J. Toxicol. Pub. Health
Vol. 14, No. 4, pp. 507-513, 1998

Cytosolic Calcium Alteration and Cell Injury by Silica in Rat Hepatocytes

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(Received June 20, 1998)
(Accepted September 28, 1998)

ABSTRACT *: The purpose of this study was to clarify the effect of silica on cytosolic free calcium mobilization and cell injury in primary cultured rat hepatocytes. Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was measured employing calcium sensitive fluorescent dye, Fura-2/AM, and cell injury was evaluated by determination of cellular ATP contents. Silica increased $[Ca^{2+}]_i$ in a concentration-dependent manner in hepatocytes (10^{-5}~10^{-2} M). Silica caused a biphasic increase in $[Ca^{2+}]_i$ which was composed of an initial rapid rise and following sustained phase. Ca^{2+} removal from the medium resulted in abolishment of initial and sustained phase of silica (10^{-2} M)-induced $[Ca^{2+}]_i$ in hepatocytes. The pretreatment with nifedipine (1 μM) attenuated silica-induced $[Ca^{2+}]_i$ increases. Silica decreased cellular ATP contents in a dose-dependent manner. This silica-induced cell injury was attenuated by the pretreatment with EGTA (100 μM) and nifedipine (1 μM). This study suggests that the elevation of $[Ca^{2+}]_i$ caused by silica may be due mainly to influx through a plasma membrane Ca^{2+} channel and hepatotoxicity by silica relate with alteration of calcium homeostasis.*

Key Words *: Silica, Cytosolic free calcium, Cell injury, Hepatocyte, Cellular ATP content*

I. INTRODUCTION

Silica, an inorganic particle, is typical fibrogenic particle which is very common in many occupations including coal mining, quarrying and sandblasting. The alveolar macrophage is the primary cell responsible for uptake and clearance of inhaled microorganisms and environmental particulates (Brain, 1985). Upon inhalation, silica dust is known to cause persistent inflammation, fibrosis, and granuloma in lung of humans and experimental animals (Davis, 1986; Ziskind *et al.*, 1976). In addition, silica also induces the alteration in other organs e.g., liver or kidney (Wang *et al.*, 1994: Newberne and Wilson, 1970).

Upon injection into circulation, silica particles have been shown to effectively block various functions of the reticuloendothelial system in liver (Levy and Wheelock, 1975).

Silica-induced hepatotoxicity has been suggested in several animal studies. The intravenous injection of saline-suspended silica induced hepatic silicosis in rats (Kanta *et al.*, 1986). In addition, the administration of inflammatory stimuli (various irritants) to laboratory animals impairs the capacity of the liver to metabolize many drugs by a reduction in the hepatic P-450 content (Beck and Whitehouse, 1974). Wang *et al.* reported that silica also signi- ficantly induced lipid peroxidation in liver of mice (Wang *et al.*, 1994).

To our knowledge, there is no report about direct hepatotoxicity of silica using the primary cultured rat hepatocytes. The purpose of this study was to expand the knowledge of silica toxicity in liver. We investigated direct effects of silica on cytosolic free calcium mobilization and cell injury using the

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primary cultured rat hepatocytes.

II. MATERIALS AND METHODS

1. Materials

Silica, nifedipine, trypan blue, Fura-2, Fura-2/AM, ethylene glycol-bis(β-aminoethyl ether) N,N,N,N-tetraacetic acid (EGTA), collagen type III (from rat tail) and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), DMEM, Ham's F-12, penicillin and streptomycin solution, and trypsin solution were purchased from Life Technologies Inc. (Gaithersburg, MD). ATP monitoring reagent was obtained from BioOrbit (Turku Finland). All other chemicals were of the highest grade available.

