusso medio di 0,84±0,25 ng cm^{-2} h^{-1} e un lag time di 1,1± 0,7 ore. Nelle celle trattate con il detergente, le concentrazioni di cromo sono molto basse e dello stesso ordine di grandezza di quelle osservate nei bianchi di controllo.

L'analisi della cute ha rivelato invece una quantità di cromo dopo 24 ore di esposizione di 3,19±1,48 μg cm^{-2} nelle celle non lavate con il detergente ed una concentrazione di 5,46±1,09 μg cm^{-2} in quelle trattate con il lavaggio.

4.1.2. Discussione

Il nostro studio ha confermato, analogamente ai dati disponibili in letteratura, un basso flusso di assorbimento percutaneo per il cromo metallico, rispetto ad altri metalli: il sudore sintetico a pH 4,5 era in grado di ossidare la polvere di Cr a Cr (III) che, a differenza del Cr (VI), attraversa la cute meno facilmente e presenta una maggiore affinità per le proteine cutanee.

Il lavaggio della cute con il detergente e la rimozione della fase donatrice dopo 30 minuti di esposizione, hanno determinato una riduzione del passaggio del metallo nel liquido ricevente e un significativo incremento della concentrazione di cromo nella cute: questo può essere attribuito da un lato alla elevata affinità del Cr (III) per i costituenti della cute e dall'altro all'azione del sodio laurilsolfato che, alterando l'integrità della membrana, facilita l'assorbimento del metallo nella cute. Questo dato deve essere tenuto in considerazione, in particolare in ambito professionale, al fine di predisporre un'adeguata protezione della cute dal contatto con sostanze tossiche, poiché anche una breve esposizione, seguita da accurata pulizia, potrebbe rappresentare un potenziale fattore di rischio per l'assorbimento. Va inoltre sottolineata l'importanza della scelta del detergente: utilizzando un prodotto comune per la decontaminazione è possibile determinare un aumento non trascurabile della penetrazione dell'agente tossico nella cute.

4.2. ASSORBIMENTO CUTANEO *IN VITRO* DI POLVERI DI COBALTO, NICHEL E CROMO ATTRAVERSO CUTE UMANA INTEGRA E LESA

(allegato II)

4.2.1. Risultati

I dati di permeazione di questo studio sono stati riassunti come mediane, valori massimi, valori minimi e 25-75esimo percentile. Le quantità medie di cobalto e nichel trovate nella fase ricevente sono significativamente più alte quando le polveri metalliche sono applicate su cute lesa rispetto alla cute integra: le mediane sono rispettivamente 0,0084 vs 3,566 µg cm^{-2} per il cobalto e 0,0072 vs 2,631 µg cm^{-2} per il nichel, mentre non sono state riscontrate differenze significative nella permeazione del cromo.

Le quantità dei tre metalli rimaste nella cute dopo le 24 ore di esposizione sono significativamente più alte nella cute lesa rispetto alla cute integra: le mediane sono rispettivamente 48,7 vs 29,6 µg cm^{-2} per il cobalto, 131 vs 82,3 µg cm^{-2} per il nichel e 62,1 vs 14,4 µg cm^{-2} per il cromo.

Le analisi delle fasi donatrici, dopo rimozione della polvere per centrifugazione e filtrazione, hanno confermato la presenza di ioni metallici in soluzione: la ionizzazione di cobalto e nichel risulta significativamente maggiore quando la sospensione è applicata alla cute lesa rispetto alla cute integra (55,6 e 129 mg L^{-1} vs. 21,3 e 57,5 mg L^{-1}), mentre il Cr non mostra differenze significative tra i due gruppi di celle (0,18 vs 0,28 mg L^{-1}).

4.2.2. Conclusioni

Questo studio ha dimostrato che polveri di cobalto, nichel e cromo, con diametro medio nell'ordine del micron, possono essere ossidate a contatto con il sudore sintetico e rilasciare ioni metallici in soluzione: tali ioni sono poi in grado di attraversare la cute, in modo variabile e dipendente sia dalla percentuale ionizzata sia dalle condizioni della cute. Le lesioni cutanee aumentano significativamente il passaggio *in vitro* di cobalto e nichel, mentre per quanto riguarda il cromo non ci sono differenze significative tra i due gruppi di celle e tra le celle trattate e i bianchi.

Per quanto riguarda invece la quantità di metallo che rimane nella pelle, la differenza tra le celle con cute integra e le celle con cute lesa è significativa per tutti i tre metalli studiati: in particolare, le quantità penetrate nella cute trattata con il cromo sono dello stesso ordine di

grandezza delle celle trattate con gli altri metalli. Questo suggerisce che la cute abbia un tropismo particolare per gli ioni trivalenti del cromo intrappolandoli nei vari strati probabilmente attraverso il legame con proteine della pelle ed evitando che permeino nella fase ricevente (Hostynek, 2003; Samitz et al., 1969).

La fase donatrice infine sembra essere influenzata dalla presenza di lesioni cutanee nel caso di cobalto e nichel: la ionizzazione dei due metalli è significativamente superiore nelle celle allestite con cute integra rispetto a quelle con cute lesa, suggerendo che il contatto della polvere a componenti cutanei esposti in seguito alla lesione possa contribuire alla ionizzazione stessa. Questa differenza non è stata notata negli esperimenti con polvere di cromo.

Questi risultati insieme ai dati già presenti in letteratura confermano che è necessario prevenire il contatto dei metalli e dei loro sali con la cute, soprattutto in presenza di lesioni cutanee anche piccole, situazione molto comune nell'ambiente di lavoro: nei nostri test piccole lesioni hanno portato ad un significativo aumento dell'assorbimento cutaneo.

4.3. PERMEAZIONE CUTANEA *IN VITRO* DI NANOPARTICELLE DI ARGENTO ATTRAVERSO CUTE UMANA INTEGRA E LESA

(Allegato IV)

4.3.1. Risultati

I dati di permeazione di questo studio sono stati riassunti come mediane, valori massimi, valori minimi e 25-75esimo percentile: essi mostrano che l'argento può passare attraverso la cute umana integra con un valore di mediana alle 24 ore di 0,46 ng cm^{-2}, mentre la permeazione attraverso la cute danneggiata è circa cinque volte maggiore con un valore di mediana di 2,32 ng cm^{-2}. Le concentrazioni dei bianchi sono tutte al di sotto del limite di rilevabilità.

Per il gruppo di celle con cute lesa è stato possibile calcolare un flusso nelle 24 ore di 0,6±0,2 ng cm^{-2} h^{-1} con un lag time inferiore a 1 ora, mentre per le celle con cute integra non è stato possibile ricavare un range di linearità date le basse concentrazioni misurate nelle fasi riceventi.

Le analisi al TEM dei campioni di cute esposti per 24 ore hanno mostrato che le nanoparticelle di argento studiate in questi test sono in grado di penetrare la barriera dello strato corneo e di raggiungere gli strati vitali più superficiali dell'epidermide.

4.3.2. Discussione

Lo studio della tossicità e delle vie di penetrazione delle nanoparticelle è iniziato solo recentemente e i dati relativi alla loro capacità di penetrare la cute sono ancora pochi. In particolare lo studio delle nanoparticelle di argento risulta interessante perché si tratta della tipologia di nanoparticelle maggiormente prodotta dall'industria con ampie applicazioni in prodotti di uso comune quali tessuti tecnici, disinfettanti ambientali, pesticidi, ecc.

I nostri dati sperimentali hanno mostrato che l'assorbimento cutaneo *in vitro* di argento, applicato in forma di nanoparticelle, attraverso la cute integra risulta molto basso, ma misurabile, mentre le lesioni cutanee aumentano la permeazione di cinque volte. Le analisi di microscopia elettronica inoltre hanno mostrato che le nanoparticelle sono in grado di raggiungere gli strati vitali dell'epidermide confermando quindi la penetrazione delle nanoparticelle in quanto tali e non solo l'assorbimento dell'argento che ionizzabile in soluzione.

Tali osservazioni sono in accordo con altri studi sull'assorbimento cutaneo di differenti

tipologie di nanoparticelle (vedi allegato III) e pongono l'attenzione sulla esigenza di valutare molto attentamente l'esposizione cutanea alle nanoparticelle, in particolare nanoparticelle metalliche, di cui conosce ancora poco. Inoltre, da un punto di vista occupazionale questi test confermano la necessità di utilizzare i dispositivi di protezione individuale per la prevenzione del contatto cutaneo con le nanoparticelle, soprattutto in presenza di abrasioni e lesioni cutanee, molto frequenti nelle normali condizioni di lavoro, che possono portare ad un aumento nell'assorbimento percutaneo.

4.4. PERMEAZIONE CUTANEA *IN VITRO* DI NANOPARTICELLE D'ORO ATTRAVERSO CUTE UMANA INTEGRA E LESA

(Allegato V)

4.4.1. Risultati

Le nanoparticelle d'oro applicate in sudore sintetico a pH 4,5 hanno portato ad un assorbimento di oro *in vitro* dopo 24 ore di esposizione pari a 60,8±25,3 ng cm^{-2} attraverso la cute integra e 55,2± 27,3 ng cm^{-2} attraverso la cute lesa.

Le nanoparticelle d'oro applicate in acqua milliQ, invece, hanno portato ad un assorbimento di oro più consistente e cioè di 214±44 ng cm^{-2} attraverso la cute integra e di 188±50 ng cm^{-2} attraverso la cute lesa.

Nel secondo esperimento è stato possibile calcolare un flusso nelle 24 ore di esposizione di 7,8±2,0 ng cm^{-2} h^{-1} e di 7,1±2,5 ng cm^{-2} h^{-1} rispettivamente per la cute integra e per la cute lesa, con un lag time per entrambe inferiore a 1 ora

La quantità di oro rimasta all'interno della cute dopo 24 ore è significativamente più alta nella cute lesa rispetto alla cute integra (rispettivamente 7,9±4,5 vs 1,8±0,7 μg cm^{-2}).

Le analisi sulle concentrazioni di oro all'interno dello strato corneo, dell'epidermide e del derma della cute integra hanno mostrato un contenuto di oro decrescente dall'esterno verso l'interno: negli "strip" di nastro adesivo è stata trovata una quantità di oro decrescente dagli 0,40±0,05 μg cm^{-2} del primo "strip" più esterno agli 0,17±0,01 μg cm^{-2} del sesto e più profondo "strip". Il contenuto di metallo nell'epidermide è di 1,78±0,68 μg cm^{-2} mentre nel derma raggiunge gli 0,05±0,02 μg cm^{-2}.

Le analisi di microscopia elettronica hanno evidenziato la presenza di nanoparticelle d'oro sia nell'epidermide che nel derma sia in campioni di cute integra che in campioni di cute lesa.

4.4.2. Discussione

I risultati di questi esperimenti *in vitro* con cute umana intera non hanno evidenziato differenze significative tra cute integra e cute lesa per quanto riguarda l'assorbimento dell'oro applicato in forma di nanoparticelle sia con il sudore sintetico che con l'acqua milliQ, mentre un aumento della concentrazione della fase donatrice ha portato ad un aumento della quantità di oro permeata nella fase ricevente. L'analisi dei vari strati della cute ha ben evidenziato un gradiente di concentrazione dall'esterno verso l'interno e una differenza significativa della

quantità di oro rimasta all'interno della cute lesa rispetto alla cute integra.

L'indagine microscopica ha confermato che anche con cute integra le nanoparticelle d'oro studiate in questi esperimenti sono in grado di attraversare lo spessore cutaneo e raggiungere gli strati vitali più profondi fino a raggiungere il derma. Questo risultato conferma che le concentrazioni d'oro ritrovate nella cute dopo mineralizzazione non derivano solo da metallo in forma ionica, ma anche da nanoparticelle in quanto tali allo stato di ossidazione (0).

Infine, i risultati di questi test suggeriscono la possibilità di usare le nanoparticelle d'oro come carrier di farmaci in formulazioni a rilascio transcutaneo e in altre applicazioni biomediche che richiedono una diffusione delle nanoparticelle nei tessuti.

La carenza di dati in letteratura e i pochi risultati ottenuti in test *in vitro* richiedono comunque una più approfondita valutazione del fenomeno dell'assorbimento cutaneo utilizzando altri tipi di nanoparticelle differenti in forma, dimensioni e rivestimento, e la valutazione accurata di tutte le variabili che possono influenzare la permeazione cutanea in funzione dell'obiettivo che si vuole raggiungere.

5. CONCLUSIONI

In conclusione, i risultati dei nostri esperimenti hanno mostrato che, *in vitro*, cobalto, nichel e cromo rilasciati da polveri metalliche e nanoparticelle di argento e oro sono in grado di permeare attraverso la cute fino alla fase ricevente, e che la presenza di lesioni cutanee, l'uso di detergenti, il pH della fase donatrice o l'entità dell'esposizione, sono in grado di volta in volta di modificare tale processo.

In particolare le lesioni cutanee determinano nei nostri esperimenti un notevole aumento nella permeazione di cobalto e nichel, applicati in forma di polveri metalliche disperse in sudore sintetico, e di argento applicato come nanoparticelle rivestite, mentre non vi sono differenze significative per quanto riguarda la permeazione del cromo da polvere metallica e dell'oro in forma di nanoparticelle. In tutti i test, però, le lesioni cutanee hanno determinato un notevole aumento della concentrazione di metallo nel tessuto cutaneo misurato dopo mineralizzazione della cute esposta.

Anche l'utilizzo di un comune detergente contenente sodio laurilsolfato e sodio lauriletere solfato nei test di assorbimento cutaneo del cromo metallico ha causato un aumento significativo della quantità di metallo misurato nella cute, nonostante la fase donatrice fosse stata rimossa dopo 30 minuti di esposizione. Questo risultato conferma l'effetto di potenziamento della permeazione che hanno questi tipi di tensioattivi (in particolare del sodio laurilsolfato) che causano modificazioni della composizione dei lipidi dello strato corneo, estraendo la matrice lipidica dello spazio intercellulare e diminuendo così la funzione di barriera della cute.

Le analisi della cute al TEM hanno inoltre dimostrato che le nanoparticelle di oro e di argento in un sistema *in vitro* sono in grado di penetrare all'interno dei vari strati cutanei. Questo significa che l'assorbimento del nichel può avvenire sia con un meccanismo di ionizzazione seguito da permeazione, già dimostrato in altri lavori (Larese et al., 2007; 2009b), sia con un meccanismo di penetrazione diretta.

I risultati di questi primi lavori sulla permeazione cutanea di nanoparticelle e l'interesse suscitato, ci hanno spinto a proseguire su questa strada e sono attualmente in corso le analisi sui test di permeazione cutanea di nanoparticelle di nichel e di cobalto.

Una delle problematiche che si incrontrano in questo tipo di studi riguarda la quantificazione delle nanoparticelle direttamente nei tessuti. Le analisi di microscopia elettronica non sono quantitative e la laboriosa preparazione dei campioni può dare luogo ad artefatti. Le analisi spettrofotometriche dopo mineralizzazione della cute, invece, forniscono solo la concentrazione totale del metallo. Con queste tecniche quindi non è possibile distinguere la percentuale di metallo penetrato in forma di nanoparticella e in forma ionica. A

questo proposito si stanno cercando soluzioni strumentali e metodologiche in grado di distinguere gli effetti diretti delle nanoparticelle da quelli indiretti dovuti al rilascio di ioni e di residui del processo di sintesi come possono essere catalizzatori o solventi.

Quindi, al fine di implementare le nostre possibilità di analisi con l'utilizzo di metodiche diverse e strumentazioni all'avanguardia, sia per quanto riguarda l'analisi microscopica dei tessuti esposti sia per studiare le formulazioni contenenti nanoparticelle, sono state avviate delle collaborazioni con laboratori di altre strutture di ricerca.

Attualmente è in corso un progetto per l'ottimizzazione dell'analisi di campioni di cute esposta a nanoparticelle metalliche tramite microtomografia (micro-CT) alla SYRMEP "beamline" (Elettra-Sincrotrone; Trieste). Questo studio ha come obiettivo la visualizzazione della distribuzione del metallo nei vari strati cutanei con tecniche d'indagine che richiedano una preparazione del campione meno invasiva rispetto alla microscopia elettronica.

Queste tecniche di visualizzazione insieme all'indagine microscopica a trasmissione (TEM) e a scansione (SEM) eseguite presso il Centro di Coordinamento e Sviluppo Progetti e Apparecchiature dell'Università di Trieste e all'analisi della concentrazione totale eseguita dopo mineralizzazione della cute con tecniche di emissione atomica (ICP-AES) e assorbimento atomico (GF-AAS) hanno lo scopo di fornire un quadro più completo dell'eventuale penetrazione e distribuzione delle nanoparticelle all'interno della cute.

Un'altra collaborazione è stata avviata con un gruppo di ricerca dell'Istituto di Fisica Applicata, Consiglio Nazionale delle Ricerche, Sesto Fiorentino (FI) per la sintesi, caratterizzazione e distribuzione nei tessuti di nanorods d'oro e della possibilità di aumentare tale penetrazione attraverso l'utilizzo del laser.

Per quanto riguarda invece la tossicità cellulare è in corso una collaborazione con un gruppo di ricerca del Dipartimento di Scienze della Vita dell'Università di Trieste per la valutazione della tossicità cellulare di nanoparticelle di argento sulla linea cellulare HaCaT, derivata da cheratinociti umani spontaneamente immortalizzati per poi proseguire con le altre nanoparticelle metalliche.

In questo tipo di studi *in vitro* ci sono ovviamente delle limitazioni dovute soprattutto alla difficoltà di ricreare tutte le condizioni di un'esposizione *in vivo*. L'utilizzo di cute espiantata, l'eccessiva idratazione nelle 24 ore, la staticità delle celle di diffusione e l'impossibilità di controllare contemporaneamente tutte le variabili, possono portare a differenze con l'effettiva situazione in un'esposizione cutanea reale. Anche questi test, però, risultanto essere molto importanti e, insieme ai dati dei test su volontari e agli studi epidemiologici, contribuiscono a definire un quadro completo della valutazione del rischio.

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ALLEGATI

ALLEGATO I

IN VITRO PERCUTANEOUS ABSORPTION OF CHROMIUM POWDER AND THE EFFECT OF SKIN CLEANSER

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ABSTRACT

The present study tried to investigate, using a synthetic sweat at pH 4.5, whether metallic chromium can pass through the skin (*in vitro*) and the effect of rapid skin decontamination with a common detergent.

A suspension of chromium powder in synthetic sweat at pH 4.5 was prepared and shaken with a stirring plate at room temperature for 30 minutes. Human skin membranes were set up in Franz-diffusion cells and 1 ml of the freshly made suspension was applied to the outer surface of the skin for 24 hours. The tests were performed without and with decontamination using the cleanser 30 minutes after the start of exposure. The appearance of metal ions in the aqueous receptor phase was quantified by Electro Thermal Atomic Absorption Spectroscopy (ETAAS) and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Speciation analysis and measurements of chromium skin content were also performed.

Chromium skin permeation was demonstrated in *in vitro* experiments using the Franz cell system, giving a permeation flux of 0.84±0.25 ng cm^{-2} h^{-1} and a lag time of 1.1±0.7 h. The cleaning procedure stop Cr permeation but its concentration into the skin significantly increased (Mann-Witney U test $P<0.03$).

The results revealed that chromium applied as powder can pass through the skin and that decontamination, done after 30 minutes of exposure, prevent Cr skin permeation but increase Cr content into the skin.

KEYWORDS: Powders; In vitro; Percutaneous absorption; Chromium; Cleanser; Skin decontamination

RIASSUNTO

Il presente studio ha lo scopo di studiare, usando sudore sintetico a pH 4.5, se il cromo metallico è in grado di passare attraverso la pelle (*in vitro*) e l'effeto di una decontaminazione rapida con un comune detergente.

Una sospensione di polvere di cromo in sudore sintetico a pH 4.5 è stata preparata e tenuta sotto agitazione a temperatura ambiente per 30 minuti. I lembi di cute umana sono stati posizionati nelle celle di diffusione di Franz ed 1 ml della sospensione è stato applicato sulla superficie esterna della cute per 24 ore. I test sono stati condotti anche operando una decontaminazione con un comune detergente dopo 30 minuti dall'inizio dell'esposizione.

La presenza di ioni metallici nella fase ricevente è stata quantificata tramite Spettroscopia di Assorbimento Atomico Elettro-Termica (ET-AAS) e Spettroscopia di Emissione Atomica al Plasma Induttivamente Accoppiato (ICP-AES). Sono state effettuate anche analisi di speciazione e misure del contenuto di cromo all'interno della cute dopo mineralizzazione.

La permeazione cutanea del cromo è stata dimostrata in esperimenti *in vitro* usando il metodo delle celle di diffusione di Franz ed è stato trovato un flusso di permeazione di 0.84 ± 0.25 ng cm^{-2} h^{-1} e un lag time di 1.1 ± 0.7 h. La procedura di pulizia ha fermato la permeazione del cromo, ma la sua concentrazione all'interno della cute è significativamente aumentata (Mann-Witney U test $P<0.03$).

I risultati hanno rivelato che il cromo applicato come polvere può permeare attraverso la cute e che la decontaminazione, effettuata dopo 30 minuti di esposizione, previene la permeazione, ma aumenta il contenuto di cromo all'interno della cute.

PAROLE CHIAVE: Polveri metalliche; In vitro; Assorbimento cutaneo; Cromo; Detergente; Decontaminazione della pelle

1. INTRODUCTION

An important research, past and ongoing, is directed towards the knowledge of the factors that can influence percutaneous absorption, in order to assess the risk of human exposure to toxics in the occupational and environmental area.

