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Research Article

CYTOTOXICITY OF ZINC OXIDE NANOPARTICLES IN HELA CANCER CELL AND ITS EFFECT TO APOPTOSIS VIA P53 AND CASPASE PATHWAY

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ABSTRACT

Cancer is the second causing death diseases in the world. Nanomedicine emerged as a promising alternative for the treatment of cancer**.** HeLa is the very commonly employed model cell line for studying human cellular and molecular biology. Nanoparticles are a special institution of substances with precise capabilities and significant applications in lots of biomedical fields. Zinc oxide nanoparticles (ZNO) showed interested biocompatibility properties such selective accumulation in tumor cells. In this study, the work Zinc oxide nanoparticles are set up through Sol-Gel approach. The combination of nanoparticles distinguished through the utilization of DLS and TEM. In- vitro anticancer activity of Zinc oxide nanoparticles towards Hela cancer cell line utilizing various parameters had been studied **.** To exert cell growth arrest against Hela cancer cell lines had been determined by using Zinc oxide nanoparticles, the proficiency antiproliferative of ZnO NPs is because of cell dying and inducing apoptosis that affirmed by the usage of acridine orange-Ethidium bromide dual staining , Genotoxicity examine through RT- PCR analysis turned into done to detect the gene expression of Caspase-8, Caspase-9, and P53. In this study, we can suggest that ZnO NPs may well be utilized to supply a new drug restitution a chemotherapy drug and an enormous medical applications .

Keywords : Zinc oxide nanoparticles, Cytotoxicity, Hela cell line , Genotoxicity, Caspase8, Caspase-9 and P53

INTRODUCTION

The high incidence and mortality of cancer patients are at this time because of the low rate of the success of treatments. A cancer affects a huge percentage of the population and 41% of cancer deaths in women are due to cervical, stomach, and breast cancers [1]. The seek for novel drugs is still a significance goal for cancer therapy. As well, the relationship of high toxicity with cancer chemotherapy drugs and their unwanted side effects enhance the requirement for novel antitumor drugs active against untreatable tumors, with scarcer side effects and/or with more therapeutic efficiency [2]. Numerous methods against the cancer have been developed including radiation, chemotherapy, surgery and immunotherapy [3]. Nanoparticles represents a new platform to produce novel cancer therapeutics [4]. nanomaterials attracted interest due to the unique physical and chemical properties, and they have been used for a broad range of biomedical applications [5]. Moreover, long-term exposure to nanoparticles such as zinc oxide (ZnO) revealed no biological impact on health and safety [6]. ZnO nanoparticles were shown to be to cytotoxic against different cancer cells [7,8], which has been demonstrated that the ZnO nanoparticles had inhibition to proliferation and found that ZnO nanoparticles can induce oxidative stress, and apoptosis in treated cancer cells [9,10]. ZnO nanoparticles (NPs) exhibit selective cytotoxicity against some types of cancer cells when the surface modified by polyacrylic acid capping that produces negatively charged significantly toxic ZnO NPs [11]. HeLa was the first human cell line established in culture and has since become the most widely used human cell line in biological research [12] . The cell line derives from a cervical cancer tumor of a patient named Henrietta Lacks, who later give up of her cancer in 1951 [13]. HeLa cells was One of the first uses to develop the vaccine against the polio virus [14]. In recent times, two Nobel prizes have been granted for discoveries where HeLa cells played a vital role, to be exact the role of telomerase in inhibiting chromosome degradation [15] and the relation between human papilloma virus and cervical cancer [16]. In this study, we utilized Zinc oxide nanoparticles synthesized by Sol-Ge methods with the study characterization of Zno nanoparticles and applied ZnO nanoparticles as a novel anticancer therapy activity against Hela cell line. Our results report in the first time that evaluated the cytotoxicity of ZnO nanoparticles induced P53 dependent, caspase independent apoptosis in Hela cell line which revealed by real-time PCR analysis .this study aimed to examine whether the death of HeLa cells is caused by apoptotic process.