2. Cell culture

Male Sprague-Dawley rats (180~200 g) for this study were purchased from Charles River. Hepatocytes were isolated by the two-step collagenase perfusion method. Briefly, animals were first anesthetized by intraperitoneal administration of 100 mg/kg phenobarbital. The liver was cannulated through the portal vein, and was washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, pH 7.4, for 15~20 min at a flow rate of 30 ml/min. It was then perfused with the same buffer containing 0.02% collagenase (type IV) and 0.075% $CaCl_2$ for 15 min at a flow rate of 15 ml/min. After the perfusion with enzyme solution, the organ was excised and placed in a petri dish. The Glisson's capsule was disrupted, and the cells were dispersed in DMEM medium containing 0.1% bovine serum albumin. The cell suspension was filtered through the three-layer of gauze, allowed sediment for 20 min, and finally, washed three times in medium by centrifugation at 50×g. After checking the cell viability using the trypan blue exclusion assay, cells were seeded onto the collagen coated 10 cm tissue culture dish (Sumitomo, Japan) at the 10^7 cells/dish. Medium used for the maintenance of the cells was DMEM/Ham's F-12 supplemented with 10% fetal bovine serum, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (10^{-7} M) and hydrocortisone (10^{-7} M). Cells were maintained in an incubator (37°C) containing an atmosphere of air with 5% CO_2 and 100% relative humidity.

3. Measurement of $[Ca^{2+}]_i$

The methods of $[Ca^{2+}]_i$ measurement have been previously described (Wahl *et al.*, 1992). The culture dishes were prepared that cover glass was attached to a 1 cm hole located at the bottom of 35 mm plastic culture dishes. Cells between the 2nd to 4th generation were harvested after trypsinization at 2 or 3 days before experiments, and seeded onto 22×22 mm cover glasses at the concentration of 10^4 cells/hole/100 μl. Cells were placed in incubator for 4 hours to be attatched to cover glass. After 4 hours, 3 ml of medium was added. Cytosolic free calcium concentration was measured at day 2 after seeding. Cells were washed with HEPES-buffered modified Hanks' solution consisting of: 127 mM NaCl; 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 1 mM $MgCl_2$; 10 mM HEPES; 1 mM $CaCl_2$ (pH 7.4) and loaded Fura-2/AM (10 μM) for 30 min at 37°C. Fluorescence-loaded cells were washed three times with the same solution for exclusion of unloaded Fura-2/AM. The fluorescence of cells was measured at room temperature using the InCaTM Imaging System from Intracellular Imaging Inc. (Cincinnati, OH, USA). The concentration of $[Ca^{2+}]_i$ was calculated from standard curve generated in situ (Wahl *et al.*, 1992).

4. Determination of cellular ATP content

Cellular ATP content was measured with micro-chemiluminescence method (Jung and Endou, 1989). After removing supernatant, 1 ml of 10% trichloroacetic acid was treated for extraction of cellular ATP. Ten μl of the extracted samples was transferred to polystyrene cuvettes (Clinicon 2174-08, Sweden) filled with 160 μl of 0.1 M tris(hydroxymethyl)-aminomethane/acetate buffer containing 0.5 mM EDTA (pH 7.75). The cuvettes were set into a luminometer (LKB-Wallac 1250, Finland). After the addition of ATP-monitoring agent (40 μl), the light intensity was measured.

5. Statistical analysis

Statistical significances were analyzed by Student's *t*-test for two-group comparison. P value less than 0.05 was considered as significantly different.

III. RESULTS

1. Silica-induced $[Ca^{2+}]_i$ changes in primary cultured hepatocytes

To know the effect of silica in liver cell, silica-induced cytosolic free calcium mobilization was studied using fluorescent dye, Fura-2. Fig. 1 showed the dose-response relationships of silica inducing the $[Ca^{2+}]_i$ mobilization in primary cultured rat hepatocytes. Exposure of fura-2-loaded cells to silica resulted in a rapid increase in $[Ca^{2+}]_i$ with a peak responding time 5~20 sec and maintained high level of sustained phase above 10 min in hepatocytes. The average resting $[Ca^{2+}]_i$ levels observed in hepatocytes were 94±8 nM. The significant $[Ca^{2+}]_i$ mobilization by silica was observed at the concentration of 10^{-5} M silica. At the concentration of 10^{-2} M, the peak values of silica-induced $[Ca^{2+}]_i$ changes in hepatocytes was 630±38 nM.

