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Genotoxicity of Aluminum Oxide (Al_2O_3) Nanoparticle in Mammalian Cell Lines

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Accepted 13 May 2009

Abstract

Nanoparticles are small-scale substances (<100 nm) with unique properties, complex exposure and health risk implications. Aluminum oxide (Al_2O_3) nanoparticles (NP) have been widely used as abrasives, wear-resistant coatings on propeller shafts of ships, to increase the specific impulse per weight of composite propellants used in solid rocket fuel and as drug delivery systems to increase solubility. However, recent studies have shown that nano-sized aluminum (10 nm in diameter) can generate adverse effects, such as pulmonary response. The cytotoxicity and genotoxicity of Al_2O_3 NP were investigated using the dye exclusion assay, the comet assay, and the mouse lymphoma thymidine kinase ($tk^{+/-}$) gene mutation assay (MLA). IC_{20} values of Al_2O_3 NP in BEAS-2B cells were determined the concentration of 273.44 μg/mL and 390.63 μg/mL with and without S-9. However IC_{20} values of Al_2O_3 NP were found nontoxic in L5178Y cells both of with and without S-9 fraction. In the comet assay, L5178Y cells and BEAS-2B cells were treated with Al_2O_3 NP which significantly increased 2-fold tail moment with and without S-9. Also, the mutant frequencies in the Al_2O_3 NP treated L5178Y cells were increased compared to the vehicle controls with S-9. The results of this study indicate that Al_2O_3 NP can cause primary DNA damage and cytotoxicity but not mutagenicity in cultured mammalian cells.

Keywords: Nanoparticle, Aluminum oxide, Comet assay, Gene mutation assay (MLA), Cytotoxicity, Genotoxicity

Nanoparticles (NPs) are small-scale substances (< 100 nm) with unique properties, complex exposure and health risk implications[1]. It is using in various fields, such as biology, pharmacology, medicine and electronics for the profit of mankinds[2].

The properties of NPs are different from those of bulk materials of the same compound, allowing them to exert novel physical and chemical functional activities[3,4]. Little is known about the effect of NPs on human health risk, particularly the effect of oxides in non-transition metals such as Al_2O_3. NPs have high surface area, so they may facilitate cellular uptake, reach potential targets such as brain, bone marrow, lymph nodes and heart and trigger harmful responsed at the cellular, subcellular and protein levels. Due to their potential toxic effects, NPs are an emerging concern for human health and environmental impacts.

The aluminum oxide (Al_2O_3, alumina) NP is one among the most abundantly produced NPs, estimated to account for approximately 20% of the 2005 world market of NPs[5]. Al_2O_3 NP which have been widely used as abrasives, wear-resistant coatings on propeller shafts of ships, to increase the specific impulse per weight of composite propellants used in solid rocket fuel and as drug delivery systems to increase solubility[6,7]. Due to the extremely small size of the NPs being used, there is a concern that they may interact directly or indirectly with macromolecules such as DNA.

A few studies have demonstrated that some compounds with bulk forms of aluminum are genotoxic both *in vitro* and *in vivo*[8-10]. And toxicity of Al_2O_3 has been reported for plants and mammalian cell lines[2,11]. However Al_2O_3 NP may differentially affect from their bulk form in cells, though chemical composition remains the same. There is a need to identify the genotoxicity of Al_2O_3 NP, because implications of NPs for cancer induction would promptly lead to risk and safety assessments.

In this study, it is used for comet assay and mouse lymphoma assay (MLA) to assess the genotoxicity of Al_2O_3 NP (< 50 nm) in mouse lymphoma cell line (L5178Y thymidine kinase $(tk)^{+/-}$-3.7.2C cells) and human bronchial epithelial cells (BEAS-2B). Comet assay which is a simple and inexpensive technique

evaluates chemicals for their ability to cause DNA strand breaks and alkali-labile sites under *in vivo* and *in vitro* conditions[12,13]. The MLA provides both rapidity and reliability of data on the genotoxicity of chemicals. The *tk* locus has been widely used to detect the ability of chemicals induce genetic damage in cultured mammalian cells.

