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Anticancer Impact and FOXM1 Regulation of Zinc Oxide Nanoparticles on HCT116 Colorectal Carcinoma Cell Line

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Abstract

The second leading cause of mortality throughout the world is colorectal cancer (CRC), and the existing treating of this situation needs to be improved. Many efforts had been focused on the therapeutic approach of cancer for a long time studying nanoparticles activity. In this study, zinc oxide nanoparticles (ZnO-NPs) were applied against the HCT116 colorectal carcinoma cell line. Methods of current work were included in vitro ZnO-NPs cytotoxicity by thiazolyl blue tetrazolium bromide (MTT) cytotoxicity test on the HCT116 cells in comparison with standard chemotherapeutic drugs that were included Doxorubicin (DOX), cyclophosphamide (CPH) and 5-fluorouracil (5-FU), furthermore, the assessment of Forehead box M1 gene (FOXM1) expression was evaluated using Livak method based on Real-time PCR technique. Results showed that ZnO-NPs were effectively and significantly inhibited the cell proliferation ($p<0.0005$) by decreasing the viability of the HCT116 cells at different concentrations involved 1, 10, 100, 500 and 1000 µg/ml, with 27.327 µg/ml half-maximal inhibitory concentration (IC50). DOX, CPH, 5-FU IC50 values were 51.112, 30.897µg/ml and 39.72 µg/ml respectively. While FOXM expression in HCT116 cell line revealed a significant effect of down regulation after treatment with different concentrations of ZnO-NPs when compared with untreated cells; (highest relative expression equal to 1.45555E-06) as compared with untreated cells (relative expression =2.462288827). Our finding that ZnO-NPs nanoparticles have an anticancer effect against HCT116 colorectal carcinoma with FOXM1down regulation

Keywords: HCT116; FOXM1; ZnO-NPs Nanoparticles; MTT Assay

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Introduction

Cancer is one of the primordial diseases that causes a high rate of mortality throughout the world, which is a global issue majorly affecting developing countries. Colorectal cancer (CRC) is a leading cause of cancer mortality, and current strategies for treating this condition need to be more improved [1]. Classical cancer therapies that are still clinically used include chemotherapy, radiotherapy as well as surgery. However, using chemotherapy or radiotherapy has proven to have significant restrictions. The initial disadvantage of chemotherapy is its lack of specificity, it also damages the adjacent normal cells/ tissues, and organs that lead to the induction of multidrug resistance (MDR) during the period of treatment, and these limitations can go as far as recurrence [2-5]. Many alternatives like Liposomes, polymeric molecules, gold, and magnetic nanoparticles have been examined as potential candidates for cancer therapy. These nanoparticles exhibit impressive properties such as versatility, functionality, biocompatibility, and other specific features. Great improvements have been made so far for the use of nano-biomaterials in cancer therapy. However, there are still many challenges ahead, and an advanced understanding of the biological features is needed to design systems with tailor-made after exposure to carcinogens [6]. Emerging evidence suggests that over expression of FOXM1 gene levels promotes subsequent cancer progression and associate with a variety of chemotherapy-resistant and aggressive human cancers, such as CRC, breast, prostate, and cervical cancers [7]. Conversely, the depletion of the FOXM1 gene is associated with carcinogenesis and growth reduction of CRC. The improvement of anticancer drug selectivity and efficacy is regarded as one of the major challenges in cancer therapy to reduce their side effects that result in the improvement of life quality for cancer patients. ZnO-NPs have been reported that induce selective killing of variable cancer cells. However, the underlying molecular mechanisms forcing the ZnO-NPs anticancer response remain unclear [8]. In this study, the effect of ZnO-NPs on FOXM1 gene down regulation against HCT116 colorectal carcinoma cell line in comparison with doxorubicin (DOX), cyclophosphamide (CPH) and 5- fluorouracil (5-FU) is considered. This work aimed was to support the hypothesis that suggests ZnO-NPs may induce cell toxicity in a specific manner, hence, received much attention for their implications in cancer therapy.

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Materials and Methods

ZnO-NPs Characterization

The nanoparticles of ZnO (Sky spring Nonmaterial, USA) were of 10-30 nm in diameter and purity of 99.8%. Their size was further confirmed with X-ray diffraction (XRD), Energy dispersive X-ray Spectroscopy (EDS) and scanning electron microscopy (SEM) as we mentioned in our data published in previous work (data not shown) [9].

