

# SiO<sub>2</sub> Nanoparticles Induced Cytotoxicity by Oxidative Stress in Human Bronchial Epithelial Cell, Beas-2B

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**Objectives:** In this study, we investigated the potential harmful effect of the exposure to silicon dioxide (SiO<sub>2</sub>) nanoparticles through in vitro toxicity assay using human bronchial epithelial cell, Beas-2B with a focus on the involvement of oxidative stress as the toxic mechanism.

**Methods:** SiO<sub>2</sub> -induced oxidative stress was assessed by examining formation of reactive oxygen species (ROS), the induction of superoxide dismutase (SOD) and heme oxygenase-1 (HO-1), as well as cytotoxicity effect was evaluation by cell viability. Subsequently, to understand the molecular mechanism of nanoparticle-induced oxidative stress, the involvement of oxidative stress-responding transcription factors, such as, nuclear factor-kappaB (NF-κB) and nuclear factor -E2-related factor-2 (Nrf-2), and mitogen-activated protein (MAP) kinase signal transduction pathway was also investigated.

**Results:** 5-d i phenyltera zolium bromide (MTT) assay results show that decrease 20% in cell viability and the number of cells in the subG1 phase increased. The increase in ROS formation was observed in SiO<sub>2</sub> nanoparticle treated cells. The expression of SOD protein was not changed, whereas that of HO-1 was increased by SiO<sub>2</sub> nanoparticle exposure. transcription factors Nrf-2 and the expression of phosphorylated form of extracellular signal-regulating kinase (ERK) was strongly induced by SiO<sub>2</sub> nanoparticle exposure.

**Conclusions:** SiO<sub>2</sub> nanoparticles exert their toxicity through oxidative stress as they cause the significant increase ROS level. SiO<sub>2</sub> nanoparticles induce induction of HO-1 via Nrf-2-ERK MAP kinase pathway. Our tested oxidative stress parameters are rather limited in terms of allowing the full understanding of oxidative stress and cellular response by SiO<sub>2</sub> nanoparticle exposure.

**Key words:** Extracellular signal-regulating kinase, Heme oxygenase-1, Nuclear factor -E2-related factor-2, Oxidative stress, Silicon dioxide nanoparticles

## INTRODUCTION

In recent years, as nanotechnology has rapidly developed, nanomaterials have been widely used in the fields of biomedicine, pharmaceutical, and other industry. Of the various manufactured nanomaterials, silicon dioxide (SiO<sub>2</sub>) nanoparticles have the potential for widespread applications. SiO<sub>2</sub> nanoparticles are being used in these fields such as chemical mechanical polishing and as additives to drugs, cosmetics, printer toners, varnishes, and food [1,2]. In recent years, the use of SiO<sub>2</sub> nanoparticles has been extended to biomedical and biotechnological fields, such as biosensors for simultaneous assay of glucose, lactate, L-glutamate, and hypoxanthine levels in rat striatum [3], biomarkers for leukemia [4,5], DNA delivery [6,7], targeted drug delivery [8] and controlled drug release for genes and proteins [9,10]