Cytotoxicity of Al_2O_3 NP

To determine the optimal concentration, cytotoxicity of Al_2O_3 NP was assessed by exposure to 0-5,000 μg/mL with 2-fold serial dilution for 2 h using trypan blue exclusion assay. The measurements of cytotoxicity were performed in the presence and absence of S-9 metabolic activation systems. Based on results of cytotoxicity assay, 20% inhibitory concentration of viability (IC_{20}) of Al_2O_3 NP was calculated. IC_{20} in BEAS-2B cells was determined the concentration of 273.44 μg/mL and 390.63 μg/mL with and without S-9, respectively. However, the L5178Y cells treated to Al_2O_3 NP was not observed cytotoxicity at the concentrations tested in this experiment, because the L5178Y cells were shown the lower cytotoxic effect than BEAS 2B cells. Previous other studies on characterization of Al_2O_3 NP showed similar results, which occurred the aggregation of the NP in water and different cell culture media with or without serum[14,15] (Figure 1).

Induction of DNA Damage of Al_2O_3 NP Using Comet Assay

One of the mechanisms of carcinogenicity is induction of DNA damage can be determined by comet assay, which is widely used for the detection and measurement of DNA strand breaks[16,17]. In this respect, to investigate whether Al_2O_3 NP and its bio-degradation products induce DNA strand breaks, the comet assay was performed in L5178Y mouse lymphoma cells and human bronchial epithelial BEAS-2B cells following guideline recommended by IWGTP[18].

Comet assay was carried out at maximum concentration of Al_2O_3 NP in soluble exposed. But Al_2O_3 NP is insoluble and aggregation in cell culture media. Therefore, Al_2O_3 NP treated to 1,250-5,000 μg/mL with 2-fold serial dilution in presence or absence of S-9 metabolic activation systems (+S-9 or −S-9) in L5178Y cells. Figure 2(A) and (B) were shown data of tail moment of Al_2O_3 NP in L5178Y cells. Al_2O_3 NP was induced DNA damage at 1,250 to 5,000 μg/mL in the +S-9 and induced at 2,500 μg/mL in the −S-9 ($P<0.05$).

Also, BEAS-2B cells were performed at the concentration from 68.38 μg/mL to 273.44 μg/mL in +S-9 and from 97.66 μg/mL to 390.63 μg/mL in −S-9. BEAS-2B cells were observed a significant increase

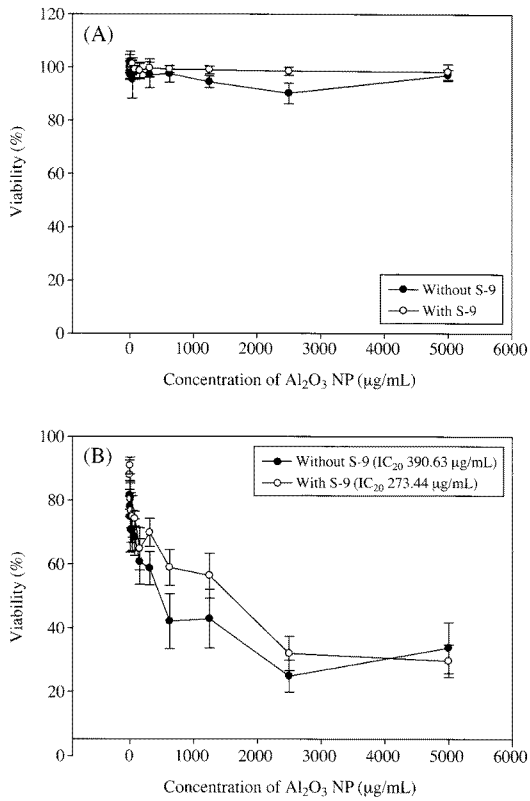

Figure 1. Cytotoxicity of Al_2O_3 NP in (A) L5178Y cells and (B) BEAS-2B cells.

of tail moment at all concentrations tested in +S-9 and −S-9 ($P<0.05$) (Figure 2(C), (D)).