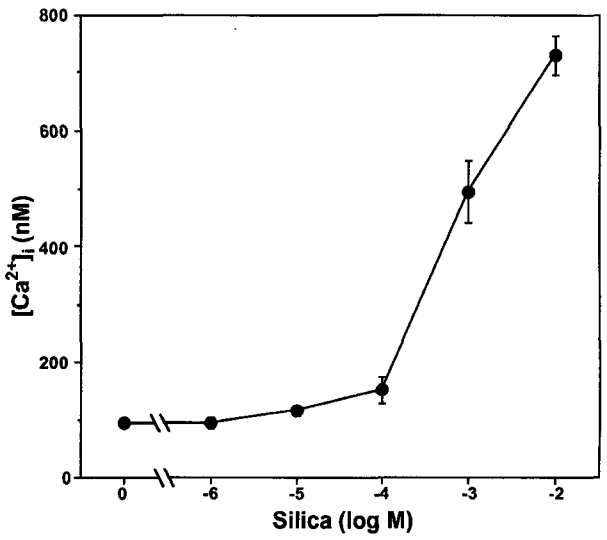

Fig. 1. Dose-response curve of silica on intracellular calcium in primary cultured rat hepatocytes. The results represented mean±SE of the peak calcium response of ten measurements obtained from 6 different cell preparations. Cells were loaded with Fura-2/AM (10 μM) for 30 min at 37°C and then silica at various concentrations in HEPES-buffered modified Hanks' solution containing 1 mM $CaCl_2$.

2. Source of silica-mediated cytosolic free calcium mobilization

To examine whether the source of an increase in $[Ca^{2+}]_i$ was from the extracellular milieu across plasma membranes through the calcium channel or from intracellular stores mediating the PI-phospholipase C (PI-PLC), the effects of extracellular calcium-free condition using calcium chealator (EGTA) and PI-PLC inhibitor (U73122) on silica-evoked $[Ca^{2+}]_i$ were examined. As shown in Fig. 2, when cells were placed in the HEPES-buffered modified Hanks' solution without $CaCl_2$ but with 100 μM EGTA 120 sec before silica addition, silica (10^{-2} M)-mediated $[Ca^{2+}]_i$ transient and sustained phases were completely abolished. The addition of $CaCl_2$ (1 mM) to bathing solution at the end of experiment showed the increment of $[Ca^{2+}]_i$ transiently and maintained sustained phase. EGTA caused no significant altering effect on resting $[Ca^{2+}]_i$ level in heptocytes. In contrast the experiment of Ca^{2+} removal, in order to evaluate a possible involvement of PLC in silica-induced $[Ca^{2+}]_i$ transient, cells were pretreated for more than 30 min with 5 μM U73122. There was no inhibitory effect of U73122 in peak and sustained phase on silica (10^{-2} M)-induced $[Ca^{2+}]_i$ in hepatocytes. In order to study relationship between silica-induced $[Ca^{2+}]_i$ mobilization and voltage-gated calcium channel, nifedipine was employed for calcium channel blocker. Silica-induced $[Ca^{2+}]_i$ transient and sustained phase were attenuated by the pretreatment with 1 μM of nifedipine for 10 min.

3. Effect of silica on cell injury

The measurement of cellular ATP content was often used for assessment of toxic effect of chemicals (Jung *et al.*, 1989). In order to investigate the toxic effect of silica, degree of cell injury was evaluated by determining cellular ATP contents. The effect of silica (10^{-6}~10^{-2} M) on change of cellular ATP contents was observed. As shown in Fig. 3, silica-induced cell injury incubated for 1 hour was increased in a concentration-dependent manner. The significant decrements of cellular ATP content in

