Evaluation of Metastasis Suppressor Genes Expression and In Vitro Anti-Cancer Effects of Zinc Oxide Nanoparticles in Human Breast Cancer Cell Lines MCF-7 and T47D

Seyed Ataollah Sadat Shandiz 1, Faryad Sharifian 2, Soraya Behboodi 3, Fatemeh Ghodratpour 2, and Fahimeh Baghbani-Arani 2*.

1. Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran
2. Department of Genetics and Biotechnology, Faculty of Biological Sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran
3. Department of Biology, Tehran Shargh (East), Payam Noor University, Tehran, Iran

Abstract

Background: Metallic nanoparticles are useful materials to be applied in biomedical research. In this study, the possible apoptotic and anti-metastatic activity of Zinc Oxide Nanoparticles (ZnONPs) was assessed in breast cancer cells.

Methods: First, in vitro cell viability was investigated by MTT assay in two human breast cancer cells (MCF-7 and T47D) and normal Human Embryonic Kidney (HEK293) cells at 37 °C overnight. Apoptosis induced by ZnONPs was evaluated by annexin V/PI staining, cell cycle analysis and caspase assay in cancerous cells. Moreover, quantitative real-time PCR was employed for the detection of two metastasis suppressor genes (KAI-1 and NM23) expression in cancerous cells.

Results: Data demonstrated that ZnONPs exert a dose-dependent inhibitory effect on the viability of T47D and MCF-7 cells, while no cytotoxic effect was observed on normal HEK293 cells. The mRNA expression levels of KAI-1 and non-metastatic protein (NM23) genes were up-regulated in ZnONP-exposed cancerous cells. ZnONPs were also found to enhance the apoptosis properties of cells by annexin V/PI staining, and caspase assay in cancerous cells. Furthermore, ZnONPs can increase sub-G1 population as compared to negative control.

Conclusion: Our findings showed that ZnONPs induce apoptotic activity and can modulate metastasis by up-regulating of KAI-1 and NM23 gene expression in two breast cancer (MCF-7 and T47D) cells.

Keywords: Apoptosis, Breast neoplasms, Cell cycle, Zinc oxide

Introduction

Breast cancer is the second most frequent malignant tumor in women, with 1.62 million new cases reported in 2012.1,2 Although conventional therapeutic options such as surgery, chemotherapy and radiation therapy have been recommended for treatment of breast cancer, all these approaches suffer from major drawbacks.3 In this regard, nanotechnology has provided considerable advancement as a promising area in medicine, drug delivery and anti-cancer applications.4

The Zinc Oxide Nanoparticle (ZnONP) has been increasingly explored for biomedical applications, particularly due to its anti-cancer properties.5 Moreover, ZnONPs have been investigated for a wide variety of applications like functioning as nanosensors, energy storage materials, nano-electronic/nano-optical devices, cosmetic products, etc.6,7 ZnONPs are being used in a broad spectrum such as gene/drug delivery and cancer therapy, with a focus on cell cytotoxicity induction at in vitro and in vivo levels.8,9

Recently, several studies have suggested the cytotoxic effects of ZnONP against several types of cancers. Wahab et al reported that about 13±2 nm ZnO nanoparticles induced significant apoptosis in HepG2 cells and human MCF-7 cells, with reactive oxygen species and oxidative stress being the major component in cytotoxicity.9,10 As more knowledge is obtained regarding alteration of gene expression during tumor progression, more potential methods are suggested for inhibiting metastatic cascades.

In this way, Metastasis Suppressor Genes (MSGs) have frequently been identified for the regulation of various cellular responses like cell invasion, tumor progression, and programmed cell death.10,11
gesting down-regulated NM23 gene expressions is related to several types of high metastatic potential cancer cells. Also, different studies have indicated that KAI1 inhibits metastasis of melanoma and breast cell lines.\(^{12,13}\) Regarding its role as a metastasis suppressor, down regulation of KAI-1 gene expression is mostly demonstrated during ovarian and prostate cancers.\(^{14,15}\) The current study investigated the apoptotic effects of ZnONPs via caspase assay, annexin V-FITC/PI staining, and cell cycle analysis against two human breast cancer cell lines. Also, real-time PCR was used to measure the expression of two metastasis suppressor genes (KAI-1 and NM23).

**Materials and Methods**

**Preparation of ZnONP**

ZnONP with an average size of 10 to 30 nm were supplied by US Research Nanomaterials, Inc. (Twig LeafLane, Houston, USA). The size and morphological characteristics of ZnONPs were determined by transmission electron microscope (Zeiss-EM10C-100 KV, Germany).

