Immunotoxicity of silicon dioxide nanoparticles with different size and electrostatic charge

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Immunotoxicity of silicon dioxide nanoparticles with different size and electrostatic charge

Directed by Professor Soo-Ki Kim

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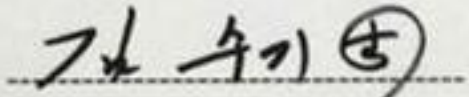
of Jae Hyun Kim is approved.



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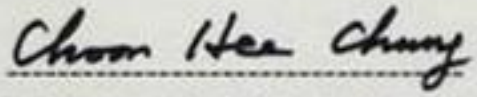

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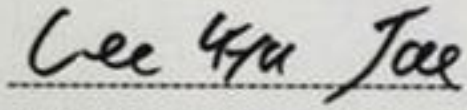

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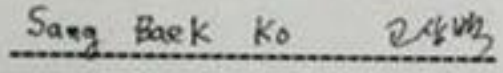

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It was out of hopes to overcome the gap of knowledge over 25 years since my graduation from medical school that I decided to enter the doctorate degree program. It has been a wonderful learning experience to get exposure to the new field of immunotoxicology and many others in modern medical science. It also was a great pleasure of mine to set the exemplary figure of continuing education for my two daughters.

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ABSTRACT

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Silicon dioxide nanoparticle (SiO_2 NP) has been widely used in biomedical field such as drug delivery and gene therapy. However, little is known about the biological effects and potential hazards of SiO2. Herein, the colloidal SiO2NPs with two different sizes (20-NM and 100-NM) and different charge (L-arginine modified: $SiO_2^{EN20[R]}$, $SiO_2^{EN100[R]}$, and negative: $SiO_2^{EN20[-]}$,$SiO_2^{EN100[-]}$) were orally administered (750 mg/kg/day) in female C57BL/6 mice for 14 days. Assessments of immunotoxicity include hematology profiling, reactive oxygen species (ROS) generation and antioxidant effect, stimulation assays for B-and T-lymphocytes, the activity of natural killer cells and cytokine profiling.

In vitro toxicity was also investigated in RAW 264.7 cell line. When cellularity of mouse spleen was evaluated, overall there is decreased on proliferation of B and T cells for all the groups fed with SiO_2 NPs, specifically the $SiO_2^{EN20[-]}$ showed the most pronounced reduction. In addition, the NO production and NK cell activity in SiO_2 NP-fed mice were significantly suppressed. Moreover, there was decreased in serum concentration of inflammatory cytokines such as interleukin (IL)-1β, IL-12 (p70), IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. To elucidate the cytotoxicity mechanism of SiO_2 in *in vivo*, *in vitro* study using RAW 264.7 cell line was performed. Both size and charge of SiO_2 using murine macrophage RAW 264.7 cells decreased cell viability dose-dependently.

Collectively, our data indicate that different sized-and charged-SiO_2 NPs would cause differential immunotoxicity. Interestingly, the small sized-and negative charge SiO2NPs showed the most potent *in vivo* immunotoxicity by way of suppressing the proliferation of lymphocytes, depressing killing activity of NK cell and decreasing the proinflammatory cytokine production, thus, leading to immunosuppression.

Key words: silicon dioxide, nanoparticle, immunotoxicity, oxidative stress, cytokines, immunosuppression

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I. INTRODUCTION

The growing fabrication and characterization of silica nano scale materials has received attention in biomedical research such as biosensors, enzyme immobilization controlled drug release and delivery and cellular uptake [1-6]. Since the emerged of these various researches on Nanomedicine, the medical applications of nanoparticles were highlighted especially for anti-cancers. Mesoporous silica nanoparticles (MSNs) attracted great attention in the last few decades for its wider plausible application in the emerging field of Nanomedicine.

MSNs is an inorganic nano-carrier that is known to be highly stable in physicochemical and

biochemical context, thus vitally important in the construction of anti-cancer medicine [6,7]. Recently, these MSNs received FDA approval as an inorganic carrier in nanomedicine and considered as one of the most promising inorganic nano-biomaterials [7, 8]. The potential applications seem endless based on the unique physicochemical characteristics of this nanomaterial like varying size, shape, chemical composition and assembly [9]. Meantime, the special physicochemical characteristics of silica posed concerns about their potential environmental and health implications [10]. To date, animal exposure with colloidal silica confirmed liver damage [11] and moderate to severe pulmonary inflammation and tissue damage primarily induced by oxidative stress and apoptosis [12,13]. Physicochemical properties play an important role in the toxic reaction of silica nanoparticles. Small particle means a larger surface area and this might indicate an increase in surface reactivity which enables them to interact with cell biomolecules [9]. The altered surface charge of NPs provides a unique way to help them to be uptake into the interior structure of cells [14].

However, most studies have focused on pulmonary and liver toxicity; rare studies have overlooked the toxicological effects of silica NPs on immune response *in vivo* [15-17]. In addition, the influence of nanoparticle properties (e.g., size, surface charge) to their potential hazards to the biological system remains to be elucidated.

In this study, we investigated the potential immunotoxicity of colloidal silicon dioxide nanoparticles (SiO_2 NPs) with two different sizes (20-nm and 100-nm) and different charge (L-arginine modified: $SiO_2^{EN2[R]}$, $SiO_2^{EN100[R]}$, and negative: $SiO_2^{EN20[-]}$, $SiO_2^{EN100[-]}$) in mice and Raw 264.7 cell lines. Accordingly, cytotoxicity was performed *in vitro* using WST-CCK8 assay. The primary indicators of

immune toxicity were also assessed *in vivo* such as body weight measurement and hematology profiles. NP-induced oxidative effect was also examined with reactive oxygen species (ROS) generation, superoxide dismutase (SOD) activity and intracellular levels of glutathione peroxidase (GPx). The cellularity of the spleen and analysis of the functional capacity of specific immune cells were evaluated by stimulation assays for B- and T- lymphocytes, and the activity of natural killer cells. In addition, we also focused on the inflammatory responses induced by NPs; therefore the concentration of cytokines was determined.

II. MATERIALS AND METHODS

1. Preparation of particle suspensions

Manufactured SiO_2 NPs were purchased from E&B Nanotech Co, Ltd (Ansan, South Korea). The colloidal SiO_2 NPs were already coated with citrate, and they had these following characteristics: the size and zeta potential of 20 nm ($SiO_2^{EN20[-]}$) and 100 nm ($SiO_2^{EN100[-]}$) are 20±2 nm; -19 mV and 90±13 nm; -40 mV, respectively. To shift the strong negative charge of SiO_2 NPs to a positive direction, their surface was modified with L-arginine (R) ($SiO_2^{EN20[R]}$, $SiO_2^{EN100[R]}$), as reported [18]. After the surface modification with L-arginine, the size and zeta potential of 20 nm and 100 nm became: 20±2 nm; −9 mV ($SiO_2^{EN20[R]}$) and 92±9 nm; −22 mV ($SiO_2^{EN100[R]}$). Then, powdered particle suspensions were prepared by dispersing the particles in 1× phosphate buffered saline (PBS), and to prevent agglomeration and to ensure uniform suspension, particles were sonicated in an ultrasonic bath (Hielscher Ultrasonics GmbH, Teltow, Germany) for 5–10 minutes.

2. Cell culture

The RAW 264.7 mouse leukemic monocyte macrophage cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's Modified Eagle's Medium (HyClone; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat-activated fetal bovine serum (FBS) (HyClone; Thermo Fisher Scientific) and 1% (v/v) antibiotic-antimycotic (Gibco®; Life Technologies) at 37℃ in a 5% CO_2 incubator.

