Toxicity of orally administered food-grade titanium dioxide nanoparticles

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Abstract
This year, France banned the application of titanium dioxide nanoparticles as a food additive (hereafter, E171) based on the insufficient oral toxicity data. Here, we investigated the subchronic toxic responses of E171 (0, 10, 100, and 1,000 mg/kg) and tried to elucidate the possible toxic mechanism using AGS cells, a human stomach epithelial cell line. There were no dose-related changes in the Organisation for Economic Cooperation and Development test guideline-related endpoints. Meanwhile, E171 deeply penetrated cells lining the stomach tissues of rats, and the IgM and granulocyte-macrophage colony-stimulating factor levels were significantly lower in the blood from rats exposed to E171 compared with the control. The colonic antioxidant protein level decreased with increasing Ti accumulation. Additionally, after 24-h exposure, E171 located in the perinuclear region of AGS cells and affected expression of endoplasmic reticulum stress-related proteins. However, cell death was not observed up to the used maximum concentration. A gene profile analysis also showed that immune response-related microRNAs were most strongly affected by E171 exposure. Collectively, we concluded that the NOAEL of E171 for 90 days repeated oral administration is between 100 and 1,000 mg/kg for both male and female rats. Additionally, further study is needed to clarify the possible carcinogenesis following the chronic accumulation in the colon.

KEYWORDS
cancer, colon, E171, microRNA, stomach, titanium dioxide nanoparticles, toxicity
1 | INTRODUCTION

Owing to its beneficial properties (i.e., bright white, poorly water soluble, and inactive), titanium dioxide (TiO₂) particles have been used to manufacture a wide range of products including cosmetics, skin care products, paints, and building materials as a pigment (called as Pigment White 6 or CI77891) for about 100 years (European Union, 1994; Weir, Westerhoff, Fabricius, Hristovski, & von Goetz, 2012). Market survey data also suggested that paints, varnishes, paper, and plastics account for ~80% of the global TiO₂ consumption (Brandessence Market Research and Consulting Pvt. Ltd., 2020). In addition, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee of Food Additives (1969) evaluated TiO₂ as a food additive and designated an acceptable daily TiO₂ intake as "not limited except for good manufacturing practice." The Scientific Committee on Food classified TiO₂ as “colours for which an acceptable daily intake was not established but which could be used in food” in 1977 (European Food Safety Authority [EFSA] Panel on Food Additives and Nutrient Sources Added to Food [ANS], 2016). Similarly, the US Food and Drug Administration (FDA) accepted the addition of ≤1% food-grade TiO₂ (called as E171 in EU) to food products without the requirement of ingredient label disclosure (US FDA, 2020). Thus, TiO₂ particles have been extensively used in the production of various foodstuffs such as chocolates, candies, chewing gum, ice cream, donuts, confectionery, and beverages, and they have been also incorporated into toothpaste and pharmaceutical products (Dorier et al., 2019).

Meanwhile, the application of TiO₂ particles in the food industry has been a major public concern for a long period of time regarding the direct exposure, especially in children who tend to like eating sweets, and this concern has been amplified with the growth of the nanotechnology industry. In addition, the International Agency for Research on Cancer (IARC) classified inhaled TiO₂ nanoparticles (NPs) as a Group 2B (potential human carcinogen), particularly for workers (IARC, 2006). France, which worked as a lead sponsor in a sponsorship program of the Organisation for Economic Cooperation and Development (OECD) working party for manufactured nanomaterials, also reviewed the health effects of food-grade TiO₂ NPs (hereafter, E171) in 2017 (The French Agency for Food, Environmental and Occupational Health & Safety, 2020), and France’s National Institute for Agricultural Research (INRA) and its colleagues reported that orally dosed E171 may cross the intestinal wall and translocate to other organs (or tissues) via the bloodstream (Bettini et al., 2017). They demonstrated that the adverse health effects of E171 are attributable to the absorption of the nanoscale but not the microscale particle fraction and that E171 disturbs the homeostasis of the immune system. Similarly, about 36% of the particles obtained from a single source of E171 were less than 100 nm in dimension, indicating a potentially significant dietary exposure to TiO₂ NPs (Tassinari et al., 2014). In addition, chronic intake of foodstuffs containing E171 initiated and promoted the expansion of preneoplastic lesions in the colon and induced a slight inflammatory microenvironment in the mucosa (Dorier et al., 2019). Therefore, France banned the use of TiO₂ as a food additive in 2020 based on inadequate evidence to guarantee its safety to humans. In this study, our objective was to identify the possible adverse health effects of ingested E171. We also investigated the potential toxic mode of action of E171 in AGS cells, a human stomach epithelial cell line.

2 | MATERIALS AND METHODS

2.1 | Characterization

E171 (HOMBITAN® FG; purity 99.5%) was purchased from Venator Germany GmbH (Duisburg, Germany) and suspended in deionized water (DW) (stock concentration of 100 mg/ml). The suspension was added to artificial gastric fluid (AGF) (Marques, Loebenberg, & Almukainzi, 2011) and cell culture medium to evaluate its stability in biological systems. Particle morphology was observed by transmission electron microscope (TEM) images (JEM-3000F, 200 kV, JEOL Ltd., Tokyo, Japan), and particle size distribution and surface charge were measured with a zeta-potential and particle size analyzer (ELSZ-1000 Photal, Otsuka Electronics, Osaka, Japan).

2.2 | Animals and housing

Five-week-old male and female specific pathogen-free Sprague Dawley rats (40 rats per sex and five rats per cage) were obtained from ORIENT BIO Inc. (Seongnam-si, Gyeonggi-do, Korea) and maintained in stainless wire cages (255 × 465 × 200 mm) under controlled environment (12/12 h light/dark cycle with 150–300 lx, temperature of 23 ± 3°C, relative humidity of 50 ± 10%, and air ventilation of 10–20 times/h). Food (PMI Nutrition International, St. Louis, MO, USA) was ad libitum given with tap water, and wood chews were provided for animal welfare. The rats were randomly assigned to one of four groups (0, 10, 100, or 1,000 mg/kg) via Pristima v. 7.4 (Xybion Medical Systems Corporation, Lawrenceville, NJ, USA). The dose levels were selected based on a previous 4-week dose range finding study and 13-week repeated study of a different TiO₂ material (P25) in rats (Heo et al., 2020). According to OECD test guideline no. 408, E171 (10 rats/sex/dose) was administered daily by oral gavage for 90 days. The control group received equal volumes of DW, and the rats were euthanized using isoflurane on necropsy. The experimental design was reviewed and assessed by the Association for the Assessment and Accreditation of Laboratory Animal Care International and the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology.

2.3 | Clinical observations and blood analysis

The health status of all rats was observed daily during the study period. The type, time of occurrence, and severity of abnormal
symptoms were recorded with the Pristima v. 7.4 system. The rats were weighed upon arrival, before randomization, weekly during pretreatment, before dosing during treatment, and before necropsy. Food consumption was recorded weekly and calculated as g/rat/day. Urinalysis was performed during the treatment period on all surviving animals. The urine was collected for ~17 h before necropsy and its volume, specific gravity (SG), color, pH, and protein (PRO), ketone body (KET), occult blood (BLD), glucose (GLU), bilirubin (BIL), nitrate (NIT), and urobinogen (URO) levels were measured with a Cobas U411 urine analyzer (Roche, Basel, Switzerland) and a urine chemical analyzer (TBA 120FR: Toshiba Corp., Tokyo, Japan). The urine was centrifuged (1,500 rpm, 5 min) and its sediment casts (epithelial cells [EPI], erythrocytes [RBC], leucocytes [WBC], and blood [BLO]) were stained and microscopically observed (Nikon Eclipse CI, Nikon, Japan). Upon necropsy, blood was drawn from the venae cavae of all rats and stored in tubes coated with ethylenediaminetetraacetic acid dipotassium and heparin. Hematological and clinical chemistry analyses were performed in an ADVIA 2100i hematology system (Siemens, Washington, DC, USA) and an automatic analyzer (TBA 120FR: Toshiba Corp., Tokyo, Japan), respectively.

2.4 | Macroscopic and microscopic findings

Forty-two tissues were taken from all rats at necropsy. The tissues were weighed, and relative organ weights were calculated using the body weights measured at necropsy. The eyes with optic nerves were fixed in Davidson’s fixative solution, and all other tissues were preserved in 10% (v/v) neutral buffered formalin. The tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined under microscope (Olympus BX53, Olympus America, USA).

