In vitro copper oxide nanoparticle toxicity on intestinal barrier

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

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Abstract (<250 words)

The use of CuO nanoparticles (NPs) has increased greatly and their potential effects on human health need to be investigated. Differentiated Caco-2 cells were treated from the apical (Ap) and the basolateral (Bl) compartment with different concentrations (0, 10, 50 and 100 µg/ml) of commercial or sonochemically synthesized (sono) CuO NPs. Sono NPs were prepared in ethanol (CuOe) or in water (CuOw), obtaining CuO NPs differing in size and shape. The effects on the Caco-2 cell barrier were assessed via trans-epithelial electrical resistance (TEER) evaluation just before and after 1, 2 and 24 h of exposure and through the analysis of cytokine release and biomarkers of oxidative damage to proteins after 24 h. Sono CuOe and CuOw NPs induced a TEER decrease with a dose-dependent pattern after Bl exposure. Differently, TEER values were not affected by the Ap exposure to commercial CuO NPs and, concerning the Bl exposure, just the lowest concentration tested (10 µg/ml) caused a TEER decrease after 24 h of exposure. An increased release of IL-8 was induced by sono CuO NPs after the Ap exposure to 100 µg/ml and by sono and commercial CuO

after the Bl exposure to all the concentrations. No effects of commercial and sono CuO NPs on IL-6 (with the only exception of 100 μg/ml Bl commercial CuO) and TNF-α release were observed. Ap treatment with commercial and CuOw NPs was able to induce significant alterations on specific biomarkers of protein oxidative damage (protein sulfhydryl group oxidation and protein carbonylation).

Short abstract (<80 words)

Differentiated Caco-2 cells were exposed from the apical and basolateral compartment to 0, 10, 50 and 100 μg/ml of sonochemical (synthesized in ethanol or in water) and commercial CuO nanoparticles (CuO NP), then trans-epithelial electrical resistance (TEER), cytokine release and biomarkers of protein oxidation were investigated. TEER was affected mostly by the Bl exposure to sono CuNPs. CuO NPs also induced a significant ($p<0.05$) release of IL-8, IL-6 and alterations in biomarkers of protein oxidation.

1. Introduction

The use of metal and metal oxide nanoparticles (NPs) has grown exponentially nowadays due to their potential applications as catalysts, sensors, components of batteries, magnetic storage media, solar cells and paints, just to mention some, covering a wide range of fields (i.e. electronics, medicine, food, agriculture, cosmetics, etc.) (Chavali & Nikolova, 2019; Madhav et al., 2017). In particular, copper oxide (CuO) NPs are becoming increasing popular since they are preferred among other NPs because of their peculiar properties like hardness, conductivity, formability and strength of alloys (Madhav et al., 2017). Cu is an essential trace element that plays many biological functions, being a fundamental structural component and regulatory cofactor of several enzymes such as cytochrome c oxidase, ceruloplasmin and copper/zinc superoxide dismutase (Horn & Barrientos, 2008). Organisms have evolved the ability to strictly control Cu homeostasis but this regulatory capability is finite thus, when the external Cu concentration is too low or too high, that condition can result in a deficiency or intoxication, respectively (Yazdankhah, Rudi, & Bernhoft, 2014). It is also historically known for its biocidal properties, with large application as bactericidal agent (Vincent, Duval, Hartemann, & Engels-Deutsch, 2018). More specifically, Cu NPs are known to exert a strong antibacterial activity due to their heat resistance, stability and large surface to volume ratio features that enable them to easily penetrate the microbial membranes (Vimbela, Ngo, Fraze, Yang, & Stout, 2017). Since the recent mounting interest in the use of copper as an antibacterial, numerous attempts for producing nanosized copper with enhanced efficacy have been performed, also for fighting against resistant strains (Baptista et al., 2018). In this regards, it has been reported that Cu antibacterial properties are mainly a result of the cellular damages induced after contact between the released Cu^{2+} ions and the bacterial membrane (Vincent et al., 2018) with a possible involvement of CuO NP ability to produce reactive oxygen species (ROS) when in contact with water (Zhang, Pornpattananangku, Hu, & Huang, 2010). ROS induce oxidative changes to cell structures with a consequent bactericidal effect and they are considered initiators of the NP-mediated cytotoxicity in eukaryotic cells. Several nanomaterials can generate ROS and that is one of the main features responsible for the NP toxicity (P. Mantecca et al., 2015). Due to copper's antibacterial performances, Cu NPs can have several applications. For instance, Cu NPs are used in food packaging to inhibit bacterial growth, and Cu NPs fixed in a polylactic acid matrix are promising toward preventing proliferation of *Pseudomonas* spp., gram-negative pathogens commonly found in processed food, which are able to develop resistance to common antibiotics (Longano et al., 2012).

