

Oral cadmium exposure affects skin immune reactivity in rats

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ABSTRACT

Skin can acquire cadmium (Cd) by oral route, but there is paucity of data concerning cutaneous effects of this metal. Cd acquired by oral route can affect skin wound healing, but the effect of Cd on other activities involved in skin homeostasis, including skin immunity, are not explored. Using the rat model of 30-day oral administration of Cd (5 ppm and 50 ppm) in drinking water, basic aspects of immune-relevant activity of epidermal cells were examined. Dose-dependent Cd deposition in the skin was observed ($0.035 \pm 0.02 \mu\text{g/g}$ and $0.127 \pm 0.04 \mu\text{g/g}$ at 5 ppm and 50 ppm, respectively, compared to $0.012 \pm 0.009 \mu\text{g/g}$ at 0 ppm of Cd). This resulted in skin inflammation (oxidative stress at both Cd doses and dose-dependent structural changes in the skin and the presence/activation of innate immunity cells). At low Cd dose inflammatory response (nitric oxide and IL-1 β) was observed. Other inflammatory cytokines (IL-6 and TNF) response occurred at 50 ppm, which was increased further following skin sensitization with contact allergen dinitro-chlorobenzene (DNCB). Epidermal cells exposed to both Cd doses enhanced concanavalin A (ConA)-stimulated lymphocyte production of IL-17. This study showed for the first time the effect of the metal which gained access to the skin *via* gut on immune reactivity of epidermal cells. Presented data might be relevant for the link between dietary Cd and the risk of skin pathologies.

1. Introduction

Cadmium (Cd) is toxic pollutant which is widely spread in the environment through industrial production and agriculture. Being non-biodegradable, Cd is persistent in the environment and is important water and food contaminant. Because of its long half-life and low rate of excretion from the organism, Cd is of both occupational and environmental concern. Despite efforts to decline anthropogenic activities which result in environmental pollution, Cd has remained global health concern greatly because of accumulation in food. This draw attention to non-occupational exposure to Cd and possible health risks (Satarug et al., 2003; Satarug and Moore, 2004).

Epidemiological data revealed that life-time exposure to environmentally-relevant levels of Cd posed a risk of variety organ diseases including chronic kidney, heart and lung diseases, and nervous tissue disorders (Adams et al., 2012; Buck Louis et al., 2012; Garcia-Esquinas et al., 2014). Experimental studies of Cd exposure by oral

route, the main way for this metal to reach the organism, showed broad spectrum of toxic effects in variety of tissues, with kidneys and liver as the most affected organs (Sugawara and Sugawara, 1974; Muller et al., 1986; Friberg et al., 1986; Jonah and Bhattacharyya, 1989; Saygi et al., 1991; Hiratsuka et al., 1999). Long-term exposure to Cd affects also male reproductive tissues (Waalkes, 2003) and exerts osteotoxicity (Ohta et al., 2000). Increased levels of Cd noted in lungs of mice exposed to oral Cd administration affected neuronal pathways involved in regulation of bronchial tone (Chandler et al., 2016).

Cd deposition was noted also in skin hair (Wibowo et al., 1986; Lauwerys et al., 1994). The metal may be taken by growing hair in the hair follicles from the blood and/or be absorbed by hair following excretion by sebaceous and sweat glands. Cd which gained access to the skin can affect physiological processes in the skin as well. In this regard, it was shown that both topical skin as well as oral exposure to Cd impaired cutaneous wound healing in mice (Lansdown et al., 2001; Mei et al., 2017). Recently explored relationship between cadmium and

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psoriasis suggested positive association between blood cadmium and severity of psoriasis (Liaw et al., 2017). This throws light on effects of Cd on other aspects of activities which maintain skin homeostasis, including skin immunity, which are not explored yet. Skin has essential immunological functions which are required for active defense against pathogens and variety of physical and chemical insults (Nestle et al., 2009). Epidermal cells are critical immune sentinels in immunosurveillance of skin exposed to external or endogenous/systemic stimuli (Nestle and Nickoloff, 2007).

The aim of this study was to examine whether oral exposure to Cd affects cutaneous immune activity. The rat model of 30-day oral administration of Cd in drinking water was used to examine basic aspects of immune-relevant activity of the epidermal cells. The obtained data showed for the first time influence of Cd which gained access to the skin via gut on skin immune system.

2. Materials and methods

2.1. Chemicals

Cadmium chloride (CdCl_2) was purchased from Serva (Serva, Feinbiochemica, Heidelberg, Germany). Concanavalin A (ConA), Lipopolysaccharide (LPS; type 0111: B4 from *Escherichia coli*), L-epinephrine, N-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide (*p*-aminobenzenesulfonamide), 3-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Chloro-2,4 dinitrochlorobenzene (DNCB) was obtained from BDH Chemicals Ltd. 2,4-dinitrobenzenesulfonic (DNTB) acid, sodium salt, was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Hydrogen peroxide (H_2O_2) was obtained from Zorka Farma, Sabac, Serbia. N,N,N',N'-ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate was obtained from USB Corporation (Cleveland, OH, USA). Sucrose was obtained from Lachner (Neratovice, Czech Republic). Dispace II was obtained from Boehringer (Manheim, Germany) and trypsin solution from Difco (Lawrence, KS, USA). Sodium nitrite (NaNO_2) and reduced L-Glutathione (GSH) were purchased from Fluka Chemie (Buchs, Switzerland). Culture medium RPMI-1640 (Biowest, Nuaillé, France) supplemented with 2 mM glutamine, 20 $\mu\text{g}/\text{ml}$ gentamycine (Galenika a.d., Zemun, Serbia) and 5% (v/v) heat inactivated fetal calf serum (Biowest, Nuaillé, France) were used. For use in experiments ConA and dispace II were dissolved in RPMI-1640 medium. All solutions for cell culture experiments were prepared under sterile conditions and were sterile filtered (Minisart, pore size 0.20 μm , Sartorius Stedim Biotech, Goettingen, Germany) before use.