MATERIALS AND METHODS

Preparation of Zinc oxide nanoparticles

The study protocol was approved by the local ethical committee at university of Bucharest/ Romania and adhered to declaration of Helsinki (EC/2018/882). The synthesis of Zinc oxide nanoparticles was done depending on Khan, et al [17] with some modification. As mention in figure (1). Zinc acetate dehydrate (4.16 g per 100 mL-1) was prepared with Distal water. Sodium hydroxide (3.5 g per 100 ml^{-1}) was prepared using de-ionized water. Solution of Sodium hydroxide was dropping above

prepared zinc acetate solution until obtained pH scale range to 12. The mixture compound required further stirring for 1 hour until the precipitate was seen. The precipitation was washed using de-ionized water and then filtered and dried it overnight in the hot air oven at 70 C**.**

Characterization of ZnO nanoparticles

Manufacturing compounds were analyzed using SEM (GENEX, USA) and TEM (Philips EM) analyses were conducted to determine the particle size and morphology. Finally, Dynamic light scattering length distributions of the nanoparticles had been identified through the Dynamic light scattering (DLS) method [18].

Maintenance of cell cultures

A human epithelial carcinoma (Hela) cell line was cultured in MEM medium with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin and then incubated at 37 °C. These cells are regularly assessed for standard growth characteristics, and they are regularly authenticated. All cell lines were cultured as adherent confluent monolayers and maintained at 37°C in a humidified atmosphere of 5% CO2 [19] . Cells were harvested after brief trypsinization with trypsin-EDTA (Capricorn-Scientific, Germany).

Cytotoxicity assays

To determine the cell killing effect of (Nano ZnO), MTT assay was used according to Suliaman, GM, et al [20] . cell viability assay was conducted on 96-well plates (SantaCruze Biotechnology, USA), Hela cancer cell line were seeded at 7000 - 10000 cells/well after 24hr or confluent monolayer is achieved, Cells were treated with (Nano ZnO) at 2 fold dilutions from (100 µg/ml - 6.25µg/ml) in culture media. Cell viability was measured at 72hrs hours of exposure by removing the medium, adding 50 µl of MTT stain and incubating for 2 hours at 37°C. After removing the stain, it washed with PBS. The absorbency was determined on a microplate reader (Biochrom, UK) at 492 nm (test wavelength); the assay was performed in triplicate**.**

Clonogenicity

The cells line was poured in 12 well plates at density of 1000 cells / ml^{-1} . After 24 hrs, the cells were treated with zinc oxide nanoparticles with IC50 concentration. After that the medium removed and the cell rinsed using PBS solution. The colonies were fixed and stained using crystal violet, then washed to remove excessive dye using Distal water and then photographed [21] .

Acridineorange –Ethidiumbromide (AO/EtBr) dualstaining

(AO/EtBr) dual staining had been utilized to recognize the cellular death. Acridine orange is taken up by each viable cells and releases green fluorescence .Ethidium bromide is taken up only by non-viable cells and releases red fluorescence through intercalation with destruction DNA [22].Cell line had been seeded with density ($1x10^4$) on the cover slide that put at the 12 well plate with ZnO nanoparticles. After 48 hrs of incubation the medium had been removed and aliquot of 20 µl of dye mixture turned into combined withi100 µL cell suspension in a well plate. After the incubation period for 15-30 min, the cover slides have been taken to see under fluorescent microscope 100X magnification [18] **.**

Gene alteration detection using Real-time quantitative PCR

Gene alteration of cell line was investigated using Real-time quantitative PCR. In this experiment, three main type of gene were measured to identify the pathway of apoptosis and the mechanism action. These genes include (P53, Caspase-9, and Caspase-8).Real-Time (RT)-PCR turned into accomplished to investigate the modifications in hippocampal expression genes. The primer sets have been designed based totally at the sequences from the NCBI database. The sequences of primers used within the quantitative RT-PCR assay that include:-
1-P53 (forward:5′-CCGTCCCAAGCAATGGATG-3′) (reverse:5′-GAAGATGACAGGGGCCAGGAG-3′)
2-Caspase-8(forward:5′-GACCACGACCTTTGAAGA GCTTC-3′) (reverse: 5′-CAGCCTCATCCGGGATATATC-3′)
3-Caspase-9 (forward: 5′-CTCTTGAGCAGTGGCTGGTC-3′) (reverse:5′-GCTGATCTATGAGCGATACT-3′)
Each RT-PCR reaction combination containing 1 µL of cDNA, 7.5 µL SYBR green, zero.3 µL Rox, 0.3 µL related primers, and the final quantity was topped up to 15 µL via adding 5.6 µL of distilled water. The assay had performed with SYBR Premix Ex. TaqTM kit. The real-time detection of emission intensity of SYBR green reacted to double-stranded DNAs and was performed via the implemented Biosystems (ABI) Prism sequence Detection system. GAPDH mRNA had been used as an inner control to identify the relative expression amount of the genes [23].