Due to the widespread application of CuO NPs, the clarification of the biological consequences of the exposure to this nanomaterial for human health is crucial. Cell-based *in vitro* methods, which are manageable, relatively rapid and cost-effective, have been regarded for toxicity screening of

new NPs (Jing, Park, Peters, & Thorne, 2015), with the vast majority of the *in vitro* studies on NP toxicity being performed exposing submerged cell cultures to NP suspensions. Several *in vivo* and *in vitro* studies have demonstrated that particles, in the ultrafine dimensional range, could exert stronger toxic and immunogenic effects in comparison with bigger particles of the same material (Titma, Shimmo, Siigur, & Kahru, 2016). As already shown the nanoform of copper is more toxic than the bulk copper (Rossetto, Melegari, Ouriques, & Matias, 2014; Shi, Abid, Kennedy, Hristova, & Silk, 2011). Various NPs can enter in contact with humans or animals through inhalation, dermal contact or ingestion. Exposure to NPs can have several adverse effects on epithelial barriers; moreover, NPs can stimulate an inflammatory response, which can lead to an increase in the epithelial permeability (mainly due to tight junction leakage), with a consequent impairment of the barrier function against microorganisms and toxic compounds (Titma et al., 2016). To cross the epithelial barrier, molecules have two main pathways: 1) entering the cell by passive or active transport, to be after exocytised from the basolateral side of the barrier (transcellular transport), or 2) filtering through the paracellular pathway (passive diffusion through the space between adjacent cells) (Chelakkot, Ghim, & Ryu, 2018). Thus, a reduction in the intestinal barrier resistance may reflect a function impairment and a possible paracellular transport of NPs (Titma et al., 2016). Studies on CuO NP toxicity performed using Caco-2 monolayers exposed to 10-200 μg CuO NPs/ml showed that the epithelial integrity, assessed by measuring the trans-epithelial electric resistance (TEER), decreased and this reduction was irreversible (Titma et al., 2016). A study evaluated the potential pro-inflammatory effects of CuO NPs on Caco-2 cells (Piret et al., 2012), showing that CuO NPs were cytotoxic and induced a marked increase in the transcripts for pro-inflammatory cytokines and chemokines, thus suggesting a pro-inflammatory effect at the intestinal level (Piret et al., 2012). A later study by another group observed an increased (concentration and time-dependent) interleukin 8 (IL-8) production after the exposure of Caco-2 cells to CuO NPs for 24-48 h (Ude et al., 2017). Given the pro-inflammatory effect of CuO NPs on Caco-2 cells, with the production of proteins able to recruit and activate immune cells implicated in intestinal inflammatory bowel disease (Piret et al., 2012), you can speculate that a chronic presence of CuO NPs in the intestinal tract could favour local intestinal inflammation. Piret and colleagues used two types of CuO NPs, with different specific surface and shape, but the same chemical composition and similar hydrodynamic diameter. Rod-shaped CuO NPs were more harmful than the spherical ones at the same concentration (Piret et al., 2012). At 100 μg/ml, rod-shaped CuO NPs completely destroyed the tissue structure after 24 h of incubation (Piret et al., 2012). In this regard, emerging literature is revealing the importance of NP shape in toxicity (Sukhanova et al., 2018), blood flow and drug delivery (Truong, Whittaker, Mak, & Davis, 2015; Ye, Shen, Yu, Wei, & Li, 2018). Specifically, the shape of NPs strongly influences their blood circulation time and vessel wall adhesion; in addition, non-spherical NPs have shown a better resistance to sequestration by the mononuclear phagocyte system in blood and are able to adhere to the vessel wall faster than spherical NPs (Ye et al., 2018). Once internalised, metallic NPs could directly mediate their toxic effects or through the release of metal ions inside the cells, serving as "Trojan horse type carriers" (Cronholm et al., 2013; Ude et al., 2017). In general, cell exposure to NPs implicates many biological effects such as ROS generation, induction of cellular oxidative stress, release of toxic metal ions (due to a partial or complete NP dissolution), protein damage, protein unfolding response, membrane leakage, inflammation (including chronic granulomatous inflammation), induction of frustrated phagocytosis, fibrogenic responses and activation of cell signaling cascades (Abdal Dayem et al., 2017; Boyles et al., 2015; He et al., 2011; Marano, Hussain, Rodrigues-Lima, Baeza-Squiban, & Boland, 2011).

