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### **Global Toxicology 2020: Food grade titanium dioxide (E171) induces systemic toxicity in mice fed with a high fat diet - Medina-Reyes EI1, UNAM. México**

#### **Abstract**

Food-grade titanium dioxide (E171) is used as additive in food products for human consumption since 1960. Recently, it was demonstrated that intragastric E171 administration enhanced colon tumor formation in experimental models and induced histological alterations in testis. This rises further concerns of E171 as a possible trigger or synergistic diet component of pathologies. Regarding to diet, the increased fat intake jumped 22.8% from 1993 to 2013 worldwide and together with the E171 as a facilitator of colon tumor formation, brings the hypothesis of exacerbated toxicity induced by E171 after oral consumption in mice fed with a high fat diet (HFD). This work aimed to evaluate the toxicity in colon, liver and testis induced by oral administration of 5 mg/kgbw E171 in mice fed with HFD for 16 weeks. Results demonstrated that mice fed with regular diet + E171 and mice fed with a HFD had higher number of adenomas, hypertrophy and hyperplasia of goblet cells and deregulation in mucin expression in colon. However, colon alterations were not exacerbated in mice fed with HFD + E171. HFD induced macrovesicular steatosis in the liver and mice fed with HFD + E171 had microvesicular steatosis which has a worse clinical prognosis. HFD mice and HFD + E171 mice had disruption of testis architecture and decrease in spermatozoa concentration and motility in the same extent. We conclude that E171 and HFD induced severe toxicity in colon and testis in the same extent but liver steatosis is

exacerbated by combination of HFD + E171 administration.

**Keywords:** biofilm, gut microbiota, immune cells, inflammation, titanium Dioxide.

#### **Introduction**

Bacterial species that occupy the colon interface with the host, advancing the turn of events and capacity of safe cells locally and foundationally. These connections are interceded by bacterially inferred metabolites, for example, short-chain unsaturated fats (SCFAs), which have been distinguished as basic inducers of invulnerable subsets (1–3) key for shielding mice from ailment advancement (2–5), underlining the job of the microbiota in gut homeostasis and host wellbeing.

The colonic epithelium goes about as a physical hindrance between the host and the gut microbiota. The emission of bodily fluid by challis cells gives a hindrance to microbial invasion. Further, Paneth cells discharge antimicrobial peptides that secure against pathogen intrusion just as direct gut microbiota structure (6). Articulation of tight intersection proteins by enterocytes likewise confines bacterial entrance. Epithelial capacity can be controlled by the gut microbiota through SCFAs, by animating bodily fluid creation (7) and tight intersection get together (8). Interestingly, dysbiosis, set apart by

impeding changes in gut microbiota arrangement, triggers expanded gut porousness and gut aggravation (9). Changes in antimicrobial peptide creation, bodily fluid layer thickness and additionally epithelial penetrability have been embroiled in the improvement of an expansive scope of illnesses, for example, colitis and colorectal malignant growth (10). These infections have additionally been connected to anomalous communications between the host epithelium and the gut microbiota through the development of biofilm. Biofilms comprise of totals of follower and planktonic microscopic organisms ensured by an extracellular network and have been seen in the proximal colon of patients determined to have such ailments (11). The components behind the arrangement and the job of biofilm in the gut are not completely seen, however biofilm arrangement has been appeared to affect both infection advancement and goals. Both in a colitis rodent model and in people, biofilm in the colon has been appeared to encourage pathobiont adherence to the epithelium and translocation to the host (12, 13). In human fiery entrail ailment, biofilm development at the site of epithelial injury recuperating has been appeared to contrarily influence mending by disabling epithelialization and tissue fix (14). At long last, an ongoing report has demonstrated that vaccination of without germ mice with biofilm positive human colon inocula was cancer-causing (15).

The recognizable proof of ecological variables that can influence gut homeostasis is in this way a basic initial phase in forestalling the improvement of purported "western way of life maladies," incorporating immune system, unfavorably susceptible and metabolic ailments. A wide scope of natural elements can influence gut homeostasis, with diet arrangement being the significant driver (16). Western-like weight control plans

enhanced in fat and basic sugars and lacking in dietary fiber have been appeared to trigger dysbiosis, expands gut penetrability and irritation (16). While the effect of these macronutrients on gut homeostasis has been broadly contemplated (17), the job of food added substances common in handled food remains ineffectively characterized. Food added substances are utilized to improve the surface, safeguarding and style of food. Food grade titanium dioxide (TiO<sub>2</sub>) or E171, is a brightening specialist present in more than 900 generally expended food items. The normal grown-up devours somewhere in the range of 0.7 and 5.9 mg of TiO<sub>2</sub> per kg of body weight (BW) every day for an amazing duration and kids are the most uncovered, expending up to 32.4 mg TiO<sub>2</sub>/kg BW/day in maximally uncovered people (18). Regardless of the way that administrative bodies don't characterize exacting rules around its utilization, new proof from creature examines has risen, featuring that TiO<sub>2</sub> may potentiate malignant growth improvement (19) and fuel incendiary gut ailment (20).

