Titanium dioxide nanoparticles translocate through differentiated Caco-2 cells monolayers, without disrupting the barrier functionality or inducing genotoxic damage

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Running title: TiO_2NPs effects on Caco-2 cell monolayers

ABSTRACT

The widespread use of titanium dioxide nanoparticles (TiO_2NPs) in commercial food products makes intestinal cells a relevant target. Accordingly, we have used the human colon adenocarcinoma Caco-2 cells to detect their potential harmful effects. Caco-2 cells can differentiate to enterocytic-like cells, forming consistent cell monolayers used as a model of intestinal barrier. Using both undifferentiated and differentiated Caco-2 cells we have explored a set of biomarkers, aiming to evaluate undesirable effects associated to TiO_2NPs exposure. Results indicate non-toxic effects in exposures ranging up to 200 µg/mL. Significant differences were observed in cell uptake, with a higher amount of incorporated TiO_2NPs in undifferentiated cells, as visualized using confocal microscopy. In well-stablished monolayers, translocation was detected by using both confocal microscopy and Transmission Electron Microscopy with Energy-dispersive X-ray spectroscopy (TEM-EDX). In spite of the observed uptake and translocation, TiO_2NPs exposure did not modify the integrity of the monolayer, as measured using TEER and LY methods. The potential genotoxic effects in differentiated cells were evaluated in the comet assay, without and with FPG enzyme to detect oxidatively damaged DNA bases. Although some changes were detected at the lower dose (10 µg/mL), no effects were observed at higher doses.

SHORT ABSTRACT

Undesirable effects associated of TiO_2NPs exposure, have been evaluated in both undifferentiated and differentiated Caco-2 cells. Significant differences were observed in cell uptake, being lower in Caco-2 cells constituting part of a well-established monolayer. TiO_2NPs translocate through the barrier but without disrupt this model of intestinal barrier and without inducing genotoxic damage

Keywords: titanium dioxide nanoparticles; differentiated Caco-2 cells; monolayer-integrity; uptake; translocation; genotoxicity.

Introduction

Titanium dioxide (TiO_2) powder is extensively used in many consumer products related with cosmetics, sunscreens, paints, and food products (Buettner & Valentine, 2011). As a human food additive, the Food and Drug Administration (USA) approved the use of TiO_2 in 1966 (CFR, 2016) and in the European Union the European Food Safety Authority (EFSA) have designed food grade TiO_2 as E171, being "E numbers" codes for substance that can be used as food additives (EFSA, 2016). Consequently, the extended use of TiO_2 as a food-grade additive/supplement ensures people exposure. Moreover, an important proportion of the E171 source corresponds to its nanoparticulate form (Weir *et al.,* 2012; Yang *et al.,* 2014).

Because of the novel properties offered by their small size, titanium dioxide nanoparticles (TiO_2NPs) are increasingly introduced in a number of commercial food products (Chaudhry *et al.,* 2008). Mainly, TiO_2NPs are used in significant amount as whitening agent, and those foods with the highest content of TiO_2NPs included candies, sweets, and chewing gums. This means that children are the part of the population with the highest exposure risk, and the realistic amount of the digested TiO_2 was averaged in 2-3 mg/Kg/bw/day for children under the age of 10 years (Weir *et al.,* (2016). Although the daily intake of TiO_2 for the overall population was estimated as 1.28 mg/kg/bw (EFSA, 2016), a recent estimation in the UK point out that the dietary consumption of TiO_2 is over 5.4 mg/person/day (Song *et al.,* 2015). All this information emphasizes the relevance of TiO_2NPs exposure and, consequently, demand strong and sound research on their potential health risks.

Until now, many *in vitro* toxicological studies using TiO_2NPs have been carried out, as reviewed (Shi *et al.,* 2013; Grande & Rucci, 2016). Nevertheless, results are conflictive assuming that potential cell damage can depend on different physico-chemical characteristics, such as size, crystal structure, and photo-activation of the used TiO_2NPs (Grande & Rucci, 2016).

Taking into account the observed discrepancies between studies, special attention must be focused on ingestion, as the main route of exposure. This means that *in vitro* studies should use as a target intestinal cell models, such as Caco-2 cells. The human colon adenocarcinoma Caco-2 cell line has been extensively used due to its capacity of undergoing spontaneous differentiation leading to the formation of a cell monolayer. This system mimics the mature small intestine enterocytes, both morphologically and functionally (Car *et al.,* 2012). Although few studies have used this *in vitro* model to evaluate TiO_2NPs effects, disruption of the normal organization of the microvilli has been reported, independently of the NPs sedimentation (Faust *et al.,* 2014). Additionally, the transport through the monolayer was assumed to be negligible (Song *et al.,* 2015); in contrast with a previous report showing a putative TiO_2NPs translocation through the *in vitro* gut monolayer (Brun *et al.,* 2014). In Figure 1, we have include a scheme hypothesizing how NPs can move from the apical to the basolateral side of the Caco-2 cell monolayer.

To clarify these contradictory results we have carried out a wide and complete study including different biomarkers to evaluate the interaction of TiO_2NPs with the *in vitro* Caco-2 monolayer. NPs translocation, as well as cellular uptake, was detected with different qualitative and quantitative techniques such as Laser Confocal Microscopy, Transmission Electron Microscopy complemented with EDX (TEM+EDX) and ICP-MS, respectively. Damage in the monolayer morphology/integrity was evaluated by measuring the transepithelial electrical resistance (TEER), quantifying the amount of Lucifer Yellow able to cross the monolayer after TiO_2NPs exposure. Finally, the comet assay was carried out to study the putative genotoxic and oxidative damage on DNA bases associated with TiO_2NPs exposures.

Material and methods

Characterization of TiO_2NPs

Titanium dioxide nanoparticles (TiO_2NPs, NM100) were supplied by the Joint Research Center (JRC, Ispra, Italy) in the frame of the EU project NanoReg, and were dispersed according to the EU Nanogenotox protocol (Nanogenotox, 2011). To proceed, TiO_2NPs were pre-wetted in 0.5% absolute ethanol and dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water. TiO_2NPs were sonicated in the dispersion medium for 16 min to obtain a stock dispersion of 2.56 mg/mL. For their characterization, in addition to the information supplied in the frame of the project (JRC), Transmission Electron Microscopy (TEM) was used on a JOEL JEM-1400 instrument (Jeol LTD, Tokyo, Japan). TEM images were processed by Image J software to calculate the size diameter, by measuring over 100 particles in random fields of view and, in addition, were used to determine morphology. Moreover, the hydrodynamic size and z-potential were evaluated by using dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) methodologies, in a Malvern Zetasizer Nano-ZS zen3600 device (Malvern, UK).