2.5 | Accumulation of E171

AGS cells (no. 21739), a human stomach epithelial cell line, was purchased from Korea Cell Line Bank and maintained in 37°C incubator with humidified atmosphere of 5% CO₂ using RPMI1640 media containing 10% fetal bovine and 1% penicillin/streptomycin. The colons obtained from rats administered the maximum dose were chopped, and AGS cells were harvested after 24-h exposure with 40 μg/ml of E171. The stomach tissues and the AGS cells were put in Karnovsky’s fixative solution (Electron Microscopy Sciences, Hatfield, PA, USA) overnight at 4°C. The samples were then fixed in a mixture of 2% (v/v) glutaraldehyde and 0.1-M sodium cacodylate buffer for 2 h, stained with 0.5% (w/v) uranyl acetate, dehydrated in graded ethanol solutions and propylene oxide, and embedded in Spurr’s resin (Electron Microscopy Sciences, Hatfield, PA, USA). The colon tissues and AGS cells were sectioned with an ultramicrotome (MT-X; RMC, Tucson, AZ, USA), stained with 2% (w/v) uranyl acetate and Reynolds’s lead citrate, and imaged with a TEM at 120 (Talos L120C, FEI, Hillsboro, OR, USA) and 80 kV (JEM1010, JEOL, Tokyo, Japan).

2.6 | Trace element determination

The tissues (colons, spleens, and kidneys) were digested in 70% (v/v) nitric acid solution using a microwave digestion system (Milestone, Sorisole, Italy). Finally, concentrations of trace elements in tissues were measured by inductively coupled plasma mass spectrometry (ICP-MS) at the Korean Basic Science Institute (Seoul, Korea).

2.7 | Immunohistochemistry

Paraffin-embedded stomach tissues were dewaxed with xylene and a graded alcohol series (100%, 95%, 70%, and 50%). After washing with phosphate-buffered saline (PBS), the tissues were placed in an antigen retrieval solution (ENZO, Seoul, Korea) and permeabilized with PBS containing Tween-20 (PBST, 1%). After blocking with 5% (v/v) bovine serum albumin in PBST (0.01%), the tissues were incubated overnight with rabbit polyclonal antibody against superoxide dismutase (SOD)-1, SOD-2 (Santa Cruz Biotechnology, Dallas, TX, USA), and cytochrome C (Cell Signaling Technology, Danvers, MA, USA) at 4°C. Following, the tissues were reacted with affinity-purified Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) and mounted with 4′,6-diamidino-2-phenylindole mounting medium. Lastly, the images were captured with an inverted phase-contrast fluorescence microscope (IX51, Olympus, Tokyo, Japan).

2.8 | Enzyme-linked immunosorbent assay

Serum was made by centrifugation (3,000 rpm, 15 min) of whole blood, and the level of immunoglobulin (Ig)A, IgE, IgG (KOMA Biotech, Seoul, Korea), IgM, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (eBioscience, San Diego, CA, USA) was measured according to manufacturer’s instruction. Finally, absorbance (450 nm) was measured using a 96-well microplate reader (PerkinElmer, Waltham, MA, USA), and the concentration in each sample was calculated based on the corresponding standard curve.

2.9 | Western blotting

The AGS cells (70%–80% of confluence) were incubated with E171 (0, 10, 20, and 40 μg/ml) for 24 h. Proteins in the cell lysates were quantified by bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO, USA), and the same amounts of proteins were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gel. Then, the proteins were transferred to nitrocellulose membranes (0.45-μm pore, GE Healthcare Life Sciences, Freiburg, Baden-Württemberg, Germany) and blocked with 5% (v/v) skim milk in PBST (0.05%). The membranes
Characterization of E171. E171 was first suspended in DW and then diluted (1:1 of volume ratio) using AGF and cell culture media. (A) Transmission electron microscope images of morphologies and crystal structures, (B) particle size distribution, and (C) zeta potential. AGF, artificial gastric fluid; DW, deionized water [Colour figure can be viewed at wileyonlinelibrary.com]
were reacted overnight at 4°C with primary mouse monoclonal antibodies against lysosome-associated membrane protein (LAMP)-1, β-actin, ER oxidoreductin (ERO)-1α, ferritin (HC), phospho-JNK, protein disulfide isomerase (PDI), eukaryotic translation initiation factor (eIF)2-α, catalase, caspase-1 (Santa Cruz Biotechnology, Dallas, TX, USA), C/EBP homologous protein (CHOP) (Cell Signaling Technology, Danvers, MA, USA), p62 (Abcam, Cambridge, UK), and rabbit monoclonal antibody against protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK), inositol-requiring enzyme (IRE)1-α (Cell Signaling Technology, Danvers, MA, USA), and rabbit polyclonal antibody against SOD-1, SOD-2, interleukin (IL)-18 (Santa Cruz Biotechnology, Dallas, TX, USA), binding immunoglobulin protein (Bip), calnexin, microtubule-associated proteins 1A/1B light chain (LC)3B (Cell Signaling Technology, Danvers, MA, USA), and goat polyclonal antibody against NACHT, LRR, and PYD domain-containing protein (NALP)3 (Abcam, Cambridge, UK). The proteins were then reacted with horseradish peroxidase (HRP)-conjugated mouse and rabbit or goat secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) and blotted in a ChemiDoc XRS+ system (Bio-Rad Laboratories, Hercules, CA, USA).

2.10 | Gene profiling analysis

The AGS cells were incubated with or without E171 (40 μg/ml) for 24 h to evaluate the effects of E171 on the gene profile. Briefly, mRNA was prepared using TRIzol solution (Invitrogen) and the microarray analysis was conducted at Macrogen (Seoul, Korea) using an Affymetrix® human 2.0ST gene chip (Illumina, San Diego, CA, USA). The data were summarized and normalized by the robust multiaverage (RMA) method in Affymetrix® Power Tools. The results of the gene-level RNA analysis were exported to perform a differentially expressed gene (DEG) analysis. For each DEG set, a hierarchical cluster analysis was conducted using complete linkage and Euclidean distance. Gene enrichment and functional annotation analyses of significant probe lists were performed via GO (http://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/). All data analyses and DEG visualizations were performed in R v. 3.3.3 (www.r-project.org).

2.11 | Statistical analysis

Statistical significance of data were analyzed using Prisitima v. 7.4. Multiple comparison methods, including Bartlett’s test, one-way analysis of variance followed by post hoc Dunnett’s test, Kruskal–Wallis (H) test followed by post hoc Dunn’s rank sum test, and Fisher’s exact test, were used to compare the data of the various treatment groups with those of the control. A p < 0.05 level was considered to be significant.

3 | RESULTS

3.1 | Characterization of E171

E171 had diameter of about 150 nm and a single anatase phase (PDF card no. 21-1272) in DW (Figure 1), and the diameter and shape were not notably altered either in AGF or cell culture media. In addition, in the same condition, E171 had highly crystalline structures with a 0.35-mm lattice spacing corresponding to the (101) plane of the anatase phase (Figure 1A). Meanwhile, the average hydrodynamic

