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Zinc supplementation alleviates OTA-induced oxidative stress and apoptosis in MDCK cells by up-regulating metallothioneins

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ABSTRACT

Aims: The present study was to investigate the protective effects of Zn supplementation in OTA-induced apoptosis of Madin-Darby canine kidney (MDCK) epithelial cells and explore the potential mechanisms. Aiming to provides a new insight into the treatment strategy of OTA-induced nephrotoxicity by nutritional regulation.
Main methods: Initially, through MTT and LDH assay revealed that Zn supplementation significantly suppressed OTA-induced cytotoxicity in MDCK cells. Then, the production of reactive oxygen species (ROS) was detected by using a DCFH-DA assay. Annexin V-FITC/PI, Hoechst 33258 staining and Flow cytometry were used to detect the apoptosis. The expressions of apoptosis-related molecules were determined by RT-PCR, Western blotting. Interestingly, OTA treatment slightly increased the levels of Metallothionein-1 (MT-1) and Metallothionein-2 (MT-2) by using RT-PCR, Western blotting assay; while Zn supplementation further improved the increase of MT-1 and MT-2 induced by OTA. However, the inhibitive effects of Zn supplementation were significantly blocked after double knockdown of MT-1 and MT-2 by using Small Interfering RNA (siRNA) Transfection method.
Key findings: Our study provides supportive data for the potential roles of Zn in reducing OTA-induced oxidative stress and apoptosis in MDCK cells.
Significance: Zn is one of the key structural components of many proteins, which plays an important role in several physiological processes such as cell survival and apoptosis. This metal is expected to contribute to the conservative and adjuvant treatment of kidney disease and should therefore be investigated further.

1. Introduction

Ochratoxin A (OTA) is one of the most prevalent and deleterious mycotoxins, which is widespread in a variety of moldy food commodities, such as grains, beans, and meat products [1–3]. Owing to its wide distribution, humans and animals are at a high risk of OTA-induced detrimental effects after the ingestion of contaminated foodstuff and feed. It has been reported that OTA has nephrotoxic, carcinogenic, immunotoxic and possibly neurotoxic, kidney has been considered as one of the main targets of OTA poisoning [4–7]. Researches have shown that the higher presence of OTA in kidney could be correlated with the progression of chronic kidney disease [8–10]. In modern society, the companion animals are often regarded as the family members and have developed strong relationship with the owners throughout their lifetime [11]. Although some surveys have revealed the incidence of OTA in commercial dog food *in vivo*, there is less report on the effect of OTA-induced nephrotoxic to canine, and the protective measure involved still remain uncleared.

Zn, as the divalent Zn^{2+} ion, generally is a component or co-factor for some vital enzymes that influence cell survival and proliferation [12]. For an instance, Zn is an essential component of Cu/Zn superoxide dismutase (SOD1) and also regulates the expression of many genes that are involved in antioxidant processes, such as metallothioneins (MTs) and glutathione peroxidase (GSH-Px) [13]. The physiological and cellular Zn^{2+} concentrations are largely regulated by MTs, Zn^{2+} importers (ZIPs proteins) and Zn^{2+} transporters (ZnTs), which play an important role in the absorption, excretion, transportation and intracellular storage of Zn [14–16].

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Table 1
Primers used for real-time quantitative PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	CACCCTGAAGTACCCCATTGAG	TTGTAGAAGGTGTGGTGCCAGAT
Caspase-3	TTCATTATTCAGGCCTGCCGAGG	TTCTGACAGGCCATGTCATCCTCA
Bcl-2	CATGCCAAGAGGGAAACACCAGAA	GTGCTTTGCATTCTTGGATGAGGG
Bax	TTCCGAGTGGCAGCTGAGATGTTT	TGCTGGCAAAGTAGAAGAGGGCAA
MT-1	AGCTGCTGTGCCTGATGTG	TATACAAACGGGAATGTAGAAAAC
MT-2	ATGGATCCCAACTGCTCCT	TGCATCTGCACTCTTTGCA

As reported, MTs are a group of small cysteine-rich proteins, which were originally discovered as a cadmium carrier in horse kidney [17]. As regulated by zinc supplementation, growing number of research have reported the protective effects of MTs against oxidative damage due to its strong free scavenging capacity [18–21]. Zn supplementation has been reported to regulate MTs expression, to alleviate OTA-induced oxidative stress and DNA damage in HepG2 cells [22]. However, questions that whether Zn supplementation could protect the MDCK cells against OTA exposure remain unanswered.

Therefore, the present study aimed to explore the effects of Zn supplementation on OTA-induced nephrotoxicity and to elucidate the potential mechanisms in MDCK cells. This study can also provide a new insight into the treatment strategy of OTA-induced nephrotoxicity by nutritional regulation.

2. Materials and methods

2.1. Cell culture and agents

The Madin-Darby canine kidney cells (MDCK cells line) were provided by the China Institute of Veterinary Drug Control. Cells were incubated in the medium of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Paisley, Scotland, U.K), added 10% heat-deactivated fetal bovine serum (FBS, Lonsa, Richmond, VA, United States), and 1% antibiotic (10,000 IU/ml penicillin and 10,000 μg/ml streptomycin) respectively. Cells were cultured in the humidified atmosphere consist of 5% CO_2 and incubated at 37 °C (Thermal Science, Waltham, MA, United States). The cells were calculated by hemocytometer as well as detached by Trypsinization. OTA, ZnSO4 and, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, United States).

