

# Effect of ZnO Nanoparticle on Cell Viability, Zinc Uptake Efficiency, and Zinc Transporters Gene Expression: a Comparison with ZnO and ZnSO<sub>4</sub>

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## ABSTRACT

Zhang X., Wang Z., Mao L., Dong X., Peng Q., Chen J., Tan C., Hu R. (2017): **Effect of ZnO nanoparticle on cell viability, zinc uptake efficiency, and zinc transporters gene expression: a comparison with ZnO and ZnSO<sub>4</sub>**. Czech J. Anim. Sci., 62, 32–41.

Zinc plays an important role in functional and structural integrity of cells. The aim of the current study was to compare cell viability, zinc uptake efficiency, and gene expression of metallothionein (*MT*), divalent metal transporter (*DMT-1*), and other important zinc transporters (*ZnTs*) under experimental treatment of TPEN (N, N, N', N'-Tetrakis (2-pyridylmethyl) ethylenediamine) (2 μM), and three zinc sources (zinc oxide nanoparticle (nano-ZnO), bulk zinc oxide (ZnO), and zinc sulfate (ZnSO<sub>4</sub>)) at different levels (25, 50, and 100 μM) in rat intestinal epithelial cell line IEC-6. Cells were classified into TPEN group and TPEN + zinc sources groups. In the present study, significantly decreased cell viability was observed in TPEN group, while supplementations with nano-ZnO at all levels and ZnO (50 and 100 μM) significantly increased the cell viability. ZnSO<sub>4</sub> at a high concentration (100 μM) inhibited cell viability. Furthermore, cells of nano-ZnO group showed the highest viability at a 25 μM concentration. The uptake efficiency of nano-ZnO is higher than that of ZnSO<sub>4</sub> and ZnO. Additionally, a significant down-regulation for *ZnT-1*, *ZnT-4*, *MT*, *DMT-1* mRNA with TPEN treatment was detected. Compared with the unchanged *ZnT-4*, all zinc treatments up-regulated the gene expressions of *ZnT-1*, *ZnT-5*, *ZnT-7*, *MT*, and *DMT-1*. Our results indicate that nano-ZnO is more effective than ZnO and ZnSO<sub>4</sub> in enhancing cell viability, and its lower cytotoxicity, higher uptake efficiency, and comparative transportation at low concentration also favour its potential use as a new zinc source in feed additives.

**Keywords:** nano-ZnO; bulk ZnO; zinc transportation; TPEN; IEC-6 cells

The intestine is the central organ for zinc absorption and transportation. Intestinal cells take up zinc from the diet, and then distribute it to different organs. Zinc is an important co-factor for many enzymes, transcription factor as well as hormones and, hence, is a vital component and intracellular mediator of the cell. In recent years, the importance of some zinc compounds supplementation in animal diets has gained recognition,

particularly in some pathological periods such as diarrhoea during post-weaning in piglets, or for enhancing productive performance (Owusu-Asiedu et al. 2003; Hojberg et al. 2005; Miller et al. 2009). However, the relative bioavailability of zinc sources used as feed additives varies substantially depending on their chemical forms and the absorption characteristics of several inorganic zinc sources are not well known.

Supported by the Sichuan Agricultural University (Shuangzhi Project).

doi: 10.17221/15/2016-CJAS

Two of the most commonly used inorganic zinc sources in feed industry up to now are zinc oxide (ZnO) and zinc sulfate (ZnSO<sub>4</sub>). Several previous studies compared the absorption characteristics of these two chemical forms using the animal test as a measure of bioavailability, however, the results were controversial. Some of these animal studies showed much lower bioavailability of zinc oxide in comparison with zinc sulfate (Sandoval et al. 1997), but some more recent studies showed that zinc oxide was effective regardless of its poor Zn bioavailability (Mavromichalis et al. 2000; Case and Carlson 2002). The studies also indicated that zinc may have a local effect in intestine, rather than a systemic effect.

Nanomaterials comprised of ZnO have generated considerable concern in recent years due to their potential use in biological applications arising from their special physicochemical properties (Stark 2011). A new form of zinc – nano-ZnO – should have an enhancing effect on zinc uptake and bioavailability. It is generally accepted now that nano-ZnO, compared to traditional Zn forms, could efficiently penetrate into cell, internalize and disrupt membrane architecture. But as a consequence of its rapid absorption characteristics, most of the studies showed cytotoxicity of nano-ZnO because of particle dissolution and zinc release in the medium or uptake in the cells leading to cell injury (Xia et al. 2008; De Berardis et al. 2010). In this regard, many recent reports have confirmed that nano-ZnO has adverse biological outcomes on the prokaryotic and eukaryotic system, while in an appropriate concentration it is nontoxic to natural biomolecules (Hanley et al. 2008; Shen et al. 2008). A majority of those studies have focused on some low tolerance cells, including immune cells, or even on some immortalized cells lines, which showed changed sensitivities to some chemicals by altering metabolic processes of cells. Thus, cytotoxicity by susceptibility to nano-ZnO appears to be affected by the cell type and is also related to other physiologically relevant parameters, including the concentration used and inherent differences in cellular uptake processes. These variable toxic effects of nano-ZnO on various types of cells indicate that a more detailed investigation of its effects on intestinal epithelial cells is needed.

