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Blood-brain barrier dysfunction induced by silica NPs *in vitro* and *in vivo*: Involvement of oxidative stress and Rho-kinase/JNK signaling pathways

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ABSTRACT

Silica nanoparticles (SiO_2-NPs) has been extensively exploited in biomedical fields and mostly designed to enter the circulatory system, however, few studies focused on the potential adverse effects of SiO_2-NPs exposure on the blood-brain barrier (BBB) that serves as a critical barrier between the central nervous system (CNS) and the peripheral circulation. This study attempts to provide an understanding of whether and how SiO_2-NPs disrupts the BBB *in vitro* and *in vivo*. Through a human BBB model, we found that SiO_2-NPs could induce tight junction loss and cytoskeleton arrangement, and increase inflammatory response and the release of vascular endothelial growth factor (VEGF) of brain microvessel endothelial cells (BMECs), which further activates astrocytes to amplify the generation of VEGF and increase the aquaporin-4 expression, and thus causing BBB disruption through a complex immunoregulatory loop between BMECs and astrocytes under SiO_2-NPs exposure. Additionally, our data show that inhibition of reactive oxygen species (ROS) and Rho-kinase (ROCK) could effectively protect the SiO_2-NPs-induced BBB dysfunction. *In vivo* studies further confirmed that SiO_2-NPs could cause the BBB paracellular opening, oxidative stress and astrocyte activation in brains of Sprague–Dawley (SD) rats. These findings demonstrate that SiO_2-NPs could disturb BBB structure and function and induce BBB inflammation, and suggest that these effects may occur through ROS and ROCK-mediated pathways, which not only improve neurotoxicity evaluation for SiO_2-NPs but also provide useful information in development of SiO_2-NPs in neuro-therapeutics and nanodiagnostics.

1. Introduction

Silica nanoparticles (SiO_2-NPs), due to its unique characteristics such as large surface area, high structural stability, easy surface functionalization, low cost of production, excellent biocompatibility and protracted circulation properties, has garnered increasing attention in the biomedical field including drug delivery, imaging, cell tracking and photothermal therapy [1]. For example, a cancer-selective SiO_2-NPs was recently approved by the US Food and Drug Administration (FDA) for a first-in-human clinical trial [2]. However, these unique features such as small size and high surface activity enabled NPs to negotiate various biological barriers and have easier access to the organ and tissue in the body [3]. Such barrier penetrability of SiO_2-NPs has been shown to occur across the placental barrier and enter fetal liver and brain [4]. The brain tissue, which has very limited regenerative capacity, is generally protected from exogenous insults by the blood-brain barrier (BBB). Once engineered NPs enter into the circulatory system as drug/gene carrier and disrupt the BBB function, it may unintentionally reach the brain and give rise to neurotoxic effects. Therefore, a fundamental understanding of the biological impact of SiO_2-NPs on BBB is of great importance for nanosafety evaluation.

The BBB not only serves as a natural barrier between the central nervous system (CNS) and the peripheral circulation, but also plays a critical role in maintaining neuronal microenvironment and brain homoeostasis. The protective properties of BBB are mainly conferred by the intricate multi-cellular vascular structure of brain microvessel endothelial cells (BMECs) coupled with astrocytes and pericytes in the brain. BMECs, the core element of the BBB, have continuous intercellular tight junctions, minimal pinocytosis

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activity and multiple specific transport systems, which greatly restrict the transport of cells and molecules in and out of the brain. Many studies have reported that some metallic NPs (*e.g.* titanium dioxide, aluminum oxide, gold and silver NPs) could cause significant pro-inflammatory response in BMECs [5–8] and increase endothelial paracellular permeability by altering endothelial tight junctions [9–11]. Relatively recent data have shown that SiO_2-NPs could pass through the *in vitro* BBB models and the permeability was mainly dependent on particle's size [12–14], and transcellular trafficking was thought to be a mechanism for the SiO_2-NPs crossing of BBB [14]. However, *in vivo* reports on this topic provide considerable controversial results. Several studies demonstrated that SiO_2-NPs administered by cerebral perfusion or dermal administration was able to pass through the BBB and reach the brain tissue [15,16], while in another study, it has been reported that dermal and oral administration of SiO_2-NPs did not enter into rats' brain by compromising the BBB [17]. Moreover, only a few studies focused on the toxicology effects of SiO_2-NPs on BBB and revealed that combination of SiO_2-NPs with several adverse factors (*e.g.*, hypertension with stress, environmental toxicants) could aggravate brain pathology and induce cerebrovascular toxicity by enhancing pro-inflammatory responses and disrupting the BBB integrity [18,19]. As a result, currently significant efforts have been directed towards investigating the penetrated ability of SiO_2-NPs in the BBB for transport of therapeutic or diagnostic agents, few studies focused on the possible adverse effects of SiO_2-NPs on BBB, particularly the detailed mechanisms of how SiO_2-NPs influences BBB has been still unclear.

