

## Mutation of p53 as a tumor suppressor gene in lung fibroblast cells exposed to nano-alumina and zinc oxide nanoparticles

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### Abstract

An increase in the broad usage of metal oxide nanoparticles in biological applications may have novel interactions with biological systems and result in emerging health problems. In this study, the effect of aluminum oxide (Al<sub>2</sub>O<sub>3</sub>, 35-45 nm) and zinc oxide (ZnO, 30 nm) nanoparticles (NPs) on the mutation of codon 248 of the p53 gene, a key gene in the tumor cell suppression, was conducted in the cellular growth medium. After 72 hours of exposure to the mentioned NPs (5, 10, 25, 50 µg/ml), lung fibroblast MRC-5 cells were evaluated through MTT assay for cytotoxicity and subsequent PCR and sequencing analysis for in vitro genotoxicity assessment. After zinc oxide nanoparticle (ZnO-NPs) treatment, cells underwent substantial cytotoxicity, and these toxicities were significant at doses of 25 and 50 µg/mL. Regarding aluminum oxide nanoparticles (nano-alumina, Al<sub>2</sub>O<sub>3</sub>-NPs), a concentration of 50 µg/mL affected the viability of MRC-5 cells. There was no significant difference in other treated groups compared to the control. Interestingly, the mutation in the 248 codons of the P53 gene was observed following 72 hours incubation of MRC-5 cells with 5 µg/mL of Al<sub>2</sub>O<sub>3</sub>-NPs. This mutation occurred in the form of the CGG to CCG, transforming the arginine codon into proline generators. The mutation in codon 248 p53 (replacement cytosine instead of guanine) will result in non-functional P53 protein production. Hence, following the modulation of p53 in lung cells, the possibility of cancer emerging will be increased. Moreover, determining the nanoparticles' accurate cytotoxic concentration is of great importance to reduce deleterious effects on the body's normal cells.

**Key words:** Aluminum oxide, Mutation, p53, Lung fibroblast MRC-5 cells, Zinc oxide

### Introduction

In the most recent decade, various nanotechnology-based products were produced in tremendous numbers for industrial and biochemical applications (Dey et al., 2008). Engineered nanomaterials, which are made out of metals, such as

alumina, silica, iron, and zinc and their oxides are the simplest commercial form of nanoparticles that have overwhelmed the worldwide market for medical applications (Balasubramanyam et al., 2009; Buzea et al., 2007; Keller et al., 2013). Nanoparticles can

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enter the body through the dermal, oral, and respiratory tract and subsequently distribute to different organs (Parveen et al., 2012; Zhang et al., 2008). A cell reaction to metal oxide nanoparticles varies based on their type, size, shape, and exposure duration. Regarding the toxicological study of metal oxide nanoparticles, their harmful influence on a wide range of cell types as well as the vital organ was approved (Ickrath et al., 2017; Jeng & Swanson, 2006; Sengul & Asmatulu, 2020).

Recent studies have shown that exposure to ZnO-NP induces oxidative stress, leading to the apoptosis mechanism in body cells. (Bai et al., 2017; Yang et al., 2020). This type of nanoparticles also prompted reactive oxygen species (ROS) formation (Li et al., 2020; Taheri, Banaee, Haghi, & Mohisani, 2017) that results in damage to cell macromolecules, including DNA (Ghosh et al., 2016). According to scientific reports, low doses of Al<sub>2</sub>O<sub>3</sub>-NPs do not have a prominent toxic effect on murine and human viable cells as well as the formation of oxidative DNA damage; however, more comprehensive studies are needed to ensure that nano-alumina has no harmful effects on the mammalian genomic regions (Demir et al., 2013; Radziun et al., 2011). P53 (also known as the guardian of the genome) is a key gene in tumor suppression that mutation in P53 is frequently found in the malignant growth of tumor cells. This mutation occurs after exposure to both endogenous and exogenous stimulators. P53 gene encodes a homo-tetramer protein called TP53, a transcription factor that is noticeably involved in cell-cycle arrest and apoptosis (Bashiri Dezfouli et al., 2020; Brown et al., 2009; Meulmeester & Jochemsen, 2008). Despite 11 exons and 393 residues polypeptides (L. Bai & Zhu, 2006), the most common mutated region in all human cancer is in exons 5–8, which code for the central core or DNA-binding domain (DBD). Residues 94–292 is the region that oncogenic p53 transposition (contains 93% of all mutations) recognized to date (Friedler et al., 2005; McCormick, 2001). Most of the presented mutations in DBD are