3. Cell viability assay

Approximately 1×10^5 of RAW 264.7 cells were seeded into each well of 96-well plates. After reaching 70% confluence, the culture medium in the plate was replaced with a freshly prepared 100 μL of SiO_2 NP suspension, which was diluted to appropriate concentrations (10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, 160 μg/mL, 320 μg/mL, and 640 μg/mL) with the culture medium, and the cells were incubated for 24 hours at 37℃ in a 5% CO_2 incubator. Cells not treated with SiO_2 NPs served as a control in the experiment. Afterwards, a commercially available CCK-8 was used to evaluate the cytotoxic effect of SiO_2 NPs. Then, 10 μL of the CCK-8 solution was added to each well and incubated at 37℃ for 1 hour. Colored supernatants were measured at 450 nm by a DTX-880 multimode microplate reader (Beckman Coulter, Inc., Brea, CA, USA). All experiments were performed in triplicate.

4. Animals and exposure

For this experiment, 6-week-old female C57BL/6 (number [n]=5) mice weighing 18±2 g were purchased from Orient Bio Inc. (Seongnam, South Korea) and were maintained at 22℃±2℃ and 40%–60% humidity under a 12:12 hours light–dark cycle. The mice were acclimatized for 1 week and randomly assigned to one of five groups: normal control (purified water alone); or one of four experimental groups treated with four types of SiO_2 NPs ($SiO_2^{EN20[R]}$, $SiO_2^{EN100[R]}$, $SiO_2^{EN20[-]}$, and $SiO_2^{EN100[-]}$), respectively. All of the animals were orally administered 750 mg/kg of the SiO_2 NP suspension daily for 2 continuous weeks. During the administration periods, the animals' body weight was recorded. The animal use protocol for this experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Wonju College of Medicine, Yonsei University Wonju Campus (Wonju-si, Gangwon-do, Republic of Korea).

5. Analysis of blood samples

Animals were sacrificed following the 2-week oral administration of SiO_2 NPs. Their blood was collected from the retro-orbital venous plexus, and the total white blood cell (WBC) count was collected in tubes coated with an anticoagulant. The blood was mixed with an automatic mixer for 5 minutes, and the WBCs and their differential counts (such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were determined by an automatic blood analyzer (Hemavet® HV950 FS; Drew Scientific, Erba Diagnostics, Inc., Dallas, Texas, USA).

6. Preparation of splenocytes

For the isolation of splenocytes, spleens were aseptically removed from recently sacrificed mice and the tissue was transferred to a tube containing 1× PBS on ice. Splenocyte suspensions were prepared by blandly squeezing the spleen between the frosted ends of the two sterile microscope slides into a 100 mm tissue culture grade Petri dish. The slides were rinsed at regular intervals with 1× PBS. The cells were formed into a single suspension using a pipette. After that, the cell suspensions were filtered through a cell strainer. Next, the cell suspensions were centrifuged at 1,500 rpm at 4℃ for 5 minutes to produce pellets. For the optimal lysis of erythrocytes, the pellets were resuspended in 5 mL of red blood cell lysis buffer and incubated on ice for 5 minutes with occasional shaking. The reaction was stopped by diluting the lysis buffer with 25 mL of 1× PBS. Thereafter, cells were spun (1,500 rpm at 4℃ for 5 minutes), and the supernatant was carefully removed. The pellet was then washed two times in 1× PBS and resuspended in Roswell Park Memorial Institute (RPMI) -1640 supplemented with 3% FBS and 1% antibiotic–antimycotic. Cells were then counted. The viability of the cells used in all the experiments was higher than 95%, as measured by the trypan blue exclusion method (Sigma-Aldrich, St Louis, MO, USA).

7. Immune cell proliferation

Single cell suspensions of lymphocytes from the spleen were prepared. Splenocytes were counted with a hemocytometer, and 1×10^5 cells per well were suspended in a 96-well flat-bottom plate in 100 μL of RPMI-1640 supplemented with 10% heat-activated FBS and 1% antibiotics. Thereafter, cells were treated with 2.5 mg/mL of concanavalin A (Con-A) (Sigma-Aldrich) and 1 mg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) with 1 μL/well. The treated cells were incubated at 37°C in a humidified atmosphere under 5% CO_2 for 2 hours. Cell proliferation was determined using a CCK-8 kit according to the manufacturer's instructions. Absorbance was measured at 450 nm by a DTX-880 multimode microplate reader.

8. NK cytotoxicity assay

The murine lymphoma cell line, YAC-1 (American Type Culture Collection), as target cells were cocultured with NK-enriched murine splenocytes. The percentage of target cells killed by effector NK cells was determined. Briefly, the dilutions of various effector cell were prepared: 1×10^5 cells/well for 100:1; 5×10^4 cells/well for 50:1; and 2.5×104 cells/well for 25:1. The target cells (1×10^3 cells/well) were then cocultured with the different effector cell dilutions prepared in a 96-well plate and incubated for 6 hours at 37℃ in a humidified incubator with 5% CO_2. The CCK-8 assay was conducted according to the manufacturer's instructions. A special highly water soluble tetrazolium salt (WST-8) (2-[2-methoxy-4-nitrophenyl]-3[4-nitrophenyl]-5[2-4disulfophenyl]-2H-tetrazolium, monosodium salt) was added to the culture. WST-8 is reduced by dehydrogenase activities in cells to give the orange formazan dye, which is soluble in the culture media. The amount of the formazan dye generated by dehydrogenase in cells is directly proportional to the number of living cells. After 1 hour of incubation with the WST-8 solution, the cell suspension was then colorimetrically measured at 450 nm by a DTX-880 multimode microplate reader, and the number of live cells in the different ratios was determined.

9. Measurement of nitric oxide (NO) production

The nitrite (NO_2^-) present in the supernatant of splenocytes was used as an indicator of nitric oxide (NO) production using the Griess reagent (Promega Corporation, Fitchburg, WI, USA). The assay relies on measuring nitrite (NO_2^-), one of the primary, stable, and nonvolatile breakdown products of NO. Briefly, 50 μL of the splenocyte supernatant was mixed with an equal volume of Griess reagent in a 96-well microtiter plate and incubated at room temperature for 15 minutes. The absorbance was read at 540 nm using a DTX-880 multimode microplate reader. The NO_2^- concentration was calculated by comparison with the representative NO_2^- standard curve generated by serial two-fold dilutions of sodium nitrate.

10. Intracellular reactive oxygen species (ROS) detection

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect the intracellular ROS level. DCFH-DA can enter into cells as a fluorescent probe, and be acetylated to form DCFH (2′,7′ dichlorofluorescin) trapped in the cells. The oxidation of DCFH (nonfluorescent) by the intracellular ROS transforms it to DCF (dichlorofluorescein) (fluorescent). The fluorescent intensity of DCF can indicate intracellular ROS quantity. The spleens of mice were collected, and splenocytes were prepared as described in the "Preparation of splenocytes" section. Splenocytes (1×10^6 cells) were incubated with 10 μmol/L of DCFH-DA for 30 minutes at 37℃ in the dark. After washing twice with PBS, the splenocytes were analyzed by a DTX-880 multimode microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

11. Antioxidant endogenous enzyme activities

The activity of SOD and GPx were measured using a BioVision kit (BioVision, Inc., Milpitas, CA, USA). Splenocyte suspensions were lysed in ice-cold radioimmunoprecipitation assay buffer, the crude cell lysate was centrifuged at 14,000 rpm for 5 minutes at 4℃, and the cell debris was discarded. The supernatant was then checked for protein concentration using the Bradford method [19]. Thereafter, supernatant was used to measure the activities of different antioxidant enzymes (SOD, GPx) according to the manufacturer's instructions (BioVision, Inc., Milpitas, CA, USA).