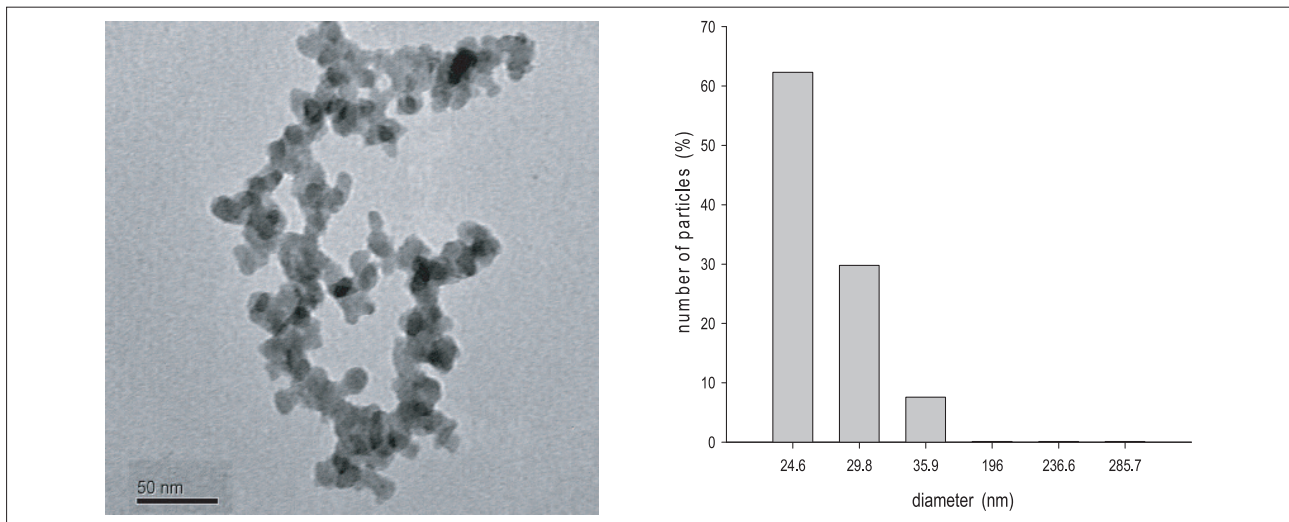
Considering their wide range of applications, the potential adverse effect of SiO<sub>2</sub> nanoparticles on human health and on the environment is of great interest. In this study, we investigated the potential harmful effect of exposure to SiO<sub>2</sub> nanoparticles by conducting an in vitro toxicity assay with a

focus on the involvement of oxidative stress. Numerous previous studies on nanoparticle toxicity, with various cell types and various nanoparticle types, reported that oxidative stress is one of the most important toxicity mechanisms related to exposure to nanoparticles [11-15]. Indeed, previous studies reported oxidative stress as the toxic mechanism of SiO<sub>2</sub> [13,16-18]

In this study, to understand the potential harmful effect of nanoparticles on human health, the oxidative stress-related toxicity was investigated by exposure to SiO<sub>2</sub> nanoparticles. SiO<sub>2</sub> -induced oxidative stress was assessed by examining formation of reactive oxygen species (ROS), the induction of superoxide dismutase (SOD) and heme oxygenase-1 (HO-

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**Figure 1.** Characterization of different sized SiO<sub>2</sub> nanoparticles using TEM and DLS methods. Particles shape were analysed by TEM (A) and the size distribution in the test media were evaluated by DLS (B).

SiO<sub>2</sub>: silicon dioxide, TEM: transmission electron microscopy, DLS: dynamic light scattering.

1), as well as cytotoxicity effect was evaluation by cell viability. Subsequently, to understand the molecular mechanism of nanoparticle-induced oxidative stress, the involvement of oxidative stress-responding transcription factors, such as, nuclear factor-kappaB (NF- $\kappa$ B) and nuclear factor -E2-related factor-2 (Nrf-2), and mitogen-activated protein (MAP) kinase signal transduction pathway was also investigated.

## MATERIALS AND METHODS

### I. Cell Culture and Nanoparticle Treatment

Human bronchial epithelial cells, Beas-2B, were maintained in DMEM / F12 (GIBCO BRL Life Technologies, Rockville, MD, USA), supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics, at 37°C in a humidified atmosphere of air and 5% CO<sub>2</sub>. SiO<sub>2</sub> nanoparticles were purchased from Sigma (St. Louis, MO, USA). Test solution of SiO<sub>2</sub> nanoparticles were prepared in the culture media and dispersed for 20 minutes using a sonicator (Branson Inc., Danbury, CT, USA) to prevent aggregation. During the testing periods, the suspension of nanoparticles was stable and uniform in the culture media. For surface area measurement, Branauer, Emmett & Teller (BET) method was used with BELSORP-mini II, a volumetric adsorption apparatus (BEL Japan, Inc., Osaka, Japan). To investigate the size and shape of SiO<sub>2</sub> nanoparticles, 20  $\mu$ L of particle suspension from test media was dried on a 400 mesh carbon-coated copper grid and imaged with a JEM 1010 transmission electron microscopy (TEM; JEOL, Tokyo, Japan) at 40-100 kV. The size

distribution of nanoparticles was evaluated using a Photal dynamic light scattering (DLS) spectrometer, DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan). The concentration we used in this study was 1 mg/L to prevent aggregation and/or precipitation of particles. The cells were treated with various sizes of nanoparticles for 24 hours for toxicological studies.