Statistical Analysis

The study data are presented as means ± standard error of the mean. Data comparison between treatment groups was accomplished by one-way analysis of variance ($P < 0.05$ considered statistically significant). Statistical analyses were performed using the Graphpad Prism 5 software package (GraphPad Software, Inc. San Diego, California).

Ethical approval

The study protocol was approved by the local ethical committee at university of Bucharest/ Romania and adhered to declaration of Helsinki (EC/2018/882)

RESULTS

Characterization of manufacturing ZnO nanoparticles
Dynamic light scattering (DLS)

Dynamic light scattering technique is used to determine Brownian motion of spherical dispersed particles and to narrate this to the hydrodynamic length of the particles dispersed within the solution via dynamic fluctuations of scattered light intensity. This scattered light intensity is similarly mathematically manipulated to relate the hydrodynamic length of the debris. A vital characteristic of Brownian motion measured with the aid of DLS is that small debris circulate quicker in assessment to large debris, and the relationship between the dimensions of a particle and its velocity due to Brownian motion is defined within the Stokes-Einstein equation. As visualized in Fig. (4-4), ZnO NPs diameter has been within the range of 10-20 nm.

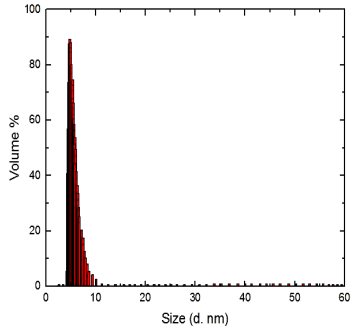

Figure 1: DLS histogram image of Zinc oxide nanoparticles

Transmission electron microscope (TEM)

To confirm and verify the physical (diameter, dispersion, morphology) parameters of the nanostructures, TEM had been employed. As seen in Fig (2), Zinc oxide nanoparticles had a spherical morphology as targeted via the selection of the relative quantity fractions inside the synthesis of the constituent blocks within the diblock copolymer and had a particularly slim size distribution with diameter 7 and 15 nm.

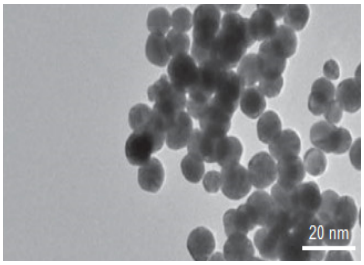

Figure 2: TEM image of Zinc oxide nanoparticles.

Anticancer

Activity Cytotoxicity determination using MTT assay

Figure (3) shows the viability results examined by MTT colorimetric assay cervical carcinoma Hela cell after 72 hours of exposure to different concentrations of ZnO nanoparticles at concentrations (100 μgmL^{-1} - 6.25 μgmL^{-1}) and then determined the cytotoxicity using MTT assay. The results demonstrated that treatment with ZnO nanoparticles inhibited the growth of cells significantly ($P \leq 0.05$) and the reduction was concentration dependent. The inhibitory concentration value (IC50) of ZnO nanoparticles was 19.3 μgmL^{-1} as visualized in Fig. (3). ZnO nanoparticles, with their specific properties which contain biocompatibility, excessive selectivity, more cytotoxicity and smooth synthesis, can be a promising anticancer agent [24].

