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## Investigating the therapeutic effect of folic acid conjugated ZnO nanoparticles on human triple negative breast cancer cell line

### ABSTRACT

Triple-negative breast cancer (TNBC) accounts for 15-20% of all invasive breast cancers and has a poor prognosis. ZnO NPs are promising anti-cancer agents. Moreover, folate alpha receptor (FR $\alpha$ ) is a potential biomarker and therapeutic target because it is significantly expressed in TNBC. Therefore, ZnO and folic acid-conjugated ZnO (F-ZnO) NPs were synthesized by the sol-gel method. NPs were characterized by DLS, zeta potential, TEM, FTIR, and ICP-MS. FA-ZnO NPs had a mean diameter of  $20 \pm 2$  nm and a surface charge of -15 mV, while ZnO NPs had a mean diameter of  $40 \pm 5$  nm and a surface charge of -5 mV. The MTT assay and trypan blue test, respectively, were used to determine the cytotoxicity and viability percentage of ZnO and F-ZnO NPs at different concentrations of 2, 4, 8, 16, 32, 64 and 128  $\mu\text{g/mL}$  for 12, 24, 48 and 72 hours (h) on the human TNBC cell line MDA-MB-231. The results indicated that both ZnO and F-ZnO NPs significantly reduced the viability of the cancer cells in a dose-dependent and time-dependent manner ( $p < 0.05$ ). The IC<sub>50</sub> values for FA-ZnO NPs were approximately 3, 3.74, 4.38 and 5.5 times higher than those for ZnO NPs at 12-, 24-, 48- and 72-hour time points, respectively. The results suggest that F-ZnO NPs have the potential to be a good option for TNBC treatment and warrant further investigations.

**Keywords:** Nanoparticle, Zinc oxide, Folic acid, Triple negative breast cancer.

### 1. INTRODUCTION

Based on the latest estimates from GLOBOCAN 2020, there were 19.3 million new cancer cases and 10 million cancer deaths worldwide in 2020. Breast cancer surpassed lung cancer as the most frequently diagnosed cancer globally in 2020. Breast cancer, along with lung, liver, stomach, and colon cancer, were the top five causes of cancer-specific mortality [1]. Breast cancer was the most prevalent malignancy in the world, with an incidence rate of 10.4 % of all cancers. It also accounted for 30 % of all new cancer diagnoses in women and was the leading cause of cancer-related death among women aged

20 to 50 years [2]. The burden of breast cancer is increasing globally.

Breast cancer can be classified into different molecular subtypes based on the genes that the cancer cells express [3,4]. These subtypes have different prognoses and treatment options. The most common subtype (73% of breast cancers) is luminal A, which is positive for estrogen receptors (ER) and/or progesterone receptors (PR), and negative for human epidermal growth factor receptor 2 (HER2) [5]. This subtype has a good prognosis and responds well to hormone therapy. Another subtype is triple-negative breast cancer (TNBC), which is negative for ER, PR, and HER2, and accounts for 10–20% of all breast cancers [6]. This subtype usually affects younger patients and certain ethnic groups, and has a poor prognosis and limited treatment options [7,8].

TNBC is a heterogeneous subtype of breast cancer with poor prognosis and low sensitivity to

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mammographic screening [9]. Heterogeneity leads to variable clinical outcomes, differential responses to neoadjuvant chemotherapy, and inconsistent survival rates [10]. Despite some responsiveness to conventional chemotherapeutic agents such as ACs, taxanes, and cyclophosphamide [11], TNBC has lower disease-free survival and overall survival than other breast cancer subtypes. TNBC lacks expression of ER, PR, and HER2 receptors, which precludes the use of hormone therapy or targeted therapy in this subtype [12], limiting the options for long-term disease management.

Nanotechnology has played an important role in diagnosing and treating cancer, including TNBC [13-15]. Researchers have extensively studied nanotechnology to enhance the efficacy of chemotherapeutic agents by promoting their bioavailability and reducing toxicity of off-target cells [16]. Some nanoparticles, such as Au, Ag, and ZnO have been used for cancer therapy and delivering anticancer agents [17-19]. Nanoparticles accumulate preferentially in the tumors by passive targeting due to the enhanced permeation and retention (EPR) effect resulting from the impaired vascular structure and defective lymphatic drainage. However, active targeting using tissue-specific ligands and biomarkers can direct nanoparticles specifically to the target tissue, enhancing therapeutic efficacy [20].

Folic acid (FA) as a targeting moiety has been widely investigated in cancer therapy to enhance accumulation and cytotoxicity of nanoparticles towards tumor cells [21,22]. It has been found that FA receptors highly expressed in malignant cells, including TNBC compared to their normal counterparts [23].

This study investigated the potential of selective toxicity of ZnO NPs and targeting of folic acid on TNBC cell line, MDA-MB-231, at different concentrations and time points. We synthesized ZnO and F-ZnO NPs by sol-gel technique and characterized them by DLS, TEM, and FTIR. Then we used MTT and cell viability assays at 12, 24, 48, and 72 hours to evaluate the effectiveness of these NPs on the human TNBC cell line.

## 2. MATERIALS AND METHODS

### 2.1. Materials

The following reagents were purchased from Sigma (Aldrich, Germany): zinc diacetate (Zn (CH<sub>3</sub>COO)<sub>2</sub> · 2H<sub>2</sub>O), folic acid (C<sub>19</sub>H<sub>19</sub>N<sub>7</sub>O<sub>6</sub>), dimethyl sulfoxide (DMSO), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), and trypan blue (TB). Gibco Co. supplied Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA, penicillin-streptomycin antibiotics, and PBS. Trypan blue

and ammonia were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade. The MDA-MB-231 cell line was acquired from Pasteur Institute (Tehran, Iran).

### 2.2. Methods

#### Zinc oxide nanoparticles (ZnO NPs) synthesis

Zinc diacetate (Zn (CH<sub>3</sub>COO)<sub>2</sub> · 2H<sub>2</sub>O) was used to synthesize ZnO NPs by the sol-gel technique. A solution of zinc diacetate (250 mg/20 mL DW) was mixed with ammonia solution (1:1/vol) for 2 hours at pH ~ 7.5, resulting in the precipitation of Zn as zinc hydroxide. The precipitate was centrifuged and re-dispersed in DW to remove excess ions. Finally, the precipitate was dried at 100°C to obtain ZnO [24].

#### Folic acid conjugated ZnO nanoparticles (FA-ZnO NPs)

FA-ZnO NPs were prepared by dissolving 0.3 g FA in 10 mL DW with a mild alkaline pH and stirring for 1 hour. In another container, 250 mg zinc acetate was dissolved in 10 mL DW and ammonia solution was added dropwise. The FA solution was then slowly added to the zinc solution and the final pH was set to 7.5. The precipitate was centrifuged and re-dispersed in DW to remove excess ions. Finally, the precipitate was dried at 100°C [24].

#### Characterization of FA-ZnO nanoparticles

The Dynamic Light Scattering Instrument (Nanoflex; ParticleMetrix, Germany) was used to measure the average diameter, polydispersity index (PDI), and zeta potential of FA-ZnO NPs in triplicate. The data were presented as means ± standard deviation (n = 3). The morphology and size of NPs were also examined by transmission electron microscopy (TEM, LEO 906 Zeiss 100KV, Germany).

The powders (as pellets in KBr, without moisture) were also characterized by Fourier transform infrared (FT-IR) spectroscopy (NEXU670 spectrometer) in the range of 400–6000 cm<sup>-1</sup>. The concentration of both bare ZnO and F-ZnO NPs dispersion was determined by inductively coupled plasma mass spectrometry (ICP-MS).

#### Cytotoxicity and viability assay

MDA-MB-231 cell line was cultured in DMEM + 10% FBS. Cell viability was assayed by seeding 5000 cells/well into 96 well plates overnight. Then they were incubated with different concentrations of bare ZnO and FA-ZnO NPs (n=3 in 200 µL per well) for 12, 24, 48, and 72 hours. After each treatment time, cells were washed twice with PBS and re-incubated for an additional 48 h. Next the medium was replaced with 10% freshly prepared

MTT (5 mg/mL) and 100  $\mu$ L culture medium without FCS per well. After 4 h incubation at 37 °C, it was replaced with DMSO (10%) per well and shaken to dissolve formazan crystals. Finally, absorbance was recorded at 570 nm.

Relative cell death was obtained by following calculation:  $R = 1 - [(A \text{ sample} - A \text{ blank}) / (A \text{ control} - A \text{ blank})]$  in which A sample and A control were absorbance of cells incubated with NPs and culture medium as control group respectively. The absorbance of MTT solution in wells without cell was considered as A blank. IC50 values were calculated using CalcuSyn version 2 software (BIOSOFT UK). Cell viability was also evaluated using trypan blue test [25]. Cells with integrated membrane were colorless while dead cells with damaged membrane were bluish-purple. Viability percentage was calculated as follows: % Viable cells: number of viable cells/ total number of cells \*100.

#### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Significant differences between different groups were determined by one-way ANOVA. Data were presented as mean  $\pm$  SEM and considered significant when  $P < 0.05$ .

### 3. RESULTS AND DISCUSSION

#### 3.1. Characterization of NPs

##### TEM studies

We measured the morphology and size of the nanoparticles using TEM. Fig. 1 shows the TEM image of F-ZnO NPs. The particles were spherical and had an average size of 20 nm. They were also uniformly dispersed, because folic acid created an electrical repulsion on their surface [19, 24]. This repulsion also made them smaller than zinc oxide nanoparticles without folic acid

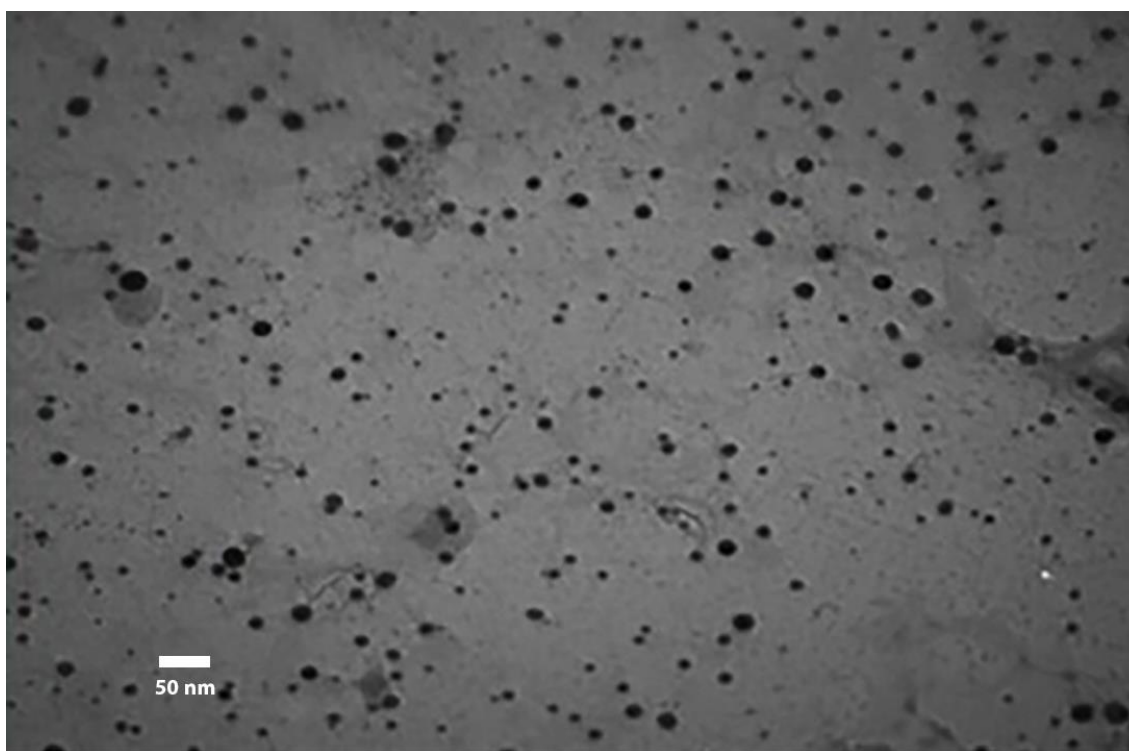


Figure 1. Transmission electron microscopy (TEM) image of folic acid conjugated zinc oxide nanoparticles (FA-ZnO NPs)

Slika 1. Slika transmisione elektronske mikroskopije (TEM) konjugovanih nanočestica cink oksida sa folnom kiselinom (FA-ZnO NPs)

##### DLS studies

Fig. 2 shows the size distribution of FA-ZnO NPs based on intensity index. The NPs have a mean diameter of  $20 \pm 2$  nm and a narrow distribution (PDI: 0.04), which indicates their stability. The presence of FA prevents the NPs from accumulating and agglomerating, and helps

them disperse well. The surface charge of FA-ZnO is -15 mV, which is due to the carboxylic acid groups of folic acid. These groups also cause electrostatic repulsion between the particles and enhance their stability. Moreover, the negative charge confirms that ZnO NPs are functionalized with FA [26].

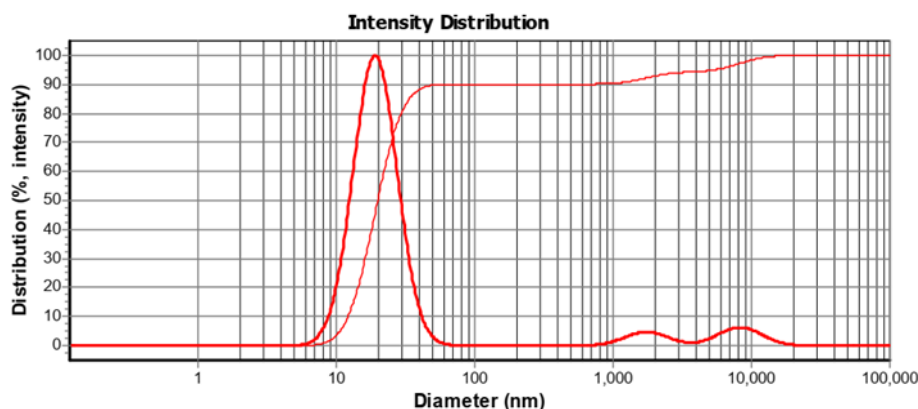


Figure 2. Particle size distribution of folic acid conjugated ZnO nanoparticles (FA-ZnO NPs) based on intensity

Slika 2. Distribucija veličine nanočestica čestica ZnO konjugovanih folnom kiselinom (FA-ZnO NPs) na osnovu intenziteta

Fourier transform Infrared (FTIR) spectroscopy study

Fig. 3 shows the FTIR spectra of ZnO NPs and FA-ZnO NPs. FTIR spectroscopy is a cost-effective and typical method to identify the functional groups and confirm the functionalization of NPs. The bands below 500  $\text{cm}^{-1}$  (431  $\text{cm}^{-1}$  and 472  $\text{cm}^{-1}$ ) in ZnO NPs spectra are related to the Zn-O stretching mode. This mode is specific to Zn-O bonds and appears in the range of 400  $\text{cm}^{-1}$  to 500  $\text{cm}^{-1}$ . The bands at 1017-1048  $\text{cm}^{-1}$  are due to the C-O stretching of zinc acetate. The strong bands at 832  $\text{cm}^{-1}$  and 921  $\text{cm}^{-1}$  are assigned to the bending mode of carbonate. These findings confirm the successful synthesis of bare ZnO NPs. The main

absorption bands at  $\sim 3000$ - $3200$   $\text{cm}^{-1}$  indicate the residual hydroxyl groups, which result from using NaOH as a precursor [27, 28]. In FA-ZnO NPs spectra, the Zn-O stretching mode shifts toward higher frequencies (446  $\text{cm}^{-1}$  and 586  $\text{cm}^{-1}$ ), which indicates an increased distance between NPs due to electrostatic repulsion.

Furthermore, FA-ZnO NPs spectra show absorption bands in the regions of  $\sim 3400$   $\text{cm}^{-1}$  (O-H mode),  $\sim 2900$   $\text{cm}^{-1}$  (C-H mode),  $\sim 1600$   $\text{cm}^{-1}$  (symmetric stretching mode of C=O), and  $\sim 1380$   $\text{cm}^{-1}$  (asymmetric stretching mode of C=O), which verify the successful conjugation of folic acid with ZnO NPs (Fig. 3) [29].

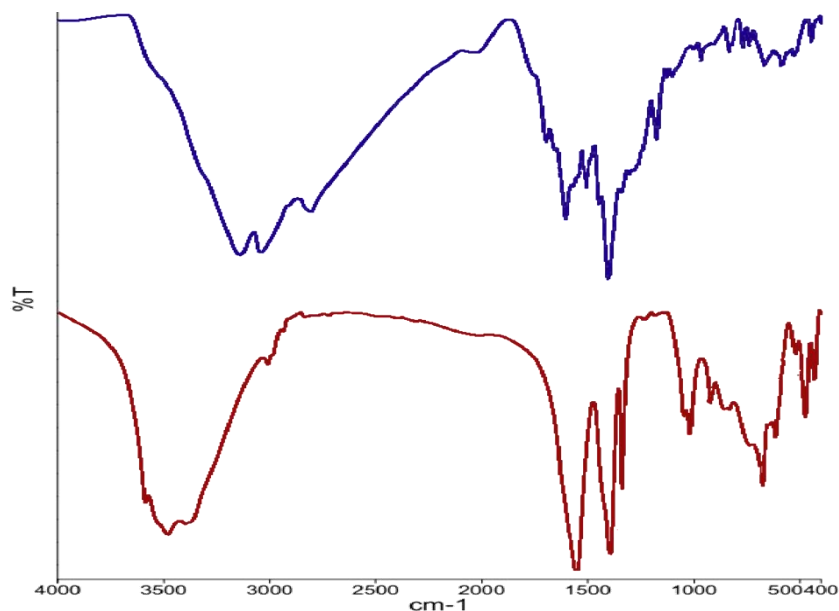


Figure 3. FTIR spectra of ZnO nanoparticles (red), and FTIR spectra of folic acid conjugated ZnO nanoparticles (blue)

Slika 3. FTIR spektri ZnO nanočestica (crveni) i FTIR spektri nanočestica ZnO konjugovanih sa folnom kiselinom (plavi)

*In vitro* studies

## Cytotoxicity and viability studies

The cytotoxicity of FA-ZnO NPs and ZnO NPs on human triple negative breast cancer cells (MDA-MB-231) was investigated using the MTT assay. This assay is a widely used method to measure the cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. It relies on the reduction of a tetrazolium salt (MTT) to insoluble purple formazan crystals by NADPH-dependent dehydrogenase enzymes in living cells (metabolically active cells). The formazan crystals can be dissolved by isopropanol or dimethyl sulfoxide and their color intensity at 590 nm is directly proportional to the number of living cells. The MTT assay can be applied to both healthy and

unhealthy cell lines to study the safety or efficacy of therapeutic agents respectively [20; 25].

Cell viability (%) was also measured by trypan blue [25]. The results showed that both types of nanoparticles inhibited cancer cell growth in a dose- and time-dependent manner at different concentrations (2, 4, 8, 16, 32, 64, and 128 µg/mL) and time points (12, 24, 48, and 72 hours) (table 1).

IC50 is a common indicator of a drug's efficacy, showing how much drug is needed to inhibit a biological process by 50%. A low IC50 value means that the drug is potent and has a large inhibitory effect at low concentrations. It also implies that the drug has less systemic toxicity when administered [30].

*Table 1. Inhibitory concentration 50% (IC50) of ZnO and folic acid conjugated ZnO (F-ZnO) nanoparticles against MDA-MB-231 as human triple negative breast cancer (TNBC) cells after various incubation times. Values expressed as means ± SD (µg/mL, n = 3)*

*Tabela 1. Inhibiciona koncentracija 50% (IC50) ZnO i nanočestica ZnO (F-ZnO) konjugovanih sa folnom kiselinom protiv MDA-MB-231 kao humanih trostruko negativnih ćelija raka dojke (TNBC) posle različitih vremena inkubacije. Vrednosti izražene kao srednje vrednosti ± SD (mg/mL, n = 3)*

Exposure times	12 h	24 h	48 h	72 h
ZnO NPs	72.8 ± 2.4	59.2 ± 0.94	52.6 ± 0.07	44.2 ± 1.43
F-ZnO NPs	23.6 ± 3.2	15.8 ± 1.26	12 ± 0.02	8.08 ± 1.13

ZnO NPs showed anti-tumor activity on MDA-MB-231 cells in a time- and concentration-dependent manner. The IC50 of ZnO NPs decreased from 72.8 µg/mL at 12 hours to 44.2 µg/mL at 72 hours, indicating the time-dependent toxicity of ZnO NPs.

FA-ZnO NPs also inhibited the growth of MDA-MB-231 cells in a time- and concentration-dependent manner, but more effectively than ZnO NPs. The IC50 of FA-ZnO NPs decreased from 23.6 µg/mL at 12 hours to 8.08 µg/mL at 72 hours, suggesting a significant improvement in tumor suppression ( $p < 0.05$ ).

The IC50 values of FA-ZnO NPs were about 3 to 5.5 times lower than those of ZnO NPs at different time points, indicating the synergistic effect of folic acid and ZnO NPs [31]. Actually, folic acid residues acted as a targeting agent for MDA-MB-231 cells [23].

Folic acid as a targeting residue led to the internalization of FA-ZnO NPs into the cells through

the folate receptor-mediated endocytosis mechanism [32]. Moreover, FA receptors were highly expressed on the triple-negative MDA-MB-231 breast cancer cells [33]. Therefore, a significant uptake of FA-ZnO NPs and a growth inhibitory effect were observed. The highest toxicity was found at 72 hours after treatment with FA-ZnO NPs, which was about 5.5 times that of bare ZnO NPs, resulting from selective accumulation in cancer cells over time. In addition, this important finding emphasized the presence of folic acid on ZnO NPs, which could specifically and selectively kill cancer cells whose folic acid receptor was overexpressed [34]. On the other hand, it would be an important finding to use the potential of these NPs in in vivo studies and finally to translate into clinical studies. Similar results were also obtained in the cell viability studies (Fig. 4, 5). The percentage of cell viability also confirmed the time- and concentration-dependent manner of ZnO and FA-ZnO NPs cytotoxicity.

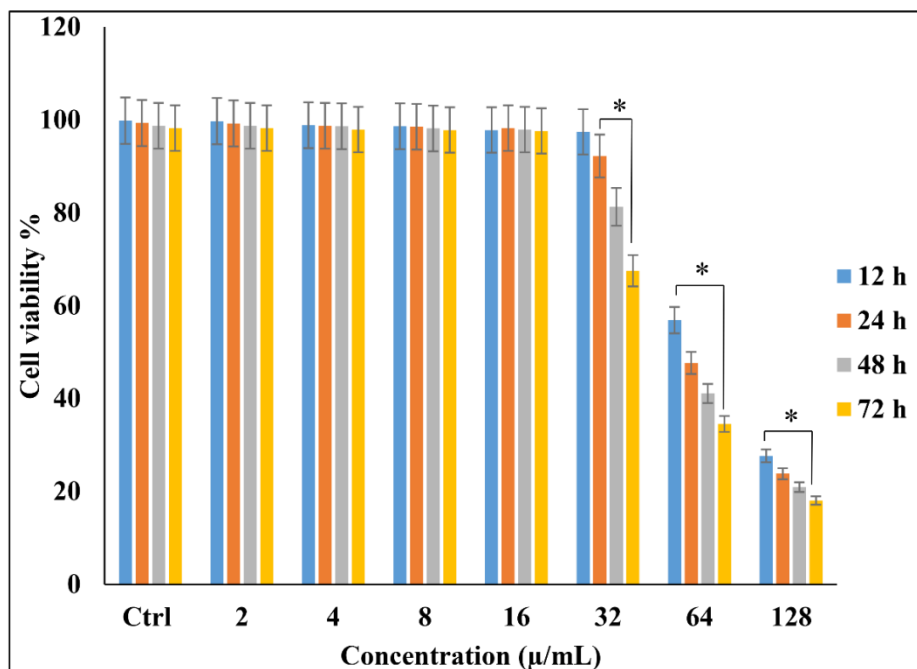


Figure 4. The effect of different concentrations of ZnO NPs on the viability of human triple negative breast cancer (TNBC) cells, MDA-MB-231 after 12, 24, 48, and 72 hours of incubation. Values expressed as means  $\pm$  SD ( $\mu\text{g/mL}$ ,  $n = 3$ ). \*  $P$ -value  $< 0.05$ . Ctr: control group

Slika 4. Efekat različitih koncentracija ZnO NPs na održivost ćelija trostrukog negativnog raka dojke (TNBC) kod ljudi, MDA-MB-231 nakon 12, 24, 48 i 72 sata inkubacije. Vrednosti izražene kao srednje vrednosti  $\pm$  SD ( $\text{mg/mL}$ ,  $n = 3$ ). \*  $P$ -vrednost  $< 0,05$ . Ctr: kontrolna grupa

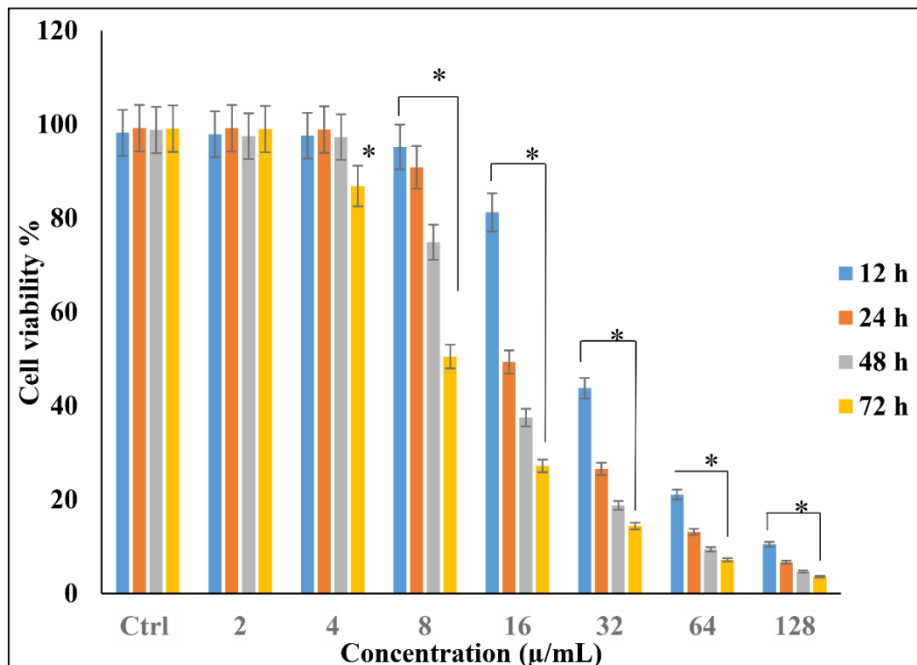


Figure 5. The effect of different concentrations of folic acid conjugated F-ZnO NPs on the viability of human triple negative breast cancer (TNBC) cells, MDA-MB-231 after 12, 24, 48, and 72 hours of incubation. Values expressed as means  $\pm$  SD ( $\mu\text{g/mL}$ ,  $n = 3$ ). \*  $P$ -value  $< 0.05$ . Ctr: control group

Slika 5. Efekat različitih koncentracija F-ZnO NPs konjugovanih sa folnom kiselinom na održivost humanih ćelija trostruko negativnih karcinoma dojke (TNBC), MDA-MB-231 nakon 12, 24, 48 i 72 sata inkubacije. Vrednosti izražene kao srednje vrednosti  $\pm$  SD ( $\text{mg/mL}$ ,  $n = 3$ ). \*  $P$ -vrednost  $< 0,05$ . Ctr: kontrolna grupa

ZnO NPs have shown higher in vitro cytotoxicity than micron-sized ZnO particles against various cancer cells, such as glioma, breast, bone, colon, leukemia and lymphoma [35-37]. For example, cancerous lymphocytes were 28–35 times more sensitive to ZnO NPs than normal ones [36]. This selectivity surpasses the ex vivo therapeutic indices (less than 10) of common chemotherapy agents like doxorubicin and carboplatin for different leukemias, lymphomas and solid tumors [36]. The cytotoxicity depends on the cell's proliferation status; the faster-growing cells are more vulnerable [36, 38]. ROS production is the main mechanism of ZnO NPs cytotoxicity [19,39], which induces apoptosis in cancer cells.

Nanoparticles are attractive for cancer treatment because they can target specific cells and have better efficacy than conventional methods such as chemotherapy, radiation therapy and surgery. ZnO NPs are especially promising because they are biocompatible, selective, cytotoxic and easy to synthesize. Zinc is a trace element in the human body and a co-factor of many enzymes that regulate cellular functions such as oxidative stress, DNA replication, DNA repair, cell cycle progression and apoptosis. Therefore, altering the zinc level in cancer cells can have detrimental effects. A possible mechanism is the imbalance between zinc-dependent protein activity and oxidative stress caused by ROS.

ZnO nanoparticles (NPs) have shown potential as an anticancer agent because they can selectively target and kill cancer cells. This is due to their selective accumulation in tumors caused by the enhanced permeability and retention (EPR) effect, electrostatic interaction and increased ROS production in cancer cells [40].

One way to deliver ZnO NPs to breast cancer cells is by using pH-sensitive nanotherapeutics that contain curcumin and are conjugated to phenylboronic acid (PBA) (ZnO-PBA-Curcumin), 40 nm. Kundu et al. developed this approach and found that it induced apoptosis in human breast cancer cells by causing oxidative stress and mitochondrial damage. The toxicity of ZnO-PBA-Curcumin was attributed to the combined effects of curcumin and ZnO [41].

The alpha-folate (FR $\alpha$ ) receptor is a membrane protein that is attached to glycosyl-phosphatidyl inositol (GPI). It is highly expressed in many tumors, such as ovarian, breast and lung cancers, but not in normal tissues. This makes it a promising target for new treatments of tumors [42]. Many researchers have been interested in using FR $\alpha$  as

a therapeutic target because of its role in tumor growth and its link to poor prognosis [42]. One type of breast cancer that shows high expression of FR $\alpha$  is triple-negative breast cancer (TNBC), which affects 80% of cases. This makes FR $\alpha$  a potential target for this type of breast cancer [43]. Studies have shown that FR $\alpha$  expression in breast cancers, including TNBCs, is related to worse outcomes and more aggressive features. High FR $\alpha$  expression also correlates with primary and metastatic tumors, suggesting that anti-FR $\alpha$  therapies could treat both types of tumors [44-45]. These findings are consistent with other studies that have shown the benefits of targeting FR $\alpha$  in different types of cancer cells [46].

This study's findings are consistent with other investigations that suggest targeting this tumor-associated antigen can benefit patients who have no available treatment options for various types of cancer.

#### 4. CONCLUSION

We demonstrate that zinc oxide nanoparticles (ZnO NPs) conjugated with folic acid (FA) can selectively kill triple-negative breast cancer cells at different concentrations and time points. Our results reveal a synergistic effect of FA and ZnO NPs that depends on both concentration and time. These findings suggest that FA-ZnO NPs have potential for further in vivo and clinical studies as a promising treatment option for triple-negative breast cancer.

#### Funding information

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## IZVOD

### istraživanje terapijskog efekta nanočestica zno konjugovanih sa folnom kiselinom na trostruko negativnu ćelijsku liniju raka dojke kod ljudi

Trostruko negativan rak dojke (TNBC) čini 15-20% svih invazivnih karcinoma dojke i ima lošu prognozu. ZnO NPs su obećavajući agensi protiv raka. Štaviše, folatni alfa receptor (FR $\alpha$ ) je potencijalni biomarker i terapijska meta jer je značajno izražen u TNBC. Zbog toga su ZnO i ZnO (F-ZnO) NPs konjugovani sa folnom kiselinom sintetisani sol-gel metodom. NP su karakterisali DLS, zeta potencijal, TEM, FTIR i ICP-MS. FA-ZnO NPs su imali srednji prečnik od  $20 \pm 2$  nm i površinsko naelektrisanje od -15 mV, dok su ZnO NPs imali srednji prečnik od  $40 \pm 5$  nm i površinsko naelektrisanje od -5 mV. MTT test i tripan plavi test, respektivno, korišćeni su za određivanje procenta citotoksičnosti i vitalnosti ZnO i F-ZnO NPs pri različitim koncentracijama od 2, 4, 8, 16, 32, 64 i 128  $\mu\text{g/mL}$  za 12, 24, 48 i 72 sata (h) na humanoj TNBC ćelijskoj liniji MDA-MB-231. Rezultati su pokazali da i ZnO i F-ZnO NPs značajno smanjuju vitalnost ćelija karcinoma na način koji zavisi od doze i vremena ( $p < 0,05$ ). Vrednosti IC<sub>50</sub> za FA-ZnO NPs bile su približno 3, 3,74, 4,38 i 5,5 puta veće od onih za ZnO NPs u vremenskim tačkama od 12, 24, 48 i 72 sata, respektivno. Rezultati sugerišu da F-ZnO NPs imaju potencijal da budu dobra opcija za tretman TNBC-a i da opravdavaju dalja istraživanja.

**Ključne reči:** nanočestica, cink oksid, folna kiselina, trostruko negativni rak dojke.

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