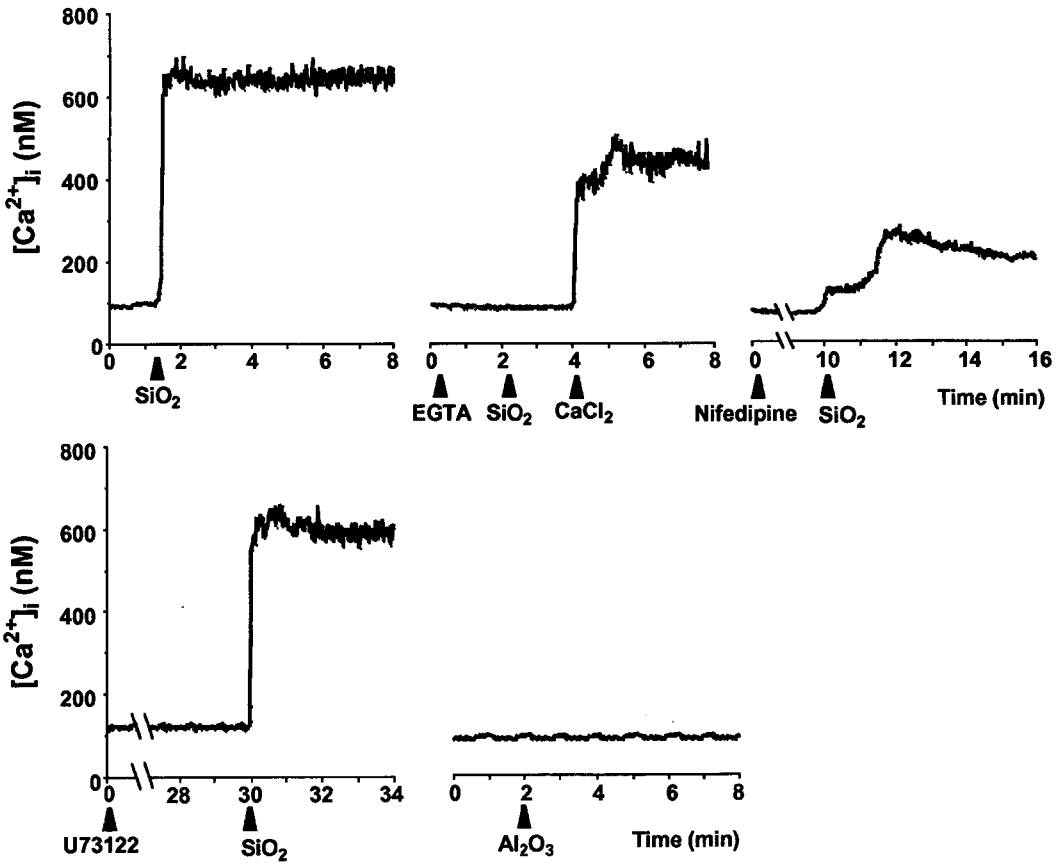

Fig. 2. Effect of extracellular calcium chelation or nifedipine on silica-induced $[Ca^{2+}]_i$ rise in primary cultured rat hepatocytes. EGTA (100 μM) was treated for 2 min in calcium-free bathing solution. Nifedipine (1 μM) was preincubated for 10 min. U73122 (5 μM) was pretreated for 30 min. Al_2O_3 (10^{-2} M) was treated for negative control. Results were representative signals of ten measurement obtained from three different cell preparations.

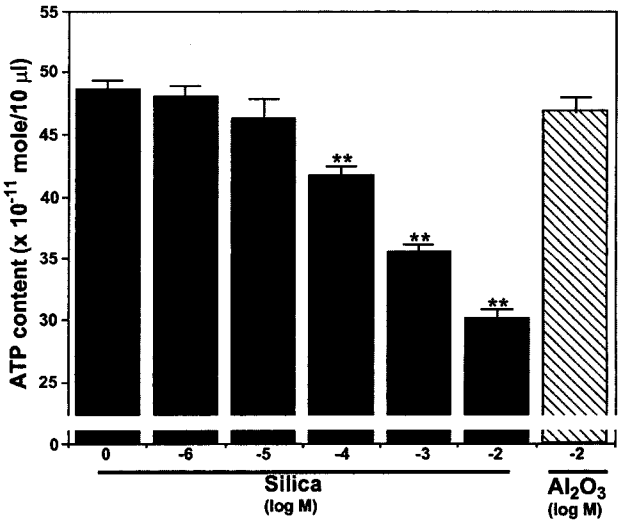

Fig. 3. Dose effect of silica on cell injury in primary cultured rat hepatocytes. Cell injury was evaluated with cellular ATP contents. Cells were incubated with indicated concentration of silica for 1 hour. The results were presented by mean±SE of triplication from four different cell preparation. **: $p<0.01$ vs. control.