### II. Cell Viability Assay

Beas-2B cells were plated on 96-well plates and the plates were incubated with the nanoparticles for 24 hours. Cell viability was measured using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-di phenyltera zolium bromide (MTT) reagent [19].

### III. Flow Cytometry

Flow cytometry was performed on the treated and control cells for analysis of the cell cycle and apoptosis. Propidium iodide (PI) stained cells were analyzed using a flow cytometer (BD Science, San Jose, CA, USA) [20]. The effect on apoptosis was determined by the increase in the proportion of subG1 hypo-diploid cells.

### IV. Formation of ROS

To detect ROS generation in nanoparticles-treated cells, a fluorometric assay using intracellular oxidation of 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma) was performed [21,22]. Cells grown to confluence were treated with SiO<sub>2</sub> nanoparticles for 24 hours, washed with PBS, and then incubated with DCFH-DA (40  $\mu$ M) for 30 minutes. Following DCFH-DA incubation, the fluorescence of

dichlorofluorescein (DCF), which is the oxidized product of DCFH-DA, was visualized using a fluorescent microscope (Nikon, Tokyo, Japan) with excitation and emission wavelengths of 485 and 530 nm, respectively.

## V. Western Blotting

For western blotting analysis, aliquots of the cell lysates were subjected to electrophoresis on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and the gel transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with a primary antibody for 1 hours, subjected to further incubation with a secondary antibody and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK). The antibodies for the detection of p38, phosphorylated p38, ERK-2, phosphorylated ERK and Nrf-2 were purchased from Santa Cruz (Santa Cruz, CA, USA), antibodies for JNK, phosphorylated JNK were from Cell Signaling (Beverly, MA, USA), antibody for HO-1 was from Stressgen (Victoria, BC, Canada) and antibody for Cu/Zn SOD was from Biodesign (Saco, ME, USA).

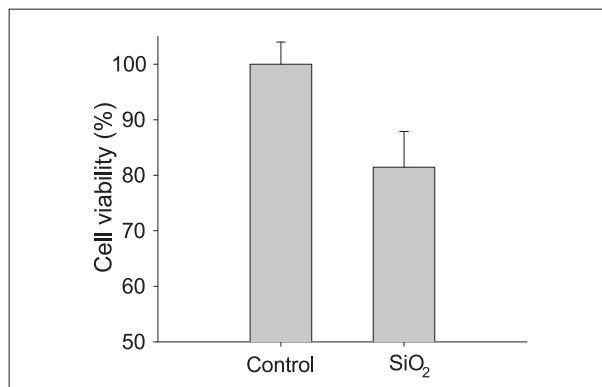
## VI. Data Analysis

Statistical differences between the control and treated cells were examined with the aid of a parametric t test using SPSS version 12.0 KO (SPSS Inc., Chicago, IL, USA). An alpha level of 0.05 was used to determine significance in all statistical analyses.

## RESULTS

### I. Characterization of SiO<sub>2</sub> Nanoparticles

The result from characterization of SiO<sub>2</sub> nanoparticles was



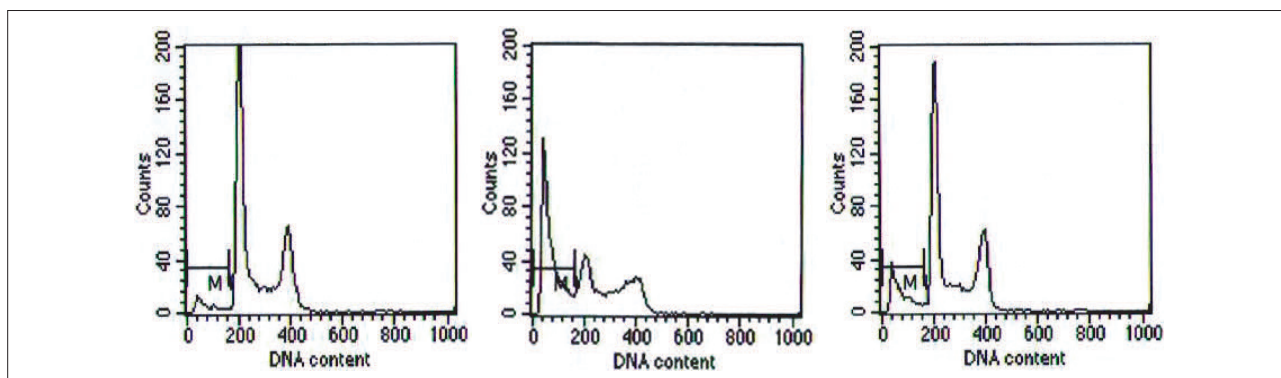
**Figure 2.** The effect of 1 mg/L SiO<sub>2</sub> nanoparticles on the cell viability investigated using MTT assay. Results were presented as relative units compared to control.