The intestine is the first tissue confronted with zinc, and an abundant uptake of zinc from the small intestine is indispensable for cellular processes

in the body. However, a high level of intracellular zinc is deleterious and poisonous to the cell. Therefore, maintaining zinc level in an appropriate variation range in tissue is of great importance. As a result, zinc metabolism is tightly regulated. Zinc homeostasis is controlled by a large group of zinc-regulatory proteins involved in zinc metabolism (zinc absorption, storage, transportation, and excretion). These key proteins include metallothionein (*MT*), divalent metal transporter (*DMT-1*), and transmembrane zinc transporters (*ZnT*). In mammalian cells, *ZnT* family consists of ten homologous SLC30 proteins (*ZnT-1–ZnT-10*) (Cousins et al. 2006) and most of the zinc transporters have been confirmed as directly or indirectly involved in absorption, storage or utilization of Zn in cells (Kambe et al. 2002; Palmiter and Huang 2004; Qin et al. 2009). *ZnT-1* was the first identified mammalian zinc transporter located in the plasma membrane and responsible for transporting cytoplasmic zinc across the membrane to the extracellular space (Qin et al. 2009). *ZnT-4* is widely expressed, but more abundantly in brain and epithelial cells (Cousins and McMahon 2000). It is very important for maintaining the body Zn homeostasis (Huang and Tepasamorndech 2013). *ZnT-5* is also highly expressed in a large number of cells, and a higher level was detected in pancreatic beta cells (Kambe et al. 2002). *ZnT-7*, which is more abundant in duodenum and jejunum, is mainly expressed in lung and small intestine. A previous study described *ZnT-7* as the Golgi apparatus protein involved in the accumulation of Zn from cytoplasm, because in the Golgi apparatus *ZnT-7* occurs at a subcellular level (Kirschke and Huang 2003). In addition, metallothionein (*MT*) is a kind of low-molecular-weight proteins, which can maintain homeostasis of some required elements (Hoadley and Leinart 1987; Cherian et al. 1994). *DMT-1* is located in the apical membrane of intestinal cells and can transport a wide range of divalent metal ions, but studies concerning Zn transportation are limited.

Little attention has been given to the uptake and regulation of *ZnTs* with ZnO nanoparticles in previous studies. Therefore, in the present study, we investigated the effects of nano-ZnO, bulk ZnO, and ZnSO<sub>4</sub> on cell viability and uptake efficiency in the rat intestinal cell line IEC-6. Moreover, we determined the mRNA expression of *ZnT-1*, *ZnT-4*, *ZnT-5*, *ZnT-7*, *MT*, *DMT-1* in response to the different forms and levels of zinc in IEC-6.

## MATERIAL AND METHODS

**Epithelial cell culture.** The rat intestinal IEC-6 cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were routinely grown in plastic tissue culture flasks (25 cm<sup>2</sup> growth area) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, and 1 × 10<sup>5</sup> U/l penicillin/streptomycin, and maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>: 95% air at 90% relative humidity. In all experiments, IEC-6 cells were seeded on tissue culture plates and monitored using phase-contrast microscopy. Cell passages were maintained between 10 and 15 for all experiments, and medium was changed every 2 days.

**Cell viability assay.** Cellular viability with or without experimental agents was determined using the water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) assay, which is based on the ability of viable cells to convert dissolved WST-8 to an insoluble orange-coloured formazan product. Briefly, IEC-6 cells propagated in 96-well plates upon reaching confluency at 37°C and 5% CO<sub>2</sub> atmosphere. The culture medium was aspirated and cells were incubated with N, N, N', N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (2 μM) and nano-ZnO, ZnO, and ZnSO<sub>4</sub> with the concentration of 25, 50, and 100 μM for 24 and 48 h, respectively. After that, CCK-8 (Beyotime Institute of Biotechnology, China) solution (10 μl/100 μl culture medium) was added and incubation for 2 h at 37°C in a humidified incubator (5% CO<sub>2</sub>) followed. Colour development at a wavelength of 450 nm was measured using an automatic plate reader Thermo Multiskan MK (Thermo Fisher Scientific Inc., USA). Relative cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = \left( \frac{\text{OD}_{450\text{nm}}(\text{sample})}{\text{OD}_{450\text{nm}}(\text{control})} \right) \times 100$$

where:

OD<sub>450nm</sub>(sample) = absorbance of treated cell sample

OD<sub>450nm</sub>(control) = absorbance of control cell sample

**Determination of zinc uptake efficiency.** IEC-6 cells propagated in 6-well plates upon reaching confluency, and the media in the wells were then replaced with IEC-6 zinc-free medium (0.82 μM

