GENE EXPRESSION AND IMMUNOLOGICAL RESPONSE IN MICE EXPOSED TO ZnO NANOPARTICLES

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Abstract

We analyzed gene expression changes in the lungs and the immunological response in splenocytes of mice exposed by inhalation of ZnO nanoparticles (NP). Adult female ICR mice were treated for three days and three months, respectively. Analysis of differential expression in genes involved in oxidative stress was conducted using quantitative RT-PCR. The potential immunotoxic and immunomodulatory effects of ZnO NP were analyzed by phenotyping and cytokine production by splenocytes after three months exposure. Three days exposure resulted in down-regulation of *GCLC*, *GSR, HMOX-1, NQO-1, NF-kB2, PTGS2* and *TXNRD1* mRNA expression; three months exposure increased the expression of these genes. Three months exposure caused a significant decrease in the percentage of granulocytes in the spleen cells, and affected the production of IL-10 and IL-6 by lipopolysaccharide-stimulated leukocytes. In summary, our study revealed changes in the expression of genes involved in the oxidative stress response following acute ZnO NP exposure. Subchronic ZnO NP exposure induced immunomodulatory effects in the spleen.

Keywords: Zinc oxide nanoparticles, inhalation, gene expression, Immune response

1. INTRODUCTION

Zinc oxide nanoparticles (ZnO NP) represent a commonly used material with a yearly production over 30000 tons. Due to their antimicrobial properties ZnO NP are applied in medical and cosmetics products, but they are also used as sunscreens, pigments, catalysts and in electronic devices. Skin is the main route of exposure of ZnO NP for consumers, while in industrial production workers are also exposed by inhalation.

The toxic effects of ZnO NP were investigated in both *in vivo* and *in vitro* studies (reviewed e.g. in [1,2]). Toxicity of ZnO NP was shown to be mostly associated with reactive oxygen species (ROS) generation and subsequent induction of oxidative stress. In aqueous solution, ZnO NP produce e.g. hydroxyl radicals, superoxide radicals or hydrogen peroxide. ROS interact with cellular membranes and induce lipid peroxidation or enter the cells and cause damage to DNA, lipids and proteins. In *in vitro* cellular models, exposure to ZnO NP results in cytotoxicity, increased oxidative stress and cytokine production, as well as genotoxicity measured by Comet assay and cytokinesis-blocked micronucleus assay [1]. Results of *in vivo* tests in animal models that are more relevant for evaluation of human risk to ZnO NP exposure depend on the exposure route. In mice, target organs for oral exposure include liver, heart, spleen, pancreas and bone [3]. In the respiratory system,

administration of ZnO NP resulted in induction of processes associated with immune response and oxidative stress. Inhalation of ZnO NP induced inflammation in the lungs of both rats and mice as indicated by an increased count of immune cells in bronchoalveolar lavage fluid and changes in the expression of immune response-related genes [4-6]. Similar effects were observed in mice following intranasal instillation [7] and in Wistar rats after intratracheal instillation of ZnO NP [8-10]. Intrapulmonary spraying of ZnO NP induced temporary epithelial hyperplasia of terminal bronchioles and interstitial pneumonitis in rats [11].

The toxicity of ZnO NP is caused by the presence of particles, as well as by their ability to dissolve in the lungs. It has been reported that the release of Zn^{2+} ions in cytosol, and their sequestration by mitochondria followed by mitochondrial dysfunction and apoptosis, plays an important role in ZnO NP toxicity [12]. The solubility of ZnO NP depends on the chemical properties of the solvent: it is lower in RPMI medium and moderately hard water than in DMEM medium [13]. After intratracheal instillation into the lungs of rats, ZnO NP remained intact at around neutral pH and rapidly dissolved under acidic conditions in the lysosomes causing lysosomal destabilization and cell death. Interestingly, no dissolution of ZnO NP was observed in artificial interstitial fluid [9].

In the present study, we aimed to analyze gene expression changes in mice exposed by inhalation to two concentrations of ZnO NP for three days and three months. Furthermore, to study the immunological consequences of ZnO NP inhalation, we phenotypically characterized the splenocytes and analyzed cytokine production in the spleen of mice exposed to ZnO NP for 3 months.

2. METHODS

2.1. Animals

Adult female ICR mice (about 6 weeks old, average weight 24 g) obtained from the Animal facility of Masaryk University (Brno, Czech Republic) were allowed to acclimate to laboratory conditions for at least 1 week before the inhalation experiments. Commercial diet and water were provided *ad libitum*. The experiments were approved by the Ethical Board of the Institute of Animal Physiology and Genetics (No. 081/2010; 29 March 2010).