**Cell culture**

A Human Embryonic Kidney (HEK293) and two breast cancer (MCF-7, T47D) cells were purchased from the National Cell Bank, Pasteur Institute of Iran. The cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS in CO\(_2\) incubator at 37°C. The cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS in CO\(_2\) incubator at 37°C.

**MTT assay**

To reveal the dose-dependent cytotoxic effects of ZnONPs, MTT assay was used. T47D, MCF-7 and HEK293 cell lines were seeded (Density rang of 1x10^4 cells per well) in 96-well micro-plates containing DMEM medium, and allowed to attach for 24 hr. After overnight incubation, the cells were treated with various concentrations of ZnONPs ranging from 1, 10, 100, to 1000 μg/ml for overnight. After the exposure was done, the MTT dye (5 mg/ml) solution was added to each well for four hr. For dissolving the MTT formazan products, the culture medium was removed from each well followed by transferring equal amount of Dimethyl Sulfoxide (DMSO). The cells were incubated with DMSO at 37°C for 30 min and absorption was calculated at 570 nm.

**Analysis of KAI-1 and NM23 gene expression**

The T47D and MCF-7 cell lines at a density of 5x 10^6 cells/ml were cultured into six-well plates followed by incubation at 37°C in CO\(_2\) incubator. After 24 hr of incubation, the cells were treated with ZnONPs for another 24 hr. Total RNA isolation from the T47D and MCF-7 cell lines was conducted using the EasyPure® RNA Kit (TransGen Biotech ER101-01, China) according to the manufacturer’s protocol. The cDNA synthesis was done using the EasyScript® First-Strand cDNA Synthesis Kit (TransGen Biotech AE301-02, China) based on the manufacturer’s protocol. The sequence of forward and reverse specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), KAI-1, and NM23 genes was designed using Primer Express Software version 3.0 (Table 1) and verified using BLAST analysis at NCBI (www.ncbi.nlm.nih.gov/blast). The GAPDH was used as the housekeeping gene for normalization within a given sample. The quantitative real-time polymerase chain reaction was performed in ABI7300 real-time PCR system (Applied Biosystems Company, USA) with SYBR GREEN® (Non-specific DNA-binding factors) based on the manufacturer’s protocol. The relative quantifications of NM23 and KAI-1 mRNA expression levels were compared with the housekeeping gene (GAPDH) using the 2^(-ΔΔCt) method.\(^{16}\)

**Caspase assay**

In vitro quantitative determination of caspase 8 and 9 proteolytic enzyme activities were evaluated in the MCF-7 and T47D cells (3-5x10^6 cells per well) based on the procedure of ApoTarget™ Caspase Colorimetric Protease Assay (Invitrogen) kit. Lysis of the cells was performed using caspase assay buffer and cells were incubated with the caspase 8 substrate (IETD-pNA) and the caspase 9 substrate (LEHD-pNA) for two hr at 37°C in a dark room. The absorbance of both untreated and treated samples was calculated at 400 or 405 nm. Fold-increase in caspase 8 and 9 activities of treated cells was evaluated by comparison with the level in the untreated control group.

**Annexin V-FITC/PI staining**

ZnONPs-induced apoptosis in cells was performed using annexin V-FITC and PI double staining detection kit (Hoffman-La Roch Ltd., Basel, Switzerland) via flow cytometry. The MCF-7 and T47D cells (1x10^6) were treated with ZnONPs in a six-well plate, for overnight incubation followed by twice washing with cold PBS and centrifugation at 3,000 rpm for 5 min. Thereafter, 500 μl of binding buffer were added to the cells. Then, the cells were stained in annexin V-FITC and PI and incubated at 25°C for 15 min in darkness. Finally, the percentages of apoptosis and necrosis of treated and untreated cells were determined by a flow cytometer (Cyflow, UK).

**Cell cycle analysis**

T47D and MCF-7 cells were treated with ICS0 concentration of ZnONPs. The two cells were harvested by