### Table 1

| Day | Male | 10 mg/kg/day | 100 mg/kg/day | 1,000 mg/kg/day | Female | Control | 10 mg/kg/day | 100 mg/kg/day | 1,000 mg/kg/day |
|-----|------|-------------|--------------|----------------|--------|---------|-------------|--------------|----------------|
| 5   | 30.7 ± 1.88 | 32.1 ± 1.11 | 30.0 ± 1.15 | 31.4 ± 1.32 | 22.8 ± 1.11 | 23.6 ± 2.08 | 22.8 ± 1.45 | 22.0 ± 0.82 |
| 11  | 33.3 ± 1.23 | 35.8 ± 0.89** | 33.5 ± 1.76 | 35.7 ± 1.89** | 24.0 ± 1.45 | 23.9 ± 2.17 | 24.1 ± 1.28 | 23.2 ± 0.75 |
| 18  | 35.9 ± 1.42 | 37.8 ± 1.94* | 36.2 ± 2.05 | 39.1 ± 1.55** | 26.5 ± 1.27 | 26.9 ± 2.35 | 26.5 ± 2.19 | 25.1 ± 1.28 |
| 25  | 36.4 ± 1.25 | 37.9 ± 2.18 | 36.5 ± 2.08 | 40.0 ± 2.8** | 26.7 ± 1.53 | 26.6 ± 2.40 | 26.6 ± 2.42 | 25.7 ± 1.12 |
| 32  | 36.4 ± 0.79 | 36.9 ± 1.81 | 36.7 ± 2.21 | 39.5 ± 2.76** | 27.2 ± 1.81 | 27.1 ± 2.34 | 27.2 ± 3.81 | 26.2 ± 1.55 |
| 39  | 36.6 ± 1.04 | 38.1 ± 2.6 | 37.7 ± 2.19 | 38.7 ± 3.97 | 27.6 ± 2.20 | 27.1 ± 2.17 | 26.5 ± 2.75 | 25.7 ± 1.86 |
| 46  | 36.8 ± 0.85 | 38.4 ± 2.47 | 37.7 ± 1.90 | 40.4 ± 3.04** | 27.7 ± 1.63 | 28.3 ± 2.75 | 27.3 ± 2.88 | 26.4 ± 1.49 |
| 53  | 36.5 ± 1.74 | 37.7 ± 2.28 | 37.4 ± 2.73 | 40.1 ± 3.07** | 27.8 ± 2.19 | 27.6 ± 2.63 | 27.2 ± 2.58 | 26.3 ± 1.16 |
| 60  | 35.8 ± 1.26 | 37.5 ± 2.32 | 36.9 ± 1.94 | 38.4 ± 2.42** | 26.5 ± 2.17 | 26.0 ± 1.91 | 26.0 ± 1.82 | 24.7 ± 1.88 |
| 67  | 35.8 ± 0.81 | 37.9 ± 2.38 | 37.1 ± 2.33 | 38.0 ± 2.91* | 27.0 ± 2.74 | 26.2 ± 1.81 | 26.0 ± 1.99 | 24.8 ± 1.27 |
| 74  | 36.1 ± 0.90 | 37.6 ± 2.43 | 37.1 ± 1.85 | 38.1 ± 2.25 | 26.6 ± 1.80 | 26.1 ± 1.77 | 25.5 ± 1.91 | 24.9 ± 1.60 |
| 81  | 36.2 ± 1.10 | 37.3 ± 2.18 | 36.7 ± 1.88 | 38.6 ± 2.33** | 25.6 ± 2.24 | 24.7 ± 1.44 | 24.2 ± 1.66 | 24.9 ± 1.19 |
| 88  | 35.1 ± 0.89 | 37.7 ± 1.82** | 36.2 ± 2.30 | 38.5 ± 2.16** | 25.4 ± 1.58 | 23.7 ± 1.77 | 24.3 ± 1.82 | 24.1 ± 1.47 |

Note. Data are represented as mean ± SD.
*Significant differences from control group (p < 0.05).**Significant differences from control group (p < 0.01).
**TABLE 2** Summary of hematological changes after dosing E171 for 90 days

| Unit       | Male                                      | Female                                      |
|------------|-------------------------------------------|---------------------------------------------|
|            | Control                                   | 10 mg/kg/day                                | 100 mg/kg/day                               | 1,000 mg/kg/day |
| RBC (×10^6/μl) | 8.8 ± 0.34                               | 8.8 ± 0.50                                 | 8.7 ± 0.41                                 | 8.8 ± 0.35 |
| HGB (g/dl)  | 15.3 ± 0.52                               | 15.5 ± 0.73                                | 15.6 ± 0.62                                 | 15.5 ± 0.49 |
| HCT (%)     | 48.9 ± 1.60                               | 49.6 ± 2.40                                | 49.7 ± 1.66                                 | 49.3 ± 1.57 |
| MCV (fl)    | 55.5 ± 1.09                               | 56.4 ± 1.59                                | 57.1 ± 1.72                                 | 56.2 ± 1.30 |
| MCH (pg)    | 17.3 ± 0.40                               | 17.7 ± 0.78                                | 18.0 ± 0.68                                 | 17.6 ± 0.58 |
| MCHC (g/dl) | 31.2 ± 0.37                               | 31.2 ± 0.60                                | 31.5 ± 0.32                                 | 31.4 ± 0.36 |
| RET (%)     | 2.0 ± 0.18                                | 2.0 ± 0.26                                 | 2.1 ± 0.34                                 | 2.1 ± 0.23 |
| RETA (10^9/L) | 174.0 ± 19.30                            | 179.1 ± 23.54                              | 185.3 ± 27.26                               | 181.8 ± 19.75 |
| PLT (10^3/μl) | 1021.4 ± 132.04                          | 983.0 ± 165.33                             | 900.2 ± 211.36                              | 964.1 ± 130.45 |
| NEU (%)     | 12.9 ± 4.34                               | 18.7 ± 6.34                                | 15.3 ± 4.57                                 | 18.7 ± 6.64 |
| LYM (%)     | 82.0 ± 4.71                               | 75.2 ± 5.89*                               | 79.3 ± 4.75                                 | 75.2 ± 6.39* |
| EOS (%)     | 1.4 ± 0.45                                | 1.5 ± 0.60                                 | 1.3 ± 0.41                                 | 1.4 ± 0.27 |
| MON (%)     | 2.7 ± 0.92                                | 3.5 ± 1.13                                 | 3.1 ± 0.82                                 | 3.4 ± 0.49 |
| BAS (%)     | 0.3 ± 0.07                                | 0.4 ± 0.14                                 | 0.4 ± 0.10                                 | 0.3 ± 0.11 |
| LUC (%)     | 0.7 ± 0.57                                | 0.7 ± 0.36                                 | 0.7 ± 0.23                                 | 0.9 ± 0.74 |
| WBC (×10^3/μl) | 10.2 ± 1.58                               | 11.6 ± 3.13                                | 11.2 ± 2.40                                | 9.9 ± 2.63 |

**Note.** Data are represented as mean ± SD.

*Significant differences from control group (*p* < 0.05).
| Unit   | Male                  |                     |                     | Female                |                     |                     |
|--------|-----------------------|---------------------|---------------------|-----------------------|---------------------|---------------------|
|        | Control  | 10 mg/kg/day  | 100 mg/kg/day   | 1,000 mg/kg/day | Control  | 10 mg/kg/day | 100 mg/kg/day | 1,000 mg/kg/day |
|        |          |          |          |              |          |          |          |              |
| GLU (mg/dl) | 102.2 ± 18.18 | 113.5 ± 25.27 | 121.4 ± 22.60 | 110.6 ± 32.01 | 131.6 ± 29.97 | 125.2 ± 19.61 | 123.5 ± 15.81 | 133.1 ± 18.23 |
| BUN (mg/dl)  | 16.0 ± 2.13    | 17.4 ± 1.21    | 16.6 ± 1.37    | 16.7 ± 0.70    | 18.9 ± 2.94    | 20.2 ± 3.29    | 20.0 ± 3.79    | 20.9 ± 2.73    |
| CREA (mg/dl) | 0.5 ± 0.03     | 0.5 ± 0.03     | 0.5 ± 0.06     | 0.5 ± 0.02     | 0.6 ± 0.07     | 0.6 ± 0.08     | 0.6 ± 0.08     | 0.6 ± 0.05     |
| TP (g/dl)    | 6.6 ± 0.28     | 6.5 ± 0.28     | 6.4 ± 0.24     | 6.6 ± 0.28     | 7.3 ± 0.41     | 7.2 ± 0.27     | 7.4 ± 0.49     | 7.3 ± 0.51     |
| ALB (g/dl)   | 4.2 ± 0.16     | 4.1 ± 0.16     | 4.1 ± 0.15     | 4.2 ± 0.16     | 4.7 ± 0.31     | 4.7 ± 0.17     | 4.8 ± 0.32     | 4.7 ± 0.34     |
| A/G (ratio)  | 1.8 ± 0.08     | 1.7 ± 0.07     | 1.8 ± 0.14     | 1.8 ± 0.10     | 1.9 ± 0.11     | 1.9 ± 0.13     | 1.9 ± 0.15     | 1.8 ± 0.15     |
| AST (IU/L)   | 112.0 ± 15.04  | 112.7 ± 11.22  | 117.8 ± 21.85  | 111.6 ± 14.52  | 122.5 ± 10.08  | 124.2 ± 23.55  | 123.0 ± 38.43  | 120.5 ± 14.49  |
| ALT (IU/L)   | 26.0 ± 4.44   | 27.6 ± 3.16    | 30.9 ± 5.16    | 26.3 ± 3.21    | 33.5 ± 11.40   | 32.2 ± 14.81   | 28.6 ± 12.84   | 41.4 ± 21.00   |
| TBIL (mg/dl) | 0.1 ± 0.02     | 0.1 ± 0.01     | 0.1 ± 0.01     | 0.1 ± 0.01     | 0.1 ± 0.01     | 0.1 ± 0.02     | 0.1 ± 0.02     | 0.1 ± 0.02     |
| GGT (IU/L)   | 0.6 ± 0.23     | 0.7 ± 0.23     | 0.7 ± 0.15     | 0.6 ± 0.31     | 1.1 ± 0.50     | 0.8 ± 0.44     | 0.8 ± 0.42     | 1.1 ± 0.41     |
| ALP (IU/L)   | 241.5 ± 48.68  | 231.9 ± 45.50  | 315.0 ± 78.00  | 248.7 ± 48.63  | 172.9 ± 44.99  | 133.8 ± 24.81  | 163.0 ± 36.61  | 141.3 ± 29.71  |
| TCHO (mg/dl) | 69.1 ± 15.91   | 69.2 ± 11.27    | 63.3 ± 18.06    | 69.4 ± 11.00    | 86.5 ± 26.34   | 76.2 ± 10.89   | 91.4 ± 18.61   | 88.0 ± 19.24   |
| TG (mg/dl)   | 38.2 ± 19.32   | 55.0 ± 24.07    | 43.5 ± 13.43    | 50.5 ± 21.66    | 40.0 ± 25.48   | 32.2 ± 11.11   | 32.0 ± 15.08   | 30.6 ± 6.43    |
| Ca (mg/dl)   | 10.6 ± 0.35    | 10.9 ± 0.39    | 10.8 ± 0.32    | 10.9 ± 0.46    | 11.4 ± 0.44    | 11.4 ± 0.32    | 11.5 ± 0.46    | 11.6 ± 0.44    |
| IP (mg/dl)   | 9.3 ± 1.19     | 9.5 ± 1.16     | 9.7 ± 0.80     | 9.9 ± 1.02     | 8.3 ± 0.91     | 8.0 ± 0.64     | 7.7 ± 1.02     | 7.9 ± 1.25     |
| K (mmol/L)   | 7.6 ± 2.02     | 7.7 ± 1.35     | 8.0 ± 1.27     | 8.0 ± 1.28     | 7.2 ± 0.74     | 6.8 ± 1.26     | 6.5 ± 0.86     | 7.3 ± 0.71     |
| CK (IU/L)    | 694.6 ± 182.76 | 631.7 ± 152.11 | 646.7 ± 233.09 | 699.3 ± 181.56 | 553.4 ± 133.95 | 639.7 ± 165.06 | 581.1 ± 200.46 | 519.2 ± 156.93 |
| PL (mg/dl)   | 99.3 ± 18.92   | 106.2 ± 15.60  | 98.2 ± 20.94   | 105.4 ± 17.04  | 160.5 ± 43.99  | 140.1 ± 15.29  | 167.5 ± 28.55  | 158.1 ± 24.52  |
| Na (mmol/L)  | 143.6 ± 2.12   | 144.2 ± 2.15   | 143.5 ± 1.78   | 144.9 ± 1.60   | 143.7 ± 1.42   | 143.9 ± 1.45   | 144.7 ± 1.16   | 144.9 ± 1.60   |
| Cl (mmol/L)  | 100.0 ± 1.25   | 101.0 ± 1.63   | 100.7 ± 1.57   | 100.8 ± 1.23   | 102.3 ± 1.77   | 102.6 ± 1.35   | 102.3 ± 1.16   | 102.7 ± 1.57   |

