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Original Article

Cyto- and genotoxicity of copper (II) oxide (CuO) nanoparticles in HeLa cells

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ABSTRACT

Background and Aims: Cancer is a widespread disease responsible for the death of millions every year. Different approaches and drugs are in use to treat cancer, however, there is a need for new drugs with low cost, high activity, and low side effect risks. Nanotechnology and nanomaterials are important to develop those drugs. Copper-based nanoparticles (NPs) are shown to have biological activity as the antibacterial, and cytotoxic potential. Copper (II) oxide (CuO) NPs are widely used among Cu-based NPs. Different studies evaluated its anticancer and cytotoxic activity; however, the results are still controversial.

Methods: It was planned to characterize the NPs using Transmission Electron Microscopy (TEM) in cell culture medium and distilled water and then to evaluate their cytotoxicity in human cervical cancer cells (HeLa) using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and neutral red uptake (NRU) assays. As one of the cytotoxicity mechanisms, the DNA damage induction potential was evaluated by Comet assay.

Results: The CuO NPs have an average diameter of about 35 nm in distilled water and 39 nm in cell culture medium. The IC50 levels of NPs were 10.7 µg/mL and 6.73 µg/mL by MTT and NRU assays, respectively. The results reveal the NPs dose-dependently increased in the DNA damage. The tail moment was 1.3-fold at 3.125 µg/mL, 2.5-fold at 6.25 µg/mL, and 3.8-fold at 12.5 µg/mL.

Conclusion: CuO NPs have high cytotoxic activity in HeLa cancerous cells. The induction of DNA damage could be an important step in the induction of cell death. Further in vivo and *in vitro* studies in need to improve the safety/low toxicity and understand the molecular mechanism of CuO-induced activity.

Keywords: Copper (II) oxide, nanoparticles, HeLa, genotoxicity, cytotoxicity

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INTRODUCTION

Cancer disease was responsible for the death of about 10 million in 2020, and it is predicted to cause the death of about 30 million in 2040 (Andleeb et al., 2021; Sung et al., 2021). Today, different approaches like hormone therapy, immunotherapy, chemotherapy, and surgeries are in use to treat/control cancer. However, research is still related to discovering and developing new chemotherapeutical agents with low side effects, low budget, and high activity (Andleeb et al., 2021). Nanotechnology and nanomaterials constitute a fertile ground for this type of research. NPs, materials with at least one dimension less than 100 nm, have superior properties, leading to wide applications in different areas (Aitken, Chaudhry, Boxall, & Hull, 2006; Sekhon 2010). Nowadays, Cu-based NPs widely used in cosmetics and medicine production and could be found in different biomedical, industrial, and commercial products such as conductors, sensors, and solar energy converters. Cu-based NPs have thermal, electrical, and catalytic properties in addition to their biological properties. (Chang, Zhang, Xia, Zhang, & Xing, 2012; Cioffi et al., 2005; Schrand et al., 2010).

The previous studies reported the accumulation of NPs in different organs (Chen et al., 2006; Kadammattil, Sajankila, Prabhu, Rao, & Rao, 2018; Lei et al., 2008; Liu et al., 2009; Meng et al., 2007) and mentioned the cellular uptake of NPs in general. And Cu-based NPs particularly lead to morphological changes in the organs and damages at the cellular level (Abudayyak, Guzel, Özhan, 2016a; 2016b; 2020; Khalid et al., 2018; Gosens et al., 2016; Thit, Selck, & Bjerregaard, 2013; 2015; Xu, Li, Xu, Xiao, & Yang, 2013). While this accumulation and cellular uptake could be evaluated negatively as a starting point for different toxic effects like hepatic damage (Chen et al., 2006; Khalid et al., 2018), nephrotic damage (Chen et al., 2006; Khalid et al., 2018; Meng et al., 2007), apoptosis in hepatocytes (Siddiqui et al., 2013; Wang et al., 2011) and inflammation in the cardiac cells (Sun et al., 2011), in the healthy organs, it also could be evaluated positively as the start of therapy for cancer and the key to cancer cells' death. Previous data enclosed the studies showed the anticancer activity of CuO NPs in different cell lines (Dadure, Mahapatra, Haldar, Potbhare, Chaudhary, 2022; Maksoudian et al., 2020; Rani & Saini, 2022). The studies vary, some of them show high activity in the cancerous cells (Abudayyak et al., 2020; Rehana, Mahendiran, Kumar, & Rahiman, 2017) the others show their safety or low toxicity (Nagajyothi et al., 2017; Oza et al., 2020). Since different factors like the cellular type play a role in the degree of toxicity in the cell, a well-known widely used HeLa was chosen to evaluate CuO NPs- induced toxicity. This will give the ability to compare the results with previous data, especially those related to biosynthesized NPs. For that, the cytotoxicity of CuO NPs was evaluated using MTT and NRU assays, and DNA damage induction using Comet assay.

