Short-term oral exposure to low doses of nano-sized TiO\textsubscript{2} and potential modulatory effects on intestinal cells

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A B S T R A C T

The present study investigated potential modulatory effects of low doses of nano-sized titanium dioxide (TiO\textsubscript{2}) on intestinal cells in vivo and in vitro. After short-term exposure to TiO\textsubscript{2} nanoparticles in rats, histopathological analysis of intestinal tissues indicated a gender-specific effect with increased length of intestinal villi in male rats only. Moreover the intestinal tissue showed nanoparticle deposition as revealed by ICP-MS determination of titanium. Increased serum testosterone levels were also detected. Considering the male-specific effects detected in vivo, the TiO\textsubscript{2} nanoparticle interaction with intestinal cells was further characterized in vitro and the modulating effect of testosterone and a hormone-induced growth factor, namely Insulin-like Growth Factor 1 (IGF-1), was also assessed. Cytotoxicity assays and analysis of Reactive Oxygen Species (ROS) production showed neither cellular alteration nor oxidative stress for nanoparticles at low concentrations, even though they were able to penetrate intestinal cells, as revealed by electron microscopy. Cell treatments with nanoparticles in association with testosterone or IGF-1 showed increased cell proliferation, compared to nanoparticles or testosterone/IGF-1 alone. Since long-term intake of TiO\textsubscript{2} nanoparticles at low doses is a relevant scenario for human exposure, attention should be given to the potential modulating activity of this nanomaterial on cell proliferation.

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1. Introduction

A healthy digestive system allows absorption of nutrients from the gut after digestion of foods, ensuring nutrient uptake and preventing the passage of larger or foreign material. Uptake of nutrients with nanoscale dimensions by the gastrointestinal (GI) tract is a physiological process but there is still limited knowledge of the effects of non naturally-occurring nano-sized particles on gut epithelium and mechanisms for their translocation across the GI tract (Powell et al., 2010).

The application of nanotechnology in food and consumers products has led to concerns that ingestion of nano-sized ingredients and additives through foods, food supplements and beverages, but also via e.g. oral drugs and toothpaste, may pose certain hazards to consumers’ health, including the altered functioning of the gut wall (Rossi et al., 2014). The consumer-safety implications from nanotechnology applications in food are intrinsically linked to the physicochemical nature of the nanomaterial, and the likelihood and extent of exposure through consumption of ‘nano-foods’. It is known that nanomaterials have much larger specific surface areas and may exhibit substantially different physicochemical and biological properties compared to their conventional, non-nanosized, counterparts with the same chemical composition. As nano-sized food ingredients and additives are likely to have a greater ability to cross the gut wall, their enhanced absorption and bioavailability would give rise to higher internal exposure, with higher plasma concentrations (from higher absorption rate), or higher area-under-the-curve exposure (from higher uptake efficiency) (Chaudhry et al., 2010). In addition, this higher internal exposure may affect distribution and retention in tissues and organs. In fact nano-sized materials generally have different tissue distribution and longer retention in tissues compared to larger-sized particles (Chaudhry et al., 2010). Toxicokinetic may change also among nanoparticles (NPs) of different size: after a single oral administration of different-sized TiO\textsubscript{2} NPs, Wang J. et al. (2007) found that larger particles (80 nm)
accumulated mainly in liver, whereas smaller particles (25 nm) accumulated preferentially in spleen and, to a lower extent, in kidney and lung.

Several reports estimate that at least 1000 consumer products containing nanomaterials are available on the market (Shukla et al., 2015; Oosthuizen et al., 2012; Singh and Ramarao, 2012). TiO$_2$ features among the most frequently applied nanomaterials, owing to its low costs, easiness to obtain at nano-sizes, and useful properties at this scale, such as reflection of UV radiations and photocatalytic properties (Wang Y.Q. et al., 2007).

TiO$_2$ in bulk form is a common food additive and excipient used in nutraceuticals, pharmaceuticals and toothpaste, and contains a fraction of the constituting particles in the nano-range (Jovanović, 2015). As a food additive (E171), it is commonly used with the crystal structures anatase and rutile as a white pigment in a variety of food items, including chewing gums, candies, chocolate and sweets, largely consumed by children (Weir et al., 2012; Peters et al., 2014). The white color is best achieved with particles of 200–300 nm, however, during the production of such particles, also particles of <100 nm (i.e. nanoparticles) are produced (Heringa et al., 2016).

Pharmaceutical/food grade TiO$_2$ has been shown to be absorbed systemically by humans following ingestion with a bi-modal pattern of uptake: it starts early (i.e. visible by 2 h following ingestion) and peaks later (i.e. 6 h following ingestion) (Pele et al., 2015). This pattern may be explained by a first absorption in the proximal small intestine (duodenum/jejunum), then followed by Peyer’s patch uptake in the more distal small intestine (ileum) (Powell et al., 2010).

