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Investigating the Effect of Yttrium Oxide Nanoparticle in U87MG Glioma and PC3 Prostate Cancer: Molecular Approaches

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ABSTRACT: Yttrium oxide (Y_2O_3) nanoparticles have very wide application areas such as biological imaging, photodynamic therapy, the material sciences, in the chemical synthesis of inorganic compounds, additives in plastic, paint, steel, optics, and iron. Potential risks to human health and the environment should be evaluated in a multi-dimensional perspective when developing nanoparticles for those applications. Therefore, in this research, we aimed to investigate changes in gene expression profiles (genes involved in different biological pathways) influenced by commonly Yttrium oxide (Y_2O_3) nanoparticle in human U87MG glioma and PC3 prostate cancer cell lines *in vitro*. The study was planned to be carried out in two stages. In the first stage, cell viability and cytotoxicity parameters were studied using 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide and lactate dehydrogenase release assays, respectively, with human U87MG glioma and human PC3 prostate cancer cell cultures. In the second stage, to obtain a clear insight into the molecular events after exposing, we examined the effects of selected Y_2O_3 nanoparticle on the expression of genes in U87MG and PC3 cell cultures using RT^2 Profiler PCR Arrays. Y_2O_3 nanoparticles have IC20 of 0,18 mg/L and 2,903 mg/L in PC3 and U87MG cell lines, respectively. Y_2O_3 nanoparticle induced up-regulation of 24 and down-regulation of 22 genes in PC3 cells and up-regulation of 53 and down-regulation of 27 genes in U87MG cells. This study of gene expression profiles affected by nanotoxicity provides critical information for the clinical and environmental applications of Y_2O_3 nanoparticles.

Keywords: Glioma, Prostate cancer cell, Cytotoxicity, *In vitro* gene expression, Nanotoxicity

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INTRODUCTION

Nanoparticles (NPs) are defined as 0D, 1D, 2D or 3D particles ranging in size from 1 to 100 nanometers, which exhibit properties different from the larger volume products of the chemically identical substance (Auffan et al., 2009; Laurent et al., 2010; Tiwari et al., 2012). With developing technology, NPs have been used in various areas such as food industry, chemical industry, agriculture, cosmetics and medicine (Lewinski et al., 2008; Kim et al., 2009; Hahn et al., 2011; Bai and Liu, 2012; Hilger 2013; Hoseinnejad et al., 2018; Shah and Rajput, 2018). Also, in recent years have been used NPs in the biomedical field for drug delivery, biosensor and imaging purposes are injected into the body or administered orally (Lewinski et al., 2008). Although it is so widely used, the toxic effects of NPs entering the body is still not fully understood (Medina et al., 2007). Some studies to date have shown that NPs may have harmful effects. For example, titanium dioxide NPs in sunscreens have been reported to cause brain degradation in mice (Long et al., 2006). Also, NPs have been revealed to cause changes in epigenetic mechanisms such as DNA methylation, histone modification and mi-RNA expression (Wong et al., 2017). In brief, *in vitro* and *in vivo* studies in rodents have confirmed the toxic effect of various NPs (carbon nanotubes, iron oxide (Fe_2O_3), yttrium oxide (Y_2O_3), cerium oxide (CeO_2), and zinc oxide (ZnO)) (Lam et al., 2004; Soto et al., 2005). On the other hand, some NPs such as yttrium oxide (Y_2O_3) are noteworthy with different properties (Kilbourn, 1994). Y_2O_3 NPs are used for optical applications in order to obtain transparent ceramics, in biological applications (photodynamic therapy and biological imaging) in plasma televisions, in the production of microwave filters, as additives in the improving of certain substances such as plastics and paints, in high temperature protection applications (Cheng, 1999; Chang and Tie, 2008; Andelman et al., 2010; Ianoş et al., 2014). It is contented that Y_2O_3 NPs used in a multifarious of applications including scanning in human body, material sciences and chemosynthesis of inorganic compounds are relatively less toxic than other nanoparticles (Schubert et al., 2006; Andelman et al., 2010). In the study of Selvaraj and colleagues, the application of Y_2O_3 NPs alone to HEK293 cells increased the intracellular ROS level (Selvaraj et al., 2014). According to study of Kennedy et al. 2009, Y_2O_3 NPs cause an inflammatory response at concentrations above 10 mg / mL. In a study conducted by Nagajyothi et al. 2018, Y_2O_3 NPs showed a strong anticarcinogenic effect on renal carcinoma cells.