TPEN was used to chelate zinc in the IEC-6 medium) with three zinc sources (nano-ZnO, bulk ZnO, and ZnSO<sub>4</sub>) at different levels (25, 50, and 100 μM). After incubation for 24 h at 37°C, the media were removed, and the cells were then washed once with 5% acetic acid and 10 mmol/l ethylenediaminetetraacetic acid (EDTA), respectively, then twice by phosphate buffered saline (PBS). After that, cells were incubated with 1 ml of ultrapure nitric acid overnight until the cell pellets were completely digested. The solutions were finally diluted to 3 ml with ultrapure water and used to analyze Zn absorption. Zn concentrations in both control and supplemented media were determined by a flame atomic absorption spectrometer NovAA 400 (Analytik Jena AG, Germany). The calculation formula of uptake efficiency is as follows:

$$\text{Uptake efficiency (\%)} = \left( \frac{\text{Zn retained in cells}}{\text{Zn added to the cell medium}} \right) \times 100$$

**Effects of incubation time.** IEC-6 cells were cultured as described above. The medium in the wells was then replaced with zinc-free medium with 50 μM nano-ZnO, ZnO, and ZnSO<sub>4</sub>, all groups were incubated at 37°C for 1, 3, 6, 12, and 24 h, respectively. Zinc concentrations in the cell sample were then determined by flame atomic absorption spectroscopy.

**RNA isolation and real-time PCR analysis.** Total RNA was isolated from both treated and untreated IEC-6 cells with Trizol reagent (Invitrogen Inc., USA) as described by the manufacturer's protocol. Reverse transcription of RNA into cDNA was performed using random hexamers, oligo (dT) primer, and PrimeScript™ RT reagent Kit (TaKaRa, China), according to the manufacturer's instructions. Real-time PCR analysis of cDNA was performed using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA) and SYBR® Premix Ex Taq™ II (TaKaRa). Relative gene expression was achieved by the Δ-Ct method. One housekeeping gene (*β-actin*) was used for normalization purpose. The reactions of PCR amplification were heated to 95°C for 10 s and immediately cycled 40 times through the 5 s denaturing step at 95°C, 20 s annealing step, and 15 s elongation step at 72°C. All primers used are listed in Table 1.

**Reagents.** DMEM, FBS, penicillin/streptomycin, and glutamine were purchased from HyClone Laboratories, Inc., USA. Nano-ZnO with 10~15 nm

doi: 10.17221/15/2016-CJAS

Table 1. Primers used in the study

Gene	Primer sequences(5'-3')	Annealing temperature (°C)	Product size (bp)
<i>ZnT-1</i>	F: GAACATGCGAGGAGTGTTCCTG R: CGTCTTCAGTACAACCCCTTCCAG	64.5	110
<i>ZnT-4</i>	F: GCAGAGGAAGGTGAAGACCAG R: GCCACAAAGCAAGAAGAGTGAG	55.0	170
<i>ZnT-5</i>	F: GGAGGAGTGGTAGTGAGTGCTGT R: TGAAGTGATAGAGCGGTGTTCC	64.5	127
<i>ZnT-7</i>	F: GCCACCCAAGTTCAATCTGTTC R: GCCGTAGAGTAGTTCCACAAAAGC	61.4	133
<i>DMT-1</i>	F: GCCCTTCACCACCTACTTTGAT R: ATCCAGCCACTGCTCCAGACT	64.5	170
<i>MT</i>	F: GGCTGCAAGAACTGCAAATG R: CACTTGTCGAGGCACCTTT	61.4	113
<i>β-actin</i>	F: AGCTGTGCTATGTTGCCCTAGAC R: CTCATTGCCGATAGTGATGACC	61.4	116

particle diameter (purity > 99%) was bestowed by the College of Chemistry and Chemical Engineering, Xiamen University (China). Zinc oxide and zinc sulfate (purity > 99% both) were purchased from Sigma Aldrich (USA). Cell counting kit solution (CCK-8) was purchased from Beyotime Institute of Biotechnology (China). RNAiso Regent, Prime-Script™ RT Reagent Kit, SYBR® Premix Ex Taq™ II were purchased from TaKaRa (China).

**Statistical analysis.** All data were expressed as means ± SD. One-way analysis of variance (ANOVA) and Dunnett's post-hoc test were used to perform comparisons by the SAS software (Statistical Analysis System, Version 9.2, 2008). Group differences resulting in *P*-values of less than 0.05 were considered to be statistically significant.

## RESULTS

**Effects of TPEN and three zinc sources on IEC-6 cell viability.** Viability of cells cultured in the presence or absence of the three zinc sources is shown in Figure 3. The results showed that viability of cells which were exposed to TPEN decreased significantly (*P* < 0.05). Compared with untreated control group, viability of cells treated with 25, 50, 100 μM nano-ZnO or 50 and 100 μM ZnO increased significantly (*P* < 0.05), but had a decreasing tendency in 100 μM concentration. Furthermore, 25 and 50 μM ZnSO<sub>4</sub> for 24 h and

25 μM for 48 h did not significantly affect cell viability (*P* > 0.05), but it induced a dramatical decrease with 100 μM concentrations similarly (*P* < 0.05). At the level of 25 μM, cell viability with nano-ZnO was significantly higher than with ZnO and ZnSO<sub>4</sub> (*P* < 0.05). When nano-ZnO and ZnO were added with the concentrations of 50 and 100 μM, cell viability was significantly higher than if ZnSO<sub>4</sub> was added (*P* < 0.05).