2.2. Cell viability assay

To measure the cell viability of OTA and $ZnSO_4$, the MDCK cells were incubated in 96-well plates (1.2×10^3 cells/well). Briefly, after pretreated with different concentrations of $ZnSO_4$ for 24 h. Subsequently, cells were exposed to the combination of OTA with $ZnSO_4$ for another 24 h. The cell viability was determined by MTT assay according to the manufacturer's instructions (Sigma, St. Louis, MO, United States) as well as absorbance was read by a spectrophotometer at 570 nm (Bio-Rad, Hercules, CA, United States). Experiments were presented as four independent experiments.

2.3. Lactate dehydrogenase activity

To detecting the release of lactate dehydrogenase (LDH), cells were incubated in 96-well plates (1.2×10^3 cells/well), pretreatment with different concentrations of $ZnSO_4$ for 24 h, then cells were exposed to OTA with $ZnSO_4$ for another 24 h. After treatment, the cell culture medium was gathered into 1.5 ml test tubes and centrifuged at 12000 rpm for 15 min at 4 °C. LDH activity was detected by using LDH kits according to the manufacturer's instructions (Jiancheng, Nanjing, Jiangsu, China).

2.4. Analysis of apoptosis by Hoechst 33258 staining and flow cytometry

The MDCK cells were cultured on 20 mm diameter round coverslips (WHB, China) at the density of 5×10^4 cells/well in 12-well plates with corresponding treatments and the intracellular ROS were measured with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma, St. Louis, MO, United States). After the culture medium was removed, the cells were washed three times with serum-free DMEM, and then cells were incubated with DCFH-DA for 20 min at 37 °C in dark. Finally, cells were washed three times with phosphate buffer saline (PBS). Samples were photographed using a Zeiss LSM 700 META confocal system. The levels of total intracellular ROS, paralleled by an increase in fluorescence intensity, was calculated as the percentage of control group cells. After being washed with cold-PBS, MDCK cells were incubated with FITC-annexin V and PI following the manufacturer's instructions (FASC Aria, BD Bioscience, San Jose, CA). The percent of apoptosis were analyzed using FACSCalibur flow cytometry (BD Biosciences).

2.5. SYBR green real-time PCR analysis

The cells were cultured at a density of 5×10^4 cells/well in 12-well plates with corresponding treatments. Total RNA was extracted using the RNAiso Plus kit following the instruction (TaKaRa, Dalian, Liaoning, China). Then, the integrity of the purified RNA was evaluated by the ratio of OD_{260}/OD_{280}. Reverse transcription was performed according to the instruction (TaKaRa, Dalian, Liaoning, China). The RT-PCR reaction was performed using a SYBR Green PCR kit (TaKaRa, Dalian, Liaoning, China) with primers (Table 1) on a 7700 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA) for 40 cycles. The relative mRNA levels were determined by the Cycler threshold (ΔCt) method, with β-actin serving as the housekeeping gene.

2.6. Western blot analysis

The cells were collected with 100 μl cold-RIPA buffer (Beyotime) to obtain the total cell lysates. Then the cell lysates were centrifuged at 12000 rpm for 10 min at 4 °C. Protein concentration was determined by using the Bicinchoninic acid (BCA) kit (Beyotime, China). The samples were added with 5× loading buffer and heated at 95 °C for 5 min. The samples were separated on 12.5% sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Molsheim, France), and incubated with primary antibodies: rabbit anti-*cleaved caspase-3*, rabbit anti-*bax*, rabbit anti-*bcl-2* and rabbit anti-β-actin from CST (Danvers, MA, United States, diluted 1:1000), as well as rabbit monoclonal anti-MT antibody from Abclonal (Nanjing, Jiangsu, China, diluted 1:1000) for 12–16 h at 4 °C, using Tris-buffered saline (TBS) containing 5% nonfat milk powder and 0.1% Tween 20 and washed for three times, each time 10 mins. The membranes were incubated with HRP-labeled goat anti-Rabbit IgG (Beyotime, China, diluted 1:10000) for 1–2 h at room temperature. Finally, immunoreactivity was detected by ECL chemoluminescence kit (Bio-Rad, Berkeley, USA). Membranes were visualized and analyzed by Luminescent Image Analyzer (Fujifilm 171 LAS-4000), and the protein bands were analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Sarasota, USA), and normalized to its respective loading

control (β-actin).

2.7. Small Interfering RNA (siRNA) transfection

The siRNA-MT (5′-GAUGCACCUCCUGCAAGAAtt-3′) were designed and synthesized by Invitrogen. The duplexes were transiently transfected into MDCK cells using X-tremeGENE transfection reagent (Roche, Basel, Switzerland) according to the manufacturer protocols. Cells were cultured in DMEM with 8% FBS without antibiotics overnight at 37 °C. Then, siRNAs and X-tremeGENE transfection reagent (1:4) were added to each well and incubated for 5 h after the confluence of cells reached 30–50%. After that, cells were incubated in DMEM with 4% FBS for OTA or $ZnSO_4$ treatments.

2.8. Statistical analysis

The data was analyzed statistically by GraphPad Prism and presented as the mean ± SEM of the indicated number of replicates. Statistical comparison was made using one-way analysis of variance (ANOVA). Results were considered significant at $P < 0.05$.

