The Anti-Tumor Effect of Zinc on Renal Cell Carcinoma by Enhancing Autophagy
(Kesan Anti-Tumor Zink ke atas Karsinoma Sel Renal dengan Mempertingkatkan Autofagi)

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ABSTRACT
Renal cell carcinoma remains one of the most common malignant tumors and is associated with significant challenges in drug therapy. Zinc has been reported to be of pharmacological importance and immense potential in the inhibition of renal cell carcinoma cells. In order to investigate the inhibitory effect of zinc on the human renal cell carcinoma cell line A498, its anti-proliferative activity was investigated using the CCK8 assay. The oxidative stress status and mitochondrial injury were detected by DCFH-DA and JC-1 staining, respectively. The expression of autophagy-related proteins was determined by western blot analysis, which aimed at exploring the antioxidant mechanisms of zinc. Over 40% yield of ROS and more than four times the number of autophagosomes were detected in the experimental group treated by zinc compared to the control group. Furthermore, zinc led to up-regulation of the Beclin 1-related signaling pathway in the late stage of autophagy degradation. Zinc demonstrated a good inhibitory effect on A498 cells with the value of IC50 similar to the values of natural products. One of the mechanisms of zinc is enhancing the Beclin 1-dependent autophagy. Taken together, with its low cost and natural properties, zinc could become a promising molecule with satisfied anti-tumor effect and low cost in the treatment of renal cell carcinoma.

Keywords: Autophagy; mitochondrial membrane potential; oxidative stress; renal cell carcinoma; zinc

INTRODUCTION
Renal cell carcinoma (RCC) is one of the most common malignant tumors in humans, and is also a quite deadly urothelial tract cancer (Bacigalupa & Rathmell 2020; Parmar et al. 2020; Song et al. 2020). The estimated new cases of RCC are 5% for males and 3% for females of all cancers in the United States (Siegel, Miller & Jemal 2020). Furthermore, its incidence increases annually. RCC emanates from the epithelium tissues of renal tubules. Whether the occurrence of RCC is sporadic or hereditary,
it relates to changes in the short arm of chromosome 3 (Srinivasan et al. 2015). In the clinic, it can be detected by abdominal computerized tomography or ultrasounds and characterized by histological phenotypes. However, the present treatments (i.e., surgical intervention, chemotherapy, radiotherapy and hormonal therapy) of RCC do not get a satisfactory curative effect. At this stage, there is no clinically proven therapy for RCC (Neha, Das & Verma 2020; Yu et al. 2022). Therefore, there is a need to improve the treatment effect of RCC.

Drug therapy, as an important tumor treatment method, can kill cancer cells quickly, and control the disease in a rapid manner. For immune drugs, interferon-α and interleukin-2 are of questionable effectiveness but with strong side-effects (Ray et al. 2012). Bevacizumab, an immunomodulator, is the first drug to be successfully approved for use in the treatment of metastatic RCC (Pili et al. 2020). Besides, T-cell receptor agonists and chimeric anti-meta receptor T-cells are trying to be utilized for RCC immunotherapy (Carlo, Voss & Motzer 2016; Escudier 2012). Additionally, chemotherapeutics plays an important role in RCC therapy. Sunitinib and pazopanib are the first-line drugs for clear-cell RCC to inhibit vascular endothelial growth factor receptor (Bersanelli et al. 2020; Lu et al. 2020). Furthermore, the second-line drugs containing everolimus, sorafenib and axitinib mainly inhibit tyrosine kinase (Sabatini et al. 2013). If first- and second-line treatments result in treatment failure, some RCC patients with sufficient financial resources can choose third-line drugs (dovitinib) to stop tumour growth (Schmidinger 2014). It seems that 5-year survival rates of RCC have been improved by drug therapy. However, it is worth noting that survival rates with advanced diseases remain acceptably low (Bacigalupa & Rathmell 2020). Thus, there is an urgent need to find new anticancer drugs with natural properties, fewer side effects, and high efficacy.