**Effects of three zinc sources on uptake efficiency in IEC-6 cells.** The uptake efficiencies of the three zinc sources in IEC-6 were the next objective of the current study. Therefore, differences in the uptake efficiency of nano-ZnO, ZnO, and ZnSO<sub>4</sub>, which were used for the performance of dose- and time-response kinetics, were confirmed by atomic absorption spectroscopy. As shown in Figure 1, cells incubated with nano-ZnO showed the highest uptake efficiency with a following order of ZnSO<sub>4</sub> and ZnO at all levels (*P* < 0.05). As for the uptake efficiencies of different levels of nano-ZnO, the highest value was observed in cells treated with the concentration of 25 μM compared to 50 and 100 μM, and this variation was similar as in the ZnO treatment. The uptake efficiency of 50 μM ZnSO<sub>4</sub> concentration was greater than that of 25 μM and 100 μM by the end of 24 h (*P* < 0.05).

To further investigate the effects of incubation time on uptake efficiency, cells were treated with 50 μM nano-ZnO, ZnO, and ZnSO<sub>4</sub> for 1, 3, 6, 12, and 24 h, respectively. As seen in Figure 2,



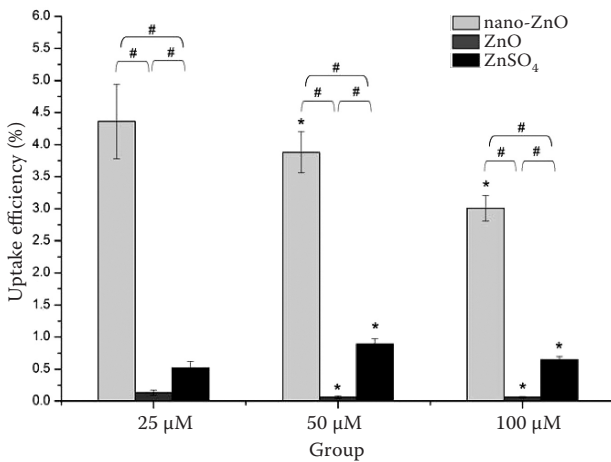


Figure 1. Uptake efficiency of nano-ZnO, feed-grade ZnO, and ZnSO<sub>4</sub> in IEC-6 cells  
 cells propagated on transwell filters were cultured with various concentrations of three zinc forms for 24 h; medium treated with 2 μM TPEN (Zn-deficient medium)  
 \*significant difference when compared with 25 μM Zn level ( $P < 0.05$ ); # significant difference when comparing between Zn sources ( $P < 0.05$ )  
 data are means  $\pm$  SD ( $n = 8$ )

the uptake efficiency of cells in nano-ZnO group was significantly higher than that of ZnSO<sub>4</sub> and ZnO from 1 h to 24 h. Our results indicated that nano-ZnO reached maximum uptake efficiency at 3 h ( $P < 0.05$ ), and then showed a significant and time-related decrease, suggesting the possible uptake saturation of nano-ZnO in the cells. In addition, there was no significant difference in uptake efficiency of ZnO from 1 h to 24 h ( $P > 0.05$ ). The cellular uptake of ZnSO<sub>4</sub> reached the highest level

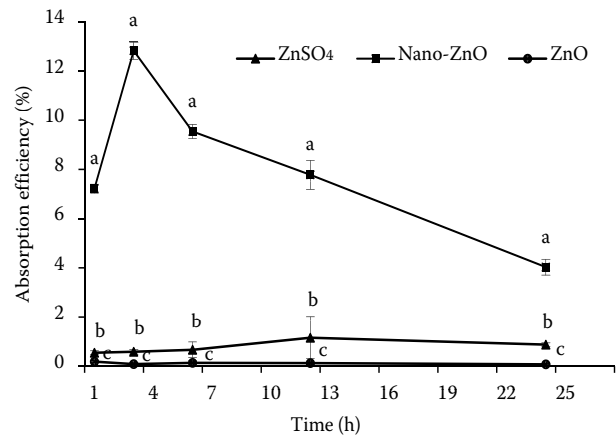


Figure 2. Absorption efficiency of nano-ZnO, feed-grade ZnO, and ZnSO<sub>4</sub> in IEC-6 cells  
 cells propagated on transwell filters were cultured with 50 μM Zn for different times as indicated; medium treated with 2 μM TPEN (Zn-deficient medium)  
 a-c significant difference when comparing between Zn sources at the same time point ( $P < 0.05$ )  
 data are means  $\pm$  SD ( $n = 8$ )

at 12 h ( $P < 0.05$ ) and decreased with increasing incubation time.

