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Silicon dioxide nanoparticle exposure affects small intestine function in an *in vitro* model

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Abstract

The use of nanomaterials to enhance properties of food and improve delivery of orally-administered drugs has become common, but the potential health effects of these ingested nanomaterials remains unknown. The goal of this study is to characterize the properties of silicon dioxide (SiO₂) nanoparticles (NP) that are commonly used in food and food packaging, and to investigate the effects of physiologically realistic doses of SiO₂ NP on gastrointestinal health and function. In this work an *in vitro* model composed of Caco-2 and HT29-MTX co-cultures, which represent absorptive and goblet cells, was used. The model was exposed to well-characterized SiO₂ NP for acute (4 hours) and chronic (5 days) time periods. SiO₂ NP exposure significantly affected iron, zinc, glucose, and lipid nutrient absorption. Brush border membrane intestinal alkaline phosphatase activity was increased in response to nano-SiO₂. The barrier function of the intestinal epithelium, as measured by transepithelial electrical resistance, was significantly decreased in response to chronic exposure. Gene expression and oxidative stress formation analysis showed NP altered the expression levels of nutrient transport proteins, generated reactive oxygen species, and initiated pro-inflammatory signaling. SiO₂ NP exposure damaged the brush border membrane by decreasing the number of intestinal microvilli, which decreased the surface area available for nutrient absorption. SiO₂ NP exposure at physiologically relevant doses ultimately caused adverse outcomes in an *in vitro* model.

Keywords

consumer products; exposure; food; SiO₂; risk assessment

Introduction

Food-grade silicon dioxide (SiO₂) or amorphous silica (coded E551) has been found to contain particles in the nano-size range (at least one dimension less than 100 nanometers) (Yang, Faust, Schoepf, Hristovski, Capco, Herckes and Westerhoff, 2016, Peters, Kramer, Oomen, Rivera, Oegema, Tromp, Fokkink, Rietveld, Marvin, Weigel, Peijnenburg and

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Bouwmeester, 2012, Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011, Auffan, Rose, Bottero, Lowry, Jolivet and Wiesner, 2009). Similar to TiO₂ NP, SiO₂ nanoparticles (NP) are used to improve the taste and texture of rich foods without adding calories, preserve food color and durability, and carry fragrances or flavors (Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011, Contado, Mejia, Lozano García, Piret, Dumortier, Toussaint and Lucas, 2016). SiO₂ NP also act as anti-caking agents to maintain flow properties of powdered mixes, seasonings, and coffee whiteners (Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011, Contado, Mejia, Lozano García, Piret, Dumortier, Toussaint and Lucas, 2016). The use of nanomaterials in food production and pharmaceuticals, however, may have unknown health effects due to unexpected biological interactions (Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011).

The gastrointestinal (GI) tract epithelium forms a selective barrier that allows essential nutrients, electrolytes, and water to pass into circulation, but must also protect from foreign antigens, microorganisms, and toxins (Groschwitz and Hogan, 2009). Ingestion of engineered nanomaterials can upset this delicate balance. A more tissue-like, anatomically and physiologically realistic *in vitro* model is needed to mimic the interactions between biological systems and NP (Chia, Tay, Setyawati and Leong, 2015, Tay, Muthu, Chia, Nguyen, Feng and Leong, 2016). A two-dimensional (2D) monolayer cell culture is phenotypically similar to the single-cell-thick *in vivo* intestinal epithelium, and is used here for nanotoxicity and nutrient absorption studies (Mahler, Esch, Tako, Southard, Archer, Glahn and Shuler, 2012, Mahler, Shuler and Glahn, 2009). A co-culture model of the two most abundant intestinal epithelial cell types (absorptive and goblet cells) on permeable supports was developed using the human Caco-2 and HT29-MTX cell lines (Wikman-Larhed and Artursson, 1995). Co-cultures of Caco-2 and HT29-MTX at the physiologically relevant ratio of 3:1, respectively, offer the best compromise between model response and the presence of a mucus layer (Mahler, Shuler and Glahn, 2009, Wikman-Larhed and Artursson, 1995). The human intestinal epithelial Caco-2 cell line forms monolayers of absorptive enterocytes with a brush border and tight junctions (TJ), and expresses carriers and receptors for nutrients, macromolecules and drugs (Wikman-Larhed and Artursson, 1995). *In vivo*, the mucus layer, composed of heavily glycosylated proteins (mucins), covers the intestinal epithelium and is secreted by goblet cells (Johansson, Sjoval and Hansson, 2013). It is necessary that goblet cells, mimicked by mucus secreting cell lines such as HT29-MTX, are incorporated into Caco-2 cell models to provide a better simulation of natural conditions (Yuan, Chen, Chai, Du and Hu, 2013). The formation of TJ between Caco-2 and HT29-H cells was verified by electron microscopy (Wikman-Larhed and Artursson, 1995). Our previous *in vitro* studies showed that the Caco-2 and HT29-MTX co-culture formed a mucus layer that completely covers the cell monolayer (Mahler, Shuler and Glahn, 2009).

NP are not uniquely reactive with human cells, but they generally have more biological reactivity than their non-nanoscale counterparts. NP composition and physicochemical properties result in different biological reactivities. Toxicity testing for NP, when performed, often focuses on acute exposures and obvious toxicity to organs or tissues. As a result, the

effects of physiologically realistic doses SiO₂ NP on more subtle aspects of GI health and function have not been fully explored. In this study the properties of SiO₂ NP, including shape, size, morphology, and zeta potential, were first determined. The intestinal model was then exposed to physiologically relevant doses of SiO₂ for both acute (4 hours) and chronic (5 days) time points. Following NP exposure, gut functionality was assessed by measuring iron (Fe), zinc (Zn), glucose, and fatty acid transport and/or uptake, intestinal alkaline phosphatase (IAP) activity, and barrier function. Next, gene expression and reactive oxygen species (ROS) formation were measured, and, finally, microvilli were visualized. It was found that SiO₂ NP exposure altered nutrient transport, ROS formation, barrier function, gene expression, and microvilli structure.

Materials and Methods

20–30nm amorphous silicon dioxide nanoparticles (Stock#: US3438, CAS#: 7631–86-9) were purchased from US Research Nanomaterials, Inc. (Houston, TX, USA). All culture flasks, plates, tubes, and pipette tips used for culturing cells were purchased from Corning Incorporated (Corning, NY, USA), and all chemicals, enzymes, and hormones were purchased from SigmaAldrich (St. Louis, MO, USA) unless otherwise stated. All glassware used for sample preparation and analysis was soaked in an acid solution of 10% hydrochloric acid (HCl) and 10% nitric acid (HNO₃) overnight, and then rinsed with 18 MΩ resistivity deionized (DI) water.

Dose Calculations

The *in vitro* NP doses were based on the approximate daily adult SiO₂ NP intake and the surface area of human small intestinal epithelium. It has been estimated that adults ingest ~35 mg of fine (0.1–1 μm diameter) to ultrafine (<100 nm diameter) silicate and aluminosilicate particles per day (Lomer, Hutchinson, Volkert, Greenfield, Catterall, Thompson and Powell, 2004). More recent work estimated nano-SiO₂ exposure to be even higher (1.8 mg/kg bw/day), although this exposure is still below the amount of silica regarded as a safety concern (21 mg/kg bw/d) (Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011). The total intestinal surface area is 2×10⁶ cm² (DeSesso and Jacobson, 2001). The chronic medium dose in this study was based on the daily SiO₂ NP surface density exposure, which is 35 mg/2×10⁶ cm² = 1.75×10⁻⁵ mg/cm². The acute medium dose is a third of the daily intake (i.e. the dose from 1 meal). The acute dosage may be a more accurate exposure estimate, as although multiple meals are consumed per day, ingested materials move through the GI tract and do not build up. A microfluidic GI tract model with periodic exposure to doses representing NP consumed in one meal would best recreate *in vivo* conditions (Mahler, Esch, Glahn and Shuler, 2009, Esch, Mahler, Stokol and Shuler, 2014).

Considering the wide variation of intake between individuals (0–254 mg/individual/d, or even up to 568 mg/individual/d) (Dekkers, Krystek, Peters, Lankveld, Bokkers, van Hoeven-Arentzen, Bouwmeester and Oomen, 2011, Lomer, Hutchinson, Volkert, Greenfield, Catterall, Thompson and Powell, 2004), high (100× physiological dose), medium (physiological dose), and low (1/100 physiological dose) doses were applied in this study.

The volume of SiO₂ NP (density 2.65 g/cm³) with a 30 nm diameter is 1.4×10^{-17} cm³ or 1.4×10^4 nm³, and the specific surface area is 75.47 m²/g. Ingestion of 35 mg/individual per day would translate to a dose of $\sim 9.3 \times 10^{14}$ particles per day per individual, with an exposure of 4.67×10^8 particles/cm² to the small intestine. Table 1 outlines the doses used for acute and chronic exposures.

Dispersion Preparation

200 mg of SiO₂ NP powder was weighed in a polystyrene weighing dish and dispersed in 20 mL 18 MΩ sterile DI water. The solution was mixed uniformly in a sterile tube with a Thermolyne Mixer (Maxi Mix II, Type 37600) for 1 min, and then serially diluted to the target concentrations in water, water with ⁵⁸Fe-ascorbate, water ⁶⁷Zn-ascorbate, minimal essential medium (MEM, Thermo Fisher Scientific, Waltham, MA USA), MEM with ⁵⁸Fe-ascorbate, MEM with ⁶⁷Zn-ascorbate, Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific), or DMEM with 10% (v/v) heat inactivated fetal bovine serum (HI-FBS, Thermo Fisher Scientific). Stable isotopes were purchased from Isoflex (San Francisco, CA). The dispersion concentration for chronic exposures was three times that for the acute exposure to represent 3 meals per day. The tubes with aqueous dispersions were sonicated (VWR® symphony™ Ultrasonic Cleaners, RF-48 W, 35k Hz operation frequency) for 30 min to break down NP agglomerates. Sonicated dispersions were used for determining NP properties or exposure to the *in vitro* model. Data were compensated for any significant changes in fluorescent or colorimetric assays due to the presence of SiO₂ NP.

