H. Türkmez et al. / Hacettepe J. Biol. & Chem., 2020, 48 (4), 309-318

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Expression Profiling and Pathway Analysis of Iron Oxide Nanoparticles Toxicity on Human Lung Alveolar Epithelial Cell Line Using Microarray Analysis

Mikrodizin Analizi ile Demir Oksit Nanoparçaciklarinin İnsan Akciğer Epitel Hücre Hatti Üzerinde Ekspresyon Profil ve Yolak Analizleri

Hasan Türkez[1], Mehmet Enes Arslan[2*], Erdal Sönmez[3], Abdulgani Tatar[4], Fatime Geyikoğlu[5] and Metin Açıkyıldız[6]

[1]Department of Medical Biology, Faculty of Medicine, Atatürk University, Erzurum, Turkey.
[2]Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey.
[3]Department of Physics, Kazım Karabekir Education Faculty, Atatürk University, Erzurum, Turkey.
[4]Department of Medical Genetics, Medical Faculty, Atatürk University, Erzurum,Turkey.
[5]Department of Biology, Faculty of Arts and Sciences, Atatürk University, Erzurum,Turkey.
[6]Department of Chemistry, Faculty of Science and Art, Kilis 7 Aralık University, Kilis, Turkey.

ABSTRACT

Toxicogenomics is a developing area searching for cellular pathways and mechanisms including cancer, immunological diseases, environmental responses, gene-gene interactions and drug toxicity. Nanoparticles (NPs) become important candidates for analyzing in toxicogenomic experiments because of their unusual properties in various biological activities. Therefore, we examined the nanotoxicity of iron oxide (Fe_2O_3) on gene expression profiling of human alveolar epithelial cells (HPAEpiC) in the study. For this aim, iron oxide nanoparticles were synthesized by zone melting method and characterized via using X-ray crystallography (XRD) and transmission electron microscope (TEM) techniques. Cell viability and cytotoxicity were determined by 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) and lactate dehydrogenase (LDH) release tests. Whole-genome microarray expression analysis was performed to explore the effects of iron oxide nanoparticles on gene expression in cultured human alveolar epithelial cells. For further analyses, these genes were functionally classified by using DAVID (The Database for Annotation, Visualization and Integrated Discovery) with gene ontology (GO) analysis. The results from this study indicated that iron oxide-mediated toxicity directly or indirectly affecting the regulation of cell proliferation, response to hormone stimulus, estrogen stimulus, cytokine activity and blood circulation by stimulating diverse genes.

Key Words
Iron oxide nanoparticles, microarray analysis, toxicogenomics, human alveolar epithelial cells, DAVID analysis.

ÖZ

Toksikogenomik, kanser, immünolojik hastalıklar, çevresel tepkiler, gen-gen etkileşimleri ve ilaç toksisitesi gibi hücresel yolları ve mekanizmaları araştıran gelişmekte olan bir alandır. Nanopartiküller (NP'ler), çeşitli biyolojik aktivitelerde sıradışı özelliklerinden dolayı toksikogenomik deneyler için önemli hale gelmişlerdir. Bu nedenle yapılan çalışmada insan alveolar epitel hücrelerinin (HPAEpiC) gen ekspresyonu profillemesi üzerinde demir oksidin (Fe_2O_3) nanotoksisitesi incelenmiştir. Bu amaçla, demir oksit nanopartikülleri kısmi erime metodu ile sentezlenmiş ve X-ışını kristalografisi (XRD) ve transmisyon elektron mikroskobu (TEM) teknikleri kullanılarak karakterize edilmiştir. Hücre canlılığı ve sitotoksisite 3-(4,5-dimetil-tiazol-2-il) 2,5-difeniltetrazolyum bromür (MTT), nötral kırmızı (NR) ve laktat dehidrojenaz (LDH) salım testleri ile tespit edilmiştir. Bütün genom mikrodizin analizi, demir oksidin nanopartikülleriyle kültürlenmiş insan alveolar epitel hücrelerinde gen ekspresyonu üzerindeki etkilerini araştırmak için uygulanmıştır. Bunların yanısıra, mikrodizin sonucunda elde edilen farklı ekspresyon düzeylerine sahip genler, gen ontolojisi (GO) analizi ile DAVID (Annotation, Visualization ve Integrated Discovery Veri Tabanı) kullanılarak işlevsel olarak sınıflandırılmıştır. Bu çalışmadan elde edilen sonuçlar, demir oksidin doğrudan veya dolaylı olarak hücre proliferasyonunun düzenlenmesini, hormon uyarımına tepkiyi, östrojen uyarısını, sitokin aktivitesini ve çeşitli genleri uyararak kan dolaşımını etkilediğini göstermiştir.

Anahtar Kelimeler
Demir oksit nanoparçacıkları, mikrodizin analizi, toksikogenomik, insan alveolar epitel hücreleri, DAVID analizi.

Article History: Received: Nov 8, 2018; Revised: Dec 18, 2019; Accepted: Jan 15, 2020; Available Online: Jun 15, 2020.

DOI: https://doi.org/10.15671/hjbc.736803

Correspondence to: M.E. Arslan, Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey.

E-Mail: enes.aslan@erzurum.edu.tr

INTRODUCTION

Recently, nano-technological developments accelerated exponentially and influenced wide spectrum areas such as disease diagnoses, cancer treatment, drug delivery, medical imaging and gene therapy. Nanoparticles (NPs) usage in these areas showed infinite potentials and also, novel applications were discovered non-stop [1-3]. Using nanotechnology in every aspect of life from medicine to nutrition was thought to be one of the most important occurrences since the industrial revolution [4]. Consumption costs for nanotechnology approached nearly US$1 trillion for now. Scientists believed that nanotechnology should be analyzed comprehensively before use in different applications because of its potential negative effects on the environment and health [5].

