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PAPER

Caseinophosphopeptides cytoprotect human gastric epithelium cells against the injury induced by zinc oxide nanoparticles

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Zinc oxide nanoparticles (ZnO NPs) are widely used as food additives, especially in nutritional foods. However, many reports have demonstrated their toxicity in humans and other biological systems. Our study has confirmed that ZnO NPs can induce apoptosis and oxidative damage on human gastric epithelium cells (GES-1). Caseinophosphopeptides (CPP) are also used as functional food additives that sequester prooxidant metals and scavenge free radicals. Herein, we investigate the combined cytotoxicity of ZnO NPs and CPP for the first time. The results show that CPP protects GES-1 cells from oxidative stress induced by ZnO NPs, decreases reactive oxygen species, diminishes the level of malondialdehyde, increases the content of glutathione and improves the activity of superoxide dismutase. Therefore, CPP can protect GES-1 cells against the ZnO NPs induced injury through the down-regulation of oxidative stress.

Introduction

In the past few years, food safety problems have attracted increasing attention, and food additive safety has become an important topic in the food safety field.[1-3] However, safety evaluation standards for food additives are based only on the toxic effects of a single additive. The combined effects of multiple additives remain unclear. Those effects include additivity, synergism, potentiation and antagonism.[2, 4] A large number of commercial products contain nanomaterials. Major metal oxide nanoparticles, including zinc, silica, copper and titanium, have promising prospects in food safety and technology.[5, 6] Although their safety has been explored,[6-8] there has been little research into the combined cytotoxicity of metal oxide nanoparticles and other food additives.

ZnO NPs are widely used as food additives.[9] The Food and Drug Administration has listed ZnO as a generally recognized as safe (GRAS) material that can be used as a food additive. ZnO NPs are added to food packing because of its excellent antibacterial ability and used for nutritional purposes,[7] they can play an important role in reducing the risk of pathogen contamination and extending the shelf life of food.[10] ZnO NPs have also been used as a dietary supplement in human and livestock because zinc can stimulate the immune system and act in an anti-inflammatory way.[11, 12] However, there are some reports that have studied the cytotoxicity of ZnO NPs, and the results demonstrated that at certain concentrations ZnO NPs were toxic to cells, including *Tetrahymena thermophila* [13], *Escherichia coli*,[9] osteoblastic MC3T3-E1 cells [14] and human lung cancer A549 cell line.[15] Hence, more attention should be paid to the safety of ZnO NPs when used as a food additive. Among bioactive peptides, CPP, which is used as a nutrition enhancer in food, has received much attention. CPP has both primary and secondary antioxidant properties that specifically involve direct free radical scavenging and sequestering of potential metal prooxidant.[16] CPP can bind to minerals such as calcium, iron and zinc and plays an important role in mineral bioavailability.[17, 18]

As indicated above, the effects of metal oxide nanoparticles and CPP in a complex system have never been investigated. In this study, the combined cytotoxicity of ZnO NPs and CPP is investigated for the first time. We studied the combined effect of ZnO NPs and CPP on the amounts of reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD). The results demonstrate that CPP can protect human gastric epithelium cells (GES-1) against ZnO NPs induced injury through the down-regulation of oxidative stress and may improve the safety of ZnO NPs in food. The related mechanism of down-regulation is also elucidated.

Results and Discussion

1. Characterization of ZnO NPs

Particle dimensions are fundamental to nano-toxicity. This principle was derived from evidence that has consistently demonstrated that NPs are able to elicit more pronounced toxicity than their larger (microparticulate) counterparts.[19] Hence, the characterization of particles prior to a toxicity study is important.[20] Accordingly, in this study, the particle sizes of ZnO NPs in the medium were determined with TEM as shown in Fig. 1a. As seen in the image, there is a polydispersity in size and morphology with both spherical and rod-like particles. The size distribution of ZnO NPs is presented in Fig. 1b by counting around 100 particles using Image J in TEM photos. Fig. 1c shows the X-ray diffraction patterns, indicating that the diffraction peaks of ZnO NPs can be indexed to hexagonal wurtzite structure of ZnO, which are in good agreement with the standard JCPDS file of ZnO (JCPDS Card No. 36-1451). The purity of ZnO NPs is 99.97% according to XRF analyses. Dynamic light scattering measurements indicate that the hydrodynamic diameter of ZnO NPs in culture medium is approximately 162 nm. The physicochemical properties of ZnO NPs are summarized in Table1.