From the results, Al_2O_3 NP was observed statistically significant differences of tail moment values compared with negative control ($P<0.05$) in both L5178Y and BEAS-2B cell line.

Al_2O_3 NP Induced Chromosomal Damage in MLA Assay

The genotoxic potentials of Al_2O_3 NP were assessed with various concentrations in the absence and presence of S-9 activation using MLA. Table 1 summarizes the results of the MLA after treatment Al_2O_3 NP at different concentrations for 3 h in L5178Y cells. Cultured medium was used as the negative control. The adequacy of the experimental conditions for detection of induced mutation was confirmed by employing positive control chemicals, methylmethanesulfonate (MMS) and cyclophosphamide (CP) for assays in the absence and presence of S-9, respectively.

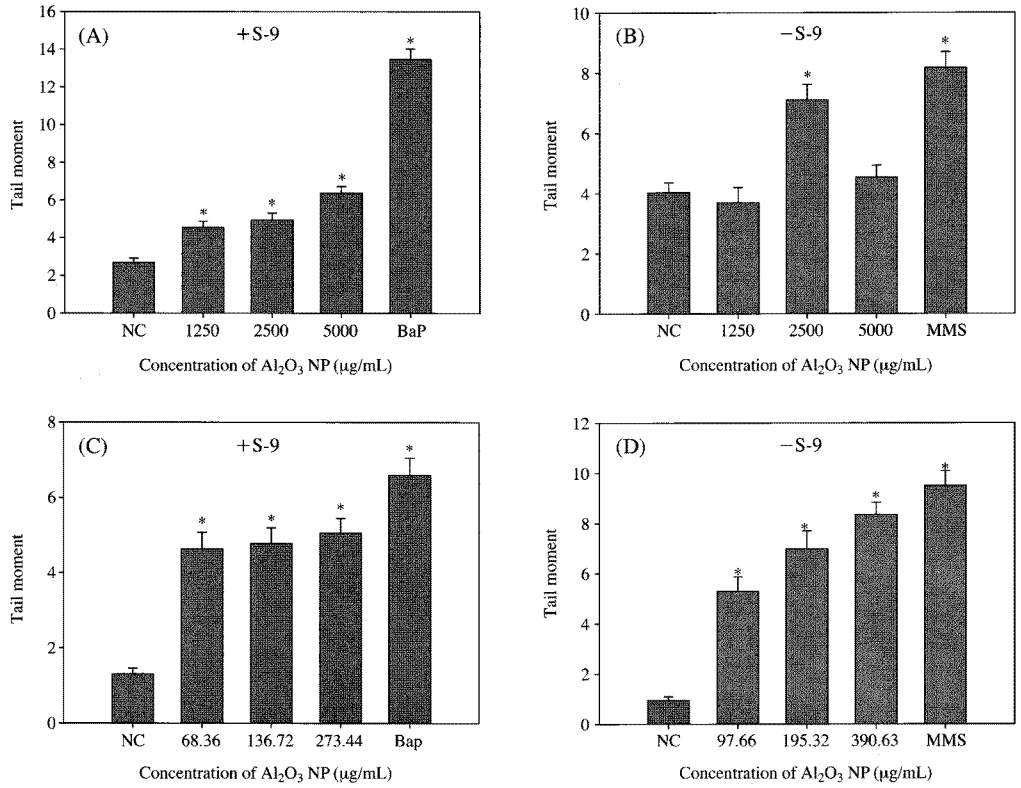

Figure 2. Results of comet assay on Al_2O_3 NP in (A) L5178Y cells with S-9, (B) L5178Y cells without S-9, (C) BEAS-2B cells with S-9 and (D) BEAS-2B cells without S-9.

