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Cytototoxic and pro-inflammatory responses induced by ZnO nanoparticles in *in vitro* intestinal barrier

Short title (up to 70 characters): ZnO nanoparticle effects on *in vitro* intestinal barrier

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Abstract (<250 word)
ZnO nanoparticles (NPs) are widely used nowadays, thus the gastrointestinal exposure to ZnO NPs is likely to be relevant and the effects on the intestinal barrier should be investigated.
Polarized Caco-2 cells were exposed from the apical (Ap) and basolateral (Bl) compartments to increasing concentrations (0, 10, 50 and 100 µg/ml) of sono and commercial ZnO NPs. The trans-epithelial electrical resistance (TEER), cell viability, pro-inflammatory cytokine release and presence of protein oxidative damages were evaluated after exposure. TEER was not significantly affected by Ap exposure to either sono or commercial ZnO NPs at any tested concentrations. After Bl exposure to sono ZnO NPs (all the concentrations) and to 100 µg/ml of commercial ZnO NPs TEER was decreased ($P<0.05$). Ap and Bl exposure to 100 µg/ml sono ZnO NPs and Ap exposure to 50 µg/ml commercial ZnO NPs induced a significant ($P<0.05$) release of IL-6. A significant ($P<0.05$) release of IL-8 was observed after Ap exposure to ZnO NPs at 100 µg/ml and after Bl exposure to sono ZnO NPs at 100 µg/ml. Ap or Bl exposure to sono or commercial ZnO NPs did not affect TNF-α secretion or protein sulfhydryl oxidation.
In conclusion, the ZnO NPs exposure from the apical compartment appeared almost safe, while the exposure through the basal compartment appeared to be more hazardous and the different NP size and crystallinity seem to affect the mode of action, but further studies are necessary to better elucidate these toxicity mechanisms.

Short Abstract (<80 words)

In this study, polarized Caco-2 cells were exposed (apical/basolateral sides) to 0, 10, 50 and 100 µg/ml of sono and commercial zinc nanoparticles (ZnO NPs). Then trans-epithelial electrical resistance (TEER), cell viability, pro-inflammatory cytokine release and presence of protein oxidative damages were evaluated. TEER was decreased ($P<0.05$) after basolateral exposure to ZnO NPs. Exposure to ZnO NPs also induced a significant ($P<0.05$) release of IL-6 and IL-8, whereas TNF-α secretion or protein sulfhydryl oxidation seemed not to be affected.

1. Introduction

Zinc oxide nanoparticles (ZnO NPs) are among the most marketed globally. According to a recent industrial survey, after SiO_2 and TiO_2, ZnO is in fact the most abundant nano metal oxide annually produced (Piccinno, Gottschalk, Seeger, & Nowack, 2012). A such considerable industrial production is likely the consequence of the multiple properties and use of this nanomaterial (NM). Among them, the antimicrobial properties dysplayed by this NM are widely exploited to synthesize new antiseptic and antifouling materials (Perelshtein, Applerot, Perkas, Wehrschetz-Sigl, Hasmann, Guebitz, & Gedanken, 2009; Al-Fori, DobretsovMyint, & Dutta, 2014). Due to this antibacterial activity, ZnO is also efficiently used in food industry either as additive or for food packaging (Espitia, Soares, Coimbra, Andrade, Cruz, & Medeiros, 2012).

In addition, Zn is an abundant trace element in the animal body and its importance on health is abundantly documented (Tapiero & Tew, 2003). Since Zn cannot be stored, a regular dietary intake is required to satisfy the physiological needs. Basing on these knowledges, Zn has been traditionally and successfully used as feed supplement. It has been demonstrated that nano formulated Zn, like nano ZnO, promotes growth: in fact, a dose dependent effect of ZnO NPs was positively correlated with the growth performance and the positive immunological responses of livestock and poultry (Yang & Sun, 2006; Sahoo, Swain, & Mishra, 2014). A recent review summarized the beneficial effects of nano ZnO and its promising application as mineral supplement to the human and livestock diet (Swain, Rao, Rajendran, Dominic, & Selvaraju, 2016).