2.2. Animals and cadmium treatment

Animal treatment and experimental procedures were carried out in compliance with the Directive 2010/ 63/EU on the protection of animals used for experimental and other scientific purposes and approved by the Ethical Committee of the Institute for Biological Research “Sinisa Stankovic” (IBISS), University of Belgrade, Serbia. Dark Agouti (DA) male rats, eight to twelve weeks of age, used in experiments, were conventionally housed at IBISS, in controlled environment (21–24 °C temperature, a 60% relative humidity and 12-h (h) light: dark cycle). Four to six animals were assigned to each treatment group per experiment, in at least two independent experiments. Rats were exposed to cadmium chloride (CdCl_2) prepared in distilled water at two concentrations, 5 ppm (5 mg/l) and 50 ppm (50 mg/l) of Cd (II) ion in a period of 30 days, while control rats were given distilled water solely. In some experiments, at day 30, 100 μl of 0.4% DNCB dissolved in acetone/olive oil was applied for 2 consecutive days to the upper part of dorsum (approximately 16 cm^2) of treated rats, as described previously (Belij et al., 2012). All rats had *ad libitum* access to standard rodent pellets and water throughout the study. Twice a week Cd solutions and

water were replaced with freshly prepared solution/water. One day before cessation of cadmium treatment (day 29th) animals were put for 24 h into metabolic cages in order to measure food consumption, urine volume and weight of feces. All functional measurements were carried out after a 30-day period of oral Cd intake, or 24 h following second DNCB application, in animals anesthetized by i.p. injection of 40 mg/kg b.w. of thiopental sodium (Rotexmedica, Tritau, Germany).

2.3. Cadmium determination

Cd content in blood and tissue of liver, kidney, intestine, hair, skin, epidermis and dermis was determined by inductively coupled plasma mass spectrometry, ICP-MS (ICAP, Q, X Thermo Scientific series 2). The entire system is controlled with Qtegra Instrument Control Software. Samples were decomposed in the microwave digestion system (Ethos One, Milestone Italy) at 180 °C, in a mix of concentrated HNO_3 (65%, Distill acid, Hotel Berghof (Germany)) and H_2O_2 (30%) by applying the following temperature program: 10 min warm-up to 180 °C and heating for 15 min. Samples were filtered and all dilutions were done using metal-free ultrapure water. All reagents were of analytical grade supplied by Merck (Darmstadt, Germany). Single-element stock solution containing 1000 mg/L of Cd was used for the standards preparation for analysis by ICP-MS. As internal standards were used: ^6Li , ^{45}Sc , ^{115}In and ^{159}Tb (VHG standards, Manchester, UK). The concentrations were expressed as μg of Cd per g of wet tissue.

2.4. Clinical biochemistry and hematology

Blood was withdrawn from abdominal artery for determination of leukocyte counts and hematologic parameters and for isolation of plasma or serum. Differential leukocyte counts and hematological parameters were determined automatically by Siemens ADVIA 120 flow cytometer (Terytown, New York, USA) using commercially available reagents. Plasma fibrinogen was measured by Siemens-Dade Behring-BCT analyzer using Multifibren U test for quantitative determination in plasma. Haptoglobin and albumin were measured in serum by BN (Dade Behring) immunochemical system for human blood proteins measured by Siemens BNII (Dade Behring) BCT analyzer. Cross-reactivity with rat blood proteins was checked using serum obtained from turpentine-induced inflammation in rat, known inflammatory model of acute phase reaction in these animals (Giffen et al., 2003). Changes in plasma or serum proteins are expressed as the relative changes (values from controls were taken as 1). Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were measured using an autoanalyzer (Ciba Corning Express, Oberline, OH) and commercially available reagents. Biochemical parameters in freshly caught urine were measured using semi quantitative Combur¹⁰ Test[®]M (Roche Diagnostics GmbH, Mannheim, Germany).

2.5. Histology

Full thickness dorsal skin (approximately 1 cm^2) tissue specimens were fixed in 4% formaldehyde (pH 6.9), and after preparation molded into paraffin wax for subsequent sectioning at 5 μm . Samples were stained with hematoxylin and eosin (H&E) and subsequently analyzed by a certified histopathologist using a Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

2.6. Measurements of skin explant's viability

Full thickness dorsal skin was excised, cleared of subcutaneous tissue, weighted, cut into pieces of approximately 0.3 cm^2 and placed in duplicate in 0.2 ml of complete culture medium. MTT reduction assay was employed for skin viability determination as described (Klein et al., 1996). MTT was added immediately, in a concentration of 0.5 mg/ml of culture, and skin explants were incubated for 3 h at 37 °C in a

humidified atmosphere of 5% CO₂. Produced formazan was dissolved by overnight incubation of skin explants in 2-methoxyethanol. Extracted chromogen was quantitated at 540 nm spectrophotometrically, using ELISA 96-well plate reader (Shimadzu Corporation, Lakewood, USA). Results are expressed as absorbance (A540nm) per g of explanted tissue.

2.7. Preparation of skin homogenates

Full thickness dorsal skin was quickly removed, weighed, frozen in liquid nitrogen and stored at -80°C until use. Skin samples were homogenized by IKA T18 Basic Homogenizer (IKA Works Inc., Wilmington NC, USA) in ten volumes of sucrose buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 250 mM sucrose) on ice. The homogenates were sonicated and then ultra-centrifuged at $100,000\times g$ for 1 h and 45 min, at 4°C . Supernatants were used for determination of antioxidant enzyme activity.

2.8. Measurement of superoxide dismutase (SOD, EC1.15.1.1) and catalase (CAT EC1.11.1.6) activity in skin homogenates

Activity of SOD was determined by the epinephrine method (Misra and Fridovich, 1972). One unit of activity was defined as the amount of enzyme necessary to decrease the rate of epinephrine auto-oxidation by 50% (pH 10.2). CAT activity was determined by the rate of H₂O₂ decomposition measured spectrophotometrically at 240 nm as described (Beutler, 1982). One unit of CAT activity was defined as the amount of enzyme that decomposed 1 mmol H₂O₂ per minute at 25°C and pH 7.0. SOD and CAT activities were measured using Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Protein concentration was determined by Lowry assay (Lowry et al., 1951).

2.9. Epidermal cell preparation, viability and culture

Skin was cut into small pieces and incubated in dispase (2.5 mg/ml) at 4°C overnight, in order to separate epidermis from the dermis. Epidermal sheets were incubated with trypsin-glucose solution (0.25% trypsin, 0.1% glucose) for 30 min at 37°C , and a suspension of epidermal cells was obtained. Epidermal cell viability was measured using MTT reduction assay as described (Oez et al., 1990). For measurements of epidermal cell cytokine production, cells were cultured (1×10^5 cells/well of 96-well plates) for 48 h with culture medium.