SiO<sub>2</sub>: silicon dioxide, MTT: 5-d i phenyltera zolium bromide.

summarized in Figure 1. The BET surface areas of was 201.01 m<sup>2</sup>/g, respectively. The TEM images indicated that silica nanoparticles had an even distribution with 20-40 nm individual particle sizes. The size distribution in the test medium was investigated using a DLS method, as the size of the nanoparticles distributed in the test medium were about 25 and 40 nm.

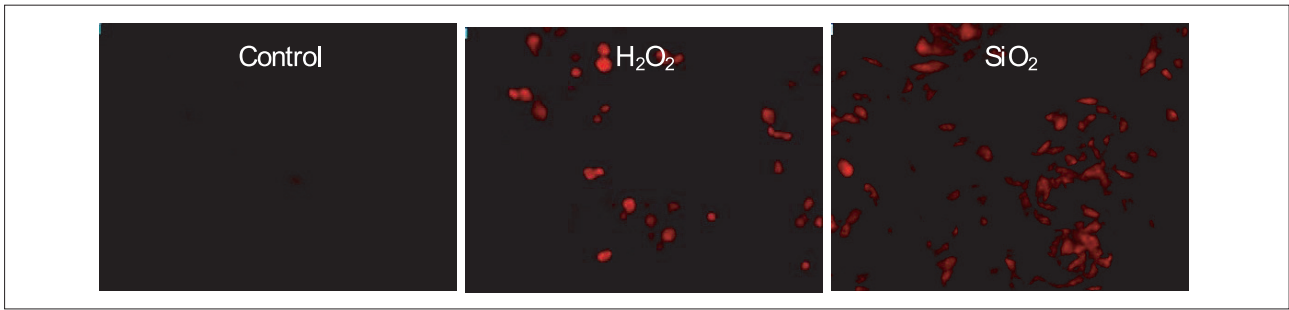
### II. Cytotoxicity of SiO<sub>2</sub> Nanoparticles

To investigate of SiO<sub>2</sub> nanoparticle induced cytotoxicity effects on Beas-2B cells, cell viability and apoptosis were examined. MTT assay results show that decrease in cell viability was observed only about 20% compared with that of the control by SiO<sub>2</sub> exposure (Figure 2). Flow cytometry analysis was conducted using PI staining to quantify the number of cells with a subdiploid DNA content (Figure 3). In Beas-2B cells exposed to SiO<sub>2</sub> nanoparticles, the number of cells in the subG1 phase increased. The degree of increase induced by SiO<sub>2</sub> nanoparticles was about 3-fold compared with that of the control.



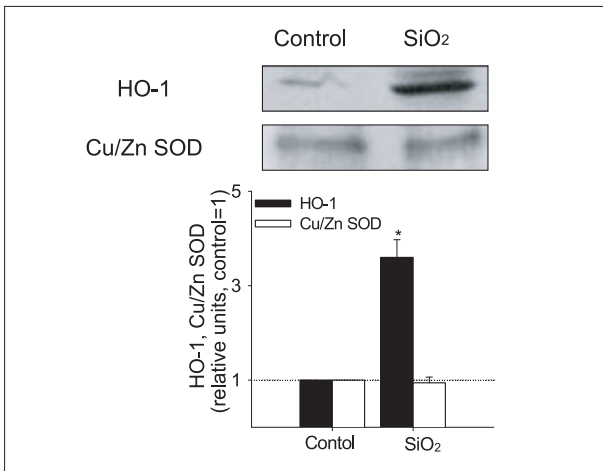
**Figure 3.** Flow cytometry analysis to quantify the number of cells with a subdiploid DNA content in 1 mg/L SiO<sub>2</sub> nanoparticles exposed Beas-2B cells. H<sub>2</sub>O<sub>2</sub> (100 uM) was used as positive control.