missense mutations leading to loss of the inhibiting function of p53 to arrest cell growth. Most of the occurring mutations in DBD have been called hotspot mutations, including six residues: Arg248, Arg273, Arg175, Gly245, Arg249, and Arg282 (Madapura et al., 2012; Paskulin et al., 2012). Among the 22 CpG site located at the DBD, three hotspot residues (175, 248, 273) account for 60% of CpG mutations (Hainaut et al., 1998). It is worth mentioning Arg 248, which make direct DNA contacts residue (Nikolova et al., 2000), is one of the most commonly mutated amino acid residues via single-base substitution (Petitjean et al., 2007) and as a result of the substitution of G to C it alters to proline. Replacement of arginine by proline contributes to a noticeable reduction in the DNA binding affinity and subsequent inactivation of the normal p53 activities.

In the present study, the mutagenicity potential of metal oxide nanoparticles (ZnO-NPs and Al<sub>2</sub>O<sub>3</sub>-NPs) in the codon 248 of TP53 were investigated in Human Medical Research Council cell strain 5 (MRC-5) / lung fibroblast cells. Moreover, to determine the properly applied doses, the cytotoxic effects of nanoparticles were evaluated. This study was conducted in the Toxicology and Animal Poisoning Research Center of the University of Tehran.

## **Materials and Methods**

### **Growth media and chemicals**

Cellular growth media of DMEM (Dulbecco's modified Eagle's medium; 4.5 gram per liter of D-glucose) were purchased from Gibco Company (USA) and were then supplemented with 1% Pen/Strep, fungicide amphotericin B (2.5 µg per mL) (Dezfouli et al., 2017), essential amino acids, and 2 mM L-glutamine. A solution of 1000 µM in phosphate buffer saline (PBS) was prepared, and the stock solution was kept at -20 degrees centigrade. Fetal calf serum (FCS) and trypsin/EDTA (0.5%) were purchased from Gibco Company (USA), and collagen was supplied from STEMCELL Technologies (Canada), and

the solvents were supplied from Merk (Germany).

#### Cell Line and Cell Culture

A lung fibroblast MRC-5 cell was provided by the Pasteur Institute of Iran (Iran). The cells were grown in DMEM (4.5 g/lit D-glucose) supplemented with 10% FCS, 1% antibiotic/ antifungal, and incubated at 37 °C under a humidified atmosphere with 5% CO<sub>2</sub>. The media was changed every two days, and the cells were passaged by trypsinization.

#### Cytotoxicity Assay

To examine the cytotoxicity of the nanoparticles (ZnO-NPs/ Al<sub>2</sub>O<sub>3</sub>), an MTT assay was used to measure the viability of MRC-5 cells. The cells were treated with different concentrations of metal oxide nanoparticles (5, 10, 25, 50 µg/ml), the cellular death rate was estimated compared to the control group after 24, 48, and 72 hours. Following treatment, cells were washed twice with phosphate-buffered saline. Then, 15 µl MTT solution was added to each well and incubated for 4 hours. To dilute the formazan crystals formed, the MTT working solution was replaced by 100 µl of DMSO followed by a 15 min shaking at room temperature. The absorption rates were read by an ELISA plate reader in wavelength of 570 nm, with 630 nm as reference wavelength (Buhrmann et al., 2020). The viability of cells (in each well) was calculated with the following formula (Bashiri Dezfouli et al. 2017):

Viability= the average absorption rate of cells/  
the average absorption rate of the control cells  
× 100

Changes in the morphological characteristics of the cells were investigated using an inverted microscope.

#### PCR and sequencing

The mutagenicity of these NPs in MRC-5 cells was assessed by the PCR assay. After 72h exposure with different concentrations (5-50 ppm) of NPs, the cells were lysed for 1 h at 4 °C in the buffer (2.5 M NaCl, 1% Triton X-100, 100 mM EDTA, and 10 mM Tris [pH 10]). DNA extraction was carried out by using a commercial kit (MBST). The