hepatocytes were observed at the concentration of 10^{-5} M to 10^{-2} M. At the maximal concentration of silica (10^{-2} M), the cell injuries in hepatocytes were 30.2±0.7 (10^{-11} mole/10 μl extract) vs. control 48.5± 0.8 (10^{-11} mole/10 μl extract). To clarify the particle specificity to hepatocyte, Al_2O_3 was utilized as negative control. No cell injury was observed in high concentration Al_2O_3 (10^{-2} M). To investigate the effect of extracellular Ca^{2+} on silica-induced cell injury, the effect of extracellular calcium removal using calcium chelator (EGTA) was observed. As shown in Fig. 4, silica (10^{-2} M)-induced cell injury (22.9±0.8×10^{-11} mole/10 μl extract) was significantly attenuated by the cotreatment with EGTA (38.0±1.6× 10^{-11} mole/10 μl extract). To examine the relationship between silica-induced cell injury and calcium influx from extracellular through the calcium channel in plasma membrane, effect of calcium channel blocker (nifedipine) was observed. As shown in Fig. 5, the

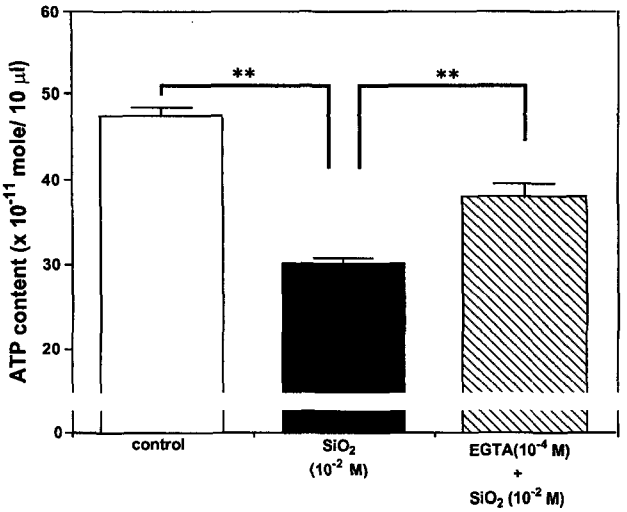

Fig. 4. Effect of extracellular calcium depletion on silica-induced cell injury in primary cultured rat hepatocytes. Cells were incubated in HEPES-buffered modified Hanks' solution without Ca^{2+} (+100 μM EGTA). The results were presented by mean±SE of triplication from four different cell preparation. **: p<0.01 vs. silica treated.

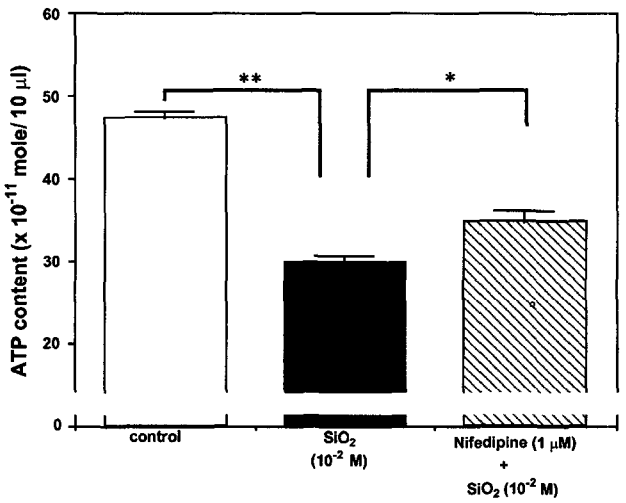

Fig. 5. Effect of nifedipine on silica-induced cell injury in primary cultured rat hepatocytes. Cells were incubated in HEPES-buffered modified Hanks' solution containing 1 mM of Ca^{2+} in the absence or presence of 1 μM nifedipine. The results were presented by mean±SE of triplication from four different cell preparation. **: p<0.01 vs. silica treated.

cotreatment with 1 μM nifedipine (a calcium channel blocker) also attenuated silica-induced cell injury ($34.7\pm1.5\times10^{-11}$ mole/10 μl extract).

IV. DISCUSSION

In alveolar macrophages, studies have indicated that silica can cause a dose-dependent increment of $[Ca^{2+}]_i$ and cell injury (Gleva *et al.*, 1990; Chen *et al.*, 1991) and that depletion of extracellular Ca^{2+} in the bathing medium can prevent silica toxicity (Kane *et al.*, 1980).

