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Original Article

In vitro platelet aggregation and oxidative stress caused by amorphous silica nanoparticles

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Abstract: Amorphous silica nanoparticles (SiNP) are being investigated for their potential use in various industrial and medical fields. Therefore, the assessment of their possible pathophysiological effect on circulating cells such as platelets is essential. We recently showed that intraperitoneal administration of SiNP causes proinflammatory and prothrombotic responses *in vivo*. However, little is known about the interaction of amorphous SiNP with platelets *in vitro*. Presently, we investigated the *in vitro* effects of SiNP (1, 5 and 25 μg/ml) on platelet aggregation, oxidative stress and intracellular calcium in mouse platelets. Incubation of platelets with SiNP caused a significant and dose-dependent platelet aggregation. Similarly, the activity of lactate dehydrogenase (as a marker of cell membrane integrity) was significantly increased by SiNP. Total antioxidant activity and lipid platelets vulnerability to *in vitro* peroxidation (measured by malondialdehyde production) were significantly increased after SiNP exposure. Additionally, SiNP exposure significantly increased the cytosolic calcium concentration. In conclusion, our *in vitro* data show that incubation of platelets with SiNP caused platelet aggregation, oxidative stress and increased intracellular calcium. This finding provides evidence on the toxicity of SiNP on platelet physiology.

Keywords: Amorphous silica nanoparticles, platelets, Oxidative stress, mice

Introduction

Amorphous silica nanoparticles (SiNP) are now being used in various fields such as industrial manufacturing, packaging, high-molecule composite materials and ceramics synthesis, drug delivery, cancer therapy and imaging [1].

Human exposure to SiNP may occur during production, storage, transportation, and consumer use [1]. Due to their very small density, SiNP can be readily evaporated into air, and can be inhaled. Following inhalation, nanoparticles have been reported to rapidly cross the alveolar capillary barrier and penetrate into to the systemic circulation and reach various organs [2-5]. Furthermore, with their medical usage, after injection or skin application, nanoparticles can be distributed into the blood and affect the circulatory cells including platelets [1]. Consequently, studying the effects of engineered nanoparticles on platelet physiology is relevant and much needed.

It has been reported that intravenous administration of SiNP induces consumptive coagulopathy in mice [6]. More recently, we have demonstrated that intraperitoneal administration of amorphous SiNP cause prothrombotic events, endothelial dysfunction and systemic inflammation in mice [7]. However, little is known about the direct *in vitro* effect of amorphous SiNP on platelets. Only a single study showed that exposure of washed human platelets *in vitro* to amorphous SiNP induced a low [NO]/[$ONOO^-$] ratio leading to platelet aggregation [8]. We recently found that the *in vitro* incubation of SiNP in whole blood caused platelet aggregation in a dose-dependent fashion [7]. However, the direct influence of SiNP on washed mouse platelets and the mechanisms related to their effects have not, as far as we are aware, been

reported. Consequently, the specific aims of this *in vitro* study were four-fold: (1) to assess the proaggregatory effects of three graded doses of SiNP on mouse platelets; (2) to evaluate the effect of SiNP on lactate dehydrogenase release by platelets; (3) to assess platelet lipid peroxidation and oxidant/antioxidant status following exposure to SiNP, including malondialdehyde (MDA) concentration and total antioxidant activity; and (4) to assess the effects of SiNP on platelet cytosolic calcium concentration.

Materials and methods

Amorphous silica nanoparticles

Amorphous silica nanoparticles (50 nm) were purchased from Polysciences (Warrington, PA, USA). Data regarding their structure, shape, size and charge were recently published [8, 9].

For electron microscopy, droplets (10 µL) of a suspension of 0.1 mg of SiNP in 500 µL were placed on matured formvar/carbon film for 30 seconds. The samples were then drained and inverted onto droplets of ultrapure water for 1 hour before being drained, dried, and examined in a Philips CM10 Transmission Electron Microscope.

Blood collection

This project was reviewed and approved by our Institutional Review Board, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Tuck ordinary mice (30-35 g, HsdOla:TO, Harlan, UK) were housed in light (12-h light:12-h dark cycle) and temperature-controlled (22 ± 1°C) rooms. They had free access to commercial laboratory chow and were provided tap water ad libitum. For blood collection, the animals were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava into syringes containing one-tenth volume Acid Citrate Dextrose (130 mM citric acid, 124 mM sodium citrate, 110 mM dextrose) anticoagulant [10].