PCR method was used to amplify a 200bp fragment containing a region of exon 7 of the p53 gene. The primer used for PCR amplification were 5'-GGCTCTGACTGTACCACCAT-3' (forward) and 5'-GGAAGAAATCGGTAAGAGG-3' (reverse). PCR amplification (Biorad) was as follows: 94 °C for 300 s; and 38 cycles of 94 °C for 45s, 52 °C for 45s, 72°C for 45s, and 1 cycle of 72°C for 300s hold. Negative controls (No template DNA) were used in each PCR run. Following amplification, agarose gel electrophoresis was performed using TBE buffer, 1.5 µl red safe, pH 8/3. Electrophoresis was conducted at 4 °C (the running buffer's temperature did not exceed 12 °C) for 40 min at an electric field strength of 0.73 V/cm (30 mA). Following the observation of PCR product bands during electrophoresis, All PCR products were sequenced based on the Sanger method.

#### Statistical Analysis

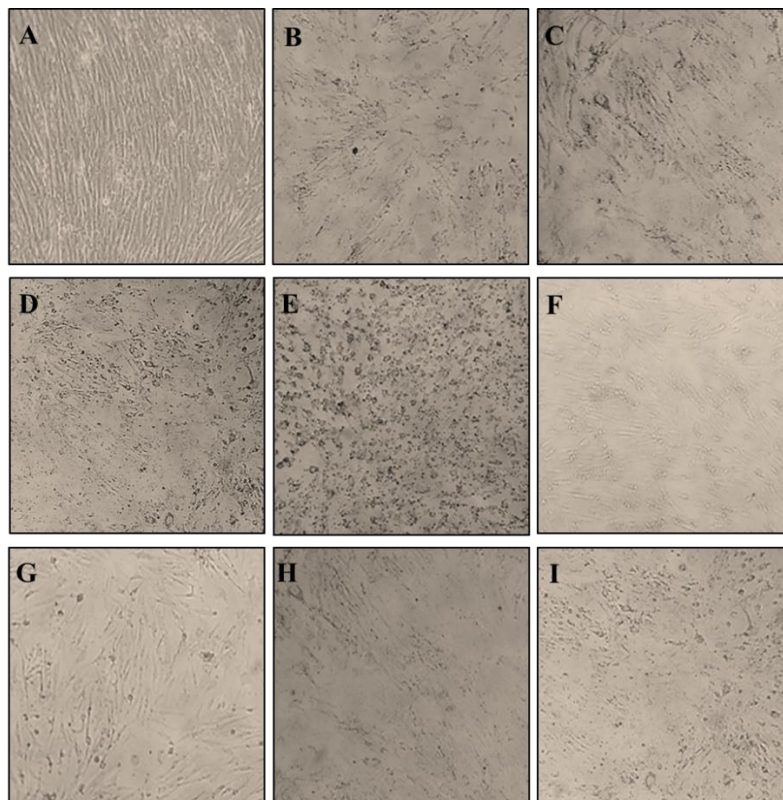
All the data from three repeats of the identical experiment were subjected to statistical analysis and exhibited as the mean ± SD. One-way analysis of variance (ANOVA) followed by all pairwise multiple comparisons employing the Tukey test was executed separately for each material. The results were set at  $P \leq 0.05$ , and cell-viability values were significantly different from those of the control value.

#### Results

The effects of different concentrations of applied nanoparticles (5, 10, 25, 50 µg/ml) on the inhibition and/or proliferation of lung fibroblast MRC-5 cells were studied in 24, 48, 72-hour using microscopic observations (Figure 1) and MTT colorimetric assays (Figure 2). As depicted in Figure 1, cell death-dependent morphological changes at doses of 25 and 50 nM were seen after 72 h exposure to ZnO-NPs. These changes were maximally observed in cells exposed to a dose of 50 µg/ml (Fig. 1). Interestingly in the presence of Al<sub>2</sub>O<sub>3</sub>-NPs, lung fibroblast cells showed less cytotoxicity than ZnO-NPs (figure 2). Following increasing the

nanoparticle concentration, morphological changes such as cytoplasmic vacuolation, cell atrophy, and decreased intercellular communication were noticeable compared to the control group. Results demonstrate that the treatment with these nanoparticles is dose/time-dependent, and a common minimum survival rate was observed at a dose of 50  $\mu\text{g/ml}$  (figure 2). The MTT assays demonstrated a significant decline in absorbance rate subsequent treatment with (5-50  $\mu\text{g/ml}$ )  $\text{Al}_2\text{O}_3$  and ZnO NPs ( $P \leq 0.05$ ) (Fig. 2). Obtained MTT results were in agreement with imaging data. The most significant cytotoxicity was observed at 50  $\mu\text{g/ml}$  concentration of both ZnO and  $\text{Al}_2\text{O}_3$  NPs with an approximately 89 and 59 percent decrease in cell viability rate, respectively, compared to the control group ( $P \leq 0.05$ ) (figure 2).