Table 1 Physicochemical characterization of ZnO NPs

Purity	Particle size (nm)		Zeta potential (mV)	
(%)	In water	In medium[a]	In water	In medium[a]
99.97	125 ±48	162 ± 76	-19.4 ±0.7	-6.4 ± 2.3

[a]Culture medium is high-glucose DMEM with 10 % fetal bovine serum. Data are presented as the mean ± S.D.

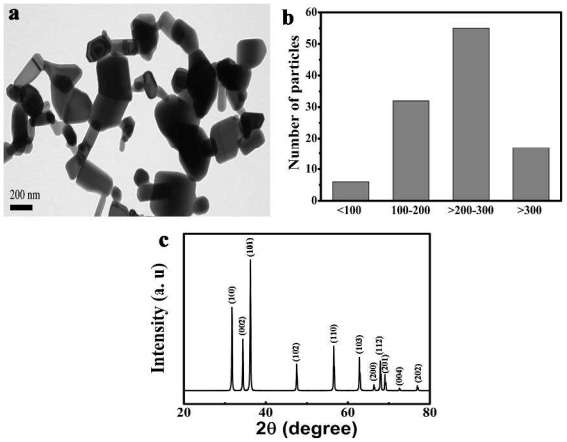

Fig. 1 Characterization of ZnO NPs: (a) TEM image; (b) size distribution of ZnO NPs; (c) X-ray diffraction. All diffraction peaks can be perfectly indexed to hexagonal wurtzite structure of ZnO (JCPDS Card No. 36-1451)

2. Cytotoxicity evaluation

Metal oxide nanoparticles have unique physicochemical properties and have multiple uses, especially as food additives. ZnO NPs have been widely used in nutritional foods, and many reports have proved its cytotoxicity and the mechanisms which are involved in the production of ROS and oxidative stress.[17, 21-23] Wang *et al.*[7] reported that Zn enters into blood circulation and accumulates mainly in the liver, lungs and kidneys after exposure to ZnO NPs. It can induce liver damage because of the Zn accumulation-induced oxidative stress led to DNA damage and apoptosis in the liver.

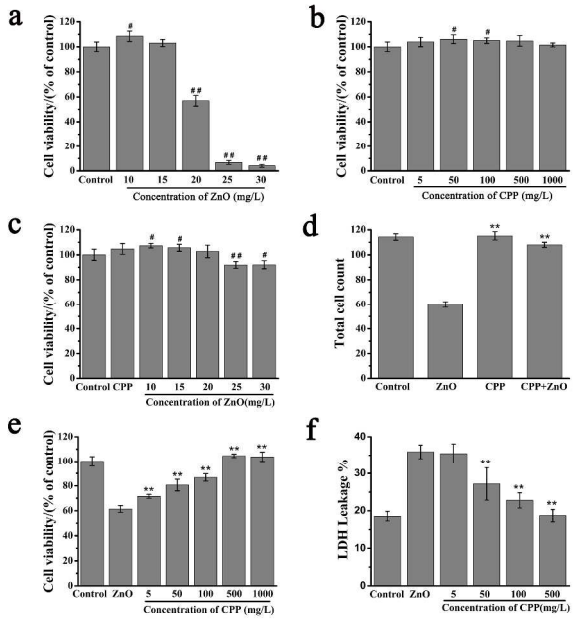

Fig. 2 Cytotoxicity evaluation of ZnO NPs, CPP and ZnO NPs & CPP treatment in GES-1 cells for 24 h: (a) different concentrations (mg/L) of ZnO NPs; (b) different concentrations (mg/L) of CPP; (c) the effect of CPP (500 mg/L) at different concentrations of ZnO NPs; (d) cell counts treatment with 20 mg/L ZnO NPs, 500 mg/L CPP, 20 mg/L ZnO NPs & 500 mg/L CPP, respectively; (e) the effects of different concentrations of CPP and ZnO NPs (20 mg/L); (f) the effects on LDH release at different concentrations of CPP with ZnO NPs (20 mg/L). Data are presented as the mean ± S.D. *$p < 0.05$ compared with cells exposed to ZnO NPs alone, $^{\#}p < 0.05$ compared with control group.