The impact of TiO<sub>2</sub> on gut homeostasis is ineffectively seen at this point proof proposes that TiO<sub>2</sub> collaborates with gut epithelial cells. In vivo and in vitro examinations have shown the collection of TiO<sub>2</sub> in the bodily fluid layer (21) and its take-up by colonic epithelial cells (22, 23). An investigation in rodents has indicated that TiO<sub>2</sub> influences insusceptible cells in the Peyer's patches related with a diminished administrative T cell extent (19). In any case, the effect of TiO<sub>2</sub> on colonic safe cells, the site where microbiota is the densest, has never been researched. While the effect of TiO<sub>2</sub> on the colonic microbiota has been recently explored in a transient report (2.5 mg TiO<sub>2</sub>/kg BW/day for multi week) (24) and utilizing a high portion (100 mg TiO<sub>2</sub>/kg BW/day) for as long as about a month (25), the effect of TiO<sub>2</sub> on the small

digestive tract microbiota is obscure.

The point of the current examination is to build up the impacts of food grade TiO<sub>2</sub> on gut homeostasis in vivo. We explored the effect of physiological dosages (2 and 10 mg TiO<sub>2</sub>/kg BW/day) and a high portion of TiO<sub>2</sub> (50 mg TiO<sub>2</sub>/kg BW/day) on mouse colonic and small digestive tract microbiota structure and capacity, epithelial capacity and mucosal aggravation following 3 a month of treatment through drinking water.

## **MATERIALS AND METHODS**

### **E171 Characterization**

#### **Size and Morphology**

Food grade TiO<sub>2</sub> was purchased from All Color Supplies PTY. Ordinary hydrodynamic estimation, polydispersity rundown and zeta ability of the TiO<sub>2</sub> nanoparticles dispersed in drinking water were settled with a Malvern Zetasizer Nano ZS at 25°C. The dissipating was assessed on numerous occasions for both size and zeta potential. The size allocation and condition of the TiO<sub>2</sub> nanoparticles dissipated in mice drinking water were settled using a NanoSight NS300 (furnished with a sCMOS camera) at 25°C. The dispersing was evaluated on various occasions (1 min for each estimation). The size spread and condition of the TiO<sub>2</sub> nanoparticles dissipated in drinking water were also explored using a Zeiss Ultra Plus separating electron amplifying focal point worked at an enlivening voltage of 10 kV. A drop of the nanoparticle dispersing was allowed to dry on a stub, after which 20 Å of platinum metal was vacillate secured onto the stub under vacuum to prevent charging. Crystal Structure and Elemental Composition

A D8 Advance Bruker diffractometer was utilized to lead the X-beam powder diffraction (XRD) examination in a level plate geometry utilizing Ni-separated Cu K $\alpha$  radiation and a Bruker Lynx eye

identifier. The XRD designs were procured from 10 to 100° 2 $\theta$  with a stage size of 0.02° and a check time of 0.1 s. Basic structure was resolved utilizing X-beam photoelectron spectroscopy (XPS) with an Al K $\alpha$  monochromator X-beam source. An overview examine was gained at 100 eV pass vitality somewhere in the range of 0 and 1,400 eV. High goals spectra for singular components were gathered at 100 Ca + 0.05 Ga. Basic structure was determined from the high-goals spectra utilizing CasaXPS with estimations done in triplicate.

### **Mice and TiO<sub>2</sub> Dosage Information**

Five to multi week-old male C57BL/6J Ausb mice from Australian Bio Resources were kept up under explicit without pathogen conditions. Every trial technique including creatures were affirmed by the University of Sydney Animal Ethics Committee under convention number 2014/696. Mice were cohoused with water and food (AIN93G; Specialty Feeds) get to not obligatory. Titanium dioxide (E171) was added to water and sonicated day by day. TiO<sub>2</sub> was controlled in drinking water at dosages of 0, 2, 10, and 50 mg TiO<sub>2</sub>/kg BW/day, which was determined dependent on the water admission estimated per confine. At week 4, mice were euthanized utilizing CO<sub>2</sub> suffocation.