| Gene     | Primer sequence                  |
|----------|----------------------------------|
| NM23     | Anti-sense: 5’-ATGGCCAACACTGTAGGCTACC-3’<br>Sense: 5’-CATGATTTTCCACCGCCGC-3’<br>Anti-sense: 5’-CTCAAGCCTTGATCAAGTCACCA-3’<br>Sense: 5’-CCACGCGGTAGAAGACATA-3’<br>Anti-sense: 5’-CCCCACTCTCCACCTTTGAC-3’<br>Sense: 5’-CATAACAGGAAATMGACCTTGACAA-3’<br>Anti-sense: 5’-CTGAGACAGCAGCCGC-3’<br>Sense: 5’-CATGATTTTCCACCGCCGC-3’<br>Anti-sense: 5’-CTCAAGCCTTGATCAAGTCACCA-3’<br>Sense: 5’-CCACGCGGTAGAAGACATA-3’<br>Anti-sense: 5’-CCCCACTCTCCACCTTTGAC-3’<br>Sense: 5’-CATAACAGGAAATMGACCTTGACAA-3’<br>|
trypsin and washed with PBS, followed by fixation in ice-cold 70% ethanol at 4°C for 30 min. The cells were washed in PBS twice and treated with 100 µg/ml of RNase, and then stained with propidium iodide for 1 hr at 25°C in the dark.

**Statistical analysis**

All data were statistically analyzed using SPSS software version 22. The values were represented as the mean±standard deviation and differences between test and control were evaluated using one-way ANOVA.

**Results and Discussion**

**TEM images**

The size and morphological characteristics of ZnONPs were determined by Transmission Electron Microscope (TEM) and it was revealed that ZnONPs images were spherical in shape (Figure 1).

**Cytotoxicity results**

The ZnONPs’ effects on the cell viability of T47D, MCF-7 and HEK293 cells were assessed using the MTT assay. The cancer and normal cells were exposed to ZnONPs for 24 hr at dose levels of 1-1000 µg/ml. The data showed that ZnONPs up to the maximum concentration of 1 µg/ml did not significantly increase the toxicity of cancer cells. In the case of MCF-7 cells, after treatment with various concentrations of ZnONPs, a decrease in cell viability was noticed. According to figure 2, it can be seen that the T47D cells showed much higher sensitivity than MCF-7 cells. Figure 2 indicates that the cell viability of HEK293 after exposure to ZnONPs showed no obvious cytotoxic effect at any concentration ($p>0.05$).

In the present study, higher mortality was observed for T47D cells than MCF-7 cells and the anti-cancer effect was enhanced following the increase of ZnONPs concentration.

**Real time PCR**

Regulation of metastasis suppressor genes plays a prominent role in the metastasis of breast cancer. Various investigators have revealed that loss of KAI-1 and NM23 gene expression has been correlated with the degree of metastatic potentials of various types of cancers 17-21. Bozdogan et al reported that NM23 and KAI1 genes were down-regulated simultaneously in tumor progression of non-small cell lung cancer 22. Moreover, Stark et al described reduction of metastasis suppressor genes including NM23, KAI1, BRMS1, and Mkk4 in breast cancer brain metastases 23.

In the current study, T47D and MCF-7 cancer cells were treated with ZnONPs at IC$_{50}$ concentration for 24 hr and quantitative real-time PCR was carried out to evaluate the mRNA levels of KAI-1 and NM23 genes. It can be seen that ZnONPs up-regulated the mRNA expression levels of these genes in T47D and MCF-7 cells. The mRNA level of KAI-1 and NM23 gene (Figure 3) was enhanced in MCF-7 cells in the treated cells compared with the untreated cells. Also, mRNA expression of KAI-1 and NM23 (Figure 4) was significantly up-regulated (3.9 and 7.6 fold, respectively) in ZnONP-exposed T47D cells as compared with the untreated control cells ($p<0.001$ for each). The current study suggested that ZnONPs could inhibit metastasis.
Anti-cancer Properties of Zinc Oxide Nanoparticles Against Breast Cancer Cells

in T47D and MCF-7 cells through the up-regulation of mRNA level of KAI-1 and NM23 genes.

Apoptotic properties

Apoptosis is a critical physiological process of cell death determined by different morphological and biochemical hallmarks.

Many studies have suggested that nanoparticles can generate ROS products and activate the caspase family of proteins followed by the liberation of cytochrome c from the inter membrane space of mitochondria. Activation of caspase proteins, as cysteine protease, is responsible for the execution phase of apoptosis. Among the many proteins involved in apoptosis, caspases' activities are critical for further investigation. The activation of caspase 8 can initiate extrinsic (Death-receptor) pathway, while the activation of caspase 9 triggers intrinsic pathway of apoptosis via mitochondria. To clarify the caspase activity through apoptosis, impact of ZnONPs on caspase 8 and caspase 9 activities was measured in T47D and MCF-7 cells.

The results showed that caspase 9 activity of MCF-7 and T47D cells was enhanced 2.30 and 1.70 times, respectively after treatment with ZnONPs, as compared with untreated cells, while no significant change in caspase 8 activity was observed in treated cells, comparing with those of the untreated cells (Figures 5 and 6). These results suggest that the intrinsic pathway in mitochondria via caspase-dependent pathway might be involved in nanoparticles-induced apoptosis.