MATERIALS AND METHODS

Materials: CuO NPs, MTT pigment, neutral red (NR) dye, dimethyl sulfoxide (DMSO), acetic acid, and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Ethylene diamine tetraacetic acid (EDTA) and Triton X-100 were obtained from Biomatik (Ontario, Canada). Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), antibiotic solution for cell culture, and trypsin-EDTA solution for cell culture were obtained from Multicell Wisent (Quebec, Canada).

NPs characterization: CuO NPs were suspended in a cell culture medium or distilled water, then NPs were dropped on a special carbon-coated mesh. NPs were analyzed by Transmission Electron Microscopy (TEM) (Jem-2100 HR, Jeol, USA). At least 100 NP were used in the calculation of the average size for each sample. Results were expressed as the mean±SD. Scanning Electron Microscopy (SEM) (JEOL JSM 5600, Jeol, USA) was used to evaluate the NPs' outer size and shape, for that powder was used directly.

Cell culture and exposure conditions: HeLa cell line (CCL-2™) was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were continued in an EMEM cell culture medium supplemented with 10% FBS and 1% antibiotic. The cells, incubated at 37 °C, 95% humidity, and 5% CO_2 conditions, were collected by trypsinization. The cells were seeded at a concentration of 10^4 cells/well and $2x10^5$ cells/well for cytotoxicity and Comet assays, respectively. Before exposure, CuO NPs were prepared freshly as 1 mg/mL suspension in a complete cell culture medium. The suspension was sonicated for 15 min to prevent their aggregation/ agglomeration, then the exposure mediums with the planned concentrations were prepared by dilution. The exposure concentrations were in the range of 1.5-60 µg/mL in cytotoxicity assays. The previous data showed that CuO NPs tested for genotoxicity at concentrations arranged 5 – 50 µg/mL for 24 hours (Abudayyak et al., 2016a; 2016b; 2020; Ahamed et al., 2010; Akhtar, Ahamed, Fareed, Alrokayan, & Kumar 2012; Wang et al., 2012). In these studies, some of the tested concentrations were higher than the IC_{50} values. For that, to be able to compare the results, in the present study where the IC_{50} was about 10.7± 0.7 µg/mL, a concentration that was previously used in data and also close to our IC_{50} value (12.5 µg/mL), the half, and quarter of it (3.125, 6.25, and 12.5 µg/mL concentrations) were used in the Comet assay. For all assays, the cells were incubated for 24 hr. The unexposed cells were accepted as growth and negative control for both cytotoxicity and genotoxicity assays. DMSO (10%), for 24 hours, was used as a positive control for cytotoxicity. Cells exposed to H_2O_2 (100) µM for 2 hours were used as a positive control in Comet assays. Cytotoxicity assays were done in triplicates and repeated in different 3 days (n = 9), Comet assay was done in duplicates and repeated in different 3 days (n = 6).

