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Silicon Dioxide Impedes Antiviral Response and Causes Genotoxic Insult During Calicivirus Replication

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Abstract

Noroviruses (NoV) are the leading cause of nonbacterial gastroenteritis in humans, and replicate extensively in the human gastrointestinal (GI) tract. Silica (also known as silicon dioxide, SiO₂) nanoparticles (NPs) used in processed foods, dairy products, and beverages also accumulate in the GI tract. We investigated the effect of silica NPs on NoV replication and host cell response during virus infection, using murine norovirus (MNV-1) infection of RAW 264.7 murine macrophages. Pretreatment with 10 µg/ml silica significantly reduced the viability of macrophages, but no cumulative effects on viability of macrophages were observed with MNV-1 infection. No difference was observed between exposure to control or silica NPs on either the quantity of viral genome copies or the production of infectious virus in macrophages infected with MNV-1. Silica NPs reduced the ability of macrophages to upregulate genes encoding bone morphogenic proteins (BMPs), chemokine ligands and cytokines for which expression levels were otherwise found to be upregulated in response to MNV-1 infection. Furthermore, silica NPs reduced the levels of proinflammatory cytokines secreted by macrophages in response to MNV infection. Finally, silica NPs with MNV-1 infection produced a genotoxic insult to macrophages. Strikingly, this genotoxic insult was also found to occur as a synergistic effect of silica NPs and feline calicivirus infection in feline kidney epithelial cells. Taken together, our study suggests important safety considerations related to reducing exposure to silica NPs affecting the GI tract in individuals infected with NoVs and possibly other foodborne viruses.

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Author Contributions

Sudhakar S. Agnihothram, Todd A. Townsend, and Marli P. Azevedo designed experiments, performed analysis and wrote the manuscript. Sheryl Anne Vermudez and Lisa Mullis performed the experiments. Mugimane G. Manjanatha previously adapted and optimized the protocol for the comet assay described in this study.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the US FDA.

Keywords

Murine Norovirus; Silica; Proinflammatory Response; Genotoxicity; Feline Calicivirus

1. INTRODUCTION

The use of nanoparticles (NPs) spans various areas, including treatment of infectious diseases, cancer therapeutics, vaccine delivery and the consumer food industry.¹ Specifically, nanotechnology has widespread applications in the food industry, to enhance the texture, taste and nutritional values of food products and prolong shelf life.^{2, 3} The increasing use of NPs in food products has resulted in increased human consumption and thus may pose a hazard to the environment and the human health.⁴ Amorphous silica is currently approved as a food additive (E 551) and is employed for various applications in the food industry.⁵ Silica is used as an anticaking agent to enhance flow properties of powdered food products and as a carrier for fragrances and flavors in food.^{5, 6} In addition, silica is also being used as a coating material in confectionery products, and in food-packaging materials, as a gas barrier for improving shelf life. The size of silica used in the food industry has now been reduced to nanoscale, making silica one of the most commonly used NPs in the food industry.^{3, 7} This extensive use of silica NPs warrants assessment of safety and toxicity potential, to make informed regulatory decisions regarding any potential hazards that may arise due to human consumption of silica NPs.

The toxicity of amorphous silica NPs has been investigated using cell-culture and animal model systems.⁸ Toxicity studies using animal models have demonstrated the capability of silica NPs to penetrate organ systems via different exposure pathways and exert adverse effects.⁹ In addition, studies using *in vitro* systems suggest that silica NPs exert cytotoxicity on various cell lines, in which the toxicities vary depending upon the route and duration of exposure, in addition to the size and surface charge.¹⁰⁻¹³ The majority of these studies have investigated silica toxicity from the perspective of occupational exposure, with the pulmonary system as the major target of toxicity.^{11, 14} Few studies have investigated the toxicity of silica NPs to cell lines of GI tract origin,^{6, 12, 13, 15} such as the gastric epithelial (GES-1) cell line and the human colorectal carcinoma (Caco-2) cell line.⁵