12. Multiplex cytokine analysis

Serum levels of selected cytokines, such as interleukin (IL)-1β, IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, were measured using a Luminex bead-based suspension array system (Bio-Rad Laboratories, Hercules, CA, USA). This Luminex-based multiplexed system combines the principle of a sandwich immunoassay with fluorescent bead-based technology. Briefly, each set of premixed beads coated with the target antibodies was added to the well, and it was incubated with the sample in a 96-well round-bottomed microtiter plate to react with specific analytes. Then, premixed detection antibodies were added to the wells followed by a fluorescently labeled reporter molecule that specifically binds the analyte. Standard curves for each cytokine were generated using the standard control concentrations provided in the kit. Each step requires a specific incubation time, with shaking at room temperature and washing steps. All washes were performed using a Bio-Plex® Pro wash station (Bio-Rad Laboratories). Finally, the samples were then read using the Bio-Plex® suspension array reader, and the raw fluorescence data were analyzed using the Bio-Plex® Manager™ software using five-parameter logistic fitting (Bio-Rad Laboratories).

13. Statistical analysis

For the cell viability assay, the half maximal effective concentration (EC_{50}) was calculated via nonlinear regression using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). For the remaining *in vivo* analysis, data values were expressed as the mean ± standard deviation. The mean values among the groups were analyzed and compared using one-way analysis of variance followed by a subsequent multiple comparison test (Tukey) with the GraphPad Prism version 5.0 software package. Differences were considered statistically significant at $P<0.05$, $P<0.01$, *and* $P<0.001$.

III. RESULTS

1. Cytotoxicity assay

After exposure for 24 hours at varying doses of SiO_2, murine macrophage RAW 264.7 cell viability detected by the CCK-8 assay resulted in an explicit dose-dependent reduction (Figure 1). Also, size- and electrostatic charge-dependent cytotoxicity of SiO2 NPs were found. The EC_{50} values after 24 hours of exposure were 71.10 ug/mL ($SiO_2^{EN20[-]}$), 211.4 ug/mL ($SiO_2^{EN100[-]}$), 233.2 ug/mL ($SiO_2^{EN20[R]}$), and 833.6 ug/mL ($SiO_2^{EN100[R]}$).

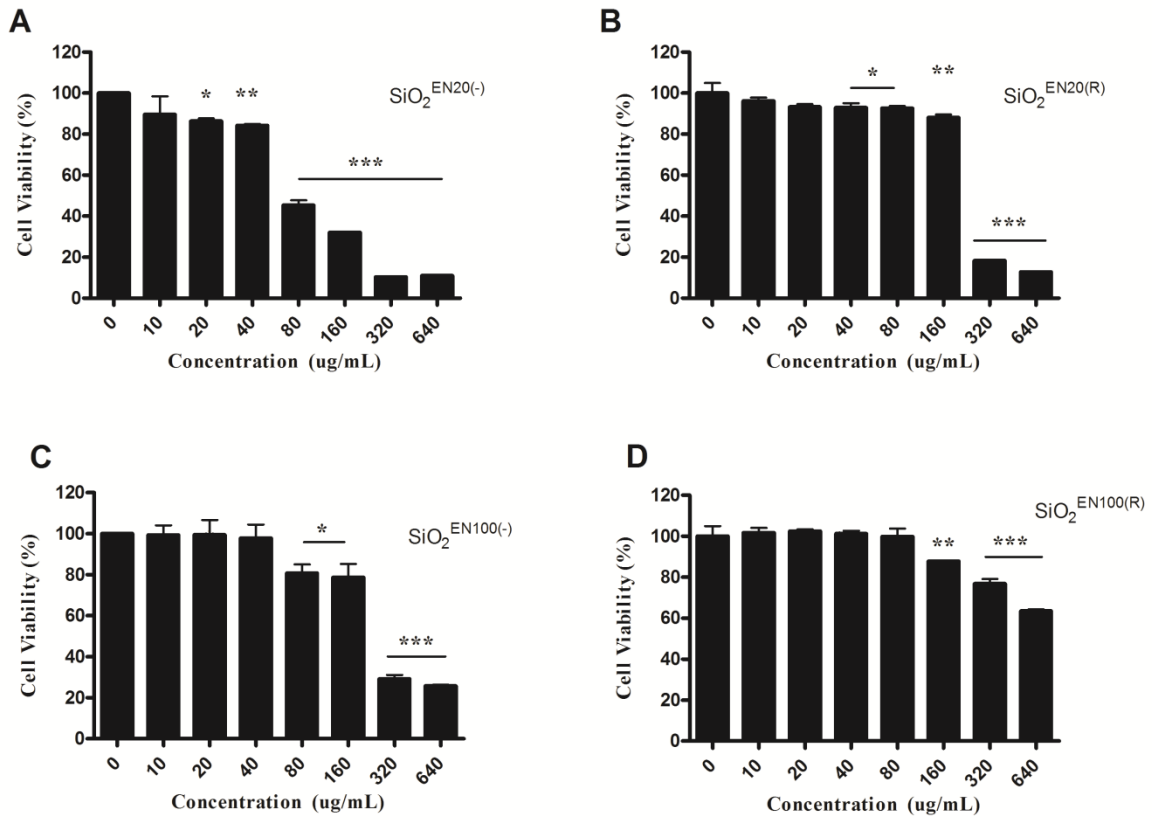

Figure 1. Effect of different sized and electrostatic charged SiO_2 NPs on the viability of murine macrophage RAW 264.7 cells. Cells were incubated with indicated concentrations of colloidal SiO_2 NPs: (A) $SiO_2^{EN20[-]}$; (B) $SiO_2^{EN20[R]}$; (C) $SiO_2^{EN100[-]}$; and (D) $SiO_2^{EN100[R]}$ for 24 hours. Cells not treated with SiO_2 NPs served as the control in the experiment. A commercially available cell viability assay cell counting kit-8 was used to evaluate the cytotoxicity effect of the SiO_2 NPs according to manufacturer's Instructions (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Data are presented as the mean ± standard deviation. *$P<0.05$; **$P<0.01$; ***$P<0.001$ versus control (0 µg/mL).

2. Body weight measurement

Body weight gain or loss may indicate an indirect immunotoxic effect. To evaluate the toxicity of SiO_2 NPs, mice were fed 750 mg/kg of SiO_2 every day for 2 weeks, and their body weight condition was monitored every 5 days for 2 weeks. After 2 weeks of feeding with SiO_2 NPs, the groups $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[R]}$ showed significant elevated body weight ($P<0.05$) compared to the untreated group (NC). However, the $SiO_2^{EN20[R]}$-fed group showed significant depressions in body weight ($P<0.05$) compared to the NC. Further, the $SiO_2^{EN100[-]}$-fed group showed no obvious difference in body weight compared to the NC (Figure 2).