### **Colonic Immune Cell Isolation and Flow Cytometry**

Bits of colon were hatched at 37°C for 40 min in Hank's Balanced Salt Solution (HBSS; Gibco) with 5 mM EDTA, 5% FBS (Gibco) and 15 mM HEPES (Gibco). Intraepithelial lymphocytes were disposed of and the rest of the tissue was brooded at 37°C for 1 h in HBSS (Gibco) with 6.7 mg/ml collagenase type IV (Gibco), 10% FBS (Gibco) and 15 mM HEPES (Gibco).

Cells were gone through a 70  $\mu$ m work

and lymphocytes enhanced by means of percoll slope of 80% and 40% (GE Life Sciences). The rundown of antibodies utilized for stream cytometry is in the Supplementary Methods. Suitability was resolved utilizing the LIVE/DEAD Fixable Blue Dead Cell stain pack (Invitrogen). Stream cytometry was performed on a LSRII stream cytometer (BD Biosciences) and information examination with FlowJo programming (Treestar Inc., Ashland, OR, USA).

### **RNA Extraction and Quantitative Real-Time PCR**

All out tissue RNA was removed utilizing TRI Reagent (Sigma) and changed over into cDNA utilizing iScript RT Supermix (BioRad) as indicated by both maker's guidelines. qPCR was performed on a LightCycler 480 (Roche) utilizing SYBR Green (Biorad) with preliminary groupings recorded in Supplementary Table 1.

### **Acetic acid derivation and Trimethylamine (TMA) Quantification**

Quantitative estimations of acetic acid derivation and TMA in plasma were controlled by atomic attractive reverberation spectroscopy (NMR). Quickly, plasma was sifted through a 3 kDa layer channel (Merck Millipore) and polar metabolites separated from the fluid period of a water:chloroform:methanol blend. Tests, containing 4,4-dimethyl-4-silapentane-1-sulfonic corrosive as an interior norm, were broke down on a Bruker 600 MHz NMR.

### **Plasma Metabolomic Screening**

A hydrophilic collaboration chromatography LC-MS/MS strategy was utilized for choline discovery in plasma as portrayed already (26). The LC was associated with an AB Sciex Triple Quad 5,500 mass spectrometer run in positive particle mode. Information examination

was done on programming Multi-Quant 3.0 for MRM Q1/Q3 top incorporation.

### **Nanolive Imaging**

*Escherichia coli* K-12 MG1655 (*E. coli*) or *Enterococcus faecalis* NCTC 775 (*E. faecalis*) were brooded for 7 h at 37°C, 5% CO<sub>2</sub> with Luria-Bertani (LB) stock containing E171 at demonstrated fixations and afterward fixed in 3% formalin short-term. Cells were resuspended in PBS and envisioned utilizing a Nanolive 3D cell adventurer. Bogus hues were applied to pictures dependent on refractive file utilizing STEVE programming.

### **Biofilm Visualization**

Biofilm Formation Assay in vitro on Cultured *E. coli*. what's more, *E. faecalis* The in vitro biofilm development examine depended on a formerly distributed convention (13). Overnight culture in quadruplicates of *E. coli* (low salt LB stock; Beckton Dickinson), *E. faecalis* (tryptone soya stock enhanced with 0.25% glucose; Sigma Aldrich) or *Staphylococcus epidermidis* NCTC 6512 (LB stock) was changed in accordance with OD of 0.5 at 600 nm and 100 µl of each bacterial culture was plated on isolated round base 96-well tissue culture plates. A further 100 µl of fitting media enhanced with TiO<sub>2</sub> was added to accomplish the showed last focuses. TiO<sub>2</sub> at the diverse last fixations in media alone was utilized as foundation controls. Plates were hatched at 37°C vigorously on a shaker (Ratek, 70 rpm) for either 24, 48, or 72 h.

### **Biofilm Formation Assay From Colonic Commensal Bacteria**

200 microliters of colon homogenates were refined in quadruplicates in level base 96-well-plates containing enhanced tryptic soy stock [sTSY: 30 g/L tryptic soy stock (Oxoid) with 5 g/L yeast separate,

5% L-cysteine, 50 mg/L hemin and 1 mg/L medianone (all from Sigma-Aldrich) to yield 0.05 mg/μl (w/v)] for 24 h, vigorously at 37°C at 70 rpm.

Tests were weakened 1:100 in new sTSY containing TiO<sub>2</sub> at demonstrated portions and brooded for 5 days. After planktonic cell expulsion, biofilm was recolored with precious stone violet (CV). Quickly, plates were washed multiple times with water, air dried and recolored with 1% CV (Sigma-Aldrich) for 30 min. After 4 washes in water and air drying, 95% ethanol was included for 15 min. Absorbance was recorded at 595 nm on a microplate peruser (Tecan Infinite M1000).