The accumulation of silica NPs in the GI tract may also result in interaction with foodborne viruses.¹⁶ These interactions may not only impact the life cycle of viruses, but also exacerbate host response and pathogenesis. Human Noroviruses (NoVs) are among the most pathogenic enteric viruses and are the leading cause of non-bacterial gastroenteritis infections throughout the world.¹⁷ They belong to the family *Caliciviridae*, and their highly infectious nature stems from their requirement for low infectious doses (as few as 10 viral particles can infect a host), their resistance to environmental stressors, and the absence of mechanisms in humans for long-term immunity to NoVs.¹⁸ NoV infection in the US occurs in approximately 19–21 million unique episodes per year, leading to 71,000 hospitalizations and causing 800 deaths, thereby imposing a serious burden on public health infrastructures and overall human health.^{19, 20} In healthy individuals, NoV infection usually manifests as a mild disease that resolves quickly, whereas many immunocompromised individuals succumb

to NoV infection.²⁰ Recent reports from the World Health Organization (WHO) have estimated that NoV infections in developing countries may result in 1.1 million hospitalizations and 218,000 deaths for immunocompromised children less than 5 years of age, with an attack rate of 56%.^{20, 21} In addition, increased risks of NoV infection have arisen via increased on cruise ships and contamination of seafood.²⁰ Together, these effects pose a significant threat to human health and the food industry.

Due to the fecal-oral transmission of NoVs and their localization to the GI tract, there is an increased probability of interactions of silica NPs with NoV. The effect of NoV-silica NP interactions on the virus cycle and host cell biology has not been investigated before. Due to the lack of a robust *in vitro* system for Human NoV infection, we utilized murine NoV (MNV-1) infection of RAW 264.7 macrophages as the model system for these studies. We tested the effect of silica NPs on cell viability, virus replication, host cell response and genotoxicity to the host. We present evidence that presence of silica NPs impaired the host response to virus infection, in addition to imparting genotoxic effects during MNV-1 replication. Furthermore, we show that the genotoxic effects of silica NP exposure also occurred during replication of a feline virus belonging to the *Caliciviridae* family.

2. MATERIALS AND METHODS

2.1. Characterization of Silica Nanoparticles

The nanoparticles were kindly provided by Dr. Huizhong Chen, Division of Microbiology, NCTR, and the size, distribution, surface charge, stability and purity of silicon dioxide particles used in this study were characterized previously using methods described by Pan et al.²²

2.2. Cell Lines, Silica NP Incubation, MNV-1 and FCV Infection

RAW 264.7 murine macrophages, and CRFK feline kidney cells were obtained from the American Type Culture Collection (ATCC), and grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO). Murine Norovirus genotype 1 (MNV-1, ATCC PTA-5935) was propagated and tittered in RAW 264.7 cells. Feline Calicivirus (FCV, ATCC VR-782) was propagated and tittered on CRFK cells.²³ RAW 264.7 macrophages were treated with silica NPs at concentrations of 10 $\mu\text{g}/\text{ml}$ for 3 h, where indicated, and then infected with MNV-1 at a multiplicity of infection (MOI) of 5 and 0.5 particles per cell as indicated. The two different MOI (5 and 0.5) were chosen for the initial viral infections to represent virus exposure in the human gut.

2.2.1. Viral RNA Extraction and Analysis of Genome Copy Number—Cell culture supernatants described were harvested at 16, 24 and 48 h post-infection to analyze the genome copy number of MNV-1. Viral RNA was extracted from 115 μl of cell culture supernatant using a MagMAXTM viral RNA isolation kit and the MagMAXTM Express Magnetic Particle Processor (Applied Biosystems/Ambion, Austin, TX). Extracted RNA samples were either analyzed immediately or stored at -80°C until use. RNA samples were analyzed for norovirus by a real-time, one-step RT-PCR, using the Rotor-Gene Multiplex

RT-PCR kit (Qiagen, Valencia, CA). The reactions were carried out in duplicate, using primers and a Cy5-labeled fluorescent probe (Integrated DNA Technology, Coralville, IA) targeting the ORF2 region (positions 6520 to 6645). MNV-1 RNA was used to generate a standard curve and 18S RNA (Applied Biosystems, Foster City, CA) was used as an internal control. RNA from mock-infected (no virus infection) RAW 264.7 cell supernatant was used as a negative control and RNase-free water was used as a non-template control. Data were transformed in gene copy number, using a standard curve generated from serial dilutions of virus stock preparations previously titrated by plaque assay. The detection limit of the assay was 2 RNA copies. Amplification was detected using a Rotor Gene Q6-plex machine from Qiagen. Raw data were analyzed using Rotor-Gene™ software. Briefly, 5 µl of RNA was transferred to a Qiagen Rotor-Gene strip tube containing 10 µl of 2× Rotor-Gene Multiplex RT-PCR master mix, 800 nM of each norovirus primer²⁴ MNVF (5'-TGCAAGCTCTACAACGAAGG-3'), MNVR (5'-CACAGAGGCCAATTGGTAAA-3'), 200 nM of probe MNVP (5'-Cy5-CCT TCC GCA CCG ATG GCA TG-IBRQ-3'), and 0.2 µl of Rotor-Gene RT mix. The thermal cycling conditions consisted of reverse transcription at 50 °C for 30 min, 95 °C for 10 min, and then 50 cycles of 94 °C for 30 sec and 48 °C for 60 sec.