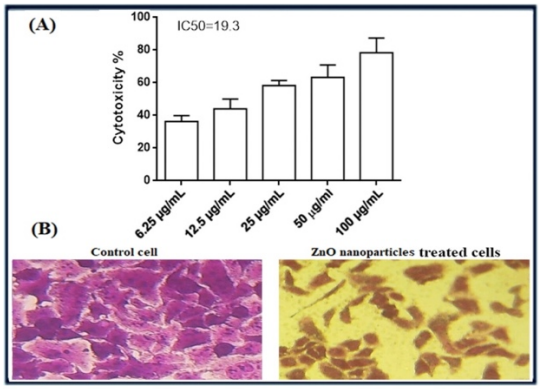

Figure 3: Antiproliferative activity of Hela cell line. (A) Cytotoxicity effect of using MTT assay, (B) imaged of treated cell by inverted phase contrast

ZnO nanoparticles have the specific capability to evoke oxidative stress in most cancers cells, which has been observed to be one of the mechanisms of cytotoxicity of ZnO nanoparticles closer to cancer cells. This property is because of the semiconductor nature of ZnO,Conduction of electricity power takes vicinity via the motion of unfastened electrons inside the valence band [25].

ZnO nanoparticles have been specific properties which includes biocompatibility, higher selectivity, improved cytotoxicity and synthesis, it can be a promising anticancer agent. ZnO nanoparticles have the particular capability to induce oxidative stress in most cancers cells, which has been observed to be one of the mechanisms of cytotoxicity of ZnO nanoparticles against most cancer cells. This residue is because of the semiconductor nature of ZnO. Conduction of electric power takes vicinity via the movement of free electrons within the valence band. However, within the case of the Nano-sized ZnO nanoparticles, electrons additionally leap to the conduction band inside the absence of UV irradiation [26]. Electrons and holes frequently recombine speedy but within the case of nanoparticles, they pass to the nanoparticle surface, in which they react with the adsorbed species [27]. This results in an elevated variety of electrons and holes within the nanoparticle surface induces ROS generation, lead to oxidative stress and ultimately cellular death whilst the anti-oxidative capability of the cell surpassed [24].

Clonogenicity assay

The figure (4) is visualized that the antiproliferative efficiency on assay cervical carcinoma Hela cell through the use of clonogenic assay had been determined to similarly verify the inhibition activity to tumor cells. Colony formation assay is an in vitro cell survival assay based totally at the capacity of a single cell to develop right into a colony [28]. This may be used to identify the effectiveness of different cytotoxic agent's. ZnO nanoparticles exhibited principal performance at the colony formation cell line at IC50 concentration. The discount of colony formation may also lead to accept as true with that the cancer cells within the continuous remedy were killed in the first 48 hrs of treatment, suggesting that ZnO nanoparticles have been taken up by way of cells and evoke the death mechanism. Consequently, the result may also display that the synthesis compound ought to set off cell death While the normal cell line.

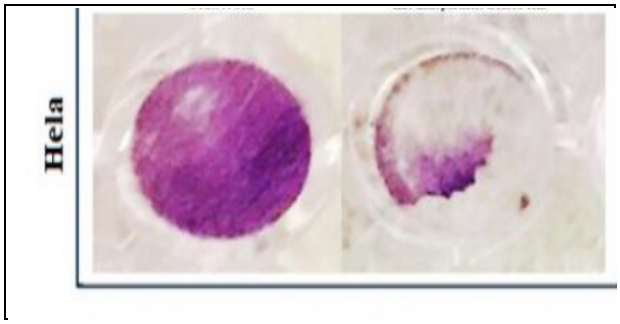

Figure 4: Clonogenic effect of Zno nanoparticle with IC50 concentrations on cell line.

Acridine orange –Ethidium bromide (AO/EtBr) dual staining

AO–EB analysis was employed to examine the changes in nuclear morphology of cervical carcinoma Hela cell. The apoptotic cells were indicated based on DNA damage. AO-EB dual staining was used to determine distinct apoptotic signs characteristics of nucleate alternations. Viable and nonapoptotic cells appeared green, while apoptotic cells appeared orange or red. As shown in figure (5), exposing cervical carcinoma Hela cell to ZnO nanoparticles caused an increase in membrane disruption and formation of lysosomes vacuoles compared to untreated control cells.

The excessive capacity to cause dying to the cell is associated with the capability of nanoparticle to penetrate via the cellular membrane and impact at the mRNA expression of suppression gene that cause increase in the production level of ROS within the cell [29]. With expanded levels of ROS and oxidative stress, ZnO NPs display a deleterious impact at the lipid, protein and nucleic acid of the cell [30].