3. Results

3.1. Cytotoxic effects of various concentration of OTA and Zinc on MDCK cells

The MDCK cells were treated with various concentrations of OTA (0–5.0 μg/ml) for 24 h and/or $ZnSO_4$ (0–400 μM) for 48 h, and then the cell viability and lactate dehydrogenase (LDH) activity were measured. As shown in Figs. 1, 0.3–5.0 μg/ml OTA induced a significant ($P < 0.01$) decrease in the cell viability. Furthermore, 0.6–5.0 μg/ml OTA induced a significant ($P < 0.01$) increase in the LDH activity compared to the control group (Fig. 1B). As shown in Fig. 1C and D, the cell viability and LDH activity were not significantly affected by $ZnSO_4$ alone treatment at the concentration of 25, 50 and 100 μM, while 150 μM or higher $ZnSO_4$ significantly ($P < 0.01$) decreased cell viability and increased LDH activity compared to the control group. Thus, 1.0 μg/ml OTA was chosen to induce cytotoxicity and $ZnSO_4$ at the safe concentrations of 25, 50 and 100 μM were chosen to identify its protective effects in the subsequent experiments.

3.2. Zinc supplementation protects MDCK cells against OTA-induced cytotoxicity

As shown in Fig. 2, OTA treatment significantly ($P < 0.05$) reduced cell viability and elevated LDH release compared to the control group. To determine the protective effects of Zn supplementation on OTA-induced cytotoxicity, MDCK cells were cultured with appropriate concentration of ZnSO4 for 24 h, and then treated with OTA for another 24 h. As shown, Zn supplementation at the concentration of 50 and 100 μM significantly ($P < 0.05$) suppressed OTA-induced decrease in cell viability and OTA-induced increase in LDH activity. However, Zn supplementation at a relatively low concentration of 25 μM did not reverse OTA-induced cytotoxicity. These results showed that Zn supplementation in a certain concentration ranges could prevent OTA-induced cytotoxicity in MDCK cells.

3.3. Zinc supplementation alleviates OTA-induced ROS accumulation and apoptosis in MDCK cells

To further explore the protective effects of Zn supplementation, intracellular reactive oxygen species probe H_2DCF-DA was applied to evaluate the production of ROS in MDCK cells. As shown in Fig. 3A, Zn supplementation significantly ($P < 0.05$) decreased the accumulation of ROS and OTA treatment significantly ($P < 0.01$) increased the production of ROS compared to the control group. Moreover, the production of ROS induced by OTA was significantly suppressed by Zn supplementation at the concentration of 50 μM ($P < 0.05$) and 100 μM

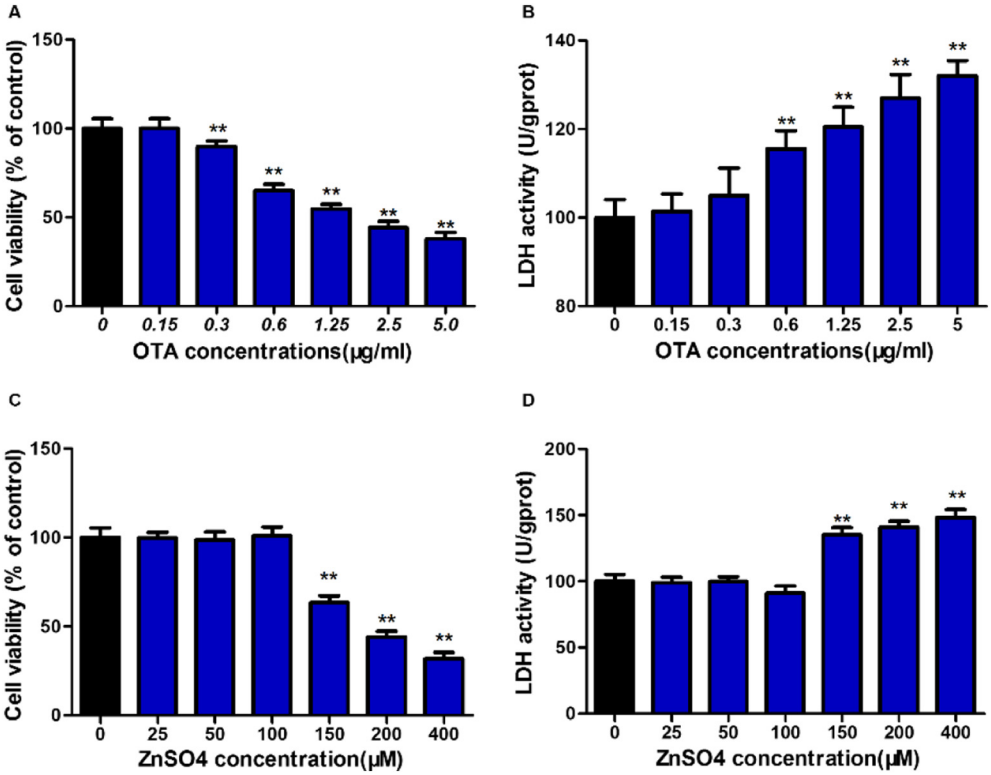

Fig. 1. Effects of various concentration of OTA and Zinc on MDCK cells. The cell viability (**A**, **C**) and LDH activity (**B**, **D**) were measured. All results are expressed as means ± SEM of four independent experiments. Significance compared with the control group $^{**}P < 0.01$.