In the light of the wide use of particulate TiO$_2$ in food and medicines and the prospective larger application of nano-sized TiO$_2$ in products that may be incidentally ingested, several studies have addressed the effects of TiO$_2$ NPs after oral exposure. A number of recent studies have shown toxic effects following oral administration of TiO$_2$ NPs in rodents (see reviews of Javicoli et al., 2012; Shi et al., 2013; Jovanović, 2015; Heringa et al., 2016) and significant tissue accumulation over time following repeated exposure due to slow tissue elimination, notwithstanding low oral bioavailability (Geraets et al., 2014). In contrast, guideline acute and subchronic oral toxicity studies in rats performed with nano-scale and pigment grade TiO$_2$ particles at very high dose levels (100–24,000 mg/kg bw/day) did not observe adverse effects (Warheit and Donner, 2015). It has been recently shown that the use of unrealistic, high doses may result, besides other drawbacks, in particle aggregation and in reduced bioavailability, highlighting that the applicability of such data for assessing potential risk for human health is questionable (Van Kesteren et al., 2015).

Based on the weight of evidence, a recent risk assessment of dietary exposure to titanium dioxide NPs via E171 found that risks cannot be excluded for adverse effects in liver, ovaries and testes: in contrast to its low costs, easiness to obtain at nano-sizes, and useful properties among the most frequently applied nanomaterials, owing to its low costs, easiness to obtain at nano-sizes, and useful properties at this scale, such as reflection of UV radiations and photocatalytic properties (Wang Y.Q. et al., 2007).

suggesting that these particles might interfere with gut functions in humans. Food-grade TiO$_2$ has been found in the ileum of healthy children and also in children having ulcerative colitis, with deposition increasing with the age (Hummel et al., 2014). TiO$_2$ deposits were also reported in patients with colon adenocarcinoma, Crohn disease and non-Crohn disease colitis (Powell et al., 1996; Thoree et al., 2008).

In a recent study, Tassinari et al. (2014) demonstrated particle deposition in internal organs, as well as reproductive and endocrine effects in rats after short-term oral exposure to TiO$_2$ NPs at dose levels consistent with real-life exposure scenarios. Male rats showed also increased testosterone serum level.

In this study we investigated TiO$_2$ NPs effects on rat gut wall in vivo in the same experimental conditions as the previous work by Tassinari et al. (2014). Based on the effects detected in vivo, the NP interaction with intestinal cells and the modulating effect of testosterone and of the hormone-induced growth factor IGF-I, was also assessed.

2. Materials and methods

2.1. Particle characterization by scanning electron microscopy and numerical data analysis

2.1.1. Nanoparticles suspensions

Two milligrams of TiO$_2$ NPs (anatase, primary size <25 nm, BET surface area 45–55 m$^2$/g, purity 99% - Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK) were weighed and suspended in ultrapure water and in DMEM culture medium to obtain suspensions for the in vivo and in vitro study, respectively. The suspensions were sonicated for 15 min under temperature-controlled conditions with a probe sonicator (Vibracell, Sonics & Materials Inc, USA, 750 W, 20 KHz, 20% amplitude, 6.5 mm probe diameter) in order to reduce agglomeration.

2.1.2. Characterization

TiO$_2$ NPs suspensions were characterized by dynamic light scattering (DLS) and electron microscopy.

On 1 ml sample suspensions in Milli-Q water and in culture medium DLS measurements by a Zetasizer Nano (Malvern Instruments, UK) were performed. After 2 min equilibration step at 37 °C, each sample underwent 10 measurements; read number and duration of each measurement were set on automatic. Intensity distribution data were considered for the analysis. Values of Hydrodynamic Diameter (Z-Average) and Polydispersity Index (Pdi) were the average of ten measurements determined by Malvern's proprietary software.

Primary size, shape, size distribution and agglomeration status of NPs in suspensions, were determined using TEM (EM 208, FEI Company, The Netherland) and scanning electron microscope (SEM) (FE-SEM Quanta Inspect, FEI Company, The Netherland) equipped with Soft Imaging System (De Berardis et al., 2010).

For TEM sample preparation, 5 µl of particle suspensions were applied directly on TEM copper grids, coated with a layer of amorphous carbon.

SEM samples were obtained transferring 15 µl of particle suspensions on to a poly-L-lysine coated microscope glass slide, and then they were coated with a thin gold film by sputtering.

2.2. In vivo studies

2.2.1. Treatment of rats and harvesting of tissue

All experiments on animals were performed according to Directive 2010/63/EU-Italian Law 4th March 2014, n. 26, and the OECD Principles on Good Laboratory Practice. Adult male and
female Sprague-Dawley rats (10/sex/group) were treated with TiO₂ NPs as described by Tassinari et al. (2014). Briefly, animals were divided into three treatment groups: 2 mg/kg bw per day or 1 mg/kg bw per day of TiO₂ NPs suspended in ultrapure water, and CTRL, vehicle only (ultrapure water), and treated per os by gavage for 5 consecutive days. The dispersions were prepared on a daily basis and were obtained by sonication of NPs for 15 min (mean potency/peak 90/180 W, room temperature). Twenty-four hours after the last treatment (day 6), male and female rats were anaesthetized with a gaseous solution of isoflurane and sacrificed by CO₂ asphyxiation. Small intestine was excised. A piece of jejunum was used for histological and the remaining part of small intestine was sampled for studying either tissue accumulation of TiO₂ NPs, determined as titanium by ICP-MS or to detect potential adverse effects.