For co-culture experiments, suprascapular and axillary lymph node cells were isolated as a source of T cells, from untreated animals, and prepared by mechanical teasing over nylon mesh (70 μm nylon, BD Bioscience, Bedford, USA) and counted by Neubauer hemacytometer (cell viability determined using a trypan blue exclusion assay, over 95%). Epidermal cells (1×10^5) were cultured (at ratio of 1:5) with skin-draining lymph node cells, with or without ConA (2.5 $\mu\text{g}/\text{ml}$). After 72 h, culture supernatant (conditioned medium) was collected by centrifugation.

2.10. Reduced glutathione (GSH) measurements in epidermal cells

Reduced glutathione (GSH) measurements content was estimated in epidermal cells (1×10^6 freshly isolated cells, lysed in 10 mM HCl), after protein precipitation by addition of 5% sulphosalicylic acid. GSH level was quantified in supernatant using DTNB in Tris-HCl (pH 8.9) and reduced glutathione as standard (Anderson, 1986) spectrophotometrically (Shimadzu Corporation, Lakewood, USA) at 412 nm. The levels of GSH were expressed as μmol of GSH/ 10^6 cells.

2.11. Griess assay

The concentration of the stable NO oxidation product, nitrite, was measured as an indicator of NO formation using Griess assay (Hibbs

et al., 1988). After 48 h culture of 1×10^5 epidermal cells per well of 96-well plates with 100 ng/ml of lipopolysaccharide (LPS), 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) and incubated for 10 min at room temperature. The absorbance was measured at dual wavelength 540 nm/670 nm by an ELISA plate reader.

2.12. Cytokine determination by ELISA

Interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor (TNF) and interleukin-10 (IL-10) concentrations were determined in media conditioned by epidermal cells. Plasma concentrations of IL-6 and TNF were measured in order to assess systemic response. Interleukin-17 (IL-17) was measured in medium obtained from co-cultures of epidermal cells and lymph node cells. Enzyme-linked immunosorbent assays (ELISA) for rat IL-1 β , IL-6 and IL-10 (R&D systems, Minneapolis, USA), rat TNF- α and mouse IL-17 cross-reactive with rat's IL-17 (eBioscience Inc., San Diego, CA, USA) were used according to manufacturer instructions. Cytokine titer was calculated by the reference to a standard curve constructed with known amounts of recombinant cytokines.

2.13. Reverse transcription—real time polymerase chain reaction (RT-PCR)

Total RNA (1 μg) isolated from epidermal cells with mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) following manufacturer's instructions, was reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Gene expression of MT1, MT2, iNOS, IL-1, IL-6, TNF and IL-10 was assessed in the samples from epidermal cells. Prepared cDNAs were amplified using Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) based on the recommendations of the manufacturer in a total volume of 20 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions were: an initial step at 50°C for 5 min, followed by a step at 95°C for 10 min and subsequent 2-step PCR program at 95°C for 15 s and 60°C for 60 s for 40 cycles. PCR primers (forward/reverse) used in the study were:

β -actin (housekeeping reporter gene): 5'-CCCTGGCTCCTAGCAC CAT-3'/5'-GAGCCACCAATCCA-CACAGA-3';

Metallothionein (MT)-1: 5'-GAACGTCAAATGCACCTCCTGC 3'/5'-CAAGACTCTGAGTTGGTCCG- 3';

Metallothionein (MT)-2: 5'- TGCAAGAAAAGCTGTGTTCC-3'/5' -TTACACCATTGTGAGGACGCC-3';

IL-1 β : 5'-CACCTCTCAAG-CAGAGCA-3'/5'-GGGTTCCATGGTGAAG TCAAC-3';

IL-6: 5'-GCCCTT CAGGAACAGCTATGA-3'/5'-TGTCACACATCA GTCCCAAG-3'; **TNF**: 5'-TCGAGT GACAAGCCCGTAGC3'/5'-CTCAGCC ACTCCAGCTGCTC-3';

iNOS: 5'-TTCCCATCGCTCCGCTG3'/5'-CCGGAGCTGTAGCACT GCA-3';

IL-10: 5'-GAA-G ACCCTCTGGATACAGCTGC-3'/5'-TGCTCCACTGC CTTGCTTTT-3'. PCR products were detected in real-time and results were analyzed with 7500 System Software (Applied Biosystems) and calculated as $2^{-\text{dCt}}$ where dCt was the difference between threshold cycle (Ct) values of specific gene and the endogenous control (β -actin).

2.14. Data display and statistical analysis

Results are expressed as means $\pm$ standard deviation (S.D.). Statistical analysis was performed using STATISTICA 7.0 (StatSoft Inc., Tulsa, OK) and statistical significance was defined by a Mann-Whitney U test. *P*-values less than 0.05 were considered significant.

Table 1
Cadmium tissue content.

Cd (µg/g)	DA Cadmium dose (ppm)		
	0	5	50
Liver	0.006 ± 0.002	1.53 ± 1.4**	2.06 ± 1.9***
Kidneys	0.013 ± 0.0005	0.41 ± 0.03**	5.21 ± 0.2***
Intestine	0.04 ± 0.005	0.22 ± 0.2*	1.06 ± 0.3***
Hair	0.15 ± 0.05	0.27 ± 0.1*	0.71 ± 0.2***
Skin			
Dorsal	0.012 ± 0.009	0.035 ± 0.02***	0.127 ± 0.04***
Ventral	0.02 ± 0.004	0.04 ± 0.02***	0.11 ± 0.03***
Epidermis (dorsal)	0.015 ± 0.008	0.034 ± 0.014**	0.120 ± 0.031***
Dermis (dorsal)	0.004 ± 0.001	0.004 ± 0.001	0.046 ± 0.007***
Blood	0.010 ± 0.001	0.023 ± 0.016**	0.026 ± 0.002***

Results are presented as mean (± SD) from at least two independent experiments (n = 4–6 rats/group). Significance at: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs. control animals (Cd dose 0 ppm); ##*P* < 0.01 and ###*P* < 0.001 vs. lower cadmium dose (5 ppm).

