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ORIGINAL ARTICLE

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Patch test–relevant concentrations of metal salts cause localized cytotoxicity, including apoptosis, in skin ex vivo

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Abstract

Background: Metal alloys containing contact sensitizers (nickel, palladium, titanium) are extensively used in medical devices, in particular dentistry and orthopaedic surgery. The skin patch test is used to test for metal allergy.

Objective: To determine whether metal salts, when applied to freshly excised skin at patch test–relevant concentrations and using a method which mimics skin patch testing, cause in changes in the epidermis and dermis.

Methods: Tissue histology, apoptosis, metabolic activity, and inflammatory cytokine release were determined for two nickel salts, two palladium salts, and four titanium salts.

Results: Patch test–relevant concentrations of all metal salts caused localized cytotoxicity. This was observed as epidermis separation at the basement membrane zone, formation of vacuoles, apoptotic nuclei, decreased metabolic activity, and (pro)inflammatory cytokine release. Nickel(II) sulfate hexahydrate, nickel(II) chloride hexahydrate, titanium(IV) bis(ammonium lactato)dihydroxide, and calcium titanate were highly cytotoxic. Palladium(II) chloride, sodium tetrachloropalladate(II), titanium(IV) isopropoxide, and titanium(IV) dioxide showed mild cytotoxicity.

Conclusion: The patch test in itself may be damaging to the skin of the patient being tested. These results need further verification with biopsies obtained during clinical patch testing. The future challenge is to remain above the elicitation threshold at noncytotoxic metal concentrations.

KEYWORDS
allergy, apoptosis, cytotoxicity, excised skin, nickel, palladium, patch test, titanium

[Correction added on 08 October after first online publication: The author Rosalien Veldhuizen was inadvertently removed from the list of authors and has been added to this current version. Her contribution has been included in the Author Contribution section.]

1 | INTRODUCTION

Metal alloys are extensively applied in medical devices, in particular in dentistry and orthopaedic surgery. These metal alloys may contain metals such as nickel, palladium, and titanium which are known to cause allergies. Clinical experience indicates that these metals may be related to type IV hypersensitivity (allergic contact dermatitis) and/or chronic inflammation of adjacent tissues due to leachables arising from metal corrosion.[1–5] This suggests that these metals may not only be contact sensitizers but also have irritant, or in extreme cases, cytotoxic properties. Although dental medical devices (eg, abutments, implants, wires) are in direct contact with the oral mucosa and orthopaedic medical devices are implanted into the body subcutaneously (eg, hip and knee implants), the gold standard for testing whether indeed an individual has an allergy to his/her implant material is still the skin patch test. This clinical diagnostic testing for suspected contact allergy is carried out by applying the metal test chemical in the form of a salt to the skin under standardized conditions (patch testing). However, it is often not taken into account that a number of different salts exist for each metal with different penetration and irritant properties which may seriously confound the interpretation of the patch test results. Also, importantly, it is not taken into account that applying these metal salts, in addition to potentially sensitizing the individual, may result in damage to the underlying skin and may even trigger cell death in the form of apoptosis. This would indicate that the patch test in itself could be damaging to the skin of the patient being tested.

Nickel is considered one of the most common sensitizers, affecting a large proportion of the European population, and even after the implementation of the EU Nickel Directive, the prevalence of nickel allergy remains high particularly among women (approximately 20%).[3,4,6] Although nickel easily corrodes in the oral environment, it is still widely used in dental devices. For example, there is still no adequate alternative to the nitinol (nickel-titanium) wire used in orthodontic treatments due to its unique properties in maintaining shape and superelasticity.[7] Although 5% hydrous nickel sulfate has been reported to give less reliable diagnostic results and therefore have low clinical relevance in patch testing,[8,9] it is still the gold standard according to the ESCD and 2.5% nickel sulfate is used in North American Guideline.[9,10] An alternative nickel salt for patch testing is nickel chloride which has been reported to show a stronger positive reaction than nickel sulfate in the patch test.[11]

Palladium is commonly found in dental devices since, due to its low price in the 1980s, it has gradually replaced gold and platinum as an appropriate component in casting alloys.[12] In a multiclinical study including 1651 patients with suspected allergy to palladium, twice as many patients (18%) tested positive to palladium allergy when 3% sodium tetrachloropalladate hydrate (86.21 mM) was used as patch test salt compared with the more frequently used 2% anhydrous palladium chloride (112.78 mM).[13] The reason for this is now thought to be due to the ability of sodium tetrachloropalladate to more easily penetrate the stratum corneum than palladium chloride.[13–15]

Titanium is combined with various elements to produce durable, lightweight alloys that are biocompatible supporting osseointegration, provide resistance against corrosion, and have a very high tensile strength. Hence, titanium and its alloys are considered to be the material of choice for dental implants and abutments.[16] Titanium is regarded as an inert metal due to its generally accepted high biocompatibility and resistance to corrosion. However, multiple cases of implant failure of titanium-based implants have been reported after surgery. Although the exact cause for this is still under debate, it may be due to the implant environment leading to corrosion of titanium products that in turn leads to immunological reactions.[17,18] Until now, there is no standard diagnostic patch test for titanium and therefore a number of different salts are currently under investigation (eg, titanium dioxide, calcium titanate, titanium bis[ammonium lactato] dihydroxide at patch test concentration 10%-20% [VUMC outpatient clinic]) and within the laboratory (titanium isopropoxide). As it has still not yet been confirmed that titanium is indeed a sensitizer, it is not possible to distinguish true negatives from false negatives.[18] However, titanium(IV)-specific lymphocytes have been generated in vitro, indicating that titanium may indeed be a sensitizer.[19]

In this study, we expand on our recently published studies describing the use of “reconstructed human skin” and “reconstructed human epidermis” (RhE) to determine the sensitizing and irritant potential of metal salts.[20–26] In the past, we have also described the influence of a common commensal microbe (*Streptococcus mitis*) on the innate immune response of both skin and gingiva to metals.[27] These combined studies indicate that nickel and palladium salts have clear irritant properties, relating to their sensitizing potency. Furthermore, nickel could be identified as a sensitizer whose potency increased when applied to the skin and gingiva in the presence of microbes. By contrast, in our in vitro studies, titanium scored as a very weak irritant nonsensitizer. The aim of this study was to determine whether metal salts, when applied to freshly excised skin at patch test–relevant concentrations and using a method which closely mimics the skin patch test, have detrimental effects in the epidermis and dermis. Tissue histology, metabolic activity, signs of apoptosis, and a triggering of inflammatory cytokine release were determined. To determine whether the observed effects were metal salt dependent, two nickel salts, two palladium salts, and four titanium salts were investigated.