2.2.2. Plaque Assay for MNV-1 and FCV—Viral titers were determined by a plaque assay using RAW 264.7 macrophage cells.²⁵ Briefly, virus supernatants were serially diluted in phosphate buffered saline, and the dilutions were inoculated on a confluent monolayer of RAW 264.7 macrophages and incubated at room temperature for 1 h. Viral inocula were removed and cells were washed twice with PBS. A 1.5% agarose overlay in DMEM was added to the cells and incubated at 37 °C. Plaques were counted at 48–72 h post incubation. Virus titers were estimated by multiplying the mean of plaque numbers in at least two wells times the reciprocal of the lowest virus dilution with visible plaques. For FCV, virus titers were determined as described previously.²³

2.3. Analysis of Gene Expression Using RT2 Profiler Array

Host gene expression was analyzed using the RT2 profiler array (Qiagen, Gaithersburg, MD). Briefly, cell pellets from various experimental conditions were collected at 16 h post infections in RNA protect (Qiagen), to preserve intact RNA. RNA extraction was performed using a miRNAEasy Kit (Qiagen), and equal amounts of RNA was used for cDNA synthesis, amplification, and gene expression analysis according to the manufacturer's protocol. Ct values for the gene list from were analyzed in duplicate, using the data analysis software provided on the Qiagen website. Gene expression values were normalized to the housekeeping genes and the expression levels were calculated as fold change compared to the mock-infected controls. The gene expression values that passed the Quality Control (QC) according to the Qiagen instructions were taken into account for analysis. The results were grouped according to functionality as directed by the manufacturer's array gene list.

2.3.1. Enzyme Linked Immunosorbent Assay (ELISA)—Cell culture supernatants collected at 24 h post infection were dispensed into 200 µl volumes and frozen at –80 °C. An ELISA test was conducted for IL-6, TNF-α and TGF-β1 according to the manufacturer's instructions (R&D Biosystems, Minneapolis, MN). Briefly, Nunc Maxisorp 96-well plates

were coated with anti-mouse IL-6 (2 $\mu\text{g/ml}$), anti-mouse TNF α (0.8 $\mu\text{g/ml}$), or TGF β 1 (4 $\mu\text{g/ml}$) (R&D BioSystems) overnight at room temperature. Before use, the plates were blocked with PBS containing 1% bovine serum albumin fraction V (BSA, Sigma) for 2 h at room temperature. Cell culture supernatant samples (100 μl) were added to the wells and the control samples were diluted in PBS/1% BSA. After 2 h incubation at RT, wells were washed 4 times with PBS containing 0.05% Tween 20. The addition of biotinylated monoclonal antibodies for each of the cytokines (IL-6, 150 ng/ml; TNF α , 50 ng/ml; TGF β 1, 75 ng/ml) were added and incubated for 2 h at room temperature. Horseradish peroxidase-conjugated streptavidin (R&D Biosystems) was added according to the recommended dilution and incubated for 30 min at room temperature. Standard curves were generated using purified recombinant IL-6, TNF α , and TGF β 1 according to the manufacturer's recommendations (R&D BioSystems) using a MaxPro-generated four-parameter curve-fit for each cytokine.

2.3.2. Trypan Blue Exclusion Assay—Macrophages treated under indicated experimental conditions were harvested by scraping, and single suspensions were prepared in cold PBS (1 $\times$, pH 7.4, GIBCO, Foster City, CA, USA). Cells were diluted in 0.4% Trypan Blue (Ambion, Foster City, CA,). Viability was measured using Cellometer Auto T4 Manual (EMD Millipore, Darmstadt, Germany).