Despite the widespread use of CuO NPs in several industrial applications, including agriculture, medicine and emerging technologies (Grigore, Biscu, Holban, Gestal, & Grumezescu, 2016; Khatoon, Mohan Mantravadi, & Nageswara Rao, 2018; Singh & Rawat, 2016; Verma & Kumar, 2019) there is a lack of knowledge (Ude et al., 2017) on potential adverse effects and biological mechanisms related to the exposure of the gastrointestinal tract to CuO NPs that needs to be

addressed to perform a complete hazard evaluation of exposure to NPs and the related potential risks for animals, humans and the ecosystem.

The aim of this study is to evaluate the *in vitro* effects of three types of CuO NPs, from commercial source and from sonochemical synthesis (ethanol- and water-based), on an *in vitro* intestinal 3D predictive model (Caloni, Cortinovis, Pizzo, & De Angelis, 2012). Caco-2 cells grown on transwell inserts were exposed to increasing concentrations of the three different types of CuO NPs, which were administered from the apical (Ap) as well as the basolateral (Bl) side.
The following major endpoints have been evaluated:

- ✓ barrier integrity;
- ✓ cytokine release;
- ✓ protein oxidative damage.

2. Materials and Methods

2.1. Nanoparticles and chemicals

Commercial Copper Oxide nanoparticles (CuOc NPs) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Sonochemical synthesized CuO NPs were prepared in ethanol (CuOe) or in water (CuOw) and the synthesis protocols, as well as the NP characterizations are available in previously published papers (Mantecca et al., 2017; Moschini et al., 2013; Perelshtein et al., 2015).
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). Ethanol was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Cell culture conditions

Caco-2 cells were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and were routinely grown in DMEM high glucose standard medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, 4 mmol/l glutamine, 1% NEAA, 10 mM HEPES and 10% heat inactivated FBS. Cells were seeded at a density of approximately 1.5×10^5 cells/filter on 1 µm pore size 12-well plate polyethylene terephthalate (PET) inserts (Millicell®, Millipore Corporation, MA, USA). After preparing a cell suspension in culture medium, 0.5 ml of the suspension were transferred into the apical (Ap) compartment and 1.5 ml of supplemented DMEM in the basolateral (Bl) compartment of each insert. Plates were then shaken gently to obtain a uniform cell distribution and transferred into the incubator at 37° C in an atmosphere of 5% CO_2. Cells were allowed to differentiate for 21 days with regular medium changes three times per week. To ensure cellular adhesion, the medium replacement was performed as gently as possible without touching the cells with the pipet tip. The culture was regularly checked with an inverted microscope to identify contaminations and/or morphological variations. Treatments were applied at the end of the differentiation period.

2.3. NP suspension preparation and characterization

To obtain a 1 mg/ml stock solution, 5 mg of CuO NPs were suspended in 5 ml of culture medium. The stock suspensions of CuO NPs were sonicated in order to reduce particle agglomeration and, immediately after sonication, used to prepare the required working concentrations of 10 µg/ml, 50 µg/ml and 100 µg/ml, by serial dilution with the culture medium.
The NP size and shape were characterized by electron transmission microscope (TEM). A small drop of NP suspension in ultrapure water was pipetted onto a Formvar-coated copper grid. Once

dried, the grids were observed using a TEM Jeol JEM 2100 Plus (JEOL, Tokyo, Japan) operating at 200 kV, equipped with an 8 megapixel Gatan (Gatan, USA) Rio Complementary Metal-Oxide-Superconductur (CMOS) camera.
The hydrodynamic behaviour of the CuO NPs suspended in cell culture medium was studied by Dynamic Light Scattering. Aliquots of 50 µg/ml NPs in DMEM medium were put in dedicated plastic cuvettes and the NP size distribution was measured using a Zetasizer Nano-ZS90 (Malvern Panalytical Ltd, UK).