2 | MATERIALS AND METHODS

2.1 | Human skin

Healthy human skin was obtained from patients undergoing plastic surgery, according to the procedures of VU University Medical Center. Human skin was used anonymously, in accordance with the *Code for Proper Use of Human Tissue*, as formulated by the Dutch Federation of Medical Scientific Societies.[28]

The excised skin was used directly after surgery; the subcutaneous fat was carefully removed using a scalpel and forceps, as previously described.[29] Pieces of skin (approximately 4 cm^2) were then placed on Transwell inserts (0.4-μm pore size; Corning, New York, USA) and cultured at the air-liquid interface. Culture medium consisted of Dulbecco's modified Eagle medium (Lonza, Basel, Switzerland)/Ham's F-12 (Gibco, Paisley, UK; 3:1), 1% Ultroser G (BioSepra S. A., Cergy-Saint-Christophe, France), 1% penicillin-streptomycin (Gibco, Paisley, UK), 1 μM/L isoproterenol (Sigma-Aldrich, Missouri, USA), 0.1 μM/L insulin (Sigma-Aldrich, Missouri, USA), and 2 ng/mL keratinocyte growth factor (Sigma-Aldrich, Missouri, USA). The skin was incubated at 37°C, 5% CO_2, and 95% relative humidity overnight.

2.2 | Chemicals and chemical exposure

A total of eight metal salts were tested (Table 1). To explore the cytotoxicity of the metals, two different metal salts were tested for nickel and palladium, and four different metal salts were tested for titanium. All chemicals were purchased from Sigma-Aldrich. Skin was topically exposed to chemicals as previously described.[20] In short, the metal salts were dissolved in distilled water or acetone olive oil (AOO; 4:1) at concentrations of 2.5%, 5%, 10%, and 20% as indicated in Table 1. The rationale behind the choice of vehicle was according to the RhE–IL-18 (interleukin-18) prevalidation study standard operating procedure, where it is described that if a chemical is water soluble, then this

TABLE 1 Test chemicals and vehicles

Formula	Name	CAS #	Vehicle	% w/v	Molarity (mM)	pH
$NiSO_4 \cdot 6H_2O$ (soluble)	Nickel(II) sulfate hexahydrate	10 101–97-0	Water	2.5	95.11	4.6
				5.0	190.22	4.3
				10	380.45	4.1
				20	760.89	3.8
$NiCl_2 \cdot 6H_2O$ (soluble)	Nickel(II) chloride hexahydrate	7791–20-0	Water	2.5	105.18	5.3
				5.0	210.36	5.2
				10	420.72	5.0
				20	841.43	4.8
$PdCl_2$ (insoluble)	Palladium(II) chloride	7647–10-1	Water	2.5	140.98	3.0
				5.0	281.96	3.0
				10	563.92	2.7
				20	1127.84	2.7
Na_2PdCl_4 (soluble)	Sodium tetrachloropalladate(II)	13820–53-6	Water	2.5	84.97	3.6
				5.0	169.95	3.4
				10	339.89	3.3
				20	679.79	3.1
$C_{12}H_{28}O_4Ti$ (soluble)	Titanium(IV) isopropoxide	546-68-9	AOO	2.5	87.96	4.7
				5.0	175.92	4.7
				10	351.84	4.7
				20	703.68	4.8
TiO_2 (insoluble)	Titanium(IV) dioxide	13463–67-7	AOO	2.5	313.01	4.8
				5.0	626.02	4.8
				10	1252.03	4.8
				20	2504.07	4.9
$C_6H_{18}N_2O_8Ti$ (soluble)	Titanium(IV) bis(ammonium lactato) dihydroxide	65104-06-5	Water	2.5	85.01	4.5
				5.0	170.02	4.5
				10	340.04	6
				20	680.09	7.5
$CaTiO_3$ (insoluble, nanoparticle)	Calcium titanate	12049-50-2	AOO	2.5	183.90	5.0
				5.0	367.81	5.0
				10	735.62	5.0
				20	1471.24	5.0

Note: The vehicles used to dissolve the chemicals were acetone olive oil (4:1) and water; water was distilled.
Abbreviations: AOO, acetone olive oil.