Caco-2 culture to differentiated cell monolayers

Dr. Isabella Angelis, from the Istituto Superiore di Sanità (ISS, Italia), pleasantly provided the human colon adenocarcinoma cell line Caco-2. This cell line was weekly maintained in Dulbecco's modified Eagle's High Glucose medium without piruvate (DMEM w/o piruvate, Life Technologies NY) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) (PAA Laboratories GmbH, Pashing, Austria) and 2.5 mg/mL plasmocin (Invivo Gen, San Diego, CA). Cells were placed in humidified atmosphere of 5% CO_2 and 95% air at 37 ºC. For experiments with undifferentiated Caco-2 cells, cells were seeded in the desired multi-well culture plate and allowed to attach for 24 h. For differentiated Caco-2 cells, cells were grown for 21 days in 12-well culture plates onto polyethylene terephthalate (PET) transwells with 1 µm pore size and an area of 1.12 cm^2. Cell culture medium was changed every 2 days. To study the

different biological-induced effects both undifferentiated and differentiated Caco-2 cells were exposed to different concentrations of TiO_2NPs for 24 h.

Cell viability after TiO_2NPs treatments

To determine the range of sub-toxic doses to be used in the whole experiment, a preliminary toxicity experiment was performed. Cell viability was determined by the Beckman counter method with a ZTM Series coulter-counter (Beckman Coulter Inc., CA). Differentiated and undifferentiated Caco-2 cells were treated for 24 h with different concentrations of TiO_2NPs ranging from 1 to 200 µg/mL. We used these two types of cells to determine the role of cell differentiation on the toxicity induced by TiO_2NPs. After treatments, cells were washed three times with i) 0.5 mL of PBS (1%) and incubated 3 min at 37 ºC with 0.25 mL of Trypsin, ii) 1.5% and iii) 1% respectively, to detach and separate them. Finally, cells were diluted in an ISOTON solution (1/100), and counted with the Beckman Cell Counter. Viability values for each concentration were calculated from averaging three independent survival experiments.

Monolayer integrity studies

To evaluate possible detrimental effects of TiO_2NPs in the integrity of the Caco-2 monolayer, trans-epithelial electrical resistance (TEER) measurements and the Lucifer Yellow assay were carried out. The TEER value of each sample was measured with an epithelial voltmeter (Millicell-ERS volt-ohm meter), prior and post TiO_2NPs treatment. TEER values were analyzed at 7, 14 and 21 days after seeding, through the processes of cell differentiation and monolayer formation. TEER measurements were done after 24 h of TiO_2NPs exposure. Monolayers with TEER values ranging 350 Ω/cm^2 were used for further experiments. TEER values were calculated according to the formula TEER = [Ω (cell inserts) - Ω (cell free inserts)] x 1.12 cm^2.

To determine the integrity of the joint junctions, the paracellular passage of Lucifer yellow (LY) was determined. Following the 24 h of TiO_2NPs treatment, Lucifer yellow was added to the apical compartment, at the concentration of 0.4 mg/mL, and placed into the

incubator during 2 h. A prompt fluorimeter (Victor III, Perkin Elmet) plate reader was used to determine the amount of LY able to cross the monolayer by 405 nm excitation and 535 nm emission.

TiO_2NPs uptake by Caco-2 cells

Laser Confocal Microscopy has shown to be a useful method to locate metallic NPs inside cells (Vila et al., 2017a). According to that, this method was used to visualize the fate of TiO_2NPs in undifferentiated and differentiated Caco-2 cells treated with 100 µg/mL of TiO_2NPs during 24h. After exposure, cells were stained with Hoechst 33342 and CellMask at 1/500 and 2/500 dilutions, respectively, during 10 min. The imaging of the stained samples was obtained by using a confocal laser scanning microscope Leica TCS SP5. TiO_2NPs were visualized by its own reflective capability looking as green spots in contrast with the blue color of nucleus and red color of cell membranes. Confocal images were processed with the software Huygens Essential 4.4.0p6 (Scientific Volume Imaging, Netherlands), and Imaris 7.2.1 (Bitplane, AG).

TiO_2NPs transport through the differentiated Caco-2 monolayer

To demonstrate the crossing of TiO_2NPs from apical to basolateral compartment, through the differentiated Caco-2 monolayer, different qualitative and quantitative methods were used. Transmission Electron Microscopy with Energy-dispersive X-ray spectroscopy (TEM-EDX) (Hitachi H 7000), Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) 7500ce (Agilent Technologies) and Fluorescent Confocal Microscopy (LEICA TSC SP5) techniques were used to detect and/or quantify those TiO_2NPs capable to cross the differentiated Caco-2 cells monolayer. Briefly, after 24 h of TiO_2NPs treatment, total medium (1.5 mL) of each basal compartment was collected in 1.5 mL microtubes. To eliminate the excess of inorganic material aggregation and crystallized proteins, samples were treated with proteinase K (100 µg/mL) and centrifuged in a speed vacuum at 37 ºC for 2 h to concentrate the amount of TiO_2NPs. This concentrate was used in the above-mentioned techniques.

Genotoxic and oxidative DNA damage quantification by using the comet assay

The potential induction of genotoxic and oxidative DNA damage to differentiated Caco-2 cells was assessed by the alkaline comet assay after 24 h of TiO_2NPs treatment. The addition of formamidopyrimidine DNA glycosylase (FPG enzyme) was used to measure oxidatively damaged DNA bases. Briefly, once treated, differentiated Caco-2 cells were washed twice with PBS, trypsinized (1% trypsin), and centrifuged at 1000 rpm for 8 min. The pellet was resuspended in PBS to obtain around 17,500 cells/25 µL, and placed in ice at 4 ºC, to avoid DNA repair. Cells were mixed with 0.75% of LMP agarose at 37 ºC and dropped (7 µL/drop and 3 drops/sample) onto Gelbond® (GB) films. In each GB two replicates of each concentration were placed (3 drops + 2 drops). Two replicates per experiment were carried out. After that, 12 samples/drops were established for each concentration. Cells on GB were lysed in cold lysis buffer at 4ºC overnight and pH 10. After that, GB were washed twice (1 x 5 min and 1 x 50 min) in enzyme buffer at 4 ºC and pH 8.0 followed by an incubation of 30 min at 37 ºC with enzyme buffer. One GB was incubated with enzyme buffer and FPG enzyme (1/10.000) and the other with a free-FPG enzyme buffer. GB were then incubated with electrophoresis buffer (alkaline buffer) for 35 min followed by the electrophoresis step for 20 min at 20 V and 300 mA at 4 ºC. Finally, GB were rinsed twice in cold PBS for 5 min, in distilled water for 1 min, fixed in absolute ethanol for at least 2 h, air-dried overnight at room temperature, and stained with SYBR Gold for 20 min. Each GB film was cut in two similar sized parts to fit into an acrylic slide (52.5 x 75 x3 mm). A coverslip of 52.5 x 75 mm was placed on top of the drops effectively sealing the samples. GB were observed using an epifluorescent Olympus BX50, and damage was quantified measuring the percentage of DNA in tail, by using the Komet 5.5 Image analysis software. One hundred randomly selected comet images were analyzed per sample. Treatments lasting for 30 min of 5 mM of potassium bromate ($KBrO_3$) and 2.5 mM of methylmethanesulfonate (MMS) were used as positive control of oxidative and genotoxic damage, respectively.

Statistical analysis

Once demonstrated the normal distribution of the parameters tested, an unpaired Student's *t*-test and an Analysis of Variance (ANOVA) were used to analyze the differences among group means and their associated procedures. In all cases, a two-sided p-value of $P<0.05$ was considered statistically significant.