Dynamic Light Scattering (DLS)

The distributions of SiO₂ NP sizes and average ζ-potentials were measured with a Zetasizer Nano ZS (Malvern Instruments Inc, Southborough, MA). Measurements were performed in Malvern disposable polycarbonate folded capillary cells with gold plated beryllium–copper electrodes (DTS1070), which were rinsed with ethanol, 18 MΩ DI water, and sample dispersions before filling. The Refractive Index (RI) of SiO₂ is 1.46, and water RI is 1.33. The viscosity of water (0.8872 mPa·s) and the dielectric constant of water (78.5 C²·N⁻¹·M⁻²) were used for measurements. The samples were equilibrated in the instrument chamber for 120 s, and measured at 25°C.

Nanoparticle Tracking Analysis (NTA)

The hydrodynamic size of SiO₂ NP was also assessed using NTA. NTA measurements were performed with a NanoSight NS300 (Malvern Instruments Limited, Worcestershire, UK), equipped with a sample chamber with 532 nm (green) laser passing through a prism-edged optical flat, computer-controlled motorized focus, and spinning disc. Stock NP powder was suspended in 18 MΩ DI water, sonicated to a homogeneous solution, and then diluted to a concentration that yields 20–50 particles/frame for optimal measurement. Samples were injected into the sample chamber at 25°C with sterile syringes until the NP dispersions reached the tip of the nozzle (Filipe, Hawe and Jiskoot, 2010). The Brownian motion of NP in water was detected and visualized by a high sensitivity sCMOS camera (Level 13, Slider Shutter 1232, Slider Gain 219), which was used to record a video, operating at 25 frames per second (Dragovic, Gardiner, Brooks, Tannetta, Ferguson, Hole, Carr, Redman, Harris, Dobson, Harrison and Sargent). NTA software simultaneously tracked the Brownian motion

of NP centers on a particle-by-particle basis throughout the 30s video frame by frame (749 frames total). The average distance of each particle was automatically calculated.

Transmission Electron Microscopy (TEM)

The primary size and morphology of SiO₂ NP were evaluated using transmission electron microscopy (TEM) on a JEOL JEM-2100F (JEOL, Peabody, MA). Stock NP powder was suspended in DI water and sonicated to a homogeneous solution. A drop of sample was loaded onto an ultrathin 400 mesh copper TEM grid (01701-F, TedPella, Redding, CA) with a plastic transfer pipette. The grids were allowed to air-dry overnight before imaging.

Scanning Electron Microscopy (SEM)

The primary size and morphology of SiO₂ NP were also assessed using a Scanning Electron Microscope (SEM, Supra 55, Zeiss, Oberkochen, Germany). Stock NP powder was loaded onto a metal mount, and then coated with carbon in a chamber with less than 10⁻⁴ atm pressure and 100 mA electrical current, resulting in a 10 nm thickness. NP samples were viewed at 200,000× magnification and 2000 eV.

Cell Culture

The human colon carcinoma Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) at Passage 17 and used in experiments at Passage 70–75. The HT29-MTX cell line was kindly provided by Dr. Thécia Lesuffleur of INSERM U560 in Lille, France, at Passage 11 and used in experiments at Passage 40–45 (Lesuffleur, Barbat, Dussaulx and Zweibaum, 1990). Caco-2 and HT29-MTX co-cultures grown for 16 days on a permeable membrane to develop a mucus layer that completely covers the monolayer and is approximately 2–10 µm thick (Mahler, Shuler and Glahn, 2009). The two types of cells were cultured in DMEM with 10% (v/v) HI-FBS at 37°C with 5% CO₂, and culture medium was changed every 2 days.

In experimental studies, Caco-2 and HT29-MTX were stained with trypan blue, counted with a hemocytometer, re-suspended at ratios of 75:25 (Caco-2/HT29-MTX), and then seeded at a density of 100,000 cells/cm² onto polycarbonate, 0.4 µm pore size, 0.33 cm² membrane, 24 well Transwell® inserts (Corning Life Sciences, Corning, NY) that were previously coated with rat tail Type I collagen (BD Biosciences, San Jose, CA, USA) at 8 µg/cm² for 1 hour at room temperature (Monopoli, Walczyk, Campbell, Elia, Lynch, Baldelli Bombelli and Dawson, 2011). Twenty-four hours before Fe or Zn transport studies, DMEM was removed from the wells, the monolayers were rinsed with phosphate-buffered saline (PBS), and the cells were cultured overnight in very low iron (<8 µg/L) MEM supplemented with 10 mM piperazine-N,N'-bis-(2-ethanesulfonic acid) (PIPES), 4 mg/L hydrocortisone, 5 mg/L insulin, 5 µg/L selenium, 34 µg/L triiodothyronine, 1% antibiotic–antimycotic solution and 20 µg/L epidermal growth factor at pH 7.0 (Glahn, Lee, Yeung, Goldman and Miller, 1998). Under these conditions, baseline cell ferritin levels were approximately 3 ng ferritin/mg cell protein.

Transepithelial Electrical Resistance (TER)

Transepithelial electrical resistance of the monolayers was measured every three days after seeding into Transwell® inserts, before and after acute exposure to NP, and every day of chronic NP exposure experiments before refreshing the NP dispersions. The TER was measured with an EVOM2 and Endohm-6 chamber from World Precision Instruments (Sarasota, FL). The Endohm chamber was soaked in 70% ethanol for 15 minutes, and then 2 mL of sterile 100 mM KCl solution was added to the chamber, which was then connected to the EVOM2. The electrode was soaked until the voltage reading was between 0–20 mV. The old KCL solution was then removed and 600 µL fresh KCl was added into the Endohm. A sterilized Calicell (World Precision Instruments) with 200µL KCL solution was inserted to the chamber. The ohm reading was adjusted to 393 ohms. After removing the Calicell, the Endohm chamber was equilibrated with 2 mL serum free DMEM for 15 minutes. After replacing the old medium with 600µL fresh medium, the TER of every sample was measured three times at different insert positions. The Transwell cultures were taken out of incubator 5 minutes prior to measurements to allow for temperature equilibration.

Acute and Chronic Exposure to NP

For acute exposures, cells were rinsed once with PBS and then 600 µL of medium was placed into the basolateral chamber of the Transwells and 100 µL of medium containing high, medium, or low doses of SiO₂ NP were placed into the apical chamber. Cells were incubated at 37 °C and 5% CO₂ on a rocking shaker (Laboratory Instrument Model RP-50, Rockville, MD) at 6 oscillations per minute for 4 hours. For chronic exposures, new SiO₂ NP dispersions were prepared in DMEM +10% HI-FBS and the apical chamber culture medium containing nano-SiO₂ dispersions was changed every 24 hours.

⁵⁸Fe and ⁶⁷Zn Uptake and Transport

For mineral uptake and transport experiments, cells were exposed to stable isotope (⁵⁸Fe, ⁶⁷Zn, Isotrex) at a concentration of 10 µM in serum free, very low mineral concentration MEM. The radiolabeled experimental medium was prepared immediately before use by combining ⁵⁸FeCl₃ (0.923 mg/mL) and 200 µL of 100 mM ascorbic acid (pH 2). The molar ratio of Fe:ascorbic acid was 1:20. The Fe (II) - ascorbate solution incubated at room temperature for 15 minutes, 334 µL of 1.5 M NaCl was added, and then 10 mL MEM was added to the solution. The zinc transport medium was prepared identically with ⁶⁷Zn. Immediately following NP exposure, 100 µL of the mineral transport medium was added to the apical chambers. Cells were incubated at 37°C and 5% CO₂ on a rocking shaker (6 oscillations/minute) for 2 hours. After 2 hours the culture medium in the bottom chamber was collected, 10 µl HNO₃ (99.999% trace metals) was added to each sample, and samples were analyzed with Inductively Coupled Plasma Mass Spectrometry (ICP-MS, ICAP Model 61E Trace Analyser; Thermo Jarrell Ash Corporation, Franklin, MA, USA).

After the two hour exposure, cells were washed twice with 200 µL in the apical chamber and 600 µL in the basolateral chamber with stop solution (130 mM NaCl, 5 mM KCl, 5 mM PIPES, pH 6.7). Removal solution (5 mM bathophenanthrolinedisulfonic acid, 130 mM NaCl, 5 mM KCl, 5 mM PIPES, 5 mM sodium dithionite) was then added to the monolayers. The removal solution has been shown to remove surface bound iron without

damaging the cell membrane (Glahn, Gangloff, Van Campen, Miller, Wein and Norvell, 1995). After 10 minutes, the removal solution was aspirated and the cells were washed twice with stop solution. The Transwell membrane was cut out and put into a microcentrifuge tube with 200 μ L of water. Cells on the membranes were lysed by sonicating (VWR® symphony™ Ultrasonic Cleaners, RF-48 W) for 15 minutes at 4°C. 10 μ L HNO₃ was added to lysed cell solutions and samples were analyzed with ICP-MS.