One of the most frequently investigated compounds for skin permeation and overall impact on human health is chromium (Cr) and its salts, probably due to the ambivalent nature of chromium as essential element, as well as a risk factor in the workplace (Cohen et al., 1993).

Chromium contact allergy is still frequent in many industrialized countries of the world. Occupational chromium dermatitis occurs among cement workers, chromium platers and metal workers, workers dealing with leather tanning and among employees in the ceramics industry (Polak, 1983; Nriagu and Nieboer, 1988).

Only tri and hexavalent Cr compounds can be considered as potential haptens because all the other Cr salts are unstable. Their ability to elicit allergic contact dermatitis depends mostly on the bio-availability of the Cr salts (Van Lierde et al., 2006).

In vitro percutaneous studies can be used to define the diffusion characteristics of xenobiotics as one way of documenting their bio-availability.

Penetration of Cr through skin has been studied in animals as well as in humans, previously analysing biopsies from skin (Liden and Lundberg, 1979) or evaluating the "disappearance measurement" of isotopes of Cr in salt formulations (Wahlberg, 1965a,b, 1968). Moreover, in 1997, Corbett et al. showed that is possible a systemic uptake of chromium in men following dermal contact with hexavalent chromium salts; but, while the absorption of chromium salts is well-known, there are few data about Cr ions release from metal Cr, and Cr metal absorption through full thickness skin.

Liden and Carter (2001) proved that nickel coins in contact with synthetic sweat release metallic ions and in a previous study of us, we demonstrated that cobalt metallic powder, when shaken in synthetic sweat, can release cobalt ions that can pass through the skin using in-vitro Franz-cell system (Larese Filon et al., 2004).

This is in accordance with the results from the *in vitro* permeation experiments which we performed with human skin, testing three important metals for allergies (Cr, Ni and Co) in the same way. Our study confirmed the capability of the sweat to oxidise Ni and Co metal powders but not Cr powder that can't to pass through the skin, suggesting that synthetic sweat used (at pH 6.5) was unable to oxidise the metal (Larese Filon et al., 2007). On the

contrary, after exposure of the skin to a donor solution containing Cr salts, Cr permeation was very high (Gammelgaard et al., 1992; Corbett et al., 1997; Hostynek, 2003; Van Lierde et al., 2006; Larese Filon et al., 2007)

The aims of this study were twofold: first to investigate, using a synthetic sweat at a lower pH (4.5), whether metallic Cr can pass through full thickness skin and, second to evaluate the effect of rapid skin decontamination using a common cleanser containing sodium lauryl sulphate (SLS).

In occupational settings skin contamination is fairly common, and the cleaning procedure not only may visibly remove most of the toxic agent, but also increase skin uptake by the penetration-enhancing effect of the surfactants. For example, sodium lauryl sulphate can increase the skin penetration of some toxic substances (Frankild et al., 1995) because it has a destructive action on stratum corneum by changing its lipid composition (Ashton et al., 1986), extracting lipids from the intercellular spaces in the stratum corneum (Imokava et al., 1989; Froebe et al., 1990), and changing alpha–keratin into beta-keratin that causes swelling and hydration of the stratum corneum (Scheuplein and Ross, 1970; Putterman et al., 1977). Larese (2006) found that a decontamination procedure using sodium lauryl sulphate after 30 minutes of application of lead oxide cause an increase of lead skin absorption.

The lack of data on skin absorption of Cr metal and the need for a better understanding of the effect of decontamination agents led us to test this metal. To do so, experiments were performed in *in vitro* Franz-cell system using human skin.

For our experiments we used the experience and the protocols employed during the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals), a three-year (2001-2004) research program funded by European Union (EDETOX, 2000).

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were analytical grade. Urea, magnesium nitrate hexahydrate, sodium hydrogenphosphate, potassium dihydrogenphosphate, diphenylcarbazide, potassium bichromate, hydrogen peroxide (30% v/v) were purchased from Carlo Erba (Milan, Italy); ammonium hydroxide (25% w/v) was bought from J. T. Baker (Deventer, Holland); lactic acid (90% v/v) from Acros Organics (Geel, Belgium); acetone, nitric acid (69% v/v) and

sulfuric acid (95% v/v) from Sigma Aldrich (Milan, Italy).

Water reagent grade was produced with a Millipore purification pack system (milliQ water).

Cr powder come from Alfa Aesar (Karlsruhe, Germany) and consisted of chromium powder, APS (Average Particle Size) <10 micron, 99.8% purity (metals basis). The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na_2HP0_4, 0.19 g of KH_2P0_4 and 9 g of NaCl into 1 liter of milliQ water (final pH = 7.35). The synthetic sweat solution used as the donor fluid consisted in 0.5% w/v sodium chloride, 0.1% w/v urea and 0.1% w/v lactic acid in milliQ water; pH was adjusted with ammonia (at pH 3.5 or 4.5 or 5.5).

2.2. Dissolution tests in synthetic sweat

As preliminary test at the study of *in vitro* percutaneous absorption of chromium powder, a research about the dissolution of chromium powder in synthetic sweat at three different pH (3.5, 4.5 e 5.5) and at two different concentrations (0.5% w/v and 5% w/v) has been performed in order to investigate the extent of ionization of this metal. To do so, 9 samples (3 for every different pH) of suspension of chromium powder in synthetic sweat for both concentrations and other 3 samples with only synthetic sweat at pH 5.5 as blank has been prepared. The volume of each suspension was 40 ml. Each suspension was put into a baker and left under moderate agitation for 24 hours at room-temperature. After 8 and 24 hours, 10 ml were removed, centrifuged for 15 minutes at 3000 rpm and the surnatant filtered two times through 0.45 μm GHP Acrodisc syringe filters (Pall Life Sciences, Ann Arbor, MI) and analysed by ICP-AES (see section 2.6.3) for the total chromium concentration and with UV-vis spectrophotometry (see section 2.6.2) for the determination of Cr(VI).

2.3. Preparation of skin membranes

Human abdominal full thickness skin was obtained as surgical waste from at least 2 donors for each experiments. Donors were man and women within a wide range of age (45÷71 years). Prior to freezing, subcutaneous fat was removed and hair shaved. All the pieces of full thickness skin were stored in freezer at -25°C for a period up to, but not exceeding, 4 months. It has been shown that this method of storage does not damage the skin since no difference in permeability was observed between fresh and frozen segments of the same skin in a separate series of experiments (Franz, 1975). Skin integrity was tested before and after each experiment using electrical conductibility by means of a conductometer

(Metrohm, 660 Conductometer, AG Oberdorfstr. 68 CH-9100 Herisau) operating at 300 Hz connected to two stainless steel electrodes (Fasano et al., 2002). The electrical conductance data, obtained in μS, were converted in KΩ cm^{-2}. Cells with a resistance lower than 3.95±0.27 KΩ cm^{-2}, were considered to be damaged 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 has a mean volume of 14.0 ml and was maintained at 32°C by means of circulation of termostated water in the jacket surrounding the cell. This temperature value has been chosen in order to reproduce the hand's physiological temperature at normal conditions. The receptor fluid consisted of phosphate buffered saline solution. The concentration of the salt in the receptor fluid is 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 cm^2 and the membranes thickness was 1 mm.

The two experiments were performed as follows.

Experiment 1

At time 0, the exposure chambers of Franz diffusion cells were each filled with 1 ml of a freshly made suspension of 2.5 g of Cr powder in 50 ml synthetic sweat at pH 4.5. Before application, the suspension was shaken with a stirring plate at room temperature for 30 minutes. The metal was studied at infinite dose (as Cr powder).

At selected intervals (1, 2, 4, 8, 16, 18, 20 and 24 hours) 1.5 ml of the dermal bathing solution was removed and analysed by means of ETAAS. Each receptor sample was immediately replaced with an equal volume of fresh physiological solution.

At the end of the experiments, the dermal bathing solution were removed and total chromium concentration was determined in each receiving chamber solution by means of ETAAS, while the donor solutions were collected and the skin was washed with 3 ml of fresh physiological solution for three times. The wash solutions were added to the donor solution for the analysis.

The experiments were performed with 6 cells at the 8 selected times for a total of 48 samples. Two additional cells were considered as blank. In the experiments we used skin from 2 different donors.

Experiment 2

At time 0, the exposure chambers of Franz diffusion cells were each filled with 1 ml of a freshly made suspension of 2.5 g of Cr powder in 50 ml synthetic sweat at pH 4.5. Before application, the suspension was shaken with a stirring plate at room temperature for 30 minutes. The metal was studied at infinite dose (as Cr powder). After 30 minutes, the donor solution was removed using a syring and three cotton balls. The skin pieces were next carefully cleaned with three cotton balls wetted with a cleanser containing sodium lauryl sulphate and sodium laureth sulphate (Ivory soap, Procter & Gamble, Cincinnati, OHIO). After the cleaning procedure the exposure chambers were filled with 1 ml of synthetic sweat at pH 4.5. At selected intervals (1, 2, 4, 8, 16, 18, 20 and 24 hours) 1.5 ml of the dermal bathing solution was removed and analysed. Each sample was immediately replaced with an equal volume of fresh physiological solution. At the end of the experiments, the dermal bathing solutions were removed and total chromium concentration was determined in each receiving chamber solution by means of ETAAS, while the donor solutions were collected and the skin was washed with 3 ml of fresh physiological solution for three times. The wash solutions were added to the donor solution for the analysis.

The experiments were performed with 4 cells at the 8 selected times for a total of 32 samples.

2.5. Skin digestion after the experiment

After the experiments, the skin was stored in freezer at –25°C. At the time of the analysis, the skin membranes were dried for 24 hours at room-temperature and then the exposed area was cut into sections and put into bakers with 25 ml of HNO_3 70% for digestion (amounts of skin were between 0.5 and 0.8 g). Afterward solutions were agitated for 20 hours at 100°C (after 2 hours they were added, drop by drop, of 5 ml of H_2O_2 till the remained solutions were of few millilitres. Then solutions were diluted to 25 ml with milliQ water for the analysis by ICP-AES.

2.6. Quantitative analysis

2.6.1. Donor and Receptor solutions

The donor solutions were collected at the end of the experiment, centrifuged for 15 minutes at 3000 rpm and the surnatant was filtrated two times through 0.45 μm GHP Acrodisc syringe filters (Pall Life Sciences, Ann Arbor, MI) and then analyzed.

The donor and the receptor solutions measurements were performed using electro-thermal

atomic absorption spectrometry (ETAAS) with Zeeman background correction. A Thermo M series AA spectrometer equipped with a GF95Z Zeeman Furnace and a FS95 Furnace Autosampler (Thermo Electron Corporation, Cambridge, UK) were used for analyses.

Cr detection limit at the analytical wavelength of 357.9 nm was 0.4 µg L^{-1}. Calibration curves were obtained using three different standards at 0.6, 3 and 6 µg L^{-1}. A 0.5% w/v solution of $Mg(NO_3)_2$ was used as modifying matrix for Cr. The samples were analyzed measuring against standard solutions for instrumental calibration. The precision of the measurements as repeatability (RSD%) for the analysis was always less than 10%.

2.6.2. Analysis of Cr(VI) in dissolution tests

The speciation analysis of Cr in the dissolution tests was performed by UV-spectrophotometry (Spectrophotometer Varian model DMS300) by means of diphenylcarbazide method: 5 ml of the sample were added of 1 ml of 1.2 M sulphuric acid and 50 µl of a solution obtained dissolving 0.25 g of 1,5-diphenylcarbazide into 50 ml of acetone, waiting for the development of the colour for 10 minutes and then measuring the absorbance at 540 nm. The determination was performed by calibration curve obtained with six standard solutions of Cr(VI) at known concentrations (0, 0.05, 0.25, 0.50, 1, 2 mg L^{-1}) obtained by dilution of a solution of $K_2Cr_2O_7$ at 1000 mg L^{-1} prepared dissolving 2.828 g of pure $K_2Cr_2O_7$ (oven-dried) in one liter of 1% v/v HNO_3 using a calibrated flask. The limit of detection (LOD) was 0.03 mg L^{-1} of Cr(VI).

2.6.3. Analysis of skin and of dissolution test solutions

Solutions obtained from the skin digestion procedure and from the dissolution tests were analysed by ICP-AES using a Spectroflame Modula E optical plasma interface (OPI) instrument (by SPECTRO, Germany) for determine the total Cr content using calibration curves (range: 0-10 mg L^{-1}) obtained by dilution of Spectrascan Chromium standard solution for ICP-AES analyses (by Teknolab A/S, Norway). The limit of detection (LOD) at the operative wavelength of 267.716 was 0.010 mg L^{-1}. The precision of the measurements as repeatability (RSD%) for the analysis was always less than 5%.

2.7. Data analysis

The concentration data (µg cm^{-3}) of metals in the receptor solution were converted to the total amount that penetrated (µg cm^{-2}), with a correction for dilution due to sample removal. To evaluate better the increase of Cr in donor solution the value of the cell at time 1 h was subtracted from the values obtained at the other times and then plotted against time. The slope

of this plot gives the "corrected" flux through skin (µg cm^{-2} h^{-1}) and the intercept of the curve in the linear steady-state region with the x-axis gives the lag time (h). Permeation coefficient was calculated from the linear steady-state region of the above plots by dividing the flux by the concentration used (Cr(III)).

Data analysis was performed using program Excel for Windows (release 2000) and Stata Corp. 2001 (release 7). Data were summarized as mean, as a measure of the central tendency and standard deviation and range, as a measure of dispersion.

All data were tested for normal distribution (Kolmogorov-Smirnov) and for homogeneity of variance (Levene-F test). The non-parametric statistical tests (Kruskal-Wallis H test and Mann-Whitney *U* test) were then applied. A *P* value <0.05 was considered significant.

3. RESULTS

The dissolution test revealed that Cr was present as ion (cromium III) in the filtered donor phase in measurable concentration that increased with the decreasing of pH (Table 1). We chose to perform the experiments at pH 4.5 in which the synthetic sweat is able to oxidise metallic chromium. Given that Cr(VI) concentration was under the limit of detection as evaluated in speciation analysis, chromium ions were presents in the trivalent form.

Table 1: Cr (mg ml^{-1}) in donor phase (ICP measurements on filtered donor phase suspensions) at 8 and 24 hours using a freshly made suspension of 2.5 g of Cr powder in 50 ml synthetic sweat at different pH.

pH	N	t = 8 h	t = 24 h
3,5	1	2.900	13.483
	2	3.181	12.423
	3	3.848	15.345
4.5	4	0.882	3.320
	5	1.879	3.572
	6	0.991	6.225
5.5	7	0.147	0.820
	8	0.224	0.918
	9	0.379	1.737
Blank 5.5	10	<0.01	<0.01
	11	<0.01	<0.01
	12	<0.01	<0.01

Figure 1 shows the permeation profile of Cr following the application of Cr powders in synthetic sweat as means and standard deviation plotted against time.

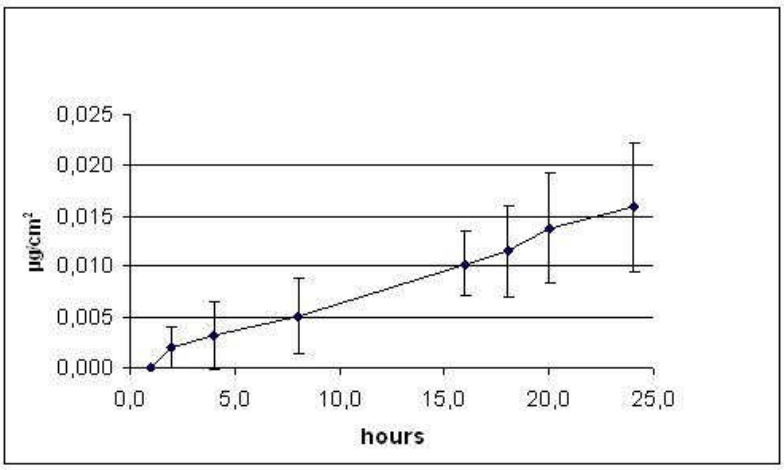

Figure 1. Permeation profile of Cr after skin application of 1 ml Cr powder suspension (2.5 g into 50 ml synthetic sweat at pH 4.5) using intact skin (means and standard deviation)

The Cr permeated the skin in little amount with a wide standard deviation. However, in experiments done without the use of the cleanser, we found a higher Cr concentration in the receiving cells, than in experiments performed using the cleaning procedure and in the blank cells in which Cr in receiving phase was below the LOD. In these latter experiments it was impossible to calculate a flux and a lag time, while in the first we calculated a flux permeation from the steady state region of 0.84±0.25 ng $cm^{-2}h^{-1}$ and a lag time of 1.1±0.7 hours (Table 2).

Table 2. Cr Flux and lag time (means and standard deviation).

Donor phase	**pH (simulated sweat)**	**Cr Flux**	**S.D.**	**LT (h)**	**S.D.**
Cr powder (1ml suspension 0.5% w/v)	4.5	0.84 (ng cm^{-2} h^{-1})	0.25	1.1	0.7
Cr powder (1ml suspension 0.5% w/v)*	6.5	nd	-	nd	-
$K_2Cr_2O_7$ * 1ml 4.5% m/v water solution	6.5	7.29 (µg cm^{-2} h^{-1})	0.25	12.5	0.9

** From Larese Filon et al. (2007) - nd: not detectable*

Considering a Cr concentration in donor phase of 0.085 ±0.033 mg L^{-1} a permeation coefficient of 0.0124 ± 0.0082 cm h^{-1} was calculated.

At the end of the experiments, we determined the total amount of Cr into the skin. The Cr skin content was higher in experiments done with the cleaning procedure and was lower when the skin was not treated with the cleanser (Mann-Whitney U test: P<0.03). Cr concentrations in receiving phase and in the skin are summarized in Table 3.

Table 3. Cr concentration in the skin and in the receiving phase at 24 hours (means and standard deviation).

	Experimental cells[1]	**Experimental cells[2]**	**Blank cells**
Cr into the skin (µg/cm^2)	3.19 (1.48)^	5.46 (1.09)*^	0.29 (0.08)
Cr in the receiving phases 24 h (µg/cm^2)	0.016 (0.005)	<LOD	<LOD

Experimental cells[1]: Cr powder exposure for 24 h. (1 ml of a suspension of 2.5 g of Cr powder in 50 ml synthetic sweat at pH 4.5) Experimental cells[2]: Cr powder exposure for 30 min (as above) and wash with cleanser. LOD: Limit of Detection. p<0.03 between Experimental cells 1 and 2. ^ p<0.002 between Experimental cells 1, 2 versus blank cells*

4. DISCUSSION

Diffusion through human skin was investigated for several metals, as chromium, due to the hazards which they present for human health in many aspects of daily life and in the workplace, but our knowledge of the process is still incomplete (Hostynek, 2003). For Cr we know that the skin absorption can be higher for hexavalent compounds (Van Lierde et al., 2006; Larese Filon et al., 2007) while trivalent chromium can pass through the skin less easily (Gammelgaard et al., 1992; Larese Filon et al., 2007). In our previous experiments we showed the high permeation profile of potassium dichromate solution while Cr powder in synthetic sweat at pH 6.5 failed to pass through the skin (Larese Filon et al., 2007). Recently, new experiments were carried out using synthetic sweat at a lower pH (pH=4.5) in order to oxidize metallic Cr to soluble Cr (III) ions. So the permeation of metallic Cr could take place if there has been previous oxidation of Cr thanks to the sweating skin action. The choice of pH 4.5 in our synthetic sweat was somewhat unusual in that many researchers *in vitro* studies choose a

pH of 6.5. However, the actual pH of human skin seems to be much more acidic, typically in the range of 4 to 5.5, and in some cases, for example during individuals' physical activity, much below this (Dyer et al., 1998). Thus, the choice of a synthetic sweat at pH 4.5 used in the present study was considered appropriate to simulate workplace conditions. The lower pH permitted us to obtain Cr(III) ions that show a low permeation profile through the skin. No significant passage of Cr into the receptor phase was found in experiments performed with cleaning procedure and in the blank cells, only in experiments done without the use of the cleanser there was a progressive increase of Cr in the receiving solution and a good reproducibility in the permeation profile of the different cells: this permits the permeation flux and lag time calculation.

These results show that Cr presents the lower permeation profile compared with Co and Ni (Larese Filon et al., 2007). This is probably due to greater rejection of the positively charged Cr(III) ions by the skin barrier, as suggested by Gammelgaard et al. (1992).

Our experiments demonstrated that the amount of chromium found in the skin layers where Cr powder was applied for 24 hours, and not washed with the cleanser, was lower than in skin exposed for only 30 minutes to Cr powder and then decontaminated. This would suggest that cleaning procedure after 30 minutes was not enough to reduce the apparently rapid initial absorption, that can occur during the first few minutes, and the ready binding of Cr to skin proteins.