2.2.2. Histological and histomorphometrical analysis

Immediately after the sacrifice, samples of rat small intestine (jejunum) were fixed in 10% buffered formalin and stored in 80% ethyl alcohol. They were embedded in paraffin, cut into 5-μm sections and stained with haematoxylin and eosin for the examination under a light microscopy (Nikon Microphot FX) with different lenses.

The quantitative histomorphometrical analyses were performed on jejunum according to De Conto et al. (2010). Briefly, slides of tissues were examined by means of an image analysis system (Nis-Elements D) applied to an optical microscope (Nikon Microphot FX). The following parameters have been analyzed: using a 10× lens, subglandular layer (lamina propria including crypt depth + lamina muscularis mucosae), villus height and widths were measured, as the mean of ten randomly selected villi and their subglandular layers. Goblet cell density was calculated as ratio between number of goblet cells and area at least five villi.

2.2.3. Apoptosis detection

Apoptosis in intestinal sections was assessed with the in situ cell death detection kit, peroxidase (TUNEL technology) (Sigma-Aldrich, Milan, Italy). Sections were previously deparaffinized with Bio Clear (Bio Optica, Milan, Italy) and processed by TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling) according to the manufacturer’s instructions. Immunostaining was visualized under a light microscope (Nikon Microphot FX).

2.3. Titanium detection in gut tissue

Sample manipulations were carried out in clean room conditions under a laminar flow box (Spectec GmbH, Erding, Germany). Identical small intestine sections were submitted to a cleaning procedure with ultrapure water to remove any GI digestion residue and placed in high-pressure Teflon containers for an overnight pre-digestion at room temperature with 5 ml HNO₃ (ultra pure grade, Carlo Erba, Rodano, Italy). After adding 2 ml of H₂O₂ (ultra pure grade, Merck, Darmstadt, Germany) samples were microwave digested by means of a Milestone Ethos Pro microwave labstation, FKV, Bergamo, Italy, using the irradiation program detailed elsewhere (Tassinari et al., 2014). Ti determination by ICP-MS was carried out using an Elan DRC II spectrometer (Perkin-Elmer, Norwalk, CT). Since all titanium isotopes suffer from heavy spectral interferences in ICP-MS, an analytical method employing the dynamic reaction mode with NH₃ as reaction gas was applied. Measurements were carried out on 59Ti and 60Ga (internal standard, both from High Purity Standards, Charleston, SC) by the method of standard addition; more details can be found in Tassinari et al. (2014). The accuracy of determinations, as assessed through spikes of known amounts of titanium in tissues (both as NP and ionic Ti), was satisfactory and within the range 88–98%.

2.4. In vitro studies

2.4.1. Cell line and TiO₂ NP treatments

The human colorectal adenocarcinoma cell line, HT-29, was obtained from the American Type Culture Collection (Rockville, MD, USA). HT-29 cells were grown as a monolayer culture in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l-glutamine and penicillin 100 units/ml and streptomycin 100 μg/ml, at 37 °C in 5% CO₂ humidified atmosphere. For cell viability, HT-29 cells were seeded in a 96-well plate (10,000 cells/well) and, at a semiconfluent state, were treated with TiO₂ NPs at different concentrations (1, 2.5, 5, and 20 μg/cm² corresponding to 1.8, 4.5, 9 and 36 μg/ml) for 6, 24 and 48 h. At each time point, cells were processed by LDH and MTT cytotoxicity tests. Before all experiments, TiO₂ NPs were suspended in cell culture medium and sonicated (mean potency/peak 90/180 W, +4 °C) for 45 min to ensure dispersion of the particles.

2.4.2. LDH leakage assay

The release of lactate dehydrogenase (LDH) was monitored with the Cytotoxicity Detection Kit (Plus) (LDH) (Roch). HT-29 cells (2 × 10⁵ cells/ml) were seeded in 24-well plates and grown for 24 h. Monolayers were then incubated with NPs (1–20 μg/cm²) for 6, 24, and 48 h. Aliquots (100 μl) of cell culture medium were collected from each well and placed in new microtiter plates. Finally, 100 μl of substrate solution was added to each well and the plates incubated for 30 min at room temperature. The absorbance at 490 nm was measured by a microplate reader (Perkin-Elmer, Massachusetts, USA). Cytotoxicity is expressed relative to the basal LDH release by untreated control cells. Results were obtained from three independent experiment performed in triplicate.

2.4.3. MTT assay

NPs cytotoxicity was determined using the 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HT-29 cells (1 × 10⁴ cells/well) were plated in a 96-well microplate and incubated at 37 °C for 24 h in a 5% CO₂ atmosphere to allow cell attachment to the surface. The cells then were treated with NPs at different concentrations (1–20 μg/cm²) and incubated at 37 °C for 6, 24, and 48 h. Then, 20 μl of a 5 mg/ml MTT solution in PBS was added to each well, and the plates were incubated for an additional 3 h. The supernatants were then carefully removed, and 100 μl of dimethyl sulfoxide were added to each well. After the formazan crystals had dissolved completely, optical density at 570 nm was determined with a spectrophotometer/fluorimeter microplate reader (Perkin-Elmer, Massachusetts, USA). The cytotoxicity (%) of the treated cells was defined as percentage of cell viability compared to control untreated cells (100% viability). Results were obtained from three independent experiment performed in triplicate.