2.3.3. Alkaline Single-Cell Gel Electrophoresis (Comet) Assay—We analyzed DNA damage and cell viability in RAW 264.7 cells following MNV-1 or FCV infection (at 5 MOI) alone, or in combination with exposure to 10 $\mu\text{g/ml}$ of silica NPs. Briefly, mock infected RAW 264.7 macrophages or CRFK cells were treated with a single 3 h exposure to a dose of 20 $\mu\text{g/ml}$ methyl methane sulfonate (MMS), used as a positive control for the comet assay. To determine if MNV-1 or FCV infection alone, and/or silica NP exposure alone, or in combination with viral infection, caused DNA damage consistent with genotoxicity in single cells, we exposed all combinations of cells to either high dosing of silica NP exposure or control treatment, infected or mock-treated RAW cells with high MOI viral infection, and subsequently performed alkaline comet assays and analysis. After dissociating RAW 264.7 cells into single cell suspensions, global cell viabilities were determined using Trypan Blue exclusion tests. For the alkaline comet assay,²⁶ 50 μl of single-cell suspensions in PBS, derived from each treatment condition were mixed with 450 μl 0.8% (diluted in PBS) low melting-point (LMP) agarose at 37 $^{\circ}\text{C}$, and 100 μl of this suspension was applied to microscope slides (Fisher Scientific, St. Louis, MO,) previously thin-coated with 1% agarose. After solidification of the LMP agarose/embedded cell mixture at 4 $^{\circ}\text{C}$, the slides were placed in freshly prepared lysis buffer (2.5 M NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100, pH 10.0) at 4 $^{\circ}\text{C}$ in the dark for 3 h. The slides then were washed once in neutralization buffer (0.4 M Tris, pH 7.5) in the dark for 5 min at 4 $^{\circ}\text{C}$ and then transferred into chilled alkaline unwinding/electrophoresis solution ($\sim$ 300 mM NaOH, 1 mM EDTA, pH $>$ 13) in the dark for 30 min to unwind DNA. Immediately following unwinding, electrophoresis was performed in the same solution at 4 $^{\circ}\text{C}$ in the dark for 30 min at 0.8 V/cm and $\sim$ 300 mA. After the slides were removed from the electrophoresis chamber, they were washed three times (5 min each) with neutralization buffer, fixed with ice-cold ethanol

(100%) and dried for 30 min. To visualize nucleic acids and facilitate scoring, the slides were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) (1:10,000 dilutions in Tris-Buffered EDTA buffer). Three slides were scored from each treatment; at least 100 representative cells were selected randomly from each slide and scored with a Nikon 501 fluorescence microscope and Comet IV digital imaging software (Perceptive Instruments, Wiltshire, UK). Percent (%) DNA in the tail, defined as the fraction of DNA in the tail divided by the total amount of DNA associated with a cell multiplied by 100, was used as the parameter for DNA damage analysis.

2.3.4. Statistical Analyses—All data were plotted and analyzed; statistical analysis was performed using Graph Pad Prism software, Microsoft Excel (Microsoft Corporation, Redmond, WA) and/or Sigma Plot (Systat Software, Inc., San Jose, CA). Two tailed *T*-tests were used to analyze statistical significance between the experimental groups.

3. RESULTS

3.1. Silica NPs are Nontoxic to Murine Macrophages

Prior to analyzing the effects of silica NPs in murine macrophages in the context of MNV-1 infection, we first examined whether the silica NPs alone exerted any effects on cell morphology and cell viability. We dosed the macrophages at two concentrations, 10 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ of silica NPs and then analyzed viability and morphology of RAW 264.7 cells. This dose range was based on our extrapolation of data indicative of the amount of daily human NP consumption.²⁷ When RAW cells were incubated with 2 or 10 $\mu\text{g/ml}$ of silica NPs and monitored for 48 h, no cytopathic effects (cell rounding, detachment and floating) was evident, as compared to the mock-infected controls (Fig. 1(A), 10 $\times$ and 20 $\times$ images). Although there were no visibly discernible cytopathic effects, preincubation with 10 $\mu\text{g/ml}$ silica NPs resulted in a ~10% reduction in cell viability ($P < 0.05$, Fig. 1(B)), as measured by a Trypan Blue exclusion assay. Preincubation with 2 $\mu\text{g/ml}$ silica NPs did not affect viability of RAW macrophages as compared to mock controls (data not shown).

