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Red Sludge-induced mRNA and miRNA Expression Alterations in Vital Organs of CBA/Ca Mice

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Abstract. *Several studies have investigated the ecological and genotoxic effects of the red mud accident in 2010 in Ajka, Hungary, but none was designed to reveal the early biological effects of red mud exposure at the level of early-responding gene expression. To address relevant questions, in the present study expression alterations of oncogenes (c-myc, K-ras), tumor suppressor genes (Bcl2, p53) and apoptosis-regulatory micro(mi) RNAs (miR-21, miR-27a, miR-93, miR-221) were analized 1, 3, 6 and 24 h after a single intraperitoneal injection of red mud to CBA/Ca mice. We observed changes in the expression of all investigated mRNAs, miR-21 and miR-221 in the liver, spleen, lung, kidney and lymph nodes of mice. An overexpression of the investigated genes was observed, but the level and the peak of the alteration differed according to examined tissue.*

Red mud or red sludge is a waste product of the Bayer process, the principal industrial means of refining bauxite in order to provide alumina as raw material for the production of aluminium. A typical plant produces one to two times as much red mud as alumina. This ratio is dependent on the type of bauxite used in the refining process. The mud primarily contains non-aluminium compounds present in the bauxite ore, left as residues after its refining along with sodium hydroxide used to dissolve aluminium oxide. The red color originates from iron(III) oxide, the main component, which can make up to 60% of the mass of the red mud. In addition to iron, the other dominant particles include silica, unleached residual aluminium, and titanium oxide. The mud is highly alkaline with a pH ranging from 10 to 13.

Red mud represents one of the biggest disposal problems of industry. Usually it is stored in large open-air ponds (1). In October 2010, approximately 1.5 million m^3 of red mud flooded Kolontár and other nearby locations in Hungary in the Ajka alumina plant accident, killing ten people and contaminating about 40 km^2 of land. The accident had deleterious ecological, physiological and financial effects. After the accident several studies examined the geochemical and toxicological properties of red mud. The team leading containment and the clean-up process tried to estimate the risk of health damage caused by the exposure. Heavy metal contamination of the soil exceeded the "B" contamination limit. Respirable heavy-metal content of dust was within the acceptable limit during a 6-month measurement period after the accident (2). Gundy *et al.* performed cytogenetical measurements in 17 victims who suffered chemical burns as well as in 35 people taking part in the clen-up (spontaneous chromosome aberration). Compared to non-exposed controls, no significant difference was found, so genotoxic effect of red mud exposure was not proved in the examined individuals (3). A study performed in 2011 reported elevated aluminium, vanadium, arsenic and molibden concentrations in a 3,076 km^2 area polluted with red mud between the Marcal and Rába rivers. Higher concentrations of chromium, gallium and nikkel were measured in a 2 km^2 area around the Ajka Alumina Plant. The concentration of certain trace elements as vanadium, arsenic, chromium and nikkel surpassed the acceptable limit for natural life circumstances in the water and the sediment of the Torna river and in the upper part of the Marcal river. The particle size showed a bi-modal distribution with 0.7- and 1.3-μm peaks. Other examinations studied the mutagenic and cytotoxic effects of red mud. Based on the results of MTT and Ames tests no genotoxical or citotoxical effects were found (4-6). However, red mud-induced alterations on gene expression have not yet been examined.

Environmental factors induce cascades of epigenetic and genomic regulatory mechanisms essential for survival and adaptation, while early-immediate responses mostly involve alterations in gene expression. Our group has in the past examined the effect of different environmental factors on the level of onco- and suppressor genes in an animal model developed in our Institute. We have reported that exposure to well-known carcinogens as 7,12-benzanthracene, N-nitroso-N-methylurea and cyclophosphamide caused the overexpression of *Ha-ras*, *c-myc* and *p53* genes already in the first 24 h (7-9).

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The role of miRNA has been verified in the pathogenesis of several diseases. Previous results underlined the role of miRNA in carcinogenesis (10). Although there are still several questions to be cleared, it is already obvious that environmental factors substantially influence miRNA expression patterns (11). In our previous studies we found that dimethylbenzanthracene and methylithrosourea exposure resulted in significant changes in the expression of microRNA playing a key role in the process of cell cycle, apoptosis and differentiation. This indicates that certain miRNA being in charge for the regulation of mRNA are suitable to predict and follow-up various environmental effects (12).

