European Review for Medical and Pharmacological Sciences 2021; 25: 1557-1563

Zinc oxide nanoparticles promotes liver cancer cell apoptosis through inducing autophagy and promoting p53

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Abstract. – OBJECTIVE: Emerging evidence has highlighted the promising potential of the application of Zinc Oxide nanoparticles (nano-ZnO) but the mechanism by how it functions in liver cancer remains elusive. We aimed to explore the effect of nano-ZnO on liver cancer cells.

MATERIALS AND METHODS: Liver cancer cells Huh7 cells were transfected with GFP-LC3, and then, treated with DMSO, Sorafenib, and nano-ZnO respectively to set blank group, Sorafenib control group, and nano-ZnO group followed by the analysis of the expression of GFP-LC3, p53, and Caspase by Western blot and RT-qPCR, cell apoptosis and viability by flow cytometry and CCK-8 assay.

RESULTS: With a diameter of nano-ZnO 14.13±0.92 nm, the amount of GFP-LC3 protein was increased after treatment of nano-ZnO. Besides, the expressions of GFP-LC3, p53, and Caspase in Sorafenib group and nano-ZnO group were significantly higher than that of control group, while their levels were highest in nano-ZnO group ($p<0.05$). In nano-ZnO group, the values of D450nm at 24 h, 48h, and 72 h were 0.56±0.06, 0.39±0.05, and 0.22±0.04, respectively, and the apoptotic rate (83.11±2.79%) was significantly lower than that of blank group and control group.

CONCLUSIONS: Nano-ZnO induced autophagy, upregulated the p53 gene, and facilitated the apoptosis of liver cancer cells, indicating that nano-ZnO might be a therapeutic approach for the treatment of liver cancer patients.

Key Words:
Nano-ZnO, Autophagy, P53 gene, Liver cancer.

Introduction

Nowadays, as a malignant tumor, liver cancer seriously threatens human life, with increasing incidence in recent years[1]. Chemotherapy is the primary treatment option to treat liver cancer currently. However, the side effect delimits the treatment outcomes[2]. Exploring other ways to treat liver cancer becomes urgent and necessary. With the rapid development of technology, nanomaterials have aroused people's attention and are widely applied for treating various diseases. *Via* regulating pharmacokinetics and pharmacodynamics[3], nanomaterials can enhance the therapeutic effect of drugs. Zinc Oxide nanoparticles (nano-ZnO) is one of the main nanomaterials for cancer treatment. Due to insufficient vessel walls and lymphatic drainage dysfunction, nano-ZnO can penetrate into cancer tissues and stay there for a while[4], which can improve the efficacy of targeting and reduces side effects simultaneously. Therefore, nano-ZnO is regarded as a major breakthrough in tumor treatment.

Autophagy is a process that maintains the stability of various substances and energy through self-digestion to ensure cell normal function. The process involves substrate identification, isolation, and transport of the substrate to lysosome. Autophagy is closely related to the occurrence and development of bacterial infections, immune system response, nerve conduction, and various diseases[5]. Consequently, autophagy is key to the normal metabolic function of liver tissue. P53 gene, as a known important tumor suppressor, modulates the cell cycle and DNA repair through transcription and translation. According to a previous study[6], mutation of the p53 gene is common in various cancer cells, leading to abnormal protein folding. The proliferation ability and reinforcement of apoptosis function of cancer cells are related to the elevation of p53 expression[7]. However, it is still unknown whether nano-ZnO enhances p53 expression by promoting autophagy. Until now, sorafenib is a rare oral molecular

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drug for treating end-stage liver cancer in clinic, as it alleviates cancer progression by inhibiting the growth of cancer cells and accelerating cell apoptosis[8]. But now, the resistance to sorafenib results in a limited application for treating liver cancer[9]. Based on the challenge and limitations mentioned above, our research aimed to assess the potential of nano-ZnO in treating liver cancer and provide a further comprehensive understanding of the mechanism of this material in cancer treatment.

Materials and Methods

Cell Culture

Huh 7 liver cancer cell line provided by Shanghai Kanglang Biotechnology Co., Ltd (Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

Characterization of Nano-ZnO

Nano-ZnO was diluted into 5 umol/ul suspension by DMSO (Sigma-Aldrich, St. Louis, MO, USA). The size, physical properties, and characteristics of ZnO were detected by the dynamic light scattering method.