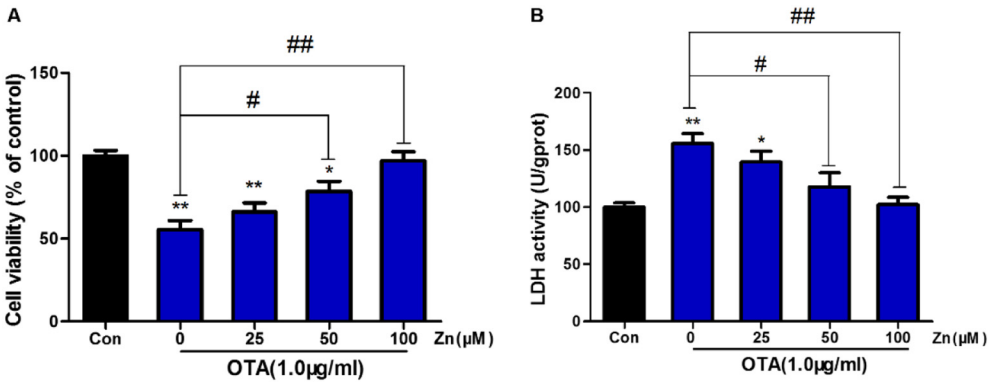

Fig. 2. Effects of Zinc supplementation on OTA-induced cytotoxicity. MDCK cells were cultured with various concentrations of $ZnSO_4$ for 24 h, then treated with 1.0 μg/ml OTA in the presence of various concentrations of $ZnSO_4$ for 24 h. Cell viability (**A**) and LDH activity (**B**) were determined. All results were expressed as means ± SEM of four independent experiments. There was a significant difference compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$. In OTA-treated groups, there was a significant difference compared with the group treated by OTA alone, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

($P < 0.01$), but Zn supplementation at a concentration of 25 μM did not significantly reduce the increase of ROS caused by OTA. These results revealed that Zn supplementation has alleviated OTA-induced ROS accumulation in MDCK cells.

To investigate the protective effect of Zn on OTA-induced apoptosis, the mRNA levels of *Bcl-2*, *Bax*, and *Caspase-3* were measured by RT-PCR and Western blotting, the apoptosis of MDCK cells were detected by Hoechst 33258 staining and flow cytometry. As shown in Fig. 3B–E, OTA treatment significantly increased the mRNA levels of *Bax, Caspase-3* and the protein expression of *Bax*, cleaved *Caspase-3*, decreased the mRNA and protein levels of *Bcl-2* ($P < 0.01$). As indicated in Fig. 3F, typical apoptotic morphological changes including nuclear chromatin condensation and fragmentation were observed. In addition, the flow cytometry analysis exhibited that OTA induced apoptosis of MDCK cells (Fig. 3G). Furthermore, Zn supplementation at the concentration of 50 or 100 μM significantly suppressed OTA-induced increase in the relative mRNA levels and the protein expression of *Bax*, *Caspase-3*, blocked OTA-induced decrease in the mRNA levels and the protein expression of *Bcl-2*. These results suggested that Zn supplementation has a significant protective effect on OTA-induced apoptosis of MDCK cells at appropriate concentrations.

3.4. Zinc supplementation up-regulates MTs levels in MDCK cells

To explore the possible mechanism involved in the protective effects of Zn supplementation in OTA-induced damage on MDCK cells, the mRNA and protein of MT-1 and MT-2 levels were measured. Interestingly, the mRNA levels of MT-1 and MT-2 significantly increased after MDCK cells were treated by OTA alone compared to the control group ($P < 0.05$). Moreover, Zn supplementation further improved OTA-induced increase in the mRNA levels of MT-1 and MT-2 (Fig. 4A and B). MTs proteins levels were examined by Western blot analysis. As shown in Fig. 4C, MTs proteins showed similar change patterns with the mRNA levels of MT-1 and MT-2 after OTA and/or Zn treatments. Even though there was no significant difference in MTs proteins between cells treated by OTA alone and cells supplemented with 25 μM Zn in the presence of OTA treatment, MTs protein significantly increased in the groups supplemented with 50 and 100 μM Zn, compared to the group treated by OTA alone ($P < 0.01$). These results indicate that OTA treatment can lead to an increase of MTs and Zn supplementation can further enhance MTs levels in MDCK cells.

3.5. MTs Knockdown abrogates the protective effect of zinc supplementation on OTA-induced ROS accumulation and apoptosis in MDCK cells

The knockdown of MTs genes led to a considerable decrease in MTs mRNA and proteins levels. There was a 70% and 72% decrease in MT-1 and MT-2 mRNA levels, respectively, and also a 65% decrease in MTs proteins levels (Fig. 5A, B). Zn supplementation significantly increased the mRNA and protein levels of MTs in cells treated with the control siRNA but not in groups treated with the MTs-specific siRNA (Fig. 5C, D) ($P < 0.01$). As shown in Fig. 6A, the inhibitive effect of Zn supplementation on OTA-induced ROS accumulation was significantly blocked when knockdown of MTs with the MTs-specific siRNA. Furthermore, the protective effects of Zn supplementation on OTA-induced apoptosis were also blocked when knockdown of MTs. As shown in Fig. 6B,D, knockdown of MTs significantly increased the mRNA and protein levels of *Bax* and decreased the mRNA and protein levels of *Bcl-2* compared with Zn and OTA combined groups ($P < 0.05$). Similarly, knockdown of MTs significantly increased the mRNA and protein levels of *Caspase-3* compared with Zn and OTA combined groups (Fig. 6C, E) ($P < 0.01$ *and* $P < 0.05$, *respectively*). As shown in Fig. 6F, G, Zn supplementation could not alleviate OTA-induced changes of chromatin and apoptotic ratio after MDCK cells were transfected with MTs-specific siRNA. These results indicated that MTs up-regulation plays an important role in the protective effect of Zn supplementation on OTA-induced ROS accumulation and apoptosis in MDCK cells.