In our present study we aimed to examine the early changes in the expression of onco- and suppressor genes (*c-myc, K-ras, p53*) and microRNAs (*miR-21, miR-27a, miR-93, miR-221*) caused by the intraperitoneal administration of red mud.

Materials and Methods

Five groups of CBA/Ca (H-2^K haplotype) male mice were used for this experiment, with six animals in each group. The animals were six-week-old (20±4 g) and were kept in isolated cages. The control group consumed the standard laboratory pellet and tap water *ad libitum*. Four groups of mice received a single intraperitoneal gavage of dried red mud, 25 mg/body weight dose, (0.5 mg/0.1 ml solved in distilled water). One, three, six and twenty-four hours after red mud injection mice were autopsied after cervical dislocation. Mice received humane care and the experiment was carried out under the approval of the Institutional Revision Board. The liver, lung, kidneys, spleen and lymph nodes of the animals were removed and 100-mg samples were obtained from each tissue of the respective groups. After homogenization of the organs, total cellular RNA was isolated using TRIZOL reagent (Invitrogen, Paisley, UK). The RNA quality was checked by denaturing gel-electrophoresis, and absorption measurement was performed at 260/280 nm (A260/A280 was over 1.8).

Ten μg RNA were dot-blotted onto Hybond N^+ nitrocellulose membrane (ECL kit, Amersham, Little Chalfont, UK) and hybridized with chemiluminescently labelled specific probes for c-*myc, p53, bcl2* and *K-ras* genes. Isolation of RNA, hybridization and detection were performed according to the manufacturer's instructions. The membranes were rehybridized with constitutively expressed beta-actin gene as a positive control. The chemiluminescent signals were detected on X-ray films, scanned into a computer and evaluated by Quantiscan software (Biosoft, Cambridge, UK). Gene expression is reported as percentage relative to the level of the expression of *β-actin* control.

Total RNA was exposed to RNAase-free DNAase and 2 μl was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Berlin, Germany). The expression of the investigated miRNAs was determined by quantitative real-time polymerase chain reaction (PCR). PCR primers were designed using the primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary). Sequence specific primers: *miR-21* forward: 5'- GCTTATCAGACTGATGTTGACTG -3', reverse: 5'-CAGCCCATCGACTGGTG-3'; *miR-27a* forward: 5'- GCAGGGCT TAGCTGCTTG-3', reverse: 5'- GGCGGAACTTAGCCACTGT-3'; *miR-93* forward: 5'-AAGTGCTGTTCGTGCAGGT-3', reverse: 5'-CTCGGGAAGTGCTAGCTCA-3', *miR-221* forward: 5'-CCTG GCATACAATGTAGATTTCTG-3', reverse: 5'-AAACCCAGCAG ACAATGTAGCT-3'. The PCR was performed on a LightCycler 480 PCR system (Roche, Berlin, Germany). The PCR reaction mixture contained: 5 μl of template cDNS, 3 μl of H_2O, 2 μl specific primer and 10 μl Master mix. The reaction mixtures were incubated in LightCycler 480 Multiwell Plate 96, for 5 min at 95˚C, followed by 55 three-step amplification cycles (95˚C for 10 s, 55˚C for 20 s, 72˚C for 15 s). miRNA expressions were determined by absolute nucleic acid quantification with 480 Light Cycler software (Roche Diagnostics GmbH, Mannheim, Germany).

The statistical calculation of differences in expression was performed using Statistical Program for Social Science 19.0 (SPSS) software (IBM, Armonk, NY, USA). Student's *t*-test was performed between control and treated groups, and *p*-values less than 0.05 were considered statistically significant.

Results

Using quantitative real-time polymerase chain reaction the expression profile of apoptosis-regulatory onco- and tumor suppressor genes and miRNAs in vital organs of CBA/Ca mice were evaluated at 1-, 3-, 6- and 24-h time point following intraperitoneal injection of red sludge.

One hour after the treatment we detected a significant up-regulation of *c-Myc* in the liver (p=0.0003) and spleen (p=0.0008), while there was a significant down-regulation in the expression of p53 in all investigated organs except for the liver (Figures 1-5). Considerably increased expression of *k-ras* gene was found in the liver (p=0.0025), lungs (p=0.0015) and kidneys (p=0.0014) 3 h after the injection of red sludge (Figures 1, 3 and 4). The expression of *bcl2* gene showed up-regulation in the spleen (p=0.0346), lungs (p=0.0415) and kidneys (p=0.0041) at the 3-h time point (Figures 2-4). The expession of *c-myc* was strongly decreased in the liver (p=0.00026) and lymph nodes (p=0.038) at the specific time point (Figures 1 and 5). In the lungs, the expression levels of all investigated mRNAs were significantly elevated 6 h after the exposure with red sludge, while the lymph nodes showed decreased expression of the same mRNAs at the same time (Figures 3 and 5). We observed a significant up-regulation of the investigated mRNAs in the liver and spleen and their down-regulation in the lymph nodes 24 h after the treatment with red sludge (Figures 1, 2 and 5).