Magnetic iron oxide NPs have been used in various applications like drug delivery, magnetic resonance imaging, tissue repair and cell separation because of its convenient surface conformations [6,7]. In researches of nano-sized magnetic particles, several types of Fe_2O_3 have been investigated (maghemite and magnetite most importantly) and these molecules were assumed to have biocompatible features and promising candidates for medical use [8]. Magnetic iron oxide NPs diffused in appropriate solvents and form homogeneous ferrofluids which could be used in different applications as a suspension [9]. These magnetic NPs could form minefields and be directed to specific regions which were used as biological carriers or magnetic resonance imaging for medical diagnosis [10].

Toxicogenomics is a rapidly growing area, which compares genomic data with toxic molecule effects. Different pathways are related to toxicity against health risk for various compounds by investigating whole-genome gene expression with respect to toxic compound exposure. Furthermore, toxic molecule databases are constituted with Toxicogenomics and health risks reports of unknown molecules can be estimated with these databases [11]. Comparable information provides novel insights about unknown compounds and their effects on gene expression and toxicity. But there must be a very large data pool to conclude this type of comparison and this type of information can only be used as preliminary information [12]. Gene expression profiles of toxic molecules are used to find parallel pathways and similar cellular mechanisms. Generally, closely related drug interactions and toxicity analysis are investigated and also irrelevant or unlikely pathways relevance can be established via Toxicogenomics [13].

For evaluating the cytotoxicity of Fe_2O_3 NPs different viability assays (MTT, LDH and NR) were carried out after incubation of 72h. LC20 value was calculated according to the LDH analysis because the results obtained from LDH analysis was found to be more accurate and correlative with the experimental replicates. Then, Fe_2O_3 NPs were applied to the cell culture at LC20 concentration and total RNAs were isolated from cultures and analyzed in microarray technologies. Finally, microarray data were investigated with The Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis and functional categories for these genes revealed to understand the effects of Fe_2O_3 NPs on biological pathways. The main aim of this article was to reveal characteristics, living cell interactions and any cytotoxic effects of Fe_2O_3 NPs, comprehensively.

MATERIALS and METHODS

Synthesis and Characterization of Iron Oxide (Fe3O4) Nanoparticles

Fe_2O_3 NPs were synthesized via the thermal decomposition of Fe(acac)3. In the reaction, 4 mmol of Fe(acac)3, 20 mmol of 1,2-hexadecanediol, 12 mmol of oleic acid, 12 mmol of oleylamine, and 40 mL of benzyl ether were mixed in mechanical activation assisted self-propagating system. The mixture was heated to 200 ˚C for 2 h. Then, the reaction solution was cooled to room temperature and 80 mL of ethanol was added. The product was collected by centrifugation and dispersed in hexane (Sigma-Aldrich®, MO, USA) with the presence of oleic acid. The sample was subjected to two cycles of precipitation in methanol and centrifugation to get pure Fe_2O_3 NPs.

The structural analysis of Fe_2O_3 nanoparticles was executed via X-ray diffraction (XRD) system by using a Rigaku diffractometer at room temperature with CuKα radiation operated at 40kV and 30 mA. The calculations were operated with a step of 0.020 in the geometry of coupled θ-2θ changed between 1000 and 8500 respectively. The molecular size and surface arrangement of Fe_2O_3 nanoparticles was observed under a scanning electron microscope (FEI inspect S50 SEM) and transmission electron microscopy (JEOL JEM-ARM200CFEG UHR-TEM).

Cell cultures

The human primary alveolar epithelial cells (HPAEpiC) and alveolar epithelial cell mediums (AEpiCM), including 500 ml of basal medium, 5 ml of epithelial cell growth supplement, 10 ml of fetal bovine serum and 5 ml of penicillin/streptomycin solution, were brought from ScienCell®. For all the cell viability assays, HPAEpiC's were growth in 48-well plates with AEpiCM and incubated in a humidified 5% CO_2 at 37°C. The cell culture was applied with Fe_2O_3 nanoparticles for 72 h at twelve different concentrations (0.625, 1.25, 2.5, 5, 10, 20, 20, 80, 160, 320, 640 and 1280 mg/L). According to previous nanotoxicity analysis, it was found to 72 h incubations with nanoparticles would result in a significant difference of cell viability and gene expression patterns. Cell cultures without Fe_2O_3 nanoparticles were investigated as a negative control group. Hydrogen peroxide (H_2O_2; 25 µM SigmaAldrich®) was used as the positive control in cytotoxicity testing.

MTT cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Thermo Fisher®, USA) was prepared and applied to cell cultures in 5 µM concentrations. As a summary, the cell cultures were incubated for 3 h with MTT solution, and dimethyl sulfoxide (DMSO) (Sigma-Aldrich®) was used as an inorganic solvent for formazan crystals which produced by viable cells. A spectroscopic plate reader was used to measure culture color intensities at 570 nm wavelength [14].

LDH cytotoxicity assay

Lactate dehydrogenase cytotoxicity assay kit (Cayman Chemical Company®, USA) was applied to the cell culture according to the manufacturer's instructions. The cells were placed to 96-well plates and wide spectrum B4C concentrations were administered to cell culture for 72h. To get rid of Fe_2O_3 in the wells, 96-well plate was centrifuged at 400 g for 5 min. After that 100 µL supernatant was transferred to a fresh 96-well plate and 100 µL of the reaction mixture was added to the samples and incubated for 30 min at room temperature. Finally, a microplate reader was used to analyze the absorbance of the cultures at 490 nm [15].