Isolation and exposure of platelets and assessment of platelet aggregation

Washed platelets were obtained as described previously [11]. The blood was centrifuged at 200*g* for 20 min at 25°C. The platelet rich plasma was separated, added with Tyrode buffer (137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 5 mM glucose, 0.4 mM Na_2HPO_4, 10 mM HEPES, 0.1% BSA), pH 6.5 in 1:7 volumetric ratio and centrifuged at 900*g* for 10 min. The platelet pellet was resuspended in 1 ml of Tyrode buffer (pH 7.0).

The platelets were exposed in Tyrode buffer (pH 7.4). Each reaction included 1 million of platelets in a total volume of 1 ml. Where indicated, control consisting of normal saline (NaCl 0.9%) containing Tween 80 (0.01%) or SiNP were incubated at 37°C at the indicated concentrations (1, 5, 25 µg/ml) for 3 min, and then stirred for another 3 min. At the end of this period, 25-µl samples were removed and fixed on ice in 225 ml cell Fix (Becton Dickinson, Franklin Lakes, NJ). After fixation, single platelets were counted in a VET ABX Micros with mouse card (ABX, Montpellier, France). The occurrence of of platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by the treatment with SiNP or saline compared with untreated platelets [12, 13]. Therefore, the degree of platelet aggregation following SiNP or saline (control) exposure was expressed as % of that obtained in untreated platelets [12, 13].

LDH leakage

One hundred µl of the platelet suspension (1 x 10^6 cells/ml) was incubated with SiNP at final concentrations of 1, 5 and 25 µg/ml, in a 24-well plate. The platelets treated with normal saline (NaCl 0.9%) containing Tween 80 (0.01%) were taken as control. The plates were incubated for 30 min at 37°C. At the end of the incubation period, the cell suspension was centrifuged, and aliquots of the resulting supernatant were subjected to LDH analysis by UV assay using a commercially available kit produced by Roche (Basel, Switzerland) in blood chemistry analyzer (Roche COBAS, Switzerland).

Measurement of markers of oxidative stress

Measurement of markers of oxidative stress was carried out as described previously with slight modifications [14, 15]. One hundred µl of the platelet suspension (1 x 10^6 cells/ml) was incubated with SiNP at final concentrations of 1, 5 and 25 µg/ml, in a 24-well plate. The platelets treated with normal saline (NaCl 0.9%) con-

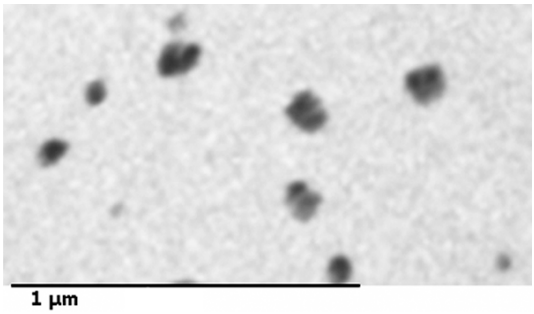

Figure 1. Transmission electron micrographs of the amorphous silica nanoparticle (SiNP) suspension showing the presence of of nanosized particles.

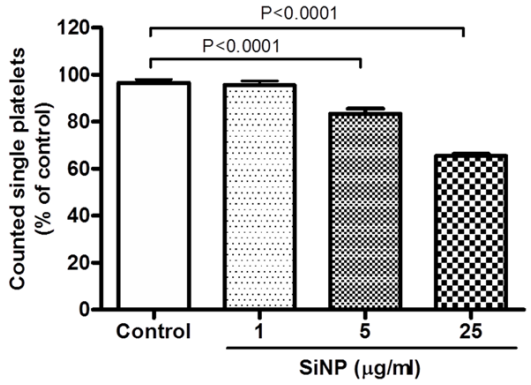

Figure 2. Effect of silica nanoparticle (SiNP) on mouse platelet aggregation *in vitro*. Platelets were incubated at 37 °C with SiNP for 3 min, stirred for another 3 min, and then single platelets were counted. Platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by SiNP or saline. The degree of platelet aggregation following SiNP or saline (control) exposure was expressed as % of that obtained in untreated platelets. Data are mean ± SEM (n = 5). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

