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Cytotoxic effect of zinc oxide nanoparticles on murine photoreceptor cells *via* potassium channel block and Na^+/K^+-ATPase inhibition

Chao Chen[1,2,*] | Wenjuan Bu[2,*] | Hongyan Ding[3] | Qin Li[4] | Dabo Wang[1] | Hongsheng Bi[5] | Dadong Guo[5]

[1]Department of Ophthalmology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

[2]Department of Ophthalmology, The First People's Hospital of Jining, Jining, Shandong Province, China

[3]Jiangsu Provincial Key Laboratory for Interventional Medical Devices, Huaiyin Institute of Technology, Huaian, Jiangsu Province, China

[4]Department of Integration of Chinese and Western Medicine, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong Province, China

[5]Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan, Shandong Province, China

Correspondence
Dadong Guo and Hongsheng Bi, Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan, Shandong Province, China.
Emails: dadonggene@163.com; azuresky1999@163.com

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Abstract

Objective: Zinc oxide (ZnO) nanoparticles can exhibit toxicity towards organisms and oxidative stress is often hypothesized to be one of the most important factors. Nevertheless, the detailed mechanism of toxicity-induced by ZnO nanoparticles has not been completely addressed. The present study aimed to investigate the toxic effects of ZnO nanoparticles on the expression and activity of Na^+/K^+-ATPase and on potassium channel block.

Materials and methods: In the present study, we explored the cytotoxic effect of ZnO nanoparticles on murine photoreceptor cells using lactate dehydrogenase (LDH) release assay, reactive oxygen species (ROS) determination, mitochondrial membrane potential ($\Delta\varphi m$) measurement, delayed rectifier potassium current recordings and Na^+/K^+-ATPase expression and activity monitoring.

Results: The results indicated that ZnO nanoparticles could increase the LDH release in medium, aggravate the ROS level within cells, collapse the $\Delta\varphi m$, block the delayed rectifier potassium current, and attenuate the expressions of Na^+/K^+-ATPase at both mRNA and protein levels and its activity, and thus exert cytotoxic effects on murine photoreceptor cells, finally damaging target cells.

Conclusion: Our findings will facilitate the understanding of the mechanism involved in ZnO nanoparticle-induced cytotoxicity in murine photoreceptor cells *via* potassium channel block and Na^+/K^+-ATPase inhibition.

1 | INTRODUCTION

Nowadays, the rapid development of nanotechnology provides novel materials with superior properties and these nanomaterials have been widely applied in the biomedical field such as gene transfection,[1,2] diagnostic tool,[3,4] drug delivery,[5,6] cosmetic products,[7] anti-cancer agents[8–10] and so on. The risks of nanomaterials to the organism, to some extent, remain a point of concern, mostly due to the lack of systematic evaluation and scientific knowledge concerning to their potential to affect health. Concerns have been raised on how the exposure to nanomaterials would influence human health.[11–13] Some investigators have performed experiments to explore the relationship between nanomaterials and human as well as ecosystem safety.

Recently, researchers have revealed that zinc oxide (ZnO) nanoparticles could exert potential genotoxicity to organisms.[14–16] The ZnO

*These authors contributed equally to this work and should be regarded as co-first authors.

nanomaterials release into the aquatic ecosystems through domestic and industrial wastewaters has the potential to induce pernicious effects on fish and other organisms.[17] Studies have also found that the genotoxicity is linked to the elevated level of reactive oxygen species (ROS),[18] disrupted calcium homeostasis,[19] the activation of the relevant signalling pathways, such as the mitogen-activated protein kinase signalling pathway,[20] the caspase-dependent signalling pathway[21] and so on. Nevertheless, the underlying mechanisms have not been well addressed.

Mitochondria are small ubiquitous organelles which play important roles in electron transport, mitochondrial transmembrane potential, cellular oxidation-reduction, especially in terms of ROS production and ROS scavenging.[22] Excessive production of ROS in mitochondria will damage many mitochondrial macromolecules, such as lipids, proteins and DNA. Overproduction of ROS will also enhance the release of cytochrome *c* (caspase activator), resulting in the impaired mitochondria leading to cellular damage or even cell death.[23] Therefore, it is crucial for the normal physiological conditions to maintain mitochondrial homeostasis within cells.

The ATP-dependent Na^+/K^+ pump can transport three Na^+ out of cells in exchange for two K^+ into the cell per molecule of ATP hydrolysed, against their respective electrochemical gradients. The appropriate Na^+ and K^+ gradients generated *via* Na^+/K^+ pump can maintain the membrane potential and are essential for cellular electric excitability.[24,25] Therefore, Na^+/K^+-ATPase will create a gradient of Na^+ and K^+, and is necessary for all living mammalian cells. Thus, dysfunction of Na^+/K^+-ATPase will induce loss of cell ion gradients, resulting in the damage of cells. Na^+/K^+-ATPase is a P-type ATPase composed of at least two subunits: a large catalytic α subunit and a regulatory, single-transmembrane-domain β subunit.[26] So far, four α isoforms (α1-4) and four β isoforms (β1-4) have been identified, and found to be encoded by four different genes, *Atp1a1-4* and *Atp1b1-4* respectively.[26–28] The α1-isoform (encoded by *Atp1a1* gene) is regarded to be uniformly expressed in all cells,[29–31] and β2-isoform (encoded by *Atp1b2* gene) of the Na^+/K^+-ATPase is predominantly expressed distinct neuronal cell types including photoreceptor cells in the retina.[32,33] Currently, the retina has been established as having a high level of "free" (loosely bound) zinc in the region of the photoreceptor terminals where it has been proposed to play a role in the modulation of glutamate release.[34]