2.4. Barrier integrity assay (Trans-Epithelial Electrical Resistance Evaluation)
Barrier impairment following exposure to CuOc NPs (Sigma-Aldrich, St. Louis, MO, USA) and sono CuOe and CuOw NPs was assessed by measuring the Trans-Epithelial Electrical Resistance (TEER). TEER values were recorded in the culture medium using an epithelial voltohmmeter (Millicell®-ERS, Millipore) just before (0 h) and 1, 2 and 24 h after treatment. For each filter, three separate measures were quickly performed. TEER values were expressed as $\Omega \times cm^2$ and calculated according to the following equation:

$$TEER = (\Omega \text{ cell monolayer} - \Omega \text{ filter cell-free}) \times \text{filter area}$$

2.5. Measurement of pro-inflammatory mediator release
After 24 h of treatment, the culture medium was collected from individual wells and frozen at -80°C for subsequent pro-inflammatory cytokine release determination. Culture medium was analyzed for the presence of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor 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) and following the manufacturer's instruction. Optical densities were read at 450 nm using an ELISA reader (Multiskan GO microplate spectrophotometer, Thermo Scientific, Waltham, MA, USA).

2.6. 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).

2.7. Western blot analysis of carbonylated proteins
Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Colombo et al., 2016). Briefly, 100 µg (1 mg/ml) of extracted 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.

2.8. Experimental Design

Caco-2 cells were treated with increasing concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) of CuOc from Sigma-Aldrich (Experiment 1) or sono CuOe and CuOw NPs (Experiment 2), by exposing the system from either Ap or Bl side. The barrier integrity was evaluated after 1, 2 and 24 h of exposure by measuring the TEER as described above (section 2.4). After 24 h of treatment, the medium was collected for IL-6, IL-8 and TNF-α determination (section 2.5) to investigate the pro-inflammatory potential.
In addition, two markers of oxidative damage on proteins (protein sulfhydryl group oxidation and protein carbonylation) were evaluated after Ap and Bl side exposure to CuOc or CuOw NPs at different concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml).

2.9. Statistical analysis

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

3. Results

3.1 CuO NPs characterization

The morphological characteristics of the CuO NPs used are reported in Figure 1, while the hydrodynamic behaviour of the same NPs suspended in DMEM at 50 µg/ml are reported in Table 1. The commercial CuO (CuOc) appeared round-shaped and smaller than 50 nm (Fig. 1a), while the sono CuOs resulted larger and leaf-like-shaped (Fig. 1b, c). Sono CuOe NPs were clearly the largest one, although their structure appeared very irregular and made of smaller crystallites, as already reported in Perelsthein et al. (2015).
The DLS analyses demonstrate that the NPs aggregate once suspended in culture medium, although the average hydrodynamic size of the different CuO NP suspensions appeared quite similar, spanning in the range of 200-300 nm, with comparable behaviour at 0h and 24h (Table 1).

3.2. Experiment 1

3.2.1. Effects of CuOc NPs on intestinal epithelial barrier integrity

The results indicate that TEER values were not significantly affected ($p> 0.05$) by Ap exposure for 1 h, 2 h or 24 h to CuOc NPs at any of the tested concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) (Figure 2). After Bl exposure, CuOc NPs were found to significantly ($p< 0.05$) decrease TEER only at the lowest concentration tested (10 µg/ml) after 24 h of exposure (Figure 2).

3.2.2. Effects of CuOc NPs on pro-inflammatory cytokine release

A significant ($p<0.05$) release of the pro-inflammatory mediator IL-6 was induced by Bl exposure to CuOc NPs at 100 µg/ml (Figure 3), whereas all concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) of CuOc NPs induced a significant ($p< 0.05$) release of IL-8 after Bl exposure (Figure 4). On the contrary, no significant release ($p> 0.05$) of TNF-α was observed after Ap or Bl exposure to all the tested concentrations of CuOc NPs (Figure 5).

3.3. Experiment 2

3.3.1. Effects of sonochemical Copper Oxide nanoparticles (CuOe and CuOw NPs) on intestinal epithelial barrier integrity

A significant ($p<0.05$) TEER decrease was observed after 24 h of Ap exposure to the highest dose (100 µg/ml) of CuOe NPs (Figure 6), whereas CuOw NPs (Figure 7) induced a significant ($p<0.05$) TEER decrease after 2 h of Ap treatment with the highest concentration (100 µg/ml).
Bl exposure to CuOe NPs was found to significantly ($p<0.05$) decrease TEER at early exposure times (1, 2h) at the concentrations of 100 and 50 µg/ml or after 24 hours of treatment (10 µg/ml) (Figure 8). Bl exposure to all doses of CuOw NPs significantly ($p<0.05$) decreased TEER starting from the first hour (50 and 100 µg/ml) or after 24 hours of treatment (10 µg/ml) (Figure 9).