Cell Transfection

After culture, cells were digested, and Opti-Mem medium was added for transfection experiment, where cells were transfected with green fluorescent protein-microtubule associated protein light chain 3 (RFP-GFP-LC3) diluted in PEI (1 μg:2 μl). The cells, then, were divided into blank group (addition of DMSO), control group (sorafenib), and experimental group (ZnO-NPS). After 1 day, the number of GFP-LC3 protein was observed under a fluorescence microscope. In this study, the quantity of GFP-LC3 protein particles over 50% of all cells was defined as autophagy.

Western Blot

The total proteins were extracted to determine the expression of autophagy marker GFP-LC3 and caspase 3. Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was then incubated with anti-Caspase3 antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then with HRP-labelled IgG antibody (Cell Signaling Technology, Danvers, MA, USA) for 2h. The membrane was developed with ECL (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by the Image Lab system.

RT-qPCR

RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and reversely transcribed into cDNA. With GAPDH as a reference, PCR amplification was carried out with 40 cycles. The experiment needs to be repeated 3 times to calculate the average Ct value, and $2^{-\Delta\Delta Ct}$ method was applied to quantify the relative expression of target genes. RT-qPCR primers were demonstrated in Table I.

CCK-8 Assay

Cells were seeded onto 96-well plates (2×10^3 cell/well). After 24 h, 48 h, and 72 h, 10 μl of Cell Counting Kit-8 (CCK-8) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added, and the optical density (D) value of cells at 450 nm (D_{450nm}) was measured by the Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) after 1 h.

Flow Cytometry

The cells were seeded onto 6-well plates in medium and suspension. After culture, cells were centrifuged at 1000 rpm for 1 hour, and the suspension was mixed with a buffer solution. Then, the cells were stained with 5 ul of phosphatidylser-

Table I. RT-qPCR primer sequence.

Gene	—	Internal reference sequence	Primer length (bp)
P53	Upstream primer	5'-TCTGTCCCTTCCCAGAAACC-3'	118
	Downstream primer	5'-TTGGGCAGTGCTCGCTTAGTGCTCC-3'	
Internal reference GAPDH	Upstream primer	5′-CTATTCGATGCCGTGTATGC-3'	226
	Downstream primer	5′-GCCTGGTCCAGACTTCTTTC-3′	

ine reagent (Thermo Fisher Scientific, Waltham, MA, USA) and propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA) and rotated for 15 min. Then, cell apoptosis was detected by flow cytometer and the apoptotic rate: cell apoptosis rate (%) = number of labeled cells (FITC+PI+)/ total number of cells×100%.

Statistical Analysis

Data were processed using SPSS 25.0 software and demonstrated as mean ± SD. Data of two groups was analyzed by unpaired Student *t*-test, while data among groups was compared by one-way analysis of variance (ANOVA), $p < 0.05$ indicated a statistically significant difference.

Results

Characterization of Nano ZnO

The dynamic light scattering method was used to detect the particle size distribution of nano-ZnO particles in DMSO solvent. The results identified nano-ZnO in the experiment group, and the diameter was 21.13 ± 0.92 nm (Figure 1).

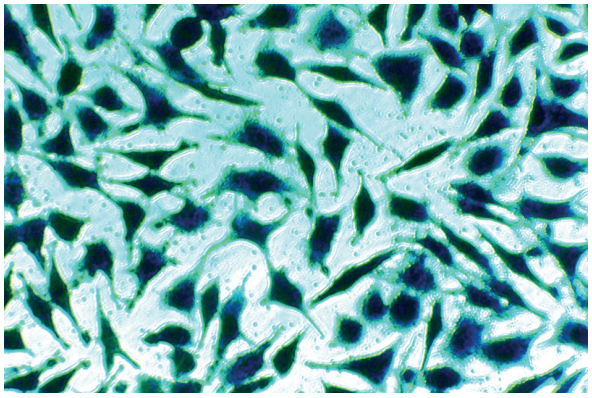

Figure 1. Characterization of nano-ZnO.

Distribution of GFP-LC3 Fluorescent Protein Particles

Under the fluorescence microscope, we found different numbers of fluorescent protein particles in cells after different treatments. The number of GFP-LC3 in nano-ZnO experimental group was significantly higher than that of the other groups, while more GFP-LC3 was observed in sorafenib control group relative to blank group (Figure 2).

GFP-LC3 and Caspase3 expression in liver cancer cells

The expressions of GFP-LC3 and apoptosis protein Caspase3 were detected by Western blot. Treatment with nano-ZnO, sorafenib or DMSO all increased the expression of GFP-LC3 and Caspase3 with the highest expression in nano-ZnO group ($p < 0.05$) (Figure 3).