2.2. Exposure to ZnO NP

The inhalation chamber used for ZnO NP exposure has been described elsewhere [14]. Briefly, the chamber made of glass and stainless steel contained separate stainless steel inhalation cages (for the control and exposed animals). Constant air flow rate, temperature, relative humidity and pressure were kept using the air-conditioning system. The flow rate ranged between 9.8 and 10.1 L/min, the temperature between 20.2 and 21.5 °C, relative humidity between 58% and 68%, and the pressure was in the range of 995-1020 hPa. The cages were illuminated for 12 h, and kept in the dark for 12 h. The mice (5 animals/ group) in two exposure groups were exposed continuously for 3 days and 3 months, using concentrations of 6.46×10^4 and 1.93×10^6 ZnO NP/cm^3, respectively; the control group was treated with the purified air alone. At the end of exposure, mice were sacrificed by cervical dislocation. Organs (lungs, spleen) were collected for biochemical and electron microscopic analyses. Lung tissue was flash-frozen in liquid nitrogen and kept at -80 °C until RNA extraction; spleen samples were kept on ice and processed within 4 h.

2.3. RNA extraction and real-time quantitative PCR

To extract RNA from lung tissue, the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) was used. The tissue (200-600 mg) was homogenized under liquid nitrogen and homogenates were transferred to tubes containing the lysis buffer provided with the kit. RNA was extracted from the homogenates according to

the manufacturer's instructions and quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). An RNA Integrity Number (RIN) ranged from 5.3 to 7.9. Isolated RNA was stored at −80 °C until further processing. One microgram of RNA from each sample was used for complementary DNA (cDNA) synthesis using the Transcriptor High Fidelity cDNA synthesis Kit (Roche, Basel, Switzerland). cDNA synthesis was run using the following conditions: 30 min at 55 °C and 5 min at 85 °C. qRT-PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were: 2 min at 95° C followed by 40 cycles of amplification (10 s at 95 °C and 60 s at 60 °C). Expression levels of target genes were normalized to the reference genes (*EIF4A2* and *CANX*). Relative changes in normalized gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method [15].

2.4. Flow cytometry characterization of spleen suspensions

Single cell suspensions prepared from spleens were washed in PBS and incubated for 30 min on ice with the following anti-mouse monoclonal antibodies (mAb; all purchased from BioLegend, San Diego, CA, USA): allophycocyanine (APC)-labeled anti-CD11b (clone M1/70), fluorescein isothiocyanate (FITC)-labeled anti-Ly6G/Ly-6C (Gr1) (clone RB6-8C5), phycoerythrin (PE)-labeled anti-CD193 (clone J073E5), FITC-labeled anti-CD19 (clone 6D5), APC-labeled anti-CD3 (clone 17A2), Alexa Fluor 700-labeled anti-CD4 (clone GK1.5), PE-labeled anti-CD8 (clone 53-6.7). Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA, USA) added to the samples 10 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (LLC, Ashland, OR, USA). Fifty thousand events from each sample were measured.

2.5. Cytokine production

Single cell suspensions were prepared from spleens of individual mice. The cells (0.6 x 10^6/ml) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA), antibiotics (penicillin, streptomycin), 10 mM Hepes buffer and 5 x 10^{-5} M 2-mercaptoethanol in 48-well tissue culture plates (Corning Inc., Corning, NY, USA), unstimulated or stimulated with 1.0 µg/ml of a T cell mitogen Concanavalin A (ConA; Sigma, St. Louis, MO, USA) or 5 µg/ml of a B cell mitogen lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA). Supernatants were harvested after a 24-h (IL-2 determination), 48-h (IFN-γ) or 72-h (IL-6, IL-10) incubation period. The production of cytokines was quantified by enzyme-linked immunosorbent assay (ELISA). Cytokine-specific capture and detection mAb purchased from PharMingen (San Jose, CA, USA) were used for the detection of IL-2 and IL-6; IFN-γ and IL-10 were measured using ELISA kits purchased from R&D Systems (Minneapolis, WN, USA), following the instructions of the manufacturers. The reaction was quantified by spectrophotometry using a Sunrise Remote ELISA Reader (Gröding, Austria).

3. RESULTS AND DISCUSSION

Inhalation exposure to ZnO NP resulted in changes of expression of genes encoding proteins associated with oxidative stress response (GCLC, and GSR, enzymes responsible for glutathione metabolism; HMOX1, a protein with anti-inflammatory properties; NQO1, a quinone reductase participating in detoxification reactions; PTGS2, a key protein in prostaglandin biosynthesis; TXNRD1, thioredoxin reductase 1) and immune response and inflammation (NF-κB2) (**Figure 1**). Three day exposure to the low concentration (6.46×10^4 NP/cm^3) resulted in down-regulation of gene expression, while exposure to the high concentration (1.93×10^6 ZnO NP/cm^3) had weaker effects mostly resulting in up-regulation of gene expression. On the other hand, three month exposure induced expression of all the analyzed genes suggesting induction of processes related to oxidative stress and inflammation.