MTT Cytotoxicity Assay

Cells of HCT116 (ATCC No CCL-247) colorectal carcinoma cell lines HCT116 were kindly provided by Dr. Hamid Naji/Pharmacology and Toxicology department/Staff of Laboratory of tissue culture/Faculty of Medicine/University of Babylon and maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.6% Penicillin-Streptomycin at 37°C in a humidified and 5% CO2 atmosphere [10]. The cytotoxicity of ZnO-NPs, DOX, CPH, and 5-FU was evaluated by MTT (3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [11-13]. Cells of HCT116 were seeded onto 96 well plates with a concentration of $l0^4$ cells/ml and incubated at the temperature of 37°C for 48-72 h. ZnO-NPs were suspended in cell culture medium and diluted to appropriate concentrations (1, 10,100, 500 and 1000 µg/ml, then the dilutions of ZnO-NPs were then sonicated using a Sonicator bath at room temperature for 10 minutes at 40 W to avoid nanoparticles (NP) agglomeration before cell exposure. When the confluent monolayer of HCT116 cells was completely formed (80%-100%), different microtiter compounds were added to cultured wells except for control in the triplicate at 100 µl volume. The plates were incubated for 48 hrs. at 37°C in 5% CO_2. Then all media were discarded from microtiter plate's wells, to remove any residual amount of composites or standard anticancer drugs due to avoid interaction with the final results. The HCT116 cell monolayer were washed 3 times with PBS solution (pH=7.2). The remaining materials (100 µl) were added to all wells included drugs/ZnO-NPs treated cells, drugs/ZnO-NPs untreated cells, and blank wells. After that, 20 µl of MTT reagent (bio WORLD, USA) was added to each well under 3-4 hrs. of incubation at the temperature of 37°C, 5% CO_2, the formazan granules were formed as a mitochondrial enzymatic process of proportional correlation with HCT116 cells viability. The formazan was solubilized by adding diluted dimethyl sulfoxide (DMSO): isopropanol (1:1) on each well included blank wells, then absorbance was read at 490 nm/reference wavelength of 630 nm by ELISA reader.

RNA Extraction

Total RNA samples were extracted using SV Total RNA Isolation System (Promega Co.; USA) from cell line HCT116 colorectal cancer following the protocol provided by the manufacturer. The estimation of nucleic acid purity and concentration of total RNA was assessed according to Usman T, et al. (2014) and Imbeaud S, et al. (2005), by using Scan drop spectrophotometer (Biodrop, UK (and RNA was considered pure if A_{260}/A_{230} equal to A_{260}/A_{280} and >1.8< 2.1 [14,15].

Reverse Transcription

RNA (1µg) was reversely transcribed using Easy Script plus ™cDNA synthesis Super Mix (ABM; Canada). The protocol was carried out in a 20 µl total volume reaction following the protocol provided by the manufacturer (abm/ Canada). The product of cDNA was then stored at -80°C until real-time analysis performance.

Real-Time RT-PCR Assay

The primer pairs of the transcription factor FOXM1 gene designed specifically by Beacon Designer™ software to cover all variant of FOXM1 gene, forward 5'-AGCAGTCTCTTACCTTCC-3', and reverse 5'-CTGGCAGTCTCTGGATAA-3', while 18SrRNA was chosen as a housekeeping gene to assess the relative expression of intended gene in qPCR, with Oligonucleotide involve forward sequence 5'-CCTGCGGCTTAATTTGACTCA-3' and reverse 5'-AGCTATCAATCTGTCAATCCTGTCC-3'. Quantitative real-time PCR assays were performed in standard duplicate using Bright Green 2X qPCR Master Mix (abm/ Canada) in 20 µl reaction volume containing 10 µl of master mix (Bright Green master mix), 0.6 µl of each forward and reverse primer mixes, 5 µl of RNase free water and 3.8µl of cDNA template on the 3005p Agilent Real-time PCR system (Agilent / USA). Real-Time PCR protocol was as follows; step 1: 95°C for 10 minutes, step 2: 95 °C for 15 Sec and 60 °C repeated 40 cycles, then in step 3: dissociation curve was used to assess the specificity of the amplified products. Samples for no-RT and no-template were also included in each run to distinguish any DNA interference or RNA contamination. The expression levels of the FOXM1 gene from the cDNA were measured by quantitative real-time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method). The fold-change in gene expression was normalized to a housekeeping gene 18S rRNA and relative to a calibrator sample [16-18].