Considering mutation detection in genomic DNA (codon 248 of p53 gene), electrophoresis results of PCR products (200bp length) after 72-hour exposure of cells to various concentrations (5-50  $\mu\text{g/ml}$ ) of exposure to NPs are shown in Fig 3. In the next step, Samples were sequenced. Following exposure to 5  $\mu\text{g/ml}$  concentration of nano-alumina, an altered allele in codon 248 was founded, representing the mutagenic characteristic of nano-alumina. Contrary to this result, in sequencing analysis of MRC-5 cells treated with ZnO-NPs, no mutagenicity on codon 248 has been observed. Based on the sequencing result, following mutation in the codon 248 region of the p53 gene, CGG altered to CCG (figure 4).



**Figure 1.** Cell death-dependent morphological changes in MRC-5 cells after 72 h exposure to metal oxide nanoparticles at different concentrations (5, 10, 25, and 50  $\mu\text{g/mL}$ ) (magnification  $\times 40$ ). (A) MRC-5 Control cells, (B) detachment and spherical formation after treatment with 5  $\mu\text{g/mL}$  ZnO-NPs concentration, (C) cytoplasm shrinkage and decrease in cell volume after treatment with 10  $\mu\text{g/mL}$  ZnO-NPs concentration, (D) chromatin condensation, cytoplasmic vacuolation, and reduction of intercellular communication after treatment with 25  $\mu\text{g/mL}$  ZnO-NPs concentration, (E) morphological change after treatment with 50  $\mu\text{g/mL}$  ZnO-NPs concentration, (F) treatment with 5  $\mu\text{g/mL}$   $\text{Al}_2\text{O}_3$ -NPs concentration, (G) treatment with 10  $\mu\text{g/mL}$   $\text{Al}_2\text{O}_3$ -NPs concentration, (H) treatment with 25  $\mu\text{g/mL}$   $\text{Al}_2\text{O}_3$ -NPs concentration, (I) morphologic evidence of toxic change after treatment with 50  $\mu\text{g/mL}$   $\text{Al}_2\text{O}_3$ -NPs concentration.

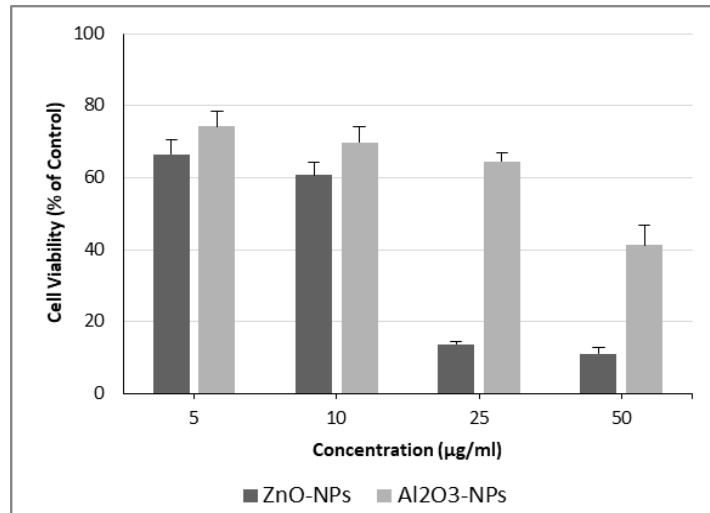


Figure 2. Comparison of the viability (%) of lung fibroblast MRC-5 cells after 72 h treated with different concentrations (5, 10, 25, and 50 µg/mL) of metal oxide nanoparticles (ZnO-NPs and Al<sub>2</sub>O<sub>3</sub>-NPs). The results are expressed as mean ± standard deviation (SD) from three individual experiments, and statistical significance is indicated as p < 0.05.

