

## Cytotoxic Effect of Titanium Dioxide-Hydroxyurea Nanocomposit on Hela Cancer Cell Line

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### ABSTRACT

**BACKGROUND AND OBJECTIVE:** The use of nanotechnology in drug delivery Not only increase the efficacy and ease of drug penetration, but also they decrease their adverse effects. In this study, TiO<sub>2</sub> nanoparticle was used as Hydroxyl Urea carrier to increase the contact surface of the drug and cells, and with pegylation of nanoparticle surface decrease immunogenicity, hence to increase drug solubility and penetration to cells. The goal of this study was to investigate cytotoxicity of synthesized TiO<sub>2</sub>-Poly Ethylene Glycol-Hydroxy Urea (TiO<sub>2</sub>-PEG-HU) nanocomposite on Hela cell-line and apoptosis induction of treated cells compared to the control group to determine the effective dose of nanodrug.

**METHODS:** In this laboratory study, the effect of TiO<sub>2</sub>-PEG-HU nano-drug was evaluated on cells bioactivity by MTT method at concentrations of 200, 400, 800, and 1800 µg/ml in 48 and 120 hours. Annexin-V/PI flowcytometry method was used to analyze apoptosis induction. Data were analyzed using uni-directional variance and independent T-test.

**FINDINGS:** Higher concentrations of TiO<sub>2</sub>-PEG-HU nanocomposite decreased cells bioactivity dependent on dosage and time. As the concentration of 1600 µg/ml of nanocomposite reduced amount of bioavailability by 1.52 times over a 120-hour period compared to the 48-hour time-effect. For both times, this reduced cell survival was `significantly different from that of the control group at the level of  $p < 0.0001$ . In addition, nano-drug significantly increased apoptosis induction 2.5 times in treated Hela cells ( $p = 0.0114$ ).

**CONCLUSION:** Nano-composite TiO<sub>2</sub>-