2.4.4. Measurement of ROS production

The generation of intracellular Reactive Oxygen Species (ROS) was monitored with a peroxide-sensitive fluorescent probe, carboxy-20,70-dichlorofluorescin diacetate (H2DCFDA) (Molecular Probe), according to the manufacturer’s guidelines. Cells, seeded in 6-well culture plates and growth at semiconfluence, were incubated with NPs (1–20 μg/cm²) for 6 and 24 h, washed with PBS, and incubated with 40 μM carboxy-H2DCFDA for 30 min at 37 °C. DCFH fluorescence was immediately measured with a fluorescent microplate reader (Perkin-Elmer, Massachusetts, USA) at excitation/emission at 488/535 nm. Cells incubated without NPs were
used as a negative control and 100 μM H₂O₂ was used as a positive control. DCHF relative fluorescence of treated cells was expressed as percentage (%) compared to control untreated cells. Results were obtained from three independent experiment performed in triplicate.

2.4.5. Measurement of mitochondrial membrane potential (MMP)
MMP was detected by using 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazo-dazo-lylcarbocyanide iodine (JC-1). This probe can selectively enter into mitochondria and reversibly change color from red to green as the membrane potential decreased. The ratio of green to red expresses the change of MMP.

Cells were treated with TiO₂ NPs (0, 1, 2.5, 5, and 20 μg/ml/cm²) for 6 and 24 h. After washing with PBS, the cells were incubated with 10 mg/ml JC-1 for 30 min. The cells were washed with PBS twice, and then analyzed by a fluorescent microplate reader (Perkin-Elmer, Massachusetts, USA). The green fluorescence intensity was determined at an excitation wavelength of 488 nm and an emission wavelength of 525 nm, whereas the red fluorescence intensity determined at an excitation wavelength of 488 nm and an emission wavelength of 590 nm H₂O₂ (100 μM) was used as a positive control. Results were obtained from three independent experiment performed in triplicate.

2.4.6. Electron microscopy
TiO₂-exposed cell specimens were analyzed by transmission electron microscopy (TEM). Cell monolayers were treated with 2.5 μg/cm² of TiO₂ NPs for 2, 6, and 24 h; then were fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h and 1% osmium tetroxide solution for 1 h at room temperature. After dehydration in ascending grades of ethanol, cells were subsequently embedded in epoxy resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed with a Philips 280 S transmission electron microscope at 80 kV.

2.4.7. Proliferation tests
Assessment of proliferation was carried out in 96-well plates at an initial cell density of 1 × 10⁴ cells/well. Cells were growth for 24 h and prior to experiments, were washed and incubated in serum-free medium for 24 h. The serum-free medium was then replaced with that containing the experimental stimuli. Testosterone (200 nM) and IGF-1 (50 nM) (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK), alone or in combination with TiO₂ NPs at 1, 2.5, 5, and 20 μg/ml/cm² concentrations were used.

Negative controls were culture medium and TiO₂ NPs only. Each stimulus was assessed in 6 separate wells and each experiment repeated on at least three occasions. Cell number was assessed after 6, 24, and 48 h by the MTT assay.

To analyze and compare the data, absorbance values were normalized to the untreated control cells such that the untreated control represented 100% mitochondrial activity, and an absorbance of 0 represented the absence of mitochondrial activity (0%). Three independent experiments were carried out.

2.5. Statistical analysis
Data are presented as means ± SD. Differences between rats treated with NPs and controls were evaluated using the Mann-Whitney U test. The statistical significance of differences between means of in vitro data was assessed by one-way analysis of variance (ANOVA). A p value < 0.05 was considered significant. When significant differences emerged from ANOVA, post-hoc multiple comparisons were made using the Bonferroni test.

3. Results

3.1. Particle characterization by dynamic light scattering and electron microscopy

3.1.1. Hydrodynamic diameter
In order to evaluate the physical characteristics of NPs relevant for their interactions with biological system we characterized the dispersion at the concentrations used in the in vitro study. At lower concentrations used in this study the size distributions obtained by DLS showed inconsistent results due to weak signal to noise ratio (data not shown). At the highest concentration, the TiO₂ NPs displayed a different behaviour when dispersed in Milli-Q water or in DMEM.

The mean hydrodynamic size, evaluated by intensity, for NPs suspensions at 36 μg/ml (corresponding to 20 μg/cm³) in Milli-Q water and in DMEM medium are displayed in Table 1. As shown by the PdI values, TiO₂ NPs in Milli-Q water were polydisperse (PdI = 0.211), whereas DMEM culture medium improved slightly the monodispersity of the suspension (PdI = 0.126). NPs suspended in culture medium joined into large agglomerates as displayed by the hydrodynamic diameter.

3.1.2. Single particle characterization by electron microscopy
Suspended of 36 μg/ml NPs in Milli-Q water and culture medium were characterized by electron microscopy. TEM analysis allowed us to detect two different morphologies for TiO₂ anatase NPs: spherical shape with primary size ranging from 20 to 60 nm and irregular shape with a length of about 60 nm and a width of about 40 nm. Moreover, these particles, suspended in either dispersion media, joined into agglomerates with different sizes, up to 2 μm of length (Fig. 1, Panel A, inset). In Fig. 1 (Panel A and B), the granulometric spectrum for the NPs in Milli-Q water and in DMEM, determined by SEM analysis, is showed.