In 30 minutes a sufficient amount of Cr has already passed into the stratum corneum or the decontamination with the cleanser was not complete and allowed penetration into the skin to continue. In this last case the cleaning procedure would have left a small amount of Cr on the surface of the skin or the stratum corneum and the following addition of synthetic sweat would have be resulted in oxidation of this small fraction to Cr(III), which penetrated into the skin. However, in our study the stratum corneum was not separated from the viable epidermis and dermis for the evaluation of the Cr skin content and, therefore, it is possible that the cleaning procedure could increase Cr concentration in the stratum corneum without increasing the penetration of Cr in the viable epidermis and dermis. The ultimate fate of chromium in the stratum corneum could be the penetration into the skin, the absorption into the systemic circulation or the loss by desquamation. In this study, when no skin cleanser was used the process of Cr penetration through the skin continued, and an increasing amount of Cr was free to permeate further through the skin to the receiving phase, while the washing with a common cleanser containing SLS let Cr skin concentration increase, but decreased permeation into the receiving phase. This could be due to several factors. First, as SLS is an amphophilic

molecule and forms aggregates known as anionic micelles that do not diffuse through the skin membranes, it is possible that Cr is retained in the skin in association with SLS micelles. Second, SLS, which is an alkaline detergent, could have led to a change of the pH of the stratum corneum and in that way would have influenced the permeation of Cr through the skin. Samitz et al. (1969) found a pH dependent diffusion of Cr(III), with the largest diffusion at pH 7 and decreased diffusion at pH 5 and 9. At last, SLS could damage the barrier properties of the skin and increase binding of Cr to skin proteins by direct action on the skin. The detergent properties of SLS could have been responsible for extracting some components of the stratum corneum, most likely proteins of the corneocyte envelope, disorganizing the lipidic matrix that cements them, opening routes of penetration within the stratum corneum and thus increasing the amount of Cr bound to skin proteins, due to the affinity of Cr for proteins. It has been reported that sodium lauryl sulphate can increase nickel penetration by reducing the barrier integrity of the skin (Frankield et al., 1995) and, in our previous work, we demonstrated that lead skin penetration increased significantly when a cleanser, containing sodium lauryl sulphate, was tested (Larese Filon et al., 2006).

Moreover, it is well known that the electrophilic nature of many metals, for example chromium (III), determines their protein reactivity, which can result in depot formation in the stratum corneum (Samitz and Katz, 1976). Such protein-metal binding can take place in all strata of the skin to the extent of building up a secondary barrier and inhibiting further diffusion (Hostynek, 2003).

In vitro experiments demonstrated that Cr(III) ions show a strong affinity for epithelial and dermal tissues, forming stable complexes which slow the rate of diffusion (Samitz et al., 1969). It has also been speculated that Cr(III) is the actual hapten and that Cr(VI), after entering the skin cells, is reduced to Cr(III) which in its turn binds onto intracellular proteins, thereby forming immunogenic complexes (Van Lierde et al., 2006).

In conclusion, although Cr metal presents a low risk of absorption, these data suggest that is necessary to prevent skin contamination and to choose carefully the decontamination measures that are provided to workers. This may be important for chromium sensitive workers exposed to detergents in daily-cleaning situations.

However, it is an *in vitro* skin model with its inherent limitations. On the one hand, several assumptions and conditions used in this *in vitro* protocol that might contribute to an upward bias in percutaneous penetration, might include a prolonged hydration of the stratum corneum with sweat and the Cr amount applied. On the other hand, workers are likely to wash more than once per day and often have mechanically damaged which the evidence suggests

would make penetration worse. Our risk analysis did not include these latter factors that would therefore lead to an underestimate of risk (Larese Filon et al., 2006).

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ALLEGATO II

IN VITRO ABSORPTION OF METAL POWDERS THROUGH INTACT AND DAMAGED HUMAN SKIN

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ABSTRACT

The bioavailability of metals, which are known as important contact allergens, is decisive for the development and the maintenance of contact dermatitis. The aim of this study was to evaluate the percutaneous penetration of metal powders of cobalt (Co), nickel (Ni) and chromium (Cr) and the effect of skin lesions on skin absorption. *In vitro* permeation experiments were performed using the Franz diffusion cells with intact and damaged human skin. Physiological solution was used as receiving phase and metal powders (Co, Ni and Cr) dispersed in synthetic sweat at pH 4.5 were applied as donor phase to the outer surface of the skin for 24 hours. The amount of each metal permeating the skin was analysed by Electro Thermal Atomic Absorption Spectroscopy (ETAAS). Donor solution analysis demonstrated that metals were present as ions. Measurements of metals skin content were also exploited. Median Co and Ni concentrations found in the receiving phase were significantly higher when Co and Ni powders were applied on the abraded skin than after application on the intact skin (3566 ng cm^{-2} and 2631 ng cm^{-2} vs 8.4 ng $cm^{-2}$and 31,0 ng cm^{-2} respectively). No significant difference was found in Cr permeation through intact and damaged skin. The measurement of metals skin content showed that Co, Ni and Cr concentrations were significantly higher in the damaged skin than in the intact skin. Co and Ni ions concentrations increased significantly when the donor solutions were applied on the damaged skin, while Cr ions concentrations did not increase. This study demonstrated that Co and Ni powders can permeate through damaged skin more easily than Cr powder, which has probably a stronger skin proteins binding capacity. Therefore, our results suggest that is necessary to prevent skin contamination when using toxic substances because a small injury to the skin barrier can significantly increase skin absorption.

KEYWORDS: Metal powders; In vitro; Percutaneous absorption; Damaged human skin; Nickel; Chromium; Cobalt

RIASSUNTO

La biodisponibilità dei metalli, riconosciuti come importanti allergeni da contatto, è decisiva per lo sviluppo e il mantenimento delle dermatiti da contatto. Lo scopo di questo studio era la valutazione della permeazione cutanea di polveri metalliche di cobalto (Co), nichel (Ni) e cromo (Cr) e l'effetto delle lesioni della pelle sull'assorbibimento cutaneo. Gli esperimenti di permeazione *in vitro* sono stati condotti usando le celle di diffusione di Franz con cute umana intera, integra e lesa. Come soluzione ricevente è stata usata soluzione fisiologica e come fasi donatrici sono state applicate alla superficie esterna della cute delle polveri di metalli (Co, Ni e Cr) disperse in sudore sintetico a pH 4,5 per 24 ore. Le quantità di ogni metallo che hanno permeato la pelle sono state analizzate tramite Spettroscopia di Assorbimento Atomico Elettro-Termica (ETAAS).

Le analisi delle soluzioni donatrici hanno dimostrato che i metalli erano presenti come ioni. Inoltre è stato analizzato, dopo mineralizzazione, il contenuto di metalli anche all'interno della cute. Le concentrazioni mediane di Co e Ni trovate nelle fasi riceventi erano significativamente più alte quando le polveri di Co e Ni sono state applicate nella cute abrasa rispetto alla cute integra (rispettivamente 3566 ng cm^{-2} e 2631 ng cm^{-2} vs 8.4 ng cm^{-2} e 31 ng cm^{-2}). Nessuna differenza significativa tra cute integra e cute lesa, invece, è stata trovata nella permeazione del Cr. Le analisi dei metalli rimasti nella pelle ha evidenziato che le concentrazioni di Co, Ni e Cr erano significativamente più alte nella cute danneggiata rispetto alla cute integra.

Infine, la concentrazione di ioni di Co e Ni aumentavano significativamente quando le fasi donatrici erano applicate alla cute danneggiata rispetto alla cute integra, mentre non vi erano differenze per quanto riguarda la ionizzazione del Cr.

Questo studio ha mostrato che le polveri di Co e di Ni possono permeare la pelle più facilmente rispetto alla polvere di Cr, il quale probabilmente viene legato in maniera più forte dalle proteine della cute. Quindi, i nostri risultati suggeriscono che è necessario prevenire la contaminazione della cute quando si usano sostanze tossiche poiché anche piccole lesioni della pelle possono aumentare significativamente l'assorbimento cutaneo.

PAROLE CHIAVE: Polveri metalliche; In vitro; Assorbimento cutaneo; Cute umana lesa; Nichel; Cromo; Cobalto

1. INTRODUCTION

In industrialized countries, skin is at high risk of exposure to chemicals and other contaminants which can be found in the environment or at a workplace. Allergic contact dermatitis affects approximately 1-10% of the general population (Guin, 1995). Nickel (Ni), cobalt (Co) and chromium (Cr) are recognized as the most common cause of contact allergy affecting 10-15% of women and a few % of men in the general population. Nickel allergy, in particular, is the most important background factor for hand eczema, which occurs in 10% of adult people (Meding and Swanbeck, 1987). A wide variety of metal objects that come into repetitive contact with the skin can release metallic ions that can diffuse through the skin and cause allergy. A limited number of experimental studies suggest that, under physiologically relevant conditions, metals may ionize and so permeate the skin. Liden and Carter (2001) found that euro coins in contact with synthetic sweat release nickel ions. Larese Filon et al. (2004, 2007), using *in vitro* Franz cell system, demonstrated that cobalt and nickel powders, when stirred in synthetic sweat, can release metallic ions that can pass through the skin.

Skin absorption of Co has been found in exposed workers and in laboratory experiments with volunteers, but there were few *in vitro* experimental data on skin absorption of this metal (Suzuki-Yasumoto and Inaba, 1976; Carson et al., 1986; Kogan and Petukhova, 1986; Scansetti et al., 1994; Linnainmaa and Kiilunen, 1997; Larese Filon et al., 2004). It is known that Co is a common sensitizer and its presence in metal objects can increase the risk of allergic contact dermatitis in sensitised people. In this latter there is a local action on the skin, but the findings by Scansetti et al. (1994) and Linnainmaa and Kiilunen (1997) suggest that this metal can permeate the skin. Studying hard metal industry workers, no correlation between Co in ambient air and Co urine concentrations was found by Scansetti et al. (1994). This result was attributed to skin contact as proved by an experiment involving voluntary skin exposure to freshly mixed or waste powder containing 5-15% of Co that identified a ten fold increase of urinary cobalt in the post exposure samples. This finding is consistent with the experiment performed by Linnainmaa and Kiilunen (1997) who observed an increase of urinary Co 4 to 6 hours following skin exposure to Co powder in 5 volunteers.

More extensive studies of nickel skin absorption have been undertaken. It was found that *in vitro* water solution of nickel salts can pass through the stratum corneum (Tanojo et al., 2001), and *in vivo* nickel powder can penetrate in depth profiles of the stratum corneum after occlusion (Hostynek, 2001). These results are consistent with earlier experiments with full

thickness human skin *in vitro*, suggesting that *in vivo* nickel ions may permeate (Tanojo et al., 2001). There are, however, a shortage of experimental data using nickel powder (Larese Filon et al., 2007).

Penetration of Cr through skin has been investigated in animals as well as in human skin biopsy specimens (Liden and Lundberg, 1979) for evaluating the "disappearance measurement" of isotopes of Cr in salt formulations (Wahlberg 1965a, 1965b, 1968). Moreover, Corbett et al. (1997) observed a systemic uptake of chromium in men following dermal contact with hexavalent chromium salts. While the absorption of chromium salts *in vitro* experiments has been widely demonstrated (Gammlgaard et al., 1992; Van Lierde et al., 2006; Larese Filon et al., 2007), little is known about Cr ions release from metal Cr and skin absorption of chromium in metal form. Our recent study performed with full thickness human skin in diffusion cells, showed that Cr powder in synthetic sweat at a low pH (pH 4.5) is able to permeate the skin (D'Agostin et al., 2007).

In order to better understand bioavailability of metals involved in skin sensitisation (Ni, Cr, Co), in this study we evaluated their skin permeation in an *in vitro* diffusion cell system.

In vitro methods are particularly useful for measuring dermal absorption of chemicals in industrial settings (e.g. cosmetic, detergent and agrochemical). Therefore, in some countries they are included among the criteria for assignment of a skin notation to chemicals (Drexler, 1998). The technical standardisation procedure has been reached on, although there is still some conflict over which guidelines to adopt (e.g., Organisation for Economic Co-operation and Development, 2000 *vs* ACGIH, American Conference of Governmental Industrial Hygienists, 1995). On the contrary, *in vivo* animal studies pose a lot of problems when extrapolating the results to humans (Poet, 2000), and theoretical models (Fiserova-Bergerova et al., 1990) tend to overestimate potential dermal exposure in occupational workplaces.

In this study, experiments were performed using intact skin as well as damaged skin to estimate the effect of skin lesions on skin absorption. Metallic powders were tested in the same way with the purpose of comparing the different ability of the three metals to permeate through human skin. To carry out the experiments, we used the experience and the protocols employed during the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals), a three-year research program (2001-2004) funded by European Union (EDETOX, 2000).

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals were analytical graded: urea, sodium chloride, magnesium nitrate, sodium hydrogenphosphate, potassium dihydrogenphosphate, diphenylcarbazide, hydrogen peroxide (30% v/v) were purchased from Carlo Erba (Milan, Italy); ammonium hydroxide (25% w/v) was bought from J. T. Baker (Deventer, Holland); lactic acid (90% v/v) from Acros Organics (Geel, Belgium); acetone, nitric acid (69.5% v/v) and sulfuric acid (95% v/v) from Sigma Aldrich (Milan, Italy).

Water reagent grade was produced with a Millipore purification pack system (milliQ water).

Ni powder came from Alfa Aesar (Karlsruhe, Germany) and consisted of Nickel powder (CAS 7440-02-0), APS (Average Particle Size) 2.2-3.0 µm, 99.9% (metals basis), C typically <0.1%.

Cr powder came from Alfa Aesar (Karlsruhe, Germany) and consisted of chromium powder (CAS 7440-47-3), APS <10 micron, 99.8% (metals basis).

Co powder came from UMICORE, Belgium and consisted of 85% particles of diameter ≤2 µm (max 5 µm) as reported in Larese et al. (2004).

The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na_2HPO_4, 0.19 g of KH_2PO_4 and 9 g of NaCl into 1 liter of milliQ water (final pH = 7.35). The synthetic sweat solution used as the donor fluid consisted in 0.5% w/v sodium chloride, 0.1% w/v urea and 0.1% w/v lactic acid in milliQ water; pH was adjusted with ammonia to pH 4.5.

2.2. Preparation of skin membranes

Human abdominal full thickness skin was obtained as surgical waste. Prior to freezing, subcutaneous fat was removed and hair shaved. All the pieces of full thickness skin were stored in freezer at -25°C for a period up to, but not exceeding, 4 months. It has been shown that this method of storage does not damage the skin since no difference in permeability was observed between fresh and frozen segments of the same skin in a separate series of experiments (Franz, 1975). Skin integrity was tested before and after each experiment using electrical conductibility by means of a conductometer (Metrohm, 660 Conductometer, AG Oberdorfstr. 68 CH-9100 Herisau) operating at 300 Hz connected to two stainless steel electrodes (Fasano et al., 2002). The electrical conductance data, obtained in µS, were

converted in KΩ cm^{-2}. Cells with a resistance lower than 3.95±0.27 KΩ cm^{-2}, were considered to be damaged as suggested by Davies et al. (2004).

Donors were men and women with a range of age from 45 to 71 years.

2.3. *In vitro* diffusion system

Percutaneous absorption studies were performed using static diffusion cells following the Franz Method (Franz, 1975). The receptor compartment has a mean volume of 14.0 ml and was maintained at 32°C by means of circulation of termostated water in the jacket surrounding the cell. This temperature value has been chosen in order to reproduce the hand's physiological temperature at normal conditions. The receptor fluid consisted of phosphate buffered saline solution. The concentration of the salt in the receptor fluid is approximately the same that can be found in the blood. The receptor 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 cm^2 and the membranes thickness was < 1 mm.

The experiments were performed as follows.

At time 0, the exposure chambers of Franz diffusion cells were each filled with 1.5 ml of a freshly made suspension of 2.5 g of nickel powder in 50 ml synthetic sweat at pH 4.5. Before application, the suspension was shaken with a stirring plate at room temperature for 30 min. The metal was studied at infinite dose (as metal powder).

The experiments were performed with six cells with intact skin and six cells with damaged skin. For the damaged skin we used an abraded skin protocol as suggested by Bronaugh and Steward (1985) modify by Larese (2006): skin was abraded by drawing the point of a 19-gauge hypodermic needle across the surface (20 marks in one direction and 20 perpendicular). Two additional cells were considered as blank. The blank cells were treated as the other cells with the exception that no metal powder was introduced to the exposure chamber.

At 24 h the dermal bathing solution was removed and nickel concentration was determined in each receiving chamber solution by means of ETAAS, while the donor solution were collected and the skin washed with 3 ml of fresh physiological solution for three times. The wash solutions were added to the donor solution for the analysis.

In each experiment, we used skin from two different donors.

The experiments were replicated with the same conditions using cobalt powder and chromium powder.

2.4. Skin digestion after the experiment

After the experiments, the skin was stored in freezer at –25°C. At the time of the analysis, the skin membranes were dried for 24 hours at room-temperature and then the exposed area was cut into sections, weighted and put into bakers with 10 ml of HNO_3 70% v/v for digestion (amounts of skin were between 0.5 and 0.8 g). Afterward, solutions were agitated for 10 hours at 100°C (after 2 hours they were added, drop by drop, of 2 ml of H_2O_2), till remained solutions were of few millilitres. Then solutions were diluted at 10 ml with milliQ water for the analysis with ETAAS.

2.5. Quantitative analysis

The metal ions content measurements were performed using Electro-Thermal Atomic Absorption Spectrometry (ETAAS) with Zeeman background correction. A Thermo M series AA spectrometer equipped with a GF95Z Zeeman Furnace and a FS95 Furnace Autosampler (Thermo Electron Corporation, Cambridge, UK) were used for analyses.

Co detection limit at the analytical wavelength of 240.7 nm was 0.4 µg L^{-1}.

Cr detection limit at the analytical wavelength of 357.9 nm was 0.2 µg L^{-1}.

Ni detection limit at the analytical wavelength of 232.0 nm was 0.2 µg L^{-1}.

We used a 0.5% w/v solution of $Mg(NO_3)_2$ as modifying matrix for Co and Cr. The samples were analysed measuring against standard solutions for instrumental calibration. The precision of the measurements as repeatability (RSD%) for the analysis was always less than 10%.

Calibration curves for the receptor fluid of the experiments performed with intact skin were obtained using three different standards at 1, 5 and 10 µg L^{-1}, while calibration curves for the receptor fluid of the experiments performed with damaged skin, for the donor solutions and for the solutions obtained from the skin digestion were obtained using three different standards at 5, 25 and 50 µg L^{-1}. The too much concentrated solutions were diluted before the analyses. Donor solutions were centrifuged for 15 minutes at 3000 rpm and the surnatant was filtered two times through 0.45 µm GHP Acrodisc syringe filters (Pall Life Sciences, Ann Arbor, MI) and then analysed.

2.6. Data analysis

The concentration data (µg cm^{-3}) of metals in the receptor solution were converted to the total amount that penetrated (µg cm^{-2}).

Data analysis was performed using the statistical package Stata software (v. 10.0).

Data were summarized using the median as a measure of the central tendency and the 25-75th percentiles, and the minimum and maximum as measures of dispersion. The Kruskal-Wallis one-way analysis of variance was used to compare metal concentrations under different experimental conditions. A *P* value of <0.05 was chosen as the limit of statistical significance.

3. RESULTS

Table 1 reports the Co, Ni and Cr amounts in the receiving cells expressed in ng cm^{-2}. In the experimental cells, Co and Ni concentrations in the receiving phase were significantly higher when Co and Ni powders were applied to the damaged skin than to the intact skin (*P*<0.02). No significant difference was found for Cr permeation through intact and damaged skin. Co and Ni penetration through damaged skin was much higher than through intact skin (3566 and 2631 ng cm^{-2} *vs* 38.4 and 31 ng cm^{-2}, respectively).

Table 1. Summary statistics for the amounts of Co, Ni and Cr (ng cm^{-2}) in the receiving cells.

Experiment	Median	25th percentile	75th percentile	Minimum	Maximum
Cobalt					
Blank cells	1.5	1.0	2.0	1.0	2.0
Intact skin	8.4	4.9	13.4	0.9	30.6
Damaged skin	3566[a]	2296	4977	100	6288
Nickel					
Blank cells	7.2	4.8	9.6	4.8	9.5
Intact skin	31.0	19.4	41.9	16.1	54.6
Damaged skin	2631[b]	1369	4961	1110	10180
Chromium					
Blank cells	4.7	4.6	4.9	4.6	4.9
Intact skin	5.0	4.5	5.5	3.5	5.5
Damaged skin	4.6	4.3	9.4	4.2	11.2

Kruskal-Wallis test: [a]P=*0.006;* [b]P=*0.004*

Table 2 reports Co, Ni and Cr concentrations in the skin. The amounts of Co, Ni and Cr found in the damaged skin after removing the skin from the test cells were significantly higher compared with Co, Ni and Cr concentrations remained in the intact skin (48.7, 131 and

62.1 µg cm^{-2} *vs* 29.6, 82.3 and 14.4 µg cm^{-2}, respectively; $P<0.03$).

Table 2. Summary statistics for the concentrations of Co, Ni and Cr (µg cm^{-2}) in the skin.

Experiment	Median	25th percentile	75th percentile	Minimum	Maximum
Cobalt					
Blank cells	0.13	0.11	0.16	0.11	0.16
Intact skin	29.6	25.2	41.4	22.4	43.9
Damaged skin	48.7[a]	41.7	89.2	40.1	209
Nickel					
Blank cells	0.14	0.08	0.20	0.08	0.20
Intact skin	82.3	42.7	125	38.8	161
Damaged skin	131[b]	118	364	110	973
Chromium					
Blank cells	0.16	0.14	0.18	0.14	0.18
Intact skin	14.4	11.3	21.0	11.2	60.6
Damaged skin	62.1[c]	50.9	103	42.9	143

Kruskal-Wallis test: [a]P=*0.014;* [b]P=*0.023;* [c]P=*0.011*

Atomic absorption spectroscopy analysis revealed that Co, Ni and Cr were present as ions in the donor phase (Table 3). The Co and Ni ions concentrations found in 1.5 ml of synthetic sweat solution were significantly higher in experimental cells with damaged skin than in those with intact skin (55.6 and 129 mg L^{-1} *vs* 21.4 and 57.5 mg L^{-1}, respectively), while Cr ions concentrations were similar in the experimental cells with either intact or damaged skin.