3.3.2. Effects of CuOe NPs and CuOw NPs on pro-inflammatory cytokine release

No significant release ($p> 0.05$) of the inflammatory mediators IL-6 and TNF-α was observed after a 24-h Ap or Bl exposure to both CuOe and CuOw NPs (Figure 10, 12). On the contrary, a significant ($p< 0.05$) release of IL-8 was induced by Ap exposure to CuOe and CuOw NPs at 100 µg/ml and by Bl exposure to all sono CuO NP concentrations (10 µg/ml, 50 µg/ml and 100 µg/ml) (Figure 11).

3.3.3. Effects of CuOc NPs and CuOw NPs on sulfhydryl groups and carbonylation of Caco-2 cell proteins

Each sample used in Western blot experiments represents the pool of three independent 12-well 24-h treated cells that were pooled together to obtain a sufficient amount of proteins for the biochemical analysis. Protein sulfhydryl groups were oxidised by both CuOc NPs and CuOw NPs only when administered on the apical side of the cell layer. The increase in NP concentration from 10 to 100 µg/mL was parallel to the decrease in protein sulfhydryl (-SH) groups (Figure 13). The experiment was performed only once with the pooled samples so it was not possible to perform a statistical analysis. However, the ratio protein sulfhydryl groups/protein, obtained from densitometric analysis of the chemiluminescent Western blot signal and the Amido Black staining on the same PVDF membrane, respectively, decreased by 25% and 60% compared to the control in protein lysates from Caco-2 cells exposed apically to, respectively, 50 and 100 µg/mL of CuOc NPs (Figure 13A). Differently, neither CuOc nor CuOw NPs induced protein sulfhydryl oxidation after basolateral exposure of Caco-2 cells (not shown).

Both CuOc NPs and CuOw NPs (in particular at 100 µg/mL) increased protein carbonylation in Caco-2 cells after apical exposure for 24h. The ratio between protein carbonylation signal and protein content, as detected by Amido Black staining, increased compared to the control in protein lysates from Caco-2 cells exposed apically to 100 µg/mL of CuOc NPs and CuOw NPs (Figure 13B). Neither CuOc nor CuOw NPs increased protein carbonylation after basolateral exposure (not shown).

4. Discussion

Evaluation of the toxic and inflammatory potential of NPs is needed in order to create a reliable database of toxicological data for hazard and risk assessment. Indeed, it should be considered that, in the nanosize range, substances acquire properties that can be completely different from those of the bulk materials (Bertero, Spicer, Coccini, & Caloni, 2019). In addition, the physicochemical properties of NPs, which influence their toxicological effects, are highly variable as they are affected by many factors, making toxicological evaluation of NPs more complex than those performed on “traditional” substances. In comparison to other NPs, like Ag and TiO_2 NPs, a relatively scarce literature concerning CuO NP risk assessment is available and even less works had focused on toxicity of ingested CuO NPs (Ude et al., 2017).

In vitro toxicological effects of CuO NPs have been demonstrated on different cell types: human lymphocytes and erythrocytes (Dey et al., 2019), airway epithelial (HEp-2) cells (Fahmy & Cormier, 2009), human alveolar epithelial cells A549 (Ivask et al., 2015), human airway smooth muscle cells (Berntsen et al., 2010), mouse pulmonary microvascular endothelial cells (Yu et al., 2010), human neuroblastoma SH-SY5Y cells (Jang, Oh, Yang, & Cho, 2016), human epithelial colorectal cells (Caco-2) and murine fibroblast cell line Balb/c 3T3 (Ivask et al., 2015). According to these works, the main mechanism involved in the toxic response elicited by CuO NPs consists in particle dissolution with a consequent release of toxic metal ions, activation of signaling cascades, induction of inflammation, ROS generation with induction of cellular oxidative stress, protein and cytoplasmic membrane damages. Previous studies using Caco-2 cells demonstrated that CuO NPs are toxic at dosages under 100 µg/mL (24 h exposure) (Titma et al., 2016), and already at ~20 µg/mL (Ivask et al., 2015), and indicated the ion release as one of the main mechanisms related to their toxicity (Ivask et al., 2015).