Al_2O_3 NP was slightly more toxic without S-9 than with S-9. Al_2O_3 NP induced the increase of toxicity in a dose-dependent manner without S-9, with a pronounced drop in the RS and RTG. The limit of 0.1 RTG is widely accepted as the maximum level of cytotoxicity for mutagenicity evaluation to avoid biologically irrelevant effect that might occur in severely stressed cells (e.g., treatments resulting in cytotoxicity >90%). Therefore 5,000 μg/mL was evaluated as the highest concentration for mutagenicity. Background mutant frequencies (MF) (87.55×10^{-6} and 25.82×10^{-6} in without S-9 and with S-9, respectively) were within the historical control range, and positive controls gave large dose-dependent increases in MF, meeting assay acceptance criteria. The treatment of Al_2O_3 NP led to a significant increase of MF at 5,000 and 2,500 μg/mL concentrations in the presence of S-9 (Table 1). And also, it was observed that small sized colonies were more than large colonies in plates with S-9 used in MLA assay (Table 2). It suggests that Al_2O_3 NP may have not mutagenic potentials but genotoxic potentials with damages of chromosomal levels.

Discussion

The results obtained in the comet assay indicated that Al_2O_3 NP was able to cause a significant and dose dependent increase in tail moment in L5178Y and BEAS-2B cells. Our study revealed significant dose-dependent increase in the mutant frequency and increase in the formation of small colonies in L5178Y cells with S-9 indicating possible chromosomal damages by Al_2O_3 NP.

Our data is in agreement with an *in vitro* genotoxicity study of Al_2O_3 NP, investigated in A549, HepG2 and NRK-52E cells with the micronucleus test, comet assay and gamma-H2AX histone foci quantification[19]. And Balasubramanyam *et al.* reported that Al_2O_3 NPs were able to cause size- and dose- dependent genotoxicity compared to Al_2O_3-bulk and control groups *in vivo* rat peripheral blood cells using micronucleus

Table 1. Toxicity and mutagenicity of Al_2O_3 NP in L5178Y $tk^{+/-}$ mouse lymphoma cells.

3 h									
	−S-9					+S-9			
Treatment (μg/mL)	%RS	RTG	Mutation Frequency ($\times 10^{-6}$)		Treatment (μg/mL)	%RS	RTG	Mutation Frequency ($\times 10^{-6}$)	
0	100.00	1.00	87.55		0	100.00	1.00	25.82	
625	92.74	0.76	64.05	NS	625	89.16	0.95	79.78	NS
1,250	100.00	1.15	70.03	NS	1,250	100.00	1.10	69.20	NS
2,500	71.54	0.93	103.65	NS	2,500	80.74	0.74	92.17	*
5,000	55.36	0.64	81.38	NS	5,000	64.24	1.02	94.73	*
Linear trend				NS	Linear trend				**
MMS					CP				
10	86.17	1.13	174.94		3	97.15	0.97	98.13	

NS: Not significant; *, **: Significant at 5% and 1% level, respectively; MMS: methylmethanesulfonate; CP: cyclophosphamide

Table 2. Plate counts and mutation frequencies for large and small colonies.

	−S−9			+S−9	
Treatment (μg/mL)	Small colonies	Large colonies	Treatment (μg/mL)	Small colonies	Large colonies
	Mean±STD	Mean±STD		Mean±STD	Mean±STD
0	14.5±43.5	0.5±0.7	0	6.0±1.4	1.0±1.4
625	13.5±33.5	2.0±2.8	625	28.5±83.5	2.5±0.7
1,250	14.5±40.7	0.0±0.0	1,250	20.0±00.0	1.0±0.0
2,500	20.0±01.4	1.0±1.4	2,500	27.5±74.9*	1.0±0.0
5,000	15.5±50.7	1.0±1.4	5,000	21.0±17.1*	3.5±0.7
MMS	46.0±62.8	1.0±0.0	CP	21.0±19.9	2.5±2.1

*: Significant at 5% level

test and comet assay[2].