Cytotoxic effect evaluation: At the end of the exposure period, MTT assay or NRU assay was applied. MTT assay is based on the evaluation of mitochondrial enzyme activity, which is accepted as a sign of cell viability. In this case, the mitochondrial enzyme of the viable cells metabolized the yellowish and water-soluble MTT to water-insoluble violet formazan crystals, while the dead cells will be unable to metabolize and so to give the violet color (Van Meerloo, Kaspers, & Cloos, 2011). NRU assay is based on the ability of viable cells to uptake by passive diffusion and then accumulate the cationic NR dye in the anionic regions in the lysosomes. Since cell death means dam-

age in lysosomes, the dead cell will be unable to accumulate the NR dyes and so to give the red color (Repetto, Del Peso, & Zurita, 2008). In the MTT assay, 25 µL of MTT solution (0.5 mg/mL) was added to each well and the plates were incubated for a further 3 hr. Then, the supernatants were thrown and 100 µL/well of DMSO was added to solve the formed violet crystals. The absorbance was measured at 590 nm using a microplate spectrophotometer system (Epoch, Germany). In the NRU assay, the exposure solutions were discharged, and 100 µL/well NR solution (50 µg/mL) was added to the plates. After incubation for 3 hours, the NR solution was discharged, and the plates were washed with warm phosphate-buffered saline (PBS) x1 twice. Then 100 µL/well of dye-solving solution (1% acetic acid, 1:1 Ethanol: Water). The absorbance was measured at 540 nm. For both assays, the cell viability and the cell death ratios were calculated compared to the negative controls. The results were expressed as the mean of concentrations that caused cell death in 50% of the cells (IC_{50}).

Genotoxic effect evaluation: The genotoxicity was evaluated by Comet assay according to the method mentioned by Collins (2004) and Speit, & Hartmann (1999). Briefly, the exposed cells were collected, washed, and adjusted to 10^6 cells/ mL. Then 100 µL of the cell suspension was mixed with 100 µL of low melting agarose and the cells spread out over slides that were previously coated with normal melting agarose. After that, coverslips were removed, and the cells in the lams were treated with a lysis solution for 12 hours and electrophoresed. Finally, the cells were treated with a normalizing solution, colored with ethidium bromide, and evaluated under the fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). The tail moment was accepted as the endpoint to evaluate the DNA damage. For each sample, at least 100 cells were evaluated. The means of the tail moments for each exposure group were calculated, then the results were calculated compared to the control group and expressed as the folds of the negative control.

Statistical analysis: The significance of results calculated compared to the control groups using one-way ANOVA, post hoc, and Dunnett test (SPSS version 28.0; SPSS Inc., Chicago, IL). $p \leq 0.05$ was considered significant.

RESULTS

NPs characterization: The average diameter of NPs in water was 35 nm, and about 98% of the particles have a diameter smaller than 60 nm. The size was higher in the complete cell culture medium, and the average diameter was 39 nm with more than 79% of the particles with a diameter smaller than 60 nm (Figure 1). Additionally, the NPs were analyzed by SEM microscopy. The SEM pictures also reveal the nano size of the particles; however, it is not enough to make calculations (Figure 2).

Cytotoxicity: The exposure to CuO NPs for 24 hours induced significant cell death that increased with the increase in concentration (Figure 3). The IC_{50} value was calculated by MTT assay to 10.7±0.7 µg/mL. While the IC_{50} calculated by NRU assay was 6.73±0.1 µg/mL.

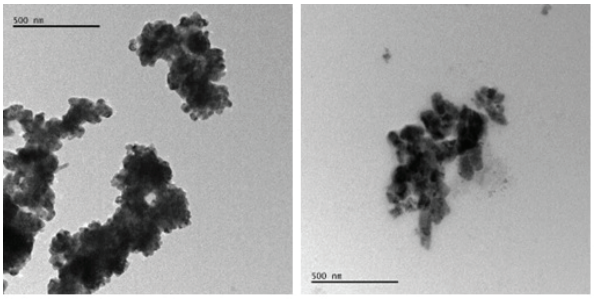

Figure 1. The TEM analysis of CuO NPs in (a) water and (b) complete cell culture medium.