Inductively Coupled Plasma Mass Spectrometry Measurement

Stable isotope ratios in culture medium in apical (top) and basolateral (bottom) chambers and cell monolayers were determined with ICP-MS. Samples were first wet ashed with HNO₃ and HClO₄. Next, 4 mL of 60/40 volume, double distilled 70% HNO₃/HClO₄ mixture and 0.25 mL of 40 mg/L Yttrium was added as an internal standard for ICP-MS to each sample. Samples were then incubated overnight at room temperature, and then heated to 120°C in an aluminum heating block for 2 hours. If the sample did not clarify with this treatment, 0.25 mL of concentrated nitric acid was added for further digestion until the temperature reached 195 °C. Next 20 mL of DI water was added, tubes were vortexed, and the solution was transferred into tubes. Concentrations of ⁵⁶Fe and ⁵⁸Fe and ⁶⁵Zn and ⁶⁷Zn were determined. ⁷²Ge was added using a mixing-T just prior to the sample entering the nebulizer, serving as an internal standard. Hydrogen at 5 mL/min was used as a reaction gas to remove polyatomic interferences of Ar-O⁺ at mass 56. The concentrations of ⁵⁸Fe and ⁶⁷Zn were determined through calibration with a certified reference material, Nickel (⁵⁸Ni and ⁶⁰Ni), which has a natural abundance ratio (⁵⁸Ni/⁶⁰Ni) of 2.59. Ions from the plasma were accelerated in an electromagnetic field and extracted into a mass spectrometer. The element concentrations were drift corrected and normalized using the Yttrium internal standard. Data output from ICP-MS is expressed as mg/kg (ppm).

Glucose Uptake and Transport

Cell monolayers in Transwell inserts were rinsed with PBS and starved in serum free, glucose free DMEM (ThermoFisher) for 1 hour. 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) glucose analog was used to model glucose uptake and transport by the small intestinal cells. 100 μ L of 100 μ M 2-NBDG in glucose-free, serum free DMEM with or without doses of NP was added to apical chambers, and then 100 μ L of medium from the basolateral chambers was collected at regular time intervals (30, 60, 90, 120, 150, 180, 210, 240 minutes). Samples were moved into black 96-well plates and replaced with 100 μ L of serum free glucose free medium in the basolateral chamber. Samples were measured in a fluorescent plate reader (Synergy 2 plate reader, Biotek, Winooski, VT, USA) at an excitation/emission spectrum of 485/528 nm. Following the 2-NBDG transport assay the Transwell membranes were cut and placed into a microcentrifuge tube with 200 μ L cell lysis buffer (a 1% sodium deoxycholate, 40 mM KCl, 20 mM Tris, and pH 7.4) (Blodgett, Kothinti, Kamyshko, Petering, Kumar and Tabatabai, 2011). After 10 min, the tube was centrifuged at 400 g for 5 minutes. 100 μ L supernatant was moved to black 96-well plates, and measured with a plate reader. A standard curve was used to convert the fluorescent density of samples in glucose free medium or cell lysate into concentration of 2-NBDG.

Intestinal Fatty Acids Uptake

Cells in a 96-well plate were immediately rinsed with 200 μ L ice-cold medium following acute or chronic exposure. Cellular uptake studies of free fatty acids were performed using fluorescent BODIPY® 500/510 C1, C12 (4, 4-Difluoro-5-Methyl-4-Bora-3a, 4a-Diaza-s-Indacene-3-Dodecanoic Acid, ThermoFisher) (Heller, Cable, Penrose, Makboul, Biswas, Cabe, Crawford and Savkovic, 2016, Choe, Jang, Park, Kim, Ahn, Park, Hong, Alitalo, Koh and Kim, 2015, Stahl, Hirsch, Gimeno, Punreddy, Ge, Watson, Patel, Kotler, Raimondi, Tartaglia and Lodish, 1999). Stock solutions of BODIPY® 500/510 C1, C12 were prepared in 5 mmol/L ethanol solution, and stored at -20°C . The analog was added to culture medium to obtain a final concentration of 50 $\mu\text{mol/L}$, 50 μL was added to each well, and cells were incubated for 10 min. The culture medium was then quickly replaced by analog-free medium, and cells were further incubated at 37°C and 5% CO_2 for 1 hour. Fluorescence intensity in each well was measured with a plate reader (excitation/emission, 490/530, green) and fluorescence images were captured with a Nikon eclipse Ti-E Inverted wide field fluorescence microscope with a $10\times$ object lens.

Gene Expression

Following NP exposure and nutrient transport studies, cell RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD). After exposure to the nanomaterials, the cells on the Transwell inserts were released from the membrane with a cell scraper and provided lysis buffer. The cell lysate was homogenized with a QIAshredder column (Qiagen) and the RNA was isolated according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using the SuperScript III RT-PCR kit with oligo(dT) primer (Biorad). Primer sequences (Guo, Martucci, Moreno-Olivas, Tako and Mahler, 2017) are available for the genes encoding duodenal cytochrome-B (DcytB), divalent metal transporter-1 (DMT1), hephaestin (HEPH), ferroportin1 (FPN1), liver-type fatty acid-binding protein (L-FABP, FABP-1), intestinal fatty acid-binding protein (FABP-2), Zinc transporter-1 (ZnT1), Zrt, Irt-like protein-1 (ZIP1), interleukin 8 (IL-8), tumor necrosis factor- α (TNF- α), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B). Endogenous sodium-D-glucose cotransporter-1 (SGLT-1/SLC5A1) and glucose transporter type 2 (GLUT2) primers were previously reported by Richter et al. (Richter, Shull, Fountain, Guo, Musselman, Fiumera and Mahler, 2018). RNA yield and purity was quantified using a NanoDrop 2000 (ThermoFisher Scientific). Samples with an $\text{OD}_{260/280}$ ratio of greater than 1.8 were considered suitable for gene expression measurements (Becker, Hammerle-Fickinger, Riedmaier and Pfaffl, 2010), and were diluted to 25 ng/ μL before converting to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time polymerase chain reaction (RT-PCR) was performed with a MiniOpticon Real-Time PCR Detection System (Biorad). The 20 μL PCR mixtures consisted of 10 μL of POWER SYBR Green PCR Master Mix (Applied Biosystem, Carlsbad, CA), 7 μL of water, and 1 μL of each primer (10 mM concentration) that was added to 1 μL of the cDNA samples. All reactions were performed in duplicates and under the following conditions: 95°C for 3 min, 50 cycles of 95°C for 60 s, 54°C for 15s, and 72°C for 30s. After the cycling process was completed, melting curves was determined from 65.0°C to 95.0°C with increments of 0.5°C for 5 seconds to ensure amplification of a single product. Gene expression was normalized to the expression of GAPDH and compared with unexposed

controls using the 2^{-Ct} method (Livak and Schmittgen, 2001). Data were analyzed with Biorad CFX Manager Software.

Reactive Oxygen Species Generation

Following NP exposure and nutrient uptake and transport, 50 μ L of CellROX® Reagent (Invitrogen), excitation/emission 485/520 (green), at a final concentration of 5 μ mol/L in MEM solution was added to the cells and incubated for 30 minutes at 37°C. Then medium was removed and cells were rinsed three times with PBS. An aliquot of 200 μ L PBS was loaded onto the cells and ROS generation was measured with a florescent plate reader.

Immunocytochemistry

Cells stained with CellROX were fixed by incubating with 4% formaldehyde (100 μ L per well) at room temperature for 50 minutes. Cells were then incubated with 0.1% Triton X-100 in PBS for 5 minutes to permeabilize, washed with PBS, and incubated with PBS containing 5% bovine serum albumin (BSA) on a rotating shaker for 1 hour. Cells were then incubated 2 hours with 25 μ L of 2.5 μ g/mL mouse anti-occludin primary antibody (Thermo Fisher Scientific, 1:200 dilution in PBS), and then 2 hours with 25 μ L 20 μ g/mL of Alexa Fluor 568 goat anti-mouse secondary antibody (Thermo Fisher Scientific, 1:100 dilution in PBS). The experiment was performed at room temperature, and cells were rinsed with PBS between each step. 25 μ L 5×10^{-3} mM solution of DRAQ5 (Thermo Fisher Scientific, 1:1000 dilution) in PBS was added and incubated for 30 minutes in the dark to counter stain DNA. After rinsing the cells with 18 M Ω DI water, the membranes were removed and mounted on a glass slide with ProLong gold mounting medium (Thermo Fisher Scientific) and cured overnight in the dark. Cells were imaged with the Leica SP5 inverted stage Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany) with the following lasers - Argon (Power 20%), HeNe 543, HeNe 594, HeNe 633. The pinhole was 83.3 μ m, and scan speed was 200 Hz. The HCX PL APO CS 63.0 $\times$ 1.40 OIL UV objective lens with a refraction index of 1.52 and numerical aperture 1.4 was used.

Alkaline Phosphatase Activity Assay

Monolayers were seeded into 24 well plates and exposed to medium or high concentrations of 30 nm SiO₂ for acute (4 hours) or chronic (5 days) time periods. Following exposure, cells were washed with 0.5 mL of PBS and then sonicated for 5 minutes at room temperature in 0.2 mL PBS. To recover the cell lysate each well was scraped into individual 1.5 mL centrifuge tubes. The alkaline phosphatase (AP) assay detects the presence of AP activity by using p-nitrophenyl phosphate (pNPP) as the substrate. The pNPP solution was made by dissolving one Tris Buffer tablet and one pNPP tablet (product code: N2770 Sigma-Aldrich) in 5mL of 18 M Ω DI water. AP hydrolyzes pNPP to p-nitrophenol, which turns bright yellow based on the concentration present. 25 μ L of cell lysate solution from each tube was added to each well of a 96-well plate. 85 μ L of the pNPP solution was then added to the wells. The plate was then incubated at room temperature for 1 hour. The absorbance was read on a plate reader at 405 nm to measure to concentration of p-nitrophenol. The Bradford assay was used to determine the total cell protein concentration. 5 μ L of cell lysate was added to a 96 well plate and then 250 μ L of Bradford Reagent was added to each well. After incubating for 15 minutes at room temperature, absorbance was read at 595 nm using a plate

reader. For each assay, a standard curve was created with p-nitrophenol (for the AP assay) or bovine serum albumin (BSA, for the Bradford assay) to calculate the unknown concentrations of p-nitrophenol or protein.