NR cell viability assay

Neutral red (NR) solution was applied to the cell cultures according to the manufacturer's recommendation (Sigma-Aldrich®, USA). For the dye uptake of viable cells lysosomes, HPAEpiC was incubated with the NR solution for 2h at 37°C. To wash cell culture, the mixture of $CaCl_2$ (0.25%) and formaldehyde (0.125%) was used and the NR solution was removed. To get rid of the NR from the

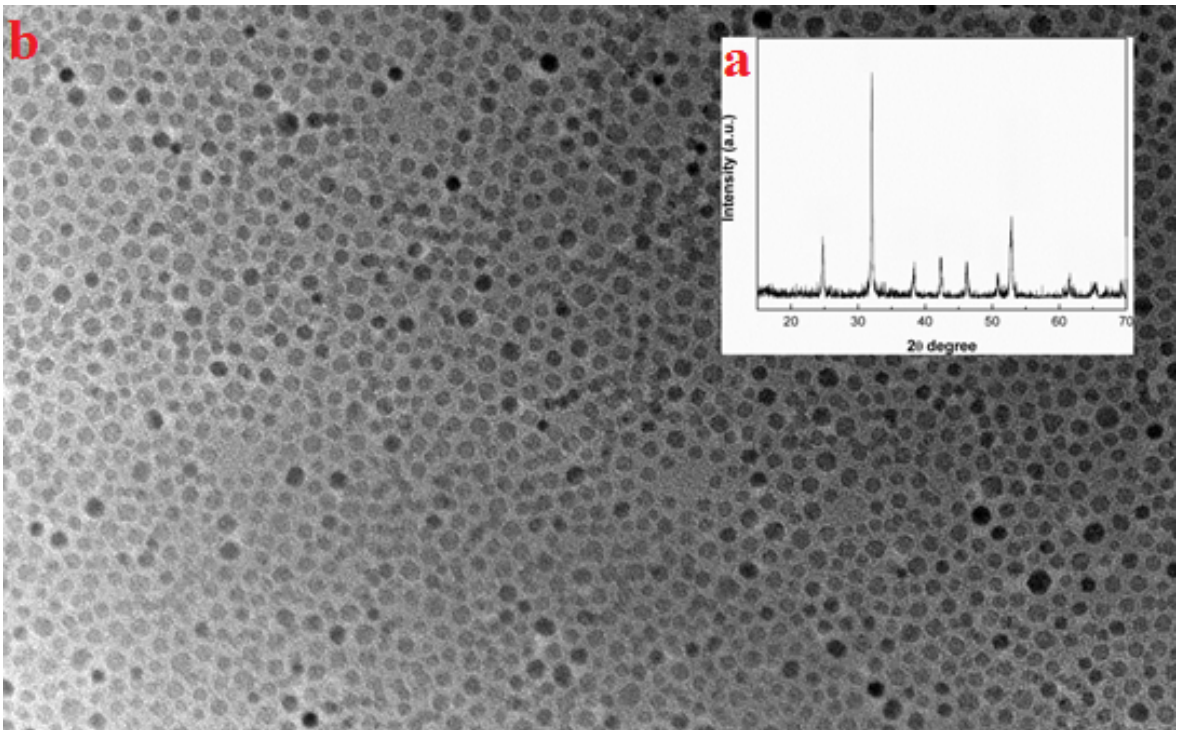

Figure 1. a-X ray diffraction pattern and b-Transmission electron microscope (TEM) image of iron oxide NPs.

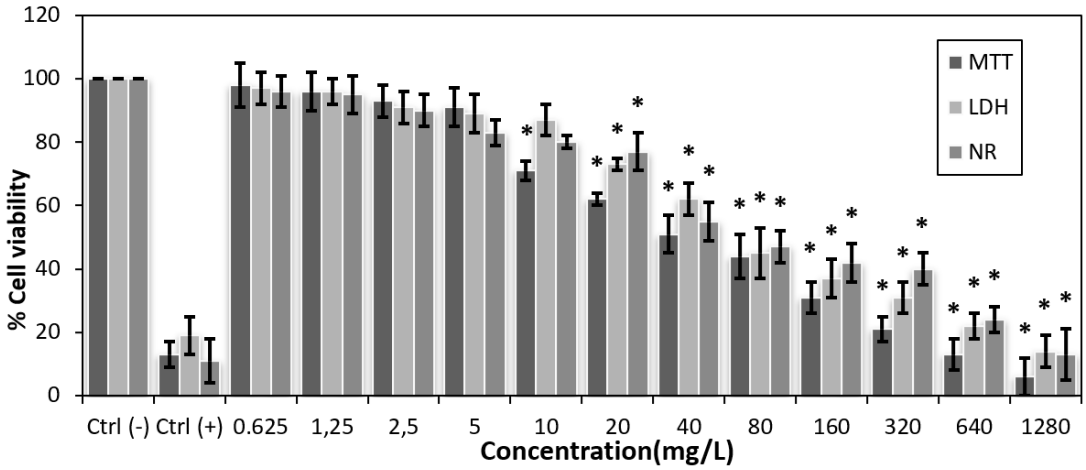

Figure 2. MTT, LDH, NR assay results of iron oxide NPs on the human lung alveolar epithelial cell line. Symbol (*) represents a statistically significant decrease in cell viability at 20 mg/L concentration, $p<0.05$.

HPAEpiC cells, a mixture of acetic acid (1%) and ethanol (50%) were added to the cell cultures and incubated at room temperature for 30 min. Finally, the microplate reader (Bio-Tek Instruments, USA) was used to analyze the optical density of each sample at 540 nm [15].