3. Results

3.1. General considerations

Rats were administered with Cd in distilled water at 5 ppm (lower dose) and 50 ppm (higher dose) during period of thirty days. Weight gain was noted in all rats during the experiment with lack of differences between control and the groups which received Cd at the end of the period of Cd consumption (215 ± 11.2 g, 216 ± 12.4 g, 219 ± 10.4 g at 0 ppm, 5 ppm and 50 ppm, respectively). No differences were observed concerning food and water intake, excretion of urine and feces between control and animals which consumed cadmium. Based on calculated water consumption, average daily Cd intake was 0.7 ± 0.1 mg/kg and 6.6 ± 1.0 mg/kg for groups receiving 5 ppm and 50 ppm of Cd, respectively. Consumption of Cd resulted in high levels of the metal in kidneys and liver (Table 1). Measurements of Cd in the hair and the skin showed dose-dependent increase of the metal compared to levels in control rats. Similar concentrations of Cd were noted in dorsal and ventral skin. Measurements of Cd concentrations in skin layers revealed increase in both epidermis and dermis, but levels were higher (*P* < 0.05) in epidermis.

No differences in the urine biochemistry were observed between groups. Increased (*P* < 0.05) levels of AST (222.5 ± 85.8 U/ml compared to 143.1 ± 31.1 U/ml in controls, *P* < 0.05) and ALT (133.5 ± 61.0 U/ml compared to 69.1 ± 9.31 U/ml in controls, *P* < 0.001) were found in plasma of rats exposed to 50 ppm of cadmium.

Generally no effects were observed in peripheral blood total and differential leukocyte counts except the increase in eosinophils numbers at both cadmium doses (Table 2). Cadmium intake resulted in lack of effects on hematological parameters and the only change which was observed, was increase in mean platelet volume. The levels of acute phase proteins stayed close to controls (considered as 1) including haptoglobin (0.9 ± 0.26 and 1.0 ± 0.15, for low and high cadmium dose, respectively), fibrinogen (0.9 ± 0.03 and 1.0 ± 0.00) and albumin (1.1 ± 0.12 and 1.2 ± 0.10). Plasma levels of TNF and IL-6 were below detection limits in all groups.

3.2. Skin superoxide dismutase (SOD) and catalase (CAT) activity

Cd administration exerted no effect on skin viability, judging by similar values of MTT reducing capacity (expressed as A_{540nm}/g of tissue) of skin from control and rats which received Cd (39.2 ± 11.8, 37.1 ± 12.8, 35.0 ± 12.9 at 0 ppm, 5 ppm and 50 ppm, respectively). Measurements of basic oxygen free radical enzyme scavengers, SOD and CAT in skin tissue homogenates showed a significant increase of the

Table 2
Peripheral blood and differential leukocyte counts.

Parameter	DA Cadmium dose (ppm)		
	0	5	50
Leukocytes (10 ⁹ /l)	9.2 ± 2.7	8.0 ± 1.4	7.8 ± 1.2
Lymphocytes (%)	52.6 ± 9.1	47.4 ± 9.2	45.5 ± 7.3
Neutrophil leukocytes (%)	35.0 ± 4.7	41.4 ± 8.0 ^{0.05*}	54.0 ± 12.5**
Monocytes (%)	1.6 ± 0.9	1.6 ± 0.6	1.3 ± 0.3
Eosinophil leukocytes (%)	0.5 ± 0.3	0.7 ± 0.2*	0.9 ± 0.2**
Basophil leukocytes (%)	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.2
Red blood cells (10 ¹² /l)	8.3 ± 0.4	8.2 ± 0.5	8.3 ± 0.4
Hg g/l	138.4 ± 9.5	138.0 ± 11.0	138.0 ± 8.3
HCT (%)	0.43 ± 0.02	0.42 ± 0.03	0.43 ± 0.02
MCV (fl)	51.4 ± 1.5	51.6 ± 0.9	52.0 ± 3.6
PLT (10 ⁹ /l)	716.0 ± 133.8	772.1 ± 98.9	710.7 ± 75.8
MPV	7.9 ± 0.8	8.0 ± 0.4	8.5 ± 0.5*
MCH (pg)	16.6 ± 0.8	16.9 ± 0.5	16.4 ± 1.3
MCHC (g/l)	321.5 ± 19.1	323.3 ± 5.3	319.6 ± 8.1
Intracellular Hg (g/l)	130.3 ± 7.9	131.5 ± 13.1	133.4 ± 8.3
Anisocytosis (%)	1.4 ± 0.4	2.2 ± 1.3*	1.7 ± 0.6
Hypochromia (%)	1.9 ± 1.4	3.3 ± 1.8	2.3 ± 1.4

Results are presented as mean (± SD) from at least two independent experiments (n = 4–6 rats/group). Significance at: **P* < 0.05 and ***P* < 0.01 vs. control animals (Cd dose 0 ppm).

activity of both enzymes at both Cd doses (Fig. 1A and B).

3.3. Skin histology

Histological analysis showed morphological and structural changes in the skin including dilatation of sebaceous follicles, newly formed vascular spaces, hyperkeratosis and hypergranulosis. Presence of neutrophils and activation of mast cells was seen in the skin of rats which received 5 ppm and 50 ppm of cadmium, respectively (Fig. 1C, D and E).

3.4. Epidermal cell cytotoxicity and stress response

To see if cadmium affects epidermal cell viability, MTT reduction assay, which is a measure of cell's metabolic viability was employed. No effects on epidermal cell viability were seen, based on similar A_{540nm} values of MTT reduction (0.27 ± 0.11 and 0.27 ± 0.10 at 5 ppm and 50 ppm, respectively, compared to 0.25 ± 0.08 at 0 ppm of Cd). Increased levels of MT-1 and MT-2 mRNA were observed at higher Cd dose (Fig. 2A). A tendency of (*P* = 0.05) and significant decrease of GSH in epidermal cells were observed at 5 ppm and 50 ppm, respectively (Fig. 2B).

3.5. Epidermal cell nitric oxide (NO) production

At lower Cd dose, epidermal cells expressed significantly higher levels of inducible form of nitric oxide synthase (iNOS) mRNA (Fig. 3A) and produced higher levels of NO compared to cells from controls (Fig. 3B). No statistically significant effect was seen at 50 ppm of cadmium.