To compare the viability of GES-1 cells after co-incubation with different concentrations of ZnO NPs, CPP and ZnO NPs & CPP, cell viability, LDH release and cell count were performed. The results are shown in Fig. 2. Dose-dependent decreases in cell viability were demonstrated using a CCK-8 assay in GES-1 cells exposed to ZnO NPs. From the data presented in Fig. 2a, the IC_{50} of ZnO NPs was approximately 20 mg/L, thus 20 mg/L ZnO NPs

was used to induce injury in GES-1 cells for subsequent experiments. García-Nebot *et al.* [17] reported that CPP can modulate the deleterious effects from oxidative challenges, preserving cell viability. In the present study, CPP alone did not exert a cytotoxic effect upon GES-1 cells and did prevent the decline of viability caused by ZnO NPs (Fig. 2b; Fig. 2c; Fig. 2e), which corresponds to the anti-oxidant ability of CPP. Similar results were also observed for LDH release and cell counts (Fig. 2d; Fig. 2f). The LDH assay indirectly measures cell death based on the amount of lactate dehydrogenase released by cells with damaged membranes.[24] LDH release in the cells was observed to increase to approximately 37 % compared with the control after ZnO NPs exposure alone. However, LDH release was reduced in a dose-dependent manner until equal to the control when CPP was co-incubated with ZnO NPs.

3. Quantitatively analyze the effects on apoptotic and necrotic GES-1 cells and DNA fluorescent staining

To quantitatively analyze the apoptotic and necrotic effects in GES-1 cells after ZnO NPs and CPP & ZnO NPs treatment, annexin V-FITC and PI assays and FACS technology were employed. Cells that are positive for annexin V-FITC indicate the release of phospholipid phosphatidylserine, an indication of the early stages of apoptosis. Cells that are positive for PI indicate cell membrane damage, which occurs either at the end stage of apoptosis, during necrosis or in dead cells. In this assay, apoptotic cells were identified as annexin V-FITC$^+$ and PI$^-$. Nonviable cells were identified as annexin V-FITC$^+$ and PI$^+$, and viable cells were identified as annexin V-FITC$^-$ and PI$^-$. In the control and CPP treated groups, no apoptosis occurred after 24 h incubation (Fig. 3a; Fig. 3b). A significant decrease in viability was observed in the 20 mg/L ZnO NPs treated group, the apoptotic cell rate was 33% and the rate of normal cells was decreased to 56% (Fig. 3c). However, cell viability increased to the control level when cells were treated with 500 mg/L CPP &20 mg/L ZnO NPs (Fig. 3d). Fig. 3e and Fig. 3f visually represent Fig. 3a-Fig. 3d.

The nuclear size and roundness is an early indicator of cell death and cell rounding is regarded as one of general cell morphology changes that indicate apoptosis.[25] To further investigate the cytoprotective effect of CPP on ZnO NPs induced toxicity, we observed cell morphological changes. Our results in Fig. 4 demonstrate that the GES-1 cells grew well in the control group (Fig. 4a; Fig. 4e; Fig. 4i). Additionally, cells treated with 20 mg/L ZnO NPs were more rounded and exhibited typical apoptotic features, which were cell body shrinkage, nuclear condensation and loss of cell membrane integrity (Fig. 4b; Fig. 4f; Fig. 4j). However, CPP & ZnO NPs treated cells displayed little cell damage (Fig. 4d; Fig. 4h; Fig. 4l). These cells exhibited normal cell morphology, integrity and nuclear size. Our results have demonstrated that CPP can preserve cell viability and normal cell morphology.