Cancer is a disease characterized by the accumulation of mutations in the cell and the ability of endless division of cells by escaping from death mechanisms (Siegel et al., 2017). In a statistical report published in 2014, it was reported that cancer caused the most death after heart disease with approximately 614348 deaths in all ages and sexes (Siegel et al., 2017). The number of people diagnosed with cancer is thought to increase day by day. Gliomas, one of the most common primary intracranial tumors, constitute 1-2% of cancer cases that are increasing each day (Weant et al., 2018; Li et al., 2018). In addition to changing the expression level of genes associated with cell cycle and metabolism have been associated with glioma formation (Ohgaki and Kleihues, 2009). Although there are many studies on the biology and genetics of gliomas, an effective treatment method has not been developed. On the other hand, prostate cancer is the most common neoplasm in elderly men and has a high morbidity and mortality rate (Pezaro et al., 2014; García-Perdomo et al., 2018). The mechanism of prostate cancer has not been fully elucidated but, age, race, the history of prostate cancer in the family, and genetic predisposition are associated with this deadly disease (Costello and Franklin, 2000). In addition, factors such as obesity, smoking, malnutrition and exposure to chemicals are among the factors that trigger the onset of prostate cancer. In addition to these risk factors, it has been reported in previous studies that factors such as defects in DNA repair pathways, damage to apoptosis mechanisms and increased

intracellular ROS levels are effective in the development and progression of prostate cancer (Khandrika et al., 2009; Kurfurstova et al., 2016).

In this study, we applied Y_2O_3 NPs to human glioblastoma cell line U87MG and prostate cancer bone metastasis cell line PC3 in order to fill the gap in literature on cytotoxic and molecular genotoxic effects of Y_2O_3 NPs. We used cell viability analysis to determine cytotoxic effects of Y_2O_3 NPs. In order to investigate the toxic effects at the molecular level, we determined the expression profiles of various genes involved in the basic metabolic processes in the cell using RT^2 Profiler PCR Arrays technique.

MATERIAL AND METHODS

Synthesis of Y_2O_3 NPs

Flower-like Y_2O_3 nanostructures were obtained by hydrothermal method. 1,91505 g of yttrium (III) nitrate hexahydrate ($Y(NO_3)_3$ 6 H_2O, %99C Merck, Darmstadt, Germany) and 0.70095 g of hexamethylenetetramine ($C_6H_{12}N_4$, %99, Merck) were added in 100 ml of distilled water and It stirred with a magnetic stirrer. This solution was put into solution autoclave and it was kept in an furnace for 10 hours at a temperature of 200 ˚C. It was allowed to cool to room temperature. Particles were separated by filter were washed several times with distilled water. Then, these particles were annealed for 1 hour at 700 °C in the furnace. The crystal structures of Y_2O_3 were investigated by Philips X'Pert Pro X-ray diffractometer (XRD), with CuK□ radiation, the surface morphologies were observed using a Zeiss EVO-LS10 scanning electron microscopy (SEM) (Sonmez et al., 2015).

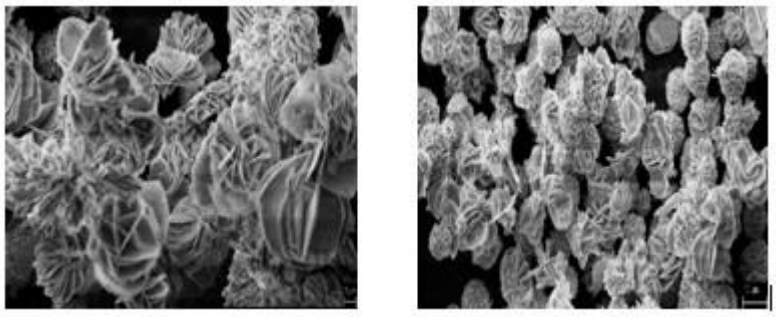

Figure 1. Flower-like Y_2O_3 NPs

Cell Cultures and Treatment of Y_2O_3 NPs

U87MG and PC3 cells supplied from ScienCell® were incubated in DMEM (Dulbecco's modified Eagle's medium-Sigma®) full medium containing 10% FBS (Sigma®) and 1% Penicillin-Streptomycin (Sigma®) in a sterile incubator with the inclusion of 5% CO_2 and 95% air at 37°C. These cells were transferred to 48 well-plate with $1x10^6$/ ml in each well and Y_2O_3 NPs were applied to PC3 and U87MG cell lines at different concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 mg/L) (Sönmez et al., 2015). After nanoparticle treatment, cells were cultured for 24 and 48 hours under the above-mentioned culture conditions.