taining Tween 80 (0.01%) were taken as control. The plates were incubated for 30 min at 37°C. At the end of the incubation period, cell suspensions were centrifuged for 2 min in an Eppendorf microcentrifuge. Platelet pellets were re-suspended in previous volume of Tyrode's buffer, pH 7.4. After that the platelets were lysed and then centrifuged at 15,000xg for 10 min. The concentrations of MDA and total antioxidant were quantified colorimetrically with commercially available kits (Cayman Chemicals, Ann Arbor, MI, USA) according to methods described by the manufacturer.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured after incubation with either vehicle or various concentrations of SiNP, as described above, according to previously described techniques [11, 14, 16]. Briefly, platelets were washed in Tyrode buffer and then loaded with the fluorescent indicator dye fura 2AM (Calbiochem; La Jolla, CA) in Tyrode buffer containing 2 mM $CaCl_2$ and 2 µM fura 2 [11]. The cells were incubated at 37°C for 10 min and washed once in Tyrode buffer containing 2 mM $CaCl_2$. The fura 2-loaded platelets were resuspended in Tyrode buffer. Then, Ca^{2+}-dependent fluorescence intensity was then monitored with a fluorimeter (model SFM 25, Kontron; Zurich, Switzerland) set at 340-nm excitation and 510-nm emission [14].

Statistics

All statistical analyses were performed with GraphPad Prism Software version 5 (San Diego, CA, USA). Comparisons between groups were performed by one way analysis of variance (ANOVA), followed by Dunnett's multiple range tests. *P*-values < 0.05 were considered as significant. All the data in the figures were reported as mean ± SEM.

Results

Particle characterization

Transmission electron microscopy analysis revealed the presence of nanosized amorphous SiNP (**Figure 1**). In our analysis, we found SiNP of around 50 nm in size. We have also seen small aggregates of probably two SiNPs with an approximate size of 100 nm. The same particles obtained from the same vendor were recently analysed by Corbalan et al. [9]. They reported the presence of significant deviations from nominal specifications which is usual in commercially supplied samples [9], and confirmed a size of around 50 nm [9].

Effect of SiNP on platelet aggregation

Compared with control group, incubation of platelets with SiNP caused significant and dose dependent proaggregatory effects (**Figure 2**). The level of significance was achieved at 5 µg/ml SiNP ($P < 0.0001$) and 25 µg/ml SiNP ($P < 0.0001$).

Effect of SiNP on LDH activity

Figure 3 shows the effect of SiNP on the activity of LDH in mouse platelets. LDH was used as

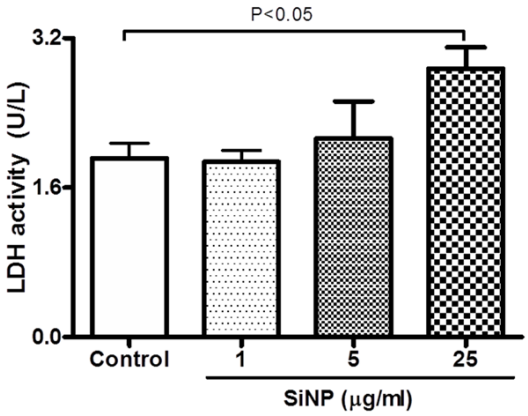

Figure 3. Effect of silica nanoparticles (SiNP) on platelet membrane integrity. Lactate dehydrogenase (LDH) activity was measured in experimental medium after incubation of platelets with various concentrations of SiNP. Data are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

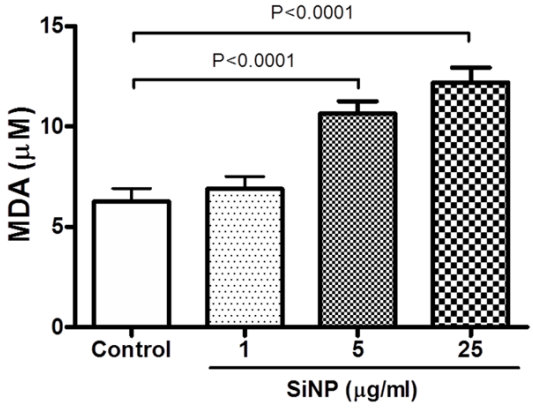

Figure 4. Effect of of silica nanoparticles (SiNP) on the concentrations of malondialdehyde (MDA) in mouse platelets. Data are mean ± SEM (n = 8 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

a marker of cell membrane integrity. Compared with control group, incubation of platelets with 25 μg/ml SiNP caused a significant increase in LDH activity ($P < 0.05$).