To better evaluate the role of metal ions in skin permeation we calculate the percentage of metal ion dose in receiving phase and into the skin (table 4): Co and Ni in receiving phase represent respectively the 0.024% and the 0.03% of ion applied for intact skin and the 3.6% and 1.27% for damaged skin. The skins contained a percentage ranging between 64.6% to 75.7% with no differences between intact and damaged skin for Co an Ni. The evaluation of metal ion percentage for Cr confirmed the very low presence of the metal in receiving phases while the skins contained the 99% per the Cr ions.

Table 3. Summary statistics for the concentrations of Co, Ni and Cr as ions (mg L^{-1}) in the donor phases.

Experiment	Median	25thpercentile	75thpercentile	Minimum	Maximum
Cobalt					
Blank cells	0.004	0.004	0.004	<LOD	0.004
Intact skin	21.4	17.5	27.2	17.4	30.6
Damaged skin	55.6[a]	47.1	83.8	44.7	86.6
Nickel					
Blank cells	0.004	0.004	0.004	<LOD	0.004
Intact skin	57.5	26.5	75.3	16.7	117
Damaged skin	129[b]	110	150	65.3	160
Chromium					
Blank cells	0.008	0.007	0.009	0.007	0.009
Intact skin	0.28	0.26	0.31	0.19	0.36
Damaged skin	0.18	0.085	0.32	0.07	0.41

Kruskal-Wallis test: [a]P=0.004; [b]P=0.011 - LOD=0.001 mg L^{-1}.

Table 4. Percentage of ion dose in different phases in relation to total ion amount.

Experiment	Receiving phases Median % (range)	Skins Median % (range)
Cobalt		
Intact skin	0.024 (0.009-0.089)	75.6 (72.0-78.4)
Damaged skin	3.6 (0.9-7.2) [a]	64.6 (46.0-90.0)
Nickel		
Intact skin	0.03 (0.01-0.08)	74.2 (53.1-91.4)
Damaged skin	1.27 (0.62-2.44) [b]	75.7 (65.0-94.0)
Chromium		
Intact skin	0.03 (0.009-0.004)	99.1 (98.6-99.9)
Damaged skin	0.01 (0.004-0.02)	99.8 (98.6-99.9)

Kruskal-Wallis test: [a]P=0.006; [b]P=0.004.

4. DISCUSSION

Even though it is known that polar organic compounds and some metals can appreciably penetrate damaged skin (Ilyin et al., 1975; Larese Filon et al., 2006), there is limited knowledge about the behaviour of Co, Ni and Cr metal powders. This study was focused on the percutaneous absorption of these metal powders through full thickness human skin (epidermis and dermis) with intact as well as abraded skin barrier in an *in vitro* diffusion cell system. *In vitro* percutaneous studies can be used to define the diffusion characteristics of xenobiotics as one way of documenting their bioavailability.

Skin absorption of metals is closely related to the capacity of the sweat to oxidise metals: our previous *in vitro* permeation experiments showed that Co powder can permeate the skin when applied using a dispersion in synthetic sweat that oxidises metallic cobalt into ions, while no absorption occurred using a dispersion in water (Larese Filon et al., 2004).

The metal oxidation has been shown in experiments treating metal objects with synthetic sweat. This method was used to determine nickel release in objects such as coins (Liden and Carter, 2001), suggesting a route of skin permeation which was until recently unknown. In our study, experiments were carried out using synthetic sweat at a low pH (pH 4.5) with the aim to oxidize metallic powders to soluble ions, so that the permeation of metallic powders could take place if there was previous oxidation of metals by means of the sweating skin action. This hypothesis was confirmed because the experiments performed by polarographic analysis revealed that metals were present as ions in the donor phase. (Larese 2004, 2007) The choice of pH 4.5 in our synthetic sweat was somewhat unusual that many researchers choose a pH of 6.5 for their simulate sweat (EEC 1998). However, the actual pH of human skin seems to be much more acidic, typically in the range of 4 to 5.5 and in some cases, for example during individuals' physical activity, much lower (Dyer et al., 1998). This is an important feature, because most chemical elements, such as chromium, become appreciably more ionized as acidity increases, approximately 10 to 100 fold per each one pH unit decrease (Zlotogorski, 1987). Thus, the choice of a simulate sweat at pH 4.5, as used in the present study, was considered appropriate to reproduce workplace conditions.

Our experiments demonstrated that metal powders are able to permeate through intact human skin in variable amounts: higher for Co and Ni, lower for Cr. However, the values obtained with intact human skin can be considered low compared to other substances which diffuse through the skin (Larese Filon et al., 1999). When the abraded skin procedure was used (Larese Filon et al., 2006), Co and Ni permeation increased significantly. Substantially

increased penetration of other metals through damaged skin has been previously reported (Ilyin et al., 1975; Larese Filon et al., 2006). In opposite, there was no significant difference in the permeation through intact and damaged skin barrier for Cr. Moreover, the amount of Cr found in the receiving phase, after exposure of the abraded skin to Cr powder for 24 hours, was considerably lower than that detected after exposure to Co and Ni powders. These findings could be explained by the different concentrations of Co, Ni and Cr ions found in the donor phases applied on intact and damaged skin. Polarographic analysis revealed that Co and Ni ions were present in the donor solutions in higher concentrations than Cr ions (Larese, 2007). The Co and Ni ion concentrations increased significantly when the donor phase was applied on the damaged skin: this finding might be explained by the increasing surface in contact with the metal and the action of some skin protein released with the damaging procedure. In our knowledge there aren't other investigation on that topic to substantiate our results.

In contrast with Co and Ni experiments, no increase in Cr ions concentration was found when Cr powder was used. This could mean that the synthetic sweat used in this study was able to oxidise only a small fraction of metallic Cr to soluble Cr ions.

A further interesting finding of this experimental investigation was the metal skin content: the amounts of Co, Ni and Cr found in the damaged skin were significantly higher than in the intact skin. Ni concentrations in intact and damaged skin were considerably higher than Co and Cr concentrations. However, the high Cr concentration which was found in the damaged skin suggests that binding of Cr in the skin interferes with diffusion of Cr through the skin for the stronger binding of Cr to skin proteins. This is confirmed by the calculation of the percentage of Cr ions into the skin that reached the 99% of the total ion amount.

It is well known that the electrophilic nature of many metals, such as chromium and nickel, determines their protein reactivity, which can result in depot formation in the stratum corneum (Samitz and Katz, 1976). Such protein-metal binding can take place in all strata of the skin to the extent of building up a secondary barrier and inhibiting further diffusion (Hostynek, 2003). The results of our study are broadly consistent with those reported in other *in vitro* experimental investigations which suggested that Cr (III) ions show a strong affinity for epithelial and dermal tissues, forming stable complexes which slow the rate of diffusion (Samitz et al. 1969).

In conclusion, the finding of the present confirmed that an injury to the skin barrier could significantly enhance Co and Ni permeation. In contrast, Cr permeation is lower both in intact than in damaged skin for the low ionisation of this metal and for his high protein

binding property. More that the 99% of the total Cr ions was into the skin: this might explain the extremely low flux of Cr through damaged skin.

In the experiments carried out with intact skin the amounts of Co, Ni and Cr found in the receiving phase were lower than those detected in our previous studies. It should be noted, however, that our previous permeation experiments were performed using a different pH for synthetic sweat and different procedures were use to remove and analyse the dermal bathing solution at selected times during a 24 hours period (Larese Filon et al., 2007; D'Agostin et al., 2007).

How accurately these *in vitro* results relate to *in vivo* workers' skin is not known. A case of intoxication after a chemical burn with increased Co in serum has been reported by Neligan (1966). In that case the cutaneous barrier was damaged and the absorption of Co was substantial. We recognise that the present *in vitro* study suggests only a simple way to simulate what occurs during real-life situations. In occupational settings workers may have their skin mechanically damaged because of cuts and abrasions which would make metal penetration substantially greater than that shown in our investigation. Moreover, it is well known that nickel allergic hypersensitivity develops more readily on exposure of damaged skin than from exposure on intact skin. The findings of our study underline the need to prevent contact with potential toxic substances and suggest that it is advisable for workers to use protective clothing, such as gloves and disposable overalls, since the granulometry of metal particles found in the work environment is similar to that used in our experiments.

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ALLEGATO III

NANOPARTICLE DERMAL ABSORPTION AND TOXICITY: A REVIEW OF THE LITERATURE

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ABSTRACT

Nanotechnologies are among the fastest growing areas of scientific research and have important applications in a wide variety of fields. The data suggest that in the future workers and consumers exposed to nanoparticles will significantly increase .

At now there are gaps in understanding about the human and environmental risk that manufactured nanoparticles pose for occupational exposed people and for consumers. There is the need for assessing the health and environmental impacts, the nanoparticles life cycle, the human exposure routes, the behaviour of nanoparticles in the body, and the risk for workers.

Possible routes of entry into the body include inhalation, absorption through the skin or digestive tract, injection, and absorption or implantation for drugs delivery systems. In particular, dermal absorption and skin penetration of nanoparticles needs a better evaluation because few and contradictory data are present in the literature, mainly on titanium dioxide. There are limited data on carbon-based nanoparticles and very few data on other metal nanoparticles increasingly used in industry. The paper reviews the literature on the percutaneous absorption of nanoparticles and their effect on skin.

KEYWORDS: Skin absorption; Nanoparticles; Review

RIASSUNTO

Le nanotecnologie sono tra le aree di ricerca in più veloce crescita con importanti applicazioni in un'ampia varietà di campi. I dati di letteratura suggeriscono che in futuro i lavoratori e i consumatori esposti alle nanoparticelle aumenteranno significativamente.

Attualmente c'è una lacuna nella comprensione dei rischi per l'uomo e per l'ambiente che presentano le nanoparticelle artificiali, sia in campo occupazionale che commerciale. C'è la necessità di valutare il loro impatto sulla salute, il loro ciclo vitale, le vie di esposizione umana, il comportamento delle nanoparticelle nel corpo e il rischio per i lavoratori.

Possibili vie d'ingresso nel corpo includono l'inalazione, l'assorbimento attraverso la cute o il tratto intestinale, l'iniezione, l'assorbimento o l'impianto di sistemi di rilascio di farmaci. In particolare, l'assorbimento cutaneo e la penetrazione della cute da parte delle nanoparticelle necessita di una miglior valutazione, dato che i dati di letteratura sono pochi e contraddittori, principalmente sull'biossido di titanio. Ci sono dati limitati su nanotubi e fullereni di carbonio, ma anche sulle nanoparticelle metalliche, il cui uso sta aumentando in campo industriale.

L'articolo revisiona la letteratura sull'assorbimento cutaneo delle nanoparticelle e il loro effetto sulla pelle.

PAROLE CHIAVE: Assorbimento cutaneo; Nanoparticelle; Review

1. INTRODUCTION

Nanomaterials are defined as materials that have at least one dimension <100 nanometers (1 nm = 10^{-9} m) and they can be divided into two large groups: ultrafine nanosized particles not intentionally produced and engineered nanoparticles produced in a controlled, engineered way (Oberdörster et al., 2005a).

Nanotechnologies are among the fastest growing areas of scientific research and have important applications in a wide variety of fields. The corresponding industries would require about two million workers in nanotechnology, and about three times as many jobs in supporting activities (Roco, 2005). Nanoscale materials are already being introduced for use in many commercially available products like cosmetics and sunscreens, pharmaceuticals, stain resistant clothing, sports equipment, automobile catalytic converters, dental bonding, cleanings products, dressings for specific wound care strategies, but many are the fields of possible future applications of nanotechnologies as drug delivery systems, nanomedicine, environmental remediation and cell imaging.

Engineered nanoparticles, because of their big surface-to-volume ratios, exhibit chemical, physical, and biological properties distinctly different from the same materials in the bulk form, but such properties may lead to adverse effects on human health and environmental systems.

Every year new products containing nanomaterials enter in the market (Woodrow Wilson International Center for Scholars, 2007) and in the next future more workers and costumers will come in contact with nanoproducts. Since the toxicological and environmental effects of these compounds are not fully known, there is the need to understand better the health and environmental impacts, the nanoparticles life cycle, the human exposure routes, the behaviour of nanoparticles into the body, and the risk for workers in order to use these new materials in a safe way (Dreher KL, 2004; EPA, 2007; Gwinn and Vallyathan, 2006; Hoet et al., 2004; Nasterlack et al., 2008; Nel et al., 2006; NIOSH, 2007; Oberdörster et al., 2005a; Oberdörster et al., 2005b).

Nanotoxicology is an emerging discipline (Oberdörster et al., 2005a) and there is a gap between the nanomaterials safety evaluation and the nanotechnology development that daily produce new materials, new synthesis, new applications, and new products ready for the market. According to the Royal Society & the Royal Academy of Engineering report (2004), nanoparticles should be treated as new chemicals from a risk-point of view because they can overcome the body's normal protective barrier given their size (Nasterlack et al., 2007;

NIOSH, 2007; Schulte et al., 2008).

Moreover ultrafine particles, different in sources and composition (Geller et al., 2002), are a component of the airborne particulate matter (Ntziachristos et al., in press; Pakkanen et al., 2001) and their absorption through inhalation and skin routes must be better studied (Ayres et al., 2008).

2. DERMAL ABSORPTION AND TOXICITY OF NANOPARTICLES

Possible routes of entry include inhalation, absorption through the skin or digestive tract (Chen et Schluesener, 2008), but also voluntary injection, absorption or implantation for drug delivery systems (Bianco et al., 2005; Guterres et al., 2007; Klumpp et al., 2006; Lademann et al., 2007).

Dermal absorption of chemicals must be considered in risk evaluation (Fiserova-Bergerova et al., 1990; Nielsen et Grandjean, 2004; Sartorelli et al., 2007). In particular, the skin is the largest organ of the body accounting for more than 10% of body mass and has an important role of barrier versus the external environment with function of protection, homeostasis maintaining, metabolism, synthesis and deposition.

Four pathways of penetration across the skin have been identified depending on physicochemical properties of the compound: intercellular, transcellular, and two transappendageal, through hair follicles and sweat glands (Scheuplein, 1967).

It is well known that small (<600 Da) lipophilic molecules can easily penetrate the skin passively (Barry, 2001), but a variety of factors can influence the extent of the dermal uptake: the skin barrier integrity, the contaminate surface, the anatomical side, and the presence of skin diseases such as allergic and irritant contact dermatitis, atopic eczema, psoriasis. Moreover mechanical flexions, irritant detergents and chemicals (Larese et al., 2006; Nielsen et al, 2007) can increase skin absorption.

There are few studies on nanoparticles skin penetration (Bronaugh, 2008; EPA, 2007; Kielhorn et al., 2006; Oberdörster et al., 2005a; SCCP, 2007) with discrepancies in results likely related to differences in techniques and methods employed, laboratory conditions and absence of standardized evaluation protocols. In addition, while the respiratory route of entry is every time a matter of concern, the skin is often considered less permeable and the risk perception by this route is very low (Donaldson et al., 2006; Geiser et al., 2003; Geys et al., 2006; Geys et al., 2007; Lam et al., 2004a; Limbach et al., 2004; Magrez et al., 2006; Muller et al., 2005; Nel et al., 2006; Oberdörster et al., 2005a; Rotoli et al., 2008; Shimada et al.,

2006; Shvedova et al., 2005). However, in the literature there are studies which suggest that the skin is an important route of entry for nanoparticles both in occupational and consumer setting.

Alvarez-Roman et al. (2004), have used confocal laser scanning microscopy to visualize the distribution of non-biodegradable, fluorescent, polystyrene nanoparticles (diameters 20 and 200 nm) across porcine skin after 0.5, 1 and 2 h of exposure in vertical diffusion cells. The surface images revealed that polystyrene nanoparticles accumulated preferentially in the follicular openings increasing in a time-dependent manner, and that the follicular localization was favoured by the smaller particle size. Tinkle et al. (2003) studied the effects of flexing movement on normal skin nanoparticles uptake showing that mechanical flexion facilitated the penetration of fluorescent dextran micrometer-sized particles that were observed in deeper dermal layers. Kim et al. (2004) found that nanoparticles administered in the dermis migrated to regional lymph nodes, potentially via skin macrophages and Langerhans cells, raising potential concern for immunomodulation.

2.1. Carbon-based nanoparticles

Carbon nanomaterials are one of the most important new classes of multifunctional nanoparticles because of their large variety of applications. This class include: (1) carbon nanotubes (CNTs), single-walled (SWCNTs) and multi-walled (MWCNTs), which have diameters ranging from a few to hundreds of nanometers, whereas their length can be up to a few micrometers, and (2) fullerenes, that have a size less than 100 nm. Modifications and derivatizations of these compounds promise a number of applications in many fields (Tasis et al., 2006).

Actually no data on dermal absorption of CNTs are present in literature while, regarding fullerenes, Rouse et al. (2007) investigated the influence of mechanical flexion on dermal absorption of fullerene amino acid-derivatized peptide nanoparticles using dermatomed porcine skin fixed to a flexing device. Confocal microscopy showed dermal penetration of the nanoparticles at 8 h in skin flexed for 60 and 90 min, while there was no evidence of penetration into the dermis of unflexed skin until 24 h.

Concerning cytotoxicity, in the last few years some studies (Tables 1 and 2) have reported possible negative effects of carbon nanomaterials on dermal cells and their possible absorption through the cutaneous barrier. Shvedova et al. (2003) investigated adverse effects of unrefined SWCNTs on cell cultures of immortalized human epidermal keratinocytes, HaCaT, finding that they can cause oxidative stress and cellular toxicity by formation of free

radicals, accumulation of peroxidative products, antioxidant depletion, and loss of cell viability. Exposure to nanotubes also resulted in ultrastructural and morphological changes in cultured skin cells. Manna et al. (2005) found an increased oxidative stress and inhibition of cell proliferation in response to treatment of keratinocytes with SWCNTs and suggest that nanotubes can activate Nuclear Factor-kappa B (NF-kB) in a dose-dependent manner. Zhang et al. (2007) investigated the effect of human epidermal keratinocytes exposure to different concentrations of 6-Aminohesanoic acid-derivatized SWCNTs. Results showed an increase of interleukin(IL)-8 release and a decrease in cell viability, suggesting a dose-dependent irritation response.

Sayes et al. (2006a) found that the cytotoxic response of human dermal fibroblasts in culture was dependent on the degree of functionalization of the SWCNTs: as the degree of sidewall functionalization increased, the SWCNTs sample became less cytotoxic. Saar et al. (2007) found that SWCNTs induced oxidative stress in human BJ Foreskin cells with an increase of the products of stress responsive genes. In another study (Tian et al., 2006), the toxic effects of five carbon nanomaterials (SWCNTs, active carbon, carbon black, MWCNTs and carbon graphite) on human fibroblast cells *in vitro* was compared. The surface area of the carbon nanomaterials was found to be the best predictor for their potential toxicity. SWCNTs induced the strongest cellular apoptosis/necrosis response. In addition, the refined SWCNTs were more toxic than their unrefined counterpart.

Herzog et al. (2007) studied the toxicity of carbon-based nanomaterials using the clonogenic assay, also called colony formation assay, in order to prevent any interaction with colorimetric indicator dyes normally used. They applied this method to test three types of carbon nanoparticles (two types of SWCNTs and one type of carbon black nanoparticles) on three different cell models including the HaCaT cells. The two types of SWCNT elicit a stronger cytotoxic response than carbon black, but all three particle types were highly effective in inhibiting cell proliferation in all three cell lines. Moreover, HaCaT cells showed decreased cell viability.

Ding et al. (2005) performed the whole genome expression array analysis based on phenotypic measurements on human skin fibroblast cell populations exposed to MWCNTs and multi-walled carbon nano-onions (MWCNOs), showed that exposure to these nanomaterials at cytotoxic doses induced cell cycle arrest and increased apoptosis/necrosis. Multiple cellular pathways were perturbed with material-specific toxigenomic profiles.

Additional studies conducted with proteomic analysis in human epidermal keratinocytes exposed to MWCNTs showed differences in expression and alterations of several proteins,

suggesting alteration of intermediate filament expression, cell cycle inhibition, altered vesicular trafficking/exocytosis and membrane scaffold protein down-regulation (Monteiro-Riviere et al., 2005b). Further two studies showed that MWCNTs, nor derivatized neither optimized for biological applications, were capable of both localizing within and initiating an irritation response in human epidermal keratinocytes (Monteiro-Riviere et al., 2005a) and that MWCNTs were able to alter the expression of protein associated with metabolism, cell signaling, stress, cytoskeletal elements and vesicular trafficking in human epidermal keratinocytes (Witzmann and Monteiro-Riviere, 2006).

Fullerene cytotoxicity seems to depend on their surface derivatization. In two different human cell lines, the lethal dose of fullerene changed over seven orders of magnitude with relatively minor alterations in fullerene structure. Oxidative damage to cell membranes was observed in all cases where fullerene exposure led to cell death (Sayes et al., 2004).

Sayes et al. (2005) found that nano-C60 colloidal suspension disrupts normal cellular functions through lipid peroxidation studying the biological effects of water-soluble fullerene aggregates on human dermal fibroblasts.

2.2. Quantum Dots

Quantum dots (QDs) are semiconductor nanocrystals consisting of a colloidal core surrounded by one or more surface coatings that give specific characteristics to this nanoparticles. These heterogeneous fluorescent nanoparticles have great potential for use as diagnostic and imaging agents in biomedicine and as semiconductors in the electronic industry, but their potential human toxicity and cytotoxicity have to be evaluated (Hardman, 2006).

Ryman-Rasmussen et al. (2006) carried out a study in which soluble QDs of two sizes with three different surface coatings were applied to porcine skin in flow-through diffusion cells. Their findings showed that QDs of different sizes, shapes, and surface coatings could penetrate intact skin in an occupationally relevant dose within the span of an average-length work day. Zhang et al. (2008) obtained different results using another type of QDs: their conclusions suggest that porcine skin penetration of QD621 is minimal and limited primarily to the outer stratum corneum layers and near hair follicles (see Table 1).