Statistical Analysis

Statistical processing (Mean, SEM and R2 and one-way ANOVA) were performed using IBM SPSS 21.0 for windows. In all tests, a P value of less than 0.05 was considered statistically significant, halve inhibitory concentration was fitted by the blotting of inhibition percentage versus the log of the concentration of any compound used. Relative quantification of Livak method was used to assess FOXM1 gene expression based on qRT-PCR relative to a housekeeping gene. Statistical significant differences were considered at P<0.05 in all calculations.

Results

Results of Anticancer assay of ZnO-NPs, DOX, CPH, and 5-FU

Anticancer activity of ZnO-NPs, DOX, CPH, and 5-FU was carried out by MTT assay on the HCT116 colorectal carcinoma cell line. After 48 hours, cell culture was examined under an inverted microscope. MTT cytotoxicity assay revealed highly significant differences between untreated cells that showed pure, confluent HCT116 cell line monolayer when compared with those treated with different concentrations of each compound (P<0.005). The ZnO-NPs compound showed significant anticancer activity against the HCT116 cell line, with IC50 value of 27.327µg/ml, which is highly significant (p<0.0001). Conventional anticancer drug DOX had shown IC50 value equal to 51.112 µg/ml and a positive Spearman correlation coefficient of 0.6929. Cyclophosphamide also revealed IC50 of 30.897 µg/ml while 5-FU showed IC50 value of 39.72 µg/ml with a positive Spearman correlation coefficient of 0.7766. There were no significant differences between ZnO-NPs and other conventional drugs according to the IC50 values. Results of ZnO-NPs, DOX, CPH, and 5-FU IC50 and R^2 were shown in table 1 and figure 1.

The values of growth inhibition percentage revealed that significant differences according to the gradual concentrations of each compound

Table 1: Cytotoxicity of ZnO-NPs presented by IC50, and R^2 values.

Compound potency and dose dependency	ZnO-NPs*	DOX	CPH	5-FU
Log IC50	1.436	1.708	1.489	1.599
IC50 (µg/ml)	27.327	51.112	30.897	39.72
R^{2**}	0.9656	0.6929	0.9839	0.7766

*ZnO-NPs=Zinc oxide nanoparticles; DOX=Doxorubicin; CPH=Cyclophosphamide; 5-FU=5-Flourouracil; IC50= Half maximal inhibitory concentration; R^2=correlation coefficient of concentration log *vs*. growth inhibition %.

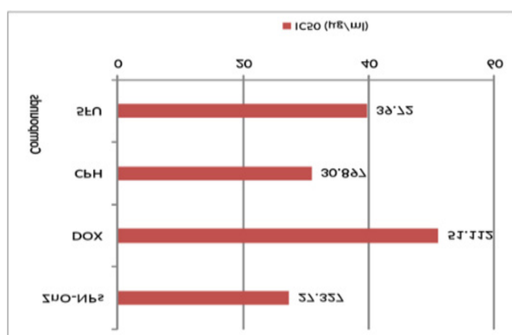

Figure 1: The inhibitory concentration 50 of ZnO-NPs presented by µg/ml as compared with DOX, CPH, and 5-FU.

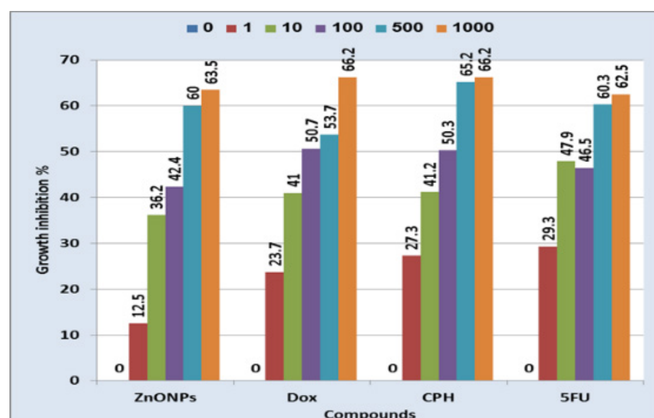

Figure 2: Values of growth inhibition percentage after treatment of HCT116 cell line with different concentrations of ZnO-NPs as compared with DOX, CPH, and 5-FU.