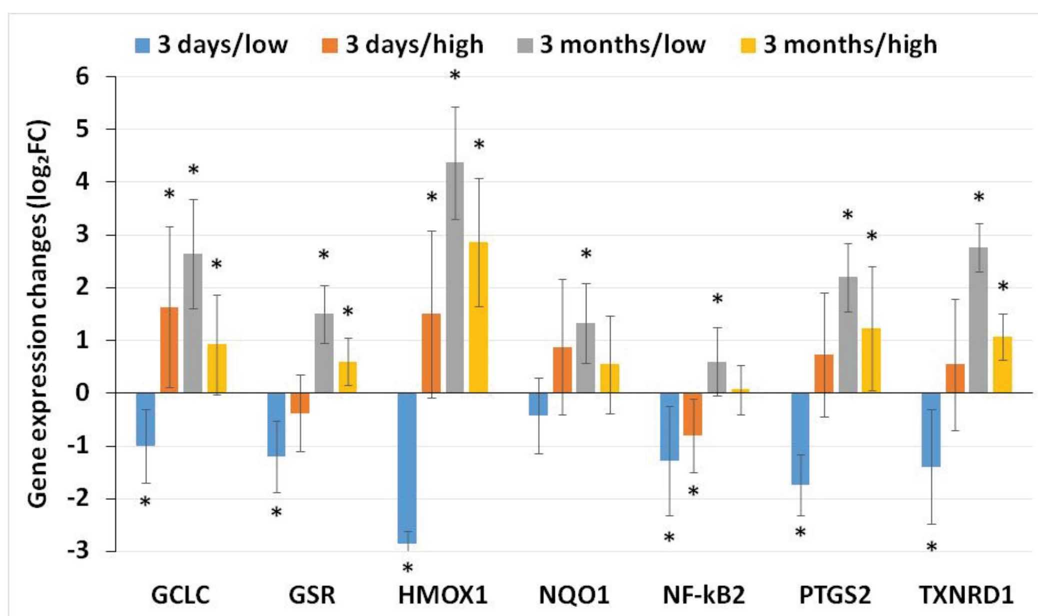

Figure 1 Gene expression changes in the lungs of mice treated with two concentrations of ZnO NP for three days and three months, respectively. Low - low concentration of ZnO NP (6.46×10^4 NP/cm^3), high - high concentration of ZnO NP (1.93×10^6 ZnO NP/cm^3). * denotes significant changes ($p<0.05$) relative to the control

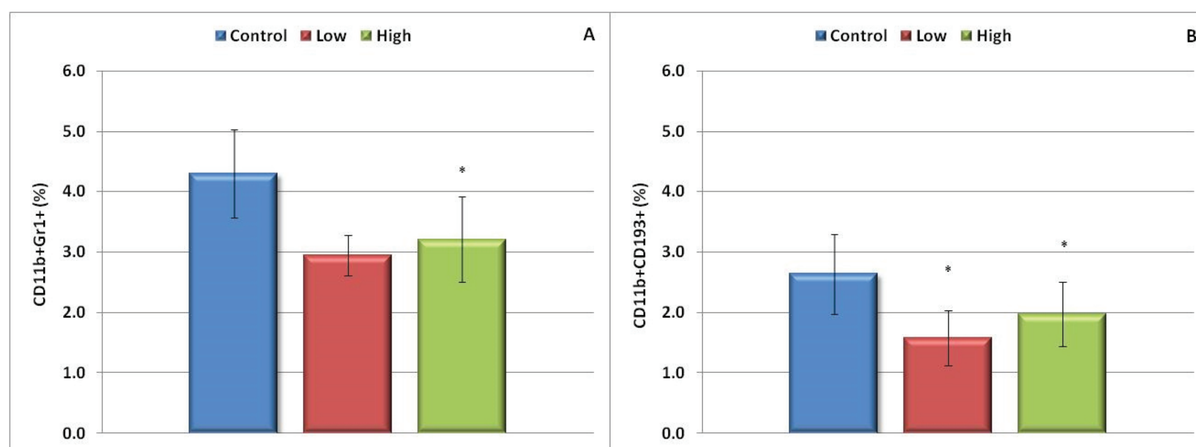

Figure 2 The effect of three months ZnO NP exposure on neutrophils (A) and eosinophils (B) (% of individual populations ± SD). * denotes a significant difference relative to the controls ($p<0.05$)