It was observed that exposure of primary cultured rat hepatocytes to silica particles revealed a concentration-dependent $[Ca^{2+}]_i$ rise. Silica-induced $[Ca^{2+}]_i$ elevation was biphasic, being transient and sustained phase (Fig. 2). The effect of silica on $[Ca^{2+}]_i$ was found to be almost instantaneous with the responding peak time 5~20 sec depending on the dose. We employed Al_2O_3 as negative control particle to clarify whether the elevation of $[Ca^{2+}]_i$ by silica in hepatocytes is caused by extracellular osmotic change or not. We could not observe any changes in $[Ca^{2+}]_i$ by Al_2O_3. This result showed that silica-induced $[Ca^{2+}]_i$ alteration was not due to extracellular osmotic changes.

Silica caused severe damage to the hepatocytes when used at high dose (10^{-2} M). Depletion of extracellular Ca^{2+} greatly inhibited both cell damage and $[Ca^{2+}]_i$ rise, suggesting the relationship, at least in part, of the two and the significance of the $[Ca^{2+}]_i$ increase as the early response leading to cell damage (Fig. 4). This observation was consistent with previous study which demonstrated that an increase in Ca^{2+} influx caused by Ca^{2+} ionophore promoted cell injury and that chelation of extracellular Ca^{2+} can greatly prevent this ionophore-induced cell injury (Pfeiffer *et al.*, 1978). In the present study, extracellular Ca^{2+} chelation by the addition of EGTA was found to have only a partial protective effect on cell injury when high dose was used, suggesting the involvement of other cytotoxic mechanisms e.g., lipid peroxidation or generation of reactive oxygen and so on.

In addition, the alteration of hepatic enzyme systems is also considerable for the effect of silica (Beck and Whitehouse, 1974). The intraperitoneal injection of silica induces the suppression of cytochrome P-450 2C11 mRNA and protein levels and inducing P-450 4A1, 4A2 and 4A3 mRNA expression. It means that silica may exert their changes in the drug-metabolizing capacity of cytochrome P-450 isozymes (Sewer *et al.*, 1997). Therefore, study of other hepatic enzyme system by silica will need to clarify the mechanism of silica hepatotoxicities.

Silica exerts its damaging effects on membranes by altering calcium homeostasis, membrane potential, and by causing membrane rupture in neutrophil and macrophage (Miles *et al.*, 1981; Vallyathan *et al.*, 1988). Investigations on the effect of extracellular Ca^{2+} removal in this study suggest that the alterations in membrane structures caused by silica can induce the influx of extracellular Ca^{2+}. To further confirm our results, two experiments using U73122 and nifedipine (a PLC inhibitor and calcium channel blocker, respectively) were conducted. The single effect or the effect of U73122 on silica-induced $[Ca^{2+}]_i$ increase were not observed in hepatocytes. This result revealed that silica-induced calcium mobilization did not relate the phospholipase C-mediated phophatidylinositol pathway. In addition, the results of nifedipine treatment shows silica-evoked calcium mobilization is related to voltage gated calcium channel (Fig. 2).

The silica-induced cell injury was significantly attenuated by EGTA or nifedipine (Figs. 4 and 5). These results reveal that silica-induced $[Ca^{2+}]_i$ mobilization and cell injury were related to the influx from extracellular calcium through the plasma membrane.

The maintenance of the $[Ca^{2+}]_i$ at a physiological level depends on energy-dependent transport systems. It has been generally accepted that the maintenance of an about 10,000-fold difference between extracellular calcium concentration and $[Ca^{2+}]_i$ requires energy in the form of ATP. It was suggested that the rise in $[Ca^{2+}]_i$ had related ATP depletion in cultured renal epithelial cells (McCoy *et al.*, 1988), isolated hepatocyte (Albano *et al.*, 1991) or cardiac myocytes (Josepphson *et al.*, 1991). To investigate this possibility, cellular ATP content of hepatocytes following silica treatment was monitored. The result showed that silica decreased cellular ATP content in a concentration-dependent manner (Fig. 3). From this result, we could speculated that silica-induced cell injury was caused by calcium influx from extracellular fluid and subsequent ATP decrement was caused by activation of Ca-H-ATPase in plasma membrane and Ca-ATPase in endoplasmic reticulum for maintaining the cytosolic calcium homeostasis. Because voltage-gated channel was opened by the change of membrane potential.

In summary, the results of this study suggest the alteration of intracellular calcium homeostasis by silica is closely related with cell injury in liver. In addition, monitoring of intracellular calcium concentration change and cellular ATP content would be thought a powerful tools for assessment of hepatotoxicity of drugs or chemicals.

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