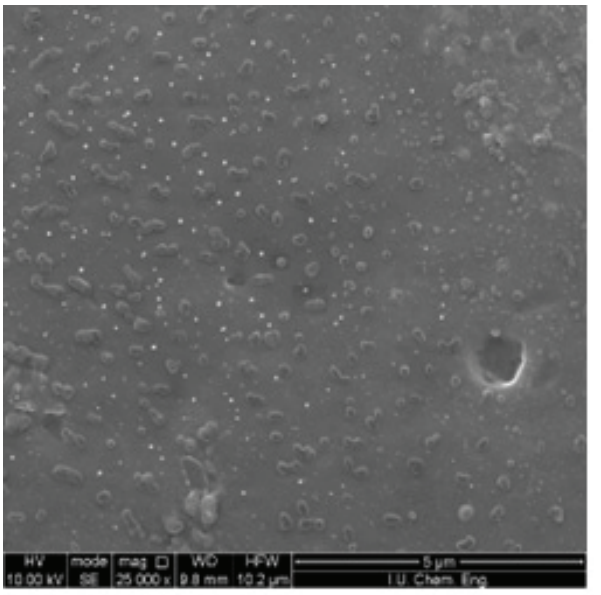

Figure 2. The SEM analysis of CuO NPs powder.

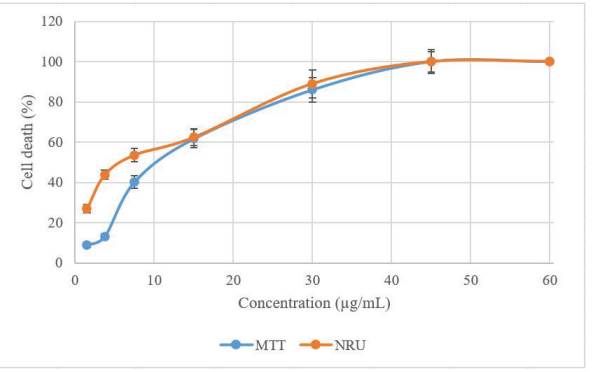

Figure 3. Cytotoxicity of CuO NPs in HeLa cell line at 1.5-60 µg/mL exposure concentrations.

DNA damage-inducing potential: Copper (II) Oxide NPs induced significant DNA damage at all exposure concentrations (Figure 4). The damage depended on concentrations. At the highest exposure concentration (12.5 µg/mL), the increase in the tail moment was 3.85-fold, while they were 2.5-fold and 1.3-fold in 6.25 µg/mL and 3.125 µg/mL, respectively, compared to the negative control group ($p < 0.05$).

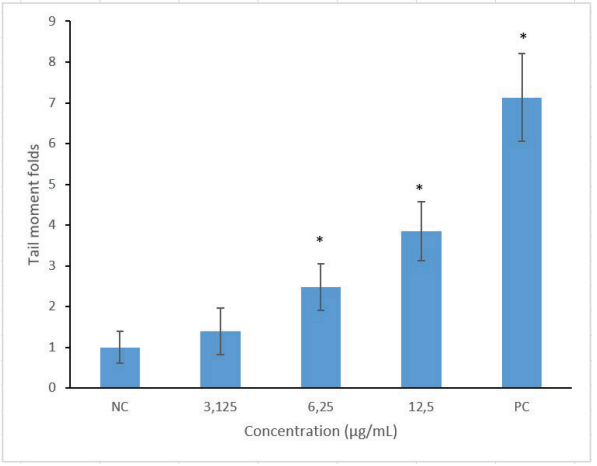

Figure 4. The DNA damage in HeLa cells exposed to CuO NPS. Cells were exposed to 3.125, 6.25, or 12.5 µg/mL CuO NPs for 24 hours. The unexposed cells and cells exposed to H_2O_2 (100 µM) for two hours were accepted as negative and positive controls, respectively. One-way ANOVA, post hoc, and Dunnett test (SPSS version 28.0) were used for the statistical analysis. *p< 0.05 was considered significant.