MTT Analysis

MTT is a kind of proliferation test that uses mitochondrial succinate dehydrogenase enzyme to measure cytotoxicity and cell viability and helps us to comment on the percentage of vitality in the environment by calorimetric measurement (Goiato et al., 2015). In this study, Colorimetric (MTT) Kit for Cell Survival and Proliferation (Millipore®, CA, USA) kit was used for MTT analysis and used solutions were prepared following the manufacturer's protocol. PC3 and U87MG cells were seeded in 48 well-plates in $1x10^6$/ml cell/well concentration and Y_2O_3 NPs were applied to cells after 24 hours at

0, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 mg/L concentrations. After nanoparticle treatment, cells were cultured for 24 and 48 hours under the above-mentioned culture conditions. In the 24th and 48th hours of treatment, MTT was added to the medium according to the manufacturer's directive, and the cells were incubated for 4 hours in 5% CO_2 incubator at 37 °C. Formazan crystals generated by living cells were dissolved by adding DMSO and absorbance measurement was taken at 570 nm. According to the results of MTT analysis, IC_{20} values for each compound were calculated according to probit analysis method (Morandi et al., 2017). Triton X-100 solution was used as positive control.

Lactate Dehydrogenase (LDH) Release

The measurement of level of extracellular lactate dehydrogenase (LDH) is widely preferred to determine the damage of cell membranes (Zheng et al., 2011). Extracellular LDH is greatly increase when the cell membranes are damaged (Fotakis and Timbrell, 2006). PC3 and U87MG cells were seeded in 48 well-plates in $1x10^6$/ml cell/well concentration and Y_2O_3 NPs were applied to cells after 24 hours at 0, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 mg/L concentrations. After nanoparticle treatment, cells were cultured for 24 and 48 hours under the above-mentioned culture conditions. The LDH kit was provided from Cell Biolabs® (CA, USA). LDH activities in cells exposed to Y_2O_3 NPs were determined following the manufacturer's protocol at 24 and 48 hours after nanoparticle treatment. Triton X-100 solution was used as positive control.

Human Molecular Toxicology Pathway Finder RT^2 Profiler PCR Array

To evaluate expression of genes involved in specific cellular processes, RNA was isolated from U87MG and PC3 cells by using PureLink RNA Mini Kit (Life Technologies®). The RNA concentrations and purities were detected using microplate reader (Biotek EPOCH), and equal amounts (1000 ng) of RNA from each sample was used to synthesize cDNA with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher®). Human Molecular Toxicology Pathway Finder RT^2 Profiler PCR array was applied according to the manufacturer's protocol. This PCR array profiled the expression of 370 genes related to 13 different cellular processes involved in response to Y_2O_3 NPs (Cat. no. 330231). The levels of expression of genes were calculated using the ΔΔCT method (Olmos-Alonso et al., 2016).

Statistical Analysis

SPSS 13.0 program was used for statistical analysis. Possible differences were determined using Student's t-test, Duncan's and ANOVA tests. The confidence interval was defined as 0.05 during the statistical analyzes.

RESULTS AND DISCUSSION

Cytotoxic Effects of Y_2O_3 NPs

Cell viability and cytotoxic effects in PC3 and U87MG cell lines of Y_2O_3 NPs were examined at 24th and 48th hours and are shown in Figure 2, 3, 4 and 5, respectively. According to the data obtained, Y_2O_3 NPs significantly reduced cell viability on PC3 and U87MG cell lines depending on dose and time. As a result of the study, IC_{20} values of Y_2O_3 NPs were determined as 0.18 mg / L for PC3 and 2.903 mg / L for U87MG cells.