Aluminium itself or related compounds are genotoxic both *in vitro* and *in vivo*[8-10]. Aluminium chloride evaluated with the micronucleus test, comet assay and chromosomal aberration anaylsis in human peripheral blood lymphocytes showed significant genotoxicity *in vitro*[20-22]. *In vivo* studies with rats revealed that aluminium sulphate and potassium aluminium sulphate induced dose-dependent increases in chromosomal aberrations[23]. Due to their potential toxic effects, manufactured NPs are an emerging concern in human health.

Toxic effects of NPs are specific to the type of base material, size, ligands and coating. There are reports that NPs can readily enter the cell membrane and accumulate in the cytoplasm, disrupt metabolism, and induce cell dysfunction or even cell death[24-26]. The accumulated Al_2O_3 NP can execute its toxicity through multiple direct or indirect mechanisms, such as mitochondrial dysfunction, oxidative stress and cell death.

The genotoxicity observed with Al_2O_3 NP may be due to pro-inflammatory effects through a reactive oxygen species (ROS)-mediated mechanism, modification of chromatin structure or liberation of DNase, a potent inducer of cytogenetic damages, from lysosomes[27-33]. Further studies are necessary to draw mechanisms by which induce genotoxicity of Al_2O_3 NP.

From these results, we suggest that Al_2O_3 NP is able to cause cytotoxicity, primary DNA damage and deletion of chromosomal levels but not mutagenicity in cultured mammalian cells. However, it is necessary to evaluate whether genotoxicity observed by treatment of Al_2O_3 NP resulted from toxicity of aluminium or nano-size of Al_2O_3. And also, there is a need to better understand the molecular mechanisms involved in the genotoxicity of Al_2O_3 NP.

Materials & Methods

Chemicals and Reagents

Nano-aluminum oxide powder (CAS No. 1344-28-1) was purchased from Sigma-Aldrich. (USA). Stock solution of used chemicals was prepared freshly in medium before use. Particles were weighed, mixed with cell culture media and resuspended by sonication

(20 min) and vortexing immediately before adding to the cells. RPMI-1640, Dulbeco's Minimum Essential Medium (DMEM), sodium pyruvate, pluronic solution, antibiotics, fetal bovine serum (FBS) and horse serum were the products of GIBCO® (California, USA). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S-9 fraction for metabolic activation system was previously reported[34]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell Culture and Cytotoxicity Tests

The mouse lymphoma cell line L5178Y and human bronchial epithelial BEAS-2B cell was employed for comet assay. L5178Y cells were cultivated in 90% RPMI-1640 with 1 mM sodium pyruvate and 0.1% pluronic, supplemented with 10% heat-inactivated horse serum and antibiotics in a humidified incubator at 37°C with 5% CO_2. The BEAS-2B cell line was cultivated in 90% DMEM with 1 mM sodium pyruvate supplemented with 10% FBS and antibiotics. For the determination of cell viability, about 10^6 cells were treated for 2 h with the chemicals. After the staining of 0.4% trypan blue (Life Technologies, MD, USA), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability of treated cultures was related to controls that were treated with the media.

Single Cell Gel Electrophoresis Assay (Comet Assay)

Preparation of L5178Y and BEAS-2B Cells for Comet Assay

For the comet assay, 8×10^5 of cells were seeded into 12 wells plate and then treated as described in the toxicity tests. After 2 h, cells were centrifuged for 5 min at 100 x g, and gently resuspended with PBS. 100 μL of the cell suspension was immediately used for the test. Cells were mixed with 100 μL of low melting point agarose (LMPA: 1%) and added to fully frosted slide which had been covered with a bottom layer of 100 μL of 1% normal melting agarose. The cell suspension was immediately covered with coverglass and the slides were then kept at 4°C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of 100 μL of 0.5% LMPA by using a coverglass and then the slide were again kept cold at 4°C for 5 min.