3.2. MNV-1 Replication Remains Unaltered in Cells Exposed to Silica NPs

We next analyzed whether pre-incubation of RAW 264.7 cells with high (10 $\mu\text{g/ml}$) or low dose (2 $\mu\text{g/ml}$) silica NPs altered MNV replication and/or the production of infectious virus particles. The MNV-induced cytopathic effect observed at 48 h post infection was used as a surrogate marker for viral replication, as MNV has been shown to cause cell rounding, shrinking and cell death following virus replication.²⁸ At 24 h post infection, MNV-1 replicated to reach peak viral titers of 8×10^6 pfu/ml when infected with 5 MOI, and $\sim 7 \times 10^6$ pfu/ml when infected with 0.5 MOI (Fig. 2(A), MNV-1 control). Replication of MNV-1 at 5 or 0.5 MOI was accompanied by rounding, shrinking and floating of cells in the monolayer, demonstrating the cytopathic effect due to MNV infection, irrespective of the MOI used (Fig. 3(A)). These cytopathic effects resulted in a significant reduction in cell viability (when compared to mock-infected) by 50% at 24 h post-infection for 5 MOI infection, as measured by a Trypan Blue exclusion assay (Fig. 3(B)). Infection with 0.5 MOI of MNV-1 also had a significant reduction in cell viability (data not shown).

Preincubation with 2 or 10 $\mu\text{g/ml}$ of silica NPs had no significant effect on the production of infectious virus particles at 24 h or 48 h post infection, when cells were infected with 5 or 0.5 MOI of MNV-1 (Fig. 2(A)). Consistent with this observation, cytopathic effects including cell rounding, shrinking and floating in the monolayer were clearly evident in cells incubated with both 10 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ of silica NPs under conditions of either 5 or 0.5 MOI infection (Fig. 3(A)).

3.3. Viral Copy Numbers in the Supernatant are Not Altered Upon Exposure to Different Doses of NPs

We next analyzed whether MNV-1 genome copy numbers in the cell culture supernatant were altered due to exposure of the RAW 264.7 cells to silica NPs followed by MNV-1 infection. Preincubation of cells with 10 -or 2- $\mu\text{g/ml}$ SiO_2 NPs, followed by 5 or 0.5 MOI MNV-1 infection, did not alter viral genome copy numbers as compared to infected controls (Fig. 2(B)).

3.4. Upregulation of Genes Encoding Cytokines, Chemokines and Growth Factors in Response to MNV-1 Infection is Impaired by the Presence of Silica NPs

The normal host response to virus infection is characterized by a change in expression of genes encoding cytokines and chemokines, which comprise the major mediators of the innate immune response to virus infection. Since silica NPs also possess immune-stimulation properties due to their foreign nature,²⁹ we hypothesized that presence of silica NPs during MNV-1 expression might alter host gene expression. Using the RT2 Profiler Array, we analyzed the expression of genes encoding various cytokines, chemokines and growth factors. Results from gene expression analysis, represented as fold change compared to mock-infected and infected controls (Fig. 4), are sorted according to the functional grouping of the genes. Whereas genes encoding the bone morphogenic proteins (BMP) were upregulated by MNV-1 infection, silica NPs caused a significant reduction in this effect (ranging from 20 to 40 fold reduction, Fig. 4(A)). Silica NPs alone caused a downregulation of the BMP-2 gene, whereas no significant effects were found for other BMP genes. Analysis of gene expression from the chemokine family ligands showed a similar trend to that observed with the BMP family. MNV-1 significantly upregulated the expression of CCL17, CXCL1 and CXCL-11 (Fig. 4(B)), which was clearly reduced by silica NPs during MNV-1 infection. The most pronounced effect was seen with CXCL1 and CXCL11, where the reduction due to silica NPs ranged from 30 to 300 fold (Fig. 4(B)). Among the genes encoding cytokines, MNV-1 caused a drastic increase (>300 fold) in IL-2 and IL-6, which again was significantly reduced by silica NPs. Finally, a similar trend was observed with the family of genes encoding growth factors, in which MNV-1 caused significant increases in *Csf2* and *Csf3*, but was reduced specifically by the presence of silica NPs (Fig. 4(D)).

3.5. Secretion of Cytokines by RAW 264.7 Macrophages is Altered by Presence of Silica NPs

Macrophages secrete several cytokines including Inter-leukin 6 (IL-6), tumor necrosis factor alpha ($\text{TNF}\alpha$), and transforming growth factor beta 1 ($\text{TGF}\beta 1$) in response to infection by microorganisms or antigenic stimulation. We hypothesized that silica NPs present during MNV-1 infection would result in alteration of cytokine secretion due to differential