*Significant differences from control group (p < 0.05).
### TABLE 4  Absolute organ weights after E171 treatment at 10, 100, and 1,000 mg/kg/day for 90 days

| Unit (g)                  | Male                          | Female                        |
|---------------------------|-------------------------------|-------------------------------|
|                           | Control 10 mg/kg/day 100 mg/kg/day 1,000 mg/kg/day | Control 10 mg/kg/day 100 mg/kg/day 1,000 mg/kg/day |
|                           | Adrenal glands                | Brain                         | Heart                        | Kidneys                      | Liver                        | Spleen                        | Thymus                       | Thyroid and parathyroid glands | Lung                          | Testes                       | Ovaries                       | Uterus/cervix                 |
|                           | 0.066 ± 0.0071 0.071 ± 0.0044 0.064 ± 0.0061 0.061 ± 0.0085 | 2.234 ± 0.1488 2.249 ± 0.0753 2.205 ± 0.0561 2.325 ± 0.0952 | 1.705 ± 0.1833 1.761 ± 0.1180 1.726 ± 0.1538 1.819 ± 0.0881 | 4.174 ± 0.5196 4.288 ± 0.5575 3.870 ± 0.4626 4.241 ± 0.3319 | 16.675 ± 2.1430 17.514 ± 2.4145 16.330 ± 2.4597 18.297 ± 2.8235 | 0.923 ± 0.1256 0.970 ± 0.1492 0.964 ± 0.1673 0.997 ± 0.1510 | 0.433 ± 0.0761 0.427 ± 0.0568 0.454 ± 0.1055 0.426 ± 0.0701 | 0.025 ± 0.0059 0.032 ± 0.0068 0.028 ± 0.0050 0.029 ± 0.0057 | 1.835 ± 0.2212 1.891 ± 0.1013 1.892 ± 0.2010 1.905 ± 0.1292 | 3.690 ± 0.3563 3.851 ± 0.1839 3.532 ± 0.4235 3.735 ± 0.4352 | -                           | -                            | -                            | -                            | -                            |

Note. Data are represented as mean ± SD.

### TABLE 5  Relative organ weights after E171 treatment at 10, 100, and 1,000 mg/kg/day for 90 days

| Unit (%body)               | Male                          | Female                        |
|---------------------------|-------------------------------|-------------------------------|
|                           | Control 10 mg/kg/day 100 mg/kg/day 1,000 mg/kg/day | Control 10 mg/kg/day 100 mg/kg/day 1,000 mg/kg/day |
|                           | Adrenal glands                | Brain                         | Heart                        | Kidneys                      | Liver                        | Spleen                        | Thymus                       | Thyroid and parathyroid glands | Lung                          | Testes                       | Ovaries                       | Uterus/cervix                 |
|                           | 0.011 ± 0.0017 0.012 ± 0.0012 0.011 ± 0.0009 0.010 ± 0.0014* | 0.384 ± 0.0342 0.379 ± 0.0306 0.379 ± 0.0380 0.371 ± 0.0345 | 0.292 ± 0.0158 0.296 ± 0.0201 0.295 ± 0.0156 0.289 ± 0.0159 | 0.714 ± 0.0618 0.717 ± 0.0524 0.660 ± 0.0536 0.674 ± 0.0432 | 2.846 ± 0.1569 2.926 ± 0.1890 2.774 ± 0.2335 2.886 ± 0.2117 | 0.158 ± 0.0210 0.163 ± 0.0228 0.164 ± 0.0187 0.158 ± 0.0217 | 0.075 ± 0.0144 0.072 ± 0.0119 0.077 ± 0.0164 0.068 ± 0.0125 | 0.004 ± 0.0013 0.005 ± 0.0014 0.005 ± 0.0007 0.005 ± 0.0010 | 0.314 ± 0.0213 0.319 ± 0.0330 0.324 ± 0.0387 0.303 ± 0.0163 | 0.635 ± 0.0819 0.649 ± 0.0590 0.603 ± 0.0595 0.593 ± 0.0638 | -                           | -                            | -                            | -                            | -                            |

Note. Data are represented as mean ± SD.