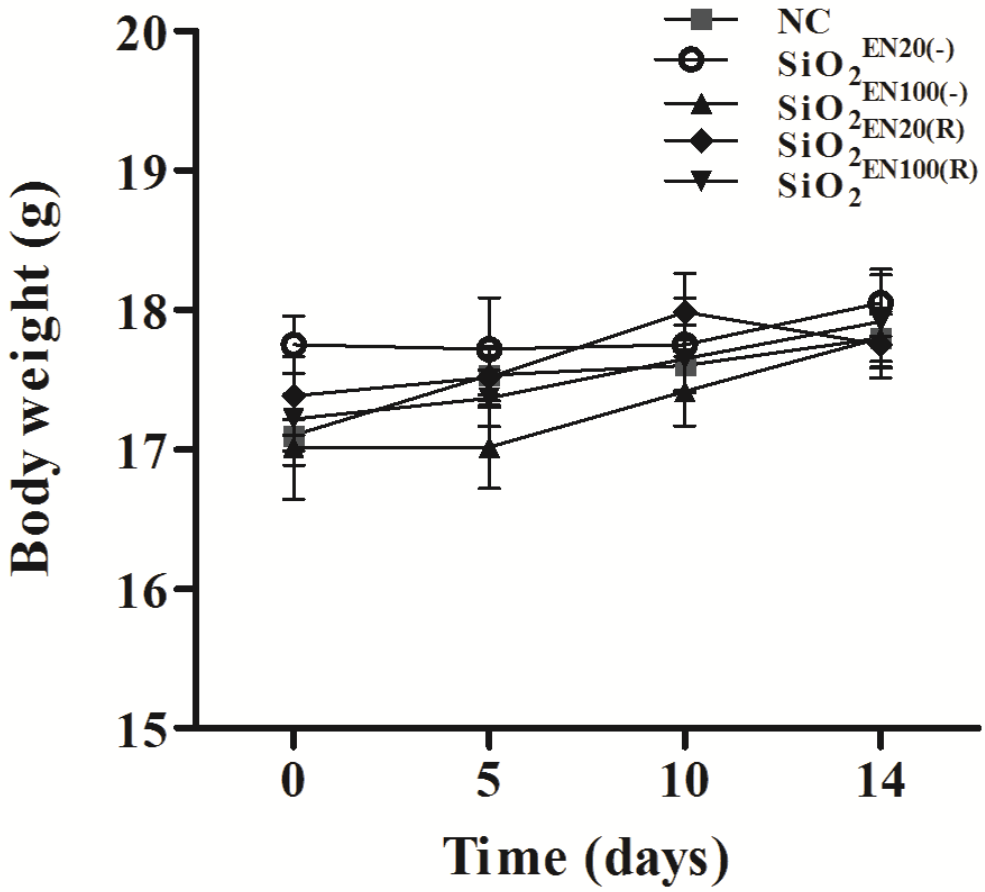

Figure 2. The effect on body weight of SiO_2 NPs fed to mice. C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges ($SiO_2^{EN[-]}$, $SiO_2^{EN[R]}$) of 750 mg/kg colloidal SiO_2 NPs for 14 days. Body weight was measured all throughout the experiment period, and it served as one of the primary indicators of SiO_2 NP immune toxicity. Data are presented as the mean ± standard deviation; n=5.

3. White blood cells (WBC) and its differential count

Total WBC counts are used to evaluate the severity of inflammation. The altered WBC counts may be indicative of direct or indirect effects of the NPs on cellular proliferation. The WBCs of mice fed orally with $SiO_2^{EN100[-]}$ for 14 days showed depressed counts, which were most prominent on the differential count of lymphocytes (Table 1, 2). Further, the $SiO_2^{EN20[-]}$-fed group showed slight decreases in WBC count and each type of WBC: lymphocytes and eosinophils. Moreover, the $SiO_2^{EN100[R]}$-fed group showed no obvious change in WBC count compared to the NC group (Table 2). However, the $SiO_2^{EN20[R]}$-fed group showed an elevated WBC count for each type of WBC.

Table 1. Total WBC and type of WBC counts in L-arginine surface modified SiO2 NP-fed mice.

WBC and members	NC	$SiO_2^{EN20(R)}$	$SiO_2^{EN100(R)}$
Total WBC, x 10^9 /L	4.030 ± 0.600	4.843 ± 1.525	4.017 ± 1.452
Neutrophil, x 10^9 /L	0.673 ± 0.129	0.828 ± 0.362	0.568 ± 0.191
Lymphocyte, x 10^9 /L	3.258 ± 0.565	3.800 ± 1.127	3.270 ± 1.190
Monocyte, x 10^9 /L	0.092 ± 0.025	0.160 ± 0.072	0.162 ± 0.087
Eosinophil, x 10^9 /L	0.003 ± 0.005	0.040 ± 0.083	0.003 ± 0.005
Basophil, x 10^9 /L	0.002 ± 0.004	0.015 ± 0.023	0.002 ± 0.004

Data are the mean ± standard deviation; n=5

Table 2. Total WBC and type of WBC counts in negatively charged SiO2 NP-fed mice.

WBC and members	NC	$SiO_2^{EN20(-)}$	$SiO_2^{EN100(-)}$
Total WBC, x 10^9 /L	6.047 ± 0.797	5.623 ± 0.737	5.087 ± 1.423
Neutrophil, x 10^9 /L	0.762 ± 0.247	0.690 ± 0.220	0.775 ± 0.327
Lymphocyte, x 10^9 /L	5.060 ± 0.721	4.768 ± 0.526	4.138 ± 1.057
Monocyte, x 10^9 /L	0.172 ± 0.056	0.153 ± 0.041	0.137 ± 0.031
Eosinophil, x 10^9 /L	0.038 ± 0.041	0.010 ± 0.011	0.025 ± 0.052
Basophil, x 10^9 /L	0.010 ± 0.011	0.000 ± 0.000	0.008 ± 0.012

Data are the mean ± standard deviation; n=5.

4. Stimulation assays for B-and T-cell lymphocytes

All immune responses are mediated by lymphocytes [20]. There are two main classes of lymphocytes: B-lymphocytes (B-cells) and T-lymphocytes (T-cells). Con-A and bacterial LPS are known to preferentially stimulate T-lymphocytes and B-lymphocytes, respectively. Stimulation assays performed on spleen-cell suspensions have been used as immune toxicity indicators. Splenocytes of mice treated with $SiO_2^{EN20[-]}$ showed significantly reduced B-cell ($P<0.01$) and slightly reduced T-cell counts (Figure 3A and B). $SiO_2^{EN20[R]}$ and $SiO_2^{EN100[R]}$ also showed significant reductions in B-cell ($P<0.001$) and T-cell counts ($P<0.05$; $P<0.001$, respectively). On the other hand, splenocytes from $SiO_2^{EN100[-]}$ mice showed significantly elevated B-cell ($P<0.05$) and T-cell counts ($P<0.001$) (Figure 3A and B).

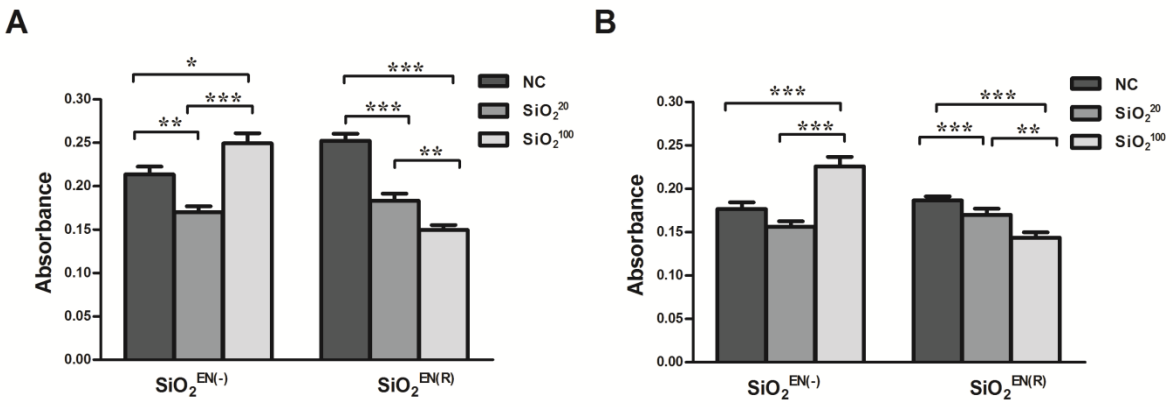

Figure 3. The effect on primary lymphocyte proliferation of SiO_2 NPs fed to mice. C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges ($SiO_2^{EN[-]}$, $SiO_2^{EN[R]}$) of 750 mg/kg colloidal SiO_2 NPs for 14 days. At the end of the treatment period, single-cell suspensions were made from spleens of mice, and the proliferation of (A) B-cells and (B) T-cells were assessed. Data are presented as the mean ± standard deviation; n=5. **P<0.05, **P<0.01, and ***P<0.001* indicate significant differences when tested with ANOVA. Tukey's test was used for post hoc tests.