4. Discussion

Ochratoxin A, one of the most deleterious mycotoxin, has led to enormous economic loss due to its widespread contamination all over the world [23]. Although toxic effects induced by OTA on pig kidney epithelial cells have been largely characterized, there remains a lack of information underlying OTA-induced cytotoxicity in MDCK cells [24]. In this study, we investigated various concentration of OTA-induced cytotoxic by MTT and LDH assay. MTT method is used for detecting cell survival and growth, which revealed that OTA could induce cytotoxicity when exposed at the lowest concentration tested (0.3 μg/ml). LDH release is a marker of plasma membrane integrity and an indicator of cell death, our results revealed that OTA could induce cytotoxicity when exposed at the lowest concentration tested (0.6 μg/ml). Because of MTT assay is generally more sensitive than LDH release assay, the significant toxicity concentrations of MTT assay was different with the significant toxicity concentrations of LDH release assay, which were consistent with some previously reported data [25]. In addition, pre-experiments showed that 1.0 μg/ml concentration was suitable in OTA-induced cytotoxic.

Previous study have demonstrated that OTA as a potent apoptotic inducer that exerts it's effects through ROS generation and activation of mitochondrion-dependent apoptosis pathways, which trigger cytochrome *c* release from the mitochondria to cytosol and activating caspases *in vitro* [26–29]. Additionally, ROS-mediated oxidative stress may be related to OTA-induced cytotoxicity and apoptosis [30,31]. In the present study, we revealed that OTA could induce oxidative stress and apoptosis by measuring ROS generation and some other parameters of apoptosis. Our present results may partly interpret the correlation between the higher presence of OTA in nephropathic canine and the progression of chronic kidney disease.

Zn, a redox inert metal, is widely considered a potential antioxidant

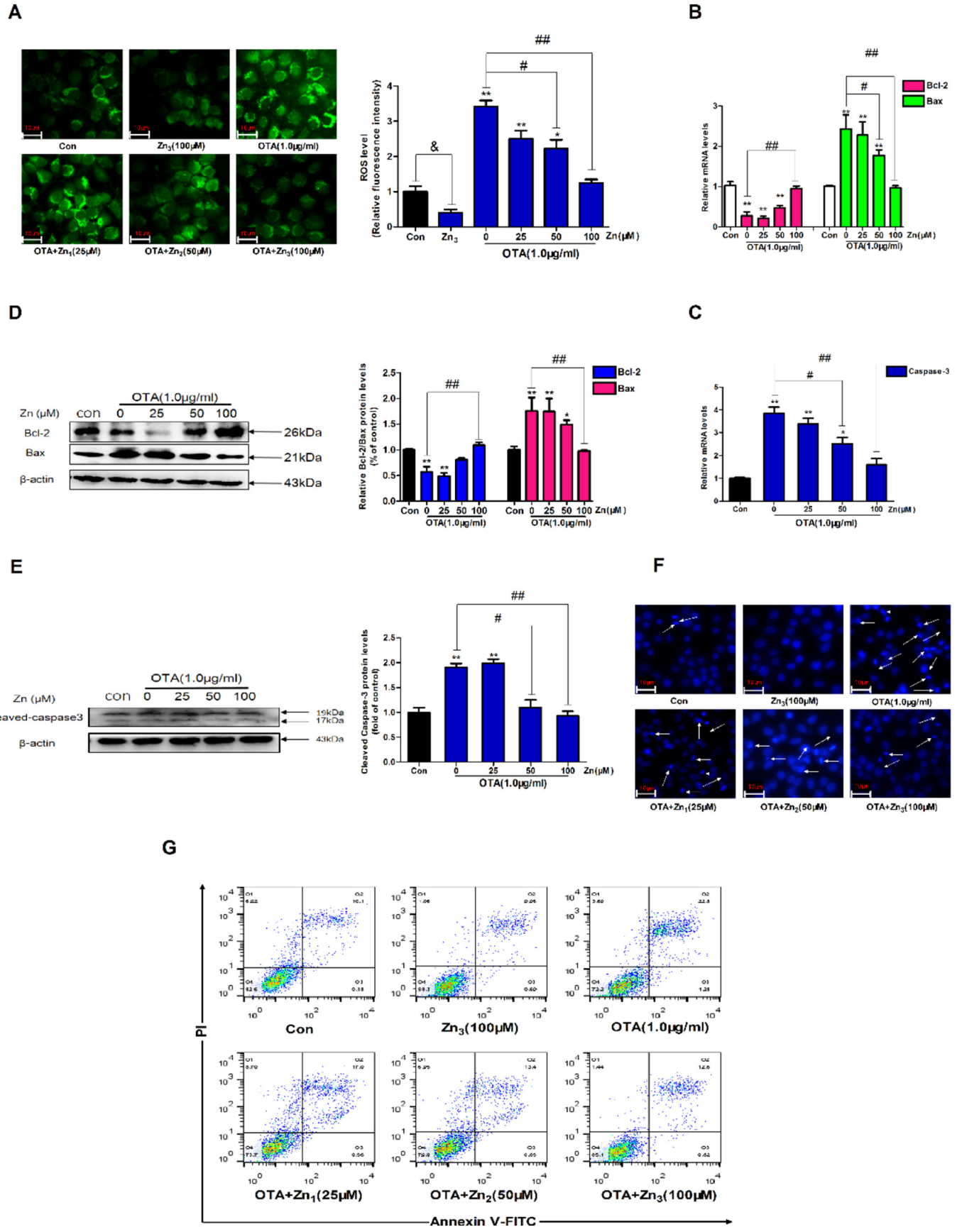

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Fig. 3. Effects of Zinc on OTA-induced ROS accumulation and apoptosis in MDCK cells. The determination of ROS levels (**A**) (Scale bar: 10 μm). The mRNA levels of *Bcl-2, Bax* (**B**) and *Caspase-3* (**C**) were detected by real-time PCR. The expression of *Bcl-2, Bax* (**D**) and cleaved *Caspase-3* (**E**) was measured by Western blotting. The nucleus was detected by Hoechst 33258 staining, Arrows denote condensed and broken nucleus (**F**) (Scale strip: 10 μm). The representative plots of flow cytometry apoptosis (**G**). The average fluorescence intensity was analyzed by Image-Pro Plus software and expressed as the percentage. All results were presented as means ± SEM of three independent experiments. Significance compared with the control group, $^{\&}P < 0.05$. In all OTA treated cells, compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, indicating a significance. In cells exposed to both OTA and $ZnSO_4$, compared with the group added with OTA, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, indicating a significant difference.