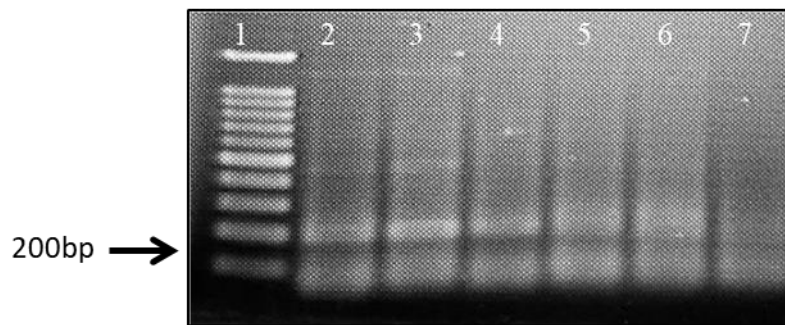


Figure 3. Electrophoresis results (2% agarose gel) of PCR products (200bp length) after 72-hour encounter to Al<sub>2</sub>O<sub>3</sub>-NPs of exon 7. Number1: DNA Ladder (100bp), Number 3, 4, 5, 6, 7, PCR products (200bp), Number7: Negative control (No template DNA).



Figure 4. Sequence alignment of exon 7 codon 248 of all samples exposed to NPs excepting NO 19 which exposed to Al<sub>2</sub>O<sub>3</sub>-NPs (5 µg/mL), showed 100% similarity. As is shown, NO 19 indicated single-base substitutions at the CGG Trinucleotide and converted to CCG representative point mutation at the second site of codon 248 P53 in MRC-5 cells.

## Discussion

The rapid growth of nanotechnology provides a broad perspective for applications of nanomaterials in various fields. Although due to their physical nature, the size of nanoparticles leads to their increasing use, resulting from undesirable interaction with various biomolecules poses risks to consumers (Sliwinska et al., 2015). Additionally, due to the positive charge of metal oxide nanoparticles, their interaction with DNA may cause irreparable outcomes like missense mutations. There are many reports regarding the association between long-term exposure to metal oxide nanoparticles and the occurrence of cell death or neoplasm transformation (Jeng & Swanson, 2006; Manke et al., 2013). Therefore, it is very important to study these nanomaterials' use in terms of health and hygiene.

For the first time, our research focused on evaluating the cytotoxic as well as mutagenicity of ZnO-NPs and Al<sub>2</sub>O<sub>3</sub>-NPs on the mutation of codon 248 of the P53 gene, a key gene in cancer suppression in human lung fibroblast cells. We used the ZnO and Al<sub>2</sub>O<sub>3</sub> nanoparticles to evaluate the cytotoxicity in human lung fibroblast cells at different concentrations. Subsequently, we used sub-lethal doses of ZnO-NPs and Al<sub>2</sub>O<sub>3</sub>-NPs to examine their DNA impact, especially on the p53 gene as a carcinogen. Based on the literature, metal oxide nanoparticles cause both cytotoxicity and induce DNA mutations that lead to different types of tumors. ZnO-NP and Al<sub>2</sub>O<sub>3</sub>-NP-treated lymphocytes exhibited a dose-dependent pattern following the cytotoxicity assessment. (Sliwinska et al., 2015). Our findings show that although exposure to a low concentration of NPs has no significant effect on cell viability, treatment with 25 µg/ml or higher doses has a delirious impact on lung fibroblast cells.

The effect of mutagenesis following DNA and RNA exposure to toxic and exogenous substances may cause teratogenic changes in the next generations. If the damage is not considered in time,

these DNA mutations may lead to malignancy and cancer (Barik & Mishra, 2019; Dixon & Kopras, 2004). Therefore, determining mutagenicity in the presence of ZnO-NP and Al<sub>2</sub>O<sub>3</sub>-NP is of great importance. Based on the findings of the p53 gene mutation in MRC5 cells, we found that exposure to low doses of nano-aluminum had mutagenic effects on the tumor suppressor gene. After sequencing, it was found that cells treated with Al<sub>2</sub>O<sub>3</sub>-NPs (concentration 5 µg / ml) contained a modified allele at codon 248, indicating the mutagenic effect Al<sub>2</sub>O<sub>3</sub>-NPs. in contrast, in ZnO-NPs treated cell, no mutagenic effect on this codon was observed. In summary, In contrast to cytotoxicity analysis of both nanoparticles (no evidence of cytotoxicity in sub-lethal doses), in sub-lethal exposure to nano-alumina and subsequently using PCR analysis on the p53 mutation region containing codon 248, we found that CGG was converted to CCG, resulting in the replacement of proline with arginine.