Zinc (Zn) is the essential trace element that plays important roles in numerous enzymatic molecules activities. Zn is physiologically relevant for the function of growth and intelligence development, immunity enhancement, increase in appetite and improvement in activities. Zn is physiologically relevant for the function (Bellia & Rizzarelli 2018). For physiological conditions, free Zn\(^{2+}\) is lowly abundant in the zinc-binding proteins, lysosomes and mitochondria. The homeostasis of Zn\(^{2+}\) is maintained by Zn transporters (ZnTs) and Zrt/Irt-like proteins (ZIPs). In the condition that a large number of Zn\(^{2+}\) enters the cells beyond the ion excretion by ZnTs, it would be bound to generate reactive oxygen species (ROS) to cause oxidative injury. Besides, Zn\(^{2+}\) would also accumulate in the mitochondria, causing organelle damage resulting from ATP depletion (Dineley, Voyyakova & Reynolds 2003; Gazaryan et al. 2007). Moreover, it has been reported that the Zn\(^{2+}\) thresholds of oxidative stress in cancer cells are lower compared to their normal counterparts (Manshian et al. 2017). Therefore, cancer cells are more sensitive to high Zn\(^{2+}\) levels, which provides a therapeutic window for targeted cancer treatment using Zn. Direct Zn administration for cancer therapy in vitro and in vivo has been applied in pancreatic cancer, esophageal cancer, colon cancer, head and neck cancer, advanced nasopharyngeal cancer, and prostate cancer (Wang et al. 2020). These results provide evidence that Zn might be a potential chemotherapeutic agent.

To the best of our knowledge, there is no study that has focused on RCC treatment using Zn. Zn as a promising pharmacological agent that requires further investigation. Therefore, in this study, we detected the cytotoxicity of Zn on RCC cells (A498 cell line) and intended to investigate its potential mechanism. Our results may provide new ideas for the drug therapy of RCC.

**MATERIALS AND METHODS**

**MATERIALS**

The human RCC cell lines A498 were purchased from ATCC (USA). Dulbecco’s modified eagle medium (high glucose) (DMEM), pancreatic, penicillin and streptomycin (PS), fetal bovine serum (FBS) were purchased from Gibco (USA). Zinc chloride (ZnCl\(_2\)) was purchased from TCI (Japan). CCK8 was obtained from Dojindo (Japan). Reactive oxygen species (ROS) assay kit (DCFH-DA method) and mitochondrial membrane potential detection kit (JC-1 method) were both purchased from Beyotime (China). Cell autophagy staining detection kit (MDC method) was obtained from Solarbio (China). Antibodies against beclin1, p62/SQSTM1, nucleoporin p62, and β-actin were purchased from Bioss (USA).

**CELL CULTURE**

A498 cells were cultured in the DMEM medium supplemented with 10% FBS and 1% PS at a temperature of 37 °C in a humidified incubator (5% CO\(_2\)). Cells that were in the exponential growth phase were used for experiments.
CCK8 ASSAY
A498 cells were seeded into 96-well plates with 5000 cells per well. After 12 h, ZnCl$_2$ solution in different concentrations (10, 20, 40, 80, 160, 320 μM) was added to the medium. Cellular viability was quantified using the CCK8 assay after successive cultivation for 24 h under a humidified atmosphere. A498 cells without any treatment were utilized as positive control to set the cellular viability as 100%. The above data were utilized to calculate 50% inhibitory concentration (IC$_{50}$) of Zn$^{2+}$ as well.

MEASUREMENT OF INTRACELLULAR ROS
A498 cells were seeded into 96-well plates with 5000 cells per well. After A498 cells adhered to the plate overnight, 20 μM of ZnCl$_2$ was added into five experimental groups at different time points (0, 6, 8, 12, 24 h), respectively. Then, the culture medium was taken out, and fresh medium without FBS containing 10 μM/L of DCFH-DA was added in to submerge the cells. A498 cells were then cultured in a humidified incubator for another 20 min. The cells were washed with DMEM three times. Their fluorescence absorbances were detected using a microplate reader at the excitation and emission wavelengths of 488 and 525 nm, respectively. A498 cells without any treatment were set as a blank control and cells treated with Rosup were set as a positive control.

DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL
A498 cells were treated similarly as above. Overall, 100 μL of the JC-1 working solution was added, which was followed by incubation for another 20 min. The cells were washed with cooled JC-1 staining buff twice. Then, 100 μL of DMEM was added. The fluorescence absorbances of JC-1 polymeride were detected using a microplate reader at the excitation and emission wavelengths of 525 and 590 nm, respectively. A498 cells without any treatment were set as a blank control and cells treated with CCCP were set as the positive control.