Considering these usages and other possible technological applications in food and feed industry, the ZnO NP ingestion and impact on the intestinal tract should be considered as a relevant exposure scenario. This ultimately calls for a careful safety evaluation of the ZnO-based NMs adopted for the food and feed industry.

The reasons above justify the rationale of the present study where the ZnO NP cytotoxic and inflammatory effects were investigated on an *in vitro* model of the intestinal barrier.

ZnO NPs differing in size and shape may indeed have different toxicity properties and many papers investigated the cytotoxicity, genotoxicity and the inflammatory effects produced by differently sized and shaped ZnO NPs (Pandurangan & Kim, 2015), also in Caco2 cells (De Angelis et al., 2012; Kang, Guan, Chen, Song, Jiang, & Zhao, 2013; Song, Guan, Lyu, Kang, Wu, & Chen, 2014).

Anyway, no conclusive results can be assumed regarding ZnO NP toxicity, since in parallel to papers reporting the low toxicity, many others indicated significant toxic effects on different cell lines (Lin, Xu, Huang, Ma, Shannon, Chen, & Huang, 2009; De Berardis, Civitelli, Condello, Lista, Pozzi, Arancia, & Meschini, 2010) and test organisms (Bondarenko, Juganson, Ivask, Kasemets, Mortimer, & Kahru, 2013), as well as remarkable inflammatory burden in exposed organs, such as the lung (Ho, Wu, Chein, Chen, & Cheng, 2011). Moreover, several studies highlighted that induction of oxidative stress was involved in the toxicity mechanism of ZnO NPs on cells of epithelial lung, liver and intestinal tract (Lin, Xu, Huang, Ma, Shannon, Chen, & Huang, 2009; Ahamed, Akhtar, Raja, Ahmad, Siddiqui, Alsalhi, & Alrokayan, 2011; Sharma, Shukla, Saxena, Parmar, Das, & Dhawan, 2009; De Berardis, Civitelli, Condello, Lista, Pozzi, Arancia, & Meschini, 2010).

In vivo studies using amphibian embryos revealed that treatments with ZnO NPs endanger gut development and produce histological lesions to the intestinal mucosa. ZnO NPs were also seen to efficiently cross the intestine epithelial barrier (Bacchetta et al., 2014; Bonfanti et al., 2015).
However, a recent study on diet supplementation with nano ZnO in mice revealed very low *in vivo* toxicity even after prolonged administration periods (Liu et al., 2017).
Although ZnO NPs are not considered among the most hazardous NMs, the large industrial production, the wide commercial use and the possible unknown effects over prolonged exposure periods suggest the opportunity to increase our knowledge on the possible adverse effects and biological mechanisms on predicted target organs.
In the present work, *in vitro* intestinal barriers made of Caco2 cells grown onto transwell inserts were exposed to increasing concentrations of two different ZnO NPs. NPs were administered from both apical and basolateral sides. The barrier integrity, cell viability, pro-inflammatory cytokine release and markers of protein oxidative damage were measured after exposure. The scope was to evaluate if ZnO NP properties and modality of interaction with the enterocytes may influence the biological responses at the intestinal barrier level.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagles Medium (DMEM) high glucose, heat inactivated fetal bovine serum (FBS), glutamine, non-essential amino acids (NEAA), N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (HEPES), penicillin/streptomycin were all purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All other chemicals were of analytical grade.

2.2. Cell culture

Caco-2 cells were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and were routinely grown in an atmosphere of 5% CO_2 at 37 °C in DMEM high glucose (4.5 g/L) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, 4 mmol/l glutamine, 1% non-essential amino acids, 10 mM HEPES and 10% heat inactivated foetal calf serum (FCS) (Caloni, Cortinovis, Pizzo, & De Angelis, 2012). The cells were seeded at 10^5 cells/filter on 1 µm pore size 12-well plate polycarbonate inserts (Millicell®, Millipore, MA, USA). Culture medium was regularly changed three times a week. The experiments were performed after 21 days of culture when the differentiation process was completed.