5. Nitric oxide (NO) production

NO is a reactive molecule that reacts with ROS to produce reactive nitrogen species; moreover, it is recognized as a mediator and regulator of immune responses [21]. NO has various physiological and pathophysiological responses, depending on its relative concentration [22]. Here, we measured the level of NO after exposure to SiO_2 NPs. Our results showed that both $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$ showed significant decreases in NO production compared to the NC (Figure 4A). However, there was no substantial difference in the NO level in $SiO_2^{EN20[R]}$ (Figure 4B) when compared to the NC group.

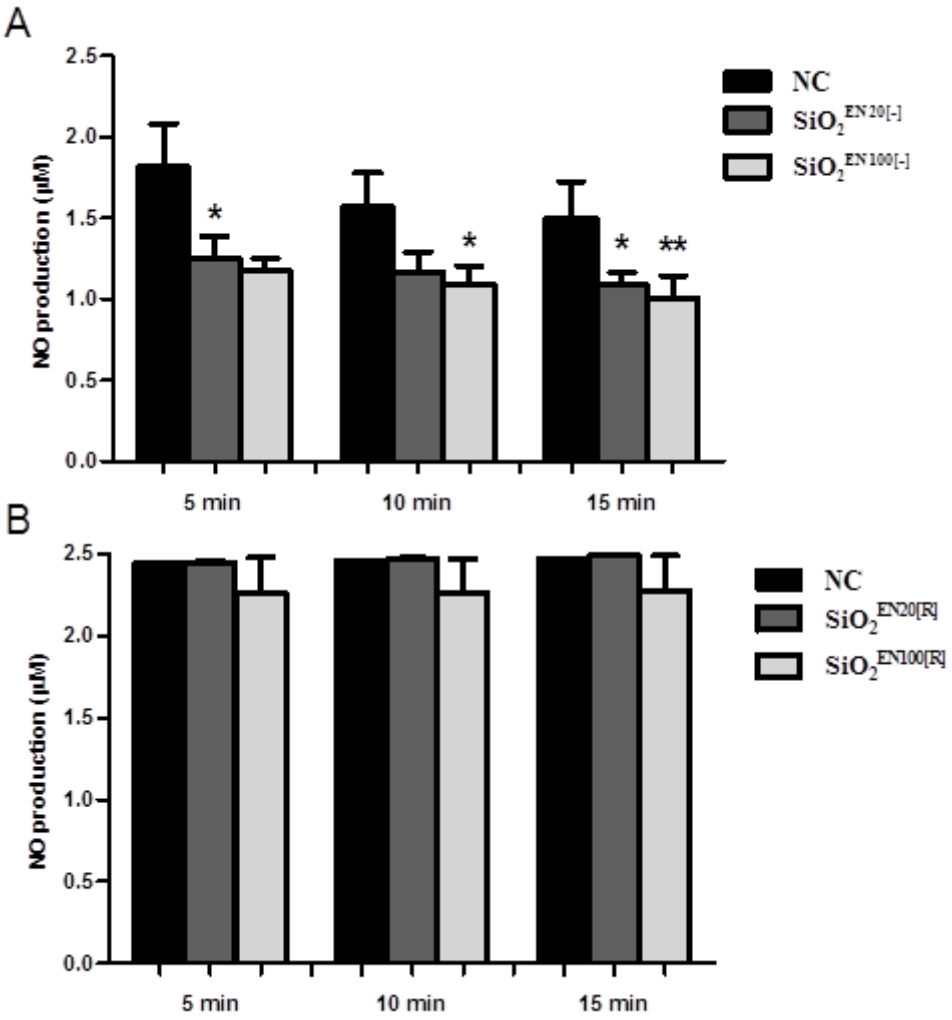

Figure 4. The effect on NO production of SiO2 NPs fed to mice. C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges ($SiO_2^{EN[-]}$, $SiO_2^{EN[R]}$) of 750 mg/kg colloidal SiO_2 NPs for 14 days. The supernatant of splenocytes from mice were harvested and accumulation was assessed using the Griess colorimetric assay according to manufacturer's instructions (Promega Corporation, Fitchburg, WI, USA), and Data are presented as the mean ± standard deviation; n=5. *$P<0.05$, and **$P<0.01$ indicate significant differences when tested with ANOVA. Tukey's test was used for post hoc tests.

6. ROS production and enzyme activities

Metal oxides mediate toxicity through oxidative stress [23]. The potential of SiO_2 NPs, as well as the influence of NP size and charge, in inducing oxidative stress was examined by measuring the ROS generation and the antioxidant activity of SOD and GPx levels in mice. We found that $SiO_2^{EN20[-]}$- and $SiO_2^{EN100[-]}$- fed mice showed a slight decrease in ROS production, while $SiO_2^{EN20[R]}$- and $SiO_2^{EN100[R]}$-fed mice showed increased production of ROS compared to the NC group (Figure 5). Furthermore, both the size and electrostatic charge of SiO_2 had no effect on intracellular activities of SOD (Figure 6A), and they had little effect on GPx (Figure 6B). There was a slight increase in the activity of $SiO_2^{EN20[R]}$- and $SiO_2^{EN100[R]}$- fed mice, which can be correlated to the slight decrease in ROS production among these groups.