Results

Characterization of TiO_2NPs

TEM images of TiO_2NPs (NM100) in stock solution revealed spherical morphology (Figure 2A). Measuring 100 particles in random fields of view we could perform a size distribution of the NPs (Figure 2B), and determine a mean size of 104.01 ± 39.42 nm (Figure 2C). Some aggregation or agglomeration of TiO_2NPs in culture medium suspension was observed, as indicated by the poly-dispersion index (PDI=0.36 ± 0.04).The DLS analysis reported values of 244.70 ± 14.40 nm (Figure 2C). No significant differences were observed between concentrations, nor between times (0 or 24 h) (data not shown).

Toxicity of TiO_2NPs

Caco-2 cells were exposed to TiO_2NPs (NM100) at concentrations ranging from 1 to 200 µg/mL, and exposures last for 24 h. To determine the role of the differentiation status, two stages of Caco-2 cells (undifferentiated and differentiated cells) were used to evaluate whether differentiation process modulates the response to the exposure. The Counter cells method was chosen to avoid the problems of interference that NPs could produce if fluorescent methods were used. Results indicate lack of toxic effects, independently of the cell status (Figure 3). As observed, cell viability remained higher than 80%, under our experimental conditions, after TiO_2NPs exposure in both, undifferentiated and differentiated Caco-2 cells. According to those results, we selected sub-toxic doses (10, 25 and 100 µg/mL) to conduct the remained experiments.

Assessment of the monolayer's integrity and permeability

NPs could have the ability to cross the monolayer, internalize into it, or produce cell damage. The alteration of the monolayer's integrity may affect not only the entry of the evaluated NP, but also the permeability to other compounds. In this way, toxic compounds that are not able to cross the monolayer under normal conditions could cross

it, if monolayer is altered. This means that this measure became an important parameter. In our case, we have determined the integrity and permeability of the monolayer after TiO_2NPs exposure by using TEER and LY assays. TEER values during 21 days of differentiation progressively increase due to the formation of tight junctions between cells, as we show in Figure 4A, reaching a value of 451.13 ± 14.99 Ω/cm^2 at day 21. Intriguingly, after 24 h of TiO_2NPs exposure (10, 25 and 100 µg/mL), no variations in TEER values were observed, demonstrating no alteration of monolayer's integrity.

Regarding monolayer's permeability evaluation, the LY assay was carried out. Lucifer Yellow is a paracellular compound able to cross the Caco-2 monolayer only when tight junctions are opened/disturbed, due to its paracellular transport. No presence of LY in the basolateral (BL) side indicates a good stability of the monolayer. Figure 5 shows that, despite an apparent increased presence of LY in the BL side, no statistical differences were found in permeability after TiO_2NPs exposure (10, 25 and 100 µg/mL), when comparing the observed values with those obtained in the untreated control.

Taking all together, our results indicate that exposure to TiO_2NPs does not alter the integrity or permeability of the monolayer of Caco-2 cells, after treatments lasting for 24 h.

Cellular uptake of TiO_2NPs

To investigate NPs internalization, confocal microscopy was used. The identification of TiO_2NPs within cells is possible due to the capability of metallic NPs to reflect polarized light. A sophisticate software allows to recover NPs and nucleus with a mask for distinguish TiO_2NPs's localization (membrane, cytoplasm or nucleus). Although confocal microscopy offers a good alternative for the qualitative observation of metallic NPs internalization, it does not allow us to distinguish between individual NPs and NPs aggregates. As in the previous studies, undifferentiated and differentiated cells were used to identify if the stage of differentiation does affects the internalization of TiO_2NPs. The highest concentration (100 µg/mL) was chosen in order to facilitate the observation

of NPs inside cells. No quantitative studies were performed in the studies using confocal microscopy.

Figure 6 contains model confocal microscopy images of undifferentiated (A, B) and differentiated (C, D) Caco-2 cells. The cell membrane was stained in red, nucleus in blue, and aggregates/agglomerates of TiO_2NPs were observed in green. As we mention before, after recovering with software mask, the localization of structures in a three-dimensional space is possible (Figure 6. B, D).

As observed, the thickness of undifferentiated cells is much lower than differentiated cells (Figure 6) because cells are not polarized and they do not express microvilli in the apical part. Undifferentiated Caco-2 cells internalized a great number of TiO_2NPs aggregates within cells (A), and within nucleus (B). By analyzing different sections of the z-axis we can observe NPs both inside the nucleus and attached to the nuclear envelope (B). On the contrary, in the differentiated state TiO_2NPs were found fundamentally in the apical membrane (C and D; pointed by green arrows). Sections of z-axis confirmed that low numbers of TiO_2NPs were internalized inside cells (D).

Translocation of TiO_2NPs through Caco-2 cells monolayer

If TiO_2NPs are able to cross the cell barrier of Caco-2, they can be collected/detected in the basolateral side. To prove this crossing action different qualitative techniques, including TEM+EDX and confocal microscopy, and quantitative techniques like ICP-MS, were used. The obtained results are shown in Figure 7. Since the aim of the study was to demonstrate the potentially of TiO_2NPs to cross the barrier, only the highest concentration (100 µg/mL) was used.

TEM+EDX technique posed some limitations in the basolateral medium analysis as large amount of organic matter and mineral salts was observed (data not shown), hindering the analysis of NPs. In spite of that, TiO_2NPs were noticed by direct TEM observation (A) and its complement with EDX analysis confirmed the presence of titanium in the composition of this structure (B). Taking advantage of the ability of metal

NPs to reflect polarized light, the basolateral medium was also analyzed by confocal microscopy. As observed, significant amounts of green spots corresponding to TiO_2NPs were observed by confocal microscopy in the basolateral medium after exposure (F), compared with untreated cells where no green signals were evident (E).

In addition, potential translocation of TiO_2NPs was chemically quantified by ICP-MS on the basolateral medium, and on the apical medium. It must be remembered that ICP-MS technique only detects the element in question, it does not distinguish between ionic and NPs form. In this case, the effects of the three concentrations (10, 25 and 100 µg/mL) were used. Our results indicate that Ti concentration values were below the detection limit of the technique (0.01 µg). This means that no significant increases of Ti were apparent in the basolateral medium (C). When the apical medium was evaluated (to test the methodology), the absolute amount of Ti (µg) in the apical chamber was relevant showing increased values when the concentration initially applied was higher (D). When the measured values were expressed as percentage of the element, with regard to the concentration initially applied, 15%, 9.2% and 12% of titanium were observed after exposures with 10, 25 and 100 µg/mL, respectively. This means that the rest of the material internalizes or remains sedimented in the cell monolayer. Taking together all the results, we can conclude that small amounts of TiO_2NPs can cross the barrier.