*Significant differences from control group (p < 0.05).
| TABLE 6 | Summary of microscopic findings at control and 1,000 mg/kg/day dosed rat for 90 days |
|----------|----------------------------------------------------------------------------------|
|          | Males (N = 10) | Females (N = 10) |
|          | Control       | 1,000 mg/kg | Control       | 1,000 mg/kg |
| Adrenal glands | Vacuolation, zona fasciculata | 2 (1>) | 3 (1>) | - | - |
|          | Granuloma     | 3 (2>) | 2 (2>) | - | 1 (1>) |
| Epididymis | Vacuolation, tubular epithelium | 1 (1>) | - | - | - |
| Eyes      | Atrophy, retina | - | 1 (2>) | - | 1 (2>) |
|          | Folds, retina | - | 1 (2>) | - | 1 (1>) |
| Heart     | Infiltration, mononuclear cell | 7 (1>) | 9 (1>) | 1 (1>) | - |
| Jejunum   | Granuloma, Peyer's patch | 1 (1>) | - | - | - |
| Kidneys   | Basophilia, tubules | 5 (1>) | 6 (1>) | 2 (1>) | 2 (1>) |
|          | 1 (2>) | 1 (2>) | - | - |
|          | Casts, hyaline | 1 (1>) | 2 (1>) | 1 (1>) | - |
|          | Cyst (s) | 2 (1>) | 2 (1>) | - | 1 (1>) |
|          | Dilation, pelvis | - | - | - | 1 (2>) |
|          | Dilation, tubules | 2 (1>) | - | 2 (1>) | 1 (1>) |
|          | Fibrosis, interstitial | 2 (1>) | 1 (1>) | 3 (1>) | 1 (1>) |
|          | - | 1 (2>) | - | - |
|          | Hyperplasia, urothelial cell | - | 1 (1>) | - | - |
|          | Infiltration, mononuclear cell, interstitial | 6 (1>) | 5 (1>) | 5 (1>) | 5 (1>) |
|          | Mineralization, corticomedullary junction | - | - | 2 (1>) | 4 (1>) |
|          | Mineralization, medulla | - | - | 1 (1>) | 1 (1>) |
| Liver     | Fibrosis | 1 (1>) | - | - | - |
|          | Infiltration, mononuclear cell | 6 (1>) | 10 (1>) | 8 (1>) | 8 (1>) |
|          | - | 2 (2>) | 1 (2>) | - |
|          | Necrosis, focal | 1 (1>) | 3 (1>) | 2 (1>) | 1 (1>) |
|          | Vacuolated area | 2 (1>) | 1 (1>) | - | - |
|          | - | 1 (2>) | 1 (2>) | - |
| Lung      | Aggregation, foamy macrophage | 1 (1>) | 3 (1>) | 2 (1>) | 6 (1>) |
|          | Eosinophilic crystal | - | 1 (1>) | - | - |
|          | Infiltration, mononuclear cell | - | - | 2 (1>) | - |
|          | Infiltration, mixed cell | 1 (1>) | 2 (1>) | - | 1 (1>) |
|          | Pigment, alveolar duct | - | 2 (2>) | - | - |
| Pancreas  | Atrophy, acinar cell | - | 1 (1>) | - | - |
|          | Fibrosis, islet | - | 2 (1>) | - | - |
|          | Infiltration, fat | - | 2 (1>) | - | - |
|          | Infiltration, mononuclear cell | - | 1 (1>) | 2 (1>) | 1 (1>) |
|          | Necrosis, single cell, acinar | 1 (1>) | - | 2 (1>) | 1 (1>) |
|          | - | 1 (2>) | 1 (2>) | - |
| Pituitary gland | Cyst(s), pars distalis | - | 1 (2>) | - | - |
|          | Vacuolation | 5 (1>) | 5 (1>) | - | - |
| Prostate  | Infiltration, mononuclear cell | 5 (1>) | 2 (1>) | - | - |
|          | - | 2 (2>) | - | - |
| Skin, inguinal | Dermatitis | - | - | 1 (2>) | - |
|          | Hyperplasia, epidermal | - | - | 1 (1>) | - |
| Spleen    | Extramedullary hemopoiesis | 1 (1>) | 1 (1>) | 1 (1>) | 1 (1>) |
| Testes    | Atrophy, tubules | 1 (1>) | - | - | - |
| Thymus    | Hyperplasia, epithelial cell | - | 2 (1>) | 6 (1>) | 6 (1>) |

Continues
diameters (Figure 1B) and the average surface charge values were quite different in the DW, AGF, and cell culture media (Figure 1C).

3.2 | Clinical observations

There were no treatment-related deaths. Abnormal clinical signs such as fur loss, scabs, scars, scratch wounds, swelling, and discolored urine were observed in the E171-dosed rats, but these effects were not dose dependent. Whereas body weight did not significantly differ among groups (Table S1), a significant increase in food consumption was observed only in male rats dosed at 1,000 mg/kg (Table 1).

3.3 | Blood analysis

As shown in Table 2, the proportion of lymphocytes in WBC slightly decreased in male rats administered the maximum E171 dose. There were no dose-related changes in any hematological (Table 2) and biochemical (Table 3) parameters, absolute organ weight (Table 4), and organ weight relative to body weight (Table 5).

3.4 | E171 accumulation in the stomach wall

There are no remarkable changes in macroscopic finding (data not shown), and dose-related histopathological lesions were also not detected (Table 6 and Figure S1). Meanwhile, we found E171 accumulation in the stomach wall of rats administered with 1,000 mg/kg of E171 for 90 days (Figures 2 and S2).

3.5 | The main routes of excretion

TiO$_2$ has low water solubility but can be dissolved in the acidic conditions such as gastric juice and lysozyme. NPs can also penetrate into

**TABLE 6** (Continued)

|                        | Males (N = 10) | Females (N = 10) |
|------------------------|---------------|------------------|
|                        | Control | 1,000 mg/kg | Control | 1,000 mg/kg |
| Thyroid glands         |         |            |         |            |
| Hypertrophy, follicular cell | 2 (1>) 3 (1>) | 1 (1>) 5 (1>) |
|                        | 3 (2>) 4 (2>) | 2 (2>) 1 (2>) |

Note. Ten rats per group were analyzed, and data were expressed as number of animals showing histological abnormalities (number of findings in individual).

**FIGURE 2** Microscopic findings in the stomach wall after treatment of E171. No remarkable findings were obtained from the 0 mg/kg dosed rat (control), whereas E171 accumulation was found at 1,000 mg/kg dosed rat.
| Elements | Tissues | Male | Control | 10 mg/kg/day | 100 mg/kg/day | 1,000 mg/kg/day | Female | Control | 10 mg/kg/day | 100 mg/kg/day | 1,000 mg/kg/day |
|---------|----------|------|---------|--------------|--------------|----------------|---------|---------|--------------|--------------|----------------|
| Ti      | Colon    | 23.77 ± 11.12 | 15.88 ± 2.30 | 15.09 ± 5.43 | 88.36 ± 68.06 |                 | 13.94 ± 1.07 | 19.19 ± 3.04 | 22.93 ± 15.92 | 69.55 ± 56.95 |
|         |         | 12.61 ± 3.78  | 9.06 ± 1.77  | 7.75 ± 1.30  | 10.46 ± 1.76  |                 | 8.04 ± 0.71  | 4.59 ± 4.39  | 8.30 ± 0.99  | 12.47 ± 4.16  |
|         | Spleen   | 7.96 ± 1.18   | 8.04 ± 1.25  | 8.66 ± 1.45  | 9.89 ± 1.66  |                 | 14.90 ± 2.27 | 20.08 ± 6.59 | 9.25 ± 8.58  | 13.73 ± 2.40  |
| Cu      | Colon    | 58.80 ± 14.37 | 47.34 ± 3.64  | 76.35 ± 71.18 | 50.36 ± 16.16 |                 | 45.09 ± 4.43 | 55.29 ± 7.54 | 51.10 ± 4.28 | 57.47 ± 7.60  |
|         | Kidney   | 278.11 ± 103.50 | 238.06 ± 69.65 | 217.14 ± 32.48 | 295.57 ± 70.45 |                 | 222.71 ± 70.10 | 183.85 ± 70.88 | 412.00 ± 151.31 | 215.23 ± 51.48 |
|         | Spleen   | 35.76 ± 2.07  | 36.53 ± 2.38  | 35.61 ± 1.75  | 40.73 ± 1.89  |                 | 45.34 ± 10.54 | 57.15 ± 19.99 | 44.93 ± 5.44 | 42.86 ± 3.39  |
| Zn      | Colon    | 760.24 ± 144.80 | 910.06 ± 377.72 | 696.52 ± 96.71 | 671.09 ± 216.05 |                 | 596.62 ± 39.06 | 728.81 ± 30.65 | 717.20 ± 49.86 | 754.97 ± 40.86 |
|         | Kidney   | 1011.85 ± 282.96 | 1057.20 ± 302.94 | 976.61 ± 436.19 | 914.13 ± 343.48 |                 | 821.49 ± 144.23 | 652.90 ± 117.79 | 840.28 ± 129.40 | 657.12 ± 52.68 |
|         | Spleen   | 632.37 ± 122.70 | 515.70 ± 78.48  | 537.50 ± 87.79  | 522.81 ± 45.71 |                 | 654.56 ± 219.42 | 734.65 ± 275.96 | 592.65 ± 97.92 | 566.02 ± 39.74 |
| Mn      | Colon    | 87.40 ± 56.83  | 59.85 ± 15.08  | 62.65 ± 32.41  | 98.20 ± 77.90  |                 | 54.41 ± 6.45  | 70.36 ± 49.15 | 52.77 ± 21.20 | 71.06 ± 34.11  |
|         | Kidney   | 22.52 ± 3.83   | 22.00 ± 2.85   | 21.96 ± 3.52   | 22.53 ± 1.59   |                 | 21.38 ± 4.15  | 17.76 ± 5.62  | 21.56 ± 3.15  | 20.99 ± 3.87  |
|         | Spleen   | 3.06 ± 4.55    | 0.00 ± 0.00    | 0.00 ± 0.00    | 5.23 ± 11.69   |                 | 8.96 ± 2.48   | 8.37 ± 5.56   | 4.95 ± 4.60   | 7.56 ± 0.59   |
| Fe      | Colon    | 557.43 ± 249.13 | 516.72 ± 144.24 | 487.96 ± 169.00 | 631.65 ± 216.66 |                 | 552.75 ± 96.13 | 594.99 ± 139.47 | 723.53 ± 195.85 | 685.56 ± 197.22 |
|         | Kidney   | 2637.77 ± 786.48 | 3076.00 ± 278.06 | 2532.63 ± 301.48 | 2671.22 ± 369.98 |                 | 3409.95 ± 931.98 | 2738.79 ± 1021.02 | 3669.08 ± 1026.18 | 3189.61 ± 589.94 |
|         | Spleen   | 4565.20 ± 14827.78 | 4097.14 ± 6214.30 | 4122.01 ± 12560.41 | 3772.32 ± 8055.82 |                 | 101149.21 ± 15471.51 | 102838.33 ± 45252.36 | 85368.19 ± 16217.55 | 79027.45 ± 4611.15 |
| Al      | Colon    | 366.51 ± 87.04  | 336.16 ± 62.58  | 316.55 ± 87.90  | 421.37 ± 165.21 |                 | 264.36 ± 53.29 | 290.99 ± 110.45 | 292.61 ± 29.18 | 324.16 ± 83.37 |
|         | Kidney   | 348.47 ± 103.00 | 363.93 ± 123.26 | 252.40 ± 31.65 | 297.47 ± 44.71 |                 | 327.22 ± 31.03 | 336.02 ± 86.53 | 339.19 ± 47.30 | 285.81 ± 32.00 |
|         | Spleen   | 353.85 ± 45.61  | 325.20 ± 73.09  | 382.98 ± 79.61  | 524.26 ± 349.04 |                 | 291.33 ± 61.33 | 358.79 ± 108.06 | 334.39 ± 44.73 | 289.92 ± 41.56 |