Alkaline Unwinding/alkaline Electrophoresis

The procedure used follows the method described by Singh *et al.*[15], with minor modification. The cells embedded in the agarose on slides were lysed for 1.5 h in reaction mixture of 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl (pH 10), and 1% Triton X-100 at 4 °C. Slides were then placed in 0.3 M NaOH and 1 mM Na_2EDTA (pH approximately 13) for 20 min to unwinding of DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

Evaluation of DNA Damage

After the electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 5 min. The slides were stained by 50 μL of 5 μg/mL ethidium bromide solution on each slide, and then covering the slide with a coverglass. Image of 200 randomly selected cells (50 cells from each of four replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis Komet 5.5 (Kinetic Imaging Limited, Liverpool, UK), determining the mean tail moment (percentage of DNA in the tail times tail length) of the 200 cells. The comparison of each test group with negative and positive control was analyzed with one way of analysis of variance (ANOVA) followed by Dunn's test. $P < 0.05$ was considered statistically significant.

L5178Y Thymidine Kinase (tk)+/−-3.7.2C Mouse Lymphoma Assay (MLA)

To prepare working stocks for gene mutation experiments, cultures were purged of $tk^{+/-}$ mutants by exposure for 1 day to THMG medium (culture medium containing 3 μg/mL thymidine, 5 μg/mL hypoxanthine, 0.1 μg/mL methotrexate and 7.5 μg/mL glycine) and then the cells were transferred to THG medium (THMG but without methotrexate) for 2 days. The purged cultures were checked for low background $tk^{+/-}$ mutants and stored in liquid nitrogen. Each experiment started with working stock. The cells were usually used on day 3 or 4 after thawing and during logarithmic growth. S-9 mixture was prepared just prior to use by combining 4 mL S-9 with 2 mL each 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP and 150 mM KCL. The concentration of S-9 mixture was 5% during treatment and the final concentration of S-9 was 2%. For treatment, cells were centrifuged and suspended at a concentration of 0.5×10^6 cells in 10 mL of medium in 15 mL polystyrene tubes. Al_2O_3 NP was tested with

and without S-9 mixture. Al_2O_3 NP at each concentration was added and these tubes were gassed with 5% CO_2 in air and sealed. The cell culture tubes were placed on a roller drum and incubated at 37°C for 3 h. At the end of the treatment period, the cell cultures were centrifuged and washed twice with fresh medium and resuspended in fresh medium. We conducted preliminary experiments to determine the solubility and cytotoxicity of Al_2O_3 NP. Cytotoxicity was determined by relative survival (RS) and relative total growth (RGT) following 3 h treatments at concentrations up to 5 mg/mL, usually regardless of solubility. The recommended highest concentration was one with a 10-20% RS and/or RTG. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al.*[35]. Simply, the treated cells in medium containing 3 μg TFT/mL for selection or without TFT for cloning efficiency were distributed at 200 μg/well into 96-well flat-bottom microtiter plates. For mutant selection, two plates were seeded with ~2,000 cell/well. For cloning sufficiency, two plates were seeded with ~1 cell/well. All plates were incubated in 5% CO_2 in air in a humidified incubator at 37°C. After 11-13 days incubation, clones were counted and the colony size distribution was determined. Mutant frequencies were calculated using a statistical package (MutantTM; UKEMS, York, UK) in accordance with the UKEMS guidelines[36].

Acknowledgements

This subject is supported by the Korea Research Foundation grants from Korea Ministry of Environment as "The Eco-technopia 21 project", KIST Core-Competence Program and Korea Research Council of Fundamental Science and Technology to Ryu, J. C. of the Republic of Korea.

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