In our study, significant apoptotic activity of ZnONPs was detected in comparison to necrosis in MCF-7 and T47D cells (Figure 7). The percentage of apoptotic cells following IC50 concentration of ZnONPs was 7.23 and 62.95 in MCF-7 and T47D cells, respectively. Previous reports suggest the apoptotic activity of ZnONPs in different types of cancers. The current investigation revealed that the apoptosis of cells by ZnONPs was related to the type of cancer cells. It was shown that T47D cells were less sensitive to ZnONPs than MCF-7 cells. The result of flow cytometry was in agreement with that of Hoechst 33258 staining and caspase assay and confirmed the high apoptotic potential of ZnONPs.

The cell cycle progression of cancer cells was evaluated with ZnONPs by flow cytometry. Figure 8 indicates that ZnONPs can increase sub-G1 population as compared to negative control. Interestingly, such data demonstrate that ZnONP induce DNA fragmentation and arrest all stages of the cell cycle. Similarly, Borumand Moghaddam et al. reported that the green synthesized ZnONPs induced apoptosis (sub-G1 stage) in MCF-7, with the IC25, IC50 and IC75 concentrations of nanoparticles at 24 hr incubation time.

Conclusion

In summary, our findings confirm the previous reports about the anti-cancer properties of ZnONPs and indicate that these nanoparticles can increase the rate of MCF-7 and T47D cancer by inducing cell death. The current research confirms that ZnONPs increase the mRNA expression levels of two metastasis suppressor genes (KAI-1 and NM23). Therefore, the nanoparticles
may interfere with the metastasis pathway through the up-regulation of KAI-1 and NM23. In our study, the caspase assay data of the two human breast cancer cells exposed to ZnONPs revealed significant increase of caspase 9 activities. Apoptosis assay via flow cytometry, cell cycle assay and cell morphology after treatment with nanoparticles can strongly confirm the anticancer properties of ZnONPs. In addition, ZnONPs can increase sub-G1 population of breast cancer cells. Overall, ZnONPs may be a potential agent for the killing of cancerous cells.

Conflict of Interest
All of the authors report no conflicts of interests in this study.

References
1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136(5):E359-86.
2. Pedersen RN, Bhaskaran K, Heide Jorgensen U, Norgaard M, Christiansen PM, Kroman N, et al. Breast cancer recurrence after reoperation for surgical bleeding. Br J Surg 2017;104(12):1665-74.
3. Lee J, Chatterjee DK, Lee MH, Krishnan S, Gold nanoparticles in breast cancer treatment: Promise and potential pitfalls. Cancer Lett 2014;347(1):46-53.

4. Rasmussen JW, Martinez E, Louka P, Wingett DG. Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. Expert Opin Drug Deliv 2010;7(9):1063-77.

5. Chandrasekaran M, Pandurangan M. In vitro selective anti-proliferative effect of zinc oxide nanoparticles against co-cultured C2C12 myoblastoma cancer and 3T3-L1 normal cells. Biol Trace Elem Res 2016;172(1):148-54.

6. Malhotra SPK, Mandal TK. Biomedical applications of Zinc Oxide nanomaterials in cancer treatment: a review. SCIREA J Chem 2016;1(2):67-89.

7. Yakimova R, Selega L, Khranovskyy V, Pearce R, Spetz AL, Udval K. ZnO materials and surface tailoring for biosensing. Front Biosci (Elite Ed) 2012;4:254-78.

8. Akhtar MJ, Ahamed M, Kumar S, Khan MM, Ahmad J, Alrokayan SA. Zinc oxide nanoparticles selectively induce apoptosis in human cancer cells through reactive oxygen species. Int J Nanomed 2012;7:845-57.

9. Wahab R, Siddiqui MA, Saquib Q, Dwivedi S, Ahmad J, Musarrat J, et al. ZnO nanoparticles induced oxidative stress and apoptosis in HepG2 and MCF-7 cancer cells and their antibacterial activity. Coll Surfaces B: Biointerfaces 2014;117(1):267-76.

10. Buxton ILO, Yokdang N. Extracellular Nm23 Signaling in breast cancer: incommodus verum. Cancers (Basel) 2011;3(3):2844-57.

11. Howlett AR, Petersen OW, Steeg PS, Bissell MJ. A novel function for the nm23-H1 gene: overexpression in human breast carcinoma cells leads to the formation of basement membrane and growth arrest. J Natl Cancer Inst 1994;86(24):1834-44.