DISCUSSION

The cellular uptake of nanoparticles was reported by different research groups as well as the research in our lab (Abudayyak et al., 2016a, 2016b, Abudayyak, Altincekic Gurkaynak, & Özhan, 2016c; Abudayyak, Guzel, Özhan, 2017; 2020). Previous data have shown that the exposure to CuO NPs and other Cu-based NPs for 24 hours induced cytotoxic effects in different cell lines such as breast cancer (MCF-7) (Alishah, Pourseyedi, Ebrahimi-pour, Mahani, & Rafiei, 2017; Mahmoud, Mohamed, Ahmed, & Akhtar, 2020), lung (A549) (Karlsson, Cronholm, Gustafsson, & Möller 2008; Wang et al 2012), hepatic cancer (HepG2) (Abudayyak et al., 2020; Siddiqui et al., 2013; Wang et al., 2011), intestinal (Caco-2) (Abudayyak et al., 2020; Piret et al., 2012), Rat kidney (NRK-52E) (Abudayyak et al., 2016b), keratinocytes (HaCaT), embryonic fibroblasts (BALB3T3) (Akhtar et al., 2012), airway epithelial (HEp-2) (Farshori et al., 2022), neuroblastoma (SH-SY5Y) (Abudayyak et al., 2016a; Chen et al., 2008), neuro-glioma (H4) (Chen et al., 2008), mouse neuroblastoma (N2A) (Perreault et al., 2012), cardiac microvascular endothelial cells (Sun et al., 2011), and primary culture of channel catfish hepatocytes cells (Wang et al., 2011). In the cell lines, the reported IC_{50} was less than 16.4 µg/mL, except for N2A cells where the cell viability was about 37% at 400 µg/mL.

Recently, the synthesis of NPs as CuO NPs using plants, yeast, and bacteria in what is called biosynthesis or green synthesis has increased dramatically. Rani & Saini (2022) reviewed more than 25 studies using herbs only, While Dadure et al. (2022) summarized 45 studies related to biosynthesized Cu-based NPs. The green synthesized CuO NPs were evaluated for their anticancer activity using MTT assay in different cell lines, HCT-116 human colon cancer cells (IC_{50}: 40 µg/mL) (Gnanavel, Palanichamy, & Roopan, 2017), AMJ-13 breast cancer cells (IC_{50}: 1.47 µg/mL), and SKOV-3 ovarian cancer cells (IC_{50}: 2.27 µg/mL) (Andleeb et al., 2021). In another study using A549 cells, the IC_{50} value for biogenic CuO NPs was 200 µg/mL (Sankar, Maheswari, Karthik, Shivashangari, & Ravikumar, 2014). The studies carried out using A549, Hep-2, MCF-7, MDA-MB-231 (human breast cancer cells), NHDF (normal human dermal fibroblast cells), and HeLa cell lines were also used to evaluate the anticancer/cytotoxic effect of green CuO NPs. The studies reported that IC_{50} values were between 18.1 µg/mL and 45.3 µg/mL, while for HeLa cells specifically were 20.3 - 45.3 µg/mL (Rehana et al., 2017). Nagajyothi et al. (2017) used HeLa cells to evaluate their green synthesized CuO NPs, after 24 hours of exposure. The IC_{50} level was higher than 500 µg/mL, besides, they noticed the ability of CuO NPs to inhibit the colony of cancerous cells (Nagajyothi, Muthuraman, Sreekanth, Kim, & Shim, 2017). Oza et al. (2020) reported no cytotoxicity of their biosynthesized CuO NPs, as they reported 80% viability at 100 µg/mL for 72 hours of exposure. The variation in the reported potential of cytotoxic effect, and so, the IC_{50} depends on different factors such as the shape, the size, the porous state, the synthesis method, and importantly the used cell line. Hela cell is one of the famous cell lines that are used for anticancer research and one of the oldest immortalized cell lines. The wide use of HeLa in different biomedical and biochemical research gives the opportunities to compare the results with the other chemicals and also with other labs (Masters 2002; Verma & Hansch 2006), however, the effect of pure, chemical, or physically synthesized, CuO NPs were not evaluated previously. For that, in the present study, CuO NPs purchased from Sigma Aldrich with known properties and wide use in research is also preferred, which gives the chance to compare the results among the cell lines. The results of this study show that CuO NPs caused cell death with IC_{50} calculated to be 10.7 µg/mL and 6.73 µg/mL by MTT and NRU assays, respectively. This indicated that HeLa cells are more sensitive toward the chemically synthesized CuO NPs compared to green NPs in the previous studies (Nagajyothi et al., 2017; Oza et al., 2020; Rehana et al., 2017). And also, HeLa cells are more sensitive than other cells used previously (Abudayyak et al., 2016a; 2016b; 2020; Akhtar et al., 2012; Alishah et al., 2017; Chen et al., 2008; Dadure et al., 2022; Farshori et al., 2022; Karlsson et al., 2008; Mahmoud et al., 2020; Perreault et al., 2012; Piret et al., 2012; Rani & Saini 2022; Sankar et al., 2014; Siddiqui et al., 2013; Sun et al., 2011; Wang et al., 2011; Wang et al 2012), except AMJ-13 and SKOV-3 cell lines (Andleeb et al., 2021).