The average diameter of NPs in Milli-Q water ranged between 70 nm and about 1.2 μm. The size distribution showed a peak between 60 nm and 90 nm corresponding to an abundance of 11% and an average diameter of about 76 nm (Fig. 1, Panel A). Overall, considering all the particles analyzed, only 13% of them had dimensions below 100 nm.

When the NPs were suspended in DMEM, the granulometric spectrum ranged between 30 nm and about 900 nm. A major peak between 30 nm and 60 nm with an abundance of 19% and an average diameter of 42 nm were observed in size distribution (Fig. 1, Panel B). The percentage of NPs with sizes below 100 nm increased to 32%.

3.2. In vivo effects on gut wall
No effects on animal health, body weight gain, and food consumption were observed during the treatment period in both sexes. Jejunum histological analysis did not show any significant qualitative changes in either 1 mg/kg or 2 mg/kg bw-treated rats vs CTRL.

In males, histomorphometrical data showed a significant and dose related increase of villus height and villus widths at 2 mg/kg bw only. Density of goblet cell resulted to be significantly and dose-
related increased in both doses in comparison to CTRL. In female jejunum, morphometry did not show any quantitative alterations in treated groups in comparison to CTRL (Table 2). The apoptosis rate was essentially unaffected in either treated group, according to TUNEL assay (data not shown).

3.3. Total titanium determination in small intestine

The analytical method used in this study allowed for the interference-free ICP-MS determination of Ti in tissues with a limit of detection (3σ) of 0.009 μg/g.

Ti levels (mean ± s.d., n = 4) in the small intestine of controls and animals treated at the lowest dose (1 mg/kg bw) were 0.08 ± 0.02 μg/g and 0.09 ± 0.02 μg/g, respectively, whereas they were 0.13 ± 0.03 μg/g (P < 0.05) in the case of animals treated at the highest dose (2 mg/kg bw).

3.4. In vitro studies

3.4.1. TiO2 NPs cytotoxicity

No statistically significant cytotoxic effect of TiO2 NPs in HT-29 cells was observed in LDH and MTT assays at all concentrations after 6, 24, and 48 h exposure (Fig. 2). No interaction was observed between dyes and NPs in a cell free system (data not shown).

3.4.2. Intracellular ROS generation induced by TiO2 NPs

The DCHF fluorescence intensity as an indication for intracellular oxidative stress showed a trend of increase after all TiO2 NPs exposure for 6 h (Fig. 3). The ROS level significantly increased with the NP concentration from 2.5 to 20 μg/cm² compared with control untreated cells. At 24 h interval time, DCHF fluorescence intensity appeared decreased compared to 6 h in all TiO2 NPs exposure samples, with significant values only for cells treated with 20 μg/cm² of TiO2 NPs compared to control cells.

Fig. 1. Size distribution of TiO2 NPs determined by SEM analysis: A) NPs in Milli-Q water; B) NPs in DMEM. Morphology of TiO2 NPs by TEM (inset).
Table 2
Histomorphometrical data of male and female rats treated for five days per os with 0 (CTRL) and 2 mg/kg bw per day of TiO2 NPs.

<table>
<thead>
<tr>
<th>Males</th>
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<th>Females</th>
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<tr>
<td></td>
<td>CTRL</td>
<td>1 mg/kg bw</td>
<td>2 mg/kg bw</td>
<td>CTRL</td>
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<td>Subglandular layer (mm)</td>
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<td>44.81 ± 7.70</td>
<td>43.58 ± 5.75</td>
<td>35.98 ± 4.81</td>
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<tr>
<td>Villus height (mm)</td>
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<td>78.86 ± 39.97</td>
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<td>53.97 ± 7.92</td>
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<td>Villus widths (mm)</td>
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<td>13.49 ± 2.94</td>
<td>15.04 ± 2.85</td>
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<tr>
<td>Density of goblet cells (n/mm²)</td>
<td>0.021 ± 0.005</td>
<td>0.031 ± 0.007*</td>
<td>0.050 ± 0.018*</td>
<td>0.046 ± 0.018</td>
<td>0.046 ± 0.013</td>
<td>0.038 ± 0.009</td>
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Mann-Whitney U test (*P < 0.05).

3.4.3. Changes of MMP induced by TiO2 NPs

The monitored changes in mitochondrial membrane potential are summarized in Fig. 4. No significant decrease of MMP was found and values in treated samples were not significantly different from the control untreated cells.

3.4.4. Electron microscopy

HT-29 cells treated with TiO2 NPs were processed for TEM (Fig. 5, Panels A–H). At early times of treatment (2–6 h) cells did not show any observable intracellular pathological differences compared to control cells (Panels A–F). At these times, electron microscopy images showed NPs agglomerates adsorbed to protrusions on the plasma membranes leading to NP engulfment inside the cells (Panels A, B). After their entry, NPs were located in the cell cytoplasm, apparently free, and in organelles such as mitochondria (Panels B–D). Some cells showed also NPs about to cross nuclear membrane (Panel E); many particles appeared located inside the nucleus by binding heterochromatin (Panel F). At late time (24 h) treatment, cells appeared yet with no dramatic morphological changes (Panels G, H). No damage to cell membranes was apparent according to cytotoxicity results and cells showed no significant ultrastructural alterations; only some mitochondria appeared swollen or damaged (Panel G) and multivesicular bodies (MVB) containing NPs agglomerates (Panel H) were observed.