In the liver, miR-93 showed a significantly decreased expression level relative to the control group (p=0.03) at the 24-h time point (Figure 6). There were no statistically significant differences detectable in the miRNA expression levels between the treated and control groups in the spleen and lungs (Figures 7 and 8). In the kidneys, we observed a marked decrease in the expression of miR-27a (p=0.0405) and miR-221 (p=0.027) at the 1- and 24-h time point (Figure 9). A significant up-regulation of miR-93 was detected three hours (p=0.0001) and 24 h (p=0.0292) after exposure in the lymph nodes (Figure 10).

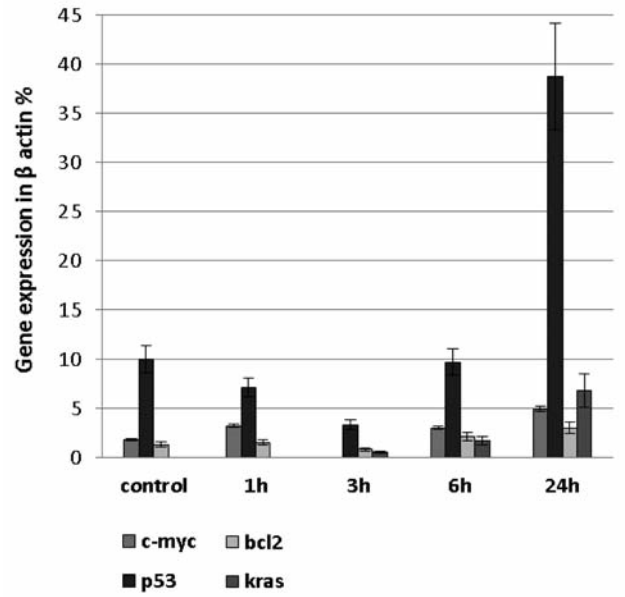

Figure 1. *Onco- and tumor-suppressor gene expressions in the liver.*

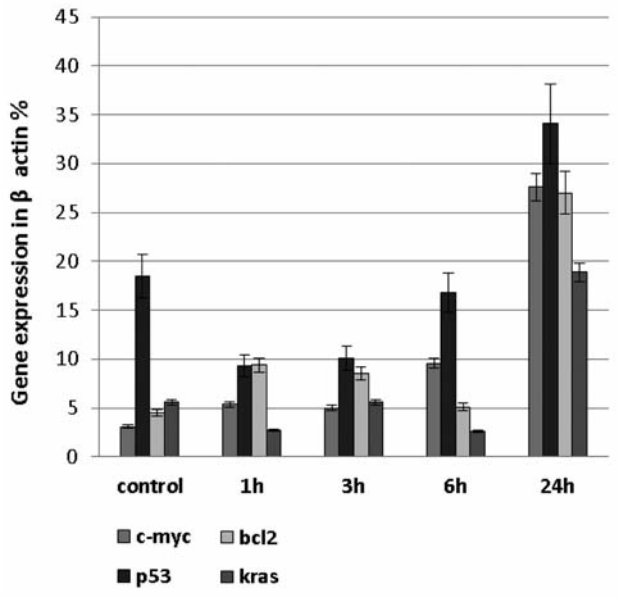

Figure 2. *Onco- and tumor-suppressor gene expressions in the spleen.*

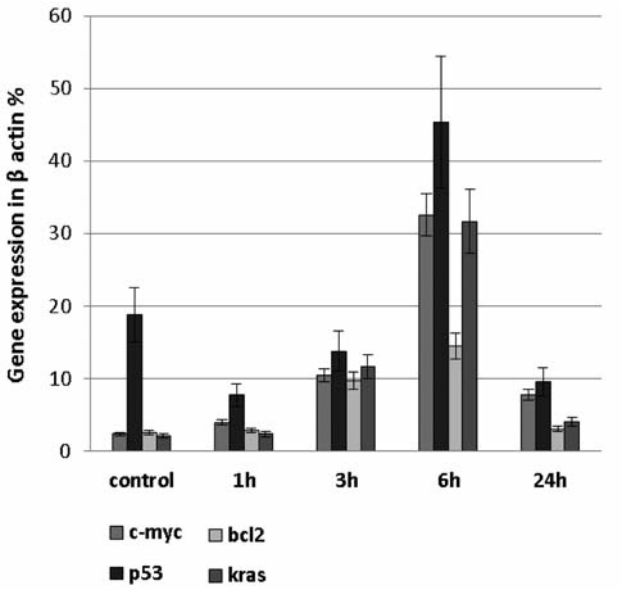