Immunological response in spleen following ZnO NP exposure

In general, no effect of ZnO NP exposure on the proportion of T or B lymphocytes was detected as evident from the comparable percentage of CD3+CD4+, CD3+CD8+ and CD19+ cells in fresh splenocytes from exposed and control mice. However, we observed a significant decrease in the proportion of eosinophils (CD11b+CD193+ cells) and neutrophils (CD11b+Gr1+ cells) in the samples from exposed animals (**Figure 2A-B**). These results suggest that three month exposure to ZnO NP has weak immunomodulatory effects on cells of innate (natural) immunity.

We further studied the production of pro-inflammatory (IL-2, IL-6, IFN-γ) and anti-inflammatory (IL-10) cytokines in unstimulated lymphocytes and T and B lymphocytes following ConA and LPS stimulation, respectively. While ZnO NP exposure did not significantly affect IL-2 and IFN-γ secretion by Con A-stimulated

T cells, the production of IL-10 and IL-6 by LPS-stimulated cells was decreased. This effect of ZnO NP inhalation was already observed on the level of spontaneous IL-6 and IL-10 production by unstimulated spleen cells (**Figure 3A-B**). These data indicate that B lymphocytes are more sensitive to ZnO NP exposure, and that subchronic inhalation of this nanomaterial might affect antibody production in the exposed mice.

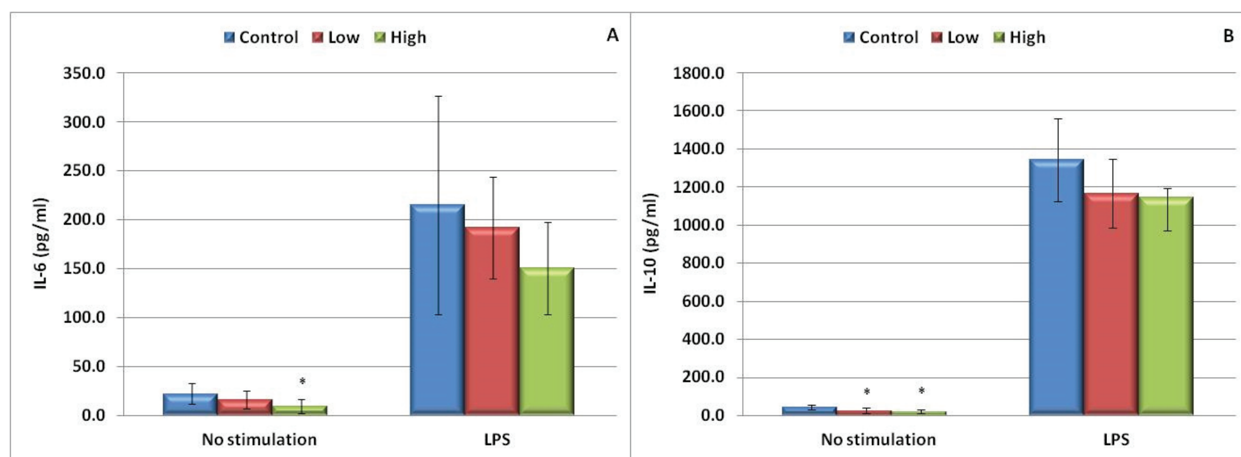

Figure 3 The production of cytokines by unstimulated spleen cells and lipopolysaccharide-stimulated B lymphocytes from the spleens of the control and exposed mice. * denotes a significant difference relative to the controls ($p<0.05$)

4. CONCLUSION

In our study we aimed to evaluate the biological consequences of acute and subchronic exposure of mice to ZnO NP. Our data showed gene expression changes in the lungs, concerning the processes associated with oxidative stress, immune response and inflammation after subchronic exposure to ZnO NP. Subchronic inhalation of ZnO NP was associated with immunomodulatory effects in the spleen. In summary, exposure to ZnO NP by inhalation caused both local and systemic effects in the experimental animals.

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REFERENCES

[1] VANDEBRIEL, R., DE JONG, W. A review of mammalian toxicity of ZnO nanoparticles. *Nanotechnology, Science and Applications,* 2012, p. 61.

[2] SHI, L.-E., LI, Z.-H., ZHENG, W., ZHAO, Y.-F., JIN, Y.-F., TANG, Z.-X. Synthesis, antibacterial activity, antibacterial mechanism and food applications of ZnO nanoparticles: a review. *Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment,* 2014, vol. 31, pp. 173-186.