Genotoxic and oxidative DNA damage induction

NPs exposure could produce several potential harmful effects on the exposed cells, being one of the most known ROS production. For this reason, DNA damage and, more specific, oxidative DNA damage detection are of crucial relevance. To assess general genotoxic damage resulting from DNA breaks, and to evaluate the oxidative damage induced at DNA bases, the alkaline comet assay, supplemented with FPG, was carried out. The effects of three concentrations (10, 25 and 100 µg/mL) were assayed in exposures lasting for 24 h after 21 days of Caco-2 differentiation. The percentage of DNA in tail was used as biomarker of effect. As observed, MMS (0.5 mM) used as a positive

control, produced a significant DNA damage increase, supporting the suitability of the assay (Figure 8A). However, no increases in DNA breaks were detected after TiO_2NPs exposure. Furthermore, when FPG enzyme was used, no oxidative DNA damage was observed (Figure 8B). FPG enzyme detects oxidized DNA bases and induces single-strand breaks after their excision; making possible the oxidative DNA damage detection, in the conditions used in the comet assay. This means that no significant induction of oxidized bases was produced after 24 h of exposure (Figure 8B). In this case, potassium bromate ($KBrO_3$), used as a positive pro-oxidant agent, induced clear and significant increases in the percentage of DNA in tail.

Discussion

The Woodrow Wilson International Center for Scholars, and the Project on Emerging Nanotechnologies created the Nanotechnology Consumer Products Inventory (CPI) in 2005. To date, the inventory has documented 1423 consumer products, from which 14 are titanium-based products and 92 contains titanium dioxide nanoparticles (TiO_2NPs) (Vance *et al.,* 2015). Since TiO_2NPs are used in processed and manufactured food-products, ingestion would be the main route of entrance, and the intestinal barrier the potential target.

In this context, we aimed to study the effects and behaviour of TiO_2NPs in Caco-2 cells monolayer, as an *in vitro* model of intestinal barrier. Caco-2 cells are able to spontaneously differentiate and polarize into enterocyte like-cells, forming a resistant monolayer which separate and mimics both small intestinal atmospheres; the mucosa and the serosa (Sambuy *et al.,* 2005). This system has been widely used in the pharmaceutical industry (Shah *et al.,* 2006) and it is proposed as a suitable tool to determine the potential translocation of ingested nanomaterials (Faust *et al.,* 2014).

As a first step we evaluated whether the enterocyte like-shape, and its morphological and functional characteristics, confers some protection against the potential toxicity of TiO_2NPs. When the cytotoxicity of undifferentiated (1-day seeded cells) and differentiated (21-days seeded cells) Caco-2 cells were evaluated, similar non-toxic effects were obtained. This lack of toxic effects would agree with previous studies using differentiated cells (Brun *et al.,* 2014; Song *et al.,* 2015; Dorier *et al.,* 2017). In spite of these non-cytotoxic effects, loss of microvilli after exposure to TiO_2NPs were reported (Faust *et al.,* 2014). Microvilli are essential for a proper enterocyte-like cell functionality as they present a wide number of enzymes involved in several cellular roles, such as mineral/nutrient transport (sodium-dependent glucose co-transporters, fatty acid-binding proteins, etc.) and nutrient digestion (Rodriguez *et al.,* 2017; Guo *et al.,* 2017; Bhattacherjee *et al.,* 2017). This loss of microvilli would translate to potential toxic effects.

When Caco-2 cells are used, as occurs in other circumstances, differences among studies are reported. These differences can be explained by the high variability that exist between laboratory clones. In fact, the presence of subpopulations throughout the differentiation step are well-known (Briske-Anderson *et al.,* 1997; Yu *et al.,* 1997).

Independently of the cytotoxicity data, the monolayer integrity is one of the principal characteristic of this *in vitro* monolayer model. Thus, variations in TEER or LY passage are indicative of disruption of integrity. In our study, the exposure to TiO_2NPs did not decrease TEER values, at any of the tested concentrations, meaning that tight junctions were non-disrupted. This is consistent with other studies where no disruption was seen after exposure to similar concentrations of TiO_2NPs (Brun *et al.,* 2014; Faust *et al.,* 2014; Janer *et al.,* 2014; Song *et al.,* 2015). To explain these results, it is possible that the induced alterations in cell junction constituents are actively repaired by the novo expression of tight junctions and adherent junction's proteins. This is suggested from the observed changes in the levels of gene expression (Brun *et al.,* 2014). Even though, monolayer integrity disruptions were observed after very high exposures to TiO_2NPs (Koeneman *et al.,* 2010; Dorier *et al.,* 2017). To confirm the results of the TEER assay, the paracellular transport was examined. Our results in the LY assay confirm that the monolayer integrity was not affected after 24 h of TiO_2NPs exposure.

Besides the apparent non-disruption of the monolayer integrity, the direct observation of the potential uptake of TiO_2NPs by Caco-2 cells is relevant in terms of toxic risk. In fact, we have already shown the TiO_2NPs uptake by BEAS-2B cells using TEM. In this case, agglomerates of TiO_2NPs were observed inside cytoplasm vesicles (Vales *et al.*, 2015). Due to the granulated nature of the Caco-2 cells cytoplasm TEM figures are difficult to interpret and, to overcome this difficulty, confocal microscopy has revealed to be a suitable tool (Vila *et al*., 2017b). Thus, when both undifferentiated and differentiated Caco-2 cells exposed to TiO_2NPs were evaluated using confocal microscopy, we could clearly detect, and specifically locate TiO_2NPs. In undifferentiated Caco-2 cells large amounts of TiO_2NPs were observed into the cytoplasm reaching the nucleus. Contrarily,

low numbers were internalized in the Caco-2 cells monolayer model. Our images demonstrate that most of the TiO_2NPs remained attached in the cells shed and extracellular matrix, probably retained in the microvilli. Our results emphasize the usefulness of the confocal microscopy to evaluate metallic NMs. In addition, they demonstrate that differentiated Caco-2 cells behave as a real barrier posing difficulties to TiO_2NPs to internalize and, eventually, crossing the enterocytic barrier. Our findings showing a differential behavior between undifferentiated and differentiated Caco-2 cells agree those using TEM (Faust *et al.,* 2014; Song *et al.,* 2015), and by micro particle-induced X-ray emission (Brun *et al.,* 2014), probably due to the internalization route of NPs and the state of Caco-2 cells differentiation. Depending on the size of the NPs there are different mechanisms of cell internalization; small NPs can enter by diffusion, but medium sized NPs are internalized by endocytosis (Bruinink *et al*., 2015). The charge of NPs and other characteristics can also modulate NPs uptake (Roy *et al.,* 2014). As above indicated we visualized TiO_2NPs into BEAS-2B cells inside vesicles (Vila *et al.,* 2017b). The endocytosis mechanisms could be modified due to the state of cell differentiation and it could explain the differential behavior between undifferentiated and differentiated Caco-2 cells. However, none of those studies demonstrated the presence of TiO_2NPs in the nucleus.

We have demonstrated that TiO_2NPs were able to cross the barrier through the cell monolayer and the semi-permeable PET membrane, by using TEM and TEM complemented with EDX analysis. This translocation was confirmed by laser scanning confocal microscopy. Surprisingly, the amount of titanium translocated to the basolateral chamber when detected by ICP-MS was beneath the ICP-MS detection limit (0.1 µg). Our ICP-MS results would agree with those from Brun *et al.* (2014) who could not detect TiO_2NPs translocation in any of the culture systems assayed (Caco-2 monolayer, Caco-2/HT-29 co-culture and Caco-2/Raji-B). In addition, very low levels of translocated TiO_2NPs were observed, independently whether they were digested or not (Song *et al.,*

2015). Our results reinforce the usefulness of using different approaches to detect NMs translocation to avoid false negative conclusions.