This study was designed to evaluate and compare the effects of CuOc NPs and sono CuO NPs synthesized in ethanol (CuOe) and in water (CuOw), at concentrations ranging from 10 to 100 µg/ml, on Caco-2 cells cultured on inserts and differentiated into an epithelial-like tissue. Indeed, differentiated Caco-2 cells are one of the most well-established human intestinal epithelial barrier model that has been extensively used in toxicological evaluations (Tan, Norhaizan, Liew, & Sulaiman Rahman, 2018). Barrier impairment, cytokine release and oxidative stress are among the endpoints that have been prioritized in the evaluation of NP toxicity using the Caco-2 cell model (Dey et al., 2019; Ude et al., 2017). Thus, in this study, we investigated 1) the Trans-Epithelial Electrical Resistance (TEER), a broadly accepted quantitative method to assess the barrier integrity and the dynamics of tight junctions (TJs) (Srinivasan et al., 2015); 2) the cytokine (IL-6, IL-8 and TNFα) release, to assess the pro-inflammatory effects of CuO NPs; 3) oxidation of protein sulfhydryl groups and protein carbonylation as markers of protein oxidative damage, in order to investigate the most likely toxicity mechanisms of CuO NPs.

Previous studies determined that CuO NPs had detrimental effects on the barrier integrity (Titma et al., 2016; Ude et al., 2017) affecting also interleukin production (Ude et al., 2017). We observed a TEER decrease at 10 µg/ml (after 24 h of Bl exposure) using commercial CuO NPs but no effects were observed after Ap exposure. Both CuOe and CuOw NPs induced TEER decrease after Bl exposure: at 10 µg/ml the effect was seen after 24 h, whereas at 50 and 100 µg/ml already after 1 or 2 h of exposure; differently, the Ap treatment induced TEER decrease only at the highest concentration (100 µg/ml) of sono CuO NPs. Based on these findings, we can state that commercial and sono CuO NPs have different toxic effects on the intestinal epithelial barrier integrity, whereas sono CuOw and CuOe showed similar effects on all the evaluated parameters. These differences between commercial and sono CuO NPs could be explained by the fact that the synthesis technique and the sonication influence the properties showed by the NPs in solution, such as the hydrodynamic size, agglomeration, aggregation, dissolution and, as a consequence, the toxicity (Kakinen, Kahru, Nurmsoo, Kubo, & Bondarenko, 2016). In particular, the irregular structure and the leaf-like morphology of the sono CuOs, might be responsible of the different toxicity profile, when compared to the regular, round-shaped commercial CuO. Although not investigated in this study, the matter of dissolution is also relevant for CuO NPs, since previous studies have shown that, although partial, the ion release from CuO NPs is an important mechanisms that determine CuO NP toxicity (Kakinen et al., 2016; Karlsson et al., 2014; Piret et al., 2012). As the TEER values of Caco-2 cells treated with sono NPs showed a decrease already after 1 h of exposure, you could assume that, along with particle dissolution, other phenomena are involved in cell toxicity induced by CuO NPs. It has been demonstrated that CuO NP dissolution could take time to occur (Ude et al., 2017). Thus, it is possible that the specific surface reactivity of the CuO NPs also plays a role in their cytotoxic effects. Moreover, another mechanism that could be involved is the so-called “Trojan horse” mechanism, which consists in the release of the ions inside the cells after