MEASUREMENT OF AUTOPHAGOSOMES
After a similar treatment method, A498 cells were washed with a wash buffer. Overall, 100 μL of DMEM containing 50 nM of MDC was added, followed by incubation for another 30 min. Then, the cells were washed with wash buffer again. Their fluorescence absorbances were detected using a microplate reader at the excitation and emission wavelengths of 338 and 500 nm, respectively.

WESTERN BLOT ANALYSIS
A498 cells were seeded onto 6-well plates with 1.2×10$^6$ cells per well and cultured for 24 h. Next, 20 μM of ZnCl$_2$ was added into the five experiment groups at different time points (0, 6, 8, 12, 24 h), respectively. The treated cells were washed with ice-cold PBS, and all cells were collected. A498 cells were blotted with primary antibodies against Beclin 1, p62/SQSTM, nucleoporin p62, or β-actin (as an internal loading control), as well as a secondary anti-IgG antibody. The immunoblots were visualized by the super signal west pico-chemiluminescence substrate and imaged by the VersaDoc imaging system.

STATISTICAL ANALYSIS
All the cell experiments were repeated six times. A one-way or two-way ANOVA test was used to determine statistical differences. Statistical significance was accepted at $P<0.05$.

RESULTS

CYTOTOXICITY OF ZN$^{2+}$
A498 cells were treated with different concentrations of Zn$^{2+}$ for 24 h. The cellular viability was found to decrease with increasing of Zn$^{2+}$ concentration (Figure 1). As derived from the standard curve, the IC$_{50}$ of Zn$^{2+}$ was 21.73 μM. Additionally, we chose the concentration (20 μM) for the subsequent experiments.

OXIDATIVE STRESS
DCFH-DA fluorescence probe can freely cross the cell membrane, and it is hydrolyzed to DCFH by intracellular esterase. DCFH does not have the ability to penetrate cell membranes and can be oxidized by ROS to form the green fluorescent substance DCF. The intensity of the green fluorescence is proportional to the level of intracellular ROS. Herein, intracellular ROS levels of A498 were significantly increased when cells were treated with Zn$^{2+}$ compared to the blank control (Figure 2). The yield of ROS increased slowly and had little difference
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in the first 18 h. At 24 h, intracellular ROS productivity of A498 cells increased significantly. These results indicate that Zn$^{2+}$ can trigger oxidative stress of A498 cells, which is correlated with treatment time. Zn will induce oxidative stress-mediated injury on A498 cells.

Mitochondrial membrane potential detection kit is used to detect the changes in mitochondrial membrane potential, and to explore the degree of mitochondrial injury. The membrane potential of healthy mitochondria

MITOCHONDRIAL INJURY

FIGURE 1. The cytotoxicity of Zn$^{2+}$ on A498 cells

FIGURE 2. The ROS productivity in A498 cells induced by Zn$^{2+}$
is relatively high. JC-1 exists as J-aggregates in mitochondrial matrix emitting red fluorescence. On the other hand, J-aggregates would disaggregate into monomers that emit green fluorescence once the mitochondrial membrane potential decreases. Figure 3 shows changes in A498 cells mitochondrial membrane potential after being treated with Zn$^{2+}$. The green fluorescence intensity was enhanced, while the red fluorescence intensity was reduced in Zn$^{2+}$ treated group. That was similar to the status in CCCP-treated group. The results showed that, similar to CCCP, the mitochondrial membrane potential was depolarized by Zn$^{2+}$.

Then, the fluorescence intensity was determined using a microplate reader (Figure 4). After treatment with Zn$^{2+}$, all of the groups showed a green fluorescence signal from A498 cells. The green fluorescence intensity of the 16 h’s experimental group was higher than the other group. That is, the amount of intracellular JC-1 monomers in this group was significantly larger. It can be inferred that Zn$^{2+}$ changed the mitochondrial membrane potential of A498 cells, which may damage the mitochondria ultimately.

**FIGURE 3.** The changes of JC-1 level in A498 cells affected by Zn$^{2+}$

**FIGURE 4.** The fluorescence intensity ratio of JC-1 aggregate (A) and JC-1 monomer (B) in A498 cells after Zn$^{2+}$ treatment
FORMATION OF AUTOPHAGOSOMES

Cells will form autophagosomes in order to phagocytize their own cytoplasm and organelles while they are being stimulated. MDC, an eosinophilic stainer, is commonly utilized to detect autophagosome formation. After the treatment with Zn\(^{2+}\), the cellular MDC fluorescence intensities of all the groups increased significantly compared to the controls (Figure 5). The experimental results demonstrate that Zn\(^{2+}\) induced formation of a large number of autophagosomes, demonstrating the considerable enhancement of autophagic activities and apoptosis of A498.