The potential genotoxic effects of TiO_2NPs have been the subject of many studies, with ambiguous results. Surprisingly, DNA damage induction has been reported in undifferentiated Caco-2 cells exposed to low concentrations (Gerloff *et al.,* 2012; Zijno *et al.,* 2015; Proquin *et al.,* 2017), but not when exposed to higher concentrations (Dorier *et al.,* 2015). This would agree with our data where only the lowest concentration tested produced a slight but statistically significant genotoxic DNA damage. This also would be consistent with our findings showing the higher TiO_2NPs concentrations tends to agglomerate in DMEM cell culture medium remaining attached to the extracellular membrane. In this way, this agglomeration would difficult their potential uptake by the cells and, in consequence, would limit the contact with the nucleus. It should be pointed out that working with NMs not always increased concentrations increases the effects, as already observed (Silva *et al.,* 2013).

Oxidative stress has become one of the most studied mechanisms of DNA damage mediated by NMs (Oberdörster *et al.,* 2005; Ayres *et al.,* 2008), and it has been suggested that TiO_2NPs generate intracellular ROS via photocatalysis (Jugan et al., 2012). In the literature, it has been described anatase as the polymorph with the highest photocatalytic activity of the two titanium polymorphs (Sclafani and Herrmann, 1996; Kim et al., 2014). Furthermore, photoactivated TiO_2NPs can generate highly reactive hydroxyl radicals ($^{\bullet}OH$) in aerated aqueous solutions, and superoxide radical anions ($O_2^{\bullet-}$) in non-aqueous media (Dodd and Jha, 2011). Although photoactivation appears to be an important mechanism of increasing cytotoxicity of TiO_2NPs, the majority of studies investigating TiO_2NPs toxicity did not take photoactivation into account. Nevertheless, the inherent capacity of TiO_2NPs to induce oxidative damage to DNA without photoactivation is unclear. With regard toTiO_2NPs micro-, but not nano-sized particles, induced significant levels of ROS in undifferentiated Caco-2 cells (Proquin *et al.,* 2017). Similarly, no significant generation of ROS was observed in Caco-2 monolayer in

exposures lasting for 24 h (Song *et al.,* 2015). Using a different model of intestinal (HT-29) cells, increases in ROS were observed after short exposure (6 h) as compared with treatments lasting for 24 h (Ammendolia *et al.,* 2017). This effect of time of exposure agree with studies using lung epithelial cell line, BEAS-2B where, although a high percentage of cell uptake was observed, no ROS detection was observed in treatments lasting for 24 h (Vila *et al.,* 2017) or when long-term exposures (up to 4 weeks) were used (Vales *et al.,* 2015). Among the harmful effects of ROS induction, damage on DNA is particularly relevant. Thus, detection of oxidized DNA bases became an important target to measure the genotoxic risk of NMs exposure. In our study, we quantified the levels of oxidative DNA damage induced by TiO_2NPs using the comet assay, in its alkaline and FPG-modified version (Azqueta & Collins, 2013). The lack of effects observed in our study agrees with those of Gerloff *et al.* (2012) where no induction of oxidized DNA bases was reported. This lack of effects can be associated with the exposure time. It is possible that the used exposure period (24 h) was enough long to induce the repair of the induced DNA lesions. This hypothesis agrees with the results of a recent study (Zijno *et al.,* 2015) where only short-term exposures (2-4 h) produced oxidized DNA bases damage. Interesting, these authors also showed a fast repair kinetics for 8-oxodG bases that were practically completely repaired after 24 h.

Altogether, our results with differentiated cells indicate that, in spite of the observed cell uptake and its translocation through the of Caco-2 cell monolayer, TiO_2NPs exposure did not affect the integrity of this barrier and, consequently, the entire system remains intact. Additionally, exposures lasting for 24 h to TiO_2NPs were not able to produce oxidative DNA damage.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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FIGURE LEGENDS

Figure 1. Scheme showing paracellular and transcellular transport of TiO_2NPs through the Caco-2 cell barrier. TiO_2NPs can move from the apical side of the transwell to the basolateral site when tight-junctions fails (paracellularly). Additionally, TiO_2NPs can enter into the cytoplasm by endocytosis, traffic through the cytoplasm, and reach the basolateral side by exocytosis.

Figure 2. TEM images of TiO_2NPs (NM100) in dried form (A). Size distribution of TiO_2NPs over 100 randomly selected particles (B). NPs average size (by TEM and DLS) and charge, by pre-wetting with 0.5% volume and steric stabilization using sterile-filtered 0.05% w/v BSA (C). Data are represented as mean ± SD.

Figure 3. Cell viability of undifferentiated (A) and differentiated (B) Caco-2 cells treated with TiO_2NPs for 24 h in a range of 0-200 μg/mL. Data are presented as a percentage of viable cells with regard to controls. Data are represented as mean ± SEM.

Figure 4. TEER values evaluated during Caco-2 cells differentiation process (A), and after 24 h of TiO_2NPs treatment (B). Data are represented as mean ± SEM. One way ANOVA was performed ***P<0.001.

Figure 5. Percentage of LY in the basolateral chamber after treatment with TiO_2NPs concentrations during 24 h. Data are represented as mean ± SEM. One way ANOVA was performed.

Figure 6. Confocal images from undifferentiated (A, B) and differentiated (C, D) Caco-2 cells treated with 100 μg/mL of TiO_2NPs. Cellular uptake was measured after 24 h of

treatment. Nucleuses are stained in blue, cell membranes in red, and TiO_2NPs in green. Arrows indicate the localization of TiO_2NPs inside the cells.

Figure7. Translocation studies of TiO_2NPs through differentiated Caco-2 monolayer. The basolateral extract was analyzed after 24 h of TiO_2NPs exposure by using TEM (A) and TEM+EDX (B); by using ICP-MS quantifying the µg of Ti in the basolateral chamber (C) and in the apical chamber (D); and by using confocal microscopy in both control (E) and TiO_2NPs treated monolayers (F).

Figure 8. Genotoxic (A) and oxidative DNA damage (B) observed after 24 h of TiO_2NPs exposure, using the comet assay. Three drops per replicate were used per concentration. Data represented as mean ± SEM. One way ANOVA was performed. ****P*<0.01; *****P*<0.001.