Mutations in the p53 gene are considered to be an interfering factor in many cancers, so identifying changes in the sequence of this gene, especially in breast cancer, is important. (Hainaut et al., 1998). Tumors with the p53 mutation have been reported to exhibit more aggressive behaviors often, so this suppressor gene has been considered as a prognostic factor in breast carcinoma (Bergh, 1999; Lewis & Parry, 2004; Powell, Soong, Iacopetta, Seshadri, & Smith, 2000). Codon 248 has been reported as a mutant in the p53 gene. In a study on breast cancer, 10 percent of the population showed a mutation in codon 248, the rate of mutation in codons 245, 175, and 273 was 40, 9, and 5 percent, respectively (Powell et al., 2000). Therefore, a mutant in the p53 gene is an important biological marker as well as prognosis of breast cancer. In the present study, the results showed that exposure to nanoparticles oxide could lead to mutations in codon 248 [(specifically by changing the sequence of CGG (arginine codon) to CAG (glutamine)].

The change in the codon 248 to form the substitution of cytosine instead of guanine in the P53 gene has not been reported. This replacement will result in producing a protein that no longer has its normal function in inhibiting the tumor. According to the results of this study, we suggest that

other mutations in the hot spots of the P53 gene (including 273 and 249) will be considered. To sum up, determining the mutagenicity impact of the metal oxide nanoparticles on the somatic cells is of prime importance in the Safety assessment of nanomaterials applied in nanomedicine.

### Conflict of Interests

The authors declare that they have no competing interests.

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## جهش ژن سرکوبگر توموری P53 در سلول‌های فیبروبلاست ریه ناشی از نانوذرات آلومینیوم و روی

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### چکیده

استفاده گسترده از نانوذرات اکسید فلز در مصارف بیولوژیکی، منجر به پیدایش اختلالات جدیدی در سیستم‌های بیولوژی و در نهایت بروز مشکلات مرتبط با سلامتی گردیده است. در این مطالعه، تأثیر نانوذرات اکسید آلومینیوم (۲۵-۳۵ نانومتر،  $Al_2O_3$ ) و اکسید روی (۳۰ نانومتر،  $ZnO$ ) بر جهش کدون ۲۴۸ ژن P53، ژن کلیدی در سرکوب سلول توموری، در محیط کشت سلولی مورد ارزیابی قرار گرفت. پس از ۷۲ ساعت مواجهه سلول‌های MRC-5 (فیبروبلاست ریه) با نانوذرات (۵، ۱۰، ۲۵ و ۵۰ میکروگرم در میلی‌لیتر)، سمیت سلولی با روش MTT و تعیین توالی با روش PCR در آزمایشگاه مورد بررسی قرار گرفتند. پس از تیمار با نانوذرات اکسید روی ( $ZnO$ -NPs)، سمیت سلولی قابل توجهی (به خصوص در دوزهای ۲۵ و ۵۰ میکروگرم/میلی‌لیتر) مشاهده شد. در ارتباط با نانوذرات اکسید آلومینیوم (نانو آلومینا)، غلظت ۵۰ میکروگرم/میلی‌لیتر بر زنده‌مانی سلول‌های MRC-5 تأثیر قابل توجهی داشت. در گروه‌های دیگر تحت تیمار، اختلاف معنی‌داری نسبت به گروه کنترل مشاهده نشد. جالب توجه این که جهش در کدون ۲۴۸ ژن P53، متعاقب ۷۲ ساعت انکوباسیون سلول‌های MRC-5 با ۵ میکروگرم/میلی‌لیتر نانوذرات اکسید آلومینیوم مشاهده شد. این تغییر از CGG به CCG رخ داده و کدون آرژنین را به مولدهای پرولین تبدیل کرد. از آن جایی که جهش در کدون ۲۴۸ (جایگزینی سیتوزین به جای گوانین) منجر به تولید پروتئین P53 غیرفعال می‌گردد، از این رو، امکان تومورزایی در سلول‌های ریه افزایش می‌یابد. علاوه بر این، تعیین غلظت سایتوتوکسیک دقیق نانوذرات برای کاهش اثرات مضر بر سلول‌های طبیعی بدن از اهمیت بسیاری برخوردار است.

**کلمات کلیدی:** آلومینیوم اکسید، جهش، P53، رده سلولی فیبروبلاست ریه (MRC-5)، روی اکسید

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