Our previous investigation has revealed that ZnO nanoparticles can inhibit murine photoreceptor-derived cell proliferation and migration *via* reducing TGF-β and MMP-9 expression.[35] In the current study, to investigate the cytotoxic effects of ZnO nanoparticles on murine photoreceptor cells (661W), we have explored the influence of ZnO nanoparticles on the lactate dehydrogenase (LDH) release of murine photoreceptor cells, mitochondrial membrane potential (Δφm) and ROS levels, potassium channel block, and Na^+/K^+-ATPase expression at both mRNA and protein levels and its activity. Our investigation will facilitate the understanding of the mechanism that is associated with the potassium channel block and decreased Na^+/K^+-ATPase expression and activity based on ZnO nanoparticle-induced cytotoxicity in murine photoreceptor cells.

2 | MATERIALS AND METHODS

2.1 | ZnO nanoparticles and preparation of ZnO nanoparticle solutions

Zinc oxide nanoparticles were obtained from Shanghai Fortunebiotech Co., Ltd (Shanghai, China) and were modified without any stabilizer molecules. The size distribution of ZnO nanoparticles characterized by a field emission scanning electron microscope ranged from 15 to 50 nm and the mean diameter was about 30 nm.[36] For the application of ZnO nanoparticles during experiments, the ZnO nanoparticle suspension was prepared by directly adding a definite amount of ZnO nanoparticles to 5 mL Dulbecco's modified Eagle's medium (DMEM) to be as a stock suspension (6250 μmol/L). Furthermore, the stock suspension was dispersed by a probe sonicator (BILON96-II; Xi'an Bilon Biotechnology Co. Ltd, Xi'an, China) for 30 minutes on ice. Subsequently, various concentrations (31.25, 62.5 and 125.0 μmol/L respectively) of ZnO nanoparticle suspensions (dissolved in DMEM) were prepared and sonicated with a probe sonicator on ice for 10 minutes, followed by vortexing for 60 seconds prior to experiments.

2.2 | Cell culture

A murine photoreceptor cell line (661W) used in the present study was provided by Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, USA). In this study, 661W cells were cultured in DMEM (Life technologies, Oklahoma City, OK 73190, USA) supplemented with 1.0 g/L of glucose, 10% foetal bovine serum (FBS Gaithersburg, MD) (HyClone, Logan, UT, USA), 100 μg/mL streptomycin and 100 U/mL penicillin. All cells were cultured at 37°C in a water-saturated incubator containing 5% CO_2 plus 95% air. Cell counts were performed using an automated cell counter (TC10; Bio-Rad, Hercules, CA, USA).

2.3 | LDH release

Lactate dehydrogenase is a cytoplasmic enzyme that exists in all living cells. If the cell membrane is damaged, LDH will be released into extracellular medium. Thus, the LDH release assay can be applied to accurately quantify the cytotoxicity of chemicals *via* the measurement of LDH released from the damaged cells. In the present study, we measured the LDH release level in extracellular medium of ZnO nanoparticle-exposed 661W cells grown in DMEM supplemented with 10% FBS using an LDH Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Nantong, China). Briefly, 661W cells (5.0×10^5 cells per well) were seeded in a six-well plate and cultured overnight at 37°C in a 5% CO_2-containing incubator, then cells were exposed to various concentrations of ZnO nanoparticles (0, 31.25, 62.5 and 125.0 μmol/L respectively). After 24-hour exposure to ZnO nanoparticles, 120 μl of supernatant under different treatment conditions was applied to react with 120 μL of the reaction mixture from the LDH Cytotoxicity Assay Kit for 30 minutes at room temperature. Supernatant from 661W cells not exposed to ZnO nanoparticles served as negative control. All procedures were performed in

accordance with the manufacturer's instructions. The absorbance of reacted LDH was assessed at 490 nm using a UV-Vis spectrophotometer (4802S; Unico [Shanghai] Instrument Co., Ltd., Shanghai, China).

2.4 | Intracellular ROS

To obtain further evidence for direct actions of ZnO nanoparticles on murine photoreceptor cells, we monitored the alterations in ROS levels after treatment with different concentrations of ZnO nanoparticles. The production of intracellular ROS was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA) by a flow cytometer (Accuri C6; Accuri Cytometers Inc., Ann Arbor, MI, USA). Briefly, cells (5×10^5 cells/well) were seeded in a six-well plate and grown overnight, then incubated with 0, 31.25, 62.5 and 125.0 μmol/L of ZnO nanoparticles for 6 hours, respectively. After harvest of cells, the cells were incubated with DCFH-DA solution (10 μmol/L) in the dark at 37°C for 30 minutes. Then cells were washed with phosphate-buffered saline (PBS) and analysed within 30 minutes using flow cytometry. The specific fluorescence signals corresponding to DCFH-DA were collected with a 525-nm band pass filter. As a rule, 2.0×10^4 cells were counted in each determination.