**Effects of three zinc sources on Zn transporters mRNA expressions in IEC-6 cells.** To test the effects of nano-ZnO, ZnO, and ZnSO<sub>4</sub> on the transcriptional level of different related zinc transporters, cells with the three Zn sources were incubated for 24 h and total RNAs were prepared. Reverse transcription PCR results showed that all of the genes were expressed in IEC-6 cells (Figures 4 and 5). Significant ( $P < 0.05$ ) decreases in

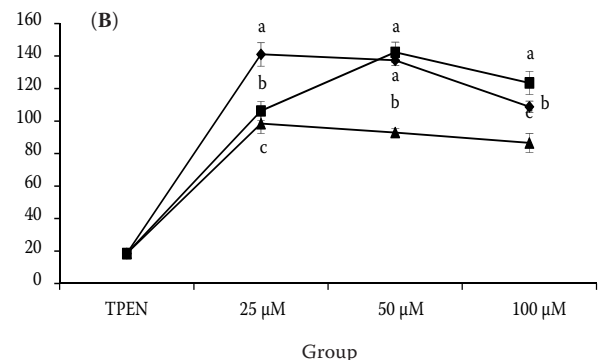
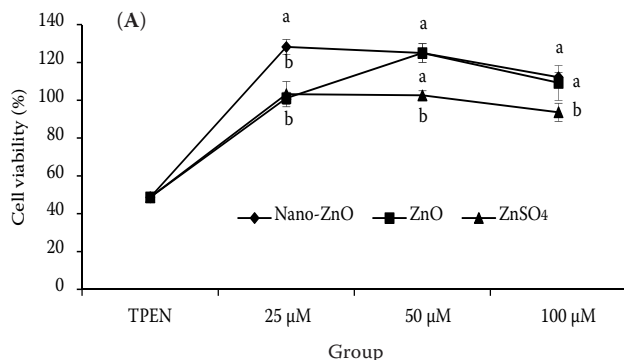


Figure 3. Effects of TPEN (Zn-deficient medium) or a combination with nano-ZnO, ZnO, and ZnSO<sub>4</sub> on cell viability (%)  
 cells propagated on transwell filters were cultured with various concentrations of three Zn forms for 24 h (A) or 48 h (B)  
 a-c different Zn sources are compared and means with different superscripts within the same Zn concentration differ significantly ( $P < 0.05$ )  
 data are means  $\pm$  SD,  $n = 8$

doi: 10.17221/15/2016-CJAS

the mRNA expressions of *ZnT-1* and *ZnT-4* were observed with supplemented TPEN, whereas the transcriptional level of *ZnT-7* was significantly increased ( $P < 0.05$ ). On the other hand, the administration of TPEN did not change the mRNA expression of *ZnT-5* ( $P > 0.05$ ).

Compared with control group, rapid and significant increases in the *ZnT-1*, *ZnT-5*, and *ZnT-7* gene expressions were observed after supplementing with different concentrations of nano-ZnO, ZnO, and ZnSO<sub>4</sub> ( $P < 0.05$ ). However, addition of different forms of zinc to the medium had no effects on the *ZnT-4* transcription level ( $P > 0.05$ ). There were no significant differences in the *ZnT-1*, *ZnT-4*, *ZnT-5*, and *ZnT-7* gene expressions between the three Zn sources at 25, 50, or 100 μM concentra-

tion, respectively ( $P > 0.05$ ). When different Zn sources of 50 μM and 100 μM were added, mRNA expression of *ZnT-1* in ZnO group was lower than that in nano-ZnO and ZnSO<sub>4</sub> groups ( $P > 0.05$ ). The mRNA expressions of *ZnT-5* with the addition of 25 μM and 100 μM nano-ZnO were higher than those with ZnSO<sub>4</sub> and ZnO, and showed a difference between ZnO and nano-ZnO at the concentration of 50 μM ( $P > 0.05$ ). In addition, the mRNA expression of *ZnT-7* with the addition of 50 μM nano-ZnO was higher than those with ZnSO<sub>4</sub> and ZnO group at the same concentration ( $P > 0.05$ ).

**Effects of three zinc sources on DMT-1 and MT mRNA expressions in IEC-6 cells.** We further investigated the mRNA expressions of *DMT-1* and