3.6. Epidermal cell cytokine responses

Cd administration resulted in increased levels of mRNA for IL-1β (though only with a tendency of an increase, *P* = 0.057, at 50 ppm), with similar pattern of protein production (significant increase at 5 ppm; numerical, *P* = 0.07, at 50 ppm) (Fig. 4 Panel A). Increased levels of mRNA for IL-6 and TNF as well as protein products were observed at higher Cd dose, solely (Fig. 4, Panel B and Panel C, respectively). To see if cadmium affects epidermal cell responsiveness to exogenous agents, skin of rats exposed to Cd was sensitized with contact

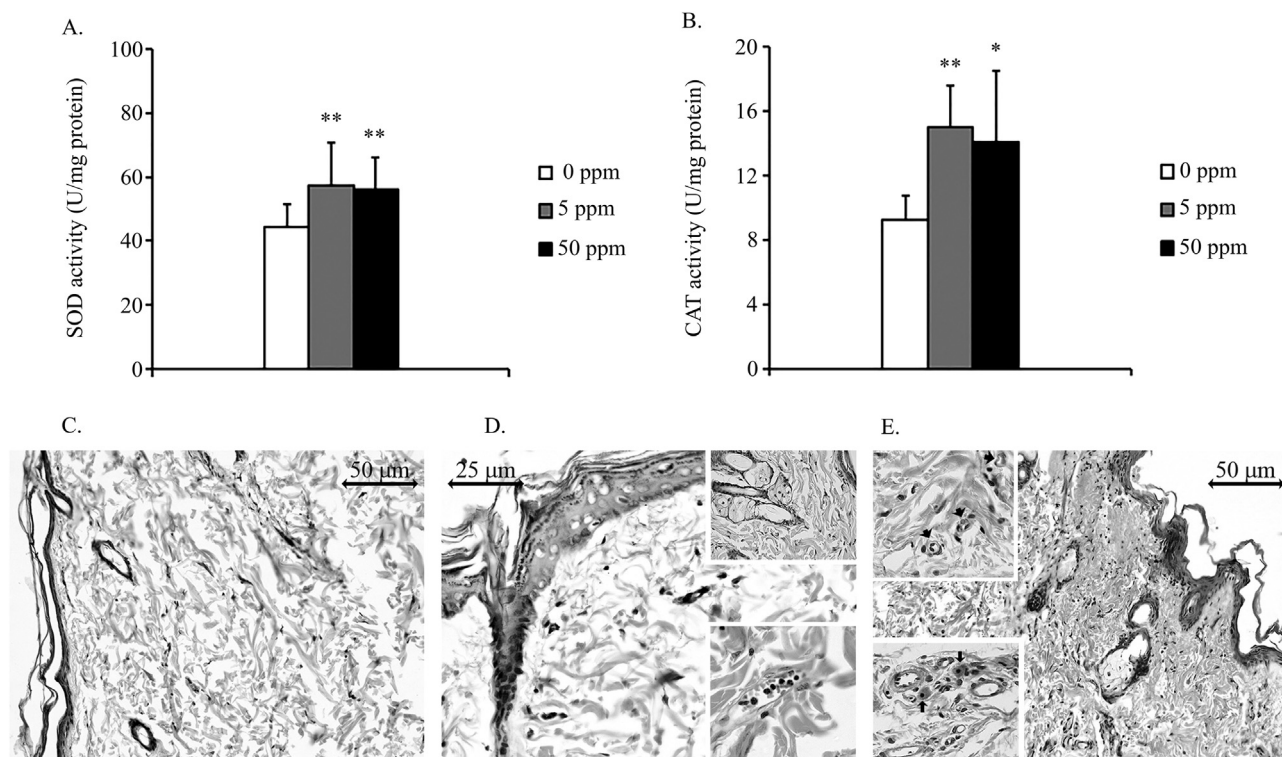


Fig. 1. Effect of oral cadmium intake on skin tissue. (A) SOD activity in the skin. (B) CAT activity in the skin. (C, D, E) Histology of skin. (C) Control group; (D) 5 ppm. Dilatation sebaceous follicles (insert, up), neutrophils in dermal vascular space (insert, down); (E) 50 ppm. Newly formed vascular spaces (insert, up), dilated lymphaticus with hyperkeratosis and hypergranulosis. Hyper-granulated mast cells (insert, down). Data are presented as mean ($\pm$ SD) from three experiments with four to six animals/group per experiment. Significance at: * $P < 0.05$ and ** $P < 0.01$ vs. control animals (Cd dose 0 ppm).