in vitro [32]. Previous studies have proved that Zn deficiency can cause cell damage and some chronic diseases [33–35]. On the contrary, Zn supplementation has been found to attenuate ethanol and acetaldehyde-induced activation of liver stellate cells [36]. However, there are few studies that have inveterated the relationship between zinc and mycotoxins. In the present study, we provided direct evidence that Zn supplementation was effective in protecting MDCK cells against OTA-induced cytotoxicity as demonstrated by the increase in the cell viability and decrease in the release of LDH.

Zn supplementation may protect cells from oxidative stress by reducing mitochondrial damage and preventing lysosome rupture [37]. As expected, we found that 1.0 μg/ml OTA caused a significant increase in ROS levels, as indicated by an increase in H_2DCF-DA fluorescence compared with that in control group, and Zn supplementation could alleviate OTA-induced increase in ROS production in a dose-dependent manner. A study reported that Zn supplementation can mitigate Cd-induced apoptosis *via* caspase-dependent pathways [38]. As we observed, Zn supplementation significantly alleviated the OTA-induced apoptosis by measuring *Bcl-2, Bax,* and *Caspase-3* and nucleus morphology in MDCK cells.

MTs are encoded by Zn-activated transcription genes group of proteins and involved in protecting cells against ROS generation [39]. MT-1 and MT-2 are the predominantly MTs exerting as the potent ROS scavengers [40]. In this study, we investigated the expression of MT-1 and MT-2 in MDCK cells using RT-PCR and western blotting. Importantly, Zn supplementation furtherly improved the increase in MT-1 and MT-2 mRNA levels and MTs protein levels induced by OTA, which is similar to the observation of OTA-treated Coco-2/TC7 cells with Zn supplementation [41]. Actually, MTs is not only a powerful scavenger of free radicals, such as ROS, superoxide anion (O_2^-), but also a protective protein from apoptosis by affecting the expression of *Caspase-3*, *Bcl-2*, and *Bax* [42,43]. To further identify the role of MTs in OTA-induced apoptosis, small interfering RNA was used for MTs knockdown. As expected, MTs Knockdown markedly abrogated the protective effect of Zn supplementation on OTA-induced ROS accumulation and apoptosis in MDCK cells. Interestingly, OTA treatment alone could also up-regulate MTs in mRNA levels and protein levels. The activation of Metal regulatory transcription factor 1 (MTF-1) may play a role in the up-regulation of MTs caused by some metals and their oxidizing compounds [44,45]. So, the up-regulation of MT-1 and MT-2 after OTA treatment could be due to the presence of oxidative compounds, such as OTA-induced peroxide.

In summary, our present study determined that OTA could induce cytotoxicity, oxidative stress, and apoptosis in MDCK cells. In this regard, Zn supplementation attenuated OTA-induced ROS production and apoptosis through the up-regulation of MTs in MDCK cells. Our study may provide supportive data for the potential protective effects of Zn in reducing OTA-induced renal toxicity.

Abbreviations

OTA	ochratoxin A
MDCK	Madin-Darby canine kidney
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
ROS	reactive oxygen species