Figure 3. *Onco- and tumor-suppressor gene expressions in the lungs.*

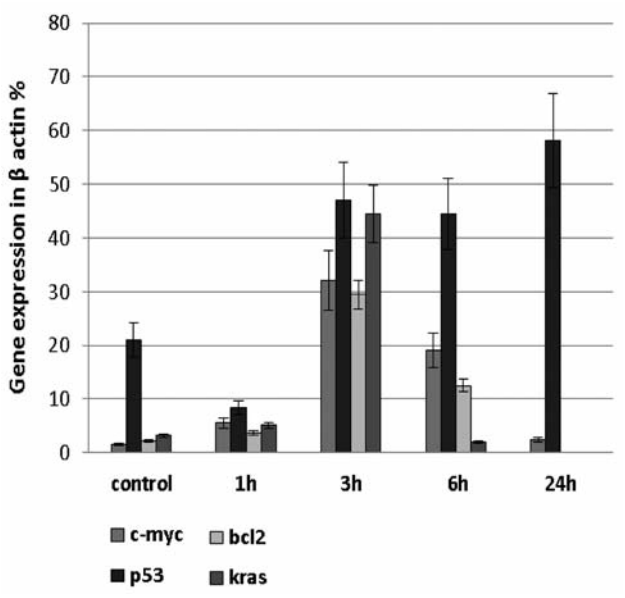

Figure 4. *Onco- and tumor-suppressor gene expressions in the kidneys.*

Discussion

To our knowledge this is the first study to examine changes in gene expression caused by red mud in an animal model. Our aim was to study the expression of mRNA and miRNA known to play a role in the control of response mechanisms to environmental influences (13, 14).

Using a real-time PCR technique the expression of onco- and tumor-suppressor genes such as *c-myc, p53, Bcl2, K-ras* was investigated in liver, spleen, lung, kidney and lymphoid tissue of CBA/Ca mice. We also investigated changes in the expression of miRNA. Their advantage compared to mRNA tests is that they may serve as useful markers to identify early environmental changes besides oncogenes and tumor