3.4.5. Modulating effect of testosterone and IGF-1 on cell proliferation

The growth levels of cells treated with combined solution of testosterone and TiO2 NPs showed comparable values for all TiO2 concentrations compared to control untreated cells until 48 h treatments. At this interval time cells treated with the lower dose of NPs (1 µg/cm²) proliferated significantly faster compared to control cells (Fig. 6). As the TiO2 NP concentration increased, the proliferation rate decreased compared to the lower concentration, but was still significantly greater compared to control untreated cells. Cells treated with 1 and 2.5 µg/cm² of NPs in combination with testosterone also showed significant higher viability at 48 h compared to cells treated with testosterone alone: this effect disappeared at higher TiO2 concentrations. When samples with combined NPs-testosterone and NPs alone are compared, a significant higher proliferation was observed for the former at all concentrations (Supplementary material 1).

Similar results were obtained upon combined treatment with TiO2 NPs and IGF-1. Growth levels were significantly higher than in control cells at 48 h with concentrations ranging from 1 to 5 µg/cm²; an initial increasing trend was already observable starting from 6 h (Fig. 7). Comparison with IGF-1 alone indicated significantly higher growth for low NPs concentrations (1 and 2.5 µg/cm²) at 48 h. The combined NPs-IGF-1 treatment elicited higher proliferation rates at 48 h when compared with the parallel samples treated with NPs alone at the two lower concentrations (Supplementary material 2).

4. Discussion

In this study, we observed an increased villus size in male rats treated per os with TiO2 NPs at 2 mg/kg bw per day for five days, whereas female rats were unaffected. The effect appeared unrelated to increased cell turnover, because no increase in apoptosis was seen in intestinal villi. Increased villus height without increased turnover may result from increased cell proliferation and accelerated migration along the villus, and it indicates both an increased absorptive surface area and increased numbers of epithelial cells. Noticeably, the intestinal cyto-architecture was generally preserved; only goblet cells appeared increased in number, suggesting a hyperplasia likely related to increased villi size.

Also amorphous silica NPs have been shown to induce proliferative effects on rat’s intestine. van der Zande et al. (2014) demonstrated a small but significant increase in villus heights and crypt depths of rat jejunum, but no significant differences in the ratio between the villus height and crypt depth in treated animals after 28-days of exposure, as compared with the controls.

Estimates of TiO2 dietary intake range from 0.1 to 1.7 mg/kg bw/day in the general adult population, with higher values for children (1–5.5 mg/kg bw/day) (Weir et al., 2012; Bachler et al., 2015; Sprong et al., 2015; EFSA, 2016; Rompelberg et al., 2016). Taking into account that about 15% (up to 36%) of TiO2 have a size <100 nm (Weir et al., 2012; Peters et al., 2014; Yang et al., 2015; Warheit and Donner, 2015; EFSA, 2016), which correspond to a very low (generally <1%) proportion of the material by mass, estimates of dietary intake of nanosized TiO2 are >2 orders of magnitude lower. It has to be noted that in the present study the proportion of particles in the nano-range, i.e. <100 nm, was 13–32% (depending on the dispersion medium), similarly to food-grade TiO2. Overall, the doses we used are comparable to those expected from real-life oral exposure of the general population taking into account the proportion of particles that are actually nano-sized, and lower than those used in many in vivo studies (e.g., Warheit and Donner, 2015). On the other hand, caution should be exercised because – apart from the dose – there are several physicochemical characteristics that have to be considered since they are likely to play a role in the toxicological properties of TiO2. For instance, the material used here is anatase-based, whereas food-grade TiO2 may contain either anatase or rutile.

Since villi turnover was calculated to be 60 h in rat’s intestine (Mayhew and Middleton, 1985), we hypothesized that the entire intestinal mucosa should be changed at least 2 times during 5 days of treatment. The ICP-MS data indicated that NPs were able to penetrate intestinal mucosa since gut bioaccumulation was relatively high, despite low doses and environmental mucosal conditions that limit NPs adsorption.

Villi alterations were observed only on male rats indicating a potential role of the endocrine system in this process. NPs can interact with the endocrine system; indeed, testosterone serum level increased in male rats treated with NPs (Tassinari et al., 2014).
An increased testosterone production can upregulate Growth Factor (GH) and IGF-1 secretion (Meinhardt and Ho, 2006), which, in turn, may elicit the increased villi proliferation observed in NP-treated males compared to control male rats in the present study. GH has been proposed to directly stimulate intestinal growth. In addition, this hormone is a major stimulus for the production of IGF-1, a growth-inducing factor that plays a role in intestinal proliferation (McMellen et al., 2010).