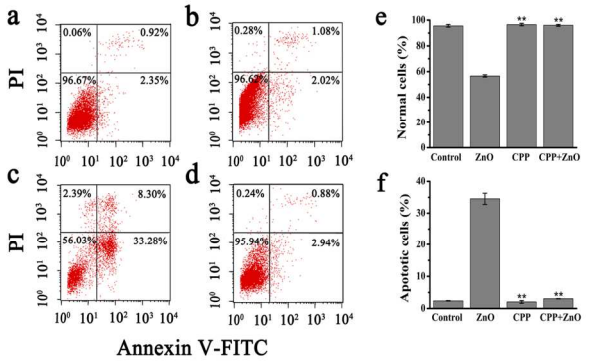

Fig. 3 The combined effect of CPP and CPP & ZnO NPs on apoptosis/necrosis of GES-1cells after 24 h incubation. (a) Control; (b) cells treated with 500 mg/L CPP; (c) cells treated with 20 mg/L ZnO NPs; (d) cells co-incubated with 500 mg/L CPP & 20 mg/L ZnO NPs. (e) normal cells from cell populations incubated with 500 mg/L CPP, 20 mg/L ZnO NPs and 500 mg/L CPP & 20 mg/L ZnO NPs for 24 h and analyzed by flow cytometry. (f) apoptotic cells from cell populations incubated with 500 mg/L CPP, 20 mg/L ZnO and 500 mg/L CPP & 20 mg/L ZnO for 24 h and analyzed by flow cytometry. Data are presented as the mean ± S.D. *p < 0.05 compared with cells exposed to ZnO NPs alone.

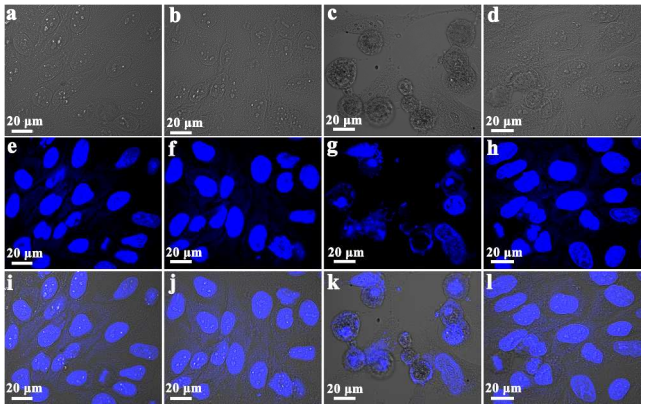

Fig. 4 Observation of ZnO NPs induced GES-1 cell morphological changes after treatment with CPP. Cells treated with 20 mg/L ZnO NPs, 500 mg/L CPP, 20 mg/L ZnO NPs & 500 mg/L CPP for 24 h, respectively. (a) Control in the bright field; (e) control stained by DAPI. (i) merge of (a) and (e); (b) cells treated with CPP in the bright-feild; (f) cells treated with CPP stained by DAPI; (j) merge of (b) and (f); (c) cells treated with ZnO NPs in the bright-feild; (g) cells treated with ZnO NPs stained by DAPI; (k) merge of (c) and (g); (d) cells treated with CPP & ZnO NPs in the bright-field; (h) cells treated with CPP &

ZnO NPs stained by DAPI; (l) merge of (d) and (h). The figures are representative of three different experiments.

4. Fluorescence assessment of zinc ion in GES-1 cells

TSQ is a commonly used Zn^{2+} sensor. Zn^{2+} binding is accompanied by the appearance of an intense fluorescence emission spectrum (excitation maximum: 360 nm, emission maximum: 490 nm). TSQ and its related fluorophores are bidentate ligands, utilizing quinoline and sulfonamide nitrogens to coordinate Zn^{2+}.[26] Some reports have shown that the toxicity of ZnO NPs was mainly associated with dissolved zinc species.[9, 27] Therefore, we explored the density of zinc ions in GES-1 cells. As shown in Fig. 5, there is no fluorescence from zinc ions except for the background fluorescent color in the control group. Under the same voltage, the cells treated with ZnO NPs were approximately 50 % round. Moreover, the concentration and fluorescence from zinc ions were evident. However, after co-incubation with ZnO NPs & CPP, cells exhibited normal cell morphology and integrity. As shown in Fig. 5f, the obvious blue of zinc ion was present in GES-1 cells, but notoxicity was induced in the cells. From the above, the protective effect of CPP against ZnO NPs was can be visualized. As early as 1998, FitzGerald *et al*.[28] summarized a review about CPP, which can complex and solubilize calcium ions preventing their precipitation, allowed the recognition of functional foods and nutraceuticals to act as mineral carriers for minerals, including calcium, iron and zinc. Esther *et al*.[17] and García-Nebot *et al*.[18] reported that CPP can bind to minerals such as calcium, iron and zinc and plays an important role in mineral bioavailability. Kitts *et al*.[16] studied the composition of CPP and its ferrous sequestering activity which confirmed that CPP can sequester iron and attributed this to the presence of a high concentration of phosphoserine residues in close proximity. Based on our results and combined the work performed by others, we infer that a number of complexes are formed by capturing Zn^{2+} when CPP is combined with ZnO NPs that contributed to the reduction of ZnO NPs cytotoxicity.