![Graph showing the fluorescence intensity ratio of MDC in A498 cells after Zn\(^{2+}\) treatment](image)

FIGURE 5. The fluorescence intensity ratio of MDC in A498 cells after Zn\(^{2+}\) treatment

THE EFFECT OF Zn\(^{2+}\) ON THE EXPRESSION AUTOPHAGY-RELATED PROTEINS

Treatment with Zn\(^{2+}\) at the concentration of 20 \(\mu\)M (Figure 6), the expression of Beclin 1, obviously increased after 16 h’s co-culture, indicating the increase in autophagic activity. Besides, both the expression of p62/SQSTM1 and nucleoporin p62 decreased at a later time, which demonstrated the increase of autophagic activity in the late stages of autophagy degradation.

![Immunoblotting (A) and densitometry (B) analysis on the expression of Beclin 1, p62/SQSTM1 and nucleoporin p62 in A498 cells treated by Zn\(^{2+}\)](image)

FIGURE 6. Immunoblotting (A) and densitometry (B) analysis on the expression of Beclin 1, p62/SQSTM1 and nucleoporin p62 in A498 cells treated by Zn\(^{2+}\).
DISCUSSION

The cytotoxicity of Zn\textsuperscript{2+} on RCC cell lines A498 was evaluated in this study. We measured intracellular ROS levels and mitochondrial membrane potential. Meanwhile, we determined the formation of autophagosomes and the expression of autophagy-related proteins to evaluate autophagy. We discovered that Zn\textsuperscript{2+} was able to induce oxidative stress and autophagy to damage mitochondria at certain concentrations, thereby killing A498 cells.

Zn is an essential trace element, and is an important factor for keeping normal human physiological functions (Leuci et al. 2020). Zn\textsuperscript{2+} has a beneficial role in preserving natural tissue barriers, such as the microvilli epithelium that prevent pathogen entry. Zn has also shown good effect on the treatment of COVID-19 (Hunter et al. 2020; Wessels, Rolles & Rink 2020). Zn acts as an anti-oxidant in order to protect retinal pigment epithelium (Pao et al. 2018). Moreover, Zn is reported to be an anti-inflammatory agent, an anti-apoptotic, a growth co-factor, and a microtubule stabilizer (Addison et al. 2019; Aziz et al. 2018; Tripon et al. 2018). On the other hand, Zn would be noxious when extracellular Zn\textsuperscript{2+} levels exceed the capacity of homeostatic control (Martínez et al. 2018). Therefore, it can be utilized for cancer chemotherapy. However, there are very few studies that focus on the cancer therapeutic effect of raw Zn. Gumulec et al. (2011) concluded that the current data regarding the impact of Zn\textsuperscript{2+} on the pathogenesis of breast cancer, and inferred that Zn\textsuperscript{2+} owned great potential in the treatment of breast cancer. Gao et al. (2020) experimentally proved that Zn\textsuperscript{2+} began to exhibit obvious cytotoxicity to human prostate cancer cells when its concentration was more than 80 μM after 48 or 72 h treatment. Herein, we discovered that Zn\textsuperscript{2+} had cytotoxicity to A498 cells when the concentration was more than 20 μM after 24 h treatment. This finding ensures the potential therapeutic effect of Zn\textsuperscript{2+} on RCC.

In view of the last five years of literatures on novel chemotherapy medications that are used to treat RCC, the IC\textsubscript{50} values for A498 cells range in 10\textsuperscript{-1}-10\textsuperscript{-9} μM: 4-Chloro-N-(3-((4-(3-(3-hydroxyphenyl)-1H-pyrazol-4-yl)pyridin-2-yl)amino)propyl)benzenesulfonamide (0.33 μM, 48 h drug exposure) (Abdel-Maksoud et al. 2019), 6-(Allylsulfanyl)-1,5-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (5 μM, 48 h drug exposure) (Malki et al. 2019), 2-(benzofuran-2-yl)ethanone (6.97 μM, 48 h drug exposure) (Ibrahim et al. 2019), 1-(2-(4-(Hydroxymethyl)piperidin-1-yl)-4-(pyridin-3-yl)phenyl)-3-(4-methylphenyl)urea (1.33 μM, 48 h drug exposure) (Al-Sanea et al. 2018), astragalin (20 μM, 24 h drug exposure) (Zhu et al. 2019), galangin (15 μM, 24 h drug exposure) (Zhu et al. 2018), mulberroside-A (20 μM, 4 h drug exposure) (Duan et al. 2019) and resveratrol (112.8 μM, 24 h drug exposure) (Zhao et al. 2018). Herein, the IC\textsubscript{50} value of Zn\textsuperscript{2+} is 21.73 μM for the 24 h drug exposure, which is close to the values of natural products. Taking the synthesis or extraction methods and costs into consideration, Zn\textsuperscript{2+} used as a RCC chemotherapeutic drug is superior.

With regards to the mechanism of inhibition, it is acceptable that higher ROS levels exceeding compensatory changes lead to apoptosis (Mir et al. 2020). Cytotoxic medicines that selectively kill cancer cells can induce the generation of ROS. Intracellular oxidative stress can be divided into three levels (Nel et al. 2006). First, increasing the level of antioxidant enzymes. Second, an increase in effective pro-inflammatory cytokines. Third, mitochondrial disorder leads to apoptosis or necrocytosis. The intracellular ROS of A498 cells were significantly increased when cells were treated with Zn\textsuperscript{2+}, compared to the blank control (Figure 2). Furthermore, results of the mitochondrial membrane potential detection kit showed that the mitochondrial membrane potential could be depolarized. The occurrence of oxidative stress in A498 cells leads to mitochondrial damage.

In addition, the number of autophagosomes increased significantly after Zn\textsuperscript{2+} treatment. This indicates that autophagy in A498 cells can be dramatically induced by oxidative stress and mitochondrial dysfunction. Autophagy is an important cellular degradation pathway that plays a central role in metabolism, as well as basic quality control. These two processes are inextricably linked to aging. It has been found that chronic autophagy inhibition confers an irreversible increase in cancer risk and uncovers a bi-phasic role of autophagy in cancer development, being both tumour suppressive and oncogenic, sequentially (Cassidy et al. 2020). Therefore, the role of autophagy is complex in cancer, which has a function in both cell death and cell survival (Camuzard et al. 2020). On the one hand, many cancer cells up-regulate autophagy, which is required to support metabolism,
The molecular regulation mechanism of autophagy is extremely complicated. Over 30 autophagy-related genes (Atg) responsive to multiple upstream signals (Rybstein et al. 2018). The process of autophagy can be divided into four stages: autophagy initiation, extension, maturation and degradation. Beclin 1 is a specific protein that plays a key role in the initiation of autophagy. Beclin 1 dissociates from Bcl2 and binds to PI3K and Atg14 to form Beclin 1-PI3K-Atg14. Then Beclin 1-PI3K-Atg14 activates other autophagy-related proteins to initiate autophagy (Al-Huseini et al. 2022; Wu et al. 2018). The expression of Beclin 1 substantially increased after 16 h Zn2+ treatment, which represents the incipient stage of autophagy. Later, the expression of p62/SQSTM1 showed a downward trend, and the expression of nucleoporin p62 continuously declined after Zn2+ treatment (Figure 6(B)). The p62 protein is a landmark protein in the late stage of autophagy degradation. The decrease in expression level represents the enhancement of autophagic activity (Shin, Park & Chung 2020). Meanwhile, the results suggested that selective autophagy had occurred, because p62 was the chaperone for selective autophagy (Hao, Wang & Jiao 2017; Jeong et al. 2019). Unfortunately, we have neither detected all the protein expressions in the entire pathway of selective autophagy, nor monitored the whole autophagy process using microscopic imaging technique. That will be the focus of our future work to better understand the toxic mechanism of Zn2+ on A498 cells. Fortunately, we have successfully prepared nanoscale zinc oxide based pH-responsive drug delivery systems (Huang et al. 2019). Not only can such drug delivery systems carry Zn2+ to target sites, but also delivery other medicine to easily realize combination therapy. This is very promising for RCC therapy.

CONCLUSION

In conclusion, the results of this current study indicate that Zn has a good inhibitory effect on RCC cells, with the value of IC50 being similar to the values of natural products. Its cytotoxic effect is due to an increase of Beclin 1-dependent autophagy induced by oxidative mitochondrial damage. Therefore, Zn was proven to be an important medicine for the treatment of RCC and should be further evaluated in vivo.

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