### Resazurin Viability Assay

Biofilm development was likewise measured dependent on Resazurin suitability examine as recently depicted (27). Quickly, culture media was evacuated and wells washed once with phosphate-cradled saline (PBS). At that point, media with 10% Resazurin (Sigma-Aldrich) was added to each well. The plates were hatched in obscurity at 37°C and fluorescence force estimated each 15 min (excitation 570 nm, outflow 585 nm). TiO<sub>2</sub> just controls were utilized to deduct foundation.

Microscopic organisms 16S rRNA Gene Amplicon Sequencing and Bioinformatics DNA from fecal examples or whole substance of small digestive tract lumen were extricated by mechanical disturbance utilizing a Fastprep (MP Biomedicals) utilizing autoclaved glass dabs (G8772 and G1145; Sigma-Aldrich) in lysis cradle [500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4% SDS] followed by 15 min brooding at 95°C. DNA was accelerated in 10M .

Hellinger-transformed data (n = 10 mice per group from 2 cages of 5 mice . Ammonium acetic acid derivation and

isopropanol and washed with 80% ethanol. Protein and RNA were evacuated utilizing the QIAamp DNA stool Minikit (Qiagen) adhering to the producer's guidelines. DNA tests were intensified over the V3-V4 district (Q5 polymerase; New England Biolabs) with these preliminaries F: 5'-ACTCCTACGGGAGGCAGCAG-3'; R: 5'-GGACTACHVGGGTWTCTAAT-3' and sequenced on an Illumina Miseq (2 × 300 bp). Information examination was performed utilizing QIIME 1.9.1 (28) utilizing default boundaries as depicted already (29). Quickly, demultiplexed combined end information were quality separated and matched utilizing the Fastq-get calculation together with no blunders permitted. Operational ordered units (OTUs) were picked utilizing 97% similitude with UCLUST, and scientific categorization was doled out with Greengenes database. The subsequent OTU table was separated by evacuating OTUs with <0.01% groupings and those identifying with Cyanobacteria or Chloroplast. Further investigation was performed with R programming (3.4.2). For measurable investigation, plenitude information was changed utilizing the Hellinger strategy. Contrasts between treatment bunches were dictated by adonis (vegetarian 2.5-2) with 9999 stages, alpha 0.05 and with the phyloseq bundle 1.25.2 (30) and Calypso 8.78 (31).

### Insights

Mann–Whitney U-test was utilized for investigation of the contrasts between the mean of gatherings and Wilcoxon combined test for matched examples. For microbiota information, huge contrasts in the general bounty of sort between treatment bunches were dictated by single direction ANOVA with post-hoc Tukey's test. Contrasts in general microbial network between treatment bunches were dictated by adonis.  $p < 0.05$  were considered measurably noteworthy.

## RESULTS

### Portrayal of Food Grade TiO<sub>2</sub> (E171)

We utilized unique light dissipating (DLS) to decide the hydrodynamic size of the E171 item utilized in this study. DLS uncovered that the TiO<sub>2</sub> nanoparticles scattered in drinking water (5 mg/ml, pH 7.8) have a normal hydrodynamic breadth of 367 nm, a polydispersity record of 0.258 and a zeta capability of  $-23.0$  mV ( $\pm 4.5$  mV). We likewise utilized nanoparticle following examination (NTA) and filtering electron microscopy (SEM) to additionally research the size and state of the TiO<sub>2</sub> nanoparticles scattered in drinking water. NTA (Supplementary Figure 1A) indicated that the TiO<sub>2</sub> nanoparticles are generally circular fit as a fiddle and range in distance across from 28 to 1,158 nm. On a number premise, the molecule size conveyance has a mean of 202 nm and a method of 138 nm and, on a weight premise, the molecule size appropriation has a mean of 363 nm and a method of 428 nm. The normal molecule distance across controlled by NTA on a weight premise (363 nm) is in acceptable concurrence with that dictated by DLS (367 nm). SEM (Supplementary Figure 1B) affirmed that the TiO<sub>2</sub> nanoparticles are generally circular fit as a fiddle and uncovered that they can be arranged into basically four gatherings (in view of diameter)—300, 150–200, 100, and 30–50 nm—which is reliable with the molecule size dissemination (on a number premise) got by NTA. TiO<sub>2</sub> was overwhelmingly in anatase structure according to producer's depiction. This was confirmed utilizing X-beam powder diffraction (information not appeared).