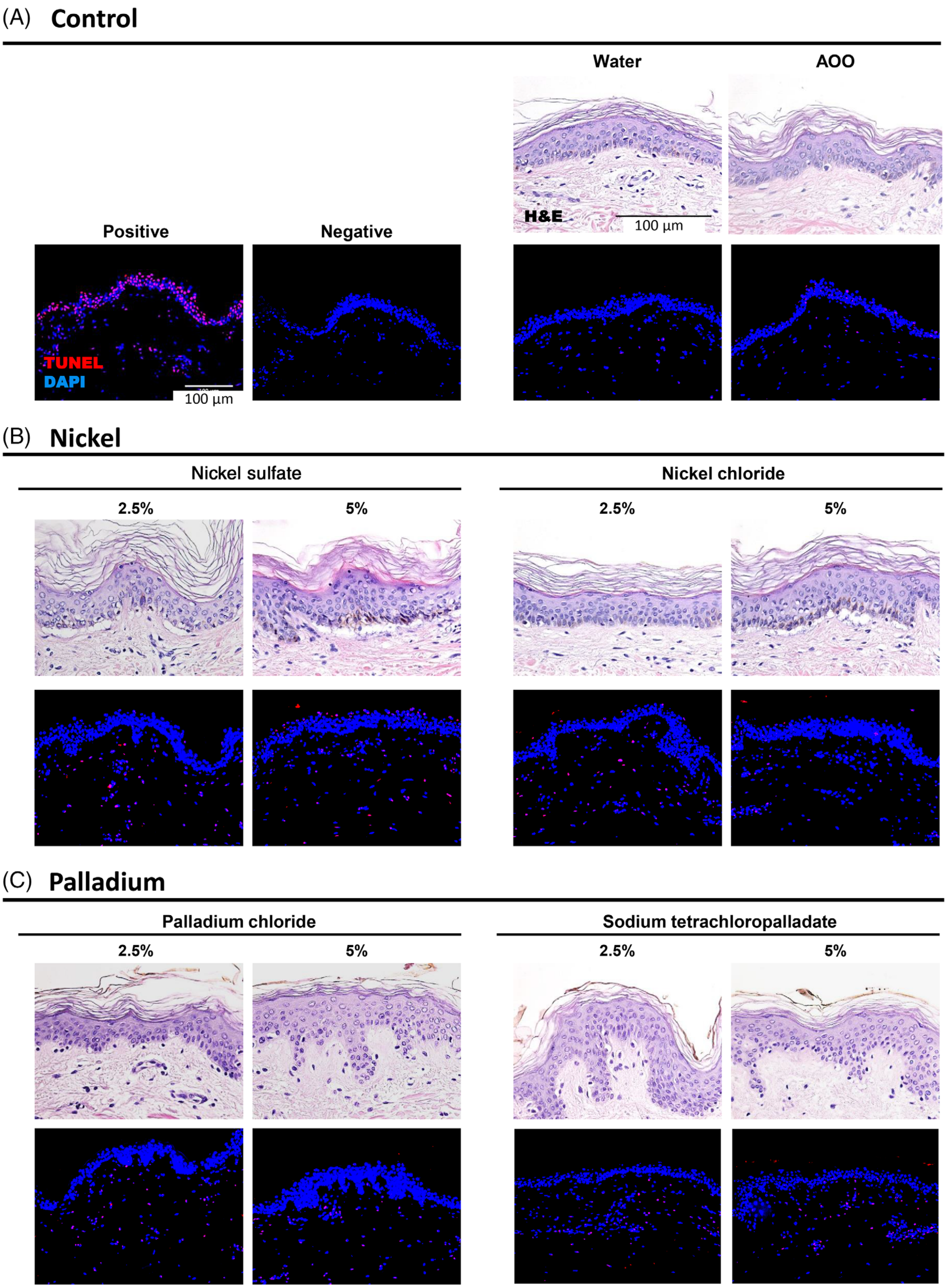

FIGURE 1 Histological assessment of metal salt cytotoxicity. Skin was exposed to vehicle or metal salts for 24 hours, processed for paraffin embedment and tissue sections (5 μm), stained with either haematoxylin and eosin stain (H&E) for assessment of histology (upper panels) or further processed with the TUNEL assay to assess apoptosis (red/purple staining nuclei), and sections were counterstained with DAPI (blue) to visualize all nuclei (lower panels). A. Control groups; B. Nickel exposure; C. Palladium exposure; D. Titanium exposure. Representative images are shown from three experiments, each performed with a separate skin donor and with an intraexperiment duplicate. Magnification bar = 100 μm. AOO, acetone olive oil; DAPI, 4′,6-diamidino-2-phenylindole; H&E, haematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling

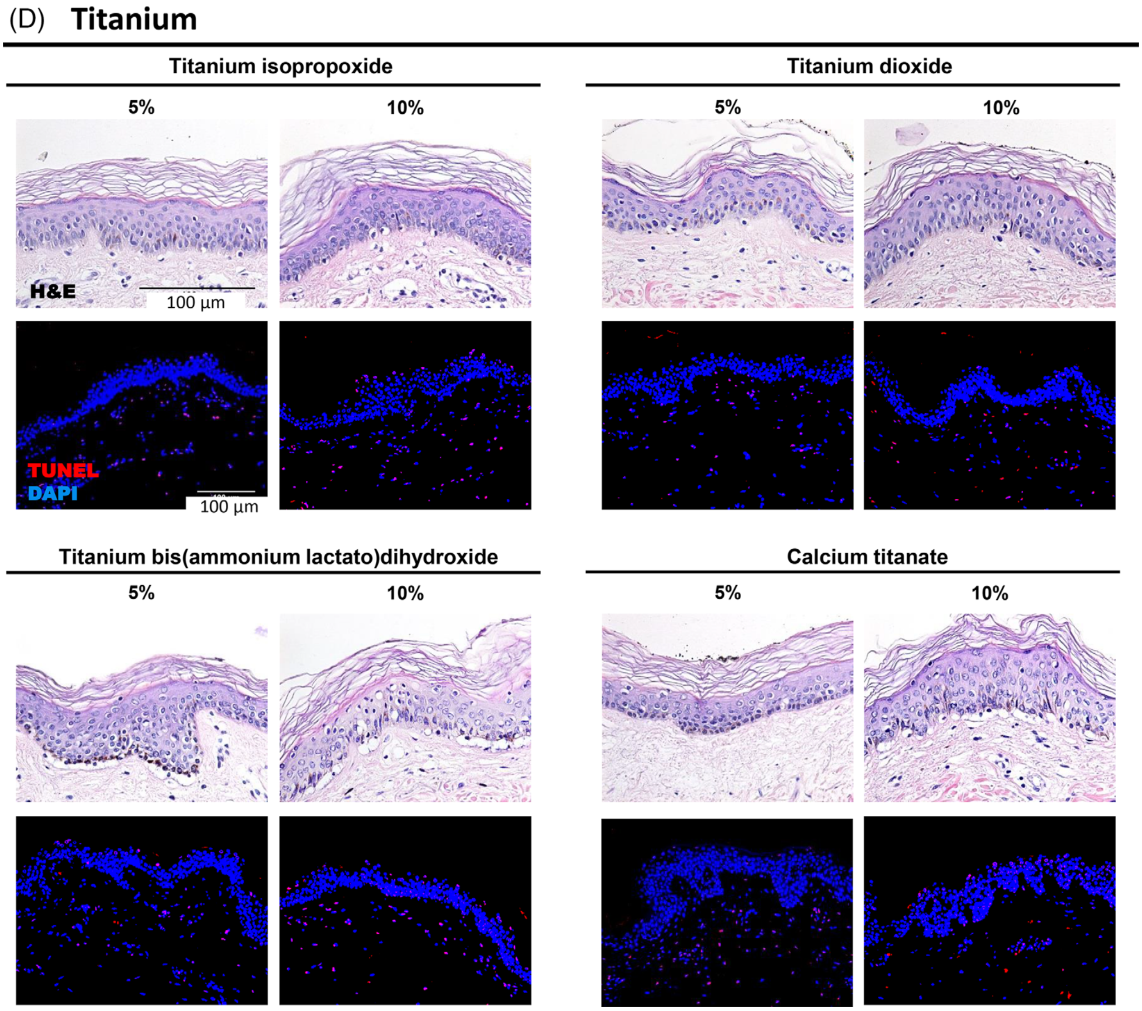

FIGURE 1 (Continued)

is the vehicle of choice, followed by AOO, which is a vehicle generally used in the local lymph node assay.[30] pH of chemicals was determined using a pHenomenal metre (pH 1100 L; VWR International, Pennsylvania, USA) for water-soluble metal salts and pH indicator paper range from 4.0 to 7.0 (Merk, New Jersey, USA) for AOO-soluble metal salts. Finn Chamber filter paper discs (18 mm; Epitest LTD Oy, HYRYLA, Finland) were impregnated with 250 μL of the vehicles (water or AOO) containing the metal salts. The filter paper discs were then applied topically to the skin stratum corneum for 24 hours. Hereafter, biopsies (3 mm in diameter) were taken and immediately analysed with the thiazolyl blue tetrazolium bromide assay (MTT assay); culture supernatants were collected and stored at −20°C for analysis by ELISA and skin tissue was processed for conventional paraffin embedment.

2.3 | MTT assay

The MTT assay (Sigma Aldrich) was used to determine mitochondrial metabolic activity by quantifying dehydrogenase activity. In short, MTT solution (2 mg/mL) was diluted in phosphate-buffered saline and pipetted (200 μL) into a 96-well plate (Corning, New York, USA). Skin biopsies (3 mm diameter) were placed in the well and incubated at 37°C for 2 hours. The biopsies were then transferred to a new 96-well plate containing 200 μL acidified isopropanol and incubated in the dark at room temperature overnight. Thereafter, 100 μL solution was removed and assessed on the spectrophotometer (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 570 nm. Results are expressed relative to vehicle-exposed skin (if a chemical interfered with the colourimetric MTT assay, it was excluded from further analysis).[22] In order to determine this, the highest concentration of the chemical (20%) was tested in the MTT assay without a skin biopsy and if a colour change was present, the chemical was excluded. In this way, calcium titanate was excluded from the MTT assay.

2.4 | Enzyme-linked immunosorbent assay

IL-1α (R&D Systems, Minnesota, USA), IL-6 (R&D Systems, Minneapolis, USA), IL-8 (Sanquin, Amsterdam, the Netherlands), IL-18 (MBL, Nagoya, Japan), and chemokine (C-C motif) ligand 20 (CCL20; R&D

Systems, Minneapolis, USA) were quantified in culture supernatants by ELISA as previously described.[26]

2.5 | Histology

Skin was fixed in 4% paraformaldehyde and processed for paraffin embedment. Tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) for histology evaluation. The stained sections were photographed using a Nikon Eclipse 80i microscope, and analysed with NIS-Elements AR 2.10 imaging software.

2.6 | TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) technique is a method to detect apoptotic DNA fragmentation and was performed essentially as described by the kit supplier (TUNEL Assay Kit – BrdU-Red ab66110; Abcam, Cambridge, UK). In short, paraffin sections (5 μm) were deparaffinized and treated with proteinase K. The DNA strand breaks were labelled with bromolated deoxyuridine triphosphate nucleotide to detect the DNA fragmentation. A positive control (treatment of sections with 2% nuclease solution [TACS-Nuclease, Trevigen, Inc]) and a negative control (omission of the DNA label step) were included when assessing chemical-exposed skin. Tissue sections were photographed using a Fluorescence microscope (Nikon Eclipse 80i, Totyo, Japan) at Ex/Em = 535/580 nm (Bru-Red) and Ex/Em = 375/460 nm (4′,6-diamidino-2-phenylindole [DAPI]).

2.7 | Statistical analysis

Different skin donors were used in each experiment; three independent experiments were performed, each with an intraexperiment duplicate. Because of the large number of metal salts being tested, different skin donors were sometimes used for testing the different metals. For analysis of MTT and cytokines, differences between

TABLE 2 TUNEL-positive nuclei in epidermis and dermis after metal salt exposure

		TUNEL positive (%)	
Chemicals	**% w/v**	**Epidermis**	**Dermis**
Control			
Positive		79.7 ± 1.94	61.9 ± 2.03
Water		0.1 ± 0.13	1.2 ± 0.19
Acetone olive oil		0.1 ± 0.14	0.2 ± 0.16
Nickel			
Nickel sulfate	2.5	2.3 ± 0.09	**18.5 ± 2.17***
	5	1.1 ± 0.67	**16.5 ± 1.05***
Nickel chloride	2.5	0.3 ± 0.25	16.9 ± 6.09
	5	1.6 ± 0.55	15.9 ± 4.39
Palladium			
Palladium chloride	2.5	0.50 ± 0.04	17.4 ± 9.21
	5	1.17 ± 0.78	17.1 ± 7.97
Sodium tetrachloropalladate	2.5	0.40 ± 0.20	10.6 ± 2.05
	5	1.50 ± 1.06	15.1 ± 6.97
Titanium			
Titanium isopropoxide	5	1.43 ± 1.22	**5.9 ± 2.21***
	10	0.85 ± 0.65	16.4 ± 6.53
Titanium dioxide	5	0.55 ± 0.11	9.5 ± 4.11
	10	1.80 ± 1.14	**15.8 ± 5.08***
Titanium bis(ammonium lactato)dihydroxide	5	1.28 ± 0.51	**12.2 ± 3.94***
	10	1.19 ± 0.61	8.8 ± 2.00.
Calcium titanate		0.98 ± 0.61	13.2 ± 5.32
	10	**5.54 ± 0.86****	10.7 ± 4.08