12. Phillips KK, White AE, Hicks DJ, Welch DR, Barrett JC, Wei LL, et al. Correlation between reduction of metastasis in the MDA-MB-435 model system and increased expression of the KAI-1 protein. Mol Carcinog 1998;21(2):111-20.

13. Wei LL, Yang X, Phillips KK, Weissman BE, Welch DR. Analysis of KAI-1 mRNA expression in human breast cancer cell lines. Proc Am Assoc Cancer Res 1996;37:529.

14. Hurst DR, Welch DR. Metastasis suppressor genes: at the interface between the environment and tumor cell growth. Int Rev Cell Mol Biol 2011;286:107-80.

15. Liu FS, Dong JT, Chen JT, Hsieh YT, Ho ES, Hung MJ. Frequent down-regulation and lack of mutation of the KAI1 metastasis suppressor gene in epithelial ovarian carcinoma. Gynecol Oncol 2000;78(1):10-15.

16. Moshfegh A, Salehzadeh A, Shandiz SAS, Shafagh M, Naemi AS, Salehi S. Phytochemical analysis, antioxidant, anticancer and antibacterial properties of the Caspian sea red Macroalgae, Laurencia caspica. Iran J Sci Technol Trans Sci 2019;43(1):49-56.

17. Foldbjerg R, Olesen P, Hougaard M, Dang DA, Hoffmann HJ, Autrup H. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol Lett 2009;190(2):156-62.

18. Arora S, Jain R, Rajiwade JM, Paknikar KM. Cellular responses induced by silver nanoparticles: In vitro studies. Toxicol Lett 2008;179(2):93-100.

19. Asare N, Instanes C, Sandberg WI, Refsnes M, Schwarze P, Kuszewski M, et al. Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. Toxicology 2012;291(1-3):65-72.

20. You J, Chang R, Liu B, Zu L, Zhou Q. Nm23-H1 was involved in regulation of KAI1 expression in high-metastatic lung cancer cells I. 9981. J Thorac Dis 2016;8(6):1217-26.

21. Yoshida BA, Sokoloff MM, Welch DR, Rinker-Shaeffer CW. Metastasis-suppressor genes: A review and perspective on an emerging field. J Natl Cancer Inst 2000;92(21):1717-30.

22. Bozdogan O, Yulug IG, Vargel I, Cavusoglu T, Karabulut AA, Karahan G, et al. Differential expression patterns of metastasis suppressor proteins in basal cell carcinoma. Int J Dermatol 2015;54(8):905-15.

23. Stark AM, Tongers K, Maass N, Mehndorff HM, Held-Feindt J. Reduced metastasis-suppressor gene mRNA-expression in breast cancer brain metastases. J Cancer Res Clin Oncol 2005;131(3):191-8.

24. Sheikh BY, Sarker MMR, Kamarudin MNA, Mohan G. Antiproliferative and apoptosis inducing effects of citral via p53 and ROS induced mitochondrial-mediated apoptosis in human colorectal HCT116 and HT29 cells line. Biomed Pharma 2017;96:834-46.

25. Asharani PV, Hande MP, Vaiyaveettil S. Anti-proliferative activity of silver nanoparticles. BMC Cell Biol 2009;10:65.

26. Turk B, Stoka V. Protease signalling in cell death: cascades versus cysteine cathepsins. FEBS Letters 2007;581(15):2761-7.

27. Puiso J, Jonkuviute D, Macioniene I, Salomskiene J, Jasutiene I, Kondrotas R. Biosynthesis of silver nanoparticles using lingonberry and cranberry juices and their antimicrobial activity, Coll Surfaces B: BioInterfaces 2014;121:214-21.

28. Krishna PG, Ananthaswamy PP, Yadavalli T, Mutta NB, Sannaiah A, Shivanna Y. ZnO nanoplatelets have selective anticancer activity. Mater Sci Eng C Mater Biol Appl 2016;62:919-26.

29. Pandurangan M, Enkhtaigan G, Kim DH. Anticancer studies of synthesized ZnO nanoparticles against human cervical carcinoma cells. Photochem J Photobiol B Biol 2016;58:206-11.

30. Mariappan P, Krishnamoorthy K, Kadarkaraithangam J, Govindasamy M. Selective toxicity of ZnO nanoparticles toward gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. Nanomedicine 2011;7(2):184-92.

31. Boroumand Moghaddam A, Moniri M, Azizi S, Abdul Rahim R, Ariff AB, Navadei M, et al. Eco-friendly formulated zinc oxide nanoparticles: induction of cell cycle arrest and apoptosis in the MCF-7 cancer cell line. Genes (Basel) 2017;8(10):281.