Figure 1

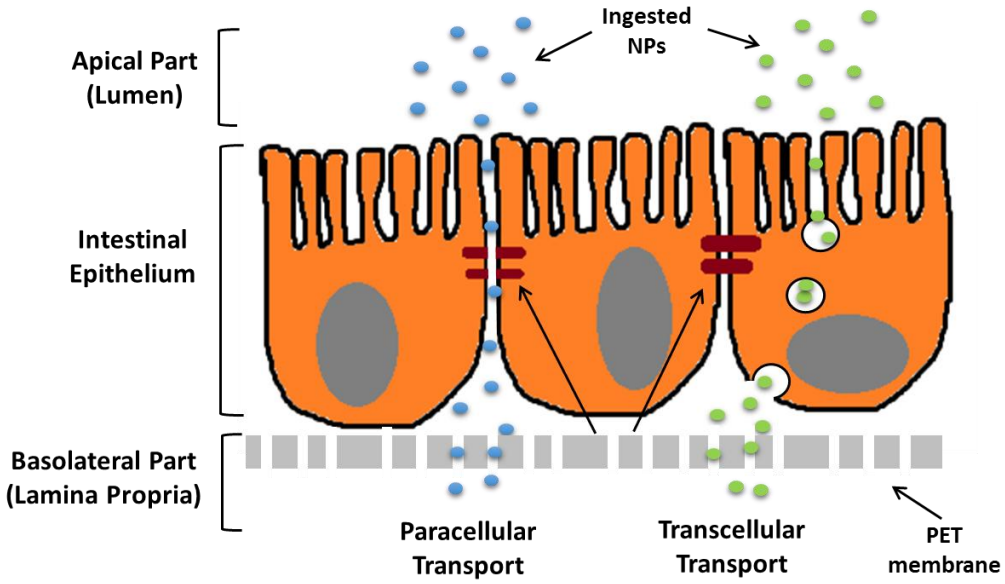
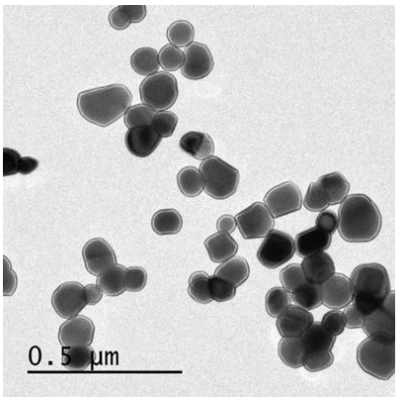
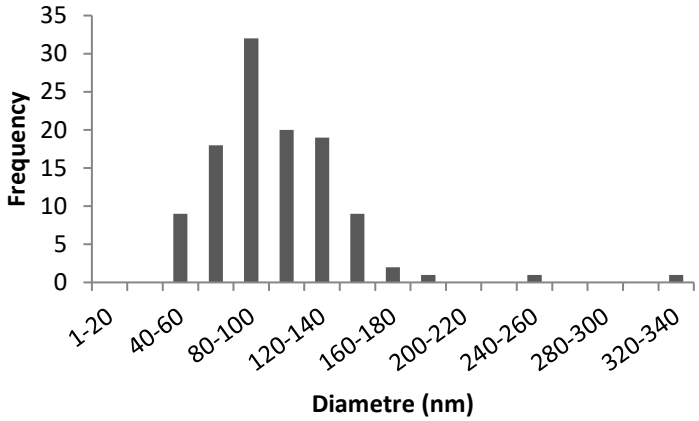

Ingested
NPs
Apical Part
(Lumen)
Intestinal
Epithelium
Basolateral Part
(Lamina Propria)
Paracellular
Transport
Transcellular
Transport
PET
membrane

Figure 2

A



B



C

	TEM	DLS	
NP	**Mean size (nm)**	**Average diameter (nm)**	**PDI**
TiO_2NPs	104.01 ± 39.42	244.70 ± 14.40	0.36 ± 0.04

Figure 3

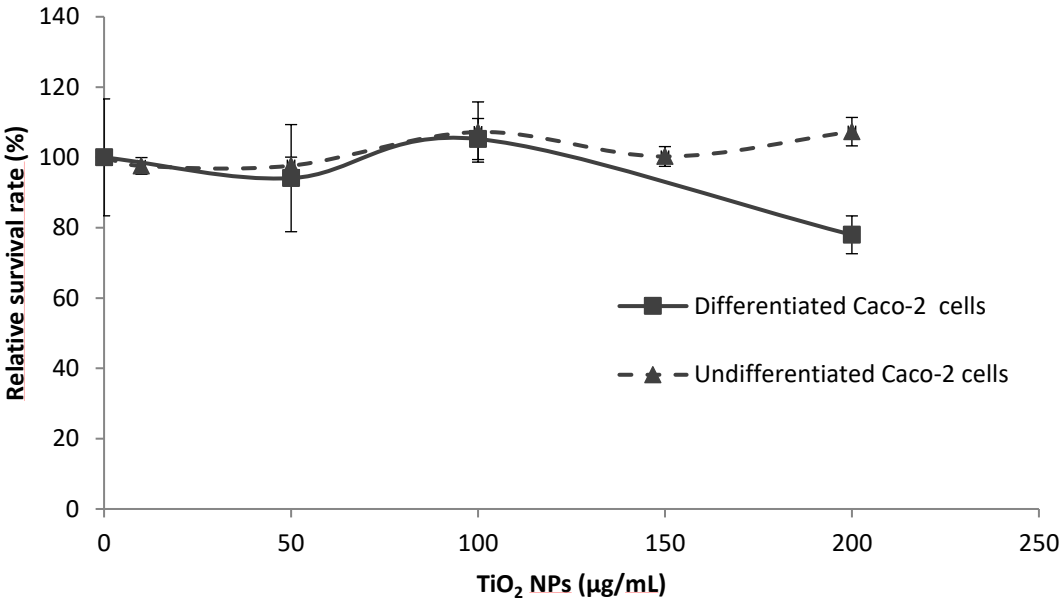
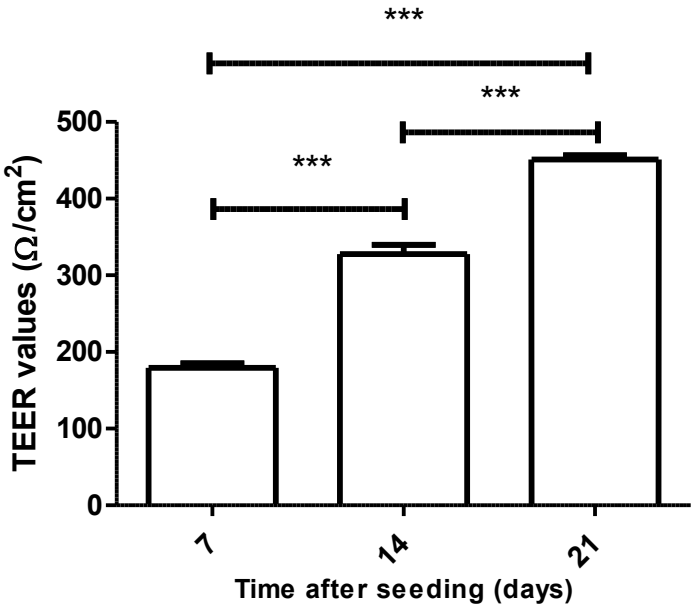

140
120
100
80
60
40
20
0
Relative survival rate (%)
0
50
100
150
200
250
TiO2 NPs (µg/mL)
Differentiated Caco-2 cells
Undifferentiated Caco-2 cells