2.3. NP preparation and characterization

The commercial ZnO NPs, catalogue #544906, were purchased from Sigma-Aldrich (Milano, Italy). The sonochemical ZnO NPs (sono ZnO) were synthesized by the Gedanken's Lab at the Department of Chemistry, Bar-Ilan Univesity (Ramat-Gan, Israel), according to the protocol already reported in previous papers (Perelshtein, Lipovsky, Perkas, Gedanken, Moschini, & Mantecca, 2015).
Five mg of ZnO NPs were weighted and suspended in 5 ml of ultrapure water to generate a stock suspension of 1 mg/ml. The suspension was sonicated for 30 min in a Ultrasonicotor Sonica (Soltec).
The primary size and shape of the ZnO NPs were characterized by TEM. Five µl of a 50 µg/ml NP suspension in ultrapure water were pipetted onto a Formvar-coated 300 mesh copper grid. The excess water was gently blotted and the grids let to dry. Once dried they were inserted into a Jeol JEM1220 electron transmission microscope operating at 80kV. Digital images were acquired by a dedicated Lerithier digital camera.
After sonication, aliquots of the stock suspensions were immediately serially diluted in cell culture medium to obtain the test concentrations.

The hydrodynamic behaviour of the ZnO NPs was studied by Dynamic Light Scattering. Aliquots of 50 µg/ml NPs in the culture medium were put in dedicated plastic cuvettes and the NP size distribution was measured using a Malvern Zetasizer Nano-ZS90.

2.4. Trans-Epithelial Electrical Resistance (TEER) measurement

Barrier impairment after exposure to ZnO NPs was assessed by measuring the Trans-Epithelial Electrical Resistance (TEER). TEER values were recorded in the culture medium at 37 °C using a pair of chopstick electrodes connected to a Millicell-ERS voltohmmeter (Millipore, MA, USA) and were expressed as $\Omega \times cm^2$. For each filter, three separate measures were collected.

2.5. Measurement of pro-inflammatory mediator release

At the end of the treatment, culture medium was collected from individual wells and frozen at -80°C for subsequent pro-inflammatory cytokine release determination. Culture medium was analysed for the presence of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) using commercially available quantitative ELISA assay kits (Human IL-6 ELISA Kit, Human IL-8 / CXCL8 ELISA Kit, Human Tumor Necrosis Factor α ELISA Kit, Sigma-Aldrich Chemical Company, St. Louis, MO, USA) according to the manufacturer's instructions. Optical density was measured at 450 nm by an ELISA reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, Waltham, MA, USA).

2.6. Experimental design

The effects of sono ZnO NPs and commercial ZnO NPs (Sigma-Aldrich, St. Louis, MO, USA) on Caco-2 barrier integrity and pro-inflammatory cytokine release were evaluated. Caco-2 cells were treated with different concentrations (i.e., 0, 10, 50 and 100 µg/ml) of sono ZnO NPs and commercial ZnO NPs from both Ap and Bl sides. The barrier integrity was assessed just before (0 h) and 1, 2 and 24 h after exposure by measuring TEER as described above (section 2.4). After 24 hours of treatment, culture medium was collected for IL-6, IL-8 and TNF-α determination (section 2.5).

2.7. Statistical analysis

Each experiment was performed in triplicate. Results are expressed as mean ± standard deviations (SD). Statistical evaluation was performed by two tailed Student's t-test. The level of significance was established at $P<0.05$.