modulation of the host cell immune response. Since there was no difference in the production of infectious virus titers or amount of viral genome copies in the supernatant between conditions of 5 or 0.5 MOI MNV-1 infection, we opted to analyze cytokine secretion with 10 $\mu\text{g}/\text{ml}$ silica NPs at 5 MOI infection. We reasoned that a 5 MOI infection would exert optimal insult to host cells with a high amount of silica NPs, which might result in significant host response. While MNV-1 infection resulted in a significant increase ($P < 0.05$) of TNF- α secretion compared to mock infection, presence of silica NPs during MNV-1 infection caused a significant (~ 1.5 fold, $P < 0.05$) reduction in the production of IL-6. This reduced amount of TNF- α was significantly higher when compared to the cells incubated with silica NPs alone, indicating the synergistic effect of MNV-1 in cells incubated with silica NPs (Fig. 5(C)). Secretion of IL-6 followed a pattern similar to TNF- α , as shown in Figure 5(A). In contrast, RAW 264.7 macrophages were found to secrete high levels of TGF- $\beta 1$ ($P < 0.05$), which was significantly reduced due to MNV-1 infection or preincubation with silica NPs (Fig. 5(B)). The synergistic effect of MNV-1 replication and silica NPs restored high levels of TGF- $\beta 1$ levels (Fig. 5(B)). Collectively, these data suggested that presence of silica during MNV-1 infection had an opposite effect on the secretion of cytokines elicited due to MNV-1 infection.

3.6. Assessment of Genotoxic Hazard Potential

We next analyzed whether silica NPs during MNV-1 infection could result in DNA damage consistent with genotoxicity, which specifically can be investigated via the alkaline comet assay (Ref. [30] and data not published). While initial viabilities varied among cell sample preparations, there was a general reduction in overall viability as a result of either MNV-1 infection or silica NPs exposure, consistent with known cytotoxic effects of these treatments.^{14, 28} Cells exposed to MMS (positive control) showed increased DNA damage, reinforcing the sensitivity and specificity of our comet assay procedure, and the susceptibility of RAW 264.7 macrophages to single-cell DNA damage. Interestingly, the presence of silica NPs caused significant DNA damage ($P < 0.05$), only in cells co-infected with virus (Fig. 6(A)).

We next analyzed whether the DNA damage caused by silica NPs during MNV-1 replication was dependent on the specific cell type (i.e., macrophage) or virus (MNV-1) used. To investigate this, we performed the alkaline comet assay on a different cell type, CRFK (feline kidney epithelial cell line), and another known surrogate for studying human NoV replication and pathogenesis (feline calicivirus).³¹ Our results corroborate that presence of silica NP induced a significant increase in DNA damage in CRFK cells in the presence of FCV, when compared to DNA damage in cells treated with silica NPs, or cells infected with FCV alone (Fig. 6(B)). These results strongly suggest that silica NPs-induced genotoxicity during virus replication occurs in other viruses of the *caliciviridae* family, irrespective of the cell type used.

4. DISCUSSION

The use of silica NPs in processed foods, anticaking agents, coating in confectionery products etc., leads to silica accumulation in the intestine.⁶ While several studies have

investigated safety of silica NPs on the intestinal cell lines, including Caco-2 and gastric epithelial fibroblasts,^{5, 12, 15} few reports have investigated the cumulative effect of silica NPs in the context of microbial infection.³² Interaction of microbial pathogens with silica NPs might exacerbate disease pathogenesis or modulate host immune response, which might culminate in immune or genotoxic insult to the cells or interfere with immune response to live vaccines. Acute human exposure to silica nanoparticles increases mortality by enhancing the permeability of silica NPs into the lungs.³² To our knowledge, this is the first report investigating the effect of silica pre-treatment of cells on MNV-1 replication, host gene expression, cytokine secretion and genotoxicity.

Prior to virus infection, we investigated the effect of preincubation of silica NPs on morphology and viability of RAW 264.7 macrophages. We observed a ~10% reduction in cell viability with the silica macrophages. Although no genotoxic insult was observed due to the silica NPs, silica did reduce the secretion of TGF- β 1. TGF- β 1 has known roles in enhancing cell survival and differentiation of immune cells,³³ while there are contrasting reports indicating the role of TGF- β 1 in apoptosis induction.³⁴ Reduction in cell viability could in part occur due to the modulation of this important growth factor. Silica did not have any cumulative effect on cell viability in the presence of MNV-1 infection. This could be because a 5 MOI of norovirus resulted in infection of most of the cells in the monolayer. The highly cytopathic nature of MNV-1 kills 50% of infected cells by 24 h, where cells susceptible to silica-induced mortality might undergo MNV-induced cell death, making the smaller effect undetectable under these conditions.