Ryman-Rasmussen et al. (2007) used human epidermal keratinocytes to assess if soluble QDs of two sizes with three different surface coatings (polyethylene glycol (PEG), PEG-amines, or carboxylic acids), induced toxic effects on skin cells. Exposure of keratinocytes to QDs significantly increased cell release of IL-1b, IL-6, and IL-8. These findings suggest that

surface coating of QDs does not influence the uptake by keratinocytes but is a primary determinant of cytotoxicity and immunotoxicity. Similar results were found by Zhang et al. (2008) using a different type of water-soluble QDs with a cadmium/selenide core and a cadmium sulfide shell coated with PEG. Another study carried out by Rouse et al. (2008) investigated the effects of applied strain on QDs uptake by human keratinocytes. Their data indicated that addition of strain resulted in an increase in cytokine production and QDs uptake, with irritation and reduction of cell viability. These data suggest that application of physiological load conditions can increase cell membrane permeability, thereby increasing nanoparticle concentration in cells.

2.3. Titanium Dioxide and Zinc Oxide

Titanium dioxide (TiO_2) and zinc oxide (ZnO) are largely present in many sunscreens formulations to protect against UV-induced skin damage. When exposed to UV radiation, TiO2 and ZnO do not undergo any chemical decomposition and for that reason they represent an alternative to chemical agents. Moreover, they offer a wider range of protection compared to other organic compounds. Actually, in many formulations TiO_2 and ZnO are included as nanosized particles because in this form they are transparent and more aesthetically acceptable to the consumers. Furthermore, TiO_2 nanoparticles are used in other several products (sport clothes, surface cleaning agents, computer devices) of the everyday life and the exposure occasions are increasing day by day.

Tan et al. (1996) performed a pilot study on percutaneous absorption of microfine TiO_2 from sunscreens applying the formulation to the skin for 2-6 weeks to 13 selected volunteers scheduled to have skin surgery for a total of 16 skin biopsies. After excision, the stratum corneum was removed from the skin samples by tape stripping and the concentration of titanium after digestion of the skin was evaluated. The titanium concentration on the skin biopsies of the subjects exposed to the microfine TiO_2 was higher than the controls.

These findings were not confirmed by other researchers who did not observe penetration of nanoparticles in the viable layers of the epidermis using different TiO_2 nanoparticle formulations and different investigative techniques. A small amount of metal oxide was only detected into the hair follicles following application to volunteers (Lademann et al, 1999).

Mavon et al. (2007) used the tape stripping method in *in vivo* experiments on volunteers after exposure to a sunscreen formulation containing TiO_2 nanoparticles. Transmission electron microscopy (TEM) and particle-induced X-ray emission (PIXE) techniques were used to localize the TiO_2 in skin sections in *in vitro* experiments by the same Authors. In this

in vivo and *in vitro* permeation study no TiO_2 was detected in the follicle, viable epidermis or dermis, and more than 90% of the applied sunscreen was recovered in the first 15 tape strippings while the remaining 10% was localized in the furrows and in the opened infundibulum.

Schulz et al. (2002), using optical and electron microscopy, proved that neither surface characteristics and particle size nor shape of the micronised pigments result in any dermal absorption of this substance. Micronised TiO_2 was solely deposited on the outermost surface of the stratum corneum, but not in deeper stratum corneum layers, the human epidermis and the dermis.

The three-year European project NANODERM involved a great number of research groups in the evaluation of skin permeation of different TiO_2 based sunscreens. The project provided many data from *in vivo* and *in vitro* experiments with human and porcine skin, with human foreskin transplanted to immunodeficient mice, and with dermal cells in culture.

Some Authors involve in the project detected a little amount of titanium dioxide in the deeper viable epidermal layers using spatially resolved ion beam analysis (PIXE, RBS, STIM and secondary electron imaging) on freeze-dried cross-sections of pig skin biopsies (Menzel et al., 2004), while other Author reported that TiO_2 nanoparticles do not penetrate through the stratum corneum of human skin transplants (Kertész et al, 2005; Kiss et al., 2008).

The project report confirmed the safety of the sunscreens formulation containing TiO_2 nanoparticles, reporting no evidence of nanoparticle transcutaneous penetration (NANODERM, 2007).

Instead, Bennat and Müler-Goymann (2000) found that different formulations had different penetration: according with their experiments, microfine TiO_2 penetrated deeper into human skin from an oily dispersion than from an aqueous one, and encapsulation of the pigments into liposomes caused a higher penetration into the skin. Furthermore, penetration was greater when applied to hairy skin, suggesting a surface penetration through hair follicles or pores.

Also Gamer et al. (2006) investigated the *in vitro* absorption of microfine ZnO and TiO_2 in cosmetic formulations through porcine skin. One ZnO formulation and two TiO_2 formulations were tested in modified Franz static dermal penetration cells. In their conclusions Authors observed that neither microfine ZnO nor micrfine TiO_2 was able to penetrate through porcine skin, and that most of the applied material was recovered in the first five tape strips, indicating that the material did not penetrate into the deeper layers of the skin.

ZnO skin absorption was investigated by Cross et al. (2007) using Franz-type diffusion

cells. Two different formulations of 26-30 nm ZnO particles and one placebo cream base formulation containing no ZnO nanoparticles were compared. Authors found that less than 0.03% of the applied zinc content was detected in the receptor phase (not significantly different following application of a placebo formulation). No particles could be detected by in the lower stratum corneum or viable epidermis by electron microscopy, suggesting that minimal nanoparticle penetration occurs through the human epidermis (see Table 1).

Some Authors suggested that nanoparticles can elicit a photocatalytic activity into the dermal layers causing formation of free radicals in skin cells, damaging DNA (Cai et al., 1992; Dunford et al., 1997; Wamer et al., 1997, Serpone et al., 2001), disrupting normal cell functions and cell viability (Sayes et al., 2006b). So the debate is just open about their safety use.

2.4. Silver and Gold Nanoparticles

Owing to their strong antibacterial activity, silver nanoparticles are largely used as a component of various commercially available products such as textiles, medical devices, contraceptives, water disinfectants, and room spray (Woodrow Wilson International Center for Scholars, 2007). Moreover, nanosilver is used for treatment of wounds and burns, as well as for coating on implants.

Some Authors suggested an increased dermal penetration of nanosilver associated with damaged skin in *in vitro* experiments (Larese et al., 2009) or following the use of nanosilver coated dressings in case of extensive burns (Trop et al, 2006).

Traditionally, silver is relatively non-toxic to mammalian but can cause argyria or argyrosis in subjects with chronic occupational exposure. Because of the extensive presence of nanosilver in textiles, wound dressing, sport clothes and other products which come in direct contact with the skin, dermal exposure must be carefully evaluated.

Keratinocytes and fibroblasts in culture were used to assess the cytotoxic effects of nanosilver released from several types of silver containing dressings (Table 2) although some laboratory and clinical studies suggested their dermal biocompatibility (Leaper, 2006; Supp et al., 2005; Muangman et al., 2006; Wright et al., 2002). The results of these studies showed that keratinocytes proliferation was significantly inhibited and cell morphology affected after exposure to extracts of nanocrystalline coated dressings (Paddle-Ledinek et al., 2006; Lam et al., 2004b). Poon and Burd (2004) found that nanosilver crystallines were toxic to both keratinocytes and fibroblasts, and that fibroblasts appeared to be more sensitive to silver than keratinocytes.

Nanogold is also an interesting nanomaterial for its applications in cell imaging, cancer therapy, tissue welding and nanomedicine. Sonavane et al. (2008) investigated the *in vitro* cutaneous penetration of three types of gold nanoparticles differing in size (15 nm, 102 nm and 198 nm) using the Franz diffusion cell method with rat skin. Gold nanoparticles showed size dependent permeation through rat skin. 15 nm gold nanoparticles showed higher permeation compared to 102 nm and 198 nm gold nanoparticles. TEM study of rat skin revealed accumulation of smaller size gold nanoparticles in deeper region of skin whereas larger particles were observed mainly in epidermis and dermis.

Regarding the cytotoxicity of gold nanoparticles, a number of studies have argued its safe use because the uptake of gold clusters (1.4 nm) by different types of cells and their interaction with DNA have been demonstrated (Connor et al., 2005; Liu et al., 2003; Tsoli et al., 2005).

Pernodet et al. (2006) investigated the effects of citrate/gold nanoparticles at different concentrations and exposure times on human dermal fibroblasts. They found that, as a result of the intracellular nanoparticle presence, actin stress fibres disappeared, thereby inducing major adverse effects on cell viability. Properties such as cell spreading and adhesion, cell growth, and protein synthesis to form the extracellular matrix were altered dramatically, suggesting that the internal cell activities were damaged.

2.5. Other Metals and Metal Oxides

Researchers are developing a number of metal and alloy nanoparticles for either various applications in industrial processes, such as catalyst, fillers, semiconductors, or for systemic drug administration, but very few data regarding their toxicity are available in literature.

It is well known that metal and metal oxide powders once placed in biologic media can release metal ions (Midander et al., 2006) that can subsequently pass through the skin (Larese et al., 2007) but little is known about skin penetration of metal nanoparticles.

Berry et al. (2004) found that underivatised iron oxide nanoparticles (8-15 nm) were rapidly endocytosed into cultured human dermal fibroblasts causing disruption to the cell cytoskeleton and a decrease in proliferation. The same nanoparticles, transferrin derivatised, stimulated cell proliferation and were not internalised, but appeared to attach to the outside of the cell membrane, most likely to cell expressed transferring receptors.

Baroli et al. (2007) demonstrated that iron-based rigid nanoparticles smaller than 10 nm were able to passively penetrate the skin through the SC lipidic matrix and hair follicle orifices, reaching the deepest layers of the SC, the stratum granulosum, and hair follicles. In

rare cases, nanoparticles were also found in the viable epidermis.

Papageorgiou et al. (2007) compared the cytotoxic and genotoxic effects of nanoparticles and micron-sized particles of cobalt-chrome alloy in cultured human fibroblasts. Nanoparticles, which caused more free radicals in an acellular environment, induced more DNA damage than micron-sized particles using the alkaline comet assay. Nanoparticles appeared to disintegrate within the cells faster than microparticles with the creation of electron dense deposits, which were enriched in cobalt. The mechanism of cell damage appeared to be different after exposure to nanoparticles and microparticles.

3. DISCUSSION AND CONCLUSIONS

Experimental findings on skin absorption and skin toxicity of nanoparticles are contradictory. More data are needed to better define and understand if skin represents a route of entry of nanoparticles into the body or a target tissue. In the final report of the project NANODERM it is stated that adverse health effects for the topical application of sunscreens containing TiO_2 nanoparticles (especially when coated) are not expected for healthy skin but several other studies on carbon-based nanoparticles and quantum dots confirm an interaction between human dermal cells and nanosized particles. The shortage of data about many types of new compounds, such as metals and metal oxides, calls for more studies to improve understanding of nanoparticle skin absorption. Quantitative data are needed because there is evidence that some nanoparticles can pass through the skin in particular conditions such as wounds, flexures sites and lesions.

Moreover, nanoparticles characterization should be essential in the future studies on dermal penetration and toxicity. Size, shape, coating, purity, presence of catalysts, extent of agglomeration and agglutination of the nanoparticles could influence the amount permeating the skin and the toxicity of the nanomaterials.

Finally, to investigate the interaction between new nanocompounds and the human skin the researchers have to take into consideration several exposure variables, such as anatomical exposure sites, extension of the exposition area, time of exposition, chronic and repeated exposure, presence of skin diseases, and the role of cleanser and penetration enhancer.

The classic investigation protocols must be adapted and re-standardized to the new nanosized compounds. Cell cultures (Bernstein and Vaughan, 1999), Franz diffusion cells, tape stripping (Escobar-Chávez et al, 2008), human skin implantations on animals, remain powerful tools to study particle interaction with human dermal tissue. Furthermore new

methods and new technique applications have to be developed (Monteiro-Riviere and Inman, 2006; SCCP, 2007). In particular microscopy techniques like Coherent anti-Stokes Raman Scattering (CARS), Transmission Electron Microscopy (TEM), Confocal Laser Scanning Microscope (CLSM) and others ion beam techniques are necessary to visualize nanoparticles into biologic structures (Moger et al., 2008).

Table 1. Nanoparticles (NPs) skin absorption studies

Study (year)	**Compound (dimensions)**	**Study type**	**Outcome**
Alvarez-Roman et al. (2004)	Polystyrene NPs (20 and 200 nm)	In vitro: diffusion cells with full-thickness porcine ear skin	NPs accumulation in follicular openings.
Baroli et al (2007)	Two different types of iron-based NPs (<10 nm)	In vitro: vertical diffusion cells with full-thickness human skin	NPs was detected into deepest layers of the epidermis, stratum granulosum, and into the hair follicles.
Bennat and Müler-Goymann (2000)	Titanium Dioxide (*)	In vivo: human volunteers - tape stripping In vitro: penetration cells with human skin and cultivated skin	Differences in penetration between different formulations. Microfine titanium dioxide penetrated probably via hair follicles and hair.
Cross et al. (2007)	Zinc Oxide (15-40 nm)	In vitro: Franz diffusion cells with human epidermis	No NPs could be detected in the lower stratum corneum or viable epidermis.
Gamer et al. (2006)	Titanium Dioxide (80 nm)	In vitro: Franz diffusion cells with porcine skin	Neither microfine zinc oxide nor micrfine titanium dioxide was able to penetrate through porcine skin, and that most of the applied material was recovered in the first five tape strips.
Kertész et al. (2005)	Titanium Dioxide (*)	In vivo: penetration via human foreskin grafts transplanted to immunodeficient mice	NPs were observed having penetrated into the corneocyte layers of stratum corneum.
Kiss et al. (2008)	Titanium Dioxide (*)	In vivo: penetration via human skin transplanted to immunodeficient mice	No evidence of penetration through the intact epidermal barrier.
Lademann et al. (1999)	Titanium Dioxide (*)	In vivo: human volunteers – tape stripping	No penetration of microparticles into viable skin tissue. Little amount was found into the hair follicles.
Larese et al. (2009)	Silver (25 nm)	In vitro: Franz diffusion cells with full-thickness human skin	Some evidence of penetration through damaged skin.
Mavon et al. (2007)	Titanium Dioxide (20 nm)	In vivo: human volunteers - tape stripping In vitro: static diffusion cells with human skin	In vivo and in vitro penetration study showed no titanium dioxide penetration into the viable skin layers.

Menzel et al. (2004).	Four different formulations containing Titanium Dioxide.	In vivo: pig skin biopsies	NPs penetrated into the stratum granulosum via intercellular space.
NANODERM (2007)	Many different formulations of Titanium Dioxide (*)	In vivo: pig skin biopsies, human healthy and psoriasic skin biopsies, human skin transplanted to immunodeficent mice	The final report concluded that no health effects are expected for topical application of sunscreens containing titanium dioxide NPs (especially when coated) on healthy skin. The situation with psoriatic, sunburned or atopic skin is less clear.
Rouse et al. (2007)	Fullerenes (0.7 nm before functionalization)	In vitro: dermatomed porcine skin fixed to a flexing device	Skin flexion increased NPs dermal penetration.
Ryman-Rasmussen et al. (2006)	Spherical Quantum Dots (4.6 nm) and ellipsoid Quantum Dots (12x6 nm)	In vitro: Porcine skin in flow-through diffusion cells	QDs of different sizes, shapes, and surface coatings could penetrate intact skin in an occupationally relevant dose.
Sonavane et al. (2008)	Gold (15 nm, 102 nm and 198 nm)	In vitro: Franz diffusion cells with rat skin	Gold NPs showed size dependent permeation As the size of the NPs increased, permeability coefficient and diffusion coefficient was found to be decreased.
Schulz et al. (2002)	Three different type of Titanium Dioxide formulations (10/15 nm, 20 nm,100 nm)	In vivo: Human volunteers – skin biopsies	Micronised titanium dioxide was solely deposited on the outermost surface of the stratum corneum but not in deeper stratum corneum layers, in the human epidermis and dermis.
Tan et al. (1996)	Titanium Dioxide (*)	In vivo: Human volunteers – Tape stripping and skin biopsies	After excision, skin analysis showed that the concentration of titanium in the subjects exposed were higher than in the controls.
Trop et al. (2006)	Silver (15 nm)	Case report: one patient treated with silver-coated wound dressing	After 1 week of local treatment in a young, previously healthy, man with 30% mixed depth burns, hepatotoxicity, argyrialike symptoms, and grayish discoloration of the patient's face appeared. Silver levels in plasma and urine were elevated.
Zhang et al. (2008)	Quantum Dots (39/40 nm)	In vitro: Porcine skin - flow-through diffusion cells	Minimal skin penetration and limited primarily to the outer Stratum Corneum layers.

Legend: NPs: nanoparticles; SWCNTs: single-walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes; MWCNOs: multi-walled carbon nano-onions; QDs: quantum dots; (*): characterization not reported or too long to be reported in the tables.

Table 2. Nanoparticles (NPs) cells toxicity studies

Study (year)	Compound (dimensions)	Type of cells	Outcome
Berry et al. (2004)	Iron Oxide (10 nm)	Human dermal fibroblasts	NPs caused disruption to cell cytoskeleton and reduced proliferation.
Ding et al. (2005)	MWCNTs and MWCNOs (*)	Human dermal fibroblasts	Exposure to NPs at cytotoxic doses induced cell cycle arrest and increased apoptosis/necrosis.
Herzog et al. (2007)	Two types of SWCNTs and Carbon Black NPs (*)	Human epidermal keratinocytes	NP inhibited cell proliferation and decreased cell viability.
Kiss et al. (2008)	Titanium Dioxide (9 nm)	Human epidermal keratinocytes - human dermal fibroblasts - primary human melanocytes - human immortalized sebaceous gland cells	NPs exerted significant and cell-type dependent effects on cellular functions, such as viability, proliferation, apoptosis and differentiation.
Lam et al. (2004b)	Silver (*)	Human epidermal keratinocytes	Proliferation was significantly inhibited and cell morphology affected.
Manna et al. (2005)	SWCNTs (*)	Human epidermal keratinocytes	NPs increased oxidative stress and inhibited cell proliferation.
Monteiro-Riviere et al. (2005a)	MWCNTs (*)	Human epidermal keratinocytes	NPs localized within the cells and initiated an irritation response, inducing the release of proinflammatory cytokine.
Monteiro-Riviere et al. (2005b)	MWCNTs (*)	Human epidermal keratinocytes	NPs caused alteration of several protein expression.
Paddle-Ledinek et al. (2006)	Silver (*)	Human epidermal keratinocytes	NPs reduced cell proliferation and affected cell morphology.
Papageorgiou et al. (2007)	Cobalt chrome alloy (30 nm)	Human dermal fibroblasts	NPs induced DNA damage, aneuploidy and cytotoxicity. NPs appeared to disintegrate within the cells with the creation of electron dense deposits which were enriched in cobalt.
Poon and Burd (2004)	Silver (*)	Human epidermal keratinocytes - human dermal fibroblasts	The contact between cells and silver released from a type of wound dressing determined a reduction in cell metabolism and vitality. Fibroblasts appeared to be more sensitive to silver than keratinocytes
Rouse et al. (2008)	Quantum Dots (6 x 12 nm)	Human epidermal keratinocytes	Applied strain caused an increase in cytokine production and QDs uptake, resulting in irritation and decreasing cell viability.

Ryman-Rasmussen et al. (2007)	Two types of Quantum Dots (4.6 nm and 6 x 12 nm, before coating)	Human epidermal keratinocytes	Exposure to QDs significantly increased cell release of interleukines. Surface coating of QDs did not influence the uptake but was a primary determinant of cytotoxicity and immunotoxicity.
Sarkar et al. (2007)	SWCNTs (*)	Human BJ Foreskin cells	NPs inducted oxidative stress and increased the expression of stress responsive genes.
Sayes et al. (2004)	Fullerenes (60 nm)	Human dermal fibroblasts	The lethal dose of NPs changed depending on their surface derivatization. Oxidative damage to cell membranes was observed in all cases where NPs exposure led to cell death.
Sayes et al. (2005)	Fullerenes (60 nm)	Human dermal fibroblasts	NPs colloidal suspension disrupted normal cellular functions through lipid peroxidation.
Sayes et al. (2006a)	SWCNTs (*)	Human dermal fibroblasts	Cytotoxic response was dependent on the degree of functionalization of the NPs: as the degree of sidewall functionalization increased, the NPs became less cytotoxic.
Sayes et al. (2006b)	Three types of Titanium Dioxide (10.1 nm, 3.2 nm, 5.2 nm)	Human dermal fibroblasts	Cytotoxicity and inflammation were observed only at relatively high concentrations. The extent to which NPs affected cellular behavior did not depend on surface area. Cytotoxicity was related to the phase composition of NP.
Shvedova et al. (2003)	SWCNTs (*)	Human epidermal keratinocytes	Exposure to NPs resulted in oxidative stress and cellular toxicity, with formation of free radicals, accumulation of peroxidative products, antioxidant depletion, loss of cell viability and ultrastructural and morphological changes.
Tian et al. (2006)	SWCNTs, active carbon, carbon black, MWCNTs and carbon graphite (*)	Human dermal fibroblasts	SWCNTs induced the strongest cellular apoptosis/necrosis. Surface area was the best predictor for the potential toxicity of these refined carbon nanomaterials.
Witzmann and Monteiro-Riviere (2006)	MWCNTs (*)	Human epidermal keratinocytes	NPs were able to alter the expression of proteins associated with metabolism, cell signaling, stress, cytoskeletal elements and vesicular trafficking.