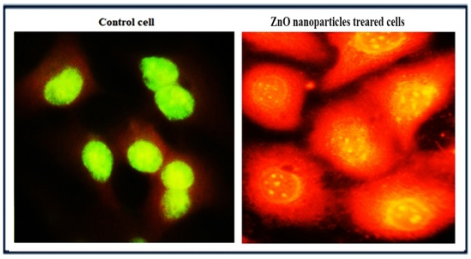

Figure 5: Fluorescence microscopy images of cervical carcinoma Hela cell stained with AO and EtBr in the presence and absence of Zinc oxide nanoparticles.

Zinc oxide nanoparticles Regulated Gene Expression

In this study, quantitative real-time PCR have been used to identify the change in the expression of the mRNA of apoptotic genes (p53, caspase-8 and caspase-9) in cells that exposed to ZnO nanoparticles for 24 hrs with concentrations (6.25µgml^{-1}, 12.5µgml^{-1}, and 25µgml^{-1}). The PCR results revealed that apoptotic arkers mRNA were altered dealt with cell lines due to ZnO nanoparticles remedy. The MRNA level of tumor suppression gene p53 turned into increase as visualized in figure (6-A) in treated cells, that the level of P53 increased dependent on the concentrations respectively. Moreover, the performance of ZnO nanoparticles at the mRNA expression of caspase-8 and caspase-9. The expression of caspase-9 turned into downregulated at 24 hrs as shown in figure (6-B) . Whilst, caspase-8 changed into upregulated in treated cells in comparison with untreated control cells at 24 hrs of treatment as visualized in figure (6-C).

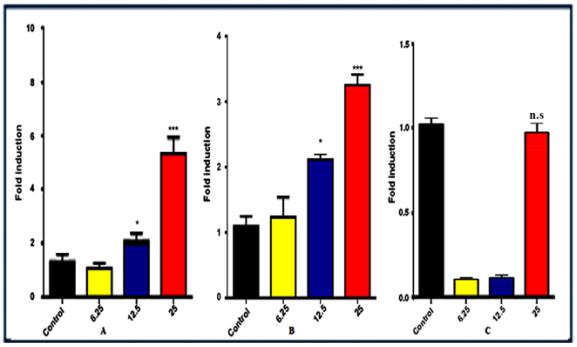

Figure 6: ZnO nanoparticles and their role in the apoptotic genes expression. (A) p53 (B) Caspase-8 (C) Caspase-9. The values represents the Mean ± S.E. **P<0.01 ,*P <0.001**

The tumor suppressor p53 gene and caspase enzyme assist to investigate cells frequently and save them from becoming cancerous [31] . If a cell indicates any form of malignancy, a DNA repair mechanism is activated to restore the altered DNA [32]. Zinc is one in every of the co-factor of those enzymes and performs a vital function in host defense in opposition to the initiation and development of cancer [33]. The particular DNAbinding domain of p53 consists of a complicated tertiary structure that's stabilized via zinc [34]. consequently, zinc performs a main function in maintaining the interest of tumor suppressor gene p53 and performs a vital role within the activation of the caspase-8 enzyme, a main enzyme accountable for apoptosis [35]. Caspase-9 is the touchy apoptosis-associated molecular target of zinc [36]. It's responsible for the activation of caspase-3 and different enzymes which are liable for nuclear membrane dissolution leading to cellular dying. Zinc performs an crucial role in response to oxidative stress, DNA replication, DNA harm repair, cell cycle development and apoptosis; as a result a deficiency of zinc ends in disruption of important homeostasis in cells [37, 38] .

CONCLUSION

Nanoparticles which incorporates ZnO had been examined and evolved for cancer therapy. Recently, numerous research concerns have proposed the use of ZnO nanoparticles as anti-cancer therapeutic drug. In this study , ZnO nanoparticles is a potential as an anticancer against HeLa cell via treatment of ZnO nanoparticles to cancer cells lead to cause significant cytotoxicity. The effect of results propose that ZnO nanoparticles had high activity within the induction of genes expression. Consequently, ZnO nanoparticles chemoprevention may be efficacious within the prevention and remedying of numerous cancers.

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