Effect of SiNP on concentrations of MDA

Figure 4 illustrates the effect of SiNP on the concentrations of MDA in mouse platelets. MDA was used to assess the susceptibility of platelet lipid to *in vitro* peroxidation. The incubation of platelets with SiNP caused a significant increase in MDA concentrations (**Figure 4**). The effect was statistically significant at 5 μg/ml ($P < 0.0001$) and 25 μg/ml ($P < 0.0001$).

Effect of SiNP on concentrations of total antioxidant activity

Figure 5 shows the effect of SiNP on total antioxidant activity. Compared with the control group, the antioxidant activity in platelets was significantly increased following the incubation of platelets with SiNP 25 μg/ml ($P < 0.0001$).

Effect of SiNP on intracellular calcium

Figure 6 represents the effect of SiNP on platelet cytosolic calcium concentration. Compared with the control group, the incubation of platelets with SiNP induced a significant increase in platelet cytosolic calcium concentration. The level of significance was achieved at 25 μg/ml ($P < 0.05$).

Discussion

In this work, we showed that *in vitro* incubation of platelets with SiNP caused platelet aggregation, oxidative stress and increased intracellular calcium.

The distinctive characteristics of nanoparticles including their very small size, large surface area and cellular penetration capacity are responsible for their specific biological responses that differ from larger particle of similar chemical composition [1, 17]. In this context, nanoparticles have been demonstrated to cause greater biological responses and particle-mediated toxicity than larger particles per given mass [17]. Exposure to silica nanoparticles, both colloidal and crystalline silica, induced more lung inflammation and injury compared with larger particles [1]. Nanoparticles can reach the systemic circulation via various routes including injection, skin penetration, ingestion or inhalation and interact with circulatory cells including platelets [1, 17]. Therefore, studies on the interaction of nanoparticles with platelets are pertinent and can possibly have significant health implica tions.

In the present study, we assessed the effect of three graded concentrations of SiNP on platelets. These concentrations are comparable

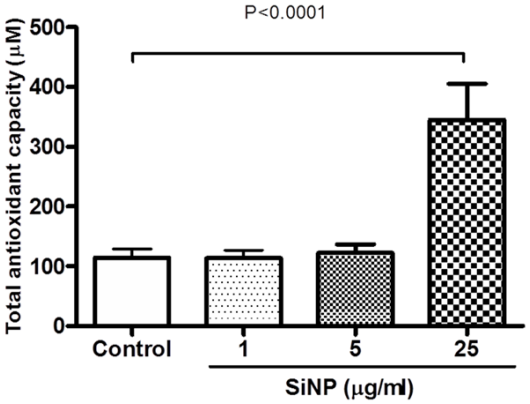

Figure 5. Effect of of silica nanoparticles (SiNP) on the total antioxidant activity in mouse platelets. Data are mean ± SEM (n = 8 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

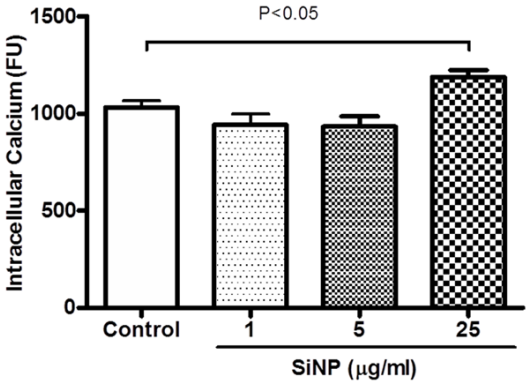

Figure 6. Effect of silica nanoparticles (SiNP) on cytosolic calcium concentration in mouse platelets. Data are mean ± SEM (n = 4-6 in each group). Statistical analysis by one-way ANOVA followed by Dunnett's multiple range tests.

with those reported in recent studies which assessed the *in vitro* effects of SiNP on human platelet aggregation (10-100 μg/ml) and mouse erythrocytes (1-125 μg/ml). Also, they are very small compared with intravenously injected SiNP in mice which consisted of 29.5-177.5 mg/kg [18] and 10-30 mg/kg [19]. Additional studies are required assess whether the concentrations of SiNP used in the current study can be reached in the blood or tissue of humans.