### Effect of Oral Administration of Food Grade TiO<sub>2</sub> on Gut Microbiota Composition

We initially decided if introduction to TiO<sub>2</sub> over a scope of physiologically pertinent dosages affected gut bacterial

networks in vivo. To accomplish this, mice were regulated TiO<sub>2</sub> by means of drinking water at dosages of either 0, 2, 10, or 50 mg TiO<sub>2</sub>/kg BW/day for 3 weeks. Sequencing of the 16S rRNA quality from fecal examples uncovered that TiO<sub>2</sub> effectsly affected bacterial decent variety as dictated by Inverse Simpson and Shannon investigations (Figures 1A,B) nor bacterial wealth (Figure 1C), equity (Figure 1D) or Faith's assorted variety (Supplementary Figure 2A) at these portions. Be that as it may, there was as yet a pattern toward decline in mice rewarded with physiological portions of TiO<sub>2</sub> (2 and 10 mg TiO<sub>2</sub>/kg BW/day). Then again, both weighted (Supplementary Figure 2B) and unweighted UniFrac (Supplementary Figure 2C) chief facilitate investigation (PCoA) gave some bunching of bacterial sythesis in control versus TiO<sub>2</sub> rewarded mice. To test this further, we performed standard correspondence examination (CCA) obliged to the 4 unmistakable TiO<sub>2</sub> fixations utilized, which uncovered critical bunching in bacterial sythesis driven by 2 mg TiO<sub>2</sub>/kg BW/day ( $p = 0.0011$ ) and 50 mg TiO<sub>2</sub>/kg BW/day ( $p = 0.0123$ ) TiO<sub>2</sub> treatment (Figure 1E). We likewise performed CCA with TiO<sub>2</sub> as a constant variable, which uncovers a portion subordinate impact of TiO<sub>2</sub> on microbiota sythesis (Supplementary Figure 2D). Treatment with TiO<sub>2</sub> altogether influenced gut microbiota arrangement freely of the pen impact (with by and large treatment impact: F-esteem = 8.2407,  $R^2 = 0.31644$ , Df = 3,  $p < 0.001$  and effect of treatment rectified for the enclosure impact: F-value = 5.8511,  $R^2 = 0.2996$ , Df = 3,  $p < 0.001$  both by adonis). We at that point decided the effect of TiO<sub>2</sub> at more profound levels and discovered huge changes at the variety level. Parabacteroides were altogether raised in TiO<sub>2</sub> rewarded mice, at a portion of 50 mg TiO<sub>2</sub>/kg BW/day (Figure 1F) while Lactobacillus and Allobaculum were essentially raised at all dosages tried (Figures 1G,H). Then again, Adlercreutzia

(Figure 1I) and Unclassified Clostridiaceae (Figure 1J) were essentially diminished in the gatherings rewarded with TiO<sub>2</sub> at the dosages of 10 and 50 mg TiO<sub>2</sub>/kg BW/day relative to the untreated gathering. These outcomes propose that TiO<sub>2</sub> minorly affected microbiota creation in vivo, while influencing not many taxa at the sort level. The gut microbiota organization in the small digestive system was additionally dissected to decide if TiO<sub>2</sub> may have a more prominent impact here than in the colon. Bacterial assorted variety files (Richness, equality, Shannon, Inverse Simpson and Faith's decent variety) were not fundamentally influenced at portions of 10 and 50 mg TiO<sub>2</sub>/kg BW/day (Supplementary Figure 2E), despite the fact that these inclined toward decline with expanding portion of TiO<sub>2</sub>. Dissimilar to in the colon, TiO<sub>2</sub> didn't altogether modify the small digestive system bacterial sythesis ( $p > 0.05$  by adonis) and weighted and unweighted UniFrac PCoA examination uncovered no conspicuous bunching (Supplementary Figures 2F,G). By and large, TiO<sub>2</sub> didn't appear to drastically affect on little intestinal microbiota structure. We additionally performed co-event examination by inspecting microbial communications from mice rewarded with either 0, 2, 10, or 50 mg TiO<sub>2</sub>/kg BW/day. We found that specific genera are reliably connected with one another paying little heed to TiO<sub>2</sub> treatment (Ruminococcus, Desulfovibrio, and Oscillospira are decidedly associated). Expanding TiO<sub>2</sub> consumption, particularly at the portion of 10 and 50 mg/kg BW/day brought about increasingly huge associations inside the system, just as expanded number of genera with noteworthy commitments. For instance, while Akkermansia was not essentially associated with the microbial system of mice directed 0, 2, or 10 mg TiO<sub>2</sub>/kg BW/day, it is included at a portion of 50 mg/kg including various co-rejection connections. These co-event diagrams are introduced in Supplementary

Figures 2H–K. These outcomes were checked utilizing the deblur pipeline (32) which settle amplicon arrangements significantly more precisely (Supplementary Figures 3A–F).