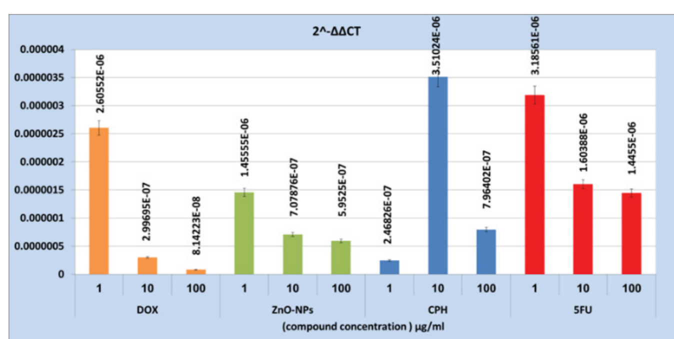

Figure 3: The relative expression of the FOXM1 gene after treatment of HCT116 cell line with different concentrations of ZnO-NPs as compared with DOX, CPH, and 5FU presented by $2^{-\Delta\Delta CT}$ with SD values.

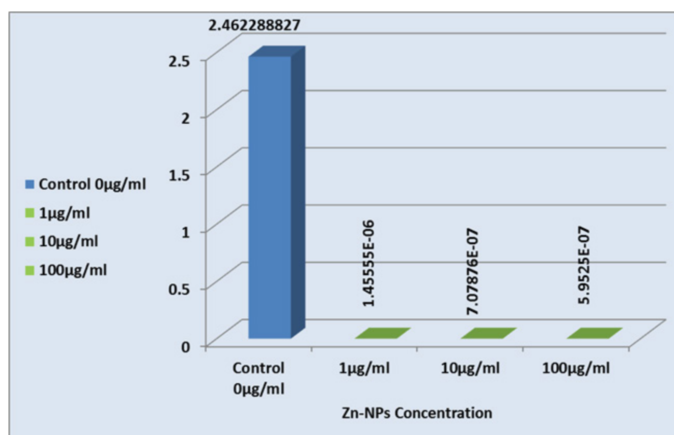

Figure 4: The relative expression of FOXM1 gene after treatment of HCT116 cell line with different concentrations of ZnO-NPs as compared with control untreated cells presented by $2^{-\Delta\Delta CT}$.

($p<0.05$). At the same time, dose-dependent variation was seen for compounds including ZnO-NPs, DOX, and CPH, while 5-FU was pointed out in a different inhibitory manner. The values of growth inhibition percentages were shown in figure 2.

Results of Foxm1 gene expression on ZnO-NPs, DOX, CPH, and 5-FU

The relative expression of FOXM1 gene after treatment of HCT116 cell line with different concentrations of ZnO-NPs (selected concentrations ranging from 1 to 100 µg/ml depending on the results of IC50 calculation in our study and other report [19] as compared with DOX, CPH, and 5FU presented by $2^{-\Delta\Delta CT}$ with SD values were shown in figure 3. Significant down regulation of FOXM1 gene expression was observed via treatment with the gradual concentration of ZnO-NPs (The highest relative expression of FOXM1 was 1.45555E-06) as compared with untreated cells (The relative expression of FOXM1 comes with 2.462288827) as shown in figure 4.

Discussion

Current treatment protocols of cancer rely on radiation and chemotherapeutic agents that work in the body by killing rapidly dividing cells. The main drawback of conventional chemotherapy is the adverse effects on the body as it lacking delivery with selectivity and/or specificity to the cancer cells, therefore they act to damage the surrounding normal healthy tissues or rapidly dividing healthy cells such as those of bone marrow, gastrointestinal tract, hair follicles, causing issues like cardiac, hepatic, pulmonary, gastrointestinal and renal toxicities [20,21]. There are many adverse effects concerning 5-FU, doxorubicin and, CPH [21-26]. Cancer multidrug resistance is a common phenomenon that results in tolerance to many conventional pharmaceutical treatments. Resistance to anticancer drugs may arise from a multivariable factor including genetic mutations and\or epigenetic changes, conserved but up regulated drug efflux, and other variable molecular and cellular mechanisms [27]. The results of the MTT cytotoxicity assay revealed highly significant differences between untreated HCT116 cells monolayer as compared with those treated with different concentrations of each compound ($P<0.005$). The MTT colorimetric assay provides accurate and reliable quantification of viability. Results showed that MTT assay was successful and valuable for the measurement of ZnO-NPs anticancer potency. The MTT tetrazolium reduction assay was the first corresponding homogeneous cell viability assay developed for the measurement of any therapeutic agent potency using a 96-well format that was valuable and suitable