Note. The values are expressed as mean ± SD.
cells in the bloodstream, and the damaged red blood cells are eliminated via the spleen. In the current study, we measured Ti concentrations in the colons, kidneys, and spleens harvested from all rats at necropsy. Importantly, the Ti concentration clearly increased only in the colons of both sexes administered with 1,000 mg/kg of E171 compared with the control, indicating that the colon is the main excretion route (Table 7). In addition, TEM images revealed that E171 accumulates in the cytosol and nuclei of various cells comprising the colon tissue and forms lamella-like structures (Figure 3). Ti accumulation can also affect the distribution of elements cross-binding with it or participating in the antioxidant response, and we found here that the colonic zinc (Zn) concentration increases in female rats exposed to E171 compared with the control. In addition, SOD proteins play a central role in inhibiting xenobiotic-induced oxidative damage and subsequent apoptosis (Fukai & Ushio-Fukai, 2011); thus, we assessed the effects of E171 on expression of SOD-1, SOD-2, and cytochrome C protein in the colonic tissues of rats in control and the maximum-dosed group. Interestingly, the expression of SOD-1 and SOD-2 proteins was clearly downregulated in the colonic tissues of both sexes and female rats, respectively. However, that of cytochrome C protein was not significantly different between groups (Figure 4).

3.6 | Effects of E171 on systemic immune response

Given that the proportion of lymphocytes in WBC decreased in rats administered with the maximum dose compared with control, we measured the GM-CSF and immunoglobulin concentrations in the blood. Importantly, the levels of GM-CSF (female) and IgM (both sexes) significantly reduced in rats administered with 1,000 mg/kg of E171 for 90 days compared with the control (Figure 5), whereas IgG, IgA, or IgE levels were not different between groups. The GM-CSF level was 46.3 ± 12.1 and 27.3 ± 9.3 pg/ml in the control and the maximum-dosed female group, respectively. The IgM level was 2,123.6 ± 176.3 and 1,926.6 ± 77.3 ng/ml in the male and female rats of the control group, respectively, whereas it was 1,886.9 ± 87.7 and

**FIGURE 3** Transmission electron microscope images of the colon tissue after treatment with 1,000 mg/kg of E171. Accumulation in cytosol (A, B) and the formation of lamella-like structure (B). E171 was also found in cytosol (C) and intracellular organelle such as mitochondria (D) [Colour figure can be viewed at wileyonlinelibrary.com]
1,696.5 ± 152.7 ng/ml for the male and female rats in the maximum-dosed group, respectively.

3.7 | Cellular response following accumulation of E171

E171 accumulated in cells lining the stomach wall of rats administered at a 1,000 mg/kg dose for 90 days; thus, we investigated the possible toxic mechanism following accumulation of E171 using AGS cells, a human stomach epithelial cell line. We first confirmed that E171 localizes in the perinuclear region of the AGS cells on 24 h after treatment (40 μg/ml) (Figures 6 and S2). The expression of the ER stress- (calnexin, IRE-1a, Bip, PERK, and Ero-1a), pyroptosis- (caspase-1, IL-18, and NALP3), autophagy- (p62 and LAMP-1), and iron metabolism-related (ferritin heavy chain) (Mumbauer, Pascual, Kolotuev, & Hamaratoglu, 2019) proteins was enhanced in the E171-treated cells compared with control accompanied by the peroxisomal (catalase) and...
FIGURE 5  (A) Effects on secretion of GM-CSF and (B) immunoglobulin (IgG, IgA, IgE, and IgM) in serum. All experiments were performed independently three times using two wells per sample, and the results were represented as mean ± SD. GM-CSF, granulocyte-macrophage colony-stimulating factor [Colour figure can be viewed at wileyonlinelibrary.com]
mitochondrial antioxidant (SOD-2) (Pias et al., 2003; Walton & Pizzitelli, 2012). Meanwhile, there were no significant changes in both the level of CHOP and phospho-JNK (which play in ER stress-triggered apoptosis; Mozzini, Cominacini, Garbin, & Fratta Pasini, 2017; Müller-Taubenberger et al., 2011) and the cytosolic antioxidant protein (SOD-1) and conversion of LC3B-I into LC3B-II (completion of autophagosome) (Figure 7). In addition, the expression levels of various microRNAs and unknown genes were markedly altered in the E171-exposed cells relative to the control (Table 8). More interestingly, cell death was not observed even at the highest concentration tested (40 μg/ml).