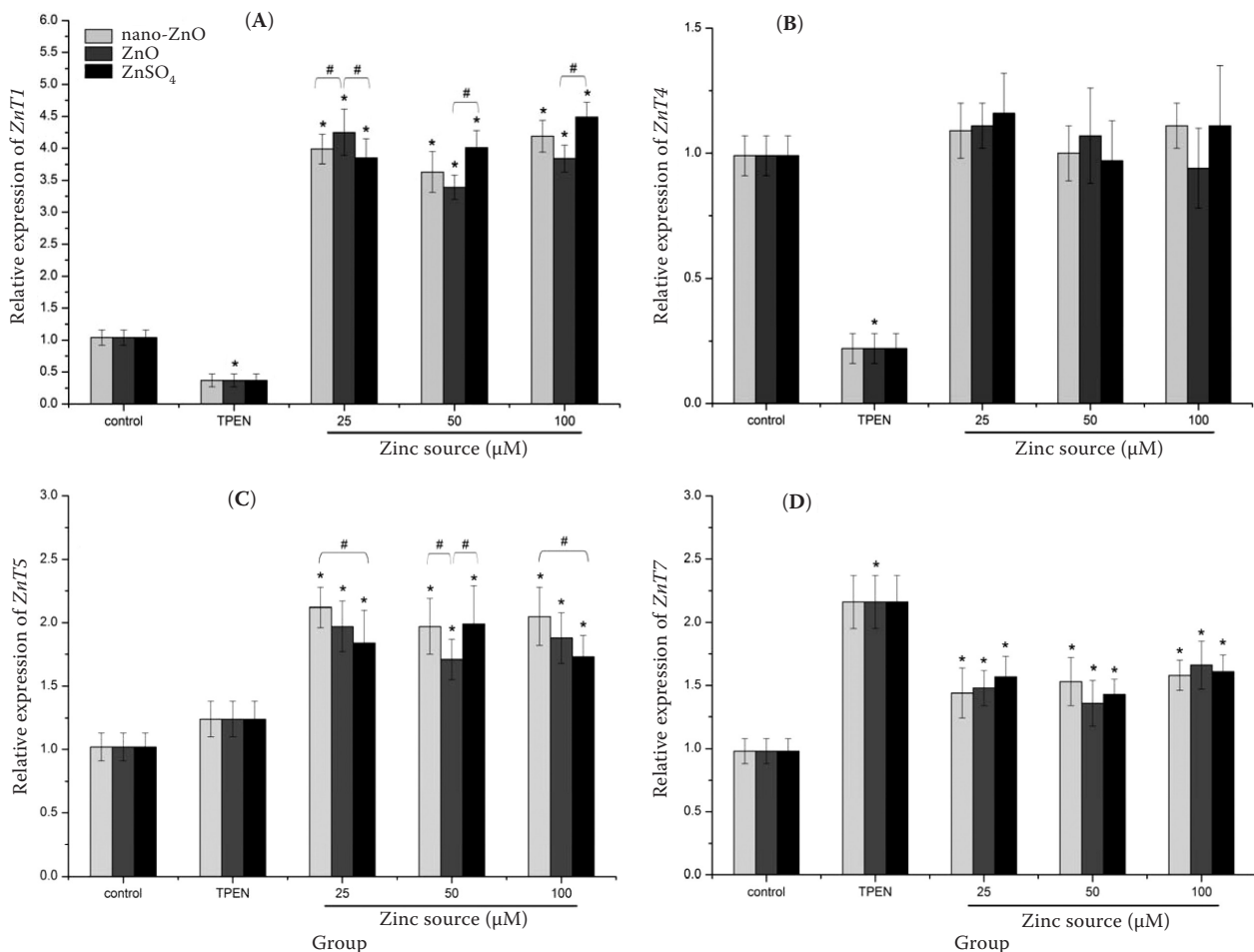


Figure 4. Effects of TPEN (Zn-deficient medium) or a combination with nano-ZnO, feed-grade ZnO, and ZnSO<sub>4</sub> on mRNA expression of *ZnT-1* (A), *ZnT-4* (B), *ZnT-5* (C), *ZnT-7* (D) in IEC-6 cells

cells propagated on transwell filters were incubated with or without (control) experimental agents for 24 h

\*significant difference when compared with control ( $P < 0.05$ )

#significant difference when comparing Zn sources within the same Zn concentration ( $P < 0.05$ )

data are means  $\pm$  SD ( $n = 8$ )

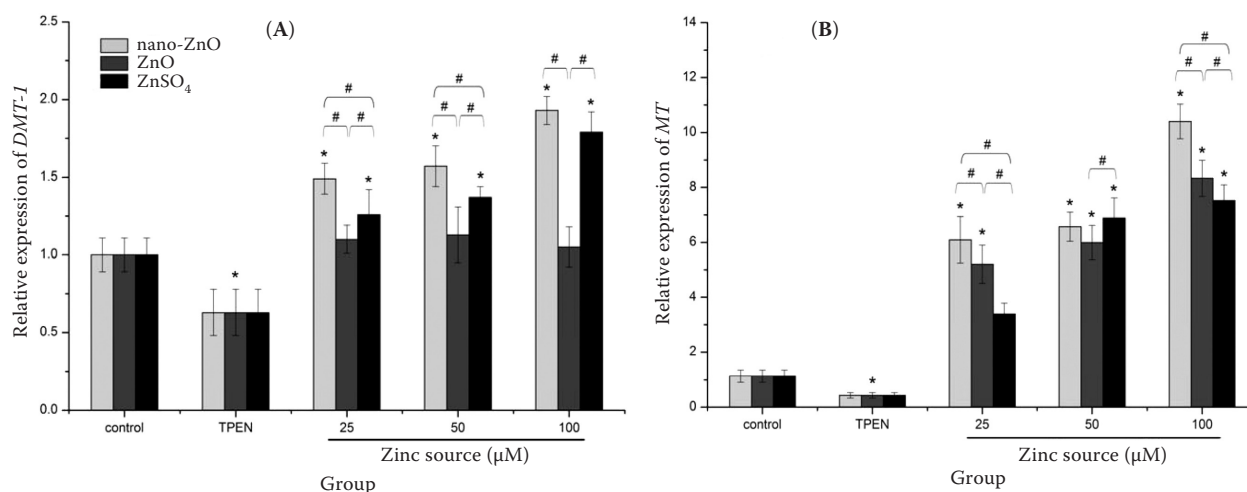


Figure 5. Effects of TPEN (Zn-deficient medium) and nano-ZnO, feed-grade ZnO, and ZnSO<sub>4</sub> on mRNA expression of divalent metal transporter (*DMT-1*) (A) and metallothionein (*MT*) (B) in IEC-6 cells

cells propagated on transwell filters were incubated with or without (control) experimental agents for 24 h

\*significant difference when compared with control ( $P < 0.05$ )