The mechanism of cellular death induction was the topic of different studies. The elevation of reactive oxygen species (ROS) and the disruption of oxidative status inside the cells, the cell arrest, the induction of apoptosis, the damages in genetic materials, and the effects on inflammatory pathways were reported in the cells exposed to CuO NPs (Maksoudian et al., 2020; Tuli et al., 2015). According to Ingle et al. (2013), Cu NPs can degrade the cellular DNA even in the absence of H_2O_2 or other outer factors necessary for oxidative reactions that make those NPs good for targeted therapy (Ingle, Duran, & Rai, 2013). In the present study, Comet assay results reveal the NPs dose-dependently increased in the DNA damage. The tail moment was 1.3-fold at 3.125 µg/mL, 2.5-fold at 6.25 µg/mL, and 3.8-fold at 12.5 µg/mL. These results confirm the results of different previous studies, it was noticed that the same nanoparticles for the same exposure period at concentrations arranged between 5 and 50 µg/mL caused DNA damage with induction of oxidative stress in NRK-52E and SHSY-5Y cells

where the DNA damages were 1.85- to 8.4-fold and 2.57- to 7.09-fold, respectively (Abudayyak et al., 2016a; 2016b). Similar results were noticed after exposure to the same NPs for the same period at concentrations between 5-20 µg/mL in Caco-2 and HepG2; In these cells, the DNA damages were between 1.2- to 7.6-fold in HepG2 cells and 5.89- to 9.6-fold in Caco-2 cell line. (Abudayyak et al., 2020).

Perreault et al. (2012) reported the induction of genotoxicity by CuO NPs at concentrations higher than 12.5 µg/mL (24 hours exposure) using a micronucleus assay in Neuro-2A cells. Similar results were reported in A549 cells after 24 hours of exposure, Akhtar et al. (2016) reported that the tail moment was 27% at 15 µg/mL, compared to 5% in the control group and Wang et al., (2012) reported an increase in the tail moment at 15 mg/L with 4.5-fold compared to the control (Akhtar et al., 2016; Wang et al., 2012). Ahamed et al. (2010) concluded that CuO NPs, at 50 µg/mL for 24 hours of exposure, can induce genotoxicity in A549 cells via oxidative stress pathway by up-regulating the expression of proteins important in DNA damage repair and cell cycle as p53 Rad51 and MSH2 (Ahamed et al., 2010). Previous data together with our results confirm the high cytotoxicity of CuO NPs in HeLa cancerous cells through damaging its genetic material, in addition to the ability to use these NPs for targeted therapy, these NPs could be used for killing those cancerous cells.

CONCLUSION

The previous data reported different results related to CuO NPs-induced toxicity in cancerous cells. This difference could be due to the variation in the cell lines that are used or could be related to the type, shape, and size of NPs, and especially the method of synthesis. The results show that CuO NPs caused cell death at relatively low concentrations (exposure concentration 1.5-60 µg/mL, $IC_{50} \leq 10.7$ µg/mL). The increase in DNA damage in the exposed cells indicates that DNA damage is one of the mechanisms of cell death in HeLa cells exposed to CuO NPs. The present results confirm the previous data that Cu-based NPs in general and CuO NPs specifically have the chance to be developed for use in targeting cancerous tissue and killing these cells. However, there is a need for further *in vitro* and *in vivo* studies to a better understanding of the mechanisms underlying CuO NPs and to approve or disapprove the safety of these NPs and their ability to be developed for use in cancer treatment.

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Conflict of Interest: The authors have no conflict of interest to declare.

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