2.5 | Mitochondrial membrane potential (Δφm)

Maintenance of the Δφm is essential for the normal performance and survival of cells. Usually, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrae thyl benzimidazolyl carbocyanine iodide (JC-1) accumulates in the mitochondria as aggregates (whose fluorescence is red) and also in the cytoplasm as monomers (whose fluorescence is green) in healthy cells. During early apoptosis, the Δφm collapses. As a consequence, JC-1 aggregates cannot accumulate within the mitochondria and dissipate into JC-1 monomers leading to loss of red fluorescence.[18] Thus, red fluorescence responds linearly to an increase in membrane potential.[37] In the present study, the alterations of Δφm in cells were measured using JC-1 probe (Beyotime Institute of Biotechnology, Nantong, China). Briefly, 661W cells (5.0×10^4) were seeded in a six-well plate and grown overnight, then incubated with 0, 31.25, 62.5 and 125.0 μmol/L of ZnO nanoparticles for 6 hours respectively. After digestion with 0.25% trypsin, cells were harvested and washed twice with cold PBS, followed by the incubation with JC-1 probe solution at 37°C for 30 minutes in the dark and washing with cold PBS. Finally, the Δφm was measured by a flow cytometer (BDVerse; BD Biosciences, Franklin Lakes, NJ, USA).

2.6 | Whole-cell patch clamp

Voltage-dependent potassium ion (K^+) channels (Kv channels) can conduct K^+ across the cell membrane in response to changes in the membrane voltage, and regulate target cell excitability by modulating the shape and frequency of action potentials, thereby having both physiological and pathophysiological implications.[38] In the current study, an automated patch clamp experiment was performed using a Nanion chip-based port-a-patch system (EPC-10, HEKA; Nanion Technologies, Munich, Germany) that can enable completely automated patch clamp recordings at room temperature (23-25°C). Briefly, 661W cells were cultured in a six-well plate overnight before patch clamp experiments. Upon the day of experiments, cells were rinsed with PBS, dissociated by exposure to 0.25% trypsin containing 1 mmol/L EDTA. After treatment with various concentrations (ie, 0, 31.25, 62.5, 125.0 μmol/L) of ZnO nanoparticles for 5 minutes, cells were used to determine the delayed rectifier K^+ current. The electrolyte solutions used had the following compositions: extracellular solutions: 10 mmol/L NaCl, 75 mmol/L CsCl, 2 mmol/L $MgCl_2$, 70 mmol/L CsF, 10 mmol/L Hepes/KOH, pH 7.2 and intracellular solutions: 10 mmol/L KCl, 75 mmol/L NaCl, 70 mmol/L NaF, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 5 mmol/L D-glucose monohydrate, 2 mmol/L EGTA, 10 mmol/L Hepes/NaOH, pH 7.4. The delayed rectifier K^+ currents were determined at a depolarizing potential of +40 mV from a holding potential of −80 mV. The Patchmaster software (Nanion Technologies, Munich, Germany) was used to record the delayed rectifier outward K^+ current.

2.7 | Quantitative PCR analysis of Na^+/K^+-ATPase mRNA levels

To investigate the effect of different concentrations of ZnO nanoparticles on the expressions of Na^+/K^+-ATPase (ie, *Atp1a1*, *Atp1b2*) mRNA, quantitative PCR (Q-PCR) was performed. Briefly, 2.0×10^5 661W cells were seeded in every well in a six-well plate and grown overnight, and were then treated with different concentrations (0, 31.25, 62.50 and 125.0 μmol/L respectively) of ZnO nanoparticles for 2 hours. After incubation, cells were harvested by trypsinization, and total cellular RNA was then extracted from 661W cells using Trizol reagent (Aidlab Biotech, Beijing, China) according to the manufacturer's instructions. After quantification of total RNA using a microspectrophotometer (K5600; Beijing Kaiao Technology Development Co., Ltd, Beijing, China), single-stranded cDNA was synthesized with 600 ng of total RNA. Furthermore, cDNA templates obtained by reverse transcription were used to quantify the gene level by Q-PCR technique with 2×Syb Green qPCR Mix (Aidlab Biotech). The target-specific primers are listed in Table 1, and the PCR programme was set as follows: 5 minutes at 95°C, followed by 50 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The fold changes were calculated using the $2^{\Delta\Delta Ct}$ method after normalization to respective endogenous GAPDH control.[39]

TABLE 1 Primers in amplification of target genes by quantitative PCR

Gene	Primer sequences
Atp1a1	F: 5′-GAGGCAGCCCAGAAACCCCAAAAC-3′
	R: 5′-TCGGCCCACTGCACTACCACAATA-3′
Atp1b2	F: 5′-GGCAGCCCTGTGTCTTCATCAAA-3′
	R: 5′-TGCGGCATTCAACATTCACCTC-3
GAPDH	F: 5′-ACGGCAAATTCAACGGCACAGTCA-3′
	R: 5′-CGGCAGAAGGGGCGGAGATG-3′