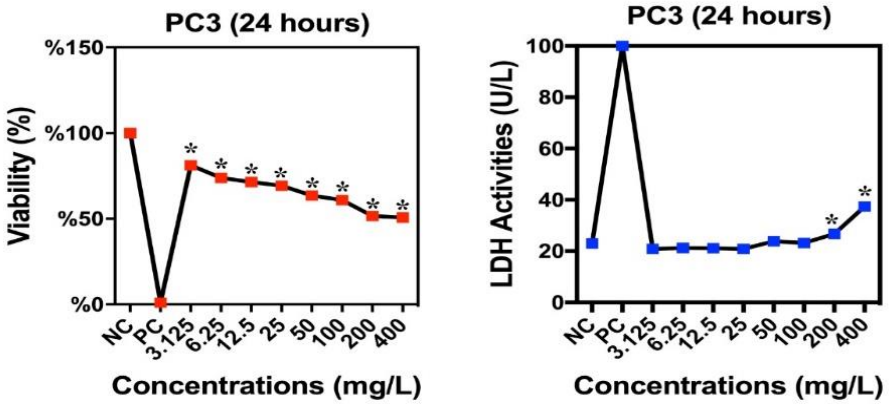

Figure 2. Percent change in viability in PC3 cell line after 24 hour Y_2O_3 exposure (NC: Negative control, PC: Positive control) (Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the $p<0.05$ level from the NC group)

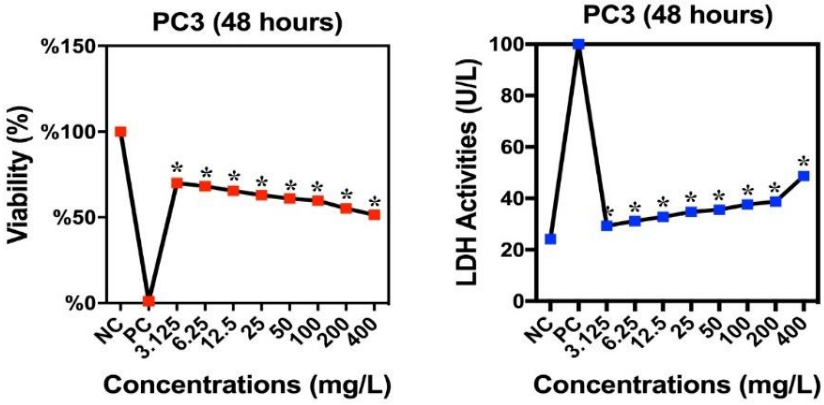

Figure 3. Percent change in viability in PC3 cell line after 48 hour Y_2O_3 exposure (NC: Negative control, PC: Positive control) (Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the $p<0.05$ level from the NC group)

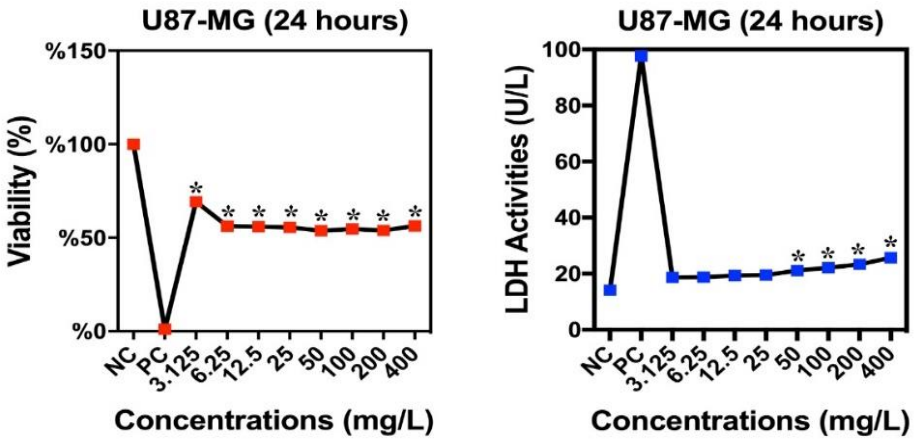

Figure 4. Percent change in viability in U87MG cell line after 24 hour Y_2O_3 exposure (NC: Negative control, PC: Positive control) (Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the $p<0.05$ level from the PC group)