Oxidative stress has been proven to play a pivotal role in BBB damage after a variety of insults such as hypoxia, drug, trauma or neurodegenerative disorders [20,21]. Likewise, inflammation is closely associated with BBB dysfunction. Several pro-inflammatory mediators (reactive oxygen species (ROS), cell adhesion molecules (CAMs), cytokines, chemokines) were reported to not only regulate the magnitude of leukocyte extravasation into brain parenchyma, but also directly cause tight junctions proteins loosening, edema formation, and leakiness of BBB *in vitro* and *in vivo* [20,22,23]. Moreover, many previous literature including ours suggested that SiO_2-NPs mediated-nanotoxicity including pulmonary toxicity, genotoxicity, cardiovascular toxicity, hepatic and brain injury was related to oxidative stress and pro-inflammatory gene activation [24–28]. To date, although oxidative stress and inflammation have been extensively studied in both BBB damage and nanotoxicity's mechanism, insufficient data is available about their roles in NPs induced increase of BBB permeability.

In our previous work, we have found that SiO_2-NPs could induce pro-inflammatory response and apoptosis of human umbilical vein endothelial cells (HUVECs) through oxidative stress via Mitogen-activated protein kinases (MAPKs) and NF-κB pathways [29,30], and other previous studies have revealed that both RhoA/Rho-kinase (ROCK) and MAPKs pathways were involved in the regulation of BBB integrity [23,31]. Depending on the context, we hypothesized that SiO_2-NPs might induce BBB inflammation and alter the BBB structure and function by oxidative stress, MAPK and ROCK signaling pathways. To test this hypothesis, we firstly developed an *in vitro* BBB model consisting of both primary human BMECs (HBMECs) and astrocytes that closely mimics *in vivo* conditions. Due to the fact that intrinsic characteristics of NPs are related to their toxicity, comparative studies of nanoscale and microscale materials are essential. Thus, utilizing this model, we focused on understanding the BBB permeability, BBB structure and function changes, and evaluation of BBB inflammation after exposure to SiO_2-NPs and silica microparticles (SiO_2-MPs) by a battery of systematic investigations including cell viability, cellular uptake, transendothelial electrical resistance (TEER), cytoskeleton arrangement, tight junctions protein expression, CAMs and aquaporin 4 (AQP4) expression. Cytokines secretion [vascular endothelial growth factor (VEGF), interleukin-6(IL-6), IL-8, IL-1β], ROS generation and Damage-associated molecular patterns (DAMPs) as well as the MAPK and ROCK signaling pathways were also investigated to elucidate the detailed mechanism of the BBB dysfunction regulated by SiO_2-NPs. To confirm the *in vitro* results, the BBB permeability, oxidative stress and astrocyte activation in brain of Sprague–Dawley (SD) rats were further evaluated by Evans blue, inductively Coupled Plasma Techniques (ICP), immunohistochemistry (IHC) and immunofluorescence (IHF) analysis. Additionally, hematological parameters were also monitored to indicate potential system toxicities.

2. Materials and methods

2.1. Preparation and characterization of silica particles

The SiO_2-NPs were synthesized by a sol-gel method according to our previous published procedure [32]. The SiO_2-MPs [Cat. No: S5631] were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The size and shape of these particles were examined under TEM (JEOL-2010, JEOL Ltd., Tokyo, Japan) and SEM (JSM-6700F, JEOL Ltd., Tokyo, Japan). Size distribution and average particle diameters were obtained from TEM images by measurement at least 150 particles using ImageJ (NIH free software). The surface area of these samples was analyzed by the Brunauer–Emmett–Teller (BET) method using a Surface Area Analyzer (ASAP2020, Micromeritics). Hydrodynamic diameter and zeta-potential of these particles in endothelial cell complete medium (ECM) (Sciencell, Carlsbad, CA, USA) were measured with a Malvern Zetasizer Nano ZS and Mastersizer Micro instrument (Malvern Instruments, Worcestershire, UK). Prior to inoculation into the *in vitro* and *in vivo* systems, both silica particles were sterilized by ethylene oxide according to previous published literature [33], and the endotoxin content of the samples was negative at the level of 0.25 EU/mL.

2.2. In vitro studies

2.2.1. Cell culture and in vitro blood-brain-barrier model

Primary HBMECs (Cat. No: cAP-0002) isolated from normal human brain tissue were purchased from Angio-proteomie (Boston, MA, USA) and grown in ECM. Primary human astrocytes (Cat. No: 1800) isolated from human cerebral cortex were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA) and grown in astrocyte complete medium (Sciencell, Carlsbad, CA, USA). The BBB *in vitro* model was constructed according to the modified procedure from previous studies [34]. In brief, HBMEC with a density of 100,000 cells/cm^2 at third passage were cultured on rat tail collagen- (Sigma, St. Louis, MO, USA) coated Transwell inserts (1.0 μm pore size, Millipore, Darmstadt, Germany) in the 12-well culture plates for 2 h, and then the astrocytes were transferred at third passage to the bottom of the 12-well plates at a concentration of 50,000 cells/cm^2, and the microplates were then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 12–14 days to form the *in vitro* model. On day 12–14, the TEER value of the HMBECs was measured, and according to previously published papers [35], the following permeability experiments were performed when TEER values were >200 Ω cm^2.

To examine the effects of ROS inhibition and ROCK/JNK blockade on the BBB model after exposure to SiO_2-NPs, HBMECs in the coculture or monoculture were respectively pretreated with 5 mM N-acetyl cysteine (NAC), or 20 μM SP600125 (JNK inhibitor) or 20 μM Y-27632 (ROCK inhibitor) (Sigma, St. Louis, MO, USA) for 1 h, and then 100 μg/mL SiO_2-NPs was added and co-incubated for 24 h,