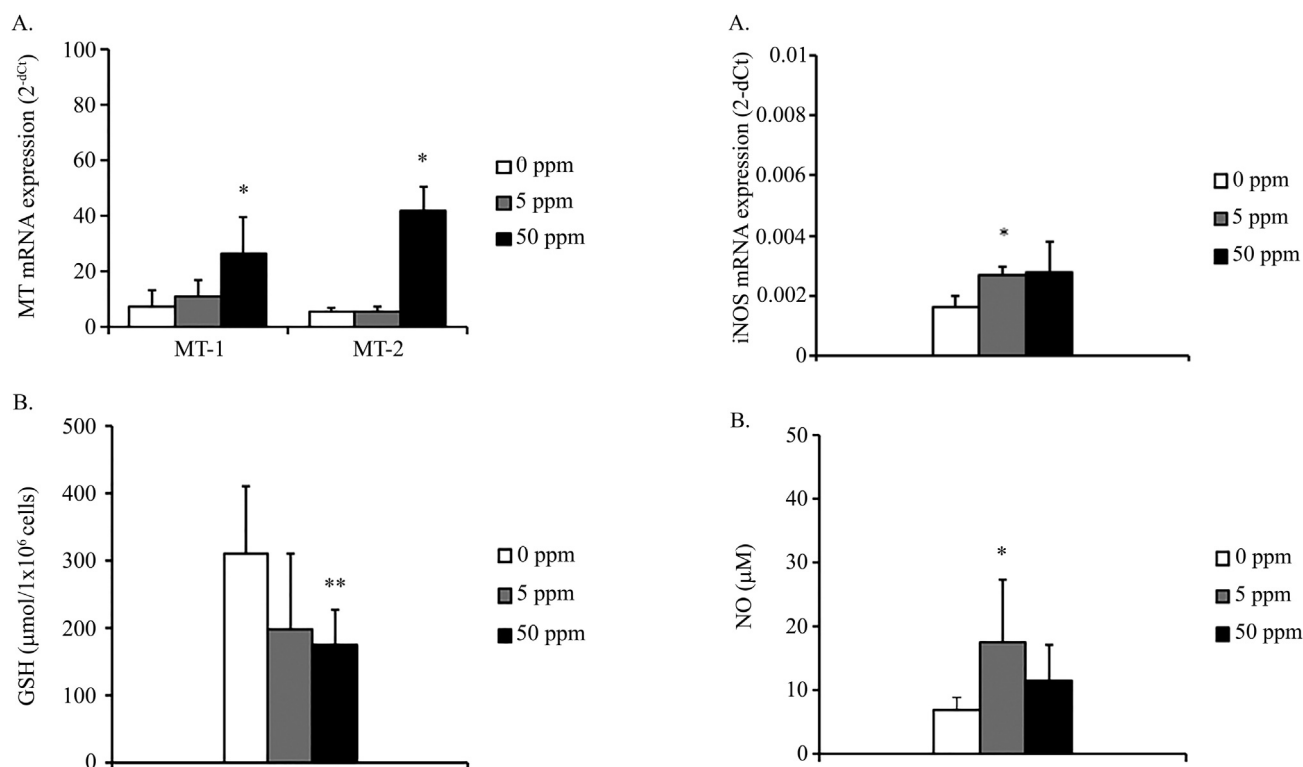


Fig. 2. Effect of oral cadmium intake on epidermal cell stress response. (A) MT-1 and MT-2 mRNA. (B) GSH. Results are presented as mean ($\pm$ SD) from at least two independent experiments (n = 4–6 rats/group). Significance at: * $P < 0.05$ and ** $P < 0.01$ vs. control animals (Cd dose 0 ppm).

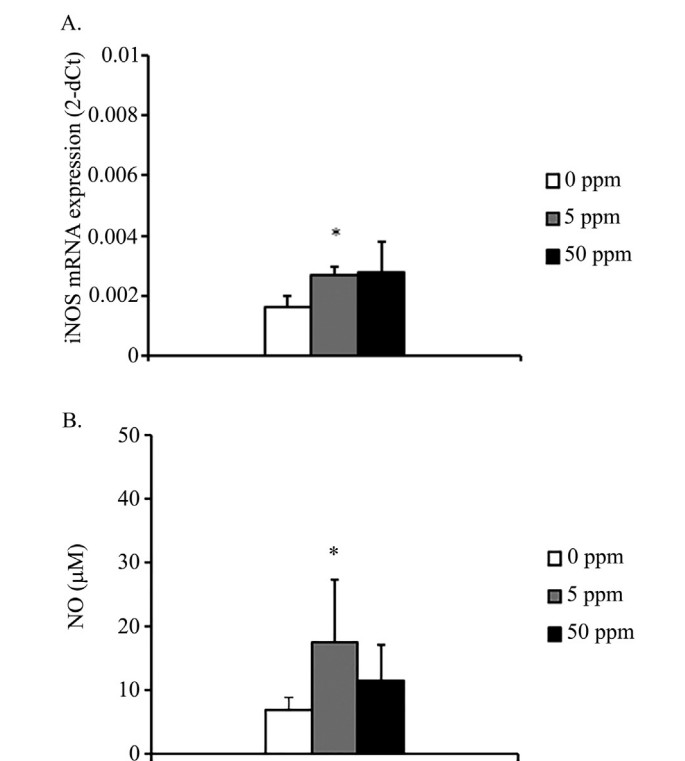


Fig. 3. Effect of oral cadmium intake on epidermal cells nitric oxide production. (A) mRNA gene expression (2^{-delta Ct}) for iNOS. (B) Nitrites concentration. Results are presented as mean ($\pm$ SD) from at least three independent experiments (n = 4–6 rats/group). Significance at: * $P < 0.05$ vs. control animals (Cd dose 0 ppm).