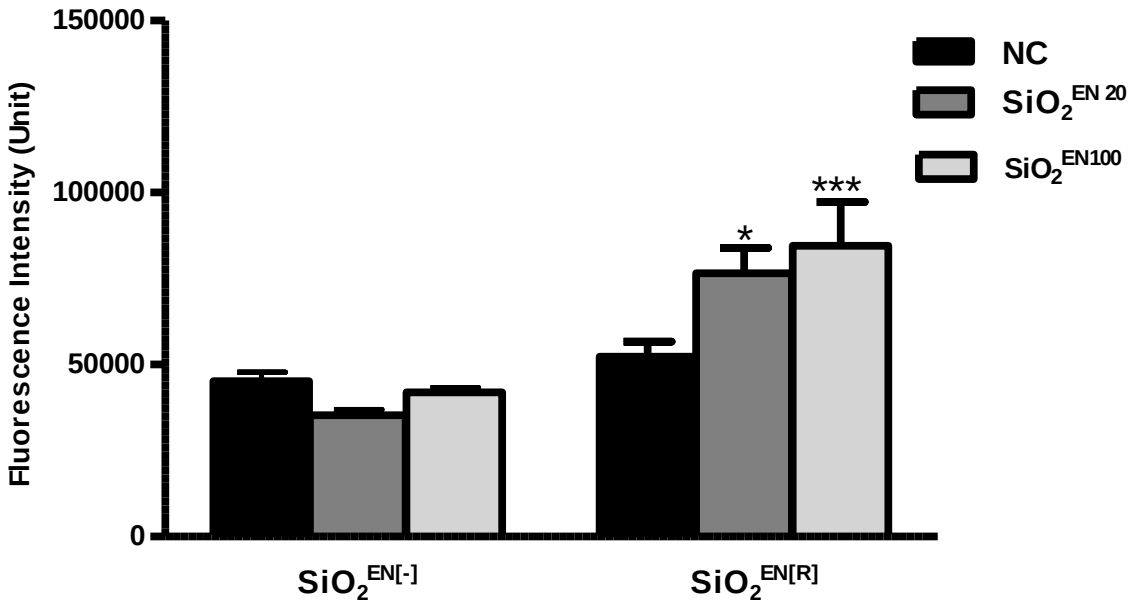

Figure 5. The effect on ROS production of SiO_2 NPs fed to mice. C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges ($SiO_2^{EN[-]}$, $SiO_2^{EN[R]}$) of 750 mg/kg colloidal SiO_2 NPs for 14 days. The supernatant of splenocytes from mice were harvested and intracellular ROS accumulation was detected by fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate using a fluorescence microplate reader. Data are presented as the mean ± standard deviation; n=5. *$P<0.05$, and **$P<0.01$ indicate significant differences when tested with ANOVA. Tukey's test was used for post hoc tests.

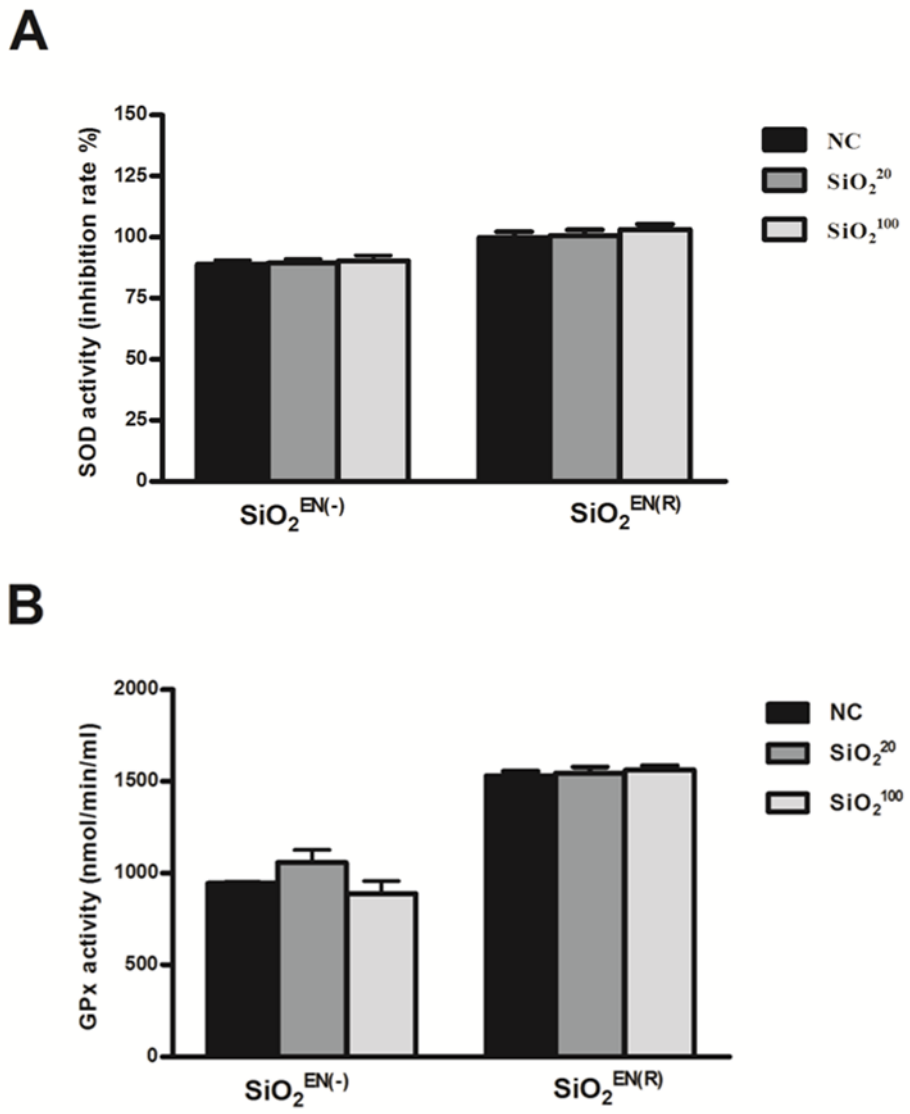

Figure 6. The effect on antioxidant activity of SiO_2 NPs fed to mice. C57BL/6 mice were treated with varying sizes (20nm, 100 nm) and charges ($SiO_2^{EN[-]}$, $SiO_2^{EN[R]}$) of 750mg/kg colloidal SiO_2 NPs for 14 days. Spleens from mice were isolated aseptically and made into single-cell suspension splenocytes. Superoxide dismutase (SOD) activity (A) and glutathione peroxidase (GPx) activity (B) were measured in splenocytes lysate by SOD activity assay kit and serum in mice by GPx activity colorimetric assay kit according to manufacturer's instructions. Data are mean ± s.d., n=5.

7. NK cell activity

NK cells are activated in response to cytokine production [24]. NK cells were incubated with target cells (YAC-1) at different ratios. In particular, $SiO_2^{EN20[R]}$ and $SiO_2^{EN20[-]}$ showed the most significant reduction (*P<0.001*) in the killing activity of the effector cell (NK), while $SiO_2^{EN100[R]}$ and $SiO_2^{EN100[-]}$ showed comparable activity with the NC group (Table 3).

Table 3. NK cell activity in SiO_2 NP-fed mice.

Effector/Target Ratio	NK cell activity (% Mean ± SD)				
	NC	$SiO_2^{EN20[-]}$	$SiO_2^{EN100[-]}$	$SiO_2^{EN20[R]}$	$SiO_2^{EN100[R]}$
100:1	76.61 ± 3.622	46. 95 ± 6.117**	71.12 ± 19.99	55.04 ±4.845***	82.50 ± 4.560
50:1	58.11 ± 1.691	40. 45 ± 3.662	48.87 ± 10.36	45.39 ±1.263	58.96 ±3.361
25:1	53.12 ± 2.229	37.51 ± 1.1160	42.91 ± 2.325	46.07 ±0.842	54.71 ±2.899

Data are presented as the mean ± SD; n=5. Nonparametric Tukey's multiple comparison test (*$P<0.01$; **$P<0.001$ versus NC).

8. Cytokines analysis

Exposure to NPs can affect the production of inflammatory and T helper (Th)1-type cytokines. Varying the size and surface charge of SiO_2 NPs differentially influenced cytokine production in the serum. Exposure with $SiO_2^{EN20[-]}$, $SiO_2^{EN100[-]}$, and $SiO_2^{EN100[R]}$ led to suppressed production of cytokines, namely IL-1β, TNF-α, IL-12p70, IL-6, and IFN-γ (Table 4, Figure 6). Conversely, $SiO_2^{EN20[R]}$ showed slight increases in IFN-γ. In addition, there were unaltered levels of Th1 cytokine (IL-12p70) and proinflammatory cytokine (TNF-α) among the group exposed to $SiO_2^{EN20[R]}$, which was in reference to the NC group (Table 4).