for high throughput screening [11,28]. ZnO-NPs compound showed significant anticancer activity against HCT116 Cell Line, with IC50 value of 27.327μg/ml, which is a significant value ($p<0.0001$) with positive dose-dependency (R^2 of ZnO-NPs =0.9656). Results have shown that ZnO-NPs from the 2nd concentration of 10 μg/mL had a significant reduction in viability of HCT116 cells ($P<0.05$). As the concentration of NPs increased, a significant reduction in cell viability was observed for HCT116 cancerous cells, these results came in assent with that mentioned by Akhtar MJ, et.al. (2012) [29]. Since FOXM1 may serve as a promising factor as a useful molecular biomarker and potential therapeutic target of CRC [30], in the current study, we aimed to evaluate the antitumor activity of ZnO-NPs in HCT116 cell line by measuring their effect on the expression levels and regulation of fork head box M1 gene (FOXM1) as indicator of apoptotic induction in tumor tissues. Results of FOXM1 mRNA relative expression in HCT116 cell line revealed a significant effect of down regulation after treatment with different concentrations of ZnO-NPs (The highest relative expression of FOXM1=1.45555E-06) as compared with untreated cells (The relative expression of FOXM1=2.462288827). Our results came following the findings of other studies that involve the FOXM1 gene is one of the most common over expressed genes in a diverse type of human solid tumors [31], including CRC [2,13]. Yang K, et al. (2015), was investigated FOXM1 protein expression in 87 CRC tissue specimens, invasive lymph nodes, and adjacent paired normal colorectal tissues by immune-histochemical analysis and transected FOXM1 specific small or short hairpin RNA (shRNA) into SW620 cells to examine the role of FOXM1on cell proliferation, colony-formation, migration and invasion in vitro [30]. They also found that the FOXM1 gene is over expressed in CRC tissues, CRC cell lines, and invasive lymph nodes. Away from the control group, our results of gene expression showed that different compounds were revealing different regulation patterns that may be varied gradually with micro titration treatment, this may be related to the fact that FOXM1 gene act to regulate genes that control G1\S-transition, S-phase progression, G2\M-transition as well as M-phase progression. Consistently, its expression and its activity are antagonistically regulated by many important proliferation and anti-proliferation signals [32]. Other mechanisms that achieved by ZnO-NPs may be as mentioned by Akhtar MJ, et.al. (2012) that suggest ZnO-NPs reveal selectively apoptotic pathway in cancer cells, which may be mediated by reactive oxygen species through Tp53 pathway, similar to the pathway of the most anticancer drugs in triggering apoptosis [29]. The quantitative RT-PCR (real-time polymerase chain reaction) results demonstrate that the exposure of HCT116 cells to ZnO-NPs results in significant down regulation of the mRNA expression level of FOXM1 gene. Results also revealed the applicable field of quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) as well as gene expression analysis remains the most sensitive technique for nucleic acid quantification, especially in CRC studies. The method of gene expression by $2^{-\Delta\Delta CT}$ has been widely used as a strategy for data analysis by quantitative real-time polymerase chain reaction (qPCR). This method is regarded as a convenient way for calculation of relative gene expression levels of different samples in that it managed directly with the threshold cycles (Cts) generated by the qPCR system for analysis and calculations Rao X, et al. (2013) [33]. We observed that ZnO-NPs have considerable effects on HCT116 cell viability as compared with untreated cells, with cytotoxic potency higher than that related to other conventional drugs (DOX, CPH, and 5-FU). Molecular data were showed that mRNA of the FOXM1 gene was significantly down regulated in ZnO-NP-treated cells. Further extensive research on other types of CRC cells and normal cells in addition to in vivo studies are needed to determine whether this is an acceptable therapeutic index in the anticancer activity of ZnO-NPs. From our findings, we concluded that FOXM1 was highly expressed in the HCT116 cell line and ZnO-NPs compound sharply down regulated this expression [34,35]. Hence, may offered a new alternative therapeutic agent with improved potency, acceptable pharmacological action, and further future investigations should be manipulated to examine this compound activity on not cancerous cells as well as experiments in vivo to be proven as useful therapeutic compound, that may consequently become an object of choice in both academia and industry.

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Conflicts of Interest

We declare no conflict of interest.

List of Abbreviations

CRC: Colorectal cancer

ZnO-NPs: Zinc oxide nanoparticles

DOX: Doxorubicin

CPH: Cyclophosphamide

5-FU: 5-fluorouracil

FOXM1: Forkhead box M1

IC50: Inhibitory concentration

CPH: Cyclophosphamide

XRD: X-ray diffraction

EDS: Energy dispersive X-ray Spectroscopy

DMSO: Dimethylsulfoxide

Cts: Threshold cycles

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