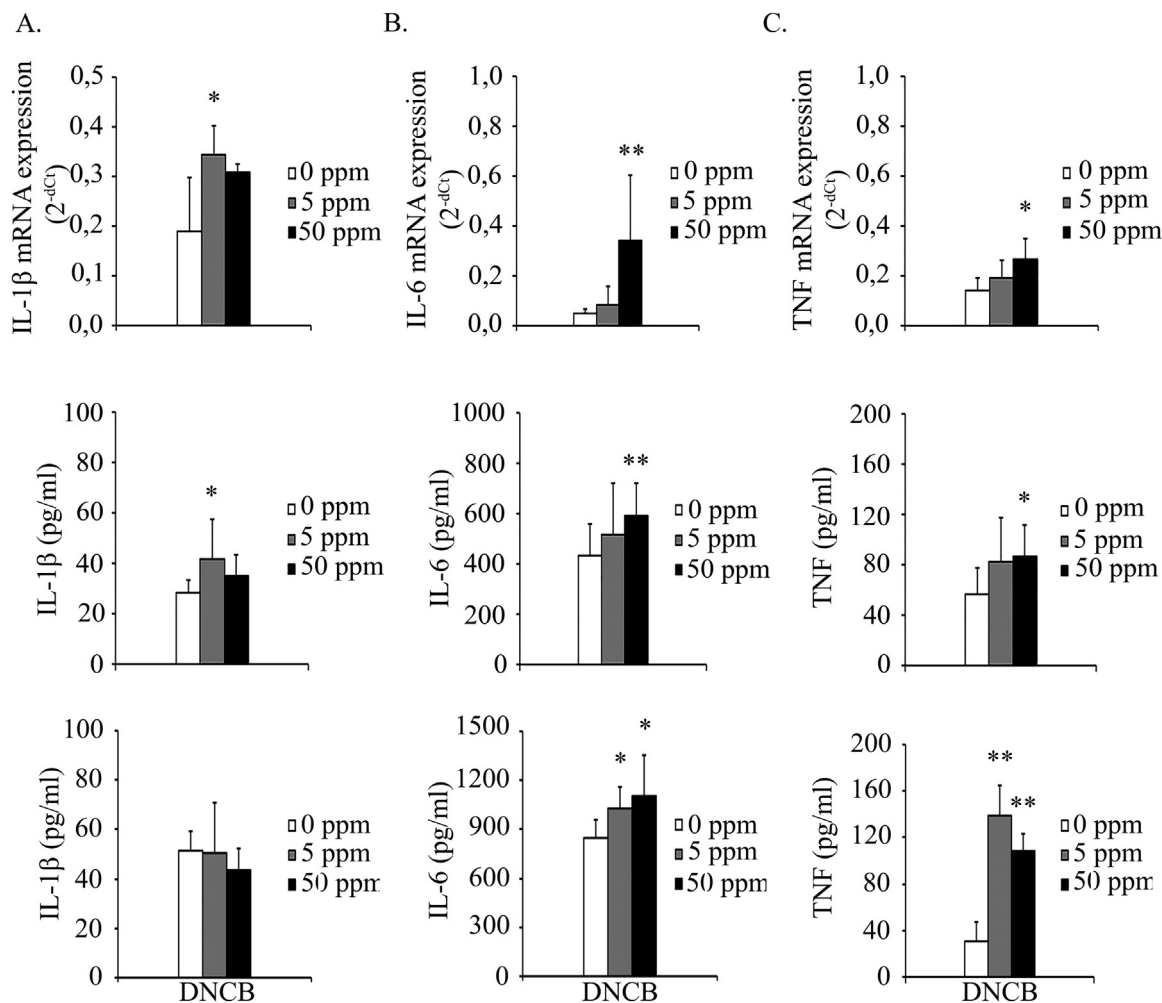


Fig. 4. Effect of oral cadmium intake on epidermal cells cytokine responses: mRNA gene expression (2^{-dCt}) (up), spontaneous production (middle) and production after *in vivo* topical application of contact allergen DNCB (down). (A) IL-1 β . (B) IL-6. (C) TNF. For DNCB treatment, data were obtained from three groups of rats: 0 ppm Cd with 0.4% DNCB, 5 ppm Cd with 0.4% DNCB and 50 ppm Cd with 0.4% DNCB. Results are presented as mean ($\pm$ SD) from at least three independent experiments ($n = 4-6$ rats/group). Significance at: * $P < 0.05$ and ** $P < 0.01$ vs. control animals (Cd dose 0 ppm/0 ppm + 0.4% DNCB).

allergen DNCB and thereafter epidermal cells were isolated and proinflammatory cytokine production was examined. As shown, further increase in both IL-6 and TNF production was seen (Fig. 4, Panels B and C, down), but no effect on IL-1 β production was seen and even a tendency ($P = 0.05$) of a decrease at 50 ppm of Cd was seen, compared to sensitized rats not exposed to cadmium (Fig. 4, Panel A, down).

To see if Cd influenced anti-inflammatory cytokine production, IL-10 response was measured. Numerical ($P = 0.070$) increase in IL-10 mRNA level (0.0024 ± 0.002 compared to 0.0012 ± 0.0007 in controls) and significantly higher ($P < 0.05$) concentrations of the cytokine protein product were observed at 50 ppm solely (166.1 ± 37.3 pg/ml compared to 115.7 ± 22.4 pg/ml in controls).

3.7. Interaction of epidermal cells with T lymphocytes

To further explore the effect of oral Cd administration on epidermal cell immune activity, cross-talk of epidermal cells and T lymphocytes was examined using co-culture system. Production of IL-17, important cytokine involved in variety of inflammatory skin diseases, was measured in co-culture of non-exposed lymph node cells and epidermal cells from cadmium-administered rats (Fig. 5). Both lymphocytes and epidermal cells produced basally low levels of IL-17 (13.3 ± 2.4 pg/ml and 36.2 ± 12.6 pg/ml, respectively), but significantly higher concentrations of the cytokine were found in cultures of epidermal cells at

50 ppm. Measurements of IL-17 produced in co-cultures of epidermal cells and lymphocytes (Fig. 5A) showed higher levels compared to individual cells, but without differences between groups. However, addition of potent T cell stimulator ConA, resulted in greater production of the cytokine in co-cultures of lymphocytes and epidermal cells from the skin of rats exposed to oral Cd compared to controls (Fig. 5B).

4. Discussion

The effect of 30-day oral intake of 5 ppm and 50 ppm of Cd on skin epidermal cell immune reactivity was examined in rats. Modulation of epidermal cell cytokine responses as well as their capacity to stimulate T cell responses was observed following oral Cd exposure. These data showed, for the first time, that Cd which gained access to the skin via intestinal absorption could affect immune-relevant epidermal cells activity.

In line with data from other studies lack of the effect of oral sub-chronic cadmium administration (Husein, 2015) on peripheral blood neutrophil and lymphocyte was shown. Increase in mean platelet volume might be considered as host attempt to preserve platelet mass, as potential of cadmium to decrease thrombocyte numbers was shown in rats exposed to 15 ppm for 4 weeks (Kocak and Akcil, 2006). Lack of the effect on acute phase/ and inflammatory cytokine response, as well as total neutrophil numbers, depict lack of systemic inflammation.

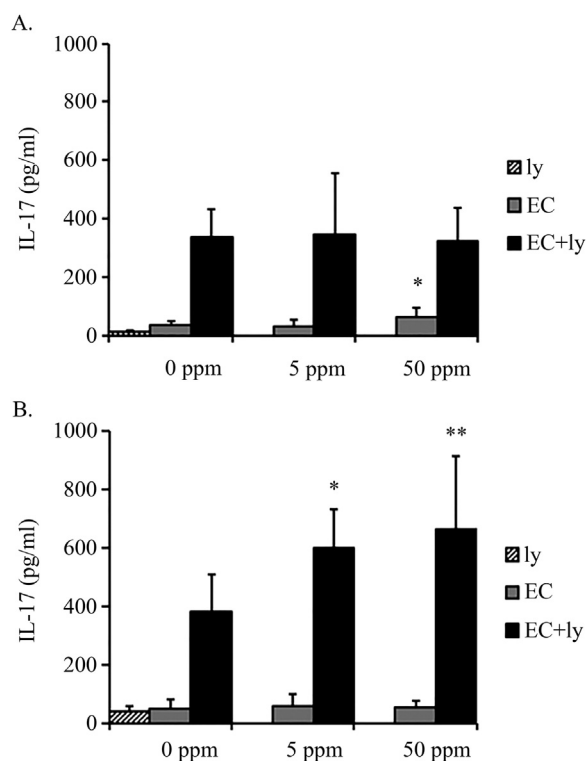


Fig. 5. Effect of oral cadmium intake on IL-17 production in co-cultures of epidermal cells and lymphocytes. (A) non-stimulated IL-17 production. (B) 2.5 µg/ml ConA stimulated IL-17 production. Results are presented as mean ($\pm$ SD) from at least two independent experiments ($n = 4-6$ rats/group). * $P < 0.05$ and ** $P < 0.01$ vs. control animals (Cd dose 0 ppm).

However, increased numbers of eosinophils might be viewed as an aspect of proinflammatory effects of cadmium. In this regard, increased allergenic properties of pollen from plants grown on cadmium-enriched soil was shown (Aina et al., 2010). Lack of changes in urine biochemical parameters depict lack of the effects of cadmium on kidneys, but exposure to higher metal concentration resulted in liver injury.