Microarray Analysis

Fe_2O_3 NPs were applied to the cell culture at lethal concentration 20 (LC20) in T150 cell culture flasks and total RNAs were isolated from cultures by using RNeasy Mini Kit (Qiagen®, USA) according to manufacturer recommendations. ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) were used to evaluate RNA purity and integrity. TargetAmp-Nano Labeling was used to amplify and purify total RNA for Illumina Expression BeadChip (EPICENTRE, Madison, USA) using to get biotinylated cRNA according to the manufacturer's instructions. By using a T7 oligo (dT) primer 500ng of total RNA was reverse-transcribed to cDNA, second-strand cDNA was synthesized, in vitro transcribed, and labeled with biotin-NTP and cRNA was quantified using the ND-1000 Spectrophotometer. Human HT-12 v4.0 Expression Beadchips were hybridized with 750 ng of labeled cRNA samples for 17h at 58°C (Illumina, Inc., San Diego, USA). Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) was used to detect array signals according to the bead array manual. Illumina bead array Reader confocal scanner was used to analyze the arrays.

Data analysis

Statistical analyzes of all viability tests were performed by using SPSS Software (version 20.0, SPSS, Chicago, IL, USA). Duncan's test was used for the analysis of the data at a significance level of 0.05. For microarray analysis, the raw data scanning and quality control checks by the visual survey were the main methods to analyze hybridization quality and overall chip performance. Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0) software provided by the manufacturer for extracting raw data. Quantile method is used for normalization and array probes transformed into the logarithm. Fold change was used to determine the statistical significance of the expression data. DAVID (http://david.abcc.ncifcrf.gov/home.jsp) analysis investigated gene enrichment and functional annotation for the significant probe list. The R 3.1.2 (www.r-project.org) software conducted all data analysis and visualization of differentially expressed genes.

RESULTS and DISCUSSION

In the last decade, nanomaterials have played an important role in a wide spectrum of production areas such as industry, medicine, food, engineering and mechanics [17,18]. Previous studies revealed that different nanoparticles could cause health problems which resulted from NPs chemical features, physical components and functional groups on their surface [19,20]. Because of NPs wide surface area with very small particle size, they might generate harmful oxyradicals (ROS) which attack DNA and other organic structure of cells [21].

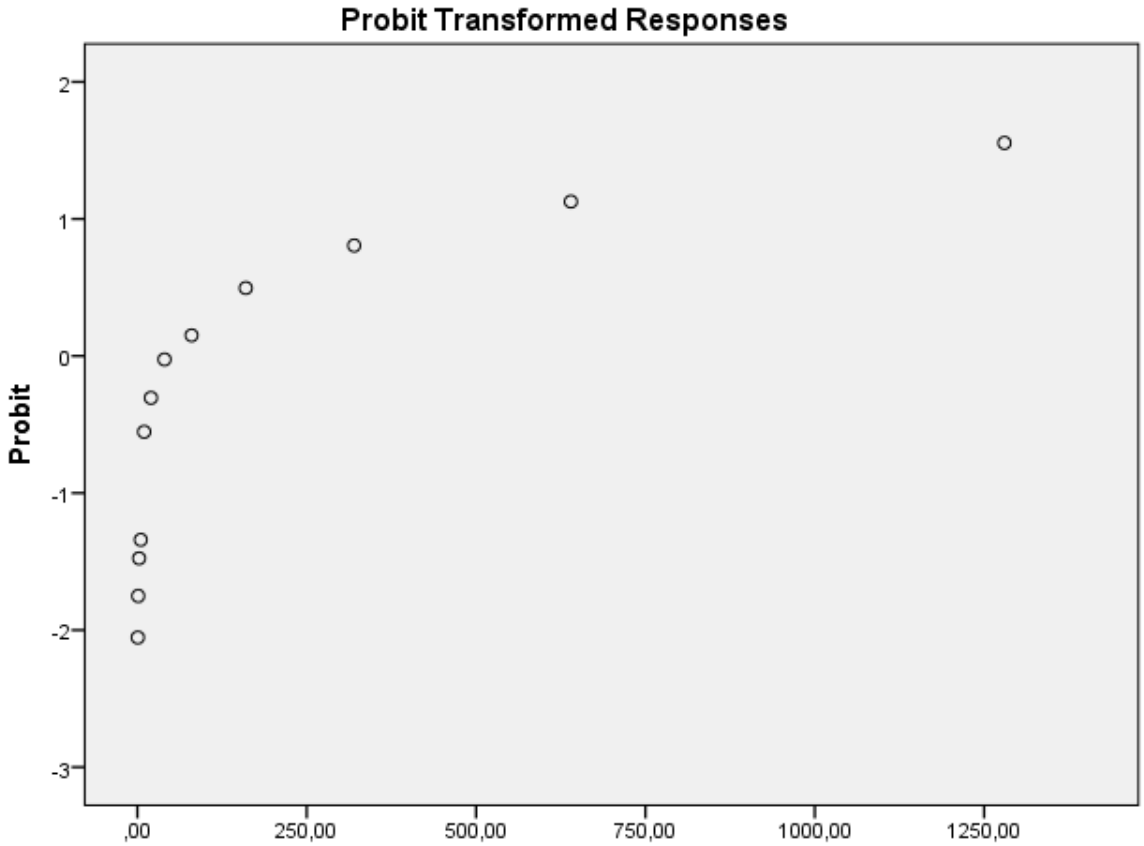

Figure 3. Probit analysis of iron oxide application obtained from LDH assay results to calculate LC20 value.