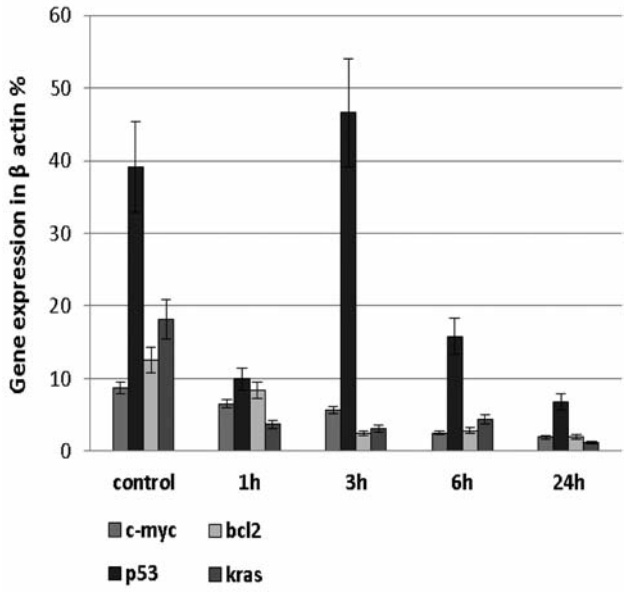

Figure 5. *Onco- and tumor-suppressor gene expressions in lymph nodes.*

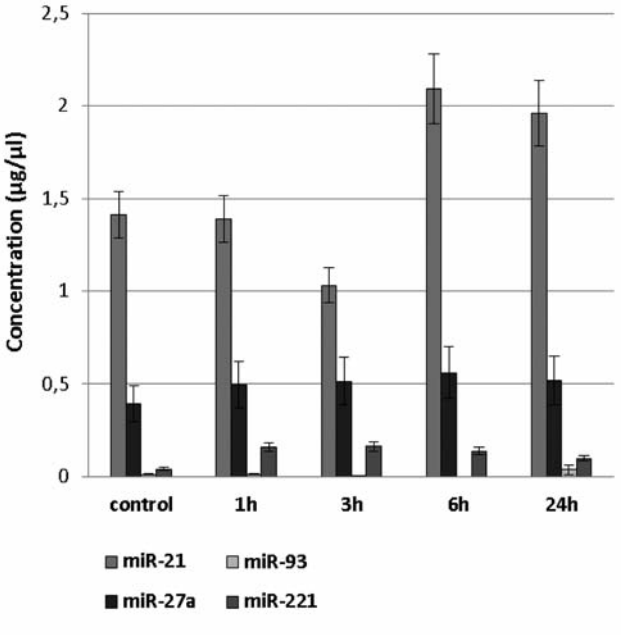

Figure 7. *miRNAs expressions in the spleen.*

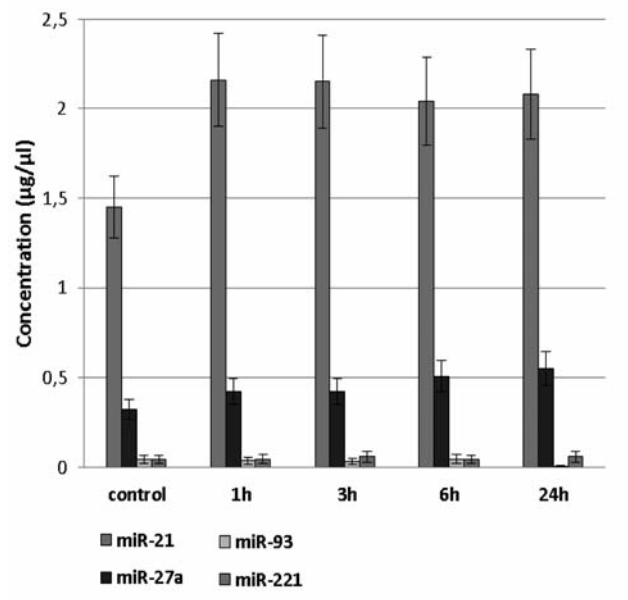

Figure 6. *miRNAs expressions in the liver.*

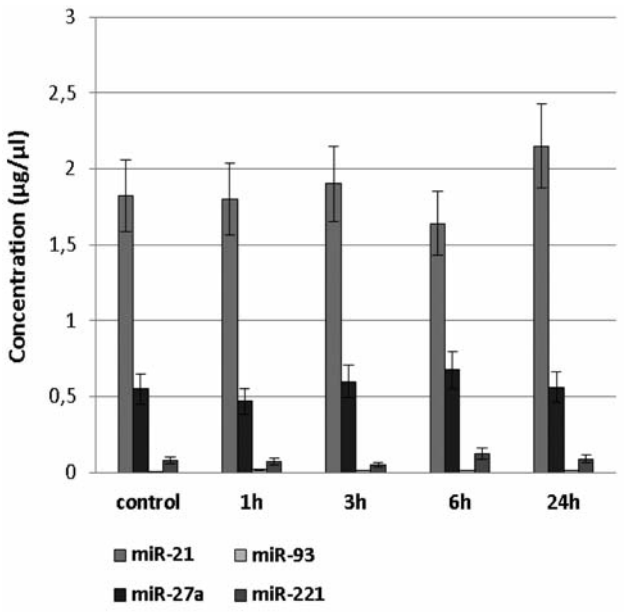

Figure 8. *miRNAs expressions in the lungs.*

suppressor genes. Moreover, as miRNA control the expression of several hundreds of such target genes, less number of molecular markers may suffice for the investigation of the effect of a certain agent. *miR-21* functions as an onkomiR, which might negatively influence the expression of numerous tumor suppresor genes such as *p53*, phosphatase and tensin homolog (*PTEN*) and programmed cell death 4 (*PDCD4*). Similarly to *miR-21*, the oncogene function of *miR-27* has been identified in the case of several types of tumors (15, 16). Among others, *miR-27* partake in the regulation of the APC gene or genes playing a role in the Wnt signal transduction pathways (17, 18). *miR-*