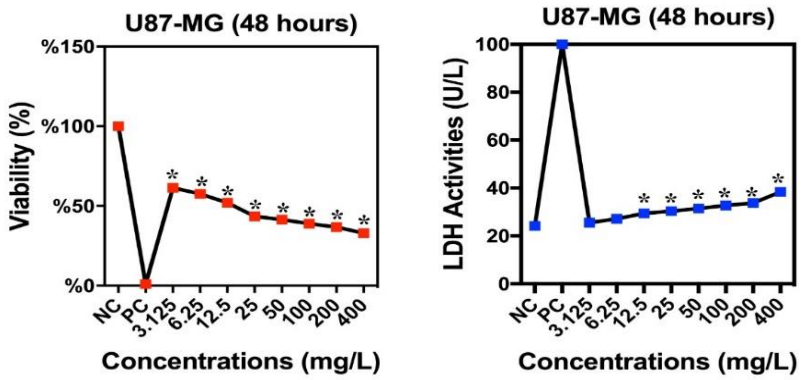

Figure 5. Percent change in viability in U87MG cell line after 48 hour Y_2O_3 exposure (NC: Negative control, PC: Positive control) (Values inside the rectangle are statistically different from the corresponding control,* symbol presents significant differences at the $p<0.05$ level from the PC group)

Effects of Y_2O_3 NPs on Gene Expression Profile

Human Molecular Toxicology Pathway Finder RT^2 Profiler PCR Array kit was used to determine the toxicity mechanisms induced by Y_2O_3 NPs in PC3 and U87MG cells. After 24 hours exposure with Y_2O_3 NPs, gene expression profiles have changed in PC3 and U87MG cell lines. There were 370 genes in the panel used, and 46 of these 370 genes in the PC3 cell line showed a significant change compared to the control, whereas a significant change was observed in the gene expression of 80 genes in the U87MG cell line. The first 10 genes that show the most change in PC3 and U87MG cell lines, respectively, are given in Table 1., Table 2., Table 3. and Table 4. In addition, these genes are stated to be genes involved in cellular events such as endoplasmic reticulum stress and unfolded protein response, cholestasis, DNA damage and repair, cytochrome P450 and phase 1 metabolism, immunotoxicity, apoptosis, necrosis, oxidative stress and antioxidant systems.

Table 1. Top 10 upregulated genes in PC3 cells treated with Y_2O_3 NPs

	Gene	Name of the Gene	Increase in Gene Expression	Gene function
1.	ASNS	Asparagine synthetase	284,0498	Endoplasmic reticulum stress and unfolded protein response
2.	PDYN	Prodynorphin	11,0043	Cholestasis
3.	CYP2C9	Cytochrome P450 family 2 member of the lower family C 9	5,8159	Cytochrome P450 and phase 1 metabolism
4.	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide	4,8232	Immunotoxicity
5.	BCL2	Apoptosis regulator	4,7568	Apoptosis
6.	HPX	Hemopexin	3,8106	Immunotoxicity
7.	CYP1A2	Cytochrome P450 family 1 sub family A member 2	3,605	Cytochrome P450 and phase 1 metabolism
8.	TRIM10	Triple motif family	3,4105	Immunotoxicity
9.	FASLG	Fas ligand	3,0738	Apoptosis
10	MAG	Myelin-associated glycoprotein	3,0105	Necrosis

Table 2. Top 10 downregulated genes in PC3 cells treated with Y_2O_3 NPs

	Gene	Name of the Gene	Decrease in Gene Expression	Gene function
1.	ATP8B1	ATPase phospholipid transport 8B1	-191,085	Cholestasis
2.	SREBF1	Sterol regulatory element binding transcription factor 1	-1,4641	Steatosis
3.	HMOX1	Heme oxygenase 1	-1,257	Oxidative stress and antioxidant systems
4.	TRIB3	Triple pseudokinase	-1,2483	Endoplasmic reticulum stress and unfolded protein response
5.	GCLM	Glutamate-cysteine ligase modifier subunit	-1,2397	Oxidative stress and antioxidant systems
6.	DNAJB9	DnaJ heat shock protein family (Hsp40) member B9	-1,2226	Endoplasmic reticulum stress and unfolded protein response
7.	ERCC2	ERCC excision repair 2, TFIIH core complex helicase subunit	-1,2058	DNA damage and repair
8.	BCL2L1	Apoptosis regulator	-1,1975	Apoptosis
9.	CDKN1A	Cyclin-linked kinase inhibitor 1A	-1,181	DNA damage and repair
10.	GRB2	Growth factor receptor bound protein 2	-1,181	Necrosis