2.8. Determination of protein thiols by means of biotin-maleimide and Western blot analysis

Biotin-maleimide [N-biotinoyl-N′-(6-maleimidohexanoyl)hydrazide] stock solution was prepared at 40 mM in DMSO and stored at -20°C. Protein samples were diluted to a final concentration of 1 mg/ml in 50 mM PBS, pH 7.4, containing 75 µM biotin-maleimide. Protein labelling was performed for 1 h at room temperature. After labelling, protein samples were mixed with an equal volume of 2× reducing Laemmli sample buffer, heated for 5 min at 90°C and analysed by SDS-PAGE using 10% (w/v) Tris–HCl polyacrylamide gels. Proteins were then transferred to PVDF membrane and biotin tag revealed with streptavidin-HRP. Briefly, PVDF membranes were washed with PBST [10 mM Na-phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20] and blocked for 1 h in 5% (w/v) non-fat dry milk in PBST. After washing three times with PBST for 5 min each, the biotin tag was probed by a 2-h incubation with 5% non-fat dry milk/PBST containing streptavidin-HRP (1: 5,000 dilution). After three washes with PBST, biotinylated proteins were visualized by ECL detection (Colombo et al., 2017).

2.9. Western blot analysis of carbonylated proteins

Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Colombo, Clerici, Garavaglia, Giustarini, Rossi, Milzani, & Dalle-Donne, 2016). Briefly, 100 µg (1 mg/ml) of plasma proteins were mixed with 20 µl of 10 mM DNPH in 2 N HCl and incubated for 60 min in the dark. Samples were then mixed with 120 µl of 20% trichloroacetic acid (TCA) and incubated for 10 min in ice. After centrifugation at 20,000 g for 15 min at 4°C, protein pellets were washed three times with 1:1 ethanol/ethylacetate to remove free DNPH. Air-dry protein pellets were resuspended in 2× reducing Laemmli sample buffer. Proteins were separated by SDS-PAGE on 12% (w/v) Tris–HCl polyacrylamide gels, transferred to PVDF membrane and detected through Western immunoblotting with anti-DNP antibody (Colombo et al., 2012; Gornati et al., 2013). Immunoreactive protein bands were visualized by ECL detection.

3. Results

3.1. ZnO NPs characterization

The morphology of the NPs used is depicted in Figure 1, while the hydrodynamic size, once suspended in the cell culture medium DMEM, is reported in Table 1. The commercial ZnO appeared smaller in size than sono ZnO NPs. Sono ZnO resulted in cubic-shaped. Most NPs showed a dimension comprised between 100 and 200 nm; only few NPs were smaller than 100 nm (Figure 1B). Commercial ZnO resulted irregularly shaped, with primary size well below 100 nm. In a previous paper, the medium primary size of the commercial ZnO, measured by TEM, was determined to be 75 nm (Bacchetta et al., 2014). Although the different size visible under the TEM, the crystallite size of the sono and commercial ZnO, measured by XRD, did not differ significantly (Perelshtein, Lipovsky, Perkas, Gedanken, Moschini, & Mantecca, 2015).
Once suspended in DMEM, both commercial ZnO and sono ZnO soon aggregated to form particle clusters, with a mean hydrodynamic size of about 300 nm and 680 nm respectively. The NP aggregation increased further after 24 h incubation time (Table 1).

3.1.1. TEER modulation, interleukins (IL-6, IL-8) and cytokine (TNF-α) release

TEER was not significantly affected by Ap exposure to either sono ZnO NPs or commercial ZnO NPs at any tested concentrations (i.e., 10, 50 and 100 µg/ml) (Figure 2A-3A). After Bl exposure, all concentrations of sono ZnO NPs were found to significantly decrease TEER starting from the first hour of treatment (Figure 2B). Differently, after Bl exposure, only the highest concentration (100 µg/ml) of commercial ZnO NPs was found to significantly decrease TEER starting from the first hour of treatment (Figure 3B). At 100 µg/ml sono ZnO NPs induced a significant release of IL-6 after both Ap and Bl exposure, whereas commercial ones induced a significant release of IL-6 only at 50 µg/ml and when applied in the Ap compartment (Figure 4). A significant release of IL-8 was observed after Ap exposure to both ZnO NPs at 100 µg/ml and after Bl exposure to sono ZnO NPs at 100 µg/ml (Figure 5). No significant release of TNF-α was observed after either Ap or Bl exposure to any sono and commercial ZnO NP concentrations (Figure 6).