Microvilli Structure

Cells were seeded in 6 well plate (Corning) with sterilized cover slides in each well that were coated with 8 $\mu\text{g}/\text{cm}^2$ rat tail Type I collagen. Monolayers were acutely or chronically exposed to SiO_2 NP after being cultured 15 days. The cells were then fixed in 4% formaldehyde and rinsed with PBS. Next, cells were dehydrated with ethanol solution (70 and 100%), transferred to hexamethyl disilazane (HMDS) with a gradient procedure (1:2 HMDS: Ethanol, 2:1 HMDS: Ethanol, 100% HMDS) and dried overnight. After carbon coating, the slides were viewed by the Inlens of a Zeiss Supra 55 Scanning Electron Microscope (Oberkochen, Germany) at 5k eV. Image analysis was performed with ImageJ (Schneider, Rasband and Eliceiri, 2012).

Statistical Analysis

All measurements of NP properties, nutrient uptake and transport were made at least 6 times for each treatment. Results are expressed as mean $\pm$ the standard error of the mean (S.E.M.). Data were analyzed with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). A t-test, paired t-test, or one-way ANOVA with Tukey's posttest were used to compare differences between means, and data were transformed when necessary to obtain equal sample variances. For the glucose transport studies, normality of distribution was determined with a D'Agostino & Pearson omnibus K2 test. Statistical significance was determined using a nonlinear regression model with replicates test for lack of fit. Out of the models tested, the quadratic model was accepted as the best fit. Curve fits were compared using the Akaike's Information Criteria (AIC) from a quadratic model. Differences between means were considered significant at $p < 0.05$.

Results

Nanoparticle Characterization

Nano- SiO_2 particles shown in a SEM image (Figure 1A) are spherical. The TEM image in Figure 1B shows that SiO_2 NP in water are agglomerating and overlapping, and that the primary size of SiO_2 NP was 34.3 ± 4.4 nm ($n=20$). This is similar to the size provided by the manufacturer. Based on the size and shape, the number of SiO_2 NP was estimated reasonably in the experimental treatment dispersions. Nanoparticle tracking analysis (NTA) indicated that the agglomerate size of SiO_2 NP was 170 ± 59 nm at a concentration of $1.21 \pm 0.06 \times 10^9$ particles/mL. Polydispersity index (PdI), generated by the software, is dimensionless and refers to the range of hydrodynamic diameter distribution. If the $\text{PdI} > 0.5$, NP will have polydisperse (polymodal) distributions (Camli, Buyukserin, Balci and Budak, 2010); if $\text{PdI} < 0.1$, there is a monodisperse distribution (Bihari, Vippola, Schultes, Praetner, Khandoga, Reichel, Coester, Tuomi, Rehberg and Krombach, 2008). The PdI of nano- SiO_2 in all dispersants was approximately 0.5, which means SiO_2 NP aggregated and were polydispersely distributed. The hydrodynamic diameters of NP were at least 219 nm with PdI of 0.173 at 1 mg/mL in water, and 796 ± 171 , 509 ± 98 , 1072 ± 161 nm at low, medium, and

high concentrations, respectively, in water (Table 1). The hydrodynamic diameters in DMEM were 330 ± 42 , 354 ± 33 , 874 ± 93 nm, and in MEM were 449 ± 127 , 478 ± 173 , 1003 ± 68 nm for low, medium, and high concentrations (Table 1). The sizes measured by NTA and dynamic light scattering (DLS) were larger than the primary particle diameters shown in TEM images because the SiO₂ NP agglomerated when suspended in water and medium (Yang, Faust, Schoepf, Hristovski, Capco, Herckes and Westerhoff, 2016). The SiO₂ nanoparticles were sonicated, but may have aggregated because the final NP solution was sonicated, not the initial solution made in DI water. As described by DeLoid et al., to generate nanoparticle dispersions in culture medium the nanomaterial should first be sonicated in deionized water with a delivered sonication energy (J/ml) equal to the material-specific critical delivered sonication energy (DSEcr), and then the NP should be diluted in cell culture medium to the desired initial concentration for application to cells (DeLoid, Cohen, Pyrgiotakis and Demokritou, 2017). The ζ potential value, which exists between surfaces of the NP and the dispersants, was in the range of -19.2 to -35.8 mV in water (Table 1). It is normally considered a stable NP solution when the ζ -potential is more negative than -30 mV because the NP strongly repel each other, which prevents particle aggregation (Hanaor, Michelazzi, Leonelli and Sorrell, 2012). In lower concentration water dispersions, the ζ potential values of SiO₂ NP also indicate that the 30 nm SiO₂ particles were aggregated (Table 1).

Tight junctions

Following acute exposures to nano-SiO₂, there were no significant effects on the transepithelial resistance (TER) values when compared to unexposed controls (Figure 1C). The acute doses of SiO₂ NP also had no effect on occludin expression (Figure 2A). In contrast, in monolayers exposed chronically to NP, after a 2-day exposure to low, medium, or high doses of nano-SiO₂ particles the TER dropped significantly below the control value (Figure 1D), which was supported by immunofluorescent stained images (Figure 2B). Chronic exposures were limited to 5 days because at day 5 the monolayers still maintained some barrier function ($\sim 160 \Omega \cdot \text{cm}^2$), which is necessary for functional studies.

Oxidative Stress

Nano-SiO₂ particle exposure induced the production of oxidative stress in intestinal epithelial cells following acute and chronic exposures. The ROS stain mean signal intensities of fluorescence significantly increased in response to ⁵⁸Fe, ⁶⁷Zn, and acute or chronic SiO₂ exposure (Figure 1 E, F, G, and H), indicating that the antioxidant defenses of the cell were overwhelmed. This was also demonstrated with confocal microscopy, where there is more green ROS staining in the low, medium, high SiO₂ exposed cultures when compared with the unexposed controls (Figure 2C). (Katayama, Xu, Fan and Mine, 2006). SiO₂ NP induced gene expression of IL-8 following chronic low and medium exposure with Zn (3.28 ± 0.41 and 3.07 ± 0.47 fold increase, respectively) (Table 2). TNF- α gene expression was upregulated 3.43 ± 0.74 fold in response to low, acute SiO₂ doses with ⁶⁵Zn, although there was no statistical significance due to large variations (Table 2). Nano-SiO₂ significantly increased TNF- α gene expression following exposure to low and high chronic doses with ⁵⁸Fe (9.61 ± 2.26 and 7.83 ± 0.85 fold increase, respectively), and, in addition, NF κ B1 was

upregulated 2.65 ± 0.47 fold in chronic, low dose cultures in response to the ROS and TNF- α signaling (Table 2).

Mineral Element Transport and Uptake

The intestinal *in vitro* model transported 1.97 ± 0.16 to 3.11 ± 0.47 ng of iron into the cell after acute exposure to SiO₂ NP (Figure 3A) and transported 1.61 ± 0.19 to 2.01 ± 0.16 ng post-chronic exposure (Figure 3B). Compared with the control group, acute and chronic exposure to SiO₂ NP resulted in significantly decreased in Fe transport (Figure 3A and B), which is representative of iron transport into the bloodstream *in vivo*. SiO₂ NP exposure did not affect Fe uptake into the cell following acute and chronic SiO₂ NP exposure, except for a significant increase in uptake after acute exposure with a low dose (Figure 3 C and D). Compared with unexposed cultures, nano-SiO₂ particles at low, medium, and high doses with ⁵⁸Fe and ⁶⁵Zn (DMT1) or only ⁶⁵Zn (Dcytb) significantly reduced gene expression of the Fe transport proteins Dcytb and DMT1. (Table 2). Low, medium, and high chronic SiO₂ NP exposure with ⁶⁵Zn significantly reduced gene expression of HEPH (0.37 ± 0.02 , 0.48 ± 0.03 , and 0.59 ± 0.05 fold decrease, respectively), and medium and high chronic exposure to SiO₂ NP with ⁵⁸Fe significantly decreased FPN1 (0.57 ± 0.05 and 0.53 ± 0.06 fold decrease, respectively) (Table 2).

The amount of ⁶⁵Zn transported following NP exposure was in the range of 12.3 ± 3.2 to 17.3 ± 2.1 ng and 14.6 ± 3.5 to 27.0 ± 7.0 ng after acute and chronic exposure, respectively (Figure 3E and F). Zn transport was not significantly altered after acute and chronic exposure to SiO₂ NP (Figure 3E and F). The unexposed monolayers took up 12.6 ± 0.7 ng ⁶⁵Zn and the amount of ⁶⁵Zn in cells was 9.1 ± 0.5 , 10.9 ± 1.0 , and 13.3 ± 1.1 ng after low, medium, and high acute exposure to SiO₂ NP (Figure 3G). The low, acute doses of SiO₂ NP decreased Zn uptake significantly. Zn uptake increased significantly after chronic nano-SiO₂ exposure (19.2 ± 0.8 , 20.7 ± 3.0 , and 19.3 ± 1.3 ng), compared with the control group (14.9 ± 0.6 ng) (Figure 3H). ZnT1 gene expression was significantly increased 1.63 ± 0.17 fold following a low, chronic dose with ⁵⁸Fe, but SiO₂ NP doses with ⁶⁵Zn did not significantly affect gene expression of ZnT1 or ZIP1 (Table 2).