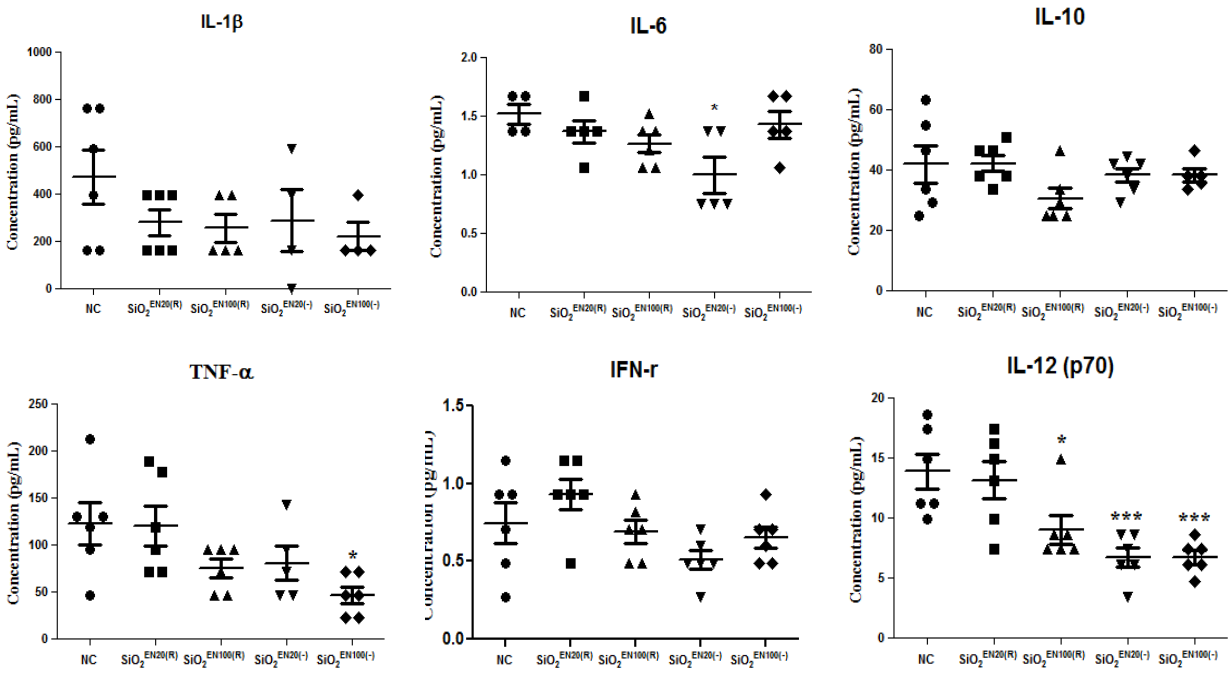

Figure 6. Serum cytokine concentration in mice exposed to SiO_2. Data are presented as the mean ± standard deviation; n=5. Nonparametric Tukey's multiple comparison test (*; $P<0.05$, **; $P<0.001$ versus NC).

Table 4. Serum cytokine concentration in mice exposed to SiO_2.

Cytokine	NC	$SiO_2^{EN20(R)}$	$SiO_2^{EN100(R)}$	$SiO_2^{EN20(-)}$	$SiO_2^{EN100(-)}$
IL-1β	473.54±277.32	279.72±130.19	255.95±130.19	287.81±260.52	220.30±118.85
IL-6	1.798±1.111	1.515±0.408	1.265±0.187	1.905±2.242	1.315±0.358
IL-10	41.993±15.239	42.192±6.610	30.587±8.558	38.268±5.874	36.087±7.070
IL-12p70	13.880±3.625	13.148±3.848	9.062±2.948*	6.702±1.997**	6.713±1.369**
IFN-γ	0.747±0.324	0.930±0.241	0.692±0.176	0.508±0.146	0.655±0.167
TNF-α	122.54±54.341	120.60±52.055	75.060±23.715	102.57±64.786	46.717±21.941*

Data are presented as the mean ± standard deviation; n=5. Nonparametric Tukey's multiple comparison test (*; $P<0.05$; **; $P<0.001$ versus NC).

IV. DISCUSSION

There is an almost unanimous opinion about the potential toxicity of NP exposure, and it includes ROS generation, proinflammatory responses, and cell death [25, 26]. However, most of the studies on NPs have focused on lung and liver toxicity, such that the toxic effect of NPs on the immune system is poorly documented [15, 16]. In addition, the influence of NP size and electrostatic charge on potential immunotoxicity remains to be elucidated. The present study addressed the immunotoxicity of different sizes and electrostatic charges of SiO_2 NPs that were fed to mice for 14 days (orally administered at doses of 750 mg/kg/day). In particular, our study shows that immune dysfunction from exposure to the varying sizes and electrostatic charges of SiO_2 would lead to immunosuppression. This was evidenced by suppressing the proliferation of lymphocytes, by depressing the killing activity of NK cells, and by decreasing inflammatory cytokine production. In addition, our data showed that different sizes and charges of SiO_2 NPs could lead to differential immunotoxicity most of the studies on NPs have focused on lung and liver toxicity, such that the toxic effect of NPs on the immune system is poorly documented [15,16].

In addition, the influence of NP size and electrostatic charge on potential immunotoxicity remains to be elucidated. The present study addressed the immunotoxicity of different sizes and electrostatic charges of SiO_2 NPs that were fed to mice for 14 days (orally administered at doses of 750 mg/kg/day). In particular, our study shows that immune dysfunction from exposure to the varying sizes and electrostatic charges of SiO_2 would lead to immunosuppression. This was evidenced by suppressing the proliferation of lymphocytes, by depressing the killing activity of NK cells, and by decreasing

inflammatory cytokine production. In addition, our data showed that different sizes and charges of SiO_2 NPs could lead to differential immunotoxicity NPs, specifically the groups fed negatively charged NPs, may show a decrease in the white pulp component of the spleen, as there is a significant decrease in lymphocyte count.

Cumulative data showed that oxidative stress is another toxic mechanism of NPs [17, 29]. Oxidative stress and NO are closely linked to inflammatory responses. NO is implicated in phagocytosis, as well as in the pathogenesis of inflammation [30, 37]. The impaired production of NO leads to undesired effects, such as inflammation and tissue damage [30]. Thus, the abnormal production of NO, such as increases or decreases upon exposure to silica NPs, may induce a proinflammatory response [30]. ROS is known to stimulate NO production. Of note, there is the decreased generation of ROS in negatively charged $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$- fed mice; therefore, there was decreased NO generation in these given groups. Further, increased production of ROS in $SiO_2^{EN20[R]}$- and $SiO_2^{EN100[R]}$- fed mice led to increased NO generation, as compared to the $SiO_2^{EN20[-]}$- and $SiO_2^{EN100[-]}$- fed groups. The NP surface charge influenced the capacity of ROS production [31, 32]. L-arginine-coated SiO_2 NPs are capable of inducing intracellular ROS; however, the decreased ROS production among the mice that were fed negatively charged SiO_2 NPs might be due to earlier or later ROS production that occurred in these groups. In addition, the decreased ROS generation among mice fed negatively charged $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$ may be due to the leakage of the fluorescent product from the cell due to membrane damage, as there is a higher tendency of cytotoxicity on negative charge, as shown by in vitro viability. Size and charge influence the cytotoxicity of engineered nanomaterials [25, 32]. When comparing the cytotoxicity of particle size and surface charge, we found that negatively charged NPs tended to be higher in toxicity,

and 20 nm NPs were the most toxic in a murine macrophage cell line (RAW 264.7) (See Figure 1). In line with our results, the reported data showed that phagocytic cells preferentially interacted with negatively charged particles [33]. Therefore, the higher toxicity of the negatively charged $SiO_2^{EN20[-]}$ is due, in part, to the stronger interaction with the macrophage cells. Given this, the in vitro cytotoxicity and in vivo ROS production showed that negatively charged SiO_2 NPs are more toxic than their negative counterparts. To further prove our findings, we selected other immunotoxicity parameters, such as cytokine profiling and NK cell killing activity.