To further investigate the mode of action of TiO₂ NPs in increasing intestinal epithelial proliferation in males, we performed a series of in vitro experiments. HT-29 cells were selected to mimic the cells in active replication of crypt/villus axis, because we hypothesized that NPs interacting with cells located near proliferating zone of crypt/villus axis could induce some alterations leading to increased size of villi. The calculated ratio between NPs and gut size of animals (Mayhew and Middleton, 1985) at our in vivo doses of 1 and 2 mg/kg bw/day was approximately 3 and 6 μg/cm²/day, respectively; considering a rate of loss because of environmental intestinal conditions, we used NPs concentrations ranging between 1 and 5 μg/cm² for in vitro studies, in addition to a dose of 20 μg/cm² as positive high dose control.

No indications of cytotoxicity were revealed at all NP concentrations, consistent with our in vivo findings and in agreement with previous studies (Koeneman et al., 2010; Gitrowski et al., 2014).

Fig. 2. Concentration and time-dependent cytotoxicity of TiO₂ NPs in HT-29 cells measured by LDH and MTT assays. The data are expressed as means ± SD from three independent experiments.
Fig. 3. Generation of reactive oxygen species (ROS) in HT-29 cells following TiO₂ NPs exposure. Data represents mean ± SD of three experiments. *p < 0.05, compared to control untreated cells.

Fig. 4. Changes of mitochondrial membrane potential in HT-29 cells treated with TiO₂ NPs at different concentrations. Data represent mean ± SD of three experiments.
TiO$_2$ NPs induced oxidative stress in dose-dependent manner at early time of exposure; however ROS levels decreased after 24 h exposure suggesting an adaptive response from cells. It was reported that TiO$_2$ NPs of <100 nm in diameter were able to generate free radicals and elevate DNA adduct formation (8-OHdG) in human lung fibroblasts (Bhattacharya et al., 2009). In addition, in A549 cells, the anatase TiO$_2$ NPs induced mitochondrial injury in a dose-dependent manner owing to ROS generation (Tang et al., 2013). Also in keratinocytes, TiO$_2$ NPs were able to induce oxidative stress that lead to cell damage and apoptosis (Shukla et al., 2011). In these cell systems toxic effects by TiO$_2$ NPs were ascribed to ROS generation that leads DNA damage and cell death; in our intestinal cells TiO$_2$ NPs induced dose-dependent ROS production but did not reduce the cell viability and were effectively internalized by the cells with cytoplasm and organelle localization. Moreover, TiO$_2$ NPs did not induce mitochondrial membrane potential changes, even though mitochondria morphological alterations appeared visible at electron microscopy. Dissipation of

**Fig. 5.** TEM micrographs of cells following exposure to 2.5 µg/cm$^2$ TiO$_2$ NPs. Panels A–B: 2 h time exposure; Panels C–F: 6 h exposure; Panels G–H: 24 h exposure.
mitochondrial membrane potential is an indicator of mitochondrial integrity and also an early step in apoptosis (Singh and Ramarao, 2012). Therefore, in our system NPs interaction with cells, in particular with mitochondria, is able to produce only a transient high level of oxidative stress that decreases during the 24 h treatment. It is possible that HT-29 cells treated with TiO2 NPs were able to react promptly against oxidative stress via antioxidant induction; in fact, several studies suggest that these systems may represent the most effective defense against oxidative stress in actively proliferating cells, but not undifferentiated or non-dividing cells (Andreoli et al., 1997; Duthie and Dobson, 1999).

We hypothesized that a testosterone increase induced by TiO2 NPs oral exposure in male rats could be related to the proliferation effects on intestinal mucosa. We further hypothesized that this could be a local, tissue-specific effect at gut level; thus we performed in vitro proliferation assays with or without testosterone or IGF-1, as hormone-regulated growth factor, in association with NPs. Results showed that TiO2 NPs in association with testosterone or IGF-1 increased cell proliferation, compared to NP or testosterone/IGF-1 alone; thus, the findings suggested a combined action of NP and sex-related endocrine status on intestinal cells.

Unexpectedly, low doses of NPs were able to induce a rate of proliferation with high significant values that decrease at the increasing NPs concentrations. Higher concentrations could induce an initial toxic effect, such as enhanced oxidative stress, counter-balancing the proliferative action. Alternatively, lower
concentrations could have a higher availability for interaction with cell molecules due to their lower agglomeration status.

The effects were more pronounced with NP + IGF-1 compared to NP + testosterone treatment. IGF-1 is an important growth factor in the intestine activity and it is the mediator of the anabolic action of GH. Sex steroids regulate GH secretion directly and indirectly through IGF-1 modulation. Testosterone stimulates GH secretion centrally and enhances its action and amplifies GH stimulation of IGF-1 (Meinhardt and Ho, 2006). Testosterone and IGF-1 levels are positively correlated in men (Erfurth et al., 1996; Pleilschiffer et al., 1996) and androgens positively modulated GH-induced stimulation of IGF-1 levels in a number of clinical studies (Hobbs et al., 1993; Hagenfeldt et al., 1992; Van Kesteren et al., 1996).

Our findings, supported by the systemic endocrine effects of TiO₂ NP, may suggest that increased testosterone production in male rats could modulate GH and IGF-1 secretion that in turn could exert proliferating activity on intestinal mucosa. The combined proliferative action of endogenous molecules and NPs may suggest that NP cell penetration and interaction with cell organelles and/or nuclear material, mainly at low doses, could induce some cellular changes by induction or modulation of cellular pathways that provide a favorable environment for the testosterone or IGF-1 action. Insoluble nanoparticle mechanisms include stimulation of IGF-1 or testosterone receptor transcription or higher receptor accessibility, as well as modulation of intracellular signaling pathways of cell proliferation.