### **Food Grade TiO<sub>2</sub> Modulates Commensal Bacterial Activity**

We and others have indicated that gut bacterial metabolites, for example, SCFAs can dramatically affect have resistant capacity and infection advancement (1–5, 33, 34). Mice rewarded with 50 mg TiO<sub>2</sub>/kg BW/day had a huge abatement in the SCFA, acetic acid derivation, in the plasma, recommending a potential effect of TiO<sub>2</sub> on have bacterial connection (Figure 2A). Such impacts on bacterial metabolites were not constrained to SCFAs as TMA, a bacterial item connected with improvement of atherosclerosis (35), was expanded at portions of 10 and 50 mg TiO<sub>2</sub>/kg BW/day (Figure 2B). TMA is a result of transformation of choline, which was additionally seen as diminished at 50 mg TiO<sub>2</sub>/kg BW/day (Figure 2C), proposing that expanded TMA was not because of an adjustment in the substrate accessibility yet possibly changes in bacterial movement.

### **Food Grade TiO<sub>2</sub> Promotes the Cluster of Commensal Bacteria and Biofilm Formation**

Microscopic organisms additionally speak with the host by means of direct associations. Studies have demonstrated that connection of biofilm on the colonic epithelium was corresponded with colorectal malignant growth, an infection where TiO<sub>2</sub> has irritating impacts (36). To investigate the likelihood that TiO<sub>2</sub> may advance biofilm development, we hatched two kinds of commensal microorganisms, *E. coli* and *E. faecalis*, within the sight of TiO<sub>2</sub>. Nanolive imaging uncovered the bunching impact of TiO<sub>2</sub> on both *E. coli*

(Figure 3A) and *E. faecalis* (Figure 3B) in vitro in a portion subordinate way. To decide if the group of microorganisms was expected to biofilm development, we acted in vitro culture of either *E. faecalis* or *E. coli* within the sight of 2, 10, or 50  $\mu\text{g/ml}$  of  $\text{TiO}_2$  for 24 or 72 h, separately. Utilizing the resazurin reasonability examine (Figure 3C), we found that  $\text{TiO}_2$  treatment altogether expanded biofilm development in the two subsets of microorganisms (Figures 3D,E) however not in *Staphylococcus epidermidis*, a strain known for its failure to shape biofilm (Supplementary Figure 4). We affirmed by confocal microscopy that  $\text{TiO}_2$  treatment expanded biofilm arrangement in both *E. coli* and *E. faecalis* (Supplementary Figure 5). To decide if such impacts were pertinent to microscopic organisms in the mind boggling condition of the gut microbiota, we brooded commensal microorganisms got from mouse colons anaerobically for 5 days with dosages of 2, 10, and 50  $\mu\text{g/ml}$  of  $\text{TiO}_2$ . Both the dosages of 10 and 50  $\mu\text{g/ml}$   $\text{TiO}_2$  fundamentally advanced biofilm development by commensal microscopic organisms (Figure 3F). These information feature that  $\text{TiO}_2$  can influence the spatial association of the gut microbiota and in this way its possible communication with the host.

### **TiO<sub>2</sub> Affects Colonic Epithelial Function**

While the effect of biofilm arrangement on the host is muddled, weakened bodily fluid creation has been related with the nearness of bacterial biofilms (11). To decide if  $\text{TiO}_2$  may affect the bodily fluid layer, we analyzed colonic Muc2 quality articulation in the colon. We found that both 10 and 50 mg  $\text{TiO}_2/\text{kg}$  BW/day diminished Muc2 articulation, proposing an unfavorable effect of  $\text{TiO}_2$  on the bodily fluid layer (Figure 4A). While biofilm development has been accounted for in colitis and colorectal malignancy (36), these maladies

have likewise been connected to expanded gut porousness (37). To test whether  $\text{TiO}_2$  influences gut penetrability, we considered the outflow of Tjp1 (encoding for zonula occludens 1), which was unaltered by  $\text{TiO}_2$  treatment (Figure 4B), recommending no effect of  $\text{TiO}_2$  on gut porousness. The other significant system of bacterial avoidance is through the arrival of antimicrobial peptides. Beta defensin is communicated dominantly in the colon and we found that Defb3 (encoding for beta-defensin-3) was raised by treatment at dosages of both 10 and 50 mg  $\text{TiO}_2/\text{kg}$  BW/day (Figure 4C). Articulations of other antimicrobial peptides, for example, granzyme B (Figure 4D), cathelin-related antimicrobial peptide (CRAMP), recovering islet-inferred protein 3 gamma (REG3 gamma) and p-lysozyme (PLYz) (Supplementary Figure 6) were unaltered. Along these lines,  $\text{TiO}_2$  treatment disables the declaration of key colonic epithelial components engaged with gut homeostasis.