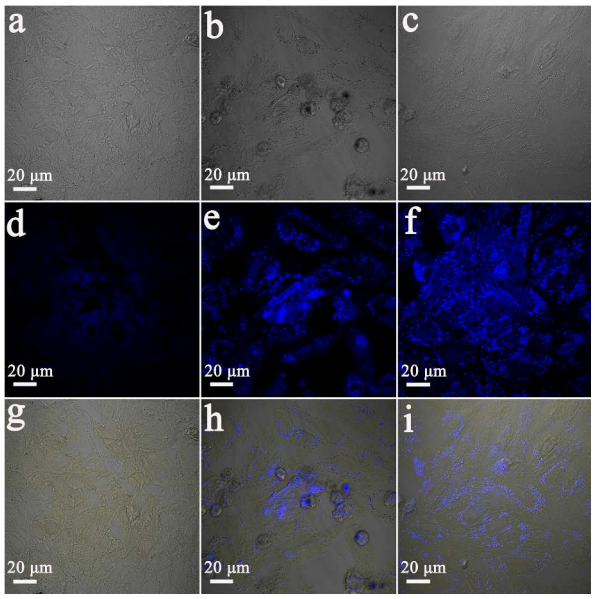

Fig. 5 Observation of zinc ion dissolved by ZnO NPs in GES-1 cells and morphological changes. Cells treated with 20 mg/L ZnO NPs, 20 mg/L ZnO NPs & 500 mg/L CPP for 24 h, respectively. (a) Control in the bright-feild; (d) control stained by TSQ; (g) merge of (a) and (d); (b) cells treated with ZnO NPs in the bright-feild; (e) cells treated with ZnO NPs stained by TSQ; (h) merge of (b) and (e); (c) cells treated with CPP & ZnO NPs in the bright-field; (f) cells treated with CPP & ZnO NPs stained by TSQ; (i) merge of (c) and (f). The figures are representative of three different experiments.

5. Intracellular redox status

Intracellular reactive oxygen species and malondialdehyde

CPP are commonly considered anti-oxidants.[16] To determine if the protective effect of CPP against ZnO NPs induced toxicity is through the down-regulation of oxidative stress, we determined the generation of ROS and the levels of MDA. Briefly, GES-1 cells exposed to 20 mg/L ZnO NPs for 24 h, resulting in a significant increase in the generation of ROS and the concentration of MDA (Fig. 6a-Fig. 6b), which is consistent with the reports published by others.[22, 23, 29] In cells treated with 500 mg/L CPP there was no generation of ROS or production of MDA. CPP was able to reduce ROS generation and MDA concentration induced by ZnO NPs treatment almost to the control level. Therefore, we confirmed that the protective effect of CPP was related to anti-oxidant mechanisms.