2.8 | Protein level and activity of intracellular Na^+/K^+-ATPase

The levels of intracellular Na^+/K^+-ATPase and their activities in murine photoreceptor cells were further assessed before and after treatment with various concentrations of ZnO nanoparticles. In brief, 661W cells (4×10^5 cells/well) were seeded in six-well plates and cultured at 37°C in an incubator overnight, then the supernatant was discarded and further supplemented with various concentrations (0, 31.25, 62.50 and 125.0 μmol/L respectively) of ZnO nanoparticles for 6 hours (final volume: 2 mL). At the indicated time, cells were harvested after digestion with 0.25% trypsin containing 1% EDTA, then the cells were collected by centrifugation at 3500 ***g*** for 15 minutes, followed by resuspension in cold PBS. Next, cells were centrifuged at 3500 ***g*** for 15 minutes, and the cell pellet was vortexed in 0.5 mL of cold PBS, sonicated on ice for 10 minutes. After centrifugation at 5000 ***g*** for 10 minutes, the levels of Na^+/K^+-ATPase from cell extracts in 100 μL of the supernatant were measured by Mouse ATPase (ATPase, Na^+/K^+ transporting) ELISA Kit in accordance with the manufacturer's instructions (Wuhan ColorfulGene Biological Technology Co., Ltd, Wuhan, China). Meanwhile, Na^+/K^+-ATPase activity (50 μL for each sample) was also measured using Na^+/K^+-ATPase Activity Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The value of optical density was determined using a 4802S UV/Vis Double Beam Spectrophotometer (Unico [Shanghai] Inc.) at 636 nm, and every experiment was repeated for three times.

2.9 | Statistical analysis

Data are presented as mean ± SD (standard deviation) from at least three independent experiments. One-way analyses of variance and *post hoc* procedures based on Newman-Keuls tests were used for significant test and $P<.05$ was considered significant.

3 | RESULTS

3.1 | Measurement of LDH release

Figure 1 shows the profile of LDH release from DMEM-grown 661W cells after exposure to various concentrations of ZnO nanoparticles for 24 hours. It was observed that compared with those observed in negative control sample (ie, cells not exposed to ZnO nanoparticles), exposure 661W cells to ZnO nanoparticles for 24 hours led to apparent increases in the levels of LDH release into DMEM, and the increases of LDH release were closely correlated with concentrations of ZnO nanoparticles incubated with cells.

3.2 | Determination of intracellular ROS

After the cells exposure to 0, 31.25, 62.5 and 125.0 μmol/L of ZnO nanoparticles for 6 hours, the ROS generation rose from 1.8 ± 0.35% to 31.7 ± 2.74%, 43.8 ± 2.97% and 61.5 ± 5.36% (Figure 2) respectively. These facts demonstrated that with the increase of concentrations of ZnO nanoparticles incubated with cells, the levels of ZnO nanoparticle-induced ROS were also elevated, indicating that the ROS production increased in a concentration-dependent manner. In addition, there existed a significant difference for the amounts of ZnO nanoparticle-induced ROS compared to that of control samples.

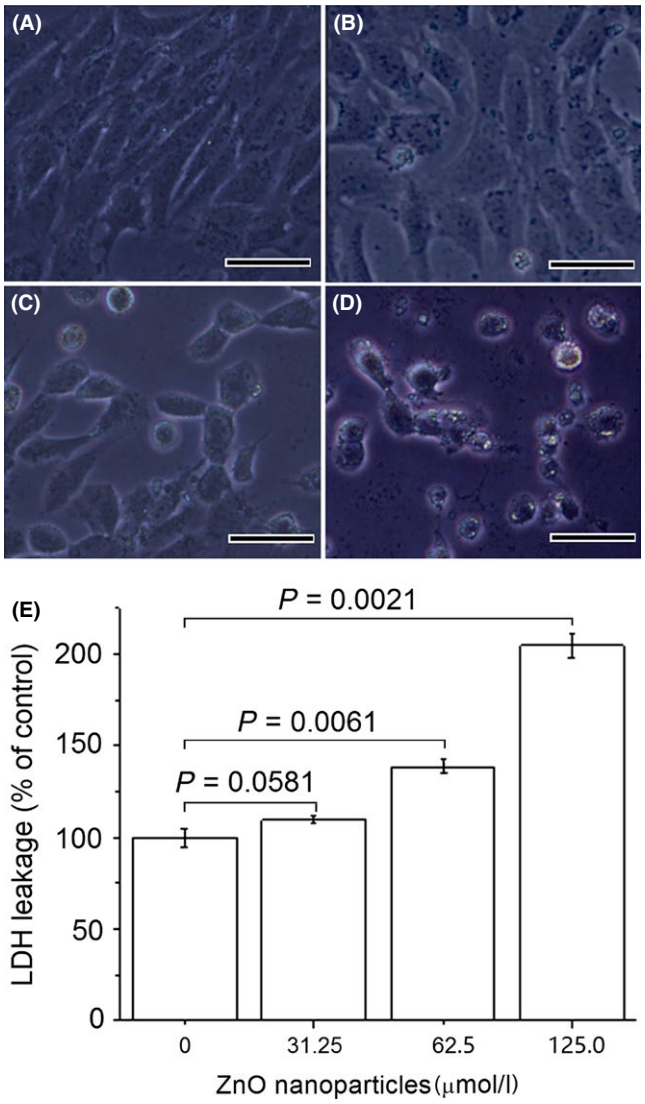

FIGURE 1 Effects of zinc oxide (ZnO) nanoparticles on cell morphology and lactate dehydrogenase (LDH) release from DMEM supplemented with 10% foetal bovine serum-grown 661W cells. Cells were exposed to different concentrations of ZnO nanoparticles (ie, 0, 31.25, 62.5, 125.0 μmol/L) for 24 h before assessing morphology and LDH release. Negative control was 661W cells not exposed to ZnO nanoparticles. (A) untreated cells; (B) cells treated with 31.25 μmol/L of ZnO NPs; (C) cells treated with 62.5 μmol/L of ZnO NPs; (D) cells treated with 125.0 μmol/L of ZnO NPs; and (E) the results of histogram analysis for LDH release. $P<.05$ is considered significant compared with negative control, NPs=nanoparticles and bar=20 μm