SiO<sub>2</sub>: silicon dioxide.



**Figure 4.** ROS induced by nanoparticles in Beas-2B cells. The cells were incubated with 1 mg/L SiO<sub>2</sub> nanoparticles for 24 hours and 40 μM DCFH-DA at 37 °C for 30 min and observed fluorescence microscope. H<sub>2</sub>O<sub>2</sub> (100 μM) was used as positive control.

ROS: reactive oxygen species, DCFH-DA: 2,7-dichlorofluorescein diacetate, SiO<sub>2</sub>: silicon dioxide.

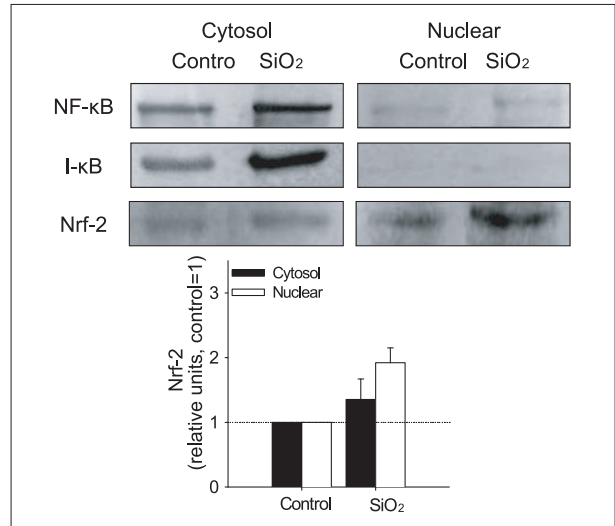


**Figure 5.** Expression of HO-1 and Cu/Zn SOD in Beas-2B cells exposed to 1 mg/L SiO<sub>2</sub> nanoparticles. Densitometric values of expression of HO-1 and Cu/Zn SOD were normalized using that of Actin and were presented as relative units compared to control. The control value was set to 1; data represent the mean ± standard error of the mean; n=3.

SOD: superoxide dismutase, SiO<sub>2</sub>: silicon dioxide, HO-1: heme oxygenase-1. \*p < 0.05 (B).

### III. SiO<sub>2</sub> Nanoparticles Induced Oxidative Stress

The formation of ROS was visualized in Beas-2B cells exposed to SiO<sub>2</sub> nanoparticles by inspection under fluorescent microscope (Figure 4). DCFH-DA staining revealed increased concentration of ROS in SiO<sub>2</sub> nanoparticles treated cells. As the increase in ROS formation was observed in SiO<sub>2</sub> nanoparticle treated cells (Figure 4), a marker for cellular defense mechanism against oxidative stress (i.e. induction of antioxidant enzymes) was investigated in Beas-2B cells exposed to SiO<sub>2</sub> nanoparticles (Figure 5). The expression of SOD protein was not changed, whereas that of HO-1 was increased by SiO<sub>2</sub> nanoparticle exposure.