Preincubation of RAW 264.7 cells with 10 μ g/ml or 2 μ g/ml silica NPs did not result in significant alteration in the formation of infectious virus particles titers at 24 and 48 h post infection, under conditions of 5 or 0.5 MOI infection. A 5 MOI of virus inoculum would mimic infection with a high virus load, with a high amount of virus particles entering the cell causing rapid progression of infection, where as a 0.5 MOI of infection would mimic infection with a low virus load, indicating lesser number of virus particles entering the cell, resulting in slow progression of virus infection. This difference was clearly depicted by the slow clearance of a monolayer with 0.5 MOI of virus. Oxidation of norovirus capsid proteins has been previously demonstrated as the underlying mechanism for reducing the effect of norovirus infectivity by titanium nanoparticles and other chemicals, including chlorine. Our results, demonstrating the inert effects of silica particles on MNV replication, indicate that silica NPs may not cause biochemical insults to structural or non-structural proteins of MNV-1. We observed no effects of silica nanoparticles on the amount of genome copies in the supernatant, which further supported our hypothesis that prior presence of silica NPs does not interfere with the biological life cycle of norovirus in macrophage populations.

Cytokines and chemokines play an integral role in establishing a proinflammatory environment during microbial infection which could lead to virus clearance and/or culminate in inflammatory damage to the host.³⁵ Genes encoding the cytokines and chemokines are differentially regulated early during virus infection, as these are the key mediators of innate immune response to microbial infection.³⁶ Our results indicated that the silica NPs reduced the ability of the host to upregulate genes, which were otherwise highly expressed during norovirus infection. In particular, silica NPs alone caused a down regulation of genes

encoding bone morphogenic proteins (BMPs). BMPs play a pivotal role in tissue remodeling and cartilage formation.³⁷ Downregulation of BMPs proteins have been implicated in progression of colon cancer,³⁸ whereas over activation of BMPs leads to inflammation of proximal portions of the intestine.³⁹ While MNV-1 infection showed up regulation of BMP genes, silica NPs during MNV-1 infection inhibited this upregulation, indicating a differential modulation of host response. These changes might exert malignant effects on host physiology and behavior, and *in vivo* studies are underway to analyze the possibility of tissue related damage.

Our results indicated that genes encoding chemokine ligands and cytokines were also upregulated during MNV-1 infection. In most RNA viruses, genomic RNA is known to be an active initiator of innate immune response, including interferon and interleukin signaling, which further results in secretion of chemokines that attract the immune cells, including neutrophils and macrophages to respond as a first line of antiviral defense.³⁵ Our observations are consistent with previous reports demonstrating induction of proinflammatory environment in macrophage cell lines, and mouse models of MNV-1 infection.⁴⁰ We observed that silica NPs reduced or inhibited the expression of genes belonging to this family of proinflammatory cytokines. Silica NPs have a size-dependent effect on immune response initiation in macrophages *in vivo*.⁴¹ The silica NPs used in this study were about 30 nm, which is the size of a MNV-1 virion.⁴² Preincubation of macrophages with silica NPs could result in partial engagement of innate immune sensors, due to a stoichiometric effect dependent on size similarity of MNV-1 and silica NPs. The surface charge of amorphous silica particles has been shown to vary depending on the pH of the environment,¹⁴ and since endosome (with acidic pH) is a hot spot for innate immune signaling,⁴³ there could be charge-dependent interaction between silica NPs and MNV-1 RNA, culminating in diminished modulation of virus-induced immune by silica NPs.

The reduction in immune activation by silica NPs during virus infection was also observed with the cytokine secretion that we tested. MNV-1 infection caused a significant increase in secretion of TNF- α and IL-6, but the NPs reduced the secretion of these cytokines. This effect could occur due to the stoichiometric competition between MNV-1 and silica in engaging the innate immune sensors. Alternatively, interaction or binding of MNV-1 RNA to silica NPs could render the virus attenuated from an immune signaling perspective, while MNV-1 still remained replication competent. Differential induction of cytokine secretion by attenuated and virulent viruses has been previously demonstrated in human rotavirus infection of gnotobiotic pigs,⁴⁴ where a virulent virus causes a peak in levels of TNF- α and IL-6 early during infection, while the attenuated virus evoked an increase at later time points during infection.⁴⁴ The levels of TGF- β 1 were reduced due to MNV-1 infection. TGF- β 1 evokes a Th-3 response to human enteric viruses.^{44, 45} The level of cytokine secretion is inversely proportional to virus replication, where enhanced virus replication reduced TGF- β 1 secretion in human rotavirus, whereas when the virus replication slows down, TGF- β secretion levels are normalized.⁴⁴ Our results showing the suppression of TGF- β 1 levels by MNV-1 are in line with these studies. In contrast, we observed that silica restored TGF- β 1 levels, while silica had minimal effects on virus replication. These observations argue that the synergistic effect of silica-MNV-1 on the host is clearly independent of virus cycle and could occur as a result of competitive modulation of host immune response.