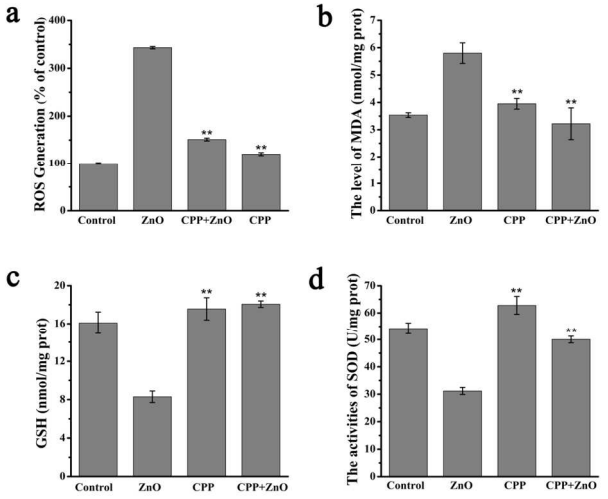

Fig. 6 Intracellular redox production after incubation with 20 mg/L ZnO NPs, 500 mg/L CPP and 20 mg/L ZnO NPs & 500 mg/L CPP for 24 h, respectively. (a) ROS generation; (b) MDA concentration; (c) GSH concentration; (d) SOD activity. Data are presented as the mean ± S.D. *p < 0.05 compared with cells exposed to ZnO NPs alone.

Intracellular glutathione and superoxide dismutase

Cell has its own defense mechanisms against the damage induced by oxidative stress,[30] it has antioxidant enzymes such as SOD, CAT and GSH-Px, and the inherent anti-oxidants such as GSH, which is the major intracellular anti-oxidant to prevent the damage induced by ROS. After 24 h exposure to 20 mg/L ZnO NPs, GSH concentration and SOD activity in cells were dramatically decreased compared with the control (Fig. 6c; Fig. 6d). GSH concentration and SOD activity in cells treated with 500 mg/L CPP were almost equal to the control level. When cells were co-incubated with 20 mg/L ZnO and 500 mg/L CPP, GSH concentration SOD activity increased to the control level. These results are in accordance with all of the above data. García-Nebot *et al.*[17] and Laparra *et al.*[31] reported that CPP can protect Caco-2 cells against H_2O_2-induced oxidative damage by preserving cell viability, increasing GSH, improving the activity of SOD, diminishing MDA and maintaining correct cell cycle progression. They also studied the total anti-oxidant capacity of CPP and proved the capacity is dose-dependent. Kibangou *et al.*[32] reported that CPP has in vitro protective effects against iron-induced lipid peroxidation. Kim *et al.*[33] evaluated the anti-oxidant capacity of CPP prepared at different pH levels and showed that the oxygen radical absorbance capacity (ORAC) against peroxyl activity might be attributed to the scavenging activity on hydroxyl radicals, as well as, the metal-chelating activity. From all of the above, we can deduce that the protection of CPP against ZnO NPs is through a down-regulation of oxidative stress and also its metal-chelating activity.

Experimental Section

ZnO samples: The food grade ZnO NPs were purchased from Wuxi Zehui Chemical industry Co., Ltd (Jiangsu, China). CPP was purchased from Beijing Weier healthy food Co., Ltd (Beijing, China).

Characterization of ZnO NPs: The crystalline phase of ZnO NPs was analyzed by XRD (Rigaku Co., Tokyo, Japan). The shape and size of ZnO NPs were investigated by TEM (JEM-200CX, JEOL, Japan). The purity of the NPs was analyzed using XRF (S4-Explorer, Bruker, Germany). The hydrodynamic size of ZnO NPs in water and culture medium and the zeta potential in water were measured on a Nanosizer (Zetasizer 3000 HS, Malvern, UK).

Cell culture: GES-1 cells were obtained from the Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from gino biological pharmaceutical technology co., Ltd (Hangzhou, China). Cells were grown to confluence in 25 cm^2 flasks supplemented with high-glucose DMEM and 10% FBS and incubated in a humidified incubator with 5 % CO_2 and 95 % air at 37 °C. Cells that were 80% confluent were used in all assays.

Cell proliferation: Cell viability assays were performed using a CCK-8 Kit (Dojindo Laboratories, Japan) following the manufacturer's instructions. The cell viability was expressed as the percentage of viable cells within the total cells. Briefly, GES-1 cells were seeded in 96-well plates (5×10^3 cells per well), after a 24 h attachment period, cells were treated with differing concentrations of ZnO NPs, CPP and ZnO NPs & CPP for an additional 24 h. The cell viability of each well was determined with the CCK-8 Kit following the instruction manual and the 450 nm absorbance of each well was measured using a microplate reader (Thermo, USA).