Figure 4

A


***
***
***
500
400
300
200
100
0
TEER values (Ω/cm2)
7
14
21
Time after seeding (days)

B

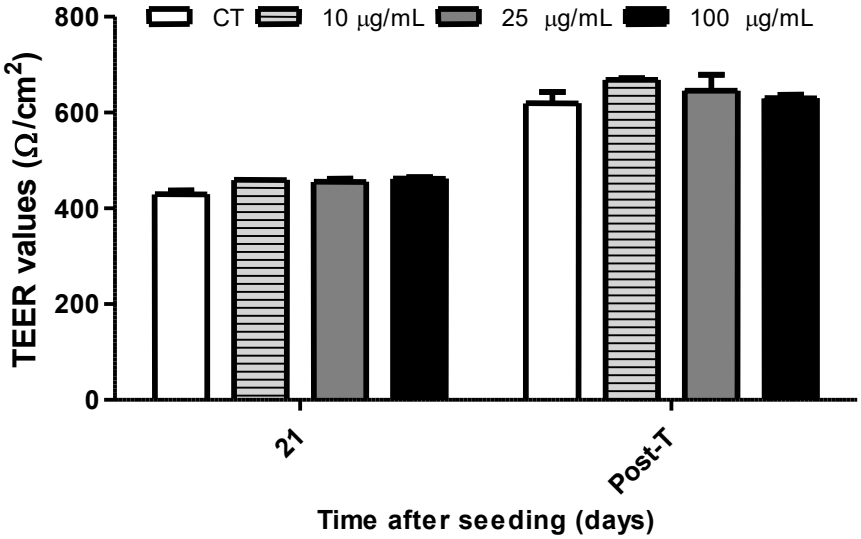

CT
10 μg/mL
25 μg/mL
100 μg/mL
800
600
400
200
0
TEER values (Ω/cm2)
21
Post-T
Time after seeding (days)