### **TiO<sub>2</sub> Contributes to Increased Colonic Macrophages and Associated Cytokines**

Diminished Muc2 has been corresponded with irritation and MUC2 insufficiency prompts unconstrained colitis (38). To test whether  $\text{TiO}_2$  may influence natural invulnerable cells in the colon, we examined myeloid insusceptible cell populaces by stream cytometry. While neutrophils (CD45+Ly6g+CD11b+) (Figure 5A) and dendritic cells (CD45+I-ab+Ly6g-F4/80-CD11c+) were unaltered (Figure 5B), macrophages (CD45+F4/80+CD8-Ly6g-I-ab+CD11b+CD103-) were essentially expanded by  $\text{TiO}_2$  at 10 and 50 mg  $\text{TiO}_2/\text{kg}$  BW/day (Figure 5C). This change was not because of an expanded enlistment of absolute monocytes (CD45+CD8-Ly6G-Ly6C+CD11b+I-ab-) (Figure 5D), recommending a potential in situ expansion of macrophages (gating systems appeared in

Supplementary Figure 7). Colonic macrophages are a significant wellspring of IL-6, TNF-alpha and IL-10, cytokines, which were additionally upregulated in the colon of TiO<sub>2</sub> rewarded mice (Figures 5E–G). We additionally watched a critical decrease in colonic crypt length by histological investigation of mice rewarded with 50 mg TiO<sub>2</sub>/kg BW/day (Figure 5H) while colon length was unaltered (information not appeared). In this way, TiO<sub>2</sub> treatment triggers changes in the colonic myeloid compartment just as auxiliary changes in the colon.

### **TiO<sub>2</sub> Promotes Increased CD8+ T Cell Infiltration in the Colon and Increased Inflammatory Cytokines**

Other cell subsets can deliver TNF-alpha, especially CD8+ T cells (39). By stream cytometry examination, we found that CD8+ T cells were fundamentally expanded from 10 mg TiO<sub>2</sub>/kg BW/day treatment (Figure 6A), as was articulation of interferon-gamma in this cell subset (Figure 6B). Expanded extents of the two macrophages and CD8+ T cells propose a condition of colonic irritation in TiO<sub>2</sub> rewarded mice which is predictable with the expanded extent of colonic Th17 cells ( $p = 0.0556$ ) (Figure 6C) just as essentially expanded articulation of IL-17A (Figure 6D). Then again, neither administrative T cells (Figure 6E) nor TGF-beta (Figure 6F) were influenced by TiO<sub>2</sub> treatment. Gating methodologies for stream cytometry investigation are appeared in Supplementary Figure 8. These discoveries show that TiO<sub>2</sub> treatment disables safe homeostasis in the colon and advances a fiery situation.

### **Conversation**

The universal use and every day utilization of TiO<sub>2</sub> by everyone warrants examination concerning its likely effect on wellbeing. After just half a month of every day TiO<sub>2</sub> utilization, we saw that colonic

homeostasis was fundamentally impeded in mice. While TiO<sub>2</sub> affected bacterial capacity by causing changes in bacterial metabolites (acetic acid derivation and TMA) and by advancing biofilm arrangement by commensal microscopic organisms, TiO<sub>2</sub> had negligible effect on gut microbiota piece. One of the significant components of physical partition among host and gut microscopic organisms was hindered by TiO<sub>2</sub>, as appeared by diminished Muc2 articulation and expanded Defb3 articulation in colonic epithelial cells. We likewise watched expanded macrophages, CD8+ T cells and Th17 T cells just as expanded fiery cytokines in the colon. This expanded aggravation was related with diminished colonic crypt length, as revealed in provocative enteral illnesses (40). Disturbance of gut homeostasis because of interminable presentation to TiO<sub>2</sub> may along these lines take action for conditions, for example, incendiary inside infections or colorectal disease.