Any invading pathogens can trigger an inflammatory response, which involves the secretion of inflammation-induced mediators such as cytokines [34]. In an inflammatory response, activated immune cells recognize the NPs by their unique physicochemical characteristics, such as their surface charge and surface properties, thus inducing the cytokines to attract more cells to eradicate the NPs [20]. Electrostatic charges of the NPs are important parameters in an inflammatory response. As noted by Tan et al [35], cationic (positively charged) engineered nanomaterials, such as liposomes, induced the secretion of cytokines, such as TNF, IL-12, and IFN-γ. In relation to this, mice fed L-arginine surface modified NPs ($SiO_2^{EN20[R]}$ and $SiO_2^{EN100[R]}$) showed induced secretion of cytokines such as IL-12, IFN-γ, and TNF-α, as compared to the anionic NP (negatively charged $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$)- fed mice. Consistent with these results, the $SiO_2^{EN20(R)}$-fed group showed increased WBC production, as compared to the negatively charged ($SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$)- fed mice (which are both in reference to their corresponding normal control group WBC levels). With respect to the relationship between the WBC level and each type of WBC (such as lymphocytes and monocytes) with inflammation, activated WBCs trigger the secretion of cytokines [36]. Schwentker et al [37] reported that NO could affect the expression

and activity of cytokines. Further, particle size and surface area are also important parameters that affect *in vivo* bioreactivity [38]. NPs are predictive in stimulating cytokine production, specifically the ultrafine particles [39]. Our study showed that induction of cytokine production is clearly seen in $SiO_2^{EN20[R]}$- fed mice. NO production was low in negatively charged $SiO_2^{EN20[-]}$- fed mice. In parallel, the secretion of cytokines (IL-1β, TNF-α, IL-12p70, and IFN-γ) in negatively charged $SiO_2^{EN20[-]}$ and $SiO_2^{EN100[-]}$- fed mice was repressed. Of these cytokines, IL-12p70 ($SiO_2^{EN20[-]}$, $SiO_2^{EN100[-]}$) and TNF-α ($SiO_2^{EN100[-]}$) were significantly decreased in concentration, as compared to the levels found among the $SiO_2^{EN20[R]}$ and $SiO_2^{EN100[R]}$- fed mice and among the mice in the NC group. Negatively charged $SiO_2^{EN20[-]}$- fed mice showed the least secretion of IFN-γ among all of the groups. These two cytokines (IL-12p70 and IFN-γ) are involved in the activation of NK cells, which were found in decreased concentrations in mice fed with negatively charged $SiO_2^{EN20[-]}$ NPs. In addition, the decreased proliferation of B-cells in negatively charged $SiO_2^{EN20[-]}$- fed mice also supports our findings, where B-cells also function to secrete cytokines such as IL-1, IL-10, IL-6, IFN-γ, and TNF-α which, in turn, activates the antigen-presenting cells [40]. Physicochemical properties such as the size and charge of NPs determine their immunotoxicity, and they may also enhance their biological reactivity [41]. The biological activity of NPs increases as the particle size decreases [42]. However, this trend can be altered by a small change in the particle's surface charge. NPs with positively charged surfaces could be more easily up-taken due to the attractive interaction to the negative cell membrane [43]. Despite using varying sizes and electrostatic charges of NPs in this study, other factors need to be considered as well, such as the different exposure periods (chronic or acute) and the different dosages (low, medium, and high) of the NPs, in order to fully discuss, in detail, the potential immunotoxicity of SiO_2. In addition, our study focused on the spleen, as it was suggested by other studies that the spleen is one of the major target organs for toxicity

[44,45]. Moreover, the spleen is also involved in the initiation of immune responses, such that lymphocyte proliferation make take place in this given organ [27]. However, to fully elucidate the biosafety of SiO_2 NPs in particular, the immune system and other target organs, such as the lymph nodes and liver, need to be tested. While this paper provides evidence on SiO_2 immunotoxicity, the underlying mechanism still needs to be elucidated.

V. CONCLUSION

Collectively, our data indicate that different sized and charged SiO_2 NPs would cause differential *in vivo* immunotoxicity. Interestingly, the mice fed with the negatively charged SiO_2 NPs exhibited higher cytotoxicity and *in vivo* immunotoxicity than their L-arginine modified charged counterparts by way of suppressing the proliferation of the lymphocytes, depressing the killing activity of NK cells, and decreasing inflammatory cytokine production, thus leading to immunosuppression. This is the first account of immunosuppression of differently sized and charged SiO_2 NPs.

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ABSTRACT IN KOREAN (국문 초록)

나노 실리카입자의 크기와 전하에 따른 면역독성 비교

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김 제 현

산화 실리콘 나노입자는 약물치료나 유전자 치료 등에서 널리 사용되고있다. 하지만 실리콘 나노입자의 생물학적 영향이나 위험성은 잘 알려져 있지 않다. 본 연구에서는 이 문제를 해결하기 위하여 서로 다른 크기 (20-nm and 100-nm)와 서로 다른 전하를 지니는 (L-arginine modified: $SiO_2^{EN20[R]}$, $SiO_2^{EN100[R]}$, and negative : $SiO_2^{EN20[-]}$,$SiO_2^{EN100[-]}$) 콜로이드 실리콘 나노입자를 이용하여 생체 및 시험관내 면역독성 연구를 시행하였다. 생체 연구로 C57BL/6 생쥐에 750mg/Kg의 용량으로 14일간 경구 투여 후, 혈액학적 분석, Reactive oxygen species (ROS) 생성, 항산화 효과, B-, T-

림프구의 증식, 자연 살해 세포의 활성도 및 여러 사이토카인 농도를 분석하였다. 시험관 면역독성 연구에서는 RAW 264.7 세포주를 이용하여 연구하였다. 면역독성결과로서 전체적으로 실리콘 나노입자를 처치한 모든 군에서 B- 와 T-림프구 수가 줄었고, 특히 작은 음전하 나노입자 $SiO_2^{EN20[-]}$ 투여군에서 가장 감소하였다. 실리콘 나노입자를 경구 투여군에서는 NO 생성과 자연살해세포능에서 모두 억제되었다. 여러 염증성 사이토카인들, 즉 Interleukin (IL)-1ß, IL-12 (p70), IL-6, tumor necrosis factor (TNF)- , and interferon (IFN)-γ 혈청농도가 유의하게 감소하였다. 실리콘 나노입자를 대식세포 RAW 264.7에 처리시 농도에 비례하여 세포 생존률이 감소하였다.

결론적으로 크기와 전하가 서로 다른 실리콘 나노입자는 시험관 및 마우스 생체내 면역독성의 차이를 보였으며, 특히 작은 크기의 음전하 실리콘 나노입자가 생체실험에서 림프구 증식억제, 자연 살해세포능 감소 및 염증성 사이토카인 농도를 감소시켜 면역억제 및 가장 강한 면역독성을 보였다.

핵심되는 말 : 산화 실리콘 나노입자, 면역독성, 산화 스트레스, 사이토카인, 면역억제