Glucose Absorption

2-NBDG transported from the apical to the basolateral chamber is representative of glucose absorption into the bloodstream, and the amount of 2-NBDG in the bottom or basolateral chamber vs. time was plotted (Figures 4A and B). There were no significant differences in 2-NBDG transport after acute exposure to SiO₂ NP (Figure 4A). Comparatively, following chronic exposure to SiO₂ NP, 1.2 μ g of 2-NBDG accumulated at the basolateral chamber (Figure 4B). The curves of 2-NBDG transport following chronic medium and high SiO₂ doses were significantly different from controls ($p = 0.027$ (medium) $p = 0.078$ (high)) following comparison using the AICs from a quadratic model (Figure 4B). The amount of 2-NBDG contained in the cell lysate of all treatments were statistically equivalent, and therefore SiO₂ NP did not significantly alter glucose uptake over a wide ranges of doses (Figure 4 C and D). SGLT1 expression was not affected by acute or chronic exposure to SiO₂ NP (Table 2). However, GLUT2 gene expression was upregulated significantly, acute exposures increased gene expression dose-dependently (1.32 ± 0.36 , 4.66 ± 0.59 , and

6.98±1.09 fold increase for low, medium and high doses, respectively). GLUT2 was not significantly affected by chronic SiO₂ NP exposure (Table 2).

Fatty Acid Absorption

SiO₂ NP significantly increased gene expression of FABP2 2.75±0.54 fold in response to a chronic, low dose with ⁵⁸Fe (Table 2). The expression of FABP1 was significantly decreased by all chronic doses of SiO₂ with ⁶⁵Zn (Table 2). SiO₂-exposed cells labeled with fluorescent fatty acid analog are representative of fatty acid uptake from the gut lumen into the small intestine. Fatty acid uptake was not significantly different after acute exposure to SiO₂ NP (Figure 4E). In contrast, uptake of fatty acids decreased after chronic exposure in response to the low dose of SiO₂ NP (Figure 4F).

Alkaline Phosphatase Activity

Acute, medium SiO₂ NP exposure significantly increased the monolayer IAP activity (Figure 4G). Chronic exposure to medium and high doses of SiO₂ also significantly increased IAP activity, and IAP activity for chronically exposed cells is ~20% higher than acutely exposed cells (Figure 4H).

Microvilli

The brush border microvilli appear as thin, finger-like projections emanating from the cell surface, as also shown in Figure 5 (Peterson, Bernent and Mooseker, 1993, Mooseker, 1985, Tilney and Mooseker, 1971, Faust, Doudrick, Yang, Westerhoff and Capco, 2014). The apical surface area covered by microvilli was significantly decreased following high acute and chronic exposure to SiO₂ NP (Figure 5, Figure 6).

Discussion

In this study, SiO₂ NP transport across the Caco-2/HT29-MTX monolayers was undetectable via ICP-MS (data not shown). The SiO₂ NP transport and uptake into the Caco-2/HT29-MTX cells was likely inhibited by the increased particle size due to agglomeration, surface charge, as well as hydrophobicity (Bouwmeester, Poortman, Peters, Wijma, Kramer, Makama, Puspitaninganindita, Marvin, Peijnenburg and Hendriksen, 2011, Bhattacharjee, Ershov, Gucht, Alink, Rietjens, Zuilhof and Marcelis, 2013, Sakai-Kato, Hidaka, Un, Kawanishi and Okuda, 2014). The cellular uptake of SiO₂ NP is particle size-dependent, especially in the range of 30–50 nm. At the 30–50 nm size range SiO₂ NP can serve as carriers in various applications (Lu, Wu, Hung and Mou, 2009, Shang, Nienhaus and Nienhaus, 2014). The mean SiO₂ NP hydrodynamic diameter was 500–1100 nm in water and medium (Table 1), which is larger than endocytic vesicles (approximately 50–150 nm) and therefore likely partitioned the SiO₂ NP to the apical surface of the cell monolayers (Liang, Lin, Whittaker, Minchin, Monteiro and Toth, 2010). SiO₂ NP suspended in cell culture medium likely form agglomerates consisting of multiple protein-coated primary particles with trapped intra-agglomerate fluid, and, therefore, agglomeration reduced the total number of particles and surface area of SiO₂ NP available for interaction with the intestinal cells. The physicochemical properties and structural characteristics of ingested SiO₂ NP, including interfacial properties, composition, charge, size, and aggregation state,

may also change significantly when digested or when interacting with different types of food (McClements, DeLoid, Pyrgiotakis, Shatkin, Xiao and Demokritou, 2016). Consumed NP are first partially digested through enzymes in saliva and mastication at a pH between 5 and 7, and then experience digestive fluids (pH 2), enzymes, and bacteria (Borel and Sabliov, 2014). The presence of a food matrix and digestion influence NP fate, transport, biokinetics, and toxicological profile, and large differences in particle size, charge, and morphology were observed in model food with and without Fe₂O₃ NP in a simulated gastrointestinal tract (DeLoid, Wang, Kapronezai, Lorente, Zhang, Pyrgiotakis, Konduru, Ericsson, White, De La Torre-Roche, Xiao, McClements and Demokritou, 2017).

TER results from junctions between epithelial cells and is composed of TJ resistance, intercellular space resistance, apical membrane resistance, and basolateral membrane resistance (Blikslager, Moeser, Gookin, Jones and Odle, 2007). In this study immunofluorescence images were qualitatively analyzed for the TJ protein occludin and TER was measured following acute and chronic NP exposures. Staining for the TJ protein occludin (Furuse, Hirase, Itoh, Nagafuchi, Yonemura and Tsukita, 1993) and measuring the TER are two common methods for evaluating epithelial monolayer integrity and TJ functionality. Chronic exposure to NP caused a significant decrease in TER, indicating that the SiO₂ NP increased the permeability of the TJ and allowed more passive diffusion between cells. An increased permeability in the TJ is the sublethal toxic outcome (Ranaldi, Marigliano, Vespignani, Perozzi and Sambuy, 2002). The TER values decreased to ~160 $\Omega \times \text{cm}^2$ following a medium, chronic SiO₂ NP exposure, but remained above 200 $\Omega \times \text{cm}^2$ following high and low exposures and therefore did not cause the epithelial cells to completely lose barrier function. In work performed by Farcal et al. SiO₂ NP did not induce any changes Caco-2 epithelium TER values during the 21 days of monitoring (7 repeated exposures at a concentration of 100 $\mu\text{g/mL}$), but short term (3 days) and long term (10 days) exposure to SiO₂ NP resulted in statistically significant cytotoxic and cytostatic effects (Farcal, Torres Andon, Di Cristo, Rotoli, Bussolati, Bergamaschi, Mech, Hartmann, Rasmussen, Riego-Sintes, Ponti, Kinsner-Ovaskainen, Rossi, Oomen, Bos, Chen, Bai, Chen, Rocks, Fulton, Ross, Hutchison, Tran, Mues, Ossig, Schnekenburger, Campagnolo, Vecchione, Pietroiusti and Fadeel, 2015). A separate *in vitro* study discovered 50 nm SiO₂ NP translocated across the intestinal barrier within a Caco-2 cell Transwell model, but *in vitro* cytotoxicity of silica particles, independent of the fluid and the concentration, was not observed at sizes that were larger than 100 nm (Sakai-Kato, Hidaka, Un, Kawanishi and Okuda, 2014).

Increased intracellular ROS contributes to increased oxidative stress in the cell (Lehman, Morris, Mueller, Salem, Grassian and Larsen, 2016, Zhang, Chen, Jiang, Wong, Yang and Zheng, 2011). ROS generation and oxidative stress can be used as a method to assess NP toxicity (Xia, Kovoichich, Brant, Hotze, Sempf, Oberley, Sioutas, Yeh, Wiesner and Nel, 2006, Liu, Rogel, Harada, Jarett, Maiorana, German, Mahler and Doiron, 2017, Tay, Setyawati and Leong, 2017, Liu, Yoo, Han, Mahler and Doiron, 2018). Antioxidant molecules, especially glutathione (GSH) (Pompella, Visvikis, Paolicchi, Tata and Casini, 2003, Helen HW and Macus Tien, 2010), scavenge peroxynitrite and hydroxyl radicals and convert hydrogen peroxide to water. NP may overwhelm the cellular mechanisms for scavenging ROS (Turrens, 2003), and result in oxidative stress on the monolayer (Huerta-

García, Pérez-Arizti, Márquez-Ramírez, Delgado-Buenrostro, Chirino, Iglesias and López-Marure, 2014). Increased oxidative species, for example ROS, results in oxidative modifications to biological macromolecules and causes DNA damage, polyunsaturated lipid oxidation, and protein degradation (Martindale and Holbrook, 2002, Imlay, 2003). In previous work, SiO₂ NP showed enhanced interaction with serum proteins and cell membranes, and induced more oxidative stress and stronger proinflammatory effects in macrophages (Di Cristo, Movia, Bianchi, Allegri, Mohamed, Bell, Moore, Pinelli, Rasmussen, Riego-Sintes, Prina-Mello, Bussolati and Bergamaschi, 2016). Similar to the amorphous SiO₂ or silica nanoparticles used here, mesoporous silica nanoparticles have also been shown to induce oxidative stress, with the mesoporosity and pore size playing a role in ROS production (Tay, Setyawati and Leong, 2017, Setyawati and Leong, 2017)

Oxidative stress induces inflammation of the gut mucosa via production of pro-inflammatory cytokines, especially IL-8 and TNF- α (Katayama, Xu, Fan and Mine, 2006). SiO₂ NP induced gene expression of IL-8 following chronic low and medium exposure with Zn (Table 2), significantly increased TNF- α gene expression following exposure to low and high chronic doses with ⁵⁸Fe, and NF κ B1 was upregulated in chronic, low dose cultures in response to the ROS and TNF- α signaling (Table 2). Activation of the NF κ B inflammatory pathways and the resultant increase in cytokine production contribute to disrupting levels and localization of TJ proteins, further decreasing intestinal barrier properties (Ma, Iwamoto, Hoa, Akotia, Pedram, Boivin and Said, 2004). In this work, the oxidative stress and activation of pro-inflammatory signaling cascades likely damaged the integrity of TJ structures and decreased barrier function.