Utilization of TiO<sub>2</sub> had no effect on microbiota decent variety in either the small digestive tract or colon. Utilizing an obliged investigation, we found that microbiota organization in the small digestive tract was unaltered while some colonic microbiota changes were driven by 2 and 50 mg TiO<sub>2</sub>/kg BW/day. Be that as it may, just a couple taxa at the class level were altogether modified in the colon, recommending that TiO<sub>2</sub> utilization is related with minor changes in bacterial networks. Likewise, TiO<sub>2</sub> may not significantly reshape the human microbiota in vivo which would affirm past in vitro discoveries in a model of improved human microbiota (41, 42). Be that as it may, treatment with TiO<sub>2</sub> over a more drawn out timeframe, as recently done by rewarding mice for 12 weeks with emulsifiers polysorbate-80 (P80) and carboxymethyl cellulose (CMC), may have an increasingly emotional effect (43).

The effect of TiO<sub>2</sub> on gut microbiota at the class level imparted a few likenesses to this investigation on emulsifiers where mice rewarded with CMC had a huge increment in *Lactobacillus* and *Allobaculum* (43). The expansion in *Lactobacillus* is especially intriguing as these microorganisms are a significant biofilm maker, proposing that TiO<sub>2</sub> may support the development of biofilm delivering microscopic organisms. Another examination proposes that TiO<sub>2</sub> may improve the development of *Lactobacillus* (44). Past investigations have demonstrated that TiO<sub>2</sub> could either tie onto the outside of microscopic organisms or microbes could take-up TiO<sub>2</sub> (45), which may trigger a barrier system adding to biofilm arrangement as we saw in vitro. We likewise found that TiO<sub>2</sub> intervened changes in the gut condition, for example, diminished Muc2 articulation, which have been appeared to support biofilm development. Since bacterially inferred SCFAs have been appeared to advance bodily fluid layer thickness, diminished acetic acid derivation at the portion of TiO<sub>2</sub> of 50 mg TiO<sub>2</sub>/kg BW/day could mostly clarified changes in bodily fluid quality articulation in mice rewarded at this portion of TiO<sub>2</sub>. TiO<sub>2</sub> may likewise straightforwardly influence the capacity of bodily fluid creating cells, as a past report proposes a productive take-up of TiO<sub>2</sub> by flagon cells in vitro (46). The bodily fluid layer is a proficient physical obstruction forestalling bacterial connection to the epithelium thus its weakness by TiO<sub>2</sub> may subsequently support bacterial connection and biofilm arrangement in the gut. So also, emulsifiers have been appeared to diminish the bodily fluid layer prompting nearer contact between commensal microscopic organisms and the epithelium (43). Be that as it may, regardless of whether emulsifiers may support biofilm development is obscure. While we didn't watch any effect of TiO<sub>2</sub> on gut porousness related qualities, Defb3 was

upregulated which may be a compensatory component to control the association with the commensal microscopic organisms.

In the colonic lamina propria, we watched a huge effect of TiO<sub>2</sub> on both intrinsic and versatile invulnerable cells with expanded macrophages, Th17 and CD8<sup>+</sup> T cells. This proinflammatory impact of TiO<sub>2</sub> is affirmed by changes in the cytokine condition with expanded IL-6, IL-17, and TNF-alpha quality articulation just as diminished colonic grave length. The later has additionally been accounted for in rodents rewarded for 100 days with 10 mg TiO<sub>2</sub>/kg BW/day (19). Our discoveries propose that a portion of the progressions instigated by TiO<sub>2</sub> happen after as meager as 30 days of day by day TiO<sub>2</sub> treatment.

In rundown, our discoveries show that TiO<sub>2</sub> significantly influences gut homeostasis in mice and that such changes can happen over some stretch of time essentially shorter than the presentation run of the mill for the human populace. These progressions were generally critical at the most noteworthy portion of 50 mg TiO<sub>2</sub>/kg BW/day, yet at the same time huge at the physiological dosages of 2 and 10 mg TiO<sub>2</sub>/kg BW/day. The master fiery condition and biofilm development actuated by TiO<sub>2</sub> incline the host to conditions, for example, provocative gut infections and colorectal malignant growth, the two of which have been demonstrated to be irritated by TiO<sub>2</sub> (19, 20). The diminished SCFA creation at the most elevated portion of TiO<sub>2</sub> has significant wellbeing suggestions as acetic acid derivation has been appeared to give insurance from colitis, colorectal malignant growth, food hypersensitivity, asthma and type 1 diabetes (2–5).

At long last, this work features the requirement for additional investigation into how TiO<sub>2</sub>, all alone and in mix with other food added substances, influences human wellbeing. Such examination

would better illuminate the guideline regarding food added substances.

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