Figure 5

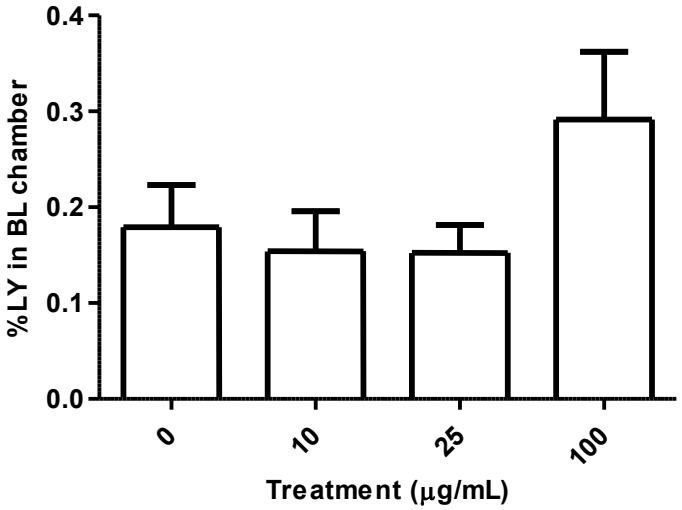


Figure 6

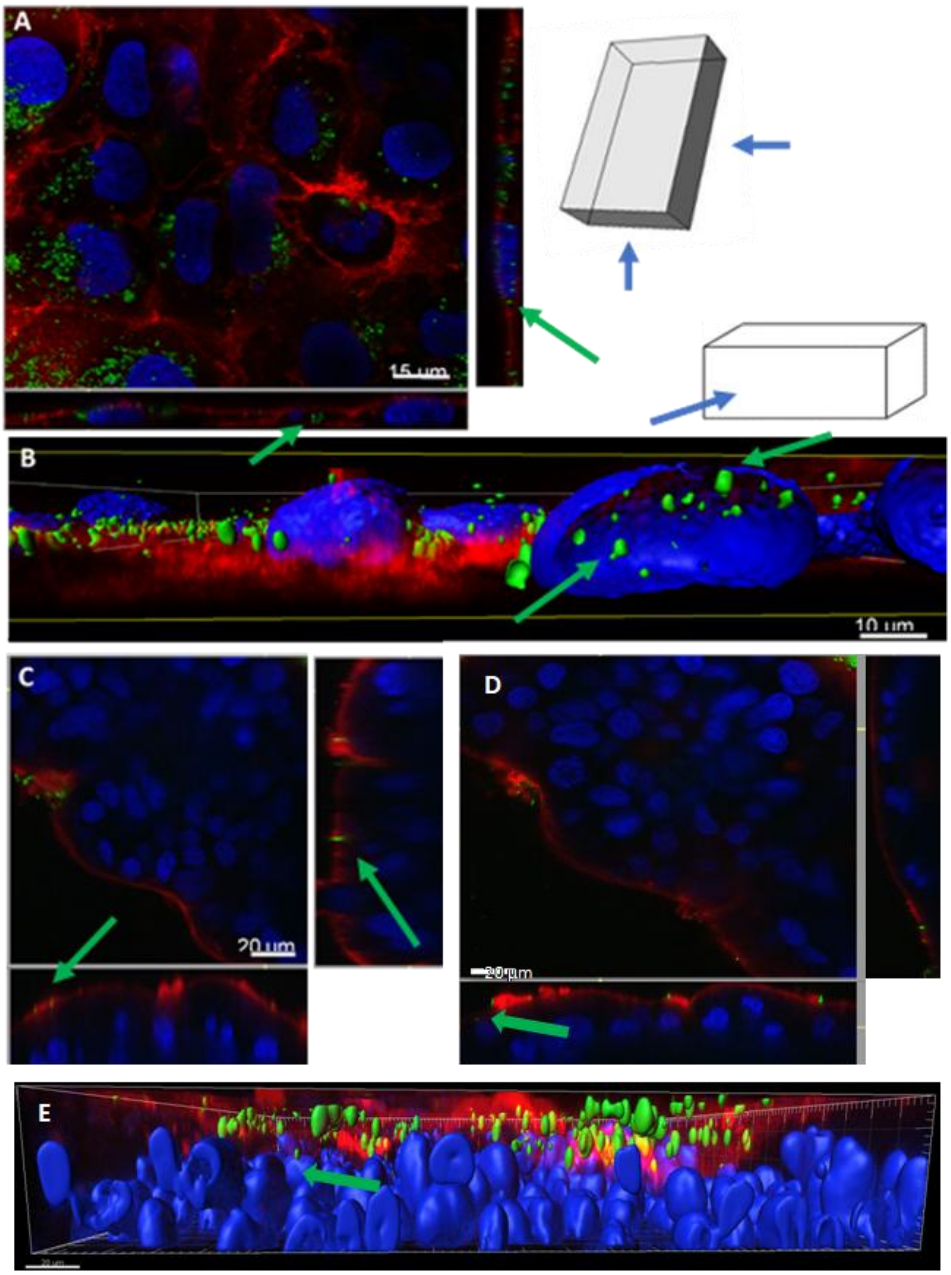

A
15 um
B
10 um
C
20 um
D
20 um
E

Figure 7

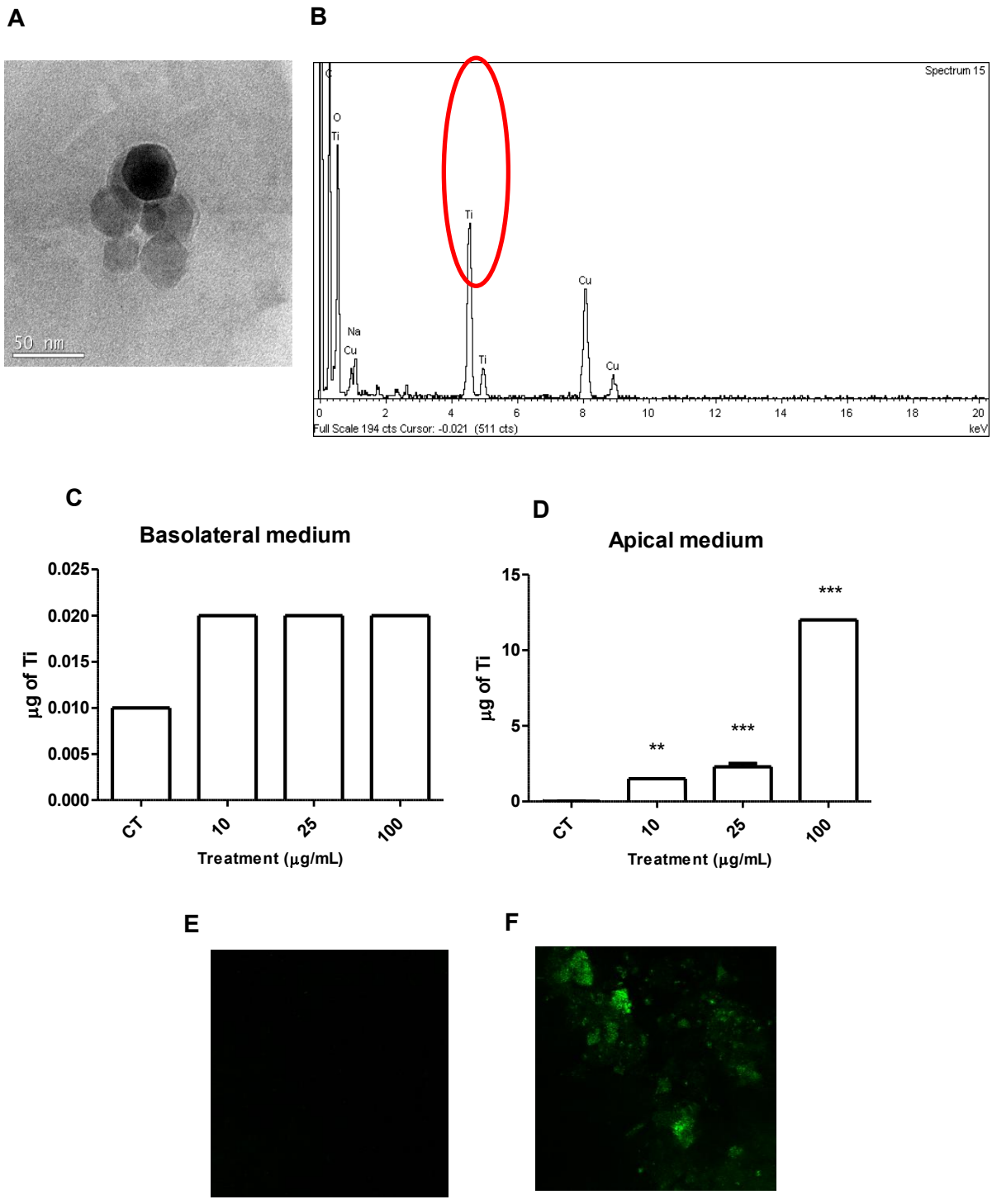
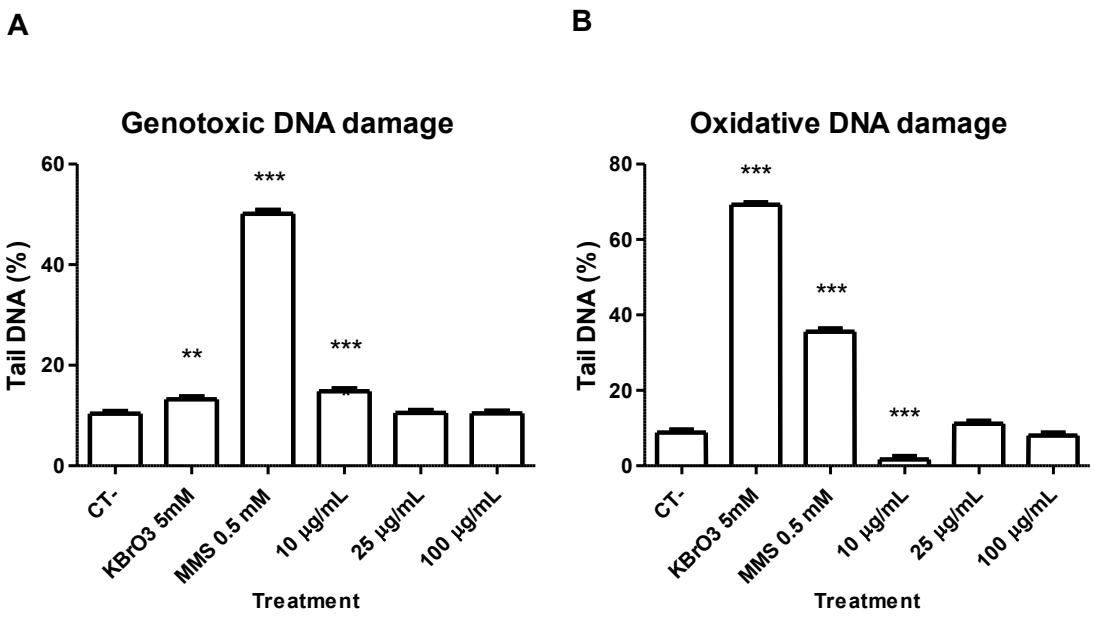

A
B
50 nm
C
O
Ti
Na
Cu
Ti
Ti
Cu
Cu
Spectrum 15
0
2
4
6
8
10
12
14
16
18
20
Full Scale 194 cts Cursor: -0.021 (511 cts)
keV
C
Basolateral medium
µg of Ti
0.025
0.020
0.015
0.010
0.005
0.000
CT
10
25
100
Treatment (µg/mL)
D
Apical medium
µg of Ti
15
10
5
0
**
***
***
CT
10
25
100
Treatment (µg/mL)
E
F

Figure 8


A
Genotoxic DNA damage
Tail DNA (%)
60
40
20
0
**
***
***
CT-
KBrO3 5mM
MMS 0.5 mM
10 µg/mL
25 µg/mL
100 µg/mL
Treatment
B
Oxidative DNA damage
Tail DNA (%)
80
60
40
20
0
***
***
***
CT-
KBrO3 5mM
MMS 0.5 mM
10 µg/mL
25 µg/mL
100 µg/mL
Treatment