Nano-ZnO Increases the Expression of p53 Gene

Results from RT-qPCR analysis demonstrated that p53 mRNA expression was elevated in the cancer cells of nano-ZnO group (14.79 ± 1.66) relative to sorafenib control group (7.84 ± 0.93), and blank group (3.05 ± 0.51) ($p < 0.05$). Treatment of sorafenib resulted in a higher level of p53 mRNA expression, compared to DMSO treatment ($p < 0.05$) (Figure 4).

D_{450nm} Value of Liver Cancer Cells Upon Treatment

The optical density values of the three groups of cells at 450 nm wavelength were detected by CCK-8 assay. Compared with blank group, D_{450nm} of the other two groups at 24 h, 48 h, and 72 h was significantly decreased ($p < 0.05$). After treatment with nano-ZnO, D_{450nm} value at 24 h, 48 h and 72 h were 0.56 ± 0.06, 0.39 ± 0.05, and 0.22 ± 0.04, which was significantly lower than sorafenib group ($p < 0.05$) (Figure 5).

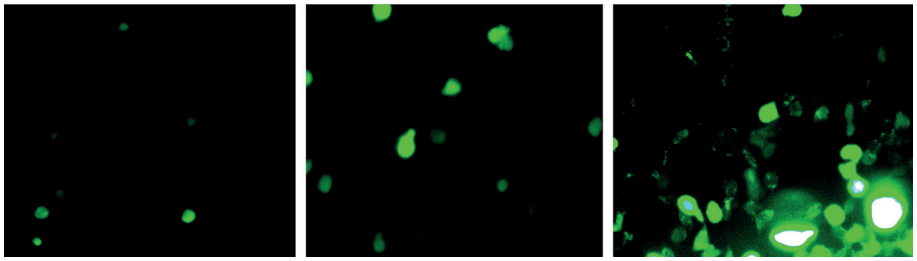

Figure 2. The distribution of GFP-LC3 fluorescent protein particles. Note: 1 Blank group; 2 Sorafenib control group; 3 Nano-ZnO group.

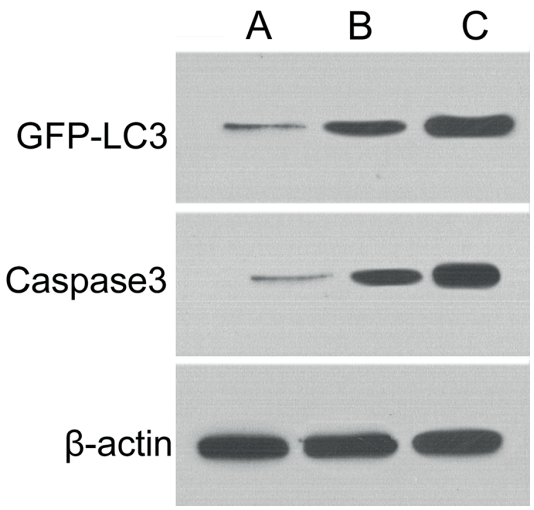

Figure 3. The expression levels of GFP-LC3 and Caspase3. Group (**A**), Blank group; Group (**B**), Sorafenib control group; Group (**C**), nano-ZnO group.

Nano-ZnO Promotes Cell Apoptosis

We then conducted flow cytometry to assess apoptosis of liver cancer cells upon treatment. According to the results, the apoptotic rate of nano-ZnO group was 83.11 ± 2.79%, which was significantly higher than that of blank group (16.39 ± 2.87%) and sorafenib control group (52.86 ± 3.02%) (Figure 6).

Discussion

Autophagy is a process in which lysosomes are activated to secrete molecules through various transmission pathways to degrade their own organelles and proteins, and other macromolecules. It can stabilize the energy and amino acids in the tissues, contributing to cell death, proliferation, and survival. Multiple stress-elicited signal transduction pathways can induce autophagy and apoptosis within the same cell, such as p53, BH3-only proteins, kinases (AKT, DAPK, and JNK)[10]. Autophagy increases the threshold of stress required for the induction of cell death and can also lead to the selective elimination of pro-apoptotic signal transducers. Caspase activation can induce the cleavage of various essential pro-autophagic proteins. Cell death frequently occurs with (or is preceded by) autophagy, but it is rarely truly mediated by autophagy. In some cases, autophagic membranes or individual autophagy-relevant proteins facilitate the activation of apoptotic or necrotic pathways, and this cell death mediated by autophagy is defined as autophagic cell death[10]. However, soluble products released by dying or dead cells can induce autophagy in neighboring cells *via* the activation of pattern recognition receptors (PRRs). Previous scholars[11] have shown that when nano-ZnO enter cells through endocytosis or cell membrane gaps, the particles induce cell oxidation and cytotoxicity. As the nano-zinc oxide particles interact with cells, the signal molecules in a stable state have interfered, and the reactive oxygen species are generated and aggregated. The concentration of reactive oxygen species rises rapidly in a short period of time, damaging intracellular DNA and protein and outer membrane of mitochondria, which affects cell productivity and further inhibits the functions of organelles. As a consequence, autophagy selectively degrades damaged organelles, proteins, and macromolecular substances, releasing energy and synthesizing important proteins that are required for amino acid raw materials[5]. Affected by nano-ZnO, autophagy signaling pathways are aberrant while excessive autophagy disturbs cell metabolism, which in turn triggers cell apoptosis[12]. In the progress of liver cancer, cell apoptosis plays a large role and thus is regarded as a reference indicator for evaluating the therapeutic effect of liver cancer[13].