The majority of absorption and chemical digestion occurs in the small intestine, and dietary iron absorption occurs primarily in the duodenum of the proximal small intestinal epithelium (Martini, 2004, Eady, Wormstone, Heaton, Hilhorst and Elliott, 2015, Galy, Ferring-Appel, Becker, Gretz, Gröne, Schümann and Hentze, 2013). Large amounts of cellular iron are stored in the intracellular iron storage protein ferritin as a compact bioavailable form (Arosio and Levi, 2010). Ferric iron (Fe³⁺) complexes are predominant in dietary nonheme iron, and are reduced to the ferrous iron (Fe²⁺) form by Dcytb at the brush border extracellular surface, and then up taken by DMT1 on the apical intestinal membrane (Lane, Bae, Merlot, Sahni and Richardson, 2015, McKie, Barrow, Latunde-Dada, Rolfs, Sager, Mudaly, Mudaly, Richardson, Barlow, Bomford, Peters, Raja, Shirali, Hediger, Farzaneh and Simpson, 2001, McCarthy and Kosman, 2015). The transmembrane ferroxidase, HEPH, oxidizes ferrous iron back to ferric iron when the ferrous iron reaches the basolateral membrane, and then the iron export protein, FPN1 transports ferric iron into the circulation (Eady, Wormstone, Heaton, Hilhorst and Elliott, 2015, Galy, Ferring-Appel, Becker, Gretz, Gröne, Schümann and Hentze, 2013, Lane, Bae, Merlot, Sahni and Richardson, 2015, McKie, Barrow, Latunde-Dada, Rolfs, Sager, Mudaly, Mudaly, Richardson, Barlow, Bomford, Peters, Raja, Shirali, Hediger, Farzaneh and Simpson, 2001, McCarthy and Kosman, 2015). Exposure to SiO₂ NP with ⁵⁸Fe tended to reduce the gene expression of Dcytb, DMT1, HEPH, and FPN1 (Table 2). The downregulation could be in response to the high iron content in the transport study medium or, for Dcytb and DMT1, because of the decrease in microvilli, which is where these proteins are expressed (Gulec, Anderson and Collins, 2014). The ⁵⁸Fe transported from the apical to basolateral chamber is representative of Fe absorption into the

bloodstream, and low acute and low, medium, and high chronic doses of SiO₂ NP decreased ⁵⁸Fe transport (Figure 3). The decrease in transport could be the result of decreased surface area for absorption due to the decreased number of microvilli in NP-exposed cultures. Similarly, chronic exposure to 30 nm TiO₂ NP significantly decreased gene expression of the Fe transport proteins Dcytb, DMT1, HEPH, and FPN1, and TiO₂ NP significantly decreased Fe transport (Guo, Martucci, Moreno-Olivas, Tako and Mahler, 2017). Oral exposure to polystyrene NP also affected iron absorption, in both an *in vitro* cell culture model and *in vivo* broiler chicken model following acute and chronic exposure (Mahler, Esch, Tako, Southard, Archer, Glahn and Shuler, 2012).

Two families of mammalian zinc transporters regulate zinc homeostasis. The ZIP family is located in proximity to the apical microvilli in differentiated Caco-2 cells, increasing intercellular zinc levels via transport of zinc into the cytoplasm from the extracellular space. ZnT-1 was the first cloned zinc transporter at the basolateral membrane of intestinal epithelial cells in the upper portion of the villus in the duodenum and jejunum, and moves zinc from the cytoplasm to the extracellular space or the lumen of organelles (Lichten and Cousins, 2009, Michalczyk and Ackland, 2013, Desouki, Franklin, Costello and Fadare, 2015, Lodemann, Gefeller, Aschenbach, Martens, Einspanier and Bondzio, 2015, Gefeller, Bondzio, Aschenbach, Martens, Einspanier, Scharfen, Zentek, Pieper and Lodemann, 2015, Ranaldi, Caprini, Sambuy, Perozzi and Murgia, 2009, Franklin, Ma, Zou, Guan, Kukoyi, Feng and Costello, 2003). ZnT1 gene expression was increased following a low, chronic exposure to SiO₂ NP, and ⁶⁵Zn uptake, but not ⁶⁵Zn transport was significantly affected by SiO₂ NP (Figure 3). The increase in zinc uptake was likely due to the increase in ROS formation and resulting inflammation. Pro-inflammatory conditions have been shown to increase Zn absorption (Pekarek and Evans, 1975, Sas and Bremner, 1979).

SGLT1 carries glucose and galactose across the enterocyte apical membrane (Lehmann and Hornby, 2016). Glucose crosses the basolateral membrane into the circulation via GLUT2, which is normally located at the basolateral membrane and translocated to the apical surface in response to high luminal glucose levels (Lehmann and Hornby, 2016, Kellett, Brot-Laroche, Mace and Leturque, 2008). SGLT1 expression was not affected by acute or chronic exposure to SiO₂ NP (Table 2). GLUT2 gene expression was increased significantly and dose-dependently by acute SiO₂ NP exposures, but was not significantly affected by chronic SiO₂ NP exposure (Table 2). 2-NBDG glucose analog transport was significantly increased following chronic medium and high doses of SiO₂ NP, which may be due to the increased permeability of the monolayers following chronic exposure.

The FABP family of fatty acid binding proteins mediates more than 95% of lipid digestion products targeting and shuttling to specific metabolic sites, and L-FABP (FABP1) and I-FABP (FABP2) are located in the absorptive intestinal villus cell (Mansbach and Gorelick, 2007, Tso, Balint, Bishop and Rodgers, 1981, Agellon, Toth and Thomson, 2002, Abumrad and Davidson, 2012, Gajda and Storch, 2015, Derikx, Evennett, Degraeuwe, Mulder, van Bijnen, van Heurn, Buurman and Heineman, 2007, Boord, Fazio and Linton, 2002). Chronic SiO₂ NP exposure significantly increased gene expression of FABP2, and the expression of FABP1 was significantly decreased by all chronic doses of SiO₂ (Table 2). I-FABP is an early marker for enterocyte cell death. It is present in the plasma of healthy individuals in

small amounts, but level rise rapidly after episodes of acute intestinal ischemia and inflammation (Derikx, Evennett, Degraeuwe, Mulder, van Bijnen, van Heurn, Buurman and Heineman, 2007). FABP2 gene expression upregulation following chronic exposure to SiO₂ NP may be due to pro-inflammatory signaling. Fatty acid uptake was not significantly changed by acute SiO₂ exposure, but low, chronic NP exposure significantly decreased fatty acid uptake (Figure 4). IAP has been shown to be a negative regulator of intestinal fat absorption (Lallès, 2010), and IAP levels were elevated following medium acute and medium and high chronic SiO₂ NP exposures.

The brush border enzyme IAP is highly concentrated in small intestinal epithelial cells and plays a critical role in intracellular homeostasis, epithelial lining function, and stress responses (Kühn, Hamarneh, Ramirez, Munoz, Morrison, Gul, Adiliaghdam and Hodin, 2016, Noda, Yamada, Tanabe, Nakaoka, Hosoi and Goseki-Sone, 2016). Additionally, IAP regulates lipid absorption, bicarbonate secretion, and duodenal surface pH (Lallès, 2010). Three primary macronutrients (carbohydrate, protein, and fat) may be related to a high level of IAP expression or activity (Lallès, 2010, Prentice, 2007). The absence of IAP results in lower levels of the junctional proteins ZO-1, ZO-2, and occludin, and *vice versa*, so the IAP enzyme plays a major role in the expression of TJ proteins in the cells (Liu, Hu, Huo, Zhang, Adiliaghdam, Morrison, Ramirez, Gul, Hamarneh and Hodin, 2016). In our study, the TER value decreased following chronic SiO₂ NP exposure and the cell monolayers may have increased IAP activity to maintain barrier function. The oxidative stress resulting from ROS and pro-inflammatory signaling cascades may have also stimulated IAP activity following acute and chronic exposure to SiO₂ NP. In previous work, 30 nm TiO₂ particles also significantly increased IAP activity (Guo, Martucci, Moreno-Olivas, Tako and Mahler, 2017).

The cytoskeletal apparatus, which is a highly ordered array of actin filaments and associated proteins, supports the apical brush border surface of the intestinal epithelial cells (Mooseker, 1985). The brush border surface is covered by a mucus layer that ranges in thickness from 10 to 100–200 μ m (jejunum to colon), and is a continuously secreted barrier that protects the underlying epithelium by trapping and coating foreign particulates and pathogens (Lundquist and Artursson, 2016, Ensign, Cone and Hanes, 2012). The mucus barrier strongly limited distribution and proximity of mucoadhesive nanoparticles, but not non-mucoadhesive nanoparticles (mucus-penetrating particles), to epithelial surfaces in both the small and large intestine (Maisel, Ensign, Reddy, Cone and Hanes, 2015). The mucus proteins bind and immobilize NP via hydrophobic interactions (Lundquist and Artursson, 2016). NP are generally much smaller than the average mucus mesh pore size, but mucus can still significantly reduce NP translocation (Walczak, Kramer, Hendriksen, Tromp, Helsper, van der Zande, Rietjens and Bouwmeester, 2015, Lai, Wang and Hanes, 2009). Norwalk (38 nm) and human papilloma virus (HPV; 55 nm) penetrate mucus and water at roughly same rate, which establishes that NP can diffuse through low viscosity pores with the a highly elastic mucin fiber matrix (Lai, Wang and Hanes, 2009). These interactions between the mucus layer and NP are why an *in vitro* model with a ~2–10 μ m thickness mucus layer was used for this study.