4 | DISCUSSION

The potential risks of nanoscale particles on the environment and human health have been continuously issued along with the great importance in future industry, and thus, nanotechnology has often been compared with a double-edged sword (Kashanian, Habibi-Rezaei, Bagherpour, Seyedarabi, & Moosavi-Movahedi, 2017; Patni & Bhatia, 2008; Solaiman et al., 2019). Meanwhile, all substance is potentially harmful to human health when it accumulates at sufficiently high concentrations, which can disturb biological homeostasis, as was first expressed by Paracelsus, a Swiss physician. Furthermore, nanoscale particles have unique physicochemical properties that differ from those of the bulk forms of the same materials. Therefore, although available information for the microscale particles is enough, biostability, interactions with biological systems, biodistributions, health effects, and the possible toxic mechanism should be carefully re-evaluated for the nanoscale particles (Fadeel & Garcia-Bennett, 2010). Here, we found that E171 is insoluble in DW, a vehicle used for dosing, and the physicochemical properties were not substantially altered in AGF or cell culture media. In addition, when orally dosed 10, 100, and 1,000 mg/kg to rats for 90 days in accordance with an OECD test guideline (OECD, 2018), any significant tissue damage was not found even in the maximum dose. Meanwhile, contrary to the
| Gene_Symbol | mRNA accession | 1st | 2nd | 3rd | AV  | SD  |
|------------|----------------|-----|-----|-----|-----|-----|
| NONHSAT092002 | 1.48 | 2.04 | 2.73 | 2.08 | 0.62 |
| NONHSAT118330 | 2.34 | 2.36 | 1.09 | 1.93 | 0.73 |
| NONHSAT118330 | 2.34 | 2.36 | 1.09 | 1.93 | 0.73 |
| NONHSAT118330 | 2.34 | 2.36 | 1.09 | 1.93 | 0.73 |
| ENST00000516096 | 1.25 | 3.36 | 1.08 | 1.90 | 1.27 |
| ENST00000458982 | 1.38 | 2.61 | 1.68 | 1.89 | 0.64 |
| ENST0000364752 | 1.60 | 1.41 | 2.65 | 1.89 | 0.67 |
| NONHSAT024019 | 1.27 | 1.64 | 2.69 | 1.87 | 0.73 |
| 1.06 | 3.33 | 1.16 | 1.85 | 1.28 |
| ENST00000410533 | 2.00 | 1.99 | 1.56 | 1.85 | 0.25 |
| ENST0000391025 | 2.54 | 1.79 | 1.05 | 1.79 | 0.74 |
| ENST00000516920 | 1.41 | 2.03 | 1.90 | 1.78 | 0.33 |
| NONHSAT027452 | 1.83 | 2.20 | 1.25 | 1.76 | 0.48 |
| ENST0000431432 | 1.29 | 1.21 | 2.77 | 1.76 | 0.88 |
| ENST0000383927 | 1.15 | 2.96 | 1.16 | 1.75 | 1.04 |
| ENST0000527159 | 1.15 | 2.84 | 1.22 | 1.74 | 0.95 |
| TAS2R50 | NM_176890 | 2.10 | 1.71 | 1.37 | 1.73 | 0.37 |
| NonHSAT025494 | 2.21 | 1.06 | 1.89 | 1.72 | 0.60 |
| Vcx3b | NM_001001888 | 2.62 | 1.16 | 1.36 | 1.72 | 0.79 |
| ENST0000426812 | 1.77 | 1.88 | 1.44 | 1.70 | 0.23 |
| NonHSAT112940 | 1.38 | 1.72 | 1.97 | 1.69 | 0.30 |
| NonHSAT021981 | 1.24 | 1.65 | 2.18 | 1.69 | 0.47 |
| ENST0000611160 | 1.80 | 2.18 | 1.06 | 1.68 | 0.57 |
| Rny4p13 | OTTHUMT00000448959 | 1.37 | 1.43 | 2.24 | 1.68 | 0.49 |
| ENST0000362700 | 1.17 | 1.81 | 2.00 | 1.66 | 0.43 |
| M1R1293 | NR_031625 | 1.46 | 1.65 | 1.88 | 1.66 | 0.21 |
| M1R4755 | NR_039911 | 1.28 | 2.41 | 1.29 | 1.66 | 0.65 |
| ENST0000435608 | 2.42 | 1.48 | 1.07 | 1.66 | 0.69 |
| NonHSAT023929 | 1.65 | 1.51 | 1.78 | 1.64 | 0.14 |
| C1Qtnf9 | NM_001303137 | 1.57 | 1.93 | 1.42 | 1.64 | 0.26 |
| ENST0000516908 | 1.43 | 1.76 | 1.73 | 1.64 | 0.18 |
| Loc105378702 | Xr_947304 | 1.16 | 2.22 | 1.54 | 1.64 | 0.54 |
| NonHSAT008674 | 1.87 | 1.15 | 1.88 | 1.63 | 0.42 |
| ENST0000560378 | 2.09 | 1.71 | 1.08 | 1.63 | 0.51 |
| ENST0000459031 | 2.39 | 1.36 | 1.13 | 1.62 | 0.67 |
| NonHSAT063651 | 1.50 | 1.80 | 1.58 | 1.62 | 0.15 |
| ENST0000459498 | 1.39 | 2.22 | 1.24 | 1.62 | 0.53 |
| Sdcbpp2 | BC030617 | 1.32 | 1.03 | 2.49 | 1.61 | 0.77 |
| Loc105374104 | Xr_924474 | 1.47 | 1.90 | 1.48 | 1.61 | 0.25 |
| NonHSAT028951 | 1.37 | 1.13 | 2.33 | 1.61 | 0.63 |
| NonHSAT017609 | 1.28 | 1.66 | 1.86 | 1.60 | 0.29 |
| ENST0000516132 | 1.25 | 1.06 | 2.47 | 1.59 | 0.76 |
| NonHSAT108903 | 1.12 | 1.96 | 1.67 | 1.59 | 0.43 |
| uc021rjm.1 | 1.74 | 1.17 | 1.84 | 1.59 | 0.36 |
| ENST0000627818 | 1.17 | 1.17 | 2.42 | 1.58 | 0.72 |

(Continues)
| Gene Symbol | mRNA accession | 1st  | 2nd  | 3rd  | AV   | SD   |
|-------------|----------------|------|------|------|------|------|
| NONHSAT119100 | 1.56 | 1.80 | 1.39 | 1.58 | 0.21 |
| ENST00000408283 | 1.61 | 1.91 | 1.21 | 1.58 | 0.35 |
| ENST00000516422 | 1.21 | 1.48 | 2.02 | 1.57 | 0.41 |
| NONHSAT141442 | 1.46 | 1.63 | 1.60 | 1.56 | 0.09 |
| NR_039783 | 1.27 | 1.81 | 1.61 | 1.56 | 0.28 |
| NR_039783 | 1.27 | 1.81 | 1.61 | 1.56 | 0.28 |
| NR_039783 | 1.27 | 1.81 | 1.61 | 1.56 | 0.28 |
| NR_039783 | 1.27 | 1.81 | 1.61 | 1.56 | 0.28 |
| NR_039783 | 1.27 | 1.81 | 1.61 | 1.56 | 0.28 |
| ENST00000411051 | 1.57 | 1.84 | 1.25 | 1.55 | 0.29 |
| CYP4F3 | NM_000896 | 1.55 | 1.59 | 1.52 | 1.55 | 0.03 |
| ENST00000391122 | 1.50 | 1.08 | 2.07 | 1.55 | 0.50 |
| NONHSAT072183 | 1.10 | 1.95 | 1.59 | 1.55 | 0.43 |
| LOC284412 | NR_029390 | 1.15 | 2.04 | 1.42 | 1.54 | 0.46 |
| LOC100652833 | XM_011543783 | 1.54 | 1.96 | 1.10 | 1.53 | 0.43 |
| LOC101927522 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| POTEH | NM_001136213 | 1.00 | 2.07 | 1.51 | 1.53 | 0.53 |
| KRTAP1-4 | NM_001257305 | 1.31 | 1.68 | 1.58 | 1.52 | 0.19 |
| LINCO00240 | NR_026775 | 1.20 | 1.84 | 1.53 | 1.52 | 0.32 |
| ENST00000410638 | 1.20 | 2.12 | 1.23 | 1.52 | 0.53 |
| ENST00000362665 | 1.62 | 1.11 | 1.81 | 1.51 | 0.36 |
| SNORA14A | NR_002955 | 1.39 | 1.92 | 1.23 | 1.51 | 0.36 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| KGF2 | POTEH | NM_001136213 | 1.00 | 2.07 | 1.51 | 1.53 | 0.53 |
| LINCO00240 | NR_026775 | 1.20 | 1.84 | 1.53 | 1.52 | 0.32 |
| SNORA14A | NR_002955 | 1.39 | 1.92 | 1.23 | 1.51 | 0.36 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| KGF2 | POTEH | NM_001136213 | 1.00 | 2.07 | 1.51 | 1.53 | 0.53 |
| LINCO00240 | NR_026775 | 1.20 | 1.84 | 1.53 | 1.52 | 0.32 |
| SNORA14A | NR_002955 | 1.39 | 1.92 | 1.23 | 1.51 | 0.36 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |
| LOC105375112 | XR_430235 | 1.27 | 1.31 | 2.00 | 1.53 | 0.41 |

List of genes that downregulated >1.5-fold compared with control

| Gene Symbol | mRNA accession | 1st  | 2nd  | 3rd  | AV   | SD   |
|-------------|----------------|------|------|------|------|------|
| ENST0000051605 | −1.63 | −1.14 | −5.04 | −2.60 | 2.12 |
| MIR3908 | NR_037470 | −1.02 | −2.18 | −4.25 | −2.48 | 1.63 |
| ENST00000408512 | −1.06 | −2.64 | −2.39 | −2.03 | 0.85 |
| KGF2 | NR_003670 | −1.25 | −3.30 | −1.40 | −1.99 | 1.14 |
| ENST00000416861 | −1.20 | −1.71 | −2.88 | −1.93 | 0.86 |
| ENST00000362381 | −2.13 | −2.50 | −1.16 | −1.93 | 0.69 |
| ENST00000362715 | −1.82 | −2.28 | −1.62 | −1.91 | 0.34 |
| MIR4302 | NR_036188 | −2.42 | −2.16 | −1.12 | −1.90 | 0.69 |
| ENST00000384211 | −1.22 | −2.37 | −2.07 | −1.89 | 0.60 |
| NONHSAT099609 | −1.06 | −1.92 | −2.65 | −1.88 | 0.79 |
| ENST00000517233 | −1.40 | −1.95 | −2.10 | −1.82 | 0.36 |
| NONHSAT073976 | −1.29 | −2.10 | −2.02 | −1.80 | 0.45 |
| ENST00000411039 | −1.04 | −2.32 | −2.00 | −1.78 | 0.67 |
| ENST00000391324 | −1.63 | −2.12 | −1.59 | −1.78 | 0.30 |
| ENST00000411248 | −1.25 | −2.47 | −1.61 | −1.78 | 0.63 |
| ENST00000384451 | −1.82 | −1.82 | −1.62 | −1.75 | 0.11 |