3.1.2. Protein sulphydryl oxidation and protein carbonylation

Each sample used in Western blot experiments represents the pool of three independent 12-wells treated cells that were pooled together to obtain a sufficient amount of proteins for the biochemical analysis. Protein sulphydryl oxidation was not significantly affected by either Ap exposure or Bl exposure to either sono ZnO NPs or commercial ZnO NPs at any tested concentration (i.e., 10, 50 and

100 µg/ml) (Figure 7). In light of the fact that the experiment was performed once with the pooled samples, it was not possible to perform a statistical analysis; however, the ratio protein carbonyls/protein, derived from densitometric analysis of the chemiluminescent Western blot signal for protein carbonylation and of the Amido Black staining on the same PVDF membrane, increased by 15% and 25% compared to control in protein lysates from Caco-2 cells exposed basolaterally to, respectively, 50 and 100 µg/ml of commercial ZnO NPs (Figure 8).

4. Discussion

It is well established that the peculiar properties of nano-sized materials, in particular their highly increased specific surface area, improve their reactivity and may influence aspects of their toxicity and inflammatory potential (Oberdörster, Oberdörster, & Oberdörster, 2005). The present study was designed to evaluate the effects of commercial ZnO NPs and sono ZnO NPs at different concentrations (10-25-50-100 µg/ml) on Caco-2 cells cultured on insert. Caco-2 cells are a widely used model of epithelial intestinal barrier and we focused the attention on the possible impairment of the barrier integrity. Previous studies with Caco-2 cells cultured on insert, exposed to ZnO NPs, demonstrated that cytotoxic effects of ZnO NPs are dose, size and time dependent (Kang, Guan, Chen, Song, Jiang, & Zhao, 2013; Song, Guan, Lyu, Kang, Wu, & Chen, 2014) and are related to their chemical composition and solubility (De Angelis et al., 2012). It was also demonstrated that oxidative stress plays an important role in ZnO NP toxicity on Caco-2 cells (Kang, Guan, Chen, Song, Jiang, & Zhao, 2013).

In this paper, we measured TEER, and evaluated the release of interleukins (IL-6, IL-8) and cytokine (TNF-α) and we assessed two markers of protein oxidative damage, protein sulphydryl oxidation and protein carbonylation. TEER, in particular, is a widely accepted quantitative technique to measure the integrity of tight junction dynamics in cell culture models of epithelial monolayers and the assessment of TEER before and after treatment with ZnO NPs is a strong indicator of the integrity of the cellular barriers. ROS formation and oxidative stress are related to inflammatory effects of ZnO NPs because they can activate pro-inflammatory signalling cascades (De Berardis, Civitelli, Condello, Lista, Pozzi, Arancia, & Meschini, 2010). Particle-induced inflammation is characterized by the recruitment and activation of phagocytic cells (e.g., monocytes and neutrophils) able to produce ROS, thus exacerbating oxidative stress and oxidative tissue damage. Herein, IL-6, IL-8 and TNF-α were measured to evaluate pro-inflammatory effects of ZnO NPs and protein sulphydryl oxidation and protein carbonylation were evaluated by Western blot to investigate oxidative protein damage.