Exposure to 30 nm SiO₂ particles significantly decreased the *in vitro* intestinal epithelium surface area covered by microvilli following acute and chronic exposure to high doses. In previous work, hematite (α -Fe₂O₃) NP (26 nm, 53 nm, 76 nm, and 98 nm) were absorbed onto cells until equilibrium (approximately 5 minutes), and this triggered a dynamic reorganization and detachment of microvilli structures on Caco-2 cell surfaces. Immediately following the NP exposure the TER was also significantly decreased, especially in response to 26 nm NP (Zhang, Kalive, Capco and Chen, 2010). In a separate study, exposure to food grade TiO₂ (E171 food additive, ~25% of the TiO₂ <100 nm) disrupted the normal organization of microvilli at a concentration of 350 ng/mL (100 ng/cm² cell surface area) (Faust, Doudrick, Yang, Westerhoff and Capco, 2014). Doses above 10 μ g/mL TiO₂ NP resulted in alteration of microvillar organization on the apical surface of the epithelium following a 10-day treatment (Koeneman, Zhang, Westerhoff, Chen, Crittenden and Capco, 2010). Both acute (4 hours) and chronic (5 days) NP exposure to physiologically realistic doses of 30 nm TiO₂ NP resulted in a decrease in absorptive cell microvilli (Guo, Martucci, Moreno-Olivas, Tako and Mahler, 2017). In a study by Wang et al., exposure to relevant levels of food-grade SiO₂ NP resulted in a net loss (43%) and disorganization of brush border microvilli (Yang, Faust, Schoepf, Hristovski, Capco, Herckes and Westerhoff, 2016). ZnO NP, which are commonly used in food packaging, affect the microvilli of *in vitro* intestinal cells at doses relevant to human exposures (Moreno-Olivas, Tako and Mahler, 2018). Chronic exposure (14 days) *in vivo* to carboxylated, polystyrene NP resulted in remodeling of the intestinal villi, which increased the surface area available for nutrient absorption (Mahler, Esch, Tako, Southard, Archer, Glahn and Shuler, 2012).

Conclusions

Characterization of 30 nm SiO₂ particles indicated that the particles were spherical and agglomerated in water and culture medium. Exposure to SiO₂ NP at physiologically relevant doses significantly affected Fe, Zn, glucose, and lipid nutrient absorption and barrier function in an *in vitro*, cell culture model of the intestinal epithelium. Significant barrier function of the *in vitro* intestinal epithelium was lost following chronic medium and high dose SiO₂ exposure. This resulted in an increase in glucose transport. SiO₂ NP decreased the number of intestinal microvilli, which decreased the surface area available for nutrient absorption. This decreased the amount of iron that was transported across the *in vitro* intestinal epithelium. SiO₂ NP also significantly increased the brush border membrane enzyme intestinal alkaline phosphatase, which may have affected fatty acid uptake. NP exposure changed the expression levels of nutrient transport proteins, and induced ROS and pro-inflammatory signaling. These results show that exposure to SiO₂ NP, which are commonly ingested from food, altered the functionality of intestinal epithelial cells after physiologically relevant exposures.

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Figure 1.

30 nm SiO₂ nanoparticle (NP) characterization, transepithelial resistance (TER), and reactive oxygen species (ROS) generation following acute (4 days) and chronic (5 days) exposure to NP. (A) Scanning electron microscopy (SEM) shows the shape of the 30 nm SiO₂ NP; (B) transmission electron microscopy (TEM) shows the primary size of SiO₂ NP in water dispersant. (C) TER of Caco-2/HT29-MTX monolayers after acute exposure to SiO₂ NP, low dose (LD), medium dose (MD), and high dose (HD), n = 12. (D) TER of Caco-2/HT29-MTX monolayers following chronic SiO₂ NP exposure, n = 12. ROS

production in response to acute (E and F) and chronic (G and H) doses of SiO₂ with 10 μM ⁵⁸Fe-ascorbate (E and F) or 10 μM ⁶⁷Zn-ascorbate (G and H), n = 3. Data is expressed as mean ± S.E.M. and statistical significance between untreated controls and nanoparticle-exposed cultures and following an unpaired student's t-test is marked with a *, p < 0.05.

Figure 2.

Confocal images of the *in vitro* epithelium stained with immunofluorescence for occludin (red), an integral plasma-membrane protein located at the tight junctions (TJ), and DNA (blue) after acute (4 hours, A) and chronic (5 days, B) exposure to 30 nm SiO₂ NP at low, medium, and high doses. Scale bars are 50 μ m. (C) ROS (green) generation after acute exposure to SiO₂ NP at low, medium, or high doses with 10 μ M ⁵⁸Fe-ascorbate. Scale bars are 20 μ m.

Figure 3. Mineral absorption across and uptake into the *in vitro* intestinal cell monolayer. ^{58}Fe transport (A, B) and uptake (C, D) and ^{67}Zn transport (E, F) and uptake (G, H) in response to acute (A, C, E, G) and chronic (B, D, F, H) exposure to 30 nm SiO_2 nanoparticles (NP). Data shown is mean $\pm$ S.E.M., statistical significance between untreated controls and nanoparticle-exposed cultures according to an unpaired student's t-test is marked with a *, $p < 0.05$, $n = 6$.

Figure 4.

Nutrient absorption across, uptake into and brush border membrane enzyme activity of the *in vitro* intestinal cell monolayer. (A) 2-NBDG glucose analog transport following acute (4 hours, A) and chronic (5 days, B) exposure to SiO₂ nanoparticles (NP). Glucose transport was significantly increased following chronic exposure to medium and high doses of NP when compared with untreated controls (n=12 across 3 transport experiments, $p < 0.02$ (medium) $p < 0.07$ (high)), but is not significantly changed with acute NP exposure or low chronic, NP exposure. Curve fits were compared using the AICs from a quadratic model. 2-

NBDG uptake after acute (C) and chronic (D) exposure to SiO₂ NP. Data is shown as mean $\pm$ S.E.M., and statistical significance between untreated controls and nanoparticle-exposed cultures according to a student's t-test is indicated with a *, $p \leq 0.05$, $n = 5$. Fluorescent BODIPY® 500/510 C1, C12 lipid uptake in response to acute (E) and chronic (F) doses of SiO₂ NP. Data is shown as mean $\pm$ S.E.M. * denotes significance according to an unpaired student's t-test, $p \leq 0.05$, $n = 20$. Intestinal alkaline phosphatase (IAP) activity in response to acute (G) and chronic (H) doses of SiO₂ NP. Data is shown as mean $\pm$ S.E.M. * denotes significance according to an unpaired student's t-test, $p < 0.05$, $n = 7$.

Figure 5. Scanning electron microscopy (SEM) images of microvilli following exposure to acute (4 hours) and chronic (5 days) low, medium, and high doses of 30 nm SiO₂ nanoparticles. Magnifications are 5,000, 10,000, and 20,000.

Figure 6. Scanning electron microscopy (SEM) images of microvilli were analyzed for microvilli density following acute (4 hours, A) or chronic (5 days, B) 30 nm SiO₂ nanoparticle (NP) exposure. Data is shown as mean±S.E.M. * denotes statistical significance between untreated controls and nanoparticle-exposed cultures according to an unpaired student's t-test, p < 0.05, n = 4.

Table 1.

Characterization of SiO₂ nanoparticles (NP) in aqueous dispersions. Hydrodynamic size, polydispersity index, and zeta potential of three 30 nm SiO₂ NP concentrations were measured. Samples were prepared in DMEM, DMEM with 10% heat inactivated (HI) fetal bovine serum (FBS), 18 MΩ deionized (DI) water and MEM with or without 10 μM ⁵⁸Fe-ascorbate or ⁶⁷Zn-ascorbate. Results are presented as mean ± S.E.M. Columns that do not share any letters are significantly different according to a one-way ANOVA with Tukey's post test (p < 0.05), n = 3 independent measurements.

Dispersants	Low Dose 2.0×10 ⁻⁷ (mg/ml) 5.4×10 ⁶ (particle/ml)			Medium Dose 2.0×10 ⁻⁵ (mg/ml) 5.4×10 ⁸ (particle/ml)			High Dose 2.0×10 ⁻³ (mg/ml) 5.4×10 ¹⁰ (particle/ml)		
	d _{h,z-average} (nm)	PdI	ζ (mV)	d _{h,z-average} (nm)	PdI	ζ (mV)	d _{h,z-average} (nm)	PdI	ζ (mV)
DMEM-10% HI-FBS	34.2±16.5 c	0.419±0.050 a	-9.76±1.10 b	33.9±16.0 c	0.440±0.049 ab	-10.1±1.3 b	35.0±15.3 c	0.451±0.043 bc	-11.4±0.7 b
DMEM	330±42 b	0.540±0.087 a	-10.1±1.8 bc	354±33 bc	0.508±0.067 ab	-6.13±0.29 b	874±93 bc	0.611±0.042 ab	-23.4±0.5 c
18 MΩ Water	796±171 b	0.531±0.051 a	-19.2±3.7 c	509±98 bc	0.469±0.030 ab	-22.1±2.6 c	1072±161 b	0.608±0.016 ab	-35.8±2.0 d
18 MΩ Water- ⁵⁸ Fe(II)-Ascorbate	942±139 b	0.508±0.037 a	12.0±1.7 a	709±170 bc	0.450±0.044 ab	11.5±1.2 a	1199±211 b	0.511±0.022 b	7.00±1.08 a
18 MΩ Water- ⁶⁷ Zn(II)-Ascorbate	882±104 b	0.574±0.039 a	12.4±1.3 a	769±119 b	0.530±0.029 ab	12.0±1.3 a	1076±130 b	0.499±0.030 b	7.97±0.66 a
MEM	449±127 bc	0.533±0.052 a	-10.7±1.9 bc	478±173 bc	0.581±0.075 a	-11.2±1.5 b	1003±68 b	0.663±0.021 a	-14.9±0.9 b
MEM- ⁵⁸ Fe(II)-Ascorbate	1899±223 a	0.382±0.024 a	-12.4±0.4 bc	1367±158 a	0.348±0.016 b	-12.9±0.3 b	1775±242 a	0.340±0.029 c	-13.1±0.7 b
MEM- ⁶⁷ Zn(II)-Ascorbate	624±115 bc	0.566±0.039 a	-13.1±2.0 bc	565±95 bc	0.595±0.056 a	-13.7±1.4 b	1109±178 b	0.617±0.028 ab	-13.8±0.8 b

Min [d_{h,z-average} (nm)]
Min [PdI] = 0
Min [ζ (mV)]

Max [d_{h,z-average} (nm)]
Max [PdI] = 1
Max [ζ (mV)]

Table 2.