Zhang et al. (2007)	SWCNTs (*)	Human epidermal keratinocytes	Exposure resulted in a dose-dependent irritation response with an increase in IL-8 release and a decrease in cell viability.
Zhang et al. (2008)	Quantum Dots (39/40 nm)	Human epidermal keratinocytes	Cell viability decreased ,and IL-6 and IL-8 release increased, both significantly. NPs were found in cytoplasmic vacuoles and at the periphery of the cell membranes.

Legend: NPs: nanoparticles; SWCNTs: single-walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes; MWCNOs: multi-walled carbon nano-onions; QDs: quantum dots; (*): characterization not reported or too long to be reported in the tables.

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ALLEGATO IV

HUMAN SKIN PENETRATION OF SILVER NANOPARTICLES THROUGH INTACT AND DAMAGED SKIN

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ABSTRACT

There is a growing interest on nanoparticle safety for topical use. The benefits of nanoparticles have been shown in several scientific fields, but little is known about their potential to penetrate the skin. This study aims at evaluating *in vitro* skin penetration of silver nanoparticles. Experiments were performed using the Franz diffusion cell method with intact and damaged human skin. Physiological solution was used as receiving phase and 70 µg cm^{-2} of silver nanoparticles coated with polyvinylpirrolidone dispersed in synthetic sweat were applied as donor phase to the outer surface of the skin for 24h. The receptor fluid measurements were performed by Electro Thermal Atomic Absorption Spectroscopy (ETAAS). Human skin penetration was also determined by using Transmission Electron Microscope (TEM) to verify the location of silver nanoparticles in exposed membranes.

Median silver concentrations of 0.46 ng cm^{-2} (range <LOD - 2.23) and 2.32 ng cm^{-2} (range 0.43-11.6) were found in the receiving solutions of cells where the nanoparticles solution was applied on intact skin (8 cells) and on damaged skin (8 cells) respectively. Twenty-four hours silver flux permeation in damaged skin was 0.6±0.2 ng cm^{-2} with a lag time < 1 hour.

Our experimental data showed that silver nanoparticles absorption through intact and damaged skin was very low but detectable, and that in case of damaged skin it was possible an increasing permeation of silver applied as nanoparticles. Moreover, silver nanoparticles could be detected in the stratum corneum and the outermost surface of the epidermis by electron microscopy.

We demonstrated for the first time that silver applied as nanoparticles coated with polyvinylpirrolidone is able to permeate the damaged skin in an in-vitro diffusion cell system.

KEYWORDS: Nanoparticles; Silver; Skin absorption; In vitro; Damaged skin

RIASSUNTO

Nella comunità scientifica si sta diffondendo un crescente interesse sulla sicurezza delle nanoparticelle per uso topico. I vantaggi delle nanoparticelle sono stati dimostrati in diversi campi scientifici, ma poco si sa circa il loro potenziale di penetrare la cute. Questo studio ha lo scopo di valutare la penetrazione cutanea *in vitro* di nanoparticelle d'argento. Gli esperimenti sono stati condotti con il metodo delle celle di diffusione di Franz con cute umana intera, integra e lesa. Come fase ricevente è stata usata soluzione fisiologica, mentre 70 µg cm^{-2} di nanoparticelle d'argento rivestite con polivinilpirrolidone e disperse in sudore sintetico sono state applicate, come fase donatrice, sulla superficie esterna della cute per 24 ore. Le analisi delle fasi riceventi sono state effettuate con Spettroscopia di Assorbimento Atomico Elettro-Termica. La penetrazione delle nanoparticelle nella cute è stata inoltre investigata tramite Microscopia Elettronica a Trasmissione (TEM) per verificare la localizzazione delle nanoparticelle d'argento nella membrana esposta.

Per quanto riguarda le quantità d'argento permeate, il valore mediano trovato è stato di 0,46 ng cm^{-2} (range <LOD – 2,23) and 2,32 ng cm^{-2} (range 0,43-11,6) rispettivamente nelle celle allestite con cute integra e nelle celle con cute lesa. Inoltre è stato calcolato un flusso di permeazione attraverso la cute lesa di 0,6±0,2 ng cm^{-2} con un lag time inferiore ad 1 ora.

I nostri dati sperimentali hanno mostrato che l'assorbimento delle nanoparticelle d'argento attraverso la cute integra e danneggiata era molto basso, ma misurabile con un aumento significativo nel caso della cute lesa.

Inoltre, le nanoparticelle d'argento sono state individuate tramite microscopia elettronica nello strato corneo e negli strati più superficiali dell'epidermide.

Per la prima volta, quindi, è stato dimostrato che l'argento applicato in foma di nanoparticelle rivestite con polivinilpirrolidone sono in grado di permeare attraverso la cute danneggiata in un sistema di diffusione *in vitro*.

PAROLE CHIAVE: Nanoparticelle; Argento; Assorbimento cutaneo; In vitro; Cute danneggiata

1. INTRODUCTION

The potential of solid nanoparticles to penetrate the stratum corneum and to diffuse into underlying structures raises a considerable health and safety issue for their topical use. The benefits of nanoparticles have been shown recently in several scientific fields, but little is known about the potential risk of contamination with nanomaterials through the cutaneous route during production or use. Nanoparticles may, just by virtue of their dimension (100 nm or less), exert biological effects: it has been demonstrated that materials at nanometric scale gain new properties that can be exploited in biotechnology, bioengineering and nanomedicine. Moreover, their surface can be changed by bioactive molecules or imaging probes that can be adsorbed, coated, conjugated or linked to them. These modified systems have been proposed for (i) cell labeling and targeting, (ii) tissue engineering, (iii) drug delivery or targeting, (iv) magnetic resonance imaging, (v) hyperthermia, (vi) magnetofection and (vii) analysis of biomolecules (Penn et al., 2003; Gupta and Gupta, 2005; Neuberger et al., 2005).

Despite a technology with great potentialities, extensive studies on nanotoxicology and nanomaterial safeness have only recently been undertaken (Hoet et al., 2004; Holsapple and Lehamn-McKeeman, 2005; Holsapple et al., 2005; Oberdoster et al., 2005; Thomas and Sayre, 2005).

Previous studies have reported that zinc oxide and titanium dioxide particles do not permeate the skin (Pflucker et al., 2001; Schultz et al., 2002), but the evidence of non-permeation was gained only indirectly using tape stripping and electron microscopy. These investigations showed that micronized titanium dioxide and zinc oxide were deposited on the external surface of the stratum corneum and could not be detected in deeper stratum corneum layers, the human epidermis and dermis. These data are consistent with the findings of Lademann et al. (1999), who revealed 17- nm titanium dioxide particles only in the upper stratum corneum and hair follicles following repeated application of an o/w emulsion to volunteers. Similar findings were reported by Dussert et al. (1997), who found no evidence of penetration of zinc oxide and titanium dioxide nanoparticles into human epidermis using electron microscopy. More recent *in vitro* studies, using pig skin exposed to microfine zinc oxide and titanium dioxide sunscreen formulations, suggested that no particles were able to penetrate porcine stratum corneum (Gamer et al., 2006). Similar results were obtained by Cross et al. (2007), who demonstrated *in vitro* that human skin penetration of zinc oxide nanoparticles was negligible.

Because of their antiseptic properties, silver nanoparticles are widely used in creams,

textiles, topical products and surgical prosthesis, but until now we have no information about their capability to penetrate or permeate the skin. Therefore, in order to assess the potential silver nanoparticles skin absorption, *in vitro* permeation studies with human skin were performed using a Franz (1975) static diffusion cell. Using this technique, we investigated the total amount of silver permeating through human skin during a 24-hours period. Experiments were run with intact skin as well as abraded skin following the Bronaugh and Steward (1985) protocol to estimate the effect of skin lesions on the permeation rate. Trasmission Electron Microscopy (TEM) was used to determine the location of silver nanoparticles in the skin layers. In the study we applied the experience and the protocol employed during the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals), a three-years (2001-2004) research program founded by European Commission (EDETOX, 2000).

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were analytical grade. Urea, magnesium nitrate hexahydrate, palladium nitrate, sodium hydrogenphosphate, potassium dihydrogenphosphate, hydrogen peroxide (30%) were purchased from Carlo Erba (Milan, Italy); ammonium hydroxide (25%) was bought from J. T. Baker (Deventer, Holland); lactic acid (90%) from Acros Organics (Geel, Belgium); nitric acid from Sigma Aldrich (Milan, Italy). Water reagent grade was produced with a Millipore purification pack system (milliQ water).

The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na_2HP0_4, 0.19 g of KH_2P0_4 and 9 g of NaCl into 1 L of milliQ water (final pH = 7.35).

The synthetic sweat solution used as the donor fluid consisted in 0.5% sodium chloride, 0.1% urea and 0.1% lactic acid in milliQ water; pH 4.5 was adjusted with ammonia.

2.2. Nanoparticle synthesis and characterization

Silver nanoparticles synthesis was carried out according to the method described by Graf et al. (2003). Synthesis included a stabilization step to prevent irreversible particle aggregation on dispersion in an aqueous medium. Stabilization was achieved by coating the nanoparticle core with polyvinylpirrolidone. A 0.14% wt silver coated nanoparticles solution in ethanol absolute 99% was used. TEM was used to characterized the nanoparticles: a drop

of the donor solution was deposited on formvar-coated grids, air-dried and observed. TEM measurements revealed that silver nanoparticles were as small as 25±7.1 nm, 25th-75th percentiles 19.5-29.3, minimum 9.8 nm, maximum 48.8 nm with 5% larger than 36.6 nm.

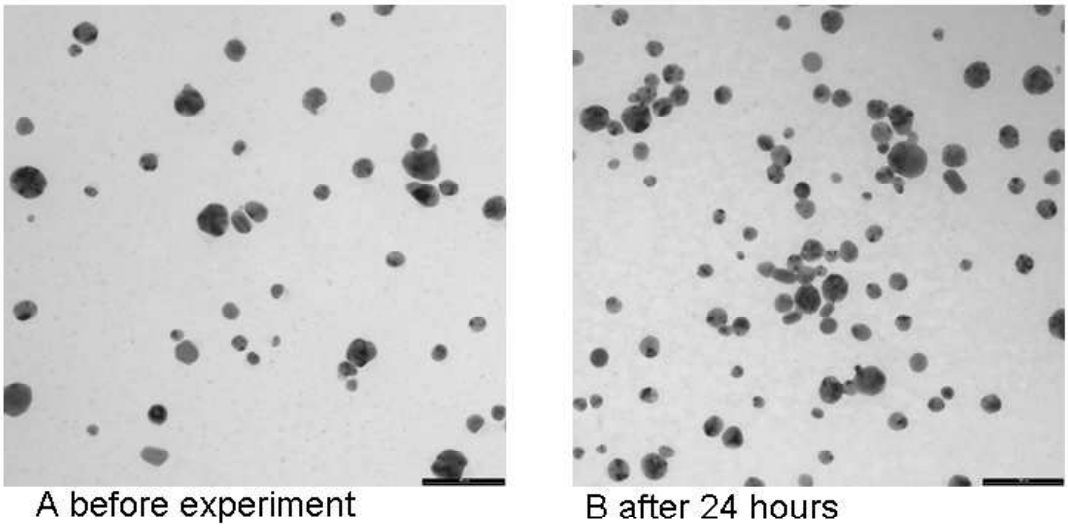

Figure 1. Nanoparticles in donor phase characterized by TEM (bar 100nm) before (A) and after (B) the experiment.

2.3. Preparation of skin membranes

Human abdominal full thickness skin was obtained as surgical waste. Prior to freezing, subcutaneous fat was removed and hair shaved. All the pieces of full thickness skin were stored in freezer at -25°C for a period up to, but not exceeding, 4 months. It has been shown that this method of storage does not damage the skin since no difference in permeability was observed between fresh and frozen segments of the same skin in a separate series of experiments (Franz, 1975). Skin integrity was tested before and after each experiment using electrical conductibility by means of a conductometer (Metrohm, 660, Metrohm AG Oberdorfstr. 68 CH-9100 Herisau) operating at 300Hz connected to two stainless steel electrodes (Fasano et al., 2002). The conductibility data, obtained in µS, were converted in KΩ cm^{-2}. Cells with a resistance lower than 3.95±0.27 KΩ cm^{-2}, were considered to be damaged as suggested by Davies et al. (2004).

Donors were men and women with a wide range of age (41÷71 years).

2.4. *In vitro* diffusion system

Percutaneous absorption studies were performed using static diffusion cells following the Franz Method (1975). The receptor compartment has a mean volume of 14.0 ml and was

maintained at 32°C by means of circulation of termostated water in the jacket surrounding the cell. This temperature value has been chosen in order to reproduce the hand's physiological temperature at normal conditions. The concentration of the salt in the receptor fluid is 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 performed as follows:

Experiment 1

At time 0, the donor chambers of eight Franz diffusion cells were filled with 70 µg cm^{-2} of silver nanoparticles dispersed in ethanol absolute 0.14 wt % and diluited 1:10 with synthetic sweat at pH 4.5 to reproduce *in vivo* condition.

At 24 h the dermal bathing solution was removed and silver concentration was determined in each receiving chamber solution. Some skin tissue pieces were analyzed with TEM.

Experiment 2

Experiment 1 was repeated using an abraded skin protocol as suggested by Bronaugh and Steward (1975): skin was abraded by drawing the point of a 19-gauge hypodermic needle across the surface (20 marks in one direction and 20 perpendicular).

Blanks. For each experiment, four cells were added as blank. The blank cells were treated as the other cells with the exception that no silver nanoparticle was introduced to the exposure chamber.

Experiment 3

At time 0, the exposure chambers of 5 Franz diffusion cells were filled with 70 µg cm^{-2} of nanoparticles solution dispersed in ethanol absolute 0.14 wt % and diluited 1:10 with synthetic sweat at pH 4.5 to reproduce *in vivo* condition (1.5 ml solution). At selected intervals (4, 8, 20, 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.

Experiment 4

Experiment 1 was repeated using an abraded skin protocol as described above. At selected intervals (4, 8, 20, 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.

2.5. Analytic Measurements

An electro-thermal atomic absorption spectrometry (GFAAS) with Zeeman background correction was used to measure the Ag concentration in receiving phase. The instrument used for analysis was a Perkin Elmer 4100ZL spectrophotometer equipped with an HGA graphite furnace and autosampler AS/71. The analytical wavelenght was of 328.1 nm. The limit of detection (LOD) for silver was 0.1 µg L^{-1}. The calibration curves were obtained using standard at 5, 10 e 20 µg L^{-1} and every sample was added up with a solution of 0.1% $Mg(NO_3)_2$ and 1g L^{-1} of $Pd(NO_3)_2$ as matrix modifier.

A 5-point standard curve was used for the analytical measurements. The correlation coefficient of the standard curve was: 0.9995 (r)

The samples were analyzed measuring against standard solutions for instrumental calibration. The CV for the analysis of each metal was always less than 5%.

2.6. 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 skin sections were washed twice (10 min each) with 0.1 M cacodylate buffer and then post fixed with 1% osmium tetroxide for 1 h at 4°C. Post fixed samples were dehydrated with an ascending ethanol series ending with 100% ethanol and then embedded in Dow 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 Ultramicrotome Leica Ultracut UCT (Leica Microsystems, Milan, Italy) equipped with a diamond blade Drukker 3mm (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 a Transmission Electron Microscope (EM208; Philips, Eindhoven, The Netherlands) with an high definition acquisition system SIS Morada and a digital image acquisition system iTEM (FEI Italia, Milan, Italy).

2.7. Data analysis

Silver concentration data (µg cm^{-3}) in the receptor solution were converted to the total amount that penetrated (µg cm^{-2}), with a correction for diluition due to sample removal.

Data analysis was performed using Excel for Windows release 2000 and the statistical software SPSS for Windows. All data were tested for normal distribution (Kolmogorov-

Smirnov). Because of non-normality of data distribution, these were reported using median, 25th and 75th percentiles, and minimum and maximum values.

The difference between independent data was assessed by means of the Mann-Whitney test. A p value of <0.05 was considered as the limit of statistical significance.

3. RESULTS

Silver nanoparticles were characterized by TEM visualization before and after the end of the experiments (Fig. 1). The dimensions were uniform in size and as small as 25 nm and stable in 24 hours.

The results of our investigations showed that silver can pass through intact human skin with a median amount of 0,46 (25-75th percentiles <LOD - 1.84; range <LOD - 2.23) ng cm^{-2} at 24 hours. Penetration of silver through damaged skin was 5 times greater than through intact skin with a wide range of results (median 2.32 ng cm^{-2}; 25-75th percentiles 0,55 – 8,67; range 0.43 - 11.6 ng cm^{-2}). Blanks were below the LOD (Table 1).

Table 1. Summary statistics for Ag content in the receiving cells. Data are given as medians, 25-75th percentiles, and minimum and maximum values (ng cm^{-2}).

	Median	**25th Percentile**	**75th Percentile**	**Minimum**	**Maximum**
Blank cells	<LOD	<LOD	<LOD	<LOD	<LOD
Intact skin	0.46*	<LOD	1.84	<LOD	2.23
Damaged skin	2.32*	0.55	8.67	0.43	11.6

**Mann-Whitney test for the difference between experimental cells and blank cells and between them p<0.05.*

Fig. 2 shows results for blank and experimental cells. In the experiment 2 with damaged skin we found a wide silver skin permeation higher than in experiment 1 performed with intact skin and in blank cells (Mann-Whitney test p<0.05).

Fig. 3 shows the permeation profile of silver following the application of silver nanoparticles in intact and damaged skin as mean and standard deviation (SD). For damaged skin we calculate a flux of 0.6±0.2 ng cm^{-2} with a lag time less than one hour for 24 hours of exposure.

Finally, the TEM investigation showed that in some slices, nanoparticles were located in

the stratum corneum and in the upper layers of the epidermis (Figure 4).

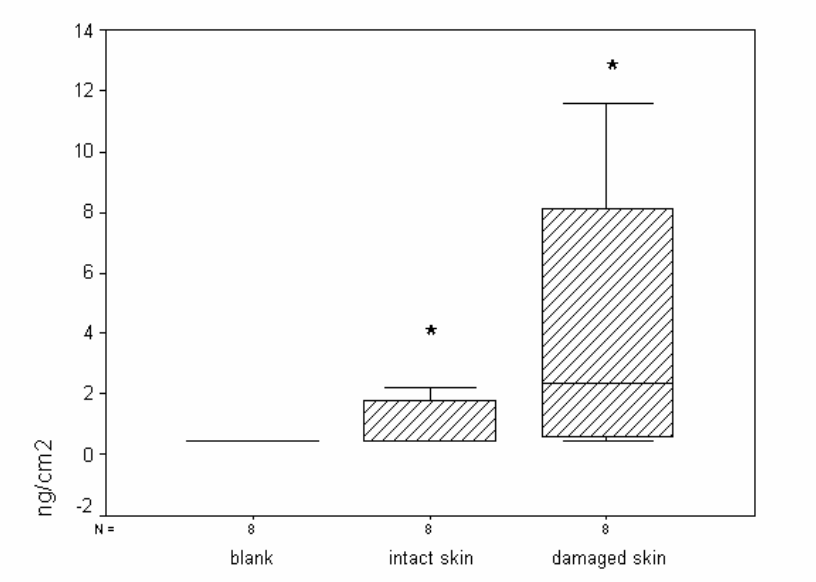

Figure 2. Silver skin penetration at 24 hours (the lines in the box plots represent the median values, the 25° and 75° percentiles).

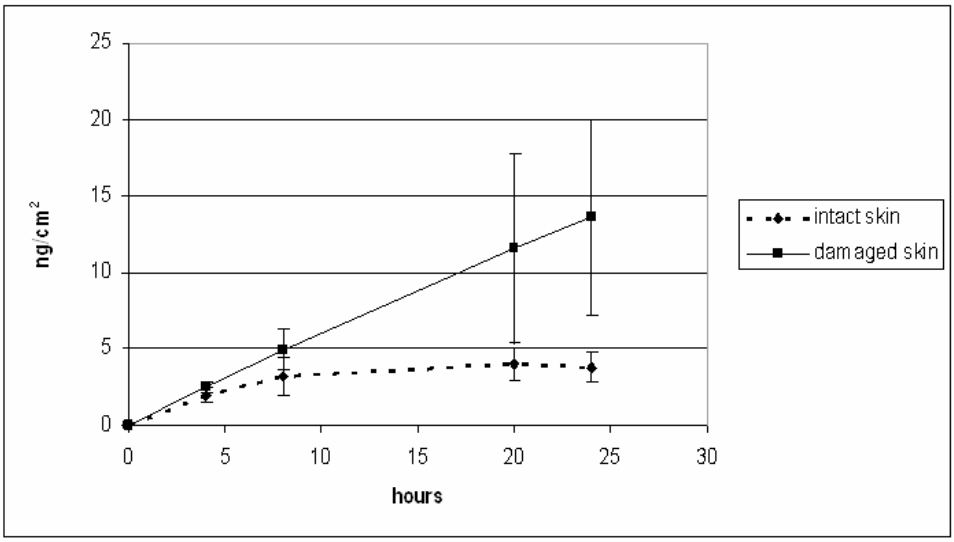

Figure 3. Silver skin penetration cumulative curve (means and SD) in the 24 hours.