Table 3. Top 10 upregulated genes in U87MG cells treated with Y_2O_3 NPs

	Gene	Name of the Gene	Increase in Gene Expression	Gene function
1.	PDYN	Prodynorphin	648,0674	Cholestasis
2.	ADM2	Adrenomeduline 2	300,2457	Endoplasmic reticulum stress and unfolded protein response
3.	CYP1A2	Cytochrome P450 family 1 subfamily A member 2	224,4111	Cytochrome P450 and phase 1 metabolism
4.	TRIM10	Triple motif family	174,8532	Immunotoxicity
5.	CYP2B6	Cytochrome P450 Family 2 Sub Family B Member 6	138,1412	Cytochrome P450 and phase 1 metabolism
6.	FASLG	Fas ligand	137,187	Apoptosis
7.	LYZ	lysosome	96,3358	Immunotoxicity
8.	ADH1C	Alcohol dehydrogenase 1C (class I), gamma polypeptide	65,7993	Immunotoxicity
9.	HPN	Hepsin	56,1028	Phospholipidosis
10.	F2	Coagulation factor II, thrombin	55,7152	Immunotoxicity

Table 4. Top 10 downregulated genes in U87MG cells treated with Y_2O_3 NPs

	Gene	Name of the Gene	Decrease in Gene Expression	Gene function
1.	MKI67	Proliferation marker Ki-67	-54,1917	Immunotoxicity
2.	UHRF1	E3 ubiquitin-protein ligase	-9,8492	Endoplasmic reticulum stress and unfolded protein response
3.	FHL2	4. LIM domain	-7,6741	Oxidative stress and antioxidant systems
4.	RAD51	Recombinase	-7,4127	DNA damage and repair
5.	BRCA1	Gene associated with DNA repair	-7,3615	DNA damage and repair
6.	HMOX1	Heme oxygenase 1	-4,1411	Oxidative stress and antioxidant systems
7.	PARP2	Poly (ADP-Ribose) polymerase 2	-3,8637	Necrosis
8.	GCLM	Glutamate-cysteine ligase modifying subunit	-3,2716	Oxidative stress and antioxidant systems
9.	CYLD	CYLD lysine 63 deubiquitase	-2,7895	Necrosis
10.	RDX	Radixin	-2,6759	Cholestasis

Although the usage area has increased with the developing technology, the detrimental effects of nanoparticles on the human health and the environment are not overlooked (Chairuangkitti et al., 2013;

Maurer-Jones et al., 2013). Nanoparticles that can be easily integrated into biological systems because of being less than 100 nm allow research and improvement in many fields such as medicine and biomedical: smart drug carriers, visualisation, biosensors, nano-sized machines, nucleic acid assays, production of DNA chips in nanoscale (Zhang et al., 2010; Veiseh et al., 2010; Li et al., 2013; Woo, 2016). However, nanoparticles that are widely used in many areas can cause irreparable damage to cells and tissues (Bañobre-López et al., 2013; Aydın et al., 2017). In order to understand the possible damages that may be caused by nano structures, we investigated the potential nanotoxicity of the Y_2O_3 NPs at potential genetic and molecular levels.