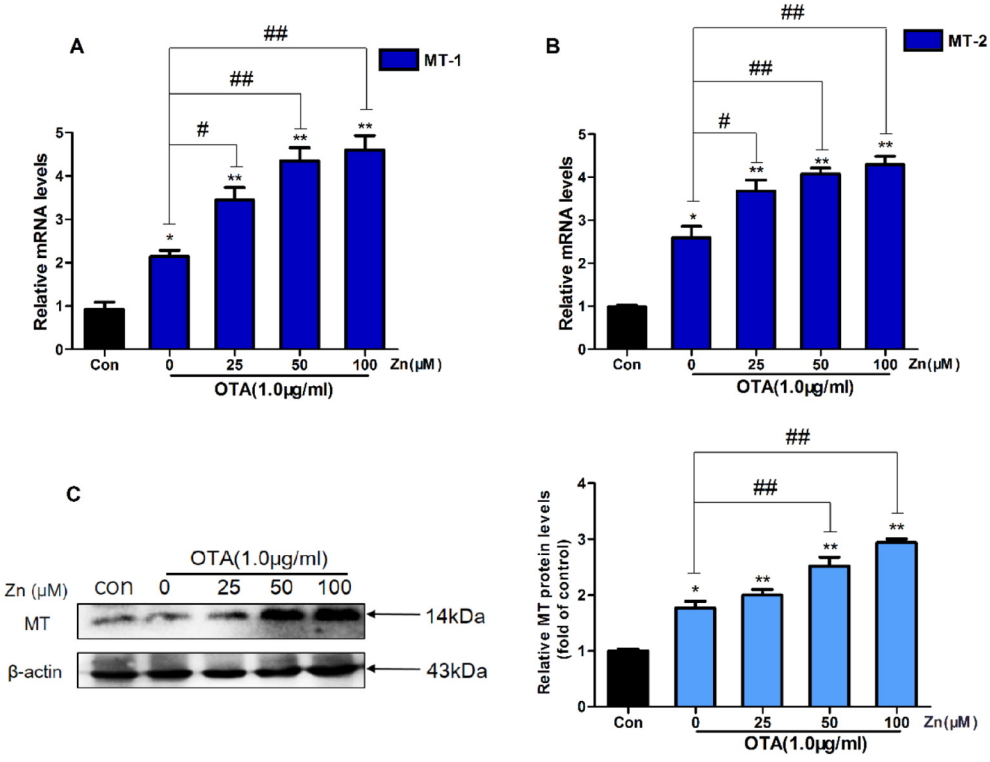

Fig. 4. Zinc supplementation could up-regulate the expression of MTs. The expression of MT-1 (**A**) and MT-2 (**B**) was detected by real-time PCR. The expression of MT protein was detected by western blotting (**C**). All the results were expressed as means ± SEM of four independent experiments. Significance compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$; in cells exposed to both OTA and $ZnSO_4$, compared with the group added with OTA, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, indicating a significant difference.

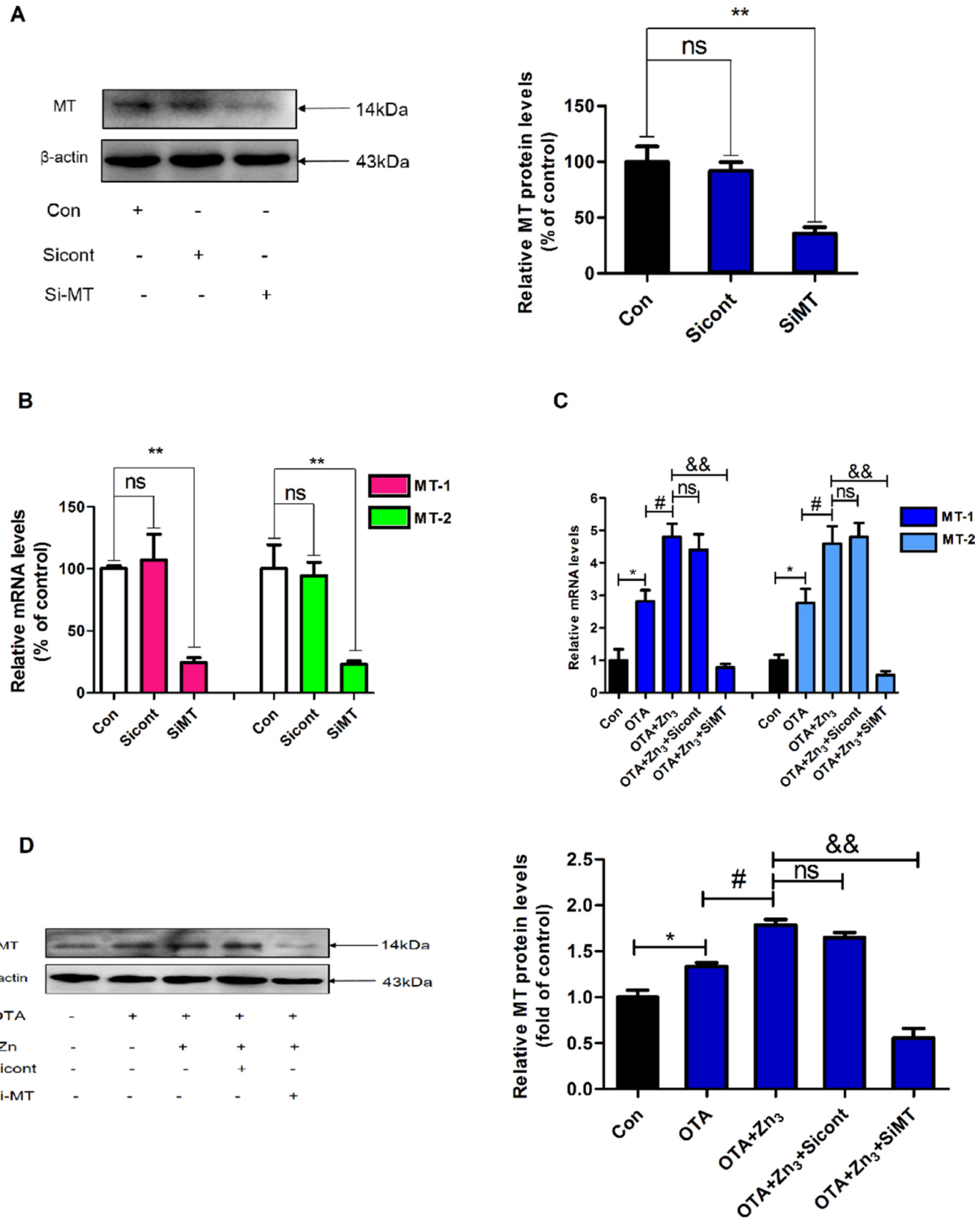

Fig. 5. The knockdown efficiency of MTs in MDCK cells. Knockdown efficiency of MT (**A**, **B**). Significance compared with the control group, $^{**}P < 0.01$. The mRNA levels (**C**) and protein levels (**D**) of MTs were detected. All the results were expressed as means ± SEM of four independent experiments. The control group had significant difference compared with the OTA alone group, $^{*}P < 0.05$, $^{**}P < 0.01$; only the OTA group had significant difference compared with the Zn_3 including OTA group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; the cells exposed to OTA + Zn_3 group had significant difference compared with those exposed to siRNA-control group, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, indicating significant differences; there was no difference between the cells exposed to siRNA-MT group and those exposed to siRNA-control group.