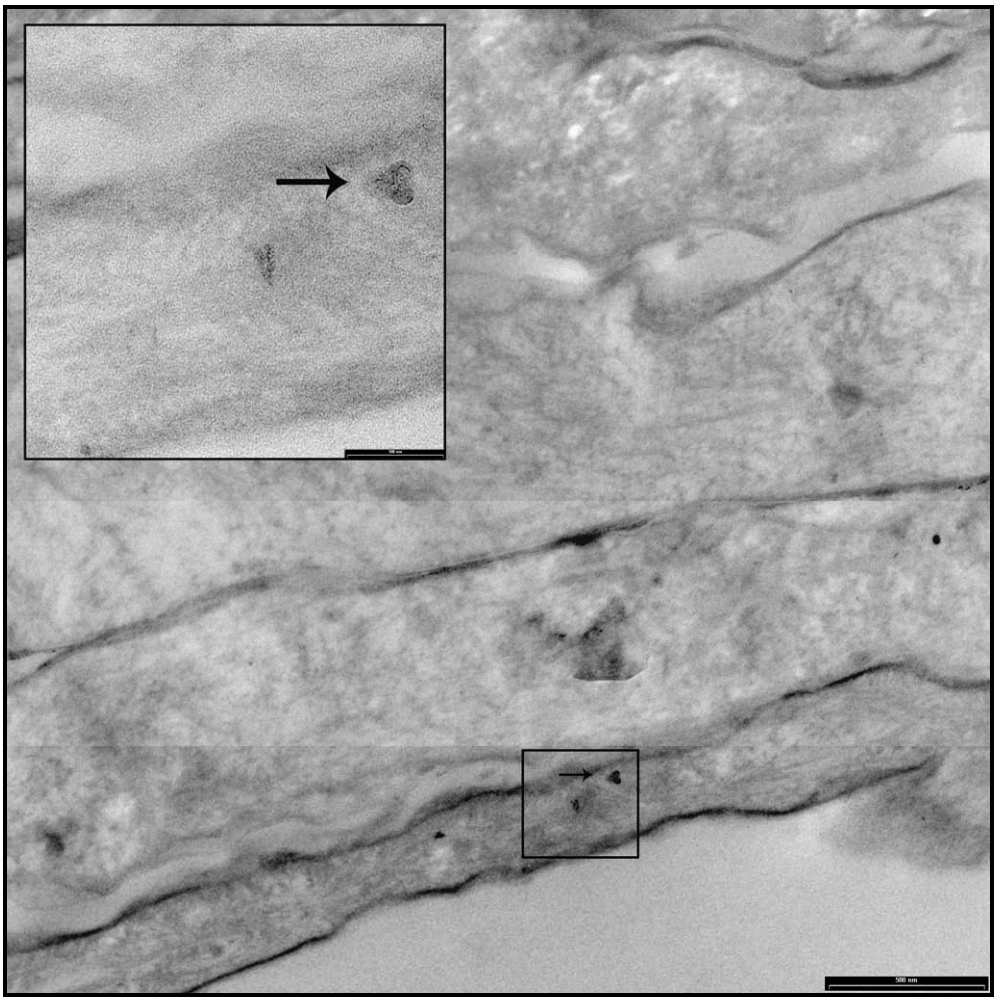

Figure 4. TEM micrograph of nanoparticles-treated skin sample. The black arrow shows nanoparticles on deep stratum corneum. Bars = 500nm and 100nm.

4. DISCUSSION

The present study investigated the skin absorption of coated silver nanoparticles using full thickness human skin (epidermis and dermis) both with intact and damaged skin barrier in an in-vitro diffusion cell system. In-vitro techniques are used all over the world to evaluate the percutaneous permeation of potentially toxic agents. Moreover, the permeability properties of the stratum corneum, that can be considered the rate-limiting step for skin absorption, are unchanged by removal from the body, so in-vitro tests give a number of advantages over all animal or human volunteer experiments (van Ravenzwaay and Leibold, 2004).

Small lipophilic molecules (<600 Da) can easily penetrate the skin passively (Barry, 2001). Also metallic ions (i.e. nickel and cobalt) are able to diffuse through human skin (Larese Filon et al., 2004, 2007). Nanoparticle dimensions are considered one of the most important parameters, because skin penetration can occur through pilosebaceus pores and

sweat gland pores: particles with diameter ranging between 7-20 nm were almost exclusively detected in the hair follicle infundibulum and below (Schaefer et al., 1990; Lauer et al., 2004; Toll et al., 2004; Meidan et al., 2005). Alvarez-Roman et al. (2004) found that 20-200 nm polystyrene nanoparticles were preferentially accumulated in follicle openings. Vogt et al. (2006) showed that 40-nm particles were able to penetrate the perifollicular dermis through the hair follicle. In contrast, for particle penetration through the intercellular transcutaneous pathway (through the stratum corneum lipidic matrix) van den Bergh et al. (1999) demonstrated that elastic particles of 100-150 nm could penetrate the stratum corneum matrix through channel-like structures remaining confined in it. Honeywell-Nguyen et al. (2004) found that elastic particles were able to reach the stratum corneum-viable epidermis junction whereas rigid particles were recovered only in the superficial layers of stratum corneum.

A recent study performed by Baroli et al. (2007) showed that metallic (iron) nanoparticles smaller than 10 nm are able to 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 do not permeate the skin.

To date, there has never been, to our knowledge, an in-vitro study of silver nanoparticles permeation through human skin, and none of previous researchers studied the effect of skin lesions on skin absorption. In fact, the amounts of silver permeating the skin found in the experiments performed with intact skin and in the blank cells were just above or below the analytical determination limit, while higher silver concentrations were found in the experiments with damaged skin. Data from this study suggest that human skin absorption of silver nanoparticles was relatively low compared with previously published absorption rates for metal powders (Larese Filon et al., 2004, 2007): silver flux was 25 times less than nickel powder flux and 200 time less than cobalt powder but only in condition of damaged skin. To note that the concentration of silver nanopaticles in donor phase was 70µg cm^{-2} while Co and Ni ions in donor phases were respectively 9 and 10 µg cm^{-2} .

However, our experiments showed not only that it is possible a low flux of silver after nanoparticles application on donor phase, but also that rigid nanoparticles smaller than 30 nm are able to penetrate passively the skin, reaching the deepest layers of the stratum corneum and the outermost surface of the epidermis. These results may suggest that a small fraction of the particles were dissolved and diffused through skin membranes as elemental silver. In accordance with this hypothesis, the solubilization and oxidation of nickel particles on the surface of the skin has been reported as the mechanism by which stratum corneum-diffusible compounds are generated and able to penetrate stratum corneum, probably by the intercellular

route (Hostynek et al., 2001).

Several factors and assumptions inherent to our in-vitro protocol may not represent real-life situations. On one hand, a limitation of this study is that it has been performed in static Franz cells where skin was static. On the other hand, flexing human epidermis has been suggested to lead to the increased translocation of small particles into the membrane (Tinkle et al., 2003).

Another aspect of our in-vitro experiments, which may not be realistic, is that the stratum corneum was exposed to simulate sweat for 24 hours. Excessive hydration is known to increase penetration of many compounds. Nevertheless, workers have often mechanically damaged skin that includes cuts and abrasions, which would make penetration substantially higher, as confirmed by this study.

In summary, the findings of this study provide the following new information on nanoparticle skin absorption: (i) there is experimental evidence for in-vitro nanoparticle skin permeation, and (ii) there is an appreciable increase in permeation using damaged skin.

This is the first time silver nanoparticles skin penetration was demonstrated: we need more data on nanoparticles skin absorption and their potential risk through the cutaneous route for occupational exposed people but also for consumers that can be repeated exposed to nanoparticles applied on the skin.

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ALLEGATO V

HUMAN SKIN PENETRATION OF GOLD NANOPARTICLES THROUGH INTACT AND DAMAGED SKIN

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Manoscritto in preparazione

ABSTRACT

The potential for solid nanoparticles to penetrate the skin lies at the centre of the debate concerning the safety for their use. Gold nanoparticles are produced for many applications in photocatalysts, photoelectrochemical materials, in medical fields as chronophores for immunoreactions. The benefits of nanoparticles have been shown in several scientific fields, but little is known about their potential to diffuse into tissues. Currently there is a lack of available data demonstrating whether manufactured nanoparticles can gain access to the epidermis and derma after skin contact even though it is known that dermally administered nanoparticles can transfer to regional lymph nodes via skin macrophages and Langerhans cells.

This study aims at evaluating *in vitro* skin penetration of gold nanoparticles. Experiments were performed using the Franz diffusion cell method with intact and damaged human skin. Physiological solution was used as receiving phase and 3ml of a solution containing 100 ppm of gold nanoparticles diluited 1:1 in water was applied as donor phase to the outer surface of the skin for 24 h. The receptor fluid measurements were performed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Human skin penetration was also determined by using Transmission Electron Microscope (TEM) to verify the location of gold nanoparticles in exposed membranes.

Mean gold content of 214±43.7 ng cm^{-2} and 187.7±50.2 ng cm^{-2} were found in the receiving solutions of cells where the nanoparticles solution was applied on intact skin (8 cells) and on damaged skin (8 cells) respectively. 24 hours gold flux permeation was 7.8±2.0 ng cm^{-2} h^{-1} in intact skin and 7.1±2.5 ng cm^{-2} h^{-1} in damaged skin, with a lag time < 1 hour. Moreover, gold nanoparticles could be detected in the stratum corneum, in the deeper layers of the epidermis and, finally in the dermis by electron microscopy.

This study showed that gold applied as nanoparticles is able to permeate the human skin in an in-vitro diffusion cell system.

KEYWORDS: Nanoparticles; Gold; Skin absorption; In vitro; Damaged skin

RIASSUNTO

La possibilità per nanoparticelle solide di penetrare all'interno della cute è uno dei punti al centro del dibattito sulla sicurezza del loro uso. Le nanoparticelle d'oro vengono prodotte per molte applicazioni, per esempio, in fotocatalisi, come cromofori per immunoreazioni o come carrier per farmaci, ecc. I vantaggi dell'uso delle nanoparticelle sono stati dimostrati in molti campi, ma poco si sa circa il loro potenziale di penetrazione e diffusione nei tessuti. Attualmente vi è una lacuna nei dati disponibili sulla capacità di nanoparticelle artificiali di raggiungere l'epidermide e il derma dopo essere venute in contatto con la cute, anche se è stato dimostrato che nanoparticelle somministrate nel derma sono in grado di migrare ai nodi linfatici trasportate dai macrofagi e dalle cellule di Langerhans.

Questo studio ha l'obiettivo di studiare la penetrazione di nanoparticelle d'oro in un sistema *in vitro*. Gli esperimenti sono stati condotti usando il metodo delle celle di diffusione di Franz con cute umana intera, integra e lesa. Come fase ricevente è stata usata soluzione fisiologica e come fase donatrice sono stati applicati, sulla superficie esterna della cute, 3 ml di una soluzione contente 100 ppm di nanoparticelle d'oro diluite in acqua milliQ, per 24 ore. Le analisi delle varie soluzioni sono state effettuate tramite Spettroscopia di Massa al Plasma Induttivamente Accoppiato (ICP-MS). Sul tessuto cutaneo sono state effettuate anche analisi di microsopia elettronica a trasmissione (TEM).

Nelle fasi riceventi è stato trovato un contenuto medio di oro di 214±44 ng cm^{-2} e di 188±50 ng cm^{-2} rispettivamente nelle celle con cute integra e nelle celle con cute lesa. Nelle 24 ore è stato calcolato un flusso di 7,8±2,0 ng cm^{-2} h^{-1} nella cute integra e 7,1±2,5 ng cm^{-2} h^{-1} nella cute lesa, con un lag time inferiore ad 1 ora. Le analisi al TEM hanno evidenziato nanoparticelle d'oro sia nell'epidermide che nel derma.

Questo studio ha mostrato che in un sistema di diffusione *in vitro* le nanoparticelle d'oro sono in grado di penetrare nello spessore cutaneo e di essere ritrovate nei vari strati della pelle.

PAROLE CHIAVE: Nanoparticelle; Oro; Assorbimento cutaneo; In vitro; Cute danneggiata

1. INTRODUCTION

Over the past decades an enormous interest in nanotechnologies has grown all over the world for their possible applications in a wide range of fields because of their small size (100 nm or less), comparable to that of biological molecules. In recent years the use of gold nanoparticles and nanorods is increasing since their valuable size and shape dependent properties are easy to control and modify and this offers powerful tools for the researchers activity (Penn et al., 2003; Gupta and Gupta, 2005; Neuberger et al., 2005; Chen et al., 2008). In particular, gold nanomaterials are rising as a lead candidate in the transport of payloads inside tissues and cells for drug delivery, gene therapy and cell imaging. Rosi et al. (2005) described the use of gold nanoparticle-oligonucleotide complexes as intracellular gene regulation agents for the control of protein expression in cells. These particles were stable against enzymatic digestion and carried oligonucleotides at a higher effective concentration than conventional transfection agents. Kawano et al. (2006) investigated the benefits of combining the use of PEG-modified cationic gold nanoparticles with electroporation for in-vivo gene delivery. The authors concluded that these modified nanoparticles maintained DNA more stably in the blood flow than in the case of naked DNA and electroporation assisted in restricted gene expression of circulating DNA in limited areas of the liver. Several other studies have been carried out on the use of modified gold nanomaterials in the imaging and therapy of tumors (Bhattacharya and P. Mukherjee, 2008; El-Sayed et al., 2006), in the direct welding of connective tissues [Gobin et al., 2005; Ratto et al., 2009), as immunostaining marker particles for electron microscopy, chromophores for immunoreactions (Mirkin et al., 1996), photocatalyst (Kamat, 2002) and Raman sensors (Tian et al., 2002).

Gold nanoparticles are stable in the body (Niidome et al., 2006), but it is datable whether gold nanoparticles are biocompatible and more data are needed to better understand their fate into the body (Lewinski et al., 2008, Takahashi et al., 2006).

Therefore, among the great number of applications, gold nanoparticles would be an interesting carrier for transdermal delivery (Chen et al., 2008), but to our knowledge, there are no data on their skin permeation in in-vitro system using human skin.

Larese et al. (2009a) demonstrated the permeation of silver nanoparticles sized 25 nm through intact and damaged human skin (0.46 ng cm^{-2} and 2.32 ng cm^{-2} respectively) using the Franz cell method (Franz., 1975), while Sonavane et al. (2008) revealed higher skin and intestine in-vitro permeation of gold nanoparticles with smaller size (15 nm) in rats. Diffusion data would permit to increase knowledge on gold nanoparticles.

To investigate the potential gold nanoparticles skin absorption in-vitro, we carried out a series of permeation experiments with human skin using a Franz static diffusion cell. Using this technique, we investigated the total amount of gold permeating through human skin during a 24-hour period. Experiments were run with intact skin as well as abraded skin following the Bronaugh and Steward (1985) protocol to estimate the effect of skin lesions on the permeation rate. In this study we applied the protocol adopted in the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals), a three-year research program (2001-2004) financially supported by the European Commission (EDETOX, 2000).

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were analytical grade Urea, magnesium nitrate hexahydrate, palladium nitrate, sodium hydrogenphosphate, potassium dihydrogenphosphate, and hydrogen peroxide (30%) were purchased from Carlo Erba (Milan, Italy); ammonium hydroxide (25%) from J. T. Baker (Deventer, Holland); lactic acid (90%) from Acros Organics (Geel, Belgium); sodium tetrachloroauric acid, trisodium citrate dihydrate, nitric acid (>69%) and hydrochloric acid (36.5-38%) from Sigma Aldrich (Milan, Italy). Water reagent grade was produced with a Millipore purification pack system (milliQ water).

The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na_2HP0_4, 0.19 g of KH_2P0_4 and 9 g of NaCl into 1 L of milliQ water (final pH = 7.35).

The synthetic sweat solution used as the donor fluid included 0.5% sodium chloride, 0.1% urea and 0.1% lactic acid in milliQ water; pH 4.5 was adjusted with ammonia.

2.2. Nanoparticle synthesis and characterization

Gold nanoparticles were synthesized by reducing tetrachloroauric acid with sodium citrate as described by Grabar et al. 1995. Trasmission Electron Microscopy (TEM) was used to evaluate nanoparticles size and stability before and after experimentsll as 12.6 ± 0.9 nm (number of nanoparticles measured: 345).

Total gold concentration was analysed by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES): 1 ml of nanoparticle suspension was treated with 1 ml of Aqua regia (a solution having concentrated nitric acid and concentrated hydrochloric acid mixed at

a volume ratio of 1:3) at room temperature and the volume was adjusted to 10 ml with milliQ water. The gold solution was measured at a wavelength of 267.595 nm. A five-point standard curve was used for the analytical measurements. The correlation coefficient of the standard curve was 0.9989. The samples were analysed by measuring against standard solutions for instrumental calibration. The coefficient of variation (CV) for the analysis of each metal was less than 3% and the limit of detection was 0.030 mg L^{-1}. The determined concentration of gold in the initial nanoparticle suspension was 100±5 mg L^{-1} (n = 3).

In order to evaluate the possible presence of gold ions in solution, 10 ml of gold nanoparticle suspension was added with 10 mg of sodium chloride to aggregate the nanoparticles and then centrifuged; the surfactant was then removed and analysed by mean of ICP-AES. Concentration of gold was under the limit of detection (0.030 mg L^{-1}).

2.3. Preparation of skin membranes

Human abdominal full thickness skin was obtained as surgical waste after the authorization of the local Ethical Committee. Prior to freezing, subcutaneous fat was removed and hair shaved by a razor. All the pieces of full thickness skin were stored in freezer at -25°C for a period up to, but not exceeding, 4 months. It has been shown that this method of storage does not damage the skin since no difference in permeability was observed between fresh and frozen segments of the same skin in a separate series of experiments (Franz, 1975). Skin integrity was tested before and after each experiment using electrical conductibility by means of a conductometer (Metrohm, 660, Metrohm AG Oberdorfstr. 68 CH-9100 Herisau) operating at 300Hz and connected to two stainless steel electrodes (Fasano et al., 2002). The conductibility data in μS were converted into KΩ cm^{-2}. Cells with a resistance lower than 3.95±0.27 KΩ cm^{-2} were considered to be damaged as suggested by Davies et al. (2004)

Donors were men and women with a wide range of age (41-71 years).

2.4. *In vitro* diffusion system

Percutaneous absorption studies were performed using static diffusion cells following the Franz method. The receptor compartment had a mean volume of 14 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 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:

Experiment 1

At time 0, the exposure chambers of 8 Franz diffusion cells were filled with 0.5 ml solution containing 100 mg L^{-1} of nanoparticles solution diluted 1:3 (15 µg cm^{-2}) with synthetic sweat at pH 4.5 to reproduce *in vivo* condition.

At 24 h the dermal bathing solution was removed and gold concentration was determined in each receiving chamber solution.

Experiment 2

Experiment 1 was repeated using an abraded skin protocol as suggested by Bronaugh 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).

Experiment 3

At time 0, the exposure chambers of 8 Franz diffusion cells were filled with 1.5 ml solution containing 100 mg L^{-1} of nanoparticles solution diluted 1:1 with milliQ water (45 µg cm^{-2}). At selected intervals (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.

Experiment 4

Experiment 3 was repeated using an abraded skin protocol as described above.

Blanks: for each experiment, four cells were added as blank. The blank cells were treated as the other cells with the exception that no gold nanoparticles was introduced to the exposure chamber.

Experiments 1 and 2 were preliminary tests to evaluate nanoparticles permeation using the same protocol previously reported (Larese et al., 2009a). Experiments 3 and 4 were performed using a higher gold concentration in milliQ water to avoid interference of the saline on gold nanoparticles .

2.5. Skin content evaluation

After the experiments, the skin pieces were removed from the Franz cells. Damaged skin samples were stored in freezer at –25 °C while intact skin samples were separated into epidermis and dermis by heat shock immerging in water at 60°C for 1 min. For 3 samples, the stratum corneum was previous removed by stripping with PVC adhesive tape (Tesa–Beiersdorf AG, Hamburg, Germany). All the skin fractions were collected and stored in freezer at –25 °C for the following digestion and analysis. At the time of the analysis, the skin membranes were dried for 24 hours at 100° C and then cut into sections and put into bakers with 25 ml of HNO_3 70% for digestion (amounts of skin were between 0.5 and 0.8 g). They were agitated for 20 hours at 100°C (after 2 hours they were added, drop by drop, of 5 ml of H_2O_2) till the solutions remained were of few millilitres. The solutions were diluted with 25 ml of milliQ water for the analysis with ICP-MS.

2.6. Analytical Measurements

The receptor fluid measurements were performed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). An Agilent 7500ce (2005) instrument equipped with a collision cell was used for analysis. Calibration curves were obtained from four different standards at 1, 5, 10, 50 μg L^{-1}. The limit of detection (LOD) was 0.001 μg L^{-1}. The correlation coefficient (r) of the standard curve was 0.9985. The CV for the analysis was always less than 3%.

2.7. 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 skin sections were washed twice (10 min each) with 0.1 M cacodylate buffer and then post fixed with 1% osmium tetroxide for 1 h at 4°C. Post fixed samples were dehydrated with an ascending ethanol series ending with 100% ethanol and then embedded in Dow 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 Ultramicrotome Leica Ultracut UCT (Leica Microsystems, Milan, Italy) equipped with a diamond blade Drukker 3mm (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 a Transmission Electron Microscope (EM208; Philips, Eindhoven, The Netherlands) with an high definition acquisition system SIS Morada and a digital image

acquisition system iTEM (FEI Italia, Milan, Italy).

2.8. Data analysis

Gold concentration data (µg cm^{-3}) in the receptor solution were converted to the total amount that penetrated (µg cm^{-2}), with a correction for dilution due to sample removal.

Data analysis was performed using the statistical software SPSS for Windows (version 15.0). All data were 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.

3. RESULTS

Gold nanoparticles characterized by TEM are reported in figure 1. The dimensions were uniform in size, as small as 12.6±0.9 nm. In the experiments 1 and 2 NPs aggregated in the donor phases. In experiments 3 and 4 NP were stable after 24 hours.