Furthermore, toxicity caused by NPs may be due to oxidative stress, but certain nanotoxicity mechanisms for different nanomaterials are still unknown [22]. In this work, Fe_2O_3 nanoparticles were chemically synthesized and properties of nanoparticles were characterized by using TEM and XRD methods. XRD studies showed that synthesized Fe_2O_3 molecules have information-relevant properties in the literature, and transmission electron microscopy (TEM) analysis identified about 10 nm of the size of the NPs. Structural analysis of the Fe_2O_3 NPs constructed with XRD which showed the highest peak between 300 and 350 corresponding to Muller indices of 220 in Figure 1a. Some of the other reflections at 2θ corresponding to Muller indices of 111, 220, 222 and 400 detected in the XRD pattern and this result correlated with the literature. According to XRD results, it could be inferenced that synthesized particles were Fe_2O_3 molecules when compared to a chemical database. In addition, the grain sizes of the boron nitride nanoparticles were calculated as 10 nm from (220) peak by using Scherrer formula [16]:

$D=0.9\lambda/\beta\cos\theta$

Where D is the mean grain size of nanoparticles which might be smaller or equal to the grain size, β was full width at half of the peak maximum (FWHM) in radians and 'θ' is Bragg's angle.

Also, transmission electron microscope (TEM) investigation supported that synthesized Fe_2O_3 NPs size nearly 10 nm (Figure 1b) and particle size quite uniform and molecules dispersion homogeneous. From these two-independent data, Scherrer calculation and TEM analysis, which were parallel to each other synthesized Fe_2O_3 molecules could be called nanoparticles with the size around 10 nm.

The results of MTT, LDH and NR analysis correlated to each other that the significant concentration level for toxicity was observed at 20 mg/L (Figure 2). The LDH assay results were used to investigate LC20 values for HPAEpiC line against Fe_2O_3 NPs exposure. According to probit analysis, LC20 value for Fe_2O_3 NPs was calculated as 20.451 mg/L (Figure 3). When figure 3 analyzed, it se-

Table 1. Up-regulated and down-regulated 50 genes subject to iron oxide application.

Iron oxide expression change (%)			
Up-regulated genes	FC	Down-regulated genes	FC
EYA4	1.68	RPS29	-1.64
PDK4	1.66	KRT19	-1.63
ADAMTS5	1.58	GPNMB	-1.51
CXCL12	1.53	SLC7A14	-1.44
LOC148430	1.53	LOC100134259	-1.43
CXCL12	1.50	PAQR5	-1.41
RRAD	1.49	KLF2	-1.40
CES1	1.47	EGR3	-1.39
SCAND2	1.46	TNFSF4	-1.39
PTGS2	1.46	TUBB2B	-1.38
CXCL5	1.46	NUP43	-1.38
G0S2	1.44	CPEB2	-1.37
CFTR	1.42	TNFSF4	-1.37
CDK2	1.37	RPL23AP13	-1.35
ATOH8	1.34	FOSB	-1.35
FOXO1	1.34	ESM1	-1.34
ASL	1.32	LDB2	-1.33
MT1M	1.32	BAALC	-1.32
CXCL12	1.32	CIDEA	-1.32
C1R	132	CCL2	-1.32
ARHGAP28	1.32	SLC14A1	-1.32
ZRSR2	1.31	NYNRIN	-1.32
ANGPT1	1.31	MGC4677	-1.31
ID2	1.30	CPA4	-1.31
CAMK2D	1.30	TMEM171	-1.31

emed that over 320 mg/L concentration Fe_2O_3 NPs were extremely lethal for the cell culture and killed nearly the whole cell population. Three different cell viability tests also gave the same results showing our analysis reliability and consistency. Three different cell viability assays were used to analyze the cytotoxic effect of Fe_2O_3 NPs independently to constitute quality control for each test to get rid of NPs interfering effect on the results. MTT, LDH and NR cytotoxicity tests on HPAEpiC cells gave similar results and LDH assay, most accurate cytotoxicity analysis, showed that approximately 20 mg/L Fe_2O_3 administration could kill 20% of cell cultures. In accordance with our findings the cytotoxicity of Fe_2O_3 nanoparticles on cultured human neuronal and glial cells was reported recently [17]. Again, the time- and dose-dependent cytotoxic effects were observed on human periodontal ligament fibroblasts (hPDLFs) and mouse dermal fibroblasts (mDFs) by iron (II, III) oxide (Fe_3O_4) nanoparticles via in vitro toxicity methods [18]. According to these results, it could be suggested that Fe_2O_3 NPs should not be used as a biocompatible material.

Subject to Fe_2O_3 treatment, most up-regulated and down-regulated 50 genes of the alveolar epithelial cells were investigated. A total of 40.000 gene probes were analyzed for fold change levels in terms of compound effects. It was found that 50 of these were changed significantly from all of the analyzed samples, 25 of

them were up-regulated and 25 of them were down-regulated. The alveolar epithelial cells gene expression changes resulted from the Fe_2O_3 NPs transaction were given in Table 1. According to microarray results, most up-regulated five genes are EYA4, PDK4, ADAMTS5, CXCL12 and LOC148430 and most down-regulated five genes are RPS29, KRT19, GPNMB, SLC7A14 and LOC100134259 respectively.