Note: Kruskal-Wallis multiple comparisons test was performed between the vehicle control and treatment groups. *$P < .05$, **$P < .01$ are considered to be statistically significant. Significant differences are highlighted in bold. Mean ± standard error of the mean of three independent experiments, with each representing a different skin donor and each with an intraexperiment duplicate is shown.
Abbreviation: TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling.

groups were determined using the Friedman test and compared with the vehicle group. The results are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software (version 8; GraphPad Software Inc, San Diego, California). For quantification of TUNEL results, QuPath software (open access; University of Edinburgh, United Kingdom) was used on TUNEL assay images. The average number of TUNEL-positive nuclei within two areas of each 5-μm TUNEL-stained tissue section (20× magnification) was determined for each of the three independent experiments. The results are presented as the mean ± SEM. Differences between groups were determined using the Kruskal-Wallis multiple comparisons test and compared with the vehicle group. Differences were considered statistically significant from the vehicle at the *$P < .05$, **$P < .01$ level.

3 | RESULTS

3.1 | Metal salts are cytotoxic and result in apoptosis when exposed to human skin

In order to determine whether patch test–relevant concentrations of metal salts are cytotoxic, salts were applied topically to the stratum corneum of freshly excised skin for 24 hours. Compared with the vehicles, clear detrimental effects on tissue histology (H&E) were observed after exposure to nickel and titanium salts and to a lesser extent after exposure to palladium salts (Figure 1).

Both nickel sulfate and nickel chloride exposure at 5% resulted in a clear separation of the epidermis from the dermis at the basement membrane zone. This was paired with condensed apoptotic cell nuclei with a typical half-moon crescent shape being observed within the epidermis and dermis. For nickel sulfate, these observations were already apparent after 2.5% salt exposure (cf. H&E staining; Figure 1A,B). Similar, but less extreme, findings were observed after exposure to palladium chloride and sodium tetrachloropalladate (2.5% and 5%) with no difference being observed between the two salts (Figure 1C). Of the four titanium salts tested, two salts showed mild cytotoxicity and few apoptotic bodies (titanium isopropoxide, titanium dioxide), whereas titanium bis(ammonium lactato) dihydroxide and calcium titanate were highly cytotoxic at concentrations of 5% and 10%, showing clear separation of the epidermis from the dermis at the basement membrane zone and numerous condensed apoptotic cell nuclei within the epidermis and dermis (Figure 1D).

To investigate the apoptotic properties of the metal salts further, the TUNEL assay was performed (Figure 1 and Table 2). The TUNEL