#significant difference when comparing Zn sources within the same concentration ( $P < 0.05$ )

data are means  $\pm$  SD ( $n = 8$ )

*MT* with the incubation of different zinc sources (Figure 5). A down-regulation of *DMT-1* and *MT* gene expressions with TPEN treatment ( $P < 0.05$ ) was registered. *DMT-1* mRNA expression was increased significantly by supplementing with nano-ZnO and ZnSO<sub>4</sub> ( $P < 0.05$ ), and no differences were observed between ZnO and control group ( $P > 0.05$ ). Moreover, when 25  $\mu$ M and 50  $\mu$ M nano-ZnO were added, the mRNA expressions of *DMT-1* were significantly higher than those of ZnO and ZnSO<sub>4</sub> groups ( $P < 0.05$ ). At the concentration of 100  $\mu$ M, the *DMT-1* mRNA expressions of nano-ZnO and ZnSO<sub>4</sub> were significantly higher ( $P < 0.05$ ) than that of ZnO. The *MT* mRNA expression was up-regulated by the three zinc sources at all concentrations ( $P < 0.05$ ). Furthermore, the results showed that the mRNA expressions of *MT* with 25  $\mu$ M and 100  $\mu$ M nano-ZnO treatments were higher than those of ZnO and ZnSO<sub>4</sub> ( $P < 0.05$ ).

## DISCUSSION

The IEC-6 cell derived from the rat intestinal epithelial cell is a good model system for studying nutrient uptake and transportation because it is difficult to investigate specific environmental parameters in a whole animal. It has been successfully and widely used in numerous experi-

mental studies for trace elements. In the current study, IEC-6 cell was used as a model to assess the effect of nano-ZnO, ZnO, ZnSO<sub>4</sub>, and TPEN on cell viability, zinc uptake efficiency, and zinc transporters gene expression. The cell viability inhibition by TPEN has already been suggested (Shen et al. 2008). Furthermore, a more recent study reported that TPEN addition noticeably lowered cell viability in HT-29 cells (Gurusamy et al. 2011). In the present study the effects of TPEN on cell viability were similar. Our results showed that viability of cells exposed to TPEN significantly decreased, and indicated that TPEN, a kind of special zinc-chelating reagent, chelated intercellular or intracellular zinc. TPEN thereby impacts the activities of key enzymes that need zinc as a co-factor.

However, no precise figure has been available up to date about the effects of nano-ZnO and ZnO on cell viability and comparison of cell viability between different kinds of inorganic zinc sources in IEC-6 cells. In the present study, viability of cells exposed to nano-ZnO increased significantly and was higher than of those exposed to ZnO at lower concentrations. Furthermore, medium treatment of ZnSO<sub>4</sub> had no significant protective effect on cell viability, and the three zinc sources at higher concentrations could even decrease it. The increase of intracellular free Zn<sup>2+</sup> which is

doi: 10.17221/15/2016-CJAS

deleterious could eventually lead to the decrease of cell viability and apoptosis (Sakabe et al. 1998), particularly in  $\text{ZnSO}_4$  treatment. While  $\text{ZnSO}_4$  is totally water-soluble and  $\text{Zn}^{2+}$  is released in large quantity, nano-ZnO and ZnO dissolution in water is very limited. Thus dissolved  $\text{Zn}^{2+}$  may gradually release from cell to meet the need of tissue, even though the dissolution of nano-ZnO would be changed under acidic conditions as well as at the presence of biological components such as amino acids and peptides (Moreau et al. 2007; Xia et al. 2008). The latter study also showed that cytotoxicity of  $\text{ZnSO}_4$  was still significantly higher than of nano-ZnO (Xia et al. 2008). The above results also demonstrated that in the IEC-6 cells, nano-ZnO at lower concentration was more bioavailable.

In our experiments, the uptake efficiency of nano-ZnO we observed was significantly higher than that of ZnO and  $\text{ZnSO}_4$  groups at all concentrations. As mentioned above, the uptake mechanism of three zinc sources differs, and the study confirmed that of nano-ZnO was more effective. Xia et al. (2008) investigated the uptake of ZnO nanoparticle in macrophages (RAW 264.7) and human bronchial epithelial cells (BEAS-2B) using inductively coupled plasma-mass spectrometry (ICP-MS) and fluorescent-labelled labelling ZnO, and it was found out that dissolution of ZnO could happen in the culture medium and endosomes.

In addition, we found that the cellular uptake of nano-ZnO reached a maximum level 3 h post-incubation and decreased with increasing incubation time. This is in line with Yu and Baek (2011) who reported that after human lung epithelial cells had been exposed to the concentration of 125  $\mu\text{g}/\text{ml}$  nano-ZnO for a given time, the uptake efficiency peaked at 4 h of nano-ZnO administration and then it decreased (Yu and Baek 2011). In contrast, the uptake efficiency of  $\text{ZnSO}_4$  seemed to peak later. Moreover, no significant differences were observed in the uptake efficiency of ZnO. The explanation of the time response of nano-ZnO may have many aspects. Intrinsically, when the concentration of nano-ZnO is below solubility, cells are mainly exposed to zinc ions, whereas when the concentration exceeds, cells are confronted with molecular nanoparticles (Cousins et al. 2006). Zinc ions of nano-ZnO were released gradually instead of rapidly. Thus, it was the reason for the higher uptake efficiency of nano-ZnO during the whole incubation time. Secondly, a self-protection

mechanism of cells could be provoked to prevent intracellular zinc excess while being exposed to high Zn levels for a long time. Furthermore, the physicochemical parameters (e.g. size, shape, surface area, electrostatic charge, materials composition, hydrophobicity) might be important factors affecting the uptake efficiency, which remains to be tested.