Only differentiated genes couldn't give enough information about the toxicological properties of a molecule on a molecular level. Thus, it was necessary to establish a link between these genes and analyzed the pathway level with two independent bioinformatics applications. DAVID analysis was used to investigate gene enrichment and functional annotation of these gene probes. According to DAVID annotation KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis; cell proliferation, hormone stimulus, cell cycle and cytokine activity pathways were mostly affected by Fe_2O_3 application (Table 2). To get further information, over 40.000 gene probes were compared via using microarray analysis and results were investigated via DAVID functional annotation pathway analysis. Investigations stated that cell proliferation, hormone stimulus, cell cycle and cytokine activity pathways were affected immensely by Fe_2O_3 exposure. When up-regulated genes analyzed individually, EYA4 gene was one of the most up-regulated ones with 68% expression change. EYA4 gene was claimed as a candidate marker for analyzing esophageal adenocarcinoma within Barrett's esophagus [23]. Also, PDK4 gene was shown to up-regulated after Fe_2O_3 exposure which activates tumorigenesis increasing of cAMP response element-binding protein (CREB) in the brain (RHEB)-mTORC1 signaling cascade [24]. Moreover, previous studies found that up-regulated ADAMTS5 gene had a stimulating effect on the migration and invasion of Non-small cell lung cancer (NSCLC) which was proved by wound healing assays and trans-well migration assays [25]. According to previous studies that correlated our results, the use of magnetic Fe_2O_3 NPs caused a disturbance in iron homeostasis, alteration in gene expression profiles, activation of signaling pathways and impairment of cell cycle regulation [26,27]. On the other hand, RPS29 gene was one of the most down-regulated genes whose mutation caused diamond-blackfan anemia (DBA-bone marrow failure syndrome) in zebrafish models [28]. Furthermore, down-regulated KRT19 was seemed to play an important role in cancer pathways and recent researchers investigated that KRT19 directly

Table 2. Most common pathways in DAVID functional annotation after iron oxide exposure. Gene numbers show common gene probes in the certain pathways.

Affected pathways	Gene numbers
Regulation of cell proliferation	9
Response to organic substance	8
Response to hormone stimulus	7
Response to endogenous stimulus	7
Positive regulation of cell proliferation	7
Response to steroid hormone stimulus	6
Response to estrogen stimulus	4
Response to hypoxia	4
Response to nutrient	4
Response to oxygen levels	4
Response to nutrient levels	4
Response to extracellular stimulus	4
Regulation of cell cycle	4
Extracellular region part	4
Response to vitamin	3
Response to peptide hormone stimulus	3
Cytokine activity	3
Blood circulation	3
Circulatory system process	3

interacts with β-catenin/RAC1 complex to regulate breast cancer properties [29]. Finally, GPNMB gene which was down-regulated in our research stimulates bone regeneration by inducing osteogenesis and angiogenesis by activating FGFR-1 signaling pathway [30]. When to take into consideration these data it could be concluded that Fe_2O_3 exposure could activate tumorigenic and metastatic genes, and prevent bone regeneration genes and increase anemia via inhibiting important genes.

STRING v10 analysis was used to show protein-protein interaction networks of differentially expressed genes on HPAEpiC to understand exposure Fe_2O_3 NPs toxicological effects on underlying molecular gene mechanisms (Figure 4). STRING analysis showed that PTGS2 and ANGPT1 proteins had a central position to link affected gene product exposure to Fe_2O_3 NPs, and probably had important roles in responding to toxicological stimulus. In the meantime, protein-protein interaction networks (STRING v10) analysis was used for a better understanding of the inter/intra-relationships between up-regulated and down-regulated genes [31]. The program revealed a relationship diagram in the aspect of activation, inhibition, co-expression and interaction of proteins. According to analysis PTGS2 and ANGPT1 proteins were positioned in the center of the diagram. Previous studies showed that PTGS2 protein was related to prostate cancer, gastric cancer and Parkinson's disease [32-34]. Also, ANGPT1 protein was found out to have important roles in the acquired paclitaxel resistance of nasopharyngeal carcinoma cells, inhibition of apoptosis in endothelial cells and angiogenesis in human mesenchymal stem cells [35–37]. Even only this information was enough to understand breakneck effects of Fe_2O_3 NPs.

In a conclusion, MTT, LDH and NR analysis revealed Fe_2O_3 NPs toxicity potential, and all of them confirmed that higher concentrations (≥20 mg/L) could lead to lethal response on the HPAEpiC cells. Microarray analysis revealed tumorigenic/anti-apoptotic, metastatic and negative regulatory effects on the development of Fe_2O_3 NPs, comprehensively. As a result, the use of Fe_2O_3 in areas such as the health, industry and food sectors were found inconvenient. On the other hand, various concerns must be addressed before reaching a definitive conclusion. These concerns include several human aspects such as toxicological features of Fe_2O_3 NPs accumulations for different organs, determining toxic dose for human health and comparable in vivo investigations.