3.3 | Alterations in Δφm

Treatment with ZnO nanoparticles appeared to decrease the red fluorescence intensity of 661W cells. As shown in Figure 3, after treatment

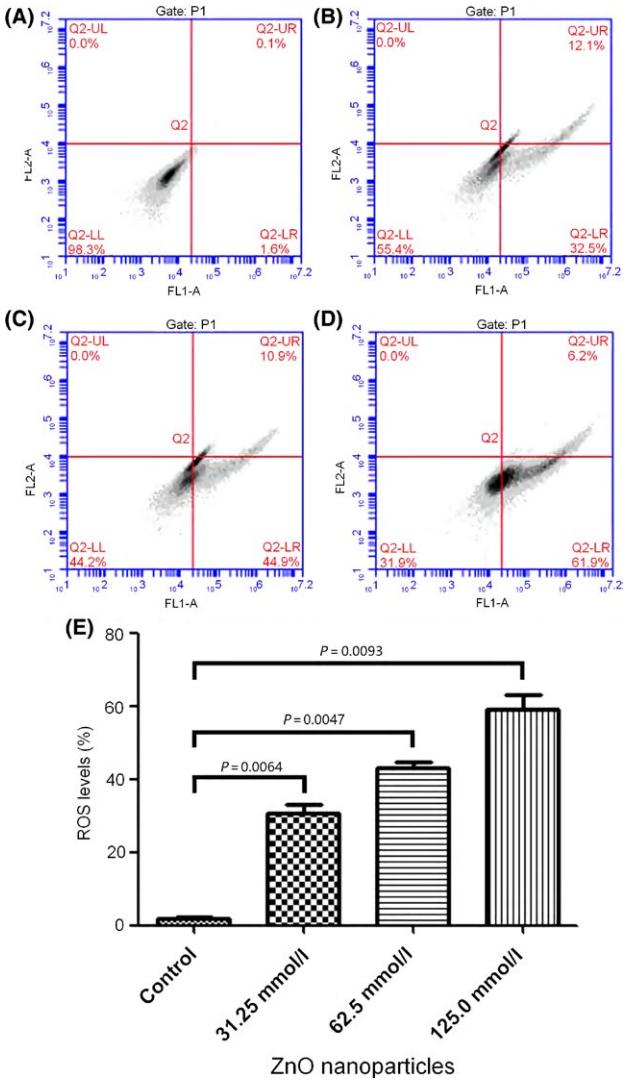

FIGURE 2 The reactive oxygen species (ROS) levels of 661W cells after exposure to different concentrations of zinc oxide (ZnO) NPs for 24 h assessed by flow cytometry. (A) Normal cells; (B) cells treated with 31.25 μmol/L of ZnO NPs; (C) cells treated with 62.5 μmol/L of ZnO NPs; (D) cells treated with 125.0 μmol/L of ZnO NPs; (E) result of histogram analysis. Data are from three independent experiments performed by flow cytometry. NPs=nanoparticles

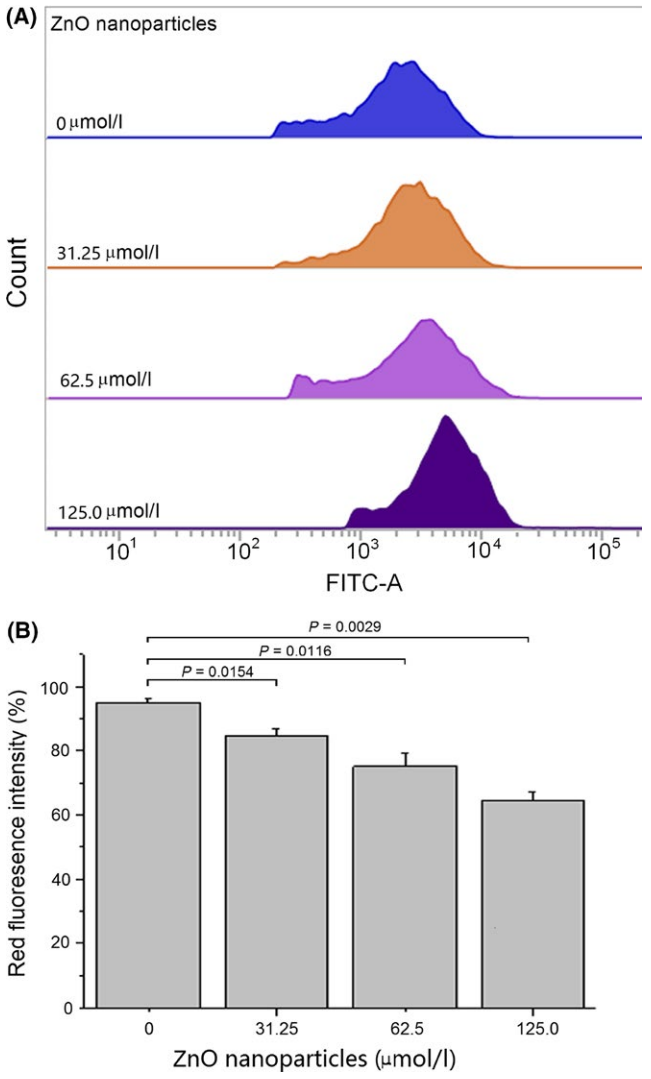

FIGURE 3 Alterations of mitochondrial membrane potential (Δφm) in murine photoreceptor cells after treatment with different concentrations of zinc oxide (ZnO) nanoparticles for 6 h. Red fluorescence represents the J-aggregates and indicates the degree of integrity of mitochondrial membrane in living cells. (A) Diagrams obtained from flow cytometry; (B) result of histogram analysis. Data are from three independent experiments performed by flow cytometry