cellular uptake (Cronholm et al., 2013; Ude et al., 2017). In addition, the two formulations exhibited different behaviour also regarding the interleukin production. While both the formulations had no effects on TNFα secretion, the Bl exposure of differentiated Caco-2 cells to 100 µg/ml of CuOc NPs induced an increase in the production of IL-6 but no effect was elicited by a similar treatment with the sono CuO NPs. Bl exposure to CuOc NPs caused an increase in the IL-8 secretion at all the concentrations tested. Instead, sono NPs affected IL-8 production both via the Ap (at the highest concentration) and the Bl (starting from the lowest concentration) exposure. Moreover, after the Ap exposure, with the increase in the NP concentration we observed a dose-dependent decrease in the amount of protein sulfhydryl groups and increase in the protein carbonyl groups. Therefore, the induction of oxidative stress could be a mechanism of toxicity shared by commercial and sono CuO NPs. According to our findings, it also seems that the toxic effects exerted by CuO NPs are influenced by the cell polarity. Indeed, both commercial and sono NPs affected differently the TEER and the interleukin production depending on the compartment of administration (Ap vs Bl). The different responses are likely due to the polarized nature of the mature Caco-2 cell monolayer: during cell differentiation and barrier formation, TJs determine the epithelial polarity with an Ap (luminal) domain and a Bl (pointing towards the lamina propria and blood vessels) domain of the plasma membrane. Ap and Bl membrane domains differ in terms of protein and lipid composition and even in the transport mechanisms; thus, it is likely that these two domains may respond differently to CuO NPs (Engevik & Goldenring, 2018). Moreover, Imai and colleagues observed that the Ap treatment of polarized Caco2 cells with Ag NPs determined an internalization and transfer of Ag NPs to the Bl side of the monolayers greater for smaller NPs than for larger ones. On the contrary, after Bl treatment, larger NP aggregates were more internalized and effective than the smaller ones (Imai et al., 2017). These authors hypothesized that, differently from the Ap exposure, when NP-cell interactions are limited by the tight junctions, from the Bl exposure the NPs gain access to the adherens junctions easier, interacting with and disrupting cadherins. A similar effect may be hypothesized also for the CuO NPs analysed in the present study, since they agglomerated in cell culture media (Tab. 1), originating larger particles which would be more effective on the intestinal barrier exposed through the Bl side.

In conclusion, according to our data, the luminal exposure to commercial CuO NPs of the Caco-2 cell system did not alter the epithelial barrier integrity and did not trigger any inflammatory response. Differently, only the highest concentration (100 µg/ml) of sono CuO NPs administered on the luminal domain induced barrier impairment and stimulated interleukin production. Conversely, Caco-2 cells seemed to be more sensitive to commercial and sono CuO NPs administered from the Bl compartment. In the future, it would be interesting to deepen our understanding of the NP uptake mechanism(s) on both apical and basolateral domains of the plasma membrane. Useful information could also come from the study of the dissolution process of commercial and sono CuO NPs and of the translocation of CuO NPs/ions across the cellular layer, also using co-cultures including immune and mucus secreting cells, since the inflammatory response seems to be an important factor involved in the cellular response to NP administration.

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7. Figure Legends

Figure 1. TEM images of different CuO NPs. a) sono CuOw; b) sono CuOe; c) commercial CuOc. Scale bars= 100 nm.

Figure 2. Effect of CuOc NPs on Caco-2 TEER values after apical and basolateral exposure. Graph shows the mean values and standard deviations (n=3). $p<0.05$.

Figure 3. Effect of CuOc 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 4. Effect of CuOc 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 5. Effect of CuOc 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).

Figure 6. Effect of sono CuOe NPs on Caco-2 TEER values after apical exposure. Graph shows the mean values and standard deviations (n=3). (*) $p< 0.05$.

Figure 7. Effect of sono CuOw NPs on Caco-2 TEER values after apical exposure. Graph shows the mean values and standard deviations (n=3). (*) $p< 0.05$.

Figure 8. Effect of sono CuOe NPs on Caco-2 TEER values after basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $p< 0.05$.

Figure 9. Effect of sono CuOw NPs on Caco-2 TEER values after basolateral exposure. Graph shows the mean values and standard deviations (n=3). (*) $p< 0.05$.

Figure 10. Effect of sono CuOe and CuOw 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).

Figure 11. Effect of sono CuOe and CuOw 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 12. Effect of sono CuOe and CuOw 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).

Figure 13. Effect of CuOc NPs and sono CuOw NPs on Caco-2 protein sulfhydryl groups (A) and protein carbonylation (B) after apical exposure for 24 h. Western blot represents the analysis of three independent pooled experiments (n=3).

8. Tables

Tab.1. DLS analyses of CuO nanoparticles. Z-average and polydispersity index (PdI) of commercial CuO NPs (CuOc), ethanol based CuO NPs (eCuO), water based CuO NPs (wCuO) suspended in culture medium (DMEM) at 50 μg/ml at time 0h and after 24h.

		0h		24h	
Sample	Primary size (nm)	z-average±SEM (nm)	PdI	z-average±SEM (nm)	PdI
CuOc	<50	268±2,57	0,18	323±1,84	0,15
CuOe	160	212±4,27	0,29	228±1,23	0,22
CuOw	70	287±15,8	0,37	233±6,21	0,19