From a physiological perspective, the cell state is optimal with an appropriate intracellular free  $\text{Zn}^{2+}$  level. However, it can be regulated via different transport proteins involved in zinc uptake, storage, and excretion that maintain the zinc homeostasis. In the current study, we examined the effects of different zinc sources on the transcriptional response of *ZnT-1*, *ZnT-4*, *ZnT-5*, *ZnT-7*, *DMT-1*, and *MT* by real-time PCR. The results show that the zinc-deficient status (TPEN addition) leads to significant down-regulation of the mRNA expressions of *ZnT-1* and *ZnT-4*, and the absent accounts of *ZnT-1* lead to an increased sensitivity of cells to zinc toxicity (Palmiter 2004).

All zinc sources treatments exhibited huge up-regulations of *ZnT-1*, *ZnT-5*, and *ZnT-7*. Conversely, *ZnT-4* gene expression did not change in any zinc treatment. This is consistent with an *in vitro* study which found that *ZnT-1* expression is markedly regulated by the dietary zinc supply (Liuzzi et al. 2001), and moreover showed that variation in zinc status does not affect *ZnT-4* mRNA levels in small intestine of rat. *ZnT-1* plays an important role in zinc efflux of cells. Thus, cells could be up-regulated at high intracellular zinc concentrations and down-regulated at low level to maintain zinc homeostasis in IEC-6 cells. Our finding also showed that there were no significant variations of mRNA expressions of *ZnT-1*, *ZnT-4*, *ZnT-5*, and *ZnT-7* between the three zinc treatments. *ZnT-1* was sensitive to zinc ions, and even though nano-ZnO can only release small amount of free  $\text{Zn}^{2+}$ , the gradually released zinc could also keep boosting the expression within the 24-hour incubation. Moreover, the regulation of *ZnT-5* and *ZnT-7* mRNA expressions via zinc is important for zinc transport from cytoplasm into the Golgi apparatus, and *ZnT-5* expression in human ileal mucosa can be inhibited by high zinc supplementary doses (Cragg et al. 2005). *ZnT-5* was reported to focus on zinc absorption, while *ZnT-7* on storage regulation. But there is no report about the effects of nano-ZnO and ZnO. In the present study, we observed huge



increases in *ZnT-7* and *ZnT-5* genes expression in IEC-6 cells upon different zinc sources or TPEN treatment and reduction at high zinc concentration. Our results also suggested that *ZnT-5* and *ZnT-7* played different roles both at absorption and storage stages.

*DMT-1* is predominantly an iron transporter such as zinc. The present study found a reduction of *DMT-1* mRNA expression after addition of TPEN, but its increase with the nano-ZnO and ZnSO<sub>4</sub> supplementation. This result is in agreement with Shen et al. (2008), who reported that mRNA expression of *DMT-1* is susceptible to low zinc concentration and resistant to TPEN treatment (Stark 2011). But the transporting mechanism of zinc regulated by *DMT-1* has not been investigated yet, thus more detailed exploration is needed. Davis et al. (1998) reported that intestinal zinc absorption was more intense in *MT* knockout mice than wild, and this result was also further supported by that *MT* was a vital component for zinc homeostasis regulation. Our results showed that compared with *DMT-1* mRNA expression, *MT* was more sensitive to zinc concentration. This was similar to a previous study, which reported that *MT* expression was down-regulated during zinc depletion (Cao et al. 2001). These findings are in line with the fact that *MT* expression was up-regulated at high zinc concentrations to boost zinc efflux, and down-regulated at low concentrations to enhance zinc shortage.

## CONCLUSION

In conclusion, the current study showed that the effect of zinc in IEC-6 cells varied with different chemical and physical forms (nano-ZnO, ZnO, and ZnSO<sub>4</sub>) and the differences concerned cell activity, uptake, and transport. Compared with the control, supplementation with the three zinc sources enhanced cell activity and uptake efficiency. Lower concentration of nano-ZnO was more bioavailable for IEC-6 cells viability. The study compared the effects of the three zinc sources on cell viability and confirmed a biphasic effect of zinc with its different chemical forms. This study also presented that supplementations with the three Zn forms could significantly increase *ZnT-1*, *ZnT-5*, *MT*, and *DMT-1*, and that *ZnT-5*, *MT*, and *DMT-1* expressions in nano-ZnO treatment were greater than those in

ZnO and ZnSO<sub>4</sub> group. In addition, the uptake and transport of nano-ZnO across the IEC-6 cell monolayers partly via zinc related transporters were induced by a released zinc ion.

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doi: 10.17221/15/2016-CJAS

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Received: 2016–01–27

Accepted after corrections: 2016–08–02