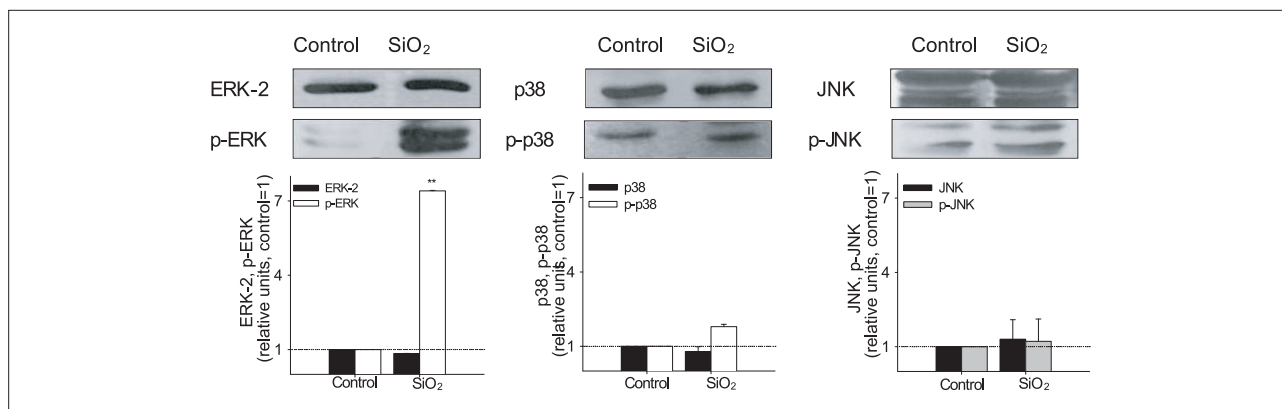
**Figure 6.** Expression of Nrf-2 in cytosolic and nuclear fraction of Beas-2B cells exposed to 1 mg/L SiO<sub>2</sub> nanoparticles. Densitometric values of expression of Nrf-2 were normalized using that of Actin and were presented as relative units compared to control.

Nrf: nuclear factor-E2-related factor-2, SiO<sub>2</sub>: silicon dioxide.

### IV. Oxidative Signaling Pathway

SiO<sub>2</sub> increased formation of ROS and the induction of antioxidant enzyme, HO-1 (Figure 4,5). In order to understand the molecular mechanism of the observed oxidative stress and related physiological alteration by SiO<sub>2</sub> nanoparticles exposure, NF-κB and Nrf-2 were examined in the cytosolic and nuclear fraction of cells treated with SiO<sub>2</sub> nanoparticles. Neither nuclear localization of NF-κB nor the degradation of cytosolic I-κB was observed, whereas nuclear localization of Nrf-2 increased in cells treated with SiO<sub>2</sub> nanoparticles (Figure 6). In our study, SiO<sub>2</sub> nanoparticles induced the translocation of Nrf-2 into the nucleus as evidenced by the results of the western blot analysis (Figure 6).

To further clarify the possible upstream oxidative signaling pathway involved in Nrf-2 activation leading to



**Figure 7.** Expression of intact and phosphorylated ERK, p38 and JNK in Beas-2B cells exposed to 1 mg/L SiO<sub>2</sub> nanoparticles. Densitometric values of expression of ERK, p38 and JNK were normalized using that of Actin and were presented as relative units compared to control. The control value was set to 1; data represent the mean  $\pm$  standard error of the mean; n=3.

ERK: extracellular signal-regulating kinase, JNK: c-Jun N-terminal kinase, SiO<sub>2</sub>: silicon dioxide.

\* $p < 0.05$ , † $p < 0.01$ .

HO-1 induction, we examined the activation of MAP kinases, known as major signaling kinases, involved in cell survival against oxidative stress through the Nrf-2 signaling pathway (Figure 7). The expression of unphosphorylated forms of MAP kinases was constant regardless of exposure of SiO<sub>2</sub> nanoparticles, whereas, the expression of phosphorylated form of ERK was strongly induced by SiO<sub>2</sub> nanoparticles. The phosphorylation of ERK was significant by SiO<sub>2</sub> nanoparticles (about 5- fold compared with that of the control). The expression level of phosphorylated p38 or JNK was not changed by SiO<sub>2</sub> nanoparticles.

## DISCUSSION

There are many studies have reported that nanoparticle toxicity deal with oxidative stress [11-14,23-27]. However, the mechanism by which oxidative stress is involved in nanotoxicity has been poorly addressed. We measured the ROS level (Figure 4), as well as the induction of antioxidant enzyme (Figure 5), provided strong evidence for the involvement of oxidative stress in SiO<sub>2</sub> nanoparticles-induced cytotoxicity. To understand the molecular mechanism of the observed SiO<sub>2</sub> nanoparticle-induced cytotoxicity by oxidative stress, activation of transcription factors (i.e NF- $\kappa$ B, Nrf-2) and signal transduction pathway (i.e. MAP kinase pathway) investigated.