Cell count: Cell counts were determined using a hemocytometer (Shanghai Qiujing Biochemical reagent and instrument Co., Ltd, China). Briefly, 4×10^5 GES-1cells were incubated in a culture flask, after a 24 h attachment period, cells were separately treated with 20 mg/L ZnO NPs, 500 mg/L CPP, 20 mg/L ZnO NPs & 500 mg/L CPP for an additional 24 h. The attached cells were then trypsinized with 25 % Trypsin-EDTA (GIBCO, Invitrogen, USA) and counted using a hemocytometer.

Cell membrane integrity: To further assess cell death by detecting cellular membrane damage, the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium was detected. Data were converted to percent viability of cells based on LDH release from positive control cells.[24] It was determined by using a commercially available kit (Dojindo Laboratories, Japan). Briefly, GES-1 cells were seeded in 96-well plates (5×10^3 cells per well), after a 24 h attachment period, cells were co-incubated with different concentrations of CPP (5-500 mg/L) & 20 mg/L ZnO for an additional 24 h. The positive control cells were treated with cell lysis buffer for 45 min before centrifugation (4000 rpm×10 min), the supernatant was transferred to a new 96-well plate, adding 50 μL work solution for 30 min and stopped with stop solution. The LDH of each well was measured at 450 nm using a microplate reader (Thermo, USA).

Annexin V-FITC and PI Assay: An annexin V-FITC (fluorescein isothiocyanate, excitation maximum: 494 nm, emission maximum: 518 nm) and propidium iodide (PI) (excitation maximum: 536 nm, emission maximum: 617 nm) assay were employed to detect apoptotic and necrotic cells. Stained cells were analyzed by two-color flow cytometry. The assay procedure was performed according to the instruction manual of the annexin V-FITC apoptosis detection kit I (BD Biosciences, USA). After co-incubation for 24 h of ZnO NPs (20 mg/L), CPP (500 mg/L) and ZnO NPs (20 mg/L) & CPP (500 mg/L), the GES-1 cells were harvested, washed twice with cold phosphate-buffered saline (PBS 0.15 M, PH=7.2) and resuspended to 1×10^6 cells/mL in binding buffer. Subsequently, 100 μL of cells was transferred to a 5 mL culture tube and 5 μL of FITC-conjugated annexin V (annexin V-FITC) and 5μL of PI were added at room temperature. After incubation for 15 min at room temperature in the dark, stained cells were diluted using binding buffer and directly analyzed by flow cytometry with the excitation wavelength for FITC is 488 nm and 543 nm for PI. (FACS, FACSCalibur, BD Biosciences, USA).

DNA fluorescent staining and confocal laser scanning microscopy studies: The fluorescent probe 4,6-diamidino-2-phenylindole (DAPI, excitation maximum: 340 nm, emission maximum: 488 nm) is a popular nuclear counter stain in multicolor fluorescent techniques. It stains nuclei specifically, with little or no cytoplasm labeling.[34] GES-1 cells were pre-cultured in culture medium containing 20 mg/L of ZnO NPs, 500 mg/L CPP and ZnO NPs (20 mg/L) & CPP (500 mg/L) for 24 h. Subsequently, cells were rinsed by PBS and DAPI working solutions. The cells were kept in a 2 mg/L DAPI working solution for 15 min in the dark at room temperature. Finally, the cells were washed twice with PBS to remove excess DAPI and examined under a confocal microscope (FV1000, Olympus Corp., Tokyo, Japan) with an excitation wavelength of 405 nm.

Fluorescence assessment of zinc ions and cell morphology: A commonly used Zn^{2+} fluorophore is 6-methoxy-8-p-toluenesulfonamido-quinoline (TSQ) (Shanghai Meilian Biotechnology Co., Ltd, China). We used a method developed by Meeusen for this assay.[26] Briefly, GES-1 cells were pre-cultured in culture medium containing 20 mg/L of ZnO NPs, 500 mg/L CPP and ZnO NPs (20 mg/L) & CPP (500 mg/L) for 24 h. The cells were then rinsed three times with PBS and stained for 30 min in an incubator using a solution of 30 μM TSQ in PBS. After the stained cells were rinsed three times with PBS, the cells were examined under a confocal microscope (FV1000, Olympus Corp., Tokyo, Japan) with an excitation wavelength of 405 nm.