with 31.25, 62.50 and 125.0 μmol/L of ZnO nanoparticles for 6 hours, the red fluorescence intensity decreased from 94.6 ± 1.2% (control) to 83.5 ± 1.6%, 75.1 ± 2.3% and 64.3 ± 1.7% respectively.

3.4 | Inhibition of the delayed rectifier outward K^+ current

As shown in Figure 4, murine photoreceptor cells alone possessed larger delayed rectifier outward K^+ current. However, after cells exposure to different ZnO nanoparticles, the delayed rectifier outward K^+ currents were decreased in a ZnO nanoparticle-dependent manner, ie, the higher the concentration of ZnO nanoparticles incubated with target cells, the less delayed rectifier outward K^+ currents of cells determined by whole-cell patch clamp.

3.5 | Decrease of Na^+/K^+-ATPase

After incubation with different concentrations of ZnO nanoparticles, the intracellular Na^+/K^+-ATPase at the both mRNA and protein levels was investigated by either Q-PCR or ELISA techniques. Figure 5A shows that the mRNA levels of intracellular Na^+/K^+-ATPase were reduced after exposure of murine photoreceptor cells to ZnO nanoparticles. The mRNA levels of intracellular Na^+/K^+-ATPase were reduced to 45.7%, 62.5% and 33.6% for Atp1a1 and 19.2%, 31.9% and 26.8% for Atp1b2, respectively, and significant differences were observed between untreated cells and ZnO nanoparticle-treated subjects.

Using the ELISA technique, we also assessed the alterations of intracellular Na^+/K^+-ATPase in protein level after murine photoreceptor

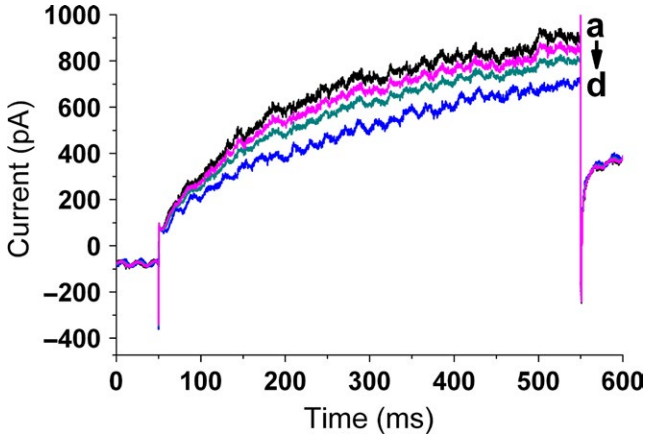

FIGURE 4 Zinc oxide (ZnO) nanoparticles induces Kv current decrease in murine photoreceptor cells. Cells were treated with different concentrations (ie, 0, 31.25, 62.5, 125.0 μmol/L) of ZnO nanoparticles for 5 min, then Kv currents were measured by using the Nanion chip-based port-a-patch system. (A) Actual traces of Kv currents in a ZnO nanoparticle-free cell; (B) representative Kv current trace of a cell after treatment with 31.25 μmol/L of ZnO nanoparticles; (C) representative Kv current trace of a cell after treatment with 62.5 μmol/L of ZnO nanoparticles; and (D) representative Kv current trace of a cell after treatment with 125.0 μmol/L of ZnO nanoparticles

cells exposure to various concentrations of ZnO nanoparticles. Figure 5B demonstrates that the expression levels of Na^+/K^+-ATPase protein were reduced after cells exposure to different concentrations of ZnO nanoparticles. We observed that the protein levels of intracellular Na^+/K^+-ATPase within photoreceptor cells were reduced from 15.03 to 11.88, 10.77 and 9.81 pg/mg after exposure to various concentrations of ZnO nanoparticles, respectively, and accompanied by a concentration-dependent manner.

3.6 | Decrease of intracellular Na^+/K^+-ATPase activity

To explore the effect of ZnO nanoparticles on the Na^+/K^+-ATPase activity, we determined the Na^+/K^+-ATPase activity following digestion. We observed that, in the supernatant obtained following trypsin digestion of the cells, the Na^+/K^+-ATPase activity was also decreased after cells exposure to different concentrations of ZnO nanoparticles compared with that of untreated murine photoreceptor cells. As shown in Figure 6, ZnO nanoparticle-treatment could result in the decreased Na^+/K^+-ATPase activity. We noted that the Na^+/K^+-ATPase activity was decreased from 0.428 to 0.302, 0.344 and 0.324 U/mg, respectively, confirming that ZnO nanoparticles could inhibit the Na^+/K^+-ATPase activity.