In this study, to provide cellular consequence of the oxidative stress response observed (Figure 4-7), and cytotoxicity test were conducted (Figure 2,3). Exposed to SiO<sub>2</sub> nanoparticles seemed to affect the cell viability and apoptosis by induction of ROS. ROS production was investigated as an initial step of oxidative stress, which revealed an increase in ROS formation in the cells exposed to SiO<sub>2</sub> compared to control.

Neither nuclear localization of NF- $\kappa$ B nor the degradation of cytosolic I- $\kappa$ B was observed, whereas nuclear localization of Nrf-2 increased in cells treated with SiO<sub>2</sub> nanoparticles (Figure 6). In our study, SiO<sub>2</sub> nanoparticles induced the translocation of Nrf-2 into the nucleus as evidenced by the results of the western blot analysis (Figure 6). The translocation of Nrf-2 into the nucleus following nanoparticles treatment was associated with the increase in HO-1 protein, which suggests that nanoparticles activate Nrf-2 in association with the upregulation of HO-1 in Beas-2B cells. However, in this study, SiO<sub>2</sub> nanoparticle-induced NF- $\kappa$ B activation was not observed, which was unexpected, as NF- $\kappa$ B is the major stress response transcription factor that has been reported to respond to a wide variety of environment stressors. Therefore, although SiO<sub>2</sub>- induced NF- $\kappa$ B activation was not observed in this study, the relative importance of NF- $\kappa$ B vs Nrf-2 signaling in terms of contribution to antioxidant response, such as HO-1 upregulation, may merit further investigation with various oxidative stress-inducing nanoparticles. To confirm SiO<sub>2</sub> nanoparticles-induced for activation of transcription factor, more direct evidence, using electrophoretic mobility shift assay (EMSA) may be needed.

The upstream signaling mechanism responsible for regulating oxidative stress is poorly defined. Most studies have focused that oxidative stress may evoke the induction of antioxidant-related transcription factors, such as Nrf-2 or NF- $\kappa$ B via basal signal transduction systems, such as MAP kinase cascade. The MAP kinase cascades are multi-functional signaling pathways that are evolutionally well conserved in all eukaryotic cells. Three MAP kinase cascades that converge on ERKs, JNKs, and p38 MAP kinases have already been characterized [28,29]. Two of those three MAP kinase cascades converge on JNKs and p38 MAP kinases are preferentially activated by cytotoxic

stresses, such as X-ray/UV irradiation, heat/osmotic shock, and oxidative/nitrosative stress [30-33]. To further clarify the possible upstream oxidative signaling pathway involved in Nrf-2 activation leading to HO-1 induction, we examined the activation of MAP kinases, known as major signaling kinases, involved in cell survival against oxidative stress through the Nrf-2 signaling pathway (Figure 7). The expression of unphosphorylated forms of MAP kinases was constant regardless of exposure of SiO<sub>2</sub> nanoparticles, whereas, the expression of phosphorylated form of ERK was strongly induced by SiO<sub>2</sub> nanoparticles.

Activation of ERK MAP kinase pathway by SiO<sub>2</sub> nanoparticles suggests that the induction of HO-1 may be mediated through Nrf-2-ERK MAP kinase signaling pathway. ERK has been known to respond to internal stimuli, such as growth factors. However, our study revealed that SiO<sub>2</sub> nanoparticles strongly induce the phosphorylation of ERK. Increase expression of phosphorylated ERK was also observed in our previous study with CeO<sub>2</sub> nanoparticles exposure [34]. These results suggest that the ERK signaling pathway also responds to environmental stressors. The activation of the ERK signaling pathway by external stimuli has already been reported [30-33].

## CONCLUSIONS

SiO<sub>2</sub> nanoparticles exert their toxicity through oxidative stress as they cause the significant increase in cellular H<sub>2</sub>O<sub>2</sub> concentrations. SiO<sub>2</sub> nanoparticles induce induction of HO-1 via Nrf-2-ERK MAP kinase pathway. Our tested oxidative stress parameters are rather limited in terms of allowing the full understanding of oxidative stress and cellular response by SiO<sub>2</sub> nanoparticle exposure. Further studies on the mechanism by which SiO<sub>2</sub> nanoparticles induce the Nrf-2-ERK MAP kinase pathway are warranted to better understand the nanoparticle-induced cytotoxicity by oxidative stress, as are studies with dose-response and time-course studies.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare on this study.

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