In our study, MTT method was used to determine the viability of cells exposed to Y_2O_3 NPs in PC3 and U87MG cell cultures while LDH method was used to determine cytotoxicity. In the MTT test, the tetrazolium salt given to the medium by the mitochondria of living cells is converted to formazan crystals (Stepanenko and Dmitrenko, 2015). The colorimetric measurement of the color resulting from MTT yields relative information about vitality (van Meerloo et al., 2011). On the other hand, the LDH test is based upon the calorimetric measurement of the molecules formed as a result of the activity of the lactate dehydrogenase enzyme leaking from the cells that lose the membrane permeability (Gaucher and Jarraya, 2015). Using MTT and LDH assays, Y_2O_3 NPs were determined to be cytotoxic to the PC3 and U87MG cell lines depending on the dose and time. In a study conducted by Zhou and his team, Y_2O_3 NPs showed dose and time dependent cytotoxic effects in primary osteoblast cells and this supports our findings (Zhou et al., 2016). Our results are in agreement with a study conducted in 2006, which has demonstrated that high concentrations (20-100 mg/mL) of Y_2O_3 NPs decreased cell viability of mouse hippocampal nerve cells (Schubert et al., 2006). In another *in vitro* study, reported by Selvaraj et al. 2014, using MTT test, Y_2O_3 NPs showed strong cytotoxicity toward human embryonic kidney (HEK293) cells. In the various studies with nanoparticles, the increased concentration and time-dependent cytotoxic effects of nanoparticles have been shown. For example, in a study using vascular endothelial cells, zinc oxide (ZnO), copper oxide (CuO) and magnesium oxide (MgO) metal nanoparticles have been shown to decrease cell viability and have cytotoxic effect due to increased dose (0.001-100 μg / ml) and time using MTT and LDH analysis (Sun et al., 2011). Ma et al., 2015 demonstrated that silver nanoparticles caused depolarization in the mitochondrial membrane, increased intracellular ROS levels, decreased cell viability and increased caspase-3 activity on murine HT22 cells (Ma et al., 2015). In a study we conducted in order to inform about the safety of nanoparticles, we demonstrated that various titanium nanoparticles have cytotoxic effects on human alveolar epithelium (HPAEpiC) and pharynx (HPPC) cells with MTT, LDH and neutral red methods (Aydın et al., 2017).

According to our research in the databases, metal oxide nanoparticles cause programmed cell death, DNA defects, increase of radicals in the cell, damage to pathways in energy metabolism, endoplasmic reticulum stress and insufficiency in immunological response (Foldbjerg et al., 2011; Vandebriel and De Jong, 2012; Manke et al., 2013; Siddiqui et al., 2013; Wahab et al., 2014; Kim et al., 2014; Lu et al., 2015; Yu et al., 2015). Gene expression profiling conducted within the scope of the investigation revealed the dynamics of expression of 370 different genes involved in some basic metabolic processes in the cell: apoptosis, necrosis, DNA damage and repair, mitochondrial energy metabolism, fatty acid metabolism, oxidative stress and antioxidant systems, heat shock proteins metabolism protein metabolism as a result of endoplasmic reticulum stress, cytochrome P450 and phase 1 metabolism, steatosis, cholestasis, phospholipidosis and immunotoxicity etc. According to our findings, Y_2O_3 NPs were found to alter the expression level of genes involved in the endoplasmic reticulum stress and unfolded protein response, cholestasis, DNA damage and repair, cytochrome P450

and phase 1 metabolism, immunotoxicity, oxidative stress and antioxidant systems and apoptosis pathways in the PC3 cell line and the expression of genes involved in the cholestasis, endoplasmic reticulum stress and unfolded protein response, cytochrome P450 and phase 1 metabolism, immunotoxicity and necrosis pathways in U87MG cells. It has been reported that the ASNS gene, which is the most expressed in the PC3 cells and encoded on the 7th chromosome, is increased in response to endoplasmic reticulum stress in the case of cytotoxicity (Lomelino et al., 2017). It is also known that expression of CYP1A2 and CYP2B6 genes responsible for the production of cytochrome P450 and phase 1 metabolism enzymes that have elevated expression in U87MG cells increases during the oxidation event (Meunier et al., 2004; Appiahopong et al., 2007). The expression level of FASLG, which causes apoptosis by activating caspase 8 cascade, increased in the U87MG cells treated with Y_2O_3 NPs (Ashkenazi and Salvesen 2014).

CONCLUSION

In conclusion, the cytotoxicity and molecular genotoxicity potential of Y_2O_3 NPs in PC3 and U87MG cells were evaluated by MTT, LDH and RT^2-PCR methods, respectively. The data obtained, the cytotoxic effects of Y_2O_3 NPs was demonstrated depend on dose and time on both cell lines and, changing the expression levels of genes involved in various metabolic processes in PC3 and U87MG cell lines after exposure with Y_2O_3 NPs. In the light of the findings of this study, since the Y_2O_3 NPs can have toxicological effects in the diagnosis and treatment of various diseases, especially cancer, the safety standards at the genetic and cellular level of Y_2O_3 NPs should be well determined. Therefore, we believe that it is of great importance to conduct *in vivo* studies to improve the clinical use of NPs.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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