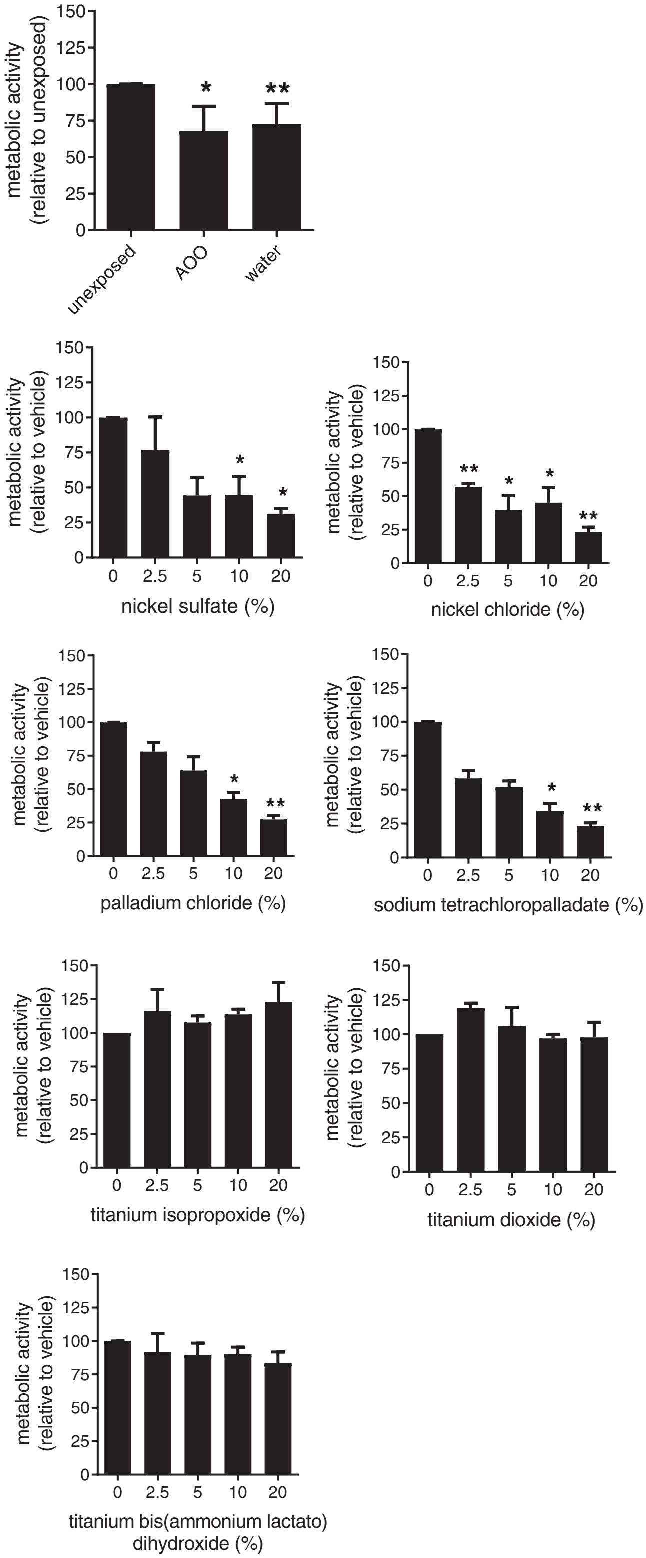

FIGURE 2 Metabolic activity of skin exposed to metal salts. Skin was exposed to vehicle or chemicals for 24 hours (see the "Materials and Methods" section and Table 1). Hereafter, the MTT assay was performed. Results are expressed relative to unexposed skin for comparison of vehicles and relative to vehicle for metal-exposed cultures. Data represent the average of three experiments, each performed with a separate skin donor and with an intraexperiment duplicate ± SEM. The Friedman multiple comparisons test was performed between the control and treatment groups. *$P < .05$, **$P < .01$ are considered to be statistically significant compared with vehicle. AOO, acetone olive oil; MTT, thiazolyl blue tetrazolium bromide; SEM, standard error of the mean

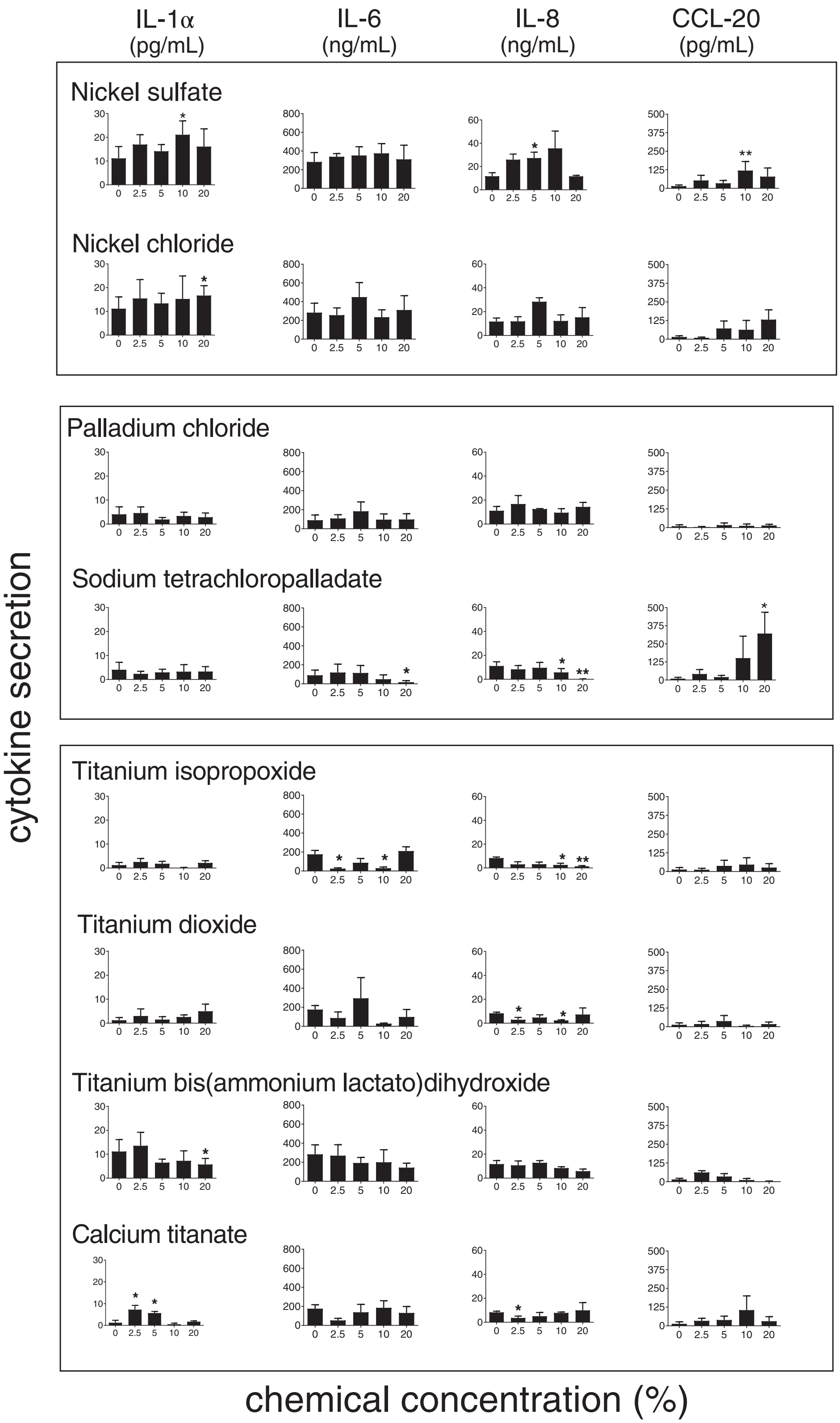

FIGURE 3 Influence of metal salts on (pro)inflammatory cytokine release. Skin was exposed to vehicle or metal salts for 24 hours. Culture supernatant was assessed by ELISA. Results are expressed as amount of protein per millilitre. Data represent the average of three experiments, each performed with a separate skin donor and with an intraexperiment duplicate ± SEM. *$P < .05$ and **$P < .01$, calculated using the Friedman multiple comparisons test, are considered to be statistically significant compared with vehicle. CCL20, IL, interleukin; SEM, standard error of the mean

assay is used to detect fragmented DNA characteristic of apoptotic cells. The positive control (skin tissue sections treated with 2% nuclease solution) shows positive "red" or "purple" staining nuclei depending on the phase in cell apoptosis, with red indicating the formation of apoptotic bodies (the final phase of the apoptosis process) and purple indicating the presence of DNA fragmentation (mixed colour of DAPI blue and red).[31,32] Sections were counterstained with DAPI to visualize intact nuclei (blue; Figure 1A). Quantification of the positive control shows approximately 80% and 60% of nuclei staining TUNEL positive in the epidermis and dermis, respectively (Table 2). Notably, numerous cell nuclei stained TUNEL positive within the epidermis or dermis after exposure to all metal salts compared with the vehicle and significance was achieved for nickel sulfate, titanium isopropoxide, titanium bis(ammonium lactato)dihydroxide, and calcium titanate (Figure 1 and Table 2). The large donor variation observed between the different batches of excised skin was most probably the reason for lack of significance being obtained for the other metal salts (Table 2). Notably, TUNEL staining was less than expected in the epidermis considering the degree of tissue destruction presented by H&E staining, with the percentage TUNEL-positive nuclei in the epidermis not exceeding 5.5% and in the dermis not exceeding 19% for the metal salts.