We have recently demonstrated that intraperitoneal administration of amorphous SiNP caused prothrombotic effects in cerebral microvessel of mice, altered endothelial function and induced systemic inflammation [7]. Moreover, we found that the *in vitro* incubation of SiNP in whole blood caused platelet aggregation in a dose-dependent fashion [7]. The last finding has prompted us to conduct the present study to specifically assess the direct effects of SiNP on washed platelets. Our data show that SiNP induced significant and dose-dependent aggregation of mouse platelets. Our results are in agreement with those of Corbalan et al. [8] who showed that exposure of human platelets *in vitro* to amorphous SiNP induced a low [NO]/[$ONOO^-$] ratio leading to platelet aggregation [8]. The last effect was greater when nanoparticle size decreased and particle concentration increased [8].

Our data show that incubation of mouse platelets with SiNP caused a significant increase of LDH activity, suggesting that SiNP can adversely affect platelet membrane integrity and induce cytolysis. Such an effect was not reported in the context of interaction of SiNP with platelets. It has been reported that incubation of endothelial cell line with SiNP cause a cytotoxic damage assessed by LDH release and a decrease in cell survival in a dose-related manner [20].

Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems leading to cell membrane impairment or DNA damage [21]. In view of the observed proaggregatory and cytotoxic effects of SiNP, we wanted to assess the mechanisms underlying these effects by verifying whether, and to what extent, would SiNP affect markers of oxidative stress, comprising MDA and total antioxidants. Our data show that SiNP cause significant and dose-dependent increase of platelet MDA which is a reflection of lipid peroxidation [21]. This finding is in line with the alteration of platelet membrane integrity assessed by LDH increase. Furthermore, we found that SiNP cause a significant increase in total antixidants. It is possible that the increase in oxidative stress induced by SiNP may have been accompanied by increased antioxidant enzymes, indicating that SiNP could trigger adaptive responses that counterbalance the potentially damaging activity of oxygen radicals. An increase of total antioxidants in bronchoalveolar lavage has been observed following the pulmonary exposure to particulate air pollution in

mice [22]. It has been reported that amorphous SiNP penetrate the platelet plasma membrane and stimulate a rapid and prolonged NO release leading to eNOS uncoupling, $ONOO^-$ over- production and eventually, a low $[NO]/[ONOO^-]$ ratio and high nitroxidative/oxidative stress [8].

Ca^{2+} is an essential second messenger in practically all cells, controlling a large range of cellular processes [23]. It is well known that platelets that have not been activated maintain a low cytosolic Ca^{2+} concentration and a steep plasma membrane Ca^{2+} gradient [23]. However, when activated by agonists, a significant increase in cytosolic Ca^{2+} is observed. The elevation in intracellular Ca^{2+} concentration contributes to various steps of cellular activation, such as reorganization of the actin cytoskeleton necessary for shape change, degranulation or inside-out activation of integrin αIIbß3, essential for platelet aggregation [23]. Our data show a significant increase in cytosolic Ca^{2+} after the incubation of platelets with 25 µg/ml SiNP. This finding is in line the proaggregatory effects induced by SiNP. This action is novel and as far as we are aware not previously reported. In human aortic endothelial cells, the possibility that nanoparticles can affect Ca^{2+} channels as well as the endothelial nitric oxide synthase that is associated with caveolae has been raised [24]. It was also speculated that SiNP are likely to stimulate a Ca^{2+} -dependent NO release in human platelets [8].

In conclusion, our data show that incubation of platelets with SiNP caused platelet aggregation, oxidative stress and increased intracellular Ca^{2+} *in vitro*. This finding provides new insights into the toxicity of SiNP on platelet physiology. Further work is warranted to uncover the mechanisms underlying our observed effects and to study the interaction of SiNP with platelet by electron microscopy.

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Disclosure of conflict of interest

No conflicts of interest to disclose.

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