Adverse effects were evident with the highest concentration (100 µg/ml) of commercial ZnO NPs. Indeed, at 100 µg/ml commercial ZnO NPs decreased TEER after Bl exposure (Figure 3) and induced a significant release of IL-8 after Ap exposure (Figure 5), in agreement with other authors (De Angelis et al., 2012). IL-8 release is evident also after Ap and Bl exposure to sono ZnO NPs at 100 µg/ml (Figure 5) as well as the release of IL-6, which is detected in both the compartments (Ap and Bl) at the same concentration (Figure 4). At all the concentrations tested (i.e., 10, 50 and 100 µg/ml) sono ZnO NPs were found to significantly decrease TEER after Bl exposure (Figure 2). No significant effect was observed on the amount of protein sulphydryls after cell exposure to ZnO NPs at any of the tested concentrations. Differently, protein carbonylation was increased compared to the control after basolateral exposure to 50 and 100 µg/ml of commercial ZnO NPs, whereas no changes were observed after apical exposure. But sono ZnO NPs did not increase protein carbonylation neither after apical nor basolateral exposure at any of the tested concentrations.

Otherwise, other results from our laboratory (data not shown) showed that the exposure to 1-10-25 µg/ml of commercial ZnO NPs or sono ZnO NPs, did not cause significant effects ($P \geq 0.05$) on TEER after 1-2-24-48 h of exposure, either from Ap *versus* Bl or from Bl *versus* Ap compartments. Similarly, no effects were observed on the release of the cytokines IL-6, IL-8 and TNF-α ($P \geq 0.05$) after 48-h

exposure, from both the Ap or the Bl side. These findings seem to suggest no impairment of the epithelial barrier with 1-10-25 µg/ml either commercial or sono ZnO NPs.
For the NPs used in our study, the primary size of commercial NPs, as reported by the manufacturers, is less than 100 nm (Table 1), whereas the exact primary size of sonochemical ones is not available. However, the actual NP sizes during exposures were over 600 nm and 900 nm when resuspended in DMEM for 24 h (Table 1) for commercial and sono, respectively. Taking all these data together, it emerges that NPs size could be an explanatory factor for differential toxicity between commercial and sono ZnO NPs. In fact, although both formulations induce a reduction in TEER at concentrations higher than 50 µg/ml after basolateral exposure, only sonochemical ZnO NPs (100 µg/ml) are able to induce an increase in IL-6 and IL-8 release after both apical and basolateral exposure.
At the same time, commercial ZnO NPs seem to have a more drastic effect on protein oxidative damage. In fact, although both formulations are not able to increase oxidation of protein sulphydryls at any tested concentrations, neither after apical nor basolateral exposure, only commercial NPs are able to increase protein carbonylation when applied basolaterally at 50 and 100 µg/ml. These data could support the hypothesis that different sizes of ZnO NPs could induce different cell responses, activating different pathways that trigger release of pro-inflammatory mediators (sonochemical ZnO NPs) or intracellular production of ROS and the consequent oxidative protein damage (commercial ZnO NPs).
In parallel to NPs size, another important factor related to effects of ZnO NPs seems to be linked to cell polarity. A different cellular response was observed when ZnO NPs were applied apically or basolaterally. A more adverse effect was observed after basolateral application, where a reduction in TEER at 50 and 100 µg/ml for both commercial and sono NPs is evident. In addition, an increased release of IL-6 and IL-8 was described after treatment with 100 µg/ml of sonochemical NPs. The different responses are probably due to the polarized structure of intestinal epithelial cells that possess an apical plasma membrane facing the lumen and a basolateral plasma membrane facing the lamina propria and blood. These two membrane domains differ in terms of protein and lipid compositions, suggesting a possible different biochemical response to ZnO NPs (Engevik & Goldenring, 2018).
The observation that ZnO NPs are toxic at higher concentrations could be in part supported by the dissolution of ZnO NPs into zinc ions. Although zinc is an essential micro-nutrient for cell functioning and survival, zinc ions in high concentrations can be toxic to many cells, including Caco-2 (Fang, Jiang, Gong, Li, Liu, & Cao, 2017). We did not measure ZnO NP dissolution, but it has been suggested in various studies for several cell culture media, including DMEM, the media we used for Caco-2 cells (Reed, Ladner, Higgins, Westerhoff, & Ranville, 2012). In addition, the analysis of dissolution of ZnO NPs in cell culture media is just one aspect of NP bioavailability. In fact, this approach does not consider the actual exposure to NPs in vivo, where NPs, in case of ingestion, have to pass through the digestion process, including pH changes, exposure to digestive enzymes, and exposure to mucus that covers the cells.
In conclusion, according to the main results of the present study, the administration of ZnO NPs to the intestinal barrier through the apical (luminal) compartment appeared almost safe, until very high NP concentrations are eventually reached. More attention should be payed to an eventual excessive NP loading that might finally lead to an increase of Zn ions and/or particles adsorption in the basal compartment, corresponding to extracellular matrix and microvessels surrounding the intestinal epithelium. This compartment in fact resulted more sensitive to ZnO NPs than the apical one. The amount of Zn ions is determined by the equilibrium constant of ZnO. If equilibrium is reached and if the amount of Zn is sufficient then for all concentrations, the same amount of Zn ions is found in the solution. It is possible that only at 100 µg/ml we reach the equilibrium and then the Zn ions cause the damage.
In future studies, it will be interesting to evaluate the dissolution process of both commercial and sonochemical ZnO NPs under experimental conditions that mimic the physiological ones, as well as the