Next the metabolic activity present within the mitochondria was investigated with the aid of the MTT assay (Figure 2). Metabolic activity decreased by approximately 30% after topical exposure to the vehicles alone. Metabolic activity further decreased in a dose-dependent manner after exposure to nickel and palladium salts in line with the cytotoxicity observed in tissue sections described above. However, for the three titanium salts which could be tested (calcium titanate interfered with the colourimetric readout of the assay), no decrease, and even a slight trend for increase in metabolic activity, was observed.

3.2 | Metal salts influence (pro) inflammatory cytokine and chemokine release

Having determined the cytotoxic properties of the different metal salts, it was next determined whether they could potentially trigger an innate immune response in the form of proinflammatory (IL-1α), inflammatory (IL-6, IL-8; Figure 3) relevant cytokine release.[26,33] Nickel sulfate and/or nickel chloride exposure resulted in increased IL-1α, IL-8, and CCL20 secretion. However, only IL-8 was increased at patch test–relevant concentrations and only after nickel sulfate (5%) exposure. Sodium tetrachloropalladate (20%), but not palladium chloride, resulted in increased CCL20 secretion. Of the four titanium salts tested, only calcium titanate resulted in increased cytokine secretion (IL-1α,), notably at the patch test–relevant concentration ($\leq$ 5%). Surprisingly, cytokine secretion decreased below vehicle levels in a number of cases, in particular after titanium salt (IL-1α, IL-6, and IL-8) and sodium tetrachloropalladate exposure (IL-6, IL-8). This decrease coincided with the levels of cytotoxicity observed in Figure 1. Baseline release of the contact sensitizer cytokine IL-18 showed large donor variation in the excised skin model, resulting in no statistically significant increase or decrease in absolute cytokine levels after metal salt exposure, but did result in a statistical fold increase relative to the vehicle after exposure to calcium titanate (data not shown).[23]

4 | DISCUSSION

In this study we show that patch test–relevant concentrations of a number of metal salts, when topically applied to excised skin, cause localized cytotoxicity. This is observed as epidermis separation at the basement membrane zone, formation of vacuoles, apoptotic nuclei, decreased metabolic activity, and (pro)inflammatory cytokine release.

The process of apoptosis, which results in cell death, includes four main phases: induction, initiation (early), execution (mid), and apoptotic (late) phases.[32,34,35] Although the appearance of apoptotic bodies is definite evidence of the final stage of apoptosis, some well-characterized morphology changes can be detected in the early stage, including chromatin condensation and crescent-shaped nuclei. These changes indicate that progression to the activation of execution caspases has occurred and that the process has become irreversible. Apoptosis is generally a slow process, which needs several days to form the final apoptotic bodies after initially triggering the process. In our study we detected mainly early apoptotic events, as we only exposed the skin for 24 hours to the metal salts. Notably, TUNEL staining was less than expected in the epidermis compared with the dermis. It is possible that because the chemicals were applied topically, the extent of tissue destruction presented by H&E staining was so extensive in the epidermis that it prevented apoptotic bodies being TUNEL stained.

It has long been recognized that the choice of salt is an important consideration in patch testing.[15,36] In a study similar to ours, Fullerton et al[36] showed that the permeation rate, and therefore the physical amount of bioavailable salt, was considerably increased when aqueous nickel chloride was used during ex vivo skin patch testing compared with aqueous nickel sulfate. In our study, detrimental histological effects, including apoptosis, were already observed at a lower nickel sulfate concentration (2.5%) compared with nickel chloride (5%). However, the metabolic activity was slightly lower in excised skin samples exposed to nickel chloride compared with nickel sulfate, when comparing the 5% aqueous solution. This would indicate that both salts do penetrate, resulting in cytotoxicity; however, the method used to assess cytotoxicity may influence the overall conclusion when comparing two different salts. Notably, nickel sulfate (5%) is the preferred patch test salt in the clinic. In a more recent study, we have shown that sodium tetrachloropalladate is the preferred salt compared with palladium chloride in detecting clinical allergy.[15] This finding is in line with our current study in which we show that sodium tetrachloropalladate has a greater impact on metabolic activity and the inflammatory response compared with palladium chloride, indicating that it has a greater ability to penetrate the skin, although both salts exerted similar degrees of detrimental histological effects, including apoptosis.

Titanium dioxide (10% or 20%) is used to determine titanium hypersensitivity even though it is accepted that false-negative test results frequently occur owing to its poor ability to penetrate the skin.[37,38] This has led the search to identify more stable, solvent-soluble, protein-reactive titanium salts with a greater ability to penetrate the skin for patch testing.[18,37,39] Of the four titanium salts tested in this study, titanium isopropoxide and titanium dioxide showed mild cytotoxicity and few apoptotic bodies, whereas titanium bis(ammonium lactato)dihydroxide and calcium titanate were highly cytotoxic, showing clear separation of the epidermis from the dermis at the basement membrane zone and numerous condensed apoptotic cell nuclei within the epidermis and dermis. It has been shown that titanium dioxide nanoparticles can induce oxidative stress signalling cascades that eventually result in cell death via apoptosis.[40–42] It has also been shown that titanium dioxide nanoparticles can cause plasma membrane damage and decreased mitochondrial activities.[43,44] Others have shown that although titanium nanoparticles can trigger apoptosis in human gastric epithelial cells, the MTS assay used in the study indicated an increase in metabolic activity after 24 hours.[45] This finding is in line with our results, as we also observed a slight increase in metabolic activity with increasing signs of apoptosis after titanium salt exposure. Mitochondria contain several proapoptotic molecules that activate cytosolic proteins to execute apoptosis, block antiapoptotic proteins in the cytosol, and directly cleave nuclear DNA, and therefore it can be expected that at the very early onset of apoptosis their activity increases rather than decreases.[46] Notably, mitochondrial activity remained high even when detrimental effects on tissue histology were observed.