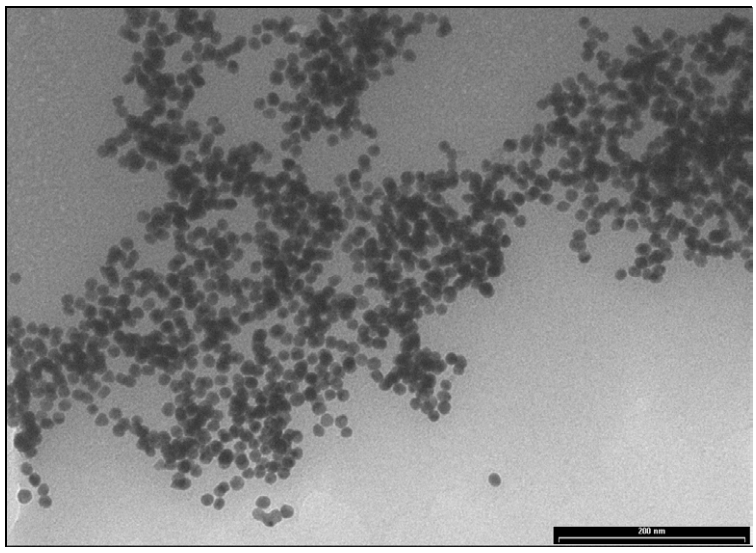

Figure 1. Gold nanoparticles in the donor phase visualized by TEM.

Gold nanoparticles applied in synthetic sweat (experiments 1 and 2) passed through both the intact and the damaged human skin with a mean amount of 60.8±25.3 ng cm^{-2} and 55.2±27.3 ng cm^{-2} at 24 hours, respectively.

Gold nanoparticles applied in milliQ water (experiments 3 and 4) revealed an higher permeation reaching in 24 hours 214±44 ng cm^{-2} and 188±50 ng cm^{-2} in intact and damaged

skin, respectively (Mann-Whitney test: $p<0.005$ between experiments 1 and 3 and 2 and 4).

Figure 2 displays the permeation profile of gold nanoparticles in intact and damaged skin (exp 3-4). 24 hours gold flux permeation was 7.8±2.0 ng cm^{-2} h^{-1} and 7.1±2.5 ng cm^{-2} h^{-1} in intact and damaged skin ($p=0.4$) respectively, with a lag time < 1 hour.

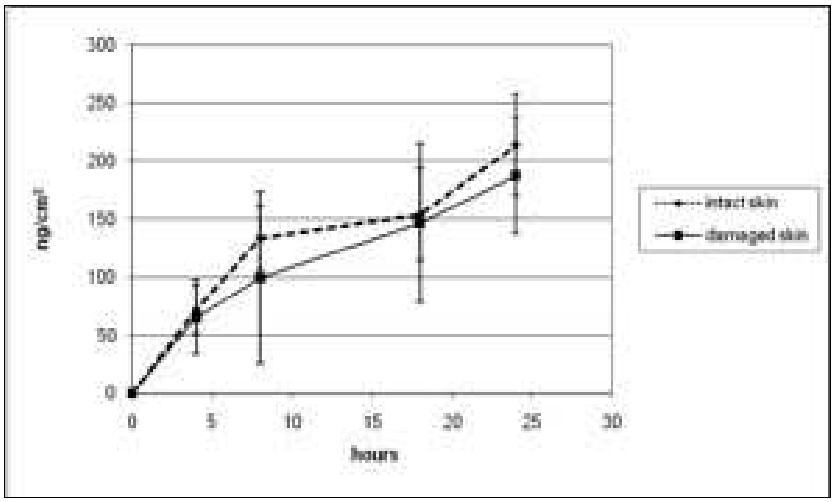

Figure 2. Gold nanoparticles permeation profiles in intact and damaged skin (exp 3 - 4).

Figure 3 shows that the gold concentration into the damage skin was significantly greater than into the intact skin (7.86±4.12 µg cm^{-2} and 1.82±0.69 µg cm^{-2}, respectively; $p<0.009$).

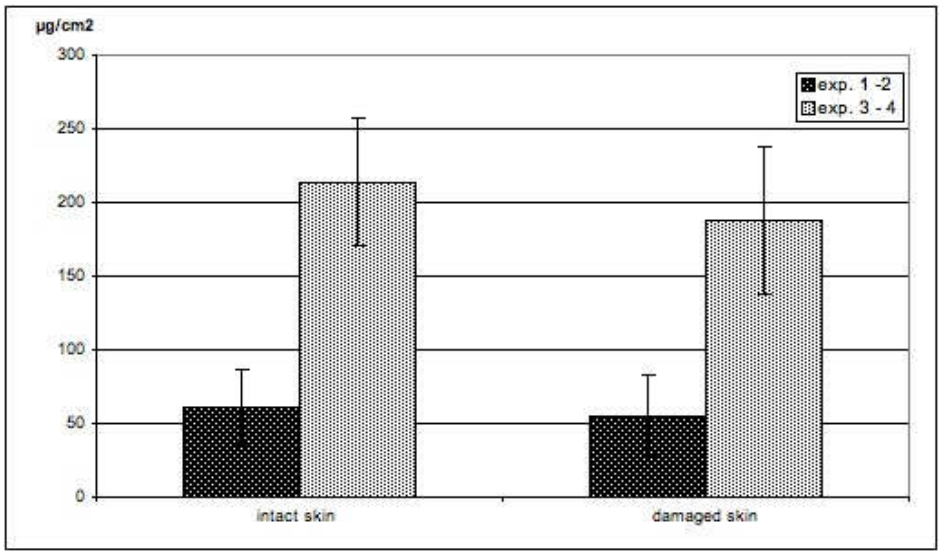

*Figure 3. Gold content (exp 1 - 2 an 3 - 4) into damaged and intact skin (means and standard deviation; * $p<0.009$)*

The measurement of gold into the stratum corneum, epidermis and dermis of the intact skin revealed a decrease in gold content from the external to the internal site ($p<0.025$).

Figure 4 shows the gold content into strip numbers ranging from 0.40±0.05 μg cm^{-2} in the superficial strip (1^{st}) to 0.17±0.01 μg cm^{-2} into the deepest strip (6^{th}) ($p<0.025$).

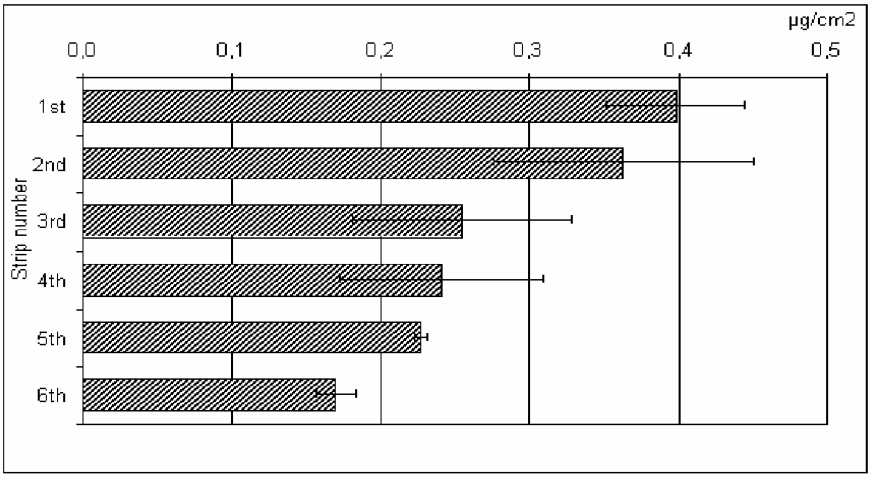

*Figure 4. Gold content (exp 3) into stratum corneum tape stripping (means and standard deviation; *p<0.025). From 1st more superficial to 6th more deep.*

The gold content into the epidermis (1.74±0.68 μg cm^{-2}) and into the derma (0.05±0.02 μg cm^{-2}) revealed a significant decrease in gold content in the deepest layers of the skin ($p<0.005$, Figure 5).

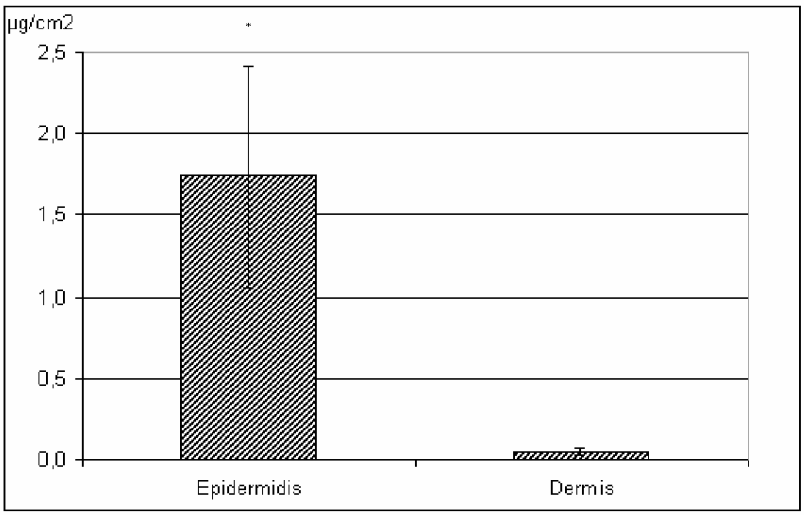

*Figure 5. Gold content (exp 3) into epidermis and dermis of intact skin samples (means and standard deviation; * p<0.005)*

The TEM investigation showed that in some slices of both intact and damaged skin, gold nanoparticles were located in the stratum corneum and in the epidermis, but also in the derma (Figure 6).

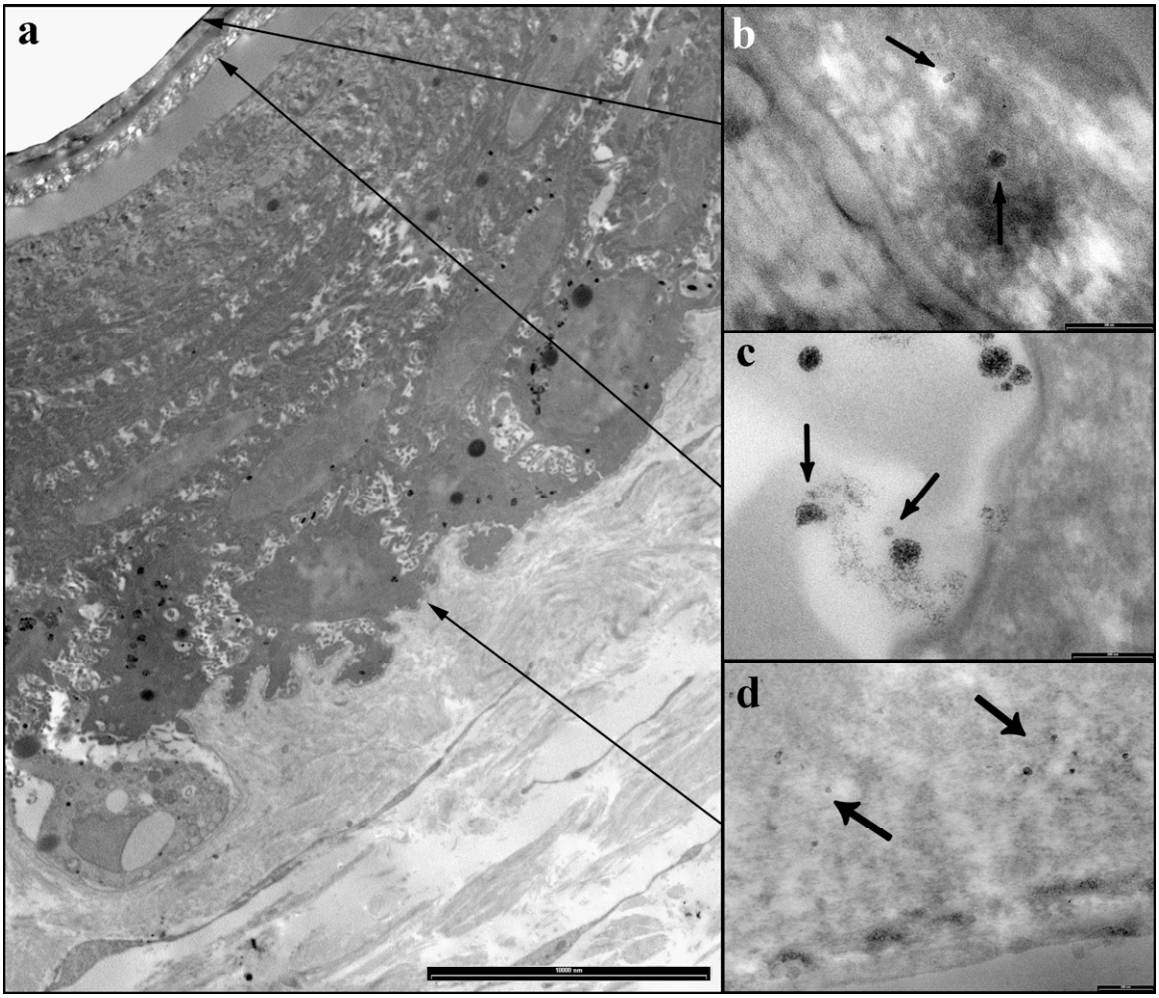

Figure 6. TEM micrographs of nanoparticles-treated intact skin sample. The black arrow shows nanoparticles on different dermal leyers. Bars: a = 10 µm; b,c,d = 200nm.

4. DISCUSSION

The present study investigated the skin absorption of gold nanoparticles using full thickness human skin (epidermis and dermis) with both intact and damaged skin barrier in an in-vitro diffusion cell system.

Nanoparticles skin permeation is something new and there are very few data available in the literature. For TiO2 the results of the three-year European project NANODERM (2007) reported no evidence of nanoparticle transcutaneous penetration (NANODERM, 2007; Kiss et al., 2008). In opposite, Baroli et al. (2007) showed that metallic (iron) nanoparticles smaller than 10 nm are able to 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. The authors, however, found that the nanoparticles

did not permeate the skin. Iron is a metal present in high concentration into human skin and this may explain the failure to observe iron nanoparticles skin permeation using Franz cells.

It is known that metallic ions (e.g. nickel and cobalt) are able to diffuse through the human skin (Larese et al.,2004; 2006) and that silver nanoparticles had a permeation profile higher in the damaged skin than in the intact skin (Larese et al., 2009a). Skin penetration can occur through pilosebaceus pores and sweat gland pores: particles with diameter ranging between 7-20 nm are almost exclusively detected in the hair follicle infundibulum and more deeper (Lauer et al., 1996; Meidan et al., 2006; Schaefer et al., 1990; Toll et al., 2004). Vogt et al. (2006) showed that 40-nm particles were able to penetrate the perifollicular dermis through the hair follicle. For gold nanoparticles a skin permeation was already reported by Sanovane et al. (2008) into rat skin and intestine; our study confirm this capability also in human skin.

The findings of this study suggest that human skin absorption of gold nanoparticles could be relevantboth in the intact and the damaged skin. Moreover, gold nanoparticles permeation seems to be greater that silver nanoparticles permeation evaluated in our previous experimental study (Larese et al., 2009a). Gold nanoparticles can easily permeate the skin and might be a valuable carrier for transdermal delivery.

The permeation results was lower in 1st and 2nd experiments probably due to the less amount of gold applied and to the fact that in some cells gold nanoparticles aggregated during the 24 hours of the experiments: this effect was related to the use of synthetic sweat in donor phases. The use of synthetic sweat in donor phases is common in this kind of experiments (Larese et al., 2004) because synthetic sweat can increase metal permeation for his ionizing action (Liden and Carter, 2001). In the experiments 3 and 4 nanoparticles were stable and permeation increased significantly.

The evaluation of gold content into the skin revealed that gold concentration decreased from the superficial to the deeper layers of the skin and a significant greater gold concentration was found in damaged skin compared to the intact skin This finding is in accord with other *in vitro* studies were damaged skin contained higher amount of silver nanoparticles (Larese et al., 2009a), cobalt, nickel and chromium powders (Larese et al., 2009b).

Microscopic investigation results showed that it is possible that rigid nanoparticles are able to penetrate passively the skin, reaching non only the deepest layers of the stratum corneum and the epidermis, but also the derma. This important finding suggests that gold nanoparticles could be absorbed in the systemic circulationa after permeation through the skin.

There are some limitations of this study that deserve attention. A limitation is that the experiments were carried out with Franz cells in which skin was static. Flexing human epidermis has been suggested to lead to the increased translocation of small particles into the membrane (Rouse et al., 2007; Tinkle et al., 2003).

Another limitation of our in-vitro experiments is that the stratum corneum was exposed for 24 hours. Excessive hydration is known to increase penetration of many compounds. Nevertheless, people have often mechanically damaged skin such as cuts and abrasions, which would make penetration substantially higher.

In conclusion, the findings of this experimental study showed that gold nanoparticles could be useful for transdermal delivery applications and that this kind of nanoparticles can permeate the skin in greater amount than other nanoparticles such us silver nanoparticles (Larese et al., 2009a). More studied are needed to evaluate skin absorption using different size and shape of gold nanoparticles.

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APPENDICE

PUBBLICAZIONI SCIENTIFICHE

D'Agostin F., **Crosera Matteo**, Adami G., Malvestio A., Rosani R., Bovenzi M., Maina G., Larese Filon F. Abstract: In vitro percutaneous absorption of chromium powder and the effect of skin cleanser. Giornale Italiano di Medicina del Lavoro ed Ergonomia 2007, 29 (3 Suppl): 452-4.

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Larese Filon F., D'Agostin F., **Crosera Matteo**, Adami G., Renzi N., Bovenzi M., Maina M. Human skin penetration of silver nanoparticles through intact and damaged skin. Toxicology 2009 (255) 33-37.

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PARTECIPAZIONE A CONGRESSI NAZIONALI ED INTERNAZIONALI

Durante il Corso di Dottorato in Scienze della Prevenzione, si è cercato di diffondere i risultati della nostra ricerca attraverso la partecipazione a congressi nazionali ed internazionali con poster, abstract e comunicazioni orali, riportate di seguito:

M. Crosera, G. Adami, F.Larese, E. Reisenhofer e M. Bovenzi "Assorbimento cutaneo in vitro di polvere ultrafine di cromo" XX Congresso Nazionale di Chimica Analitica, S. Martino al Cimino (VT), 16-20 Settembre 2007.

G. Adami, S. Cozzi, **M. Crosera**, F. Lo Coco e E. Reisenhofer "Determinazione mediante titolazione amperometrica con europio(III) di specie complessanti nell'acqua di mare del Golfo di Trieste" XX Congresso Nazionale di Chimica Analitica, S. Martino al Cimino (VT), 16-20 Settembre 2007.

F. D'Agostin, **M. Crosera**, G. Adami, A. Malvestio, R. Rosani, M. Bovenzi, G. Maina, F. Larese Filon. "Assorbimento percutaneo di polvere di cromo ed effetti della detersione: risultati di una indagine in vitro" 70° Congresso Nazionale SIMLII (Società Italiana di Medicina del Lavoro e Igiene Industriale). Roma, 12-15 dicembre 2007.

F. Larese Filon, F. D'Agostin, **M. Crosera**, G. Adami, R. Rosani, C. Romano, M. Bovenzi, G. Maina. "Assorbimento percutaneo di nanoparticelle di argento in un sistema in vitro" 70° Congresso Nazionale SIMLII (Società Italiana di Medicina del Lavoro e Igiene Industriale). Roma, 12-15 dicembre 2007.

F. Larese Filon, F. D'Agostin, M. Bovenzi, **M. Crosera**, G. Adami, C. Romano, G. Maina. "Human skin penetration of silver nanoparticles: in-vitro assessment" Perspective in Percutaneous Penetration, PPP2008, Eleventh International Conference, La Grande-Motte, France, 25-29 marzo 2008.

F. Larese Filon, F. D'Agostin, **M. Crosera**, M. Bovenzi, G. Adami, G. Maina. "Human skin penetration of silver nanoparticles" Nanotox 2008, 2nd International Conference on Nanotoxicology, Zurigo, 7-10 Settembre 2008.

M. Crosera, F. Larese Filon, F. D'Agostin, M. Bovenzi, G. Maina, G. Adami "Assorbimento cutaneo in vitro di polveri di cobalto, nichel e cromo attraverso cute umana integra e lesa" XXI Congresso Nazionale di Chimica Analitica, Arcavacata di Rende (CS), 21-25 Settembre 2008.

C. Zanette, C. Florio, **M. Crosera**, G. Adami, M. Bovenzi, F. Filon Larese. "Effetto citotossico di nanoparticelle d'argento su cheratinociti umani HaCaT in coltura" XV Congresso Nazionale della Società Italiana di Tossicologia, Verona, 19-22 gennaio 2009.

C. Zanette, C. Florio, **M. Crosera**, G. Adami, M. Bovenzi, F. Filon Larese. "Cytotoxicity of silver nanoparticles on human HaCaT keratinocytes" OEESC2009, Occupational and Environmental Exposure of the Skin to Chemicals, Edimburgo, 14-17 giugno 2009.

F. Larese Filon, **M. Crosera**, G. Adami, F. D'Agostin, M. Bovenzi, G. Maina. "Human skin penetration of gold nanoparticles: an in-vitro assessment" OEESC2009, Occupational and Environmental Exposure of the Skin to Chemicals, Edimburgo, 14-17 giugno 2009.

M. Crosera, F. Larese Filon, M. Bovenzi, G. Maina, E. Reisenhofer, G. Adami. "Human skin penetration of gold nanoparticles through intact and damaged skin" XXIII Congresso Nazionale della Società Chimica Italiana, Sorrento, 5-10 Luglio 2009.

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