4 | DISCUSSION

Zinc oxide nanoparticles have offered great promise in many industrial and biomedical applications. However, investigations have also shown

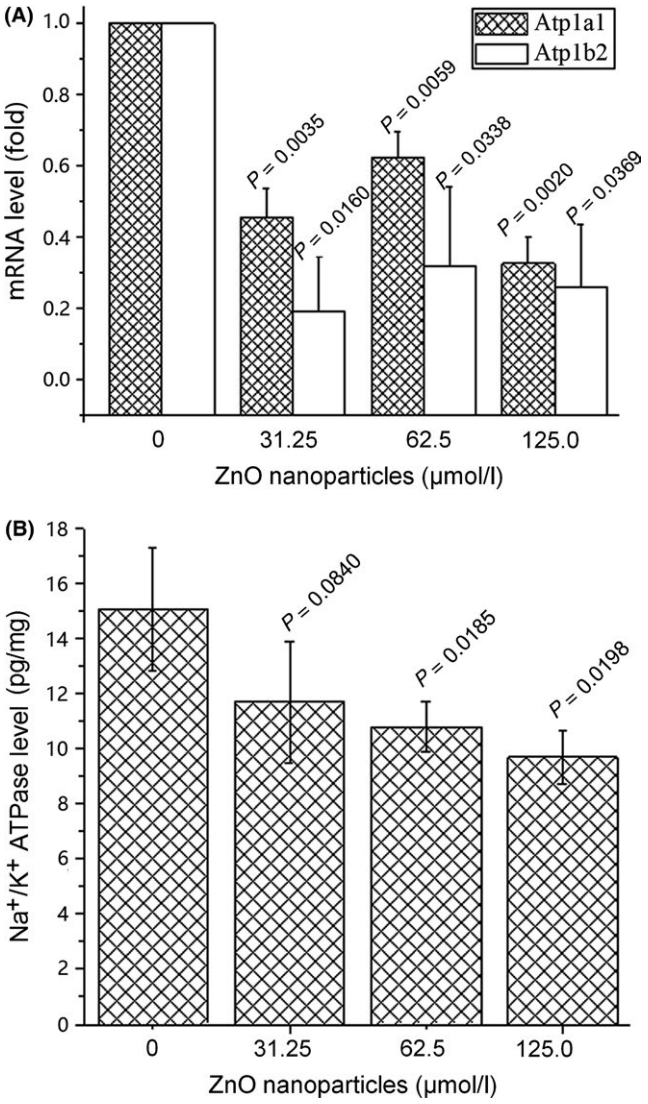

FIGURE 5 Expressions of Na+/K+-ATPase at mRNA and protein levels after treatment with different concentrations of zinc oxide (ZnO) nanoparticles. Cells were seeded in six-well plates and cultured overnight, then treated with different concentrations (ie, 0, 31.25, 62.5, 125.0 μmol/L) of ZnO nanoparticles either for 2 h (for mRNA measurement) or for 6 h (for protein measurement). Next, alterations of Na+/K+-ATPase in both mRNA (A) and protein (B) levels were determined using quantitative PCR and ELISA techniques respectively. Results were presented as mean ± SD (n=3)

that ZnO nanoparticles could exert cytotoxic effect on target cells, and the underlying mechanism is involved in oxidative stress.[10,18] Currently, LDH release assay is an appropriate and possibly preferable means of measuring cellular cytotoxic reactions, which can measure the activity of LDH released into the medium from dead cells.[40] In the present study, we observed that ZnO nanoparticles could induce LDH release into the medium in a concentration-dependent manner (Figure 1), causing an elevation of LDH level in medium, indicating that ZnO nanoparticles can apparently induce murine photoreceptor cell damage.

Metabolism of oxygen within cells generates potentially deleterious ROS. Usually, the rate and magnitude of oxidant formation is balanced by the rate of oxidant elimination under normal physiological

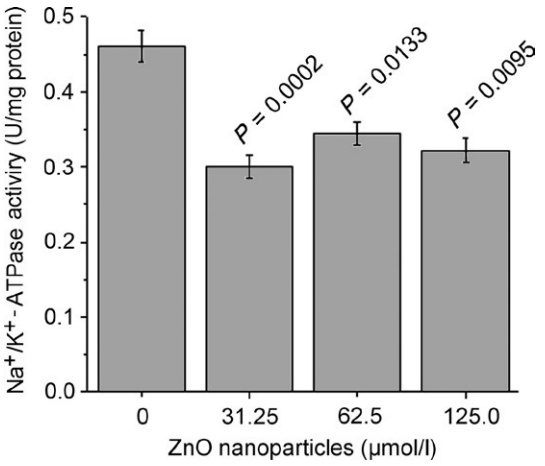

FIGURE 6 Measurement of Na+/K+-ATPase activity of murine photoreceptor cells after treatment with different concentrations of ZnO nanoparticles. Cell were treated with different concentrations (0, 31.25, 62.5 and 125.0 μmol/L respectively) of ZnO nanoparticles for 6 h, then cells were digested with 0.25% trypsin, washed with PBS, sonicated on the ice for 15 min, and centrifuged at 5000 ***g*** at 4°C for 10 min, finally the supernatant was used to determine Na+/K+-ATPase activity. Data were presented as mean ± SD of n=3