(Continues)
Titanium Dioxide Manufacturers’ Association’s claim that it is not readily absorbed by the human body, E171 markedly penetrated and accumulated in the stomach walls of rats exposed to 1,000 mg/kg of E171. Moreover, E171 penetrated the plasma membranes of AGS cells derived from stomach epithelial tissue and also formed lamella-like structures (Cheong et al., 2007) and autophagosome-like vacuoles in the colon and the AGS cells, respectively. Lysosomes serve for both degradation of materials taken up from outside the cells and digestion of the cell’s own components with enzymes that are active at the acidic conditions. In addition, lamella bodies and autophagosomes are

### Table 8 (Continued)

| Gene Symbol | mRNA accession | 1st  | 2nd  | 3rd  | AV   | SD  |
|-------------|----------------|------|------|------|------|-----|
| ENST0000408498 | NONHSAT016100 | −1.06 | −1.71 | −2.47 | −1.75 | 0.71 |
| IGL5 | OTTHUMT00000321640 | −1.38 | −2.46 | −1.39 | −1.74 | 0.62 |
| NONHSAT040114 | ENST0000459100 | −1.03 | −2.07 | −2.02 | −1.71 | 0.59 |
| ENST0000517232 | NONHSAT104835 | −1.96 | −1.71 | −1.42 | −1.70 | 0.27 |
| NONHSAT126341 | ENST0000517038 | −1.04 | −1.86 | −2.10 | −1.67 | 0.56 |
| MIR4718 | NR_039869 | −1.51 | −1.11 | −2.34 | −1.65 | 0.63 |
| ANKRD30BP2 | ENST0000507630 | −1.11 | −2.12 | −1.72 | −1.65 | 0.51 |
| MIR811A1 | NONHSAT121656 | −1.19 | −1.51 | −2.17 | −1.63 | 0.50 |
| LINCO1372 | NR_108104 | −1.68 | −1.15 | −2.04 | −1.62 | 0.45 |
| MIR1203 | NONHSAT118989 | −1.28 | −1.23 | −2.32 | −1.61 | 0.60 |
| MIR1203 | NONHSAT112144 | −1.77 | −1.60 | −1.41 | −1.59 | 0.18 |
| MIR811A1 | NM_001256532 | −1.88 | −1.62 | −1.26 | −1.59 | 0.31 |
| MIR1203 | ENST0000516265 | −1.32 | −1.07 | −2.36 | −1.58 | 0.69 |
| CEP152 | XM_011521374 | −1.26 | −1.33 | −2.14 | −1.58 | 0.49 |
| MIR3160-1 | NONHSAT088447 | −1.36 | −1.77 | −1.58 | −1.57 | 0.21 |
| MIR3160-1 | NR_036117 | −1.38 | −1.45 | −1.86 | −1.56 | 0.26 |
| MIR3160-1 | NONHSAT126341 | −1.24 | −1.14 | −2.31 | −1.56 | 0.65 |
| HNRNPA3 | NM_194247 | −1.04 | −2.56 | −1.06 | −1.56 | 0.44 |
| MIR3663 | NONHSAT137860 | −1.09 | −1.69 | −1.86 | −1.55 | 0.41 |
| LOC10193818 | ENST00005383917 | −1.33 | −2.04 | −1.26 | −1.54 | 0.43 |
| ARHGEF25 | ENST00005464602 | −1.04 | −2.06 | −1.53 | −1.54 | 0.51 |
| LOC102724571 | ENST00004365420 | −1.01 | −1.61 | −2.00 | −1.54 | 0.50 |
| NR_037436 | NR_132738 | −1.39 | −1.48 | −1.73 | −1.53 | 0.19 |
| ARHGEF25 | ENST0000442086 | −1.48 | −1.47 | −1.65 | −1.53 | 0.10 |
| NONHSAT087882 | NONHSAT087882 | −1.30 | −1.78 | −1.50 | −1.53 | 0.24 |

Note. Data indicate the values of three independent experiments, average (AV), and SD.
associated with early defense mechanisms against foreign bodies and are characteristic of various lysosomal storage diseases. Therefore, we propose that the NOAEL of E171 for 90-day repeated oral dosing is less than 1,000 mg/kg.

According to epidemiological evidence, the incidences of colorectal and gastric cancer are globally increasing. Moreover, as mentioned above, inhaled TiO2 NPs were classified as a Group B2 carcinogen by IARC, and France has banned the use of E171 as a food additive until the safety has been empirically and clinically verified. Although ingested NPs are excreted mainly via the feces, they can be resorbed from the kidney depending on their biostability, and NPs that have entered immune cells in the bloodstream can accumulate in the spleen. A previous study suggested that 90-day orally dosed TiO2 NPs caused inflammatory response and liver dysfunction via oxidative stress accompanying notable accumulation in the liver (Cui et al., 2012). A 100-day repeated dosed E171 also promoted microinflammation and initiated preneoplastic lesions in the colon, and it altered the expression of genes involved in innate and adaptive immune response and oxidative stress (Blevins et al., 2019). In the current study, Ti concentrations clearly increased in the colonic tissues, altering the tissue level of the antioxidant protein (SOD-1 and SOD-2). We also found that the proportion of lymphocytes in WBC was clearly lower in rats exposed to E171 compared with the control and that the GM-CSF and IgM levels notably reduced in the blood of rats in the same group. GM-CSF regulates myelopoiesis in physiological steady state and modulates immunity under inflammatory conditions including autoimmune disease (Becher, Tugues, & Greter, 2016; Bhattacharya et al., 2015). In addition, IgM is the antibody that is produced mainly in the spleen in response to initial antigen exposures. Hence, we hypothesize that part of E171 may be dissolved under the acidic conditions of the stomach and that the rest may form aggregates with diet or other particles. Also, it may affect antioxidant capacity being resorbed during the stay in the colon (Lomer, Thompson, & Powell, 2002; Park, Yoon, Choi, Yi, & Park, 2009; Proquin et al., 2017). In addition, we hypothesize that chronic E171 intake might impair host’s defense function against foreign bodies.

The reassessment of E171 as a putative carcinogen may be crucial in the determination and establishment of its safety (Armand et al., 2016; Falck et al., 2009; Kang, Kim, Lee, & Chung, 2008; Warheit, Brown, & Donner, 2015). Accumulated clinical and empirical evidence has demonstrated that TiO2 NPs induce ER stress by promoting oxidative stress. In addition, chronic ER stress may be associated with tumor development by triggering inflammatory responses (Lin, Jiang, Chen, Zhao, & Wei, 2019), and it could also be involved in immunosuppression (Salminen, Kaarniranta, & Kauppinen, 2020). In this study, we found that E171 did not affect the expression of SOD-1 and SOD-2 proteins in AGS cells. In addition, E171 penetrated the cells comprising the colon tissue and localized to the perinuclear regions of AGS cells. Meanwhile, dead cells were not observed even in cells treated at the maximum concentration (40 μg/ml). Furthermore, the expression of ER stress-related proteins increased in E171-treated cells compared with control, and microarray analysis demonstrated that expression of several microRNAs is the most affected following exposure to E171. In particular, the expression of microRNA 3908 was the most downregulated. More interestingly, many of the affected genes were those whose function is unknown. MicroRNAs are noncoding RNAs that are involved in post-transcriptional regulation by affecting both the stability and translation of mRNA. Previous studies have suggested that microRNA 3908 inhibits cancer progression by inducing apoptosis (Liu, Chen, & Zhang, 2017) and that bitter-taste receptor genes (such as TAS2R50) can be involved in progression of colorectal neoplasia (Schembre, Cheng, Wilkens, Albright, & Marchand, 2013). In addition, SOD-2 transforms toxic mitochondrial superoxide into nontoxic products, inhibiting apoptosis, and ferritin heavy chain also protects cells against free radical accumulation (Mumbauer, Pascual, Kolotuev, & Hamaratoglu, 2019). Furthermore, ER stress can initiate pyroptosis and inflammasome formation (Lebeauin et al., 2015). Therefore, further study is required to elucidate the adverse health effects following chronic accumulation in the stomach and colons (Proquin et al., 2017).

In conclusion, we suggest that NOAEL of 90-day repeated orally dosed E171 is between 100 and 1,000 mg/kg for both sexes of rats and that further study is needed to clarify the possible carcinogenesis following the chronic accumulation in the colons.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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