Another observation was the occurrence of a genotoxic insult that was exclusively a synergistic effect of MNV-1 and silica, as the presence of either one alone did not impart genotoxicity. While silica NPs have been shown to exert genotoxic insults,⁴⁶ macrophages were resistant to silica-induced genotoxicity. This difference could occur due to a difference in subcellular localization of silica NPs. The replication complexes (RCs) of MNV-1 localize in cellular compartments adjacent to the nucleus.⁴⁷ Any charge based interaction of silica NPs with the RCs can result in altered localization,⁴⁷ and the membrane permeabilization properties of silica could result in silica being localized in the nucleus in the presence of MNV-1 infection. This could impart DNA damage as measured by comet tails. Our results showing FCV-silica induced genotoxicity in CRFK cells demonstrates that this phenomenon is independent of the cell type, but may occur with other enteric viruses increasing the membrane permeability and imparting DNA damage.

Taken together, our study for the first time unravels several interesting effects of silica NP accumulation during enteric virus infection, and suggests safety considerations of evaluating NP toxicity in the context of virus infection. Studies are underway to analyze these effects in a mouse model of MNV infection.

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Figure 1.

Effect of silica NPs on cell morphology and viability of RAW 264.7 cells. (A) Bright-field images (10× and 20×) of RAW 264.7 macrophages taken at 48 h post incubation with 10 µg/ml or 2 µg/ml of SiO₂NPs, shown in comparison to mock-infected cells. (B) Percentage cell viability calculated using a Trypan Blue exclusion assay, with cells harvested at 24 h post incubation. Error bars represent SEM. ** $P < 0.05$ as analyzed by a Students *T* test.

Figure 2.

Effect of Preincubation with silica NPs on production of infectious virus particles and viral genome copy numbers. (A) Infectious virus titers determined by plaque assay from cell culture supernatants harvested at 24 h and 48 h post infection, from cells incubated with 10 μ g/mL and 2 μ g/mL silica NPs for 3 h, and infected with 5 MOI and 0.5 MOI. (B) Viral genome copies determined by a real time PCR assay from cell culture supernatants harvested at 24 h and 48 h post infection, from cells incubated with 10 μ g/mL and 2 μ g/mL silica NPs (indicated as 20 μ g and 2 μ g in the figure) for 3 h, and infected with 5 MOI and 0.5 MOI MNV-1.

Figure 3.

Cytopathic effect and cell viability in macrophages preincubated with silica NPs and infected with MNV. (A) Bright-field images (40 $\times$) of RAW 264.7 macrophages preincubated with 10 $\mu\text{g}/\text{mL}$ (indicated as 10 μg in the figure) of silica NPs for 3 h followed by 5 or 0.5 MOI MNV infection, shown in comparison with MNV infection alone. (B) Percentage cell viability calculated using a Trypan Blue exclusion assay with cells harvested at 24 h post incubation. Error bars represent SEM. ** $P < 0.05$ as analyzed by a Students T test.

Figure 4.

Expression of genes related to immune response to MNV-1 infection in the presence of silica NPs. Fold change of BMP family of genes (A), chemokine ligands (B), cytokines (C), and growth factors (D) shown in comparison to mock-infection, as analyzed by the RT2 profiler array.

Figure 5.

Production of proinflammatory cytokines in RAW 264.7 cells incubated with silica NPs and infected with 5 MOI of MNV. Levels of IL-6 (A), TNF α (B), and TGF β 1 (C) as measured by an ELISA from culture supernatants harvested at 24 h from cells incubated with 10 μ g/mL silica NPs (indicated as 10 μ g in the figure) or infected with 5 MOI MNV-1. Error bars represent SEM. ** $P < 0.05$ as analyzed by a Students T test.

Figure 6.

Effect of silica NPs on genotoxic insults to cells. (A) Genotoxic insult, shown as % tail DNA under indicated experimental conditions, with MNV-1 in RAW 264.7 macrophages, as measured by the comet assay. (B) Genotoxic insult, shown as % tail DNA under indicated experimental conditions, with FCV in CRFK epithelial cells, as measured by the comet assay. Note that of silica NPs enhanced the genotoxic insult under both conditions. Error bars represent SEM. ** $P < 0.05$ as analyzed by a Students T test.