In line with data in mice (Mei et al., 2017) we observed Cd deposition in skin of rats which consumed Cd in drinking water and also showed that there was no regional (dorsal vs ventral skin) differences in deposition of this metal in the skin. Higher Cd concentration in epidermis compared to dermis, most probably resulted from binding of the metal to SH group of cysteine in epidermal keratins (Vallee and Ulmer, 1972; Fasanya-Odeyemi et al., 1998). Increased levels of SOD and CAT activity, enzymes which are part of host antioxidant defense, most probably resulted from the attempt of the skin to counter-attack increased production of reactive oxygen species and to limit tissue damage (Kim et al., 1998; Morales et al., 2006). Hyperkeratosis noted in the skin of rats which acquired Cd via oral route reflects Cd toxicity to the skin as shown in the case of dermal Cd administration in rats (Fasanya-Odeyemi et al., 1998). Presence of neutrophils as well as hyper-granulated mast cells in the skin of rats which consumed Cd, most probably reflected response to tissue injury induced by increased reactive oxygen species production. These leukocytes might be affected by Cd itself, as this metal influence granulocyte migration (Hernandez and Macia, 1996). Presence of activated mast cells, skin sentinels and responders to variety of environmental stimuli (Theoharides et al., 2012) might reflect response to Cd deposited in the skin (Schedle et al., 1995).

Although studies *in vitro* showed that Cd affected keratinocyte viability (Bae et al., 2001; Son et al., 2010), no reduction of epidermal cell viability was observed. However, Cd acquired in the skin by oral route exerted toxicity to epidermal cells judging by increase of both forms of

metallothionein (MT) and a decrease in GSH, the sensitive biomarkers of toxicity of this metal (Nzengue et al., 2011; Hofmann et al., 2014). Increase of MT-1 and MT-2 mRNA at higher Cd dose solely as well as more pronounced decrease of GSH at this dose reflect greater toxicity of 50 ppm of Cd. Changes in these stress proteins most probably reflect establishment of epidermal cell protection as described for keratinocyte exposed to Cd *in vitro* (Nzengue et al., 2008, 2009). In line with data which showed NO production by macrophage cell line exposed to Cd *in vitro* (Hassoun and Stohs, 1996) as well as by some tissues (liver, heart and testis) *in vivo* (Chen et al., 2003), our study showed that epidermal cells could also respond to Cd by production of this mediator. Stimulation of NO production, long known inflammatory mediator (Cirino et al., 2006), depicts proinflammatory effect of Cd in the skin. Increased production of NO by epidermal cells from skin which acquired Cd by oral exposure to 5 ppm, resulted from increased expression of iNOS. Lack of the effect of higher Cd dose on NO production might be ascribed to inhibition of iNOS, as shown for intestinal epithelial cell line (Cavicchi et al., 2000).

Increases in epidermal cell production of IL-1 β , IL-6 and TNF also showed pro-inflammatory potential of Cd in the epidermis. Epidermal cells seemed more prone to respond to Cd by expression and production of IL-1, as increase in mRNA as well as protein product was noted already at 5 ppm. In corroboration, our preliminary data showed increase of mRNA for IL-33, another member of IL-1 family of cytokines (Liew et al., 2010). Gene expression ($2^{-\Delta C_t}$) of IL-33 mRNA was as follows: 0.014 ± 0.005 , 0.025 ± 0.006 and 0.028 ± 0.008 at 0 ppm, 5 ppm and 50 ppm of Cd, respectively ($P < 0.05$ vs 0 ppm of Cd for both doses). Lack of dose-dependence in the IL-1 response suggest that higher Cd dose might exert inhibitory effects as well. Increased epidermal cell pro-inflammatory cytokine response most probably have resulted from keratinocyte's activity. Normal keratinocytes as well as keratinocyte cell lines are capable to respond *in vitro* by cytokine production to variety of insults including metals (Luster et al., 1995; Schmalz et al., 1998; Ermolli et al., 2001) and our study showed that epidermal cells can respond to Cd acquired by oral route by production of inflammatory cytokines. Cytokine production might represent the response to oxidative injury-related changes in the epidermis. However, direct effect of Cd deposited in the epidermis should not be excluded, given the long-known potential of this metal to stimulate cytokine production in epithelial cells (Kayama et al., 1995; Hyun et al., 2007; Lag et al., 2010). Increase in IL-10 showed that Cd can induce this anti-inflammatory cytokine as well. It might have resulted from the effect of the metal itself, as stimulation of human lung epithelial cells IL-10 production by this metal was reported (Odeyemi et al., 2015).

Epidermal cells, keratinocytes in the first place, play pivotal role in skin immunity as initiators of cutaneous inflammation in healthy skin and as participants in inflammatory/immunological skin diseases (Nestle et al., 2009; Albanesi et al., 2005). Cd increase IL-1 β , IL-6 and TNF production by epidermal cells from skin of exposed rats, which might therefore be important for inflammatory skin diseases induction and/or progression. Increase of TNF and IL-6 production by epidermal cells following skin sensitization with DNCB speaks in favor of these assumptions and showed the potential of cadmium to increase responsiveness to external inflammatory stimuli. Both cytokines are involved in inflammation induced by skin irritation (Corsini and Galli, 1998; Ku et al., 2009). Lack of an increase of IL-1 might have been related to increase of IL-6 as this cytokine was shown to inhibit IL-1 in some settings (Schindler et al., 1990; Xing et al., 1997).

Increased levels of IL-17 in co-cultures of lymph node cells with epidermal cells from skin which acquired Cd, depicted impact of this metal on immune potential of epidermal cells. It might have resulted from epidermal cell-derived cytokines, particularly IL-6 which is required for differentiation of IL-17 producing cells (Zhou et al., 2007; Nishihara et al., 2007). Production of IL-17 by minor populations of epidermal cells might have also contributed to observed response (Mabuchi et al., 2011) and higher levels of the cytokine in cultures of

epidermal cells which acquired Cd after consumption of 50 ppm supported such considerations. Given the involvement of IL-17 in variety of inflammatory diseases (Miossec, 2009), higher levels of IL-17 in epidermal cell-lymph node cell co-cultures might be of significance for inflammation-based skin diseases.

5. Conclusions

Data presented in this study showed that Cd which gained access to the skin via oral route modulated inflammatory/immune activity of epidermal cells. These novel data might be relevant for the link between dietary Cd and the risk of skin pathologies/skin disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

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