In this study, under a fluorescence microscope, the production of the fluorescent GFP-LC3 was detected, suggesting the phenomena of autophagy in the liver cancer cells. We noticed various amount of GFP-LC3 in the three groups, where the nano-ZnO group exhibited the highest amount, meaning the highest autophagy in the group rela-

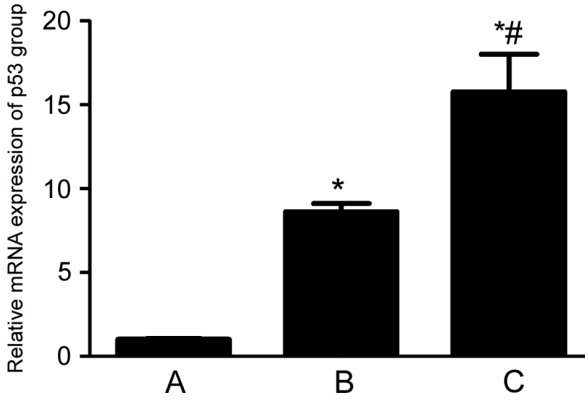

Figure 4. The expression level of P53 gene mRNA. Group (A), Blank group; Group (B), Sorafenib control group; Group (C), nano-ZnO group. *$p < 0.05$ *vs*. blank group; $p < 0.05$ *vs*. sorafenib control group.

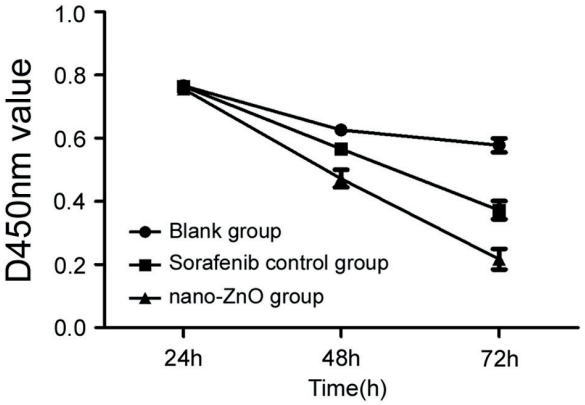

Figure 5. D_{450nm} values of three groups of cells.

tive to the other two groups. Consistently, Western blot analysis indicated the highest expression of GFP-LC3 in the nano-ZnO-treated cells (p<0.05). Normally, liver cancer cells have certain autophagy. After treatment with sorafenib and nano-ZnO, autophagy was stimulated, especially upon nano-ZnO treatment, which was consistent with the results of Prasad[14]. It is probably that nano-ZnO particles are highly targeted[15] and are not easily metabolized after entering cancer tissues. Then nano-ZnO might cause the accumulation of reactive oxygen species in cells for a period of time to promote autophagy. Sorafenib is highlighted to inhibit the activity of endothelial growth factor receptor[16] and type III tyrosine kinase[17], leading to inhibition of the growth of cancer cells.

Our study exhibited a consistent trend of the expressions of the apoptotic protein Caspase3 and the apoptotic gene p53. Upon nano-ZnO treatment, Caspase3 and p53 were significantly decreased, compared to that sorafenib and DMSO treatment ($p < 0.05$). CCK-8 assay and flow cytometry were performed to detect the optical density and cell apoptosis rate of the three groups of cells after 24 h, 48 h, 72 h. D_{450nm} value of the nano-ZnO group at each time was significantly lower than that of sorafenib control group and blank group, accompanied by highest apoptotic rate. The D_{450nm} value can reflect cell activity and cell proliferation. Thus, it is suggested that treatment of nano-ZnO can significantly inhibit cell prolifera-