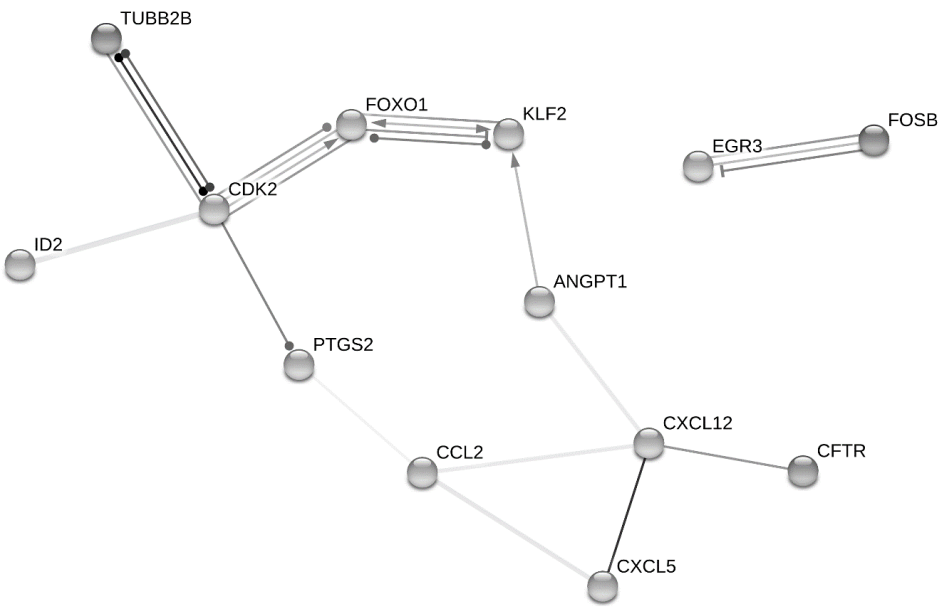

Figure 4. Protein-protein interaction networks (STRING v10) analysis of differentially expressed genes on HPAEpiC against exposure to iron oxide NPs.

Acknowledgments

This research was supported by National Boron Research Institute (BOREN) (Grant number: Ç0391).

References

1. M.R. Gwinn, V. Vallyathan, Nanoparticles: Health effects - Pros and cons, Environ. Health Perspect., 114 (2006) 1818-1825.
2. C. Corot, P. Robert, J.M. Id??e, M. Port, Recent advances in iron oxide nanocrystal technology for medical imaging, Adv. Drug Deliv. Rev., 58 (2006) 1471-1504.
3. A. Srinivas, P.J. Rao, G. Selvam, A. Goparaju, B.P. Murthy, N.P. Reddy, Oxidative stress and inflammatory responses of rat following acute inhalation exposure to iron oxide nanoparticles, Hum. Exp. Toxicol. 31 (2012) 1113-1131.
4. A. Aston, Beaming in On Nano Gold., BusinessWeek, (2005) 122.
5. M.C. Roco, Environmentally responsible development of nanotechnology, Environ. Sci. Technol., 39 (2005) 106A-112A.
6. S. Gupta, C.A. Pattillo, S. Wagh, Effect of remittances on poverty and financial development in sub-Saharan Africa, World Dev., 37 (2009) 104-115.
7. P. Kucheryavy, J. He, V.T. John, P. Maharjan, L. Spinu, G.Z. Goloverda, V.L. Kolesnichenko, Superparamagnetic iron oxide nanoparticles with variable size and an iron oxidation state as prospective imaging agents, Langmuir, 29 (2013) 710-716.
8. U. Cornell, R.M., Schwertmann, The iron oxides: structure, properties, reactions, occurrence and uses, Mineral Mag. 61 (2003) 740-741.
9. M. Babincová, P. Babinec, C. Bergemann, High-gradient magnetic capture of ferrofluids: Implications for drug targeting and tumor embolization, Zeitschrift Fur Naturforsch. Sect. C J. Biosci., 56 (2001) 909-911.
10. B. Bonnemain, Superparamagnetic agents in magnetic resonance imaging: physicochemical characteristics and clinical applications. A review., J. Drug Target., 6 (1998) 167-174.
11. S.J. Bulera, S.M. Eddy, E. Ferguson, T. a Jatkoe, J.F. Reindel, M.R. Bleavins, F. a De La Iglesia, RNA expression in the early characterization of hepatotoxicants in Wistar rats by high-density DNA microarrays., Hepatology, 33 (2001) 1239–1258.
12. T.P. Reilly, M. Bourdi, J.N. Brady, C.A. Pise-Masison, M.F. Radonovich, J.W. George, L.R. Pohl, Expression profiling of acetaminophen liver toxicity in mice using microarray technology, Biochem. Biophys. Res. Commun., 282 (2001) 321-328.
13. J.F. Waring, R. Ciurlionis, R. a. Jolly, M. Heindel, R.G. Ulrich, Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity, Toxicol. Lett., 120 (2001) 359-368.
14. H. Turkez, F. Geyikoglu, Y.I. Mokhtar, B. Togar, Eicosapentaenoic acid protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced hepatic toxicity in cultured rat hepatocytes, Cytotechnology. Ahrenhoers, (2012) 15-25.
15. H. Turkez, E. Sönmez, A. Di Stefano, Y.I. Mokhtar, Health risk assessments of lithium titanate nanoparticles in rat liver cell model for its safe applications in nanopharmacology and nanomedicine., Cytotechnology, 68 (2016) 291-302.
16. G. Gudage, J.C. Vyas, R. Sharma, Studies and correlation among the structural, optical and electrical parameters of spray-deposited tin oxide (SnO_2) thin films with different substrate temperatures, Phys. B Condens. Matter., 403 (2008) 3724-3729.
17. N. Fernández-Bertólez, C. Costa, M.J. Bessa, M. Park, M. Carriere, F. Dussert, J.P. Teixeira, E. Pásaro, B. Laffon, V. Valdiglesias, Assessment of oxidative damage induced by iron oxide nanoparticles on different nervous system cells, Mutat. Res. Toxicol. Environ. Mutagen., 845 (2019) 402989.
18. Ç. Dönmez Güngüneş, Ş. Şeker, A.E. Elçin, Y.M. Elçin, A comparative study on the in vitro cytotoxic responses of two mammalian cell types to fullerenes, carbon nanotubes and iron oxide nanoparticles, Drug Chem. Toxicol., 40 (2017) 215-227.
19. R. Hardman, A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors, Environ. Health Perspect., 114 (2006) 165-172.
20. Y. Wang, L. Deng, A. Caballero-Guzman, B. Nowack, Are engineered nano iron oxide particles safe? an environmental risk assessment by probabilistic exposure, effects and risk modeling, Nanotoxicology, 10 (2016) 1545-1554.
21. D.M. Brown, M.R. Wilson, W. MacNee, V. Stone, K. Donaldson, Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines, Toxicol. Appl. Pharmacol., 175 (2001) 191-9.
22. N. Lewinski, V. Colvin, R. Drezek, Cytotoxicity of nanoparticles, Small, 4 (2008) 26-49.
23. H. Zou, N.K. Osborn, J.J. Harrington, K.K. Klatt, J.R. Molina, L.J. Burgart, D.A. Ahlquist, Frequent methylation of Eyes absent 4 gene in Barrett's esophagus and esophageal adenocarcinoma, Cancer Epidemiol. Biomarkers Prev., 14 (2005) 830-834.
24. Z. Liu, X. Chen, Y. Wang, H. Peng, Y. Wang, Y. Jing, H. Zhang, PDK4 protein promotes tumorigenesis through activation of cAMP-response element-binding protein (CREB)-Ras homolog enriched in brain (RHEB)-mTORC1 signaling cascade, J. Biol. Chem., 289 (2014) 29739-29749.
25. J. Gu, J. Chen, J. Feng, Y. Liu, Q. Xue, G. Mao, L. Gai, X. Lu, R. Zhang, J. Cheng, Y. Hu, M. Shao, H. Shen, J. Huang, Overexpression of ADAMTS5 can regulate the migration and invasion of non-small cell lung cancer, Tumour Biol., 37 (2016) 8681-8689.
26. N. Singh, G.J.S. Jenkins, R. Asadi, S.H. Doak, Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION), Nano Rev. 1 (2010) 5358.
27. S. Rajiv, J. Jerobin, V. Saranya, M. Nainawat, A. Sharma, P. Makwana, C. Gayathri, L. Bharath, M. Singh, M. Kumar, A. Mukherjee, N. Chandrasekaran, Comparative cytotoxicity and genotoxicity of cobalt (II, III) oxide, iron (III) oxide, silicon dioxide, and aluminum oxide nanoparticles on human lymphocytes in vitro, Hum. Exp. Toxicol., 35 (2015) 170-183.
28. L. Mirabello, E.R. Macari, L. Jessop, S.R. Ellis, T. Myers, N. Giri, A.M. Taylor, K.E. McGrath, J.M. Humphries, B.J. Ballew, M. Yeager, J.F. Boland, J. He, B.D. Hicks, L. Burdett, B.P. Alter, L. Zon, S.A. Savage, Whole-exome sequencing and functional studies identify RPS29 as a novel gene mutated in multicase Diamond-Blackfan anemia families, Blood, 124 (2014) 24-32.
29. S.K. Saha, H.Y. Choi, B.W. Kim, A.A. Dayem, G.-M. Yang, K.S. Kim, Y.F. Yin, S.-G. Cho, KRT19 directly interacts with β-catenin/RAC1 complex to regulate NUMB-dependent NOTCH signaling pathway and breast cancer properties, Oncogene, 36 (2017) 332-349.
30. X. Hu, P. Zhang, Z. Xu, H. Chen, X. Xie, GPNMB enhances bone regeneration by promoting angiogenesis and osteogenesis: potential role for tissue engineering bone, J. Cell. Biochem., 114