This hypothesis implies that NPs orally administered in rats interact with enterocytes in the proliferating zone of crypt/villus axis. We found that, after exposure at a dose of 2 mg/kg bw, intestinal male rats mucosa contained TiO₂ NPs as revealed by ICP-MS data. This finding indicates that NPs are able to penetrate the intestinal mucosa and they could also interact with the proliferating zone of crypt/villus axis. The low dose levels used in our study, as compared to previous studies, might have led to a lower grade of agglomeration, thus facilitating bioaccessibility and local effects in the villus epithelium.

Particle surface charge can play also a crucial role in modulating the NP absorption (Fröhlich and Robleg, 2012). Net neutral or positive surface charge prevents mucosahesion, favoring penetration, whereas passage of negatively charged compounds is hindered. TiO₂ NPs of many commercial preparations have an isoelectric point at pH 6.0–7.0, and their surface is negatively charged at pH > 7 and positively charged at pH < 6 (Bae et al., 2003; Fernández-Ibáñez et al., 2003; Guny et al., 2006). The pH of the proximal small intestine is between 6 and 7 in humans (Evans et al., 1983) and 4–6 in mice and rats (McConnell et al., 2008; Lucas, 1983). Even taking into account the anatomical and physiological differences between rats and humans, it is reasonable to assume that a positive surface charge of TiO₂ NPs can favor intestinal absorption in both species. It should be considered that pathological events or perturbation of the small intestinal environment often determines a decrease of the intraluminal pH in humans and such conditions favor the existence of a positive charge on TiO₂ NPs surface.

Moreover, interaction with proliferating cells could explain the NPs accumulation pattern in rat intestinal tissue: NPs uptake occurs more efficiently in undifferentiated cells compared to non-dividing differentiated cells, such as enterocytes located on villus tip. Studies using differentiated CaCo-2 cells as intestinal model showed lower cellular uptake of TiO₂ NPs (Janter et al., 2014). In addition, cells with NPs inside cytoplasm might disseminate NPs between daughter cells that migrate along crypt/villus axis towards villus tip. It was observed in fact that TiO₂ particles were retained after 7 days of subculture of intestinal cells from mouse biopsies; then, after uptake, particles are divided and retained between daughter cells (Urrutia-Ortega et al., 2016).

The increased mucosal proliferation in normal rat intestine after TiO₂ NPs administration and the parallel in vitro data suggest that these NPs may induce mucosal hyperplasia. In general, to maintain gut homeostasis, increased proliferation is counterbalanced by an increased enterocyte apoptosis, which also promotes disposal of genetically aberrant stem cells and prevents tumorigenesis. We cannot exclude that over time the normal control mechanisms might counteract the increased villous proliferation in rats orally exposed to TiO₂ NPs. However, the significant increase observed after a short exposure time suggests that the induced hyperplasia may persist.

Enhanced cell proliferation, when insufficiently counter-weighted by apoptosis, increases the rate of endogenous mutations in tumor suppressor genes and oncogenes (Kinzler and Vogelstein, 1996). Hyperproliferation and abnormal distribution of replicating cells along the length of the colonic crypts have been described in the apparently normal mucosa of subjects prone to develop colon cancer (Lipkin, 1988; Anti et al., 1993).

Villous hyperplasia was observed most commonly as an adaptive response in the small intestine following extensive intestinal resection (Gleson et al., 1971). Increase in epithelial cell proliferation is thought to be crucial for repair and regeneration processes in recovering bowel. However, the compensatory hyperplasia characterized by increased villous height and crypt depth might predispose to the development of neoplasia in the residual adapted bowel (Williamson et al., 1980). As shown by Botelho et al. (2014), TiO₂ NPs induce tumor-like phenotypes in human gastric epithelial cells by stimulation of proliferation and apoptosis inhibition; in addition, in a mouse model of colitis-associated cancer food grade TiO₂ exacerbated tumor formation in a colitis-associated cancer model in Balb/c mice (Urrutia-Ortega et al., 2016).

The possible role of TiO₂ NPs in carcinogenesis is related to their ability to induce DNA adducts; the International Agency for Research on Cancer (IARC) classified TiO₂ as a Group 2B carcinogen (possibly carcinogenic to humans) based on mechanistic and animal studies addressing exposure by inhalation (IARC, 2010). Differently from other NPs, DNA adducts induced by TiO₂ in intestinal cells were efficiently repaired (Zijno et al., 2015) but erroneous repair may cause mutations and ultimately lead to cell transformation.

Since dietary intake of TiO₂ NPs occurs frequently and continuously albeit at low doses, a repeated effect on intestinal mucosa could lead to an increased risk of tumor development or to progression of existing tumoral processes. Even though further investigations are needed, our results prompt toward a greater attention to TiO₂ NPs as a factor that could be involved in proliferative processes that can lead to carcinogenesis in the gut.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.01.031.

Appendix A. Supplementary data

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References


proliferation patterns as predictors of adenomatous colorectal polyp recurrence. Gut 34, 525–530.