Gene expression in response to acute (4 hours) or chronic (5 days) exposure to 30 nm SiO₂ nanoparticles (NP) with 10 μ M ⁵⁸Fe-ascorbate, 10 μ M ⁶⁷Zn-ascorbate, or 100 μ M 2-NBDG. Results are presented as mean $\pm$ S.E.M. Tables that do not share any letters are significantly different according to a one-way ANOVA with Tukey's post test ($p < 0.05$), $n = 3$ independent measurements.

Genes		Acute Exposure (4 hours)				Chronic Exposure (5 days)				High AU
		Control	Low	Medium	High	Control	Low	Medium	High	
DMT1	+Zn	a 1.01 $\pm$ 0.04	a 1.00 $\pm$ 0.03	ab 1.28 $\pm$ 0.29	b 1.82 $\pm$ 0.20	a 1.01 $\pm$ 0.09	c 0.20 $\pm$ 0.02	b 0.38 $\pm$ 0.02	b 0.52 $\pm$ 0.03
	+Fe	a 1.05 $\pm$ 0.22	a 0.91 $\pm$ 0.21	a 1.30 $\pm$ 0.34	a 1.21 $\pm$ 0.08	a 1.00 $\pm$ 0.02	c 0.39 $\pm$ 0.03	bc 0.64 $\pm$ 0.09	ab 0.89 $\pm$ 0.11	
DCYTB	+Zn	a 1.01 $\pm$ 0.05	a 1.00 $\pm$ 0.07	a 1.12 $\pm$ 0.15	a 2.55 $\pm$ 0.57	a 1.02 $\pm$ 0.10	b 0.50 $\pm$ 0.07	b 0.45 $\pm$ 0.01	b 0.43 $\pm$ 0.02	
	+Fe	a 1.05 $\pm$ 0.23	a 1.31 $\pm$ 0.37	a 1.06 $\pm$ 0.16	a 1.63 $\pm$ 0.34	ab 1.05 $\pm$ 0.16	b 2.41 $\pm$ 0.66	ab 1.26 $\pm$ 0.10	a 1.01 $\pm$ 0.12	
FPN1	+Zn	a 1.02 $\pm$ 0.09	a 1.20 $\pm$ 0.16	a 1.03 $\pm$ 0.13	a 1.29 $\pm$ 0.14	a 1.05 $\pm$ 0.21	ab 0.90 $\pm$ 0.13	b 0.57 $\pm$ 0.05	b 0.53 $\pm$ 0.06	
	+Fe	a 1.07 $\pm$ 0.26	a 0.85 $\pm$ 0.09	a 1.53 $\pm$ 0.58	a 0.96 $\pm$ 0.12	a 1.03 $\pm$ 0.10	a 1.34 $\pm$ 0.11	a 1.07 $\pm$ 0.10	a 0.96 $\pm$ 0.11	
HEPH	+Zn	a 1.04 $\pm$ 0.13	a 0.92 $\pm$ 0.10	a 0.95 $\pm$ 0.23	a 1.32 $\pm$ 0.16	a 1.01 $\pm$ 0.11	c 0.37 $\pm$ 0.02	bc 0.48 $\pm$ 0.03	b 0.59 $\pm$ 0.05	
	+Fe	a 1.06 $\pm$ 0.25	a 0.81 $\pm$ 0.12	a 1.51 $\pm$ 0.46	a 1.40 $\pm$ 0.12	a 1.03 $\pm$ 0.11	a 1.09 $\pm$ 0.21	a 0.96 $\pm$ 0.14	a 0.75 $\pm$ 0.11	
ZnT1	+Zn	a 1.02 $\pm$ 0.09	a 0.93 $\pm$ 0.08	a 0.98 $\pm$ 0.11	a 1.00 $\pm$ 0.13	a 1.04 $\pm$ 0.17	b 1.63 $\pm$ 0.17	a 0.81 $\pm$ 0.06	a 0.69 $\pm$ 0.12	
	+Fe	a 1.04 $\pm$ 0.20	a 1.13 $\pm$ 0.33	a 1.18 $\pm$ 0.19	a 1.33 $\pm$ 0.32	a 1.05 $\pm$ 0.14	a 0.84 $\pm$ 0.10	a 1.02 $\pm$ 0.08	a 0.68 $\pm$ 0.10	
ZIP1	+Zn	a 1.04 $\pm$ 0.12	a 1.19 $\pm$ 0.12	a 1.17 $\pm$ 0.26	a 1.51 $\pm$ 0.24	ab 1.01 $\pm$ 0.06	a 1.64 $\pm$ 0.35	b 0.56 $\pm$ 0.06	ab 0.95 $\pm$ 0.07	
	+Fe	a 1.03 $\pm$ 0.17	a 1.47 $\pm$ 0.52	a 0.51 $\pm$ 0.11	a 1.22 $\pm$ 0.24	a 1.13 $\pm$ 0.22	a 2.58 $\pm$ 1.37	a 1.99 $\pm$ 0.20	a 1.40 $\pm$ 0.29	
FABP1	+Zn	a 1.05 $\pm$ 0.14	a 0.99 $\pm$ 0.07	a 1.23 $\pm$ 0.19	a 1.03 $\pm$ 0.22	a 1.06 $\pm$ 0.22	a 1.19 $\pm$ 0.18	a 1.01 $\pm$ 0.11	a 1.08 $\pm$ 0.15	
	+Fe	a 1.17 $\pm$ 0.47	a 0.76 $\pm$ 0.20	a 1.11 $\pm$ 0.37	a 0.70 $\pm$ 0.01	a 1.07 $\pm$ 0.16	b 0.61 $\pm$ 0.09	b 0.44 $\pm$ 0.04	b 0.36 $\pm$ 0.05	
FABP2	+Zn	a 1.04 $\pm$ 0.19	a 0.92 $\pm$ 0.09	a 1.14 $\pm$ 0.14	a 1.23 $\pm$ 0.31	a 1.02 $\pm$ 0.12	b 2.75 $\pm$ 0.54	ab 2.13 $\pm$ 0.38	ab 1.38 $\pm$ 0.14	
	+Fe	a 1.02 $\pm$ 0.16	a 0.66 $\pm$ 0.16	a 1.15 $\pm$ 0.36	a 1.12 $\pm$ 0.17	a 1.08 $\pm$ 0.18	a 2.19 $\pm$ 0.59	a 1.05 $\pm$ 0.14	a 0.91 $\pm$ 0.15	
IL8	+Zn	a 1.06 $\pm$ 0.26	a 1.53 $\pm$ 0.57	a 1.41 $\pm$ 0.77	a 1.94 $\pm$ 0.87	a 1.04 $\pm$ 0.16	b 3.28 $\pm$ 0.41	b 3.07 $\pm$ 0.47	ab 2.19 $\pm$ 0.26	
	+Fe	a 1.01 $\pm$ 0.09	a 1.47 $\pm$ 0.56	a 0.80 $\pm$ 0.24	a 0.88 $\pm$ 0.20	a 1.14 $\pm$ 0.26	ab 1.66 $\pm$ 0.11	b 2.28 $\pm$ 0.41	ab 1.96 $\pm$ 0.19	
TNF α	+Zn	a 1.07 $\pm$ 0.29	a 3.43 $\pm$ 0.74	a 2.49 $\pm$ 0.87	a 1.66 $\pm$ 0.49	a 1.02 $\pm$ 0.12	a 0.87 $\pm$ 0.21	b 0.38 $\pm$ 0.05	a 0.37 $\pm$ 0.04	
	+Fe	a 1.00 $\pm$ 0.05	a 1.79 $\pm$ 0.54	a 1.79 $\pm$ 0.95	a 1.93 $\pm$ 1.22	a 1.33 $\pm$ 0.43	b 9.61 $\pm$ 2.26	a 2.22 $\pm$ 0.22	b 7.83 $\pm$ 0.85	
NF κ B1	+Zn	a 1.03 $\pm$ 0.10	a 1.06 $\pm$ 0.06	a 0.99 $\pm$ 0.09	a 1.76 $\pm$ 0.35	a 1.02 $\pm$ 0.10	b 2.65 $\pm$ 0.47	a 1.21 $\pm$ 0.04	a 1.02 $\pm$ 0.07	
	+Fe	a 1.01 $\pm$ 0.13	a 1.70 $\pm$ 0.54	a 1.20 $\pm$ 0.25	a 1.43 $\pm$ 0.37	a 1.04 $\pm$ 0.11	a 1.41 $\pm$ 0.19	a 1.34 $\pm$ 0.15	a 1.30 $\pm$ 0.19	
GLUT2	+2NBDG	a 1.27 $\pm$ 0.39	a 1.32 $\pm$ 0.36	b 4.66 $\pm$ 0.59	b 6.98 $\pm$ 1.09	a 1.05 $\pm$ 0.15	a 0.82 $\pm$ 0.04	a 0.80 $\pm$ 0.03	a 0.74 $\pm$ 0.14	
SGLT1	+2NBDG	a 1.03 $\pm$ 0.13	a 1.28 $\pm$ 0.26	a 0.93 $\pm$ 0.09	a 0.91 $\pm$ 0.09	a 1.01 $\pm$ 0.07	a 0.83 $\pm$ 0.06	a 0.91 $\pm$ 0.03	a 0.80 $\pm$ 0.16	Low AU