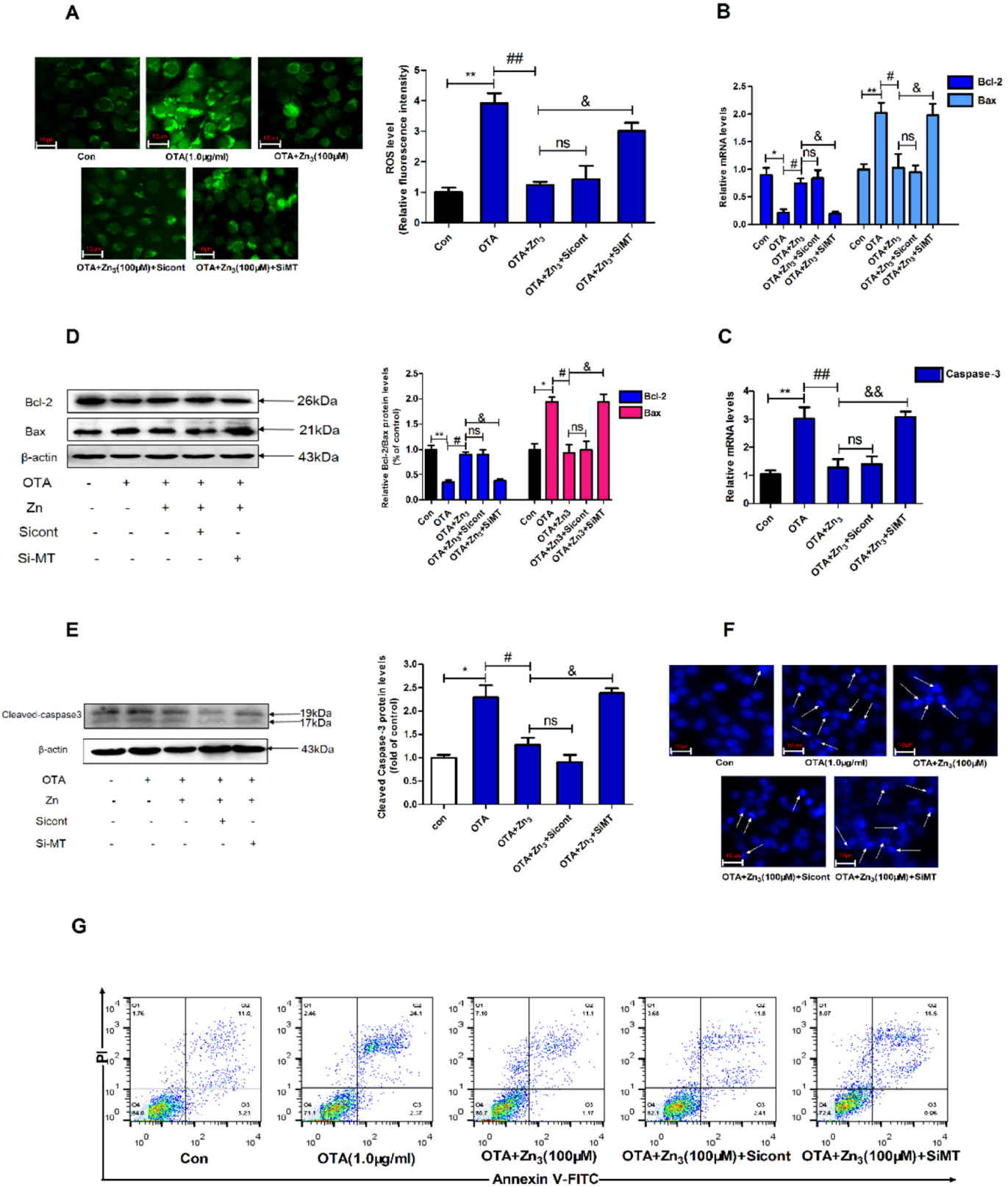

Fig. 6. MTs Knockdown aggravates the OTA-induced ROS accumulation and apoptosis in MDCK cells. The average fluorescence intensity was analyzed by Image-Pro Plus software and expressed as the percentage (set as 100%) (Scale bar: 10 μm) (**A**). The mRNA levels of *Bcl-2, Bax* and, *caspase-3* were detected (**B**, **C**). The protein levels of *Bcl-2, Bax* and cleaved *Caspase-3* was detected by western blotting (**D**, **E**). Hoechst 33258 staining (Scale bar: 10 μm) was used to detect apoptosis, Arrows denote condensed and broken nucleus (**E**). The representative plots of flow cytometry apoptosis (**G**). All the results were expressed as means ± SEM of four independent experiments. The control group had significant difference compared with the OTA group, $^{*}P < 0.05$,$^{**}P < 0.01$; only the OTA group had significant difference compared with the Zn_3 including OTA group, $^{\#}P < 0.05$,$^{\#\#}P < 0.01$; the cells exposed to siRNA-MT group had significant difference compared with those exposed to OTA + Zn_3 group, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, indicating significant differences. There was no difference between the cells exposed to siRNA-MT group and those exposed to siRNA-control group.

SOD	superoxide dismutase
GSH-Px	glutathione peroxidase
ZIPs	Zn^{2+} importers
ZnTs	Zn^{2+} transporters
LDH	lactate dehydrogenase
PBS	phosphate buffer saline
TBS	Tris-buffered saline
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium salt-Polyacrylamide gel electrophoresis
BCA	Bicinchoninic acid
ECL	enhanced chemiluminescence
DCFH-DA	2′,7′-dichlorofluorescein diacetate
MT	metallothionein
MTF-1	Metal regulatory transcription factor 1
O_2^-	superoxide anion
siRNA	small interfering RNA
ANOVA	one-way analysis of variance
SEM	standard error mean

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Declaration of competing interest

The authors declare that they have no competing interests.

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