[3] WANG, B., FENG, W., WANG, M., WANG, T., GU, Y., ZHU, M., OUYANG, H., SHI, J., ZHANG, F., ZHAO, Y., CHAI, Z., WANG, H., WANG, J., Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice, *Journal of Nanoparticle Research* .2008, vol. 10, pp. 263-276.

[4] MORIMOTO Y., IZUMI H., YOSHIURA Y., TOMONAGA T., OYABU T., MYOJO T., KAWAI K., YATERA K., SHIMADA M., KUBO M., YAMAMOTO K., KITAJIMA S., KURODA E., KAWAGUCHI K., SASAKI T. Evaluation of pulmonary toxicity of zinc oxide nanoparticles following inhalation and intratracheal instillation, *International Journal of Molecular Sciences.* 2016, vol. 17, p. 1241.

[5] CHUANG, H.-C., JUAN, H.-T., CHANG, C.-N., YAN, Y.-H., YUAN, T.-H., WANG, J.-S., CHEN, H.-C., HWANG, Y.-H., LEE, C.-H., CHENG, T.-J. Cardiopulmonary toxicity of pulmonary exposure to occupationally relevant zinc oxide nanoparticles, *Nanotoxicology*. 2014, vol. 8, pp. 593-604.

[6] ADAMCAKOVA-DODD, A., STEBOUNOVA, L.V., KIM, J.S., VORRINK, S.U., AULT, A.P., O'SHAUGHNESSY, P.T., GRASSIAN, V.H., THORNE, P.S. Toxicity assessment of zinc oxide nanoparticles using sub-acute and sub-chronic murine inhalation models, *Particle and Fibre Toxicology,* 2014, vol. 11, p. 15.

[7] SAPTARSHI, S.R., FELTIS, B.N., WRIGHT, P.F., LOPATA, A.L. Investigating the immunomodulatory nature of zinc oxide nanoparticles at sub-cytotoxic levels in vitro and after intranasal instillation in vivo, *Journal Nanobiotechnolog*y. 2015, vol. 13, p. 6.

[8] FUKUI, H., IWAHASHI, H., ENDOH, S., NISHIO, K., YOSHIDA, Y., HAGIHARA, Y., HORIE, M. Ascorbic acid attenuates acute pulmonary oxidative stress and inflammation caused by zinc oxide nanoparticles, *Journal of Occupational Health*. 2015, vol. 57, pp. 118-125.

[9] CHO, W.-S., DUFFIN, R., HOWIE, S.E.M., SCOTTON, C.J., WALLACE, W.A.H., MACNEE, W., BRADLEY, M., MEGSON, I.L., DONALDSON, K. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn2+ dissolution inside lysosomes, *Particle and Fibre Toxicology*. 2011, vol. 8, p. 27.

[10] CHO, W.-S., DUFFIN, R., POLAND, C.A., HOWIE, S.E.M., MACNEE, W., BRADLEY, M., MEGSON, I.L., DONALDSON, K. Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing, *Environmental Health Perspectives,* 2010, vol. 118, pp. 1699-1706.

[11] XU, J., FUTAKUCHI, M., ALEXANDER, D.B., FUKAMACHI, K., NUMANO, T., SUZUI, M., SHIMIZU, H.,. OMORI, T, KANNO, J., HIROSE, A., TSUDA, H. Nanosized zinc oxide particles do not promote DHPN-induced lung carcinogenesis but cause reversible epithelial hyperplasia of terminal bronchioles, *Archives of Toxicology,* 2014, vol. 88, pp. 65-75.

[12] KAO, Y.-Y., CHEN, Y.-C,. CHENG, T.-J,. CHIUNG, Y.-M,. LIU, P.-S. Zinc oxide nanoparticles interfere with zinc ion homeostasis to cause cytotoxicity. *The Journal of Toxicological Sciences* 2012, vol. 125, pp. 462-472.

[13] REED, R.B,. LADNER, D.A., HIGGINS, C.P., WESTERHOFF, P., RANVILLE, J.F. Solubility of nano-zinc oxide in environmentally and biologically important matrices, *Environmental Toxicology and Chemistry.* 2012, vol. 31, pp. 93-99.

[14] DUMKOVA, J., VRLIKOVA, L., VECERA, Z., PUTNOVA, B., DOCEKAL, B., MIKUSKA, P., FICTUM, P., HAMPL, A., BUCHTOVA, M. Inhaled cadmium oxide nanoparticles: their in vivo fate and effect on target organs, *International Journal of Molecular Sciences.* 2016, vol. 17.

[15] LIVAK, K.J., SCHMITTGEN, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods.* 2001, vol. 25, pp. 402-8.