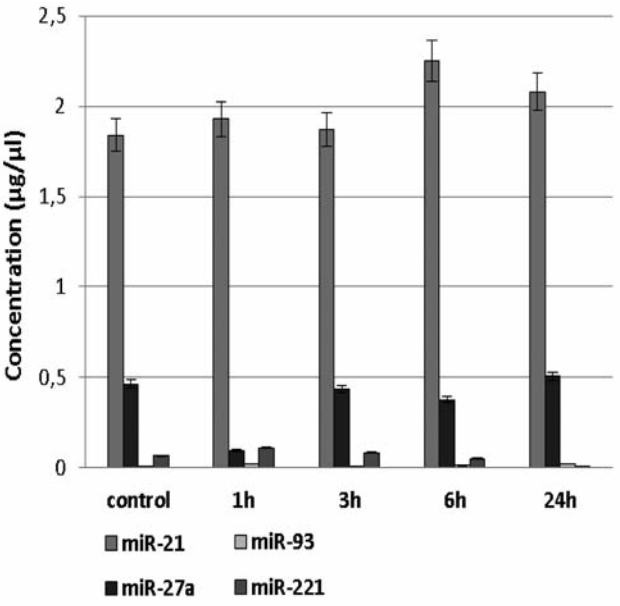

Figure 9. *miRNAs expressions in the kidneys*.

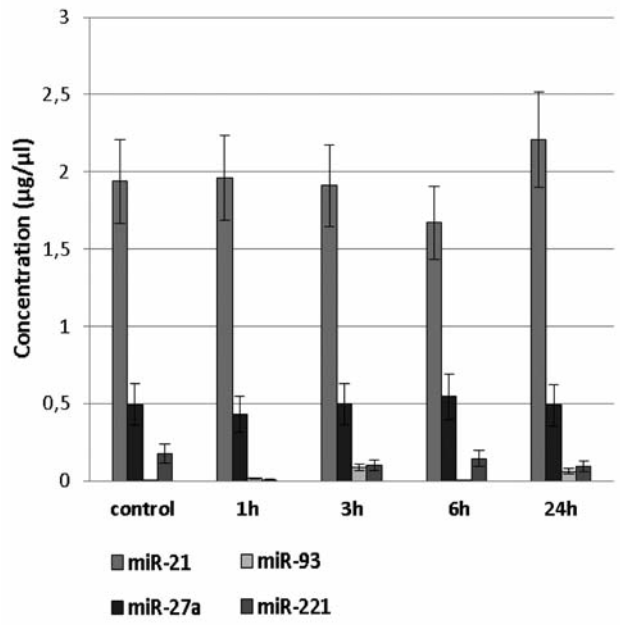

Figure 10. *miRNAs expressions in the lymph nodes*.

221 is also one of the most often investigated miRNAs that controls the function of genes playing a role in the proliferation of endothelial cells (*CD117*) and in the process of angiogenesis (19). *miR-93* has been proved to play a role in the control of the *TP53INP1* gene (20). Moreover, the overexpression of miR-93 has been associated with the pathogenesis of various tumors (21).

Based on our results we can state that the expression of the analyzed genes showed significantly different expressions in the investigated organs. In the liver, we found the overexpression of the *p53* gene 24 h after the exposure, while among the tested microRNA, *miR-21* showed elevated expression.

In the spleen, the expression of *miR-21* and *miR-221* was found to be elevated compared to controls, and after 24 h we observed an overexpression of *K-ras, c-myc, p53* and *Bcl2* genes. We further detected an overexpression of the four investigated onco- and tumor-suppressor genes 3 and 6 h after the administration of red mud in the kidneys, and 6 h after treatment in the lungs. The expression levels of *miR-21* were raised 24 h after exposure in the lungs, and after 6 h in the kidneys. In the case of *miR-221* we observed an elevation after 1, 3 and 6 h in lung-and kidney tissues. On the other hand, unlike other tissues, samples from lymph nodes exhibited a decreased expression of the investigated onco-suppressor genes.

Our present study is the first to prove early gene expression changes in mice after intra-peritoneal administration of red mud. We proved that the intra-peritoneal administration of red mud changed the expression of several mRNAs and miRNAs holding a role in the processes of cell proliferation, differentiation, signal transduction and apoptosis. As our investigation was limited to the first 24 h after the administration of red sludge, it is not known whether the observed effect is permanent or temporary, thus the gene expression changes caused by red sludge require longer-term investigations with a greater number of genes analyzed.

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