Here we have compared different salts for the same metal. Although many in vitro studies describe metal salt cytotoxicity in, for example, dendritic cell, T-cell, and keratinocyte assays,[47–51] very few studies compare cytotoxicity of different salts for the same metal. In the past we have compared the same four titanium salts in our MUTZ-3-derived Langerhans cell assay, RhE model, and reconstructed human skin model with integrated Langerhans cells and found that only titanium bis(ammonium lactato)dihydroxide scored as a weak irritant with regard to Langerhans cell phenotype; however, changes in histology were not investigated and no change in metabolic activity (MTT assay) was observed in these studies.[22,24] In line with this study, in the RhE study we also found that metabolic activity decreased more after sodium tetrachloropalladate exposure than palladium chloride exposure and after nickel chloride exposure compared with nickel sulfate exposure.[22] The results in our present study cannot be explained by a difference in molarity or solubility, as more cytotoxicity was observed for different salts of the same metal when similar amounts or less molarity was used independent of solubility or the vehicle used, for example, upon comparing titanium isopropoxide and titanium dioxide with the more cytotoxic titanium bis(ammonium lactato) dihydroxide and calcium titanate (Table 1). Neither can they be explained by differences in pH of the dissolved metal salts, as the most acid salts (eg, palladium) were least cytotoxic and palladium chloride is more acidic than sodium tetrachloropalladate (Table 1).

In our study, a dose-response in cytokine secretion does not always occur. Cytokines are generally released as an inflammatory response at subcytotoxic concentrations. Therefore, bell-shaped cytokine dose release curves can be observed when high cytotoxicity is reached at high chemical concentrations, resulting in death of cells which would produce the cytokines. Alternatively, for other cytokines which are stored intracellularly, a typical dose-response may be observed as the membrane becomes permeable. From our study we do not distinguish between newly produced cytokines and intracellularly stored cytokines, and furthermore donor variation between the skin samples results in large experimental variation.

As with all in vitro and ex vivo studies, the limitations of our study should be recognized and discussed. The skin was used within 24 hours after surgery and further incubated for 24 hours at 37°C in a culture incubator at 95% humidity. These culture conditions may decrease barrier competency compared with intact human skin. Therefore, the metal salts may be able to penetrate more easily in our model than if they were directly applied to the skin of a volunteer or patient. However, in the clinics, patch test salts are applied under occlusion which also creates a localized humid environment similar to our culture incubator. We should also consider the vehicles and method of application used in our study. We used Finn Chamber filter paper discs (18 mm; Epitest LTD Oy, Finland) which were impregnated with the metal salt dissolved in either water or AOO to enable maximal solubility of the chemical and slow release of the chemical from the paper disc into the excised skin. Typically, during human skin patch testing, the chemical is mixed with petrolatum[52] before applying to the same Finn Chamber filter discs and then applied to the skin for 48 hours. Therefore, even though the time of exposure and release kinetics of our ex vivo study cannot be directly compared with the human patch test situation, we do still show that by applying similar chemical concentrations to those used in clinics, a localized cytotoxic effect on the skin does occur.

Whereas this study focusses on exposure of metal salts to the skin, metals are incorporated into many medical devices, particularly those used in dentistry. The oral cavity is considered to be a much more hostile environment than the skin, due to the presence of an extensive microbiome and also saliva which contains corrosive compounds such as hydrogen, chloride ions, sulfide compounds, dissolved oxygen, enzymes, and free radicals.[1] Metal alloys will corrode with time after prolonged contact with the mucosa, releasing metal ions into the surrounding tissue. The resulting typical clinic manifestations of the oral mucosa are xerostomia, metal taste, burning sensation, stomatitis, and lichenoid lesions.[2,53,54] It is most possible that these clinical manifestations are partly due to metal ion–induced cytotoxicity including apoptosis.

In conclusion, metal salts applied to excised skin show localized cytotoxic effects. Whether this also occurs in vivo, and whether penetrated metal ions would also result in systemic effects, remains currently unknown. Further verification of our results from biopsies obtained during clinical patch testing is now required. The aim of patch testing is to have a chemical concentration that is high enough to elicit an allergic skin reaction in sensitized individuals, but low enough not to sensitize nonallergic people, even after repeated testing. Besides, it should be as nonirritative as possible to facilitate patch test reading. Therefore, it may be considered to explore the possibility of buffering the salts to provide a more neutral nonirritative pH, or reducing further the concentration of metal salts routinely used in clinical patch testing by exchanging the salt for one that is more skin permeable. The challenge will be to remain above the elicitation threshold at noncytotoxic metal concentrations. Alternatively, focusing on in vitro lymphocyte cytokine and transformation tests which are showing promising results for identifying people with allergy to metals is an option.[55,56]

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Yan Zhang: Data curation; formal analysis; investigation; methodology; writing - original draft; writing-review & editing. **Niels de Graaf:** Conceptualization; methodology; writing-review & editing. **Rosalien Veldhuizen:** Involved in original experiment design, performing and data collection of some experimentals and data interpretation. **Sanne Roffel:** Formal analysis; investigation; methodology; writing-review & editing. **S W Spiekstra:** Conceptualization; data curation; methodology. **Thomas Rustemeyer:** Conceptualization; investigation; methodology; supervision; writing-review & editing. **Cees J. Kleverlaan:** Conceptualization; investigation; methodology; writing-review & editing. **Albert Feilzer:** Conceptualization; funding acquisition; investigation; project administration; writing-review & editing. **Hetty Bontkes:** Conceptualization; investigation; methodology; supervision; writing-review & editing. **Dongmei Deng:** Conceptualization; formal analysis; writing - original draft; writing-review & editing.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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