conditions.[41] It has been confirmed that numerous environmental factors, including engineered nanoparticles, can lead to overproduction of ROS and oxidative stress.[42] Studies have demonstrated that metal oxide nanoparticle-induced toxicity is primarily mediated by increased ROS production. Fu et al.[43] found that compared to their bulk-size counterparts, engineered nanomaterials will lead to the production of higher levels of ROS and induction of oxidative damage because of their small size, high specific surface area and high surface reactivity. Regarding ZnO nanoparticles, our investigation revealed that exposure of murine photoreceptor cells to ZnO nanoparticles could lead to the overproduction of ROS (Figure 2). Similarly, Yoo et al.[44] observed that treatment with positively charged ZnO nanoparticles made Hela cells generate excessive ROS *via* adsorption and endocytosis of the nanoparticles and further induce cell death, suggesting the strong inducible role of ZnO nanoparticles in the production of ROS to the organisms. Thus, the overgeneration of ROS will trigger endoplasmic reticulum stress and subsequently cause cell damage, finally inducing target cell apoptosis and/or necrosis.[21]

Mitochondria are critical subcellular components that play a crucial role in energy generation (adenosine triphosphate, ATP), intermediary metabolism and cell death. $\Delta\varphi m$ is critical for maintaining the physiological function of the respiratory chain for mitochondria to generate ATP. Normally, a healthy cell has higher $\Delta\varphi m$. Mitochondrial dysfunction has been proven to cause the collapse of $\Delta\varphi m$, lead to mitochondrial depolarization and release of several apoptogenic proteins into the cytosol, participate in the induction of apoptosis, which may be crucial to the apoptotic pathway.[45] In the meantime, mitochondria are also major sources of ROS within the cell. Therefore, the ability to determine $\Delta\varphi m$ and ROS can provide important clues about the physiological status of the cell and the function of the mitochondria.[46] In this paper, we found that exposure to ZnO nanoparticles causes a collapse of mitochondrial inner transmembrane potential, and the higher concentrations of ZnO nanoparticles incubated with target cells, the lower $\Delta\varphi m$ of target cells possessed (Figure 3). This result indicated that $\Delta\varphi m$ collapse is a critical event in inducing murine photoreceptor cell death after exposure to ZnO nanoparticles.

Voltage-dependent potassium channels allow for the selective permeability of K^+ in a membrane potential-dependent manner, playing critical roles in maintaining normal physiological status of cells.[47] Zhao et al.[48] observed that using the whole-cell patch clamp technique, ZnO nanoparticles could increase the current-voltage curve of delayed rectifier potassium current from +20 to +90 mV on rat hippocampal CA3 pyramidal neurons. By contrast, results from the electrophysiological experiments demonstrated that ZnO nanoparticles could block the ion permeation pathway of voltage-dependent potassium channels, influence the physiological exchange between intracellular sodium ions and extracellular potassium ions, collapse the membrane potential, and disrupt the balance of intracellular microenvironment, finally resulting in the cell damage. These findings indicate that the electrophysiological behaviour varied from the tissues. The electrophysiological property of a single cell may be more sensitive than that of tissue.

Na^+/K^+-ATPase is essential for establishing and maintaining hyperpolarized membrane potentials, and the activity of Na^+/K^+-ATPase is responsible for transmembrane ion gradients and is fundamental to cell function and survival. Failure of Na^+/K^+-ATPase activity has been recognized in the pathogenesis of neurodegeneration.[49] At present, it is known that loss of Na^+/K^+-ATPase not only eradicates visual function but also leads to age-dependent degeneration in photoreceptors.[50] As a kind of enzyme, Na^+/K^+-ATPase is sensitive to alterations in the redox status of cells.[51] In our study, we observed that cells exposed to ZnO nanoparticles could cause the elevation of LDH levels and the increment of ROS level, resulting in the occurrence of oxidative stress within cells. If excessive oxidative stress within cells cannot be eliminated in time, it will inhibit both the expressions and activities of Na^+/K^+-ATPase, disrupt the balance of intracellular and extracellular ions between Na^+ and K^+ and block the delayed rectifier outward K^+ currents as well as membrane potential, cause the imbalance of physiological homeostasis within cells, and finally damage the cells. However, Sawosz et al.[52] found that injection of silver nanoparticles into chicken embryos at the beginning of embryogenesis could increase the expressions of Na^+/K^+-ATPase, affecting cell differentiation; meanwhile, Bessemer et al.[53] also revealed that exposure of freshwater fish to ZnO nanoparticles could increase the gill Na^+/K^+-ATPase activity, and this result may be attributed to the increased epithelial permeability or structural remodelling. Based on above-mentioned findings, we infer that the differences of Na^+/K^+-ATPase activity after exposure to nanoparticles may be interpreted as the difference of tissues exposed to ZnO nanoparticles and the variance of nanomaterials.

5 | CONCLUSIONS

In conclusion, ZnO nanoparticles can apparently promote the intracellular LDH release into medium, elevate the ROS levels within

cells and collapse the $\Delta\varphi$m of murine photoreceptor cells. Exposure of murine photoreceptor cells to ZnO nanoparticles will decrease the delayed rectifier potassium currents and attenuate the expressions of Na^+/K^+-ATPase and its activity, and thus disrupt the balance of intracellular microenvironment, finally exhibiting cytotoxic effect on murine photoreceptor cells. Taken together, our findings facilitate the understanding of cytotoxic effect of ZnO nanoparticles mediated by potassium channel block and Na^+/K^+-ATPase inhibition on murine photoreceptor cells.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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