(2013) 2729-37.

31. D. Szklarczyk, A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K.P. Tsafou, M. Kuhn, P. Bork, L.J. Jensen, C. von Mering, STRING v10: protein-protein interaction networks, integrated over the tree of life., Nucleic Acids Res., 43 (2015) D447-52.
32. M.S. Fawzy, A.R. Elfayoumi, R.H. Mohamed, I.R.A. Fatah, S.F. Saadawy, Cyclooxygenase 2 (rs2745557) Polymorphism and the susceptibility to benign prostate hyperplasia and prostate cancer in egyptians, Biochem. Genet., 54 (2016) 326-336.
33. Y. Dai, Y. Wu, Y. Li, Genetic association of cyclooxygenase-2 gene polymorphisms with Parkinson's disease susceptibility in Chinese Han population, Int. J. Clin. Exp. Pathol., 8 (2015) 13495-13499.
34. Y. Zhang, H. Su, K. Pan, L. Zhang, J. Ma, L. Shen, J.-Y. Li, W.-D. Liu, I. Oze, K. Matsuo, Y. Yuasa, W. You, Methylation status of blood leukocyte DNA and risk of gastric cancer in a high-risk Chinese population, Cancer Epidemiol. Biomarkers Prev., 23 (2014) 2019-2026.
35. J. Hua, Z.-G. He, D.-H. Qian, S.-P. Lin, J. Gong, H.-B. Meng, T.-S. Yang, W. Sun, B. Xu, B. Zhou, Z.-S. Song, Angiopoietin-1 gene-modified human mesenchymal stem cells promote angiogenesis and reduce acute pancreatitis in rats, Int. J. Clin. Exp. Pathol., 7 (2014) 3580-3595.
36. J. Zhao, L. Chen, B. Shu, J. Tang, L. Zhang, J. Xie, X. Liu, Y. Xu, S. Qi, Angiopoietin-1 protects the endothelial cells against advanced glycation end product injury by strengthening cell junctions and inhibiting cell apoptosis, J. Cell. Physiol., 230 (2015) 1895-1905.
37. W. Li, Y. You, X. Zhang, Y. Song, H. Xiang, X. Peng, J. Qin, G. Tan, Amplification of chromosome 8q21-qter associated with the acquired paclitaxel resistance of nasopharyngeal carcinoma cells, Int. J. Clin. Exp. Pathol., 8 (2015) 12346-12356.