Measurement of reactive oxygen species and malondialdehyde: ROS were measured using the oxidation-sensitive fluorophore 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Jiancheng Bioengineering Institute, China). DCFH-DA is a nonfluorescent compound that is freely taken up by cells and hydrolyzed by esterases to 2′,7′-dichlorofluorescein (DCFH). DCFH is then oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides, thereby indicating the level of intracellular ROS. Briefly, cells were harvested and incubated with 20 mM DCFH-DA dissolved in cell-free medium at 37 °C for 30 min and then washed three times with PBS. Cellular fluorescence was quantified using a fluorescence spectrophotometer (F-700, Hitachi Corp., Japan) at an emission wavelength of 525 nm. During the entire procedure with DCFH-DA, the plate was kept out of light to minimize the fading of the fluorophore. Intracellular ROS production was expressed as a percentage of control cell ROS levels.

MDA, a product of lipid peroxidation (LPO), was analyzed using an assay kit (Jiancheng Bioengineering Institute, China). In brief, cells were harvested by trypsinization and cellular extracts were prepared by sonication (Vcx-130PB, Sonics Corp., USA) in extracting solution, and then the assay was performed according to the manufacturer's instructions for the MDA assay kit. The same procedure was used to lyse the cells and protein

concentrations were determined using a BCA assay kit (Jiancheng Bioengineering Institute, China), in the following assays unless otherwise indicated. The concentration of MDA can be measured at a wavelength of 530 nm using a microplate reader (Thermo, USA) by reacting with thiobarbituric acid (TBA) to produce a stable chromophore. MDA levels were expressed as nmol/mg protein.

Measurement of glutathione and superoxide dismutase: GSH is a natural antioxidant in cells. However, it decreases under conditions of oxidative stress because of free radical scavenging or the reduction of peroxides.[35] The concentration of GSH was analyzed by an assay kit (Jiancheng Bioengineering Institute, China) and measured at a wavelength of 405 nm using a microplate reader (Thermo, USA). All procedures were in compliance with the manufacturer's instructions.

SOD is an important enzyme in mammalian cells. The SOD activity assay is based on its ability to inhibit the oxidation of oxymine by O_2^- produced in the xanthine-xanthineoxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 450 nm by 50%. The activity of SOD is analyzed by an assay kit (Jiancheng Bioengineering Institute, China) and measured at a wavelength of 450 nm using a microplate reader (Thermo, USA). All procedures were in compliance with the manufacturer's instructions.

Statistical Analysis: Results were expressed as the mean ± standard deviation (S.D.) from three to six individual experiments. The statistical significance was analyzed by t-tests of one-way analysis of variance (ANOVA). Difference was considered significant if $p<0.05$.

Conclusions

The CPP and ZnO NPs have shown promise for use as functional food ingredients. Taking into account the results obtained here, the IC_{50} in GES-1 cells for ZnO NPs is a dose of approximately 20 mg/L, with the toxic effects being induced by oxidative stress. CPP demonstrated a cytoprotective effect on GES-1 cells against ZnO NPs induced oxidative stress, preserving cell viability, decreasing ROS, diminishing the level of MDA, increasing the content of GSH and improving the activity of SOD. From all of these facts, we can deduce that CPP can protect GES-1 cells against ZnO NPs induced oxidative stress and improve the safety of ZnO NPs.

Acknowledgments

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The table of contents entry

Caseinophosphopeptides cytoprotect human gastric epithelium cells against the injury induced by zinc oxide nanoparticles

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Caseinophosphopeptides can protect GES-1 cells against the zinc oxide nanoparticles induced injury through the down-regulation of oxidative stress.

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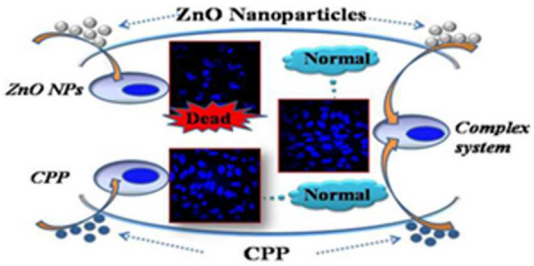

Page Headings
Left page: Y. Wang* et al.
Right page: Combined cytotoxicity evaluation of ZnO NPs and CPP