biological pathways at the base of the different sensitivity observed in the apical and basal cell membrane domains. In addition, since the inflammatory one seemed to be the main cell response to ZnO NPs, the use of in vitro intestinal barriers including immune cells may be suggested for improving the predictivity of the biological model and the hazard of orally administered nanosized ZnO.

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7. Figure Legends

Figure 1. TEM images of commercial ZnO (A) and sono ZnO (B)

Figure 2. Effect of sono ZnO NPs on Caco-2 TEER values after apical (Panel A) and basolateral (Panel B) exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

Figure 3. Effect of commercial ZnO NPs on Caco-2 TEER values after apical (Panel A) and basolateral (Panel B) exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

Figure 4. Effect of sono and commercial ZnO NPs on Caco-2 IL-6 release after a 24-h apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

Figure 5. Effect of sono and commercial ZnO NPs on Caco-2 IL-8 release after a 24-h apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

Figure 6. Effect of sono and commercial ZnO NPs on Caco-2 TNF-α release after a 24-h apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $P < 0.05$.

Figure 7. Effect of sono and commercial ZnO NPs on protein sulphydryl oxidation after apical and basolateral exposure of Caco-2 cells. Each lane represents the pool of three protein extracts obtained from three polycarbonate filters on a 12-well plate. Western blot showing reduced protein thiol signal after ECL development (upper panel) and Amido black staining for protein loading (lower panel) are shown.

Figure 8. Effect of sono and commercial ZnO NPs on protein carbonylation after apical and basolateral exposure of Caco-2 cells. Each lane represents the pool of three protein extracts obtained from three polycarbonate filters on a 12-well plate. Western blot showing carbonylated protein (PCO) signal after ECL development (upper panel) and Amido black staining of PVDF membrane for protein loading (lower panel) are shown.

8. Tables

Table 1. Hydrodynamic size of commercial (com) and sonochemical (sono) ZnO NPs suspended at 50 ug/ml in cell culture medium (DMEM) after 0 and 24 h of incubation at 37°C.

NPs	Primary size, nm	Medium	Hydrodynamic size, nm (pdi)	
			0 h	24 h
com ZnO NPs	< 100	DMEM	299 ± 25 (0.5)	603 ± 79 (0.5)
sono ZnO NPs	n.a.	DMEM	680 ± 236 (0.9)	953 ± 178 (0.9)

9. Figures

(Figures should be uploaded as separate figure files. Upload each illustration as a separate file in either .tiff or .eps format, with the figure number and the top of the figure indicated. Compound figures e.g. 1a, b, c should be uploaded as one figure. Tints are not acceptable. All illustrations must be supplied at the correct resolution: Black and white and colour photos - 300 dpi; Graphs, drawings, etc - 800 dpi preferred/600 dpi minimum)