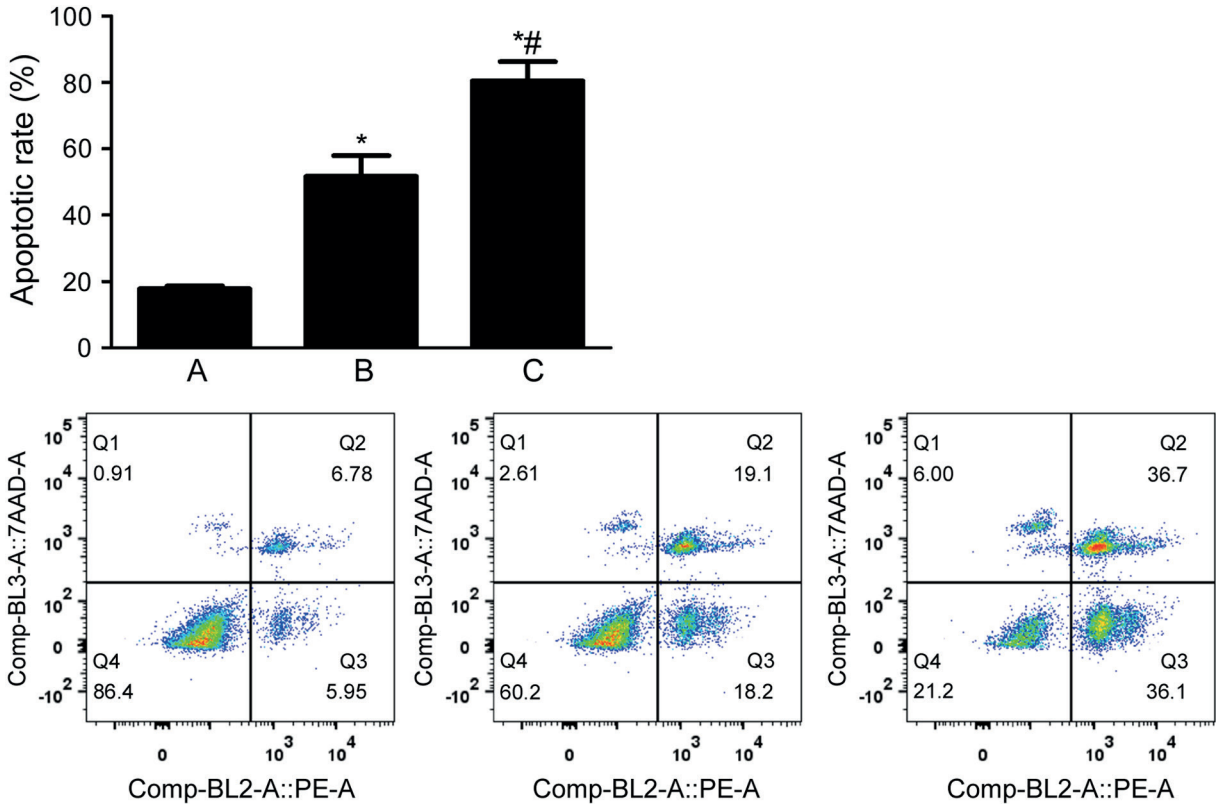

Figure 6. Apoptosis rate of liver cancer cells upon treatment. Group A, Blank group; Group B, Sorafenib control group; Group C, nano-ZnO group. *$p < 0.05$ *vs*. blank group; #$p < 0.05$ *vs*. sorafenib control group.

tion and promote apoptosis. It might be due to the fact that nano-ZnO enter the cell by changing the mitochondrial membrane potential[18], increasing the permeability of the outer mold, and activating the expression of P53 and Caspase3. As an important tumor suppressor gene, p53 is expressed through transcription and translation, specifically inhibiting the expression of Bcl-2[19] and promoting the apoptosis of cancer cells. Caspase 3 activates apoptosis, promotes the release of cytochrome C, activates apoptotic protease activator 1, decomposes DNA polymerase, and damages cell DNA, thereby reducing the proliferation of cancer cells and increasing apoptosis[20,21].

Conclusions

Our study for the first time shows that in liver cancer cells, nano-ZnO can promote autophagy, upregulate the expression of P53 and Caspase3, and trigger the apoptosis, thereby inhibiting liver cancer cells growth and proliferation, which is the novelty of our study. This method of treating liver cancer with nano-ZnO is worthy of being further explored in clinic.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by Hubei Province Natural Science Foundation (NO.2020CFB508), Hubei Province Health and Family Planning Scientific Research Project (No. WJ2019M008), Wuhan Municipal Science and Technology Bureau of applied basic research project (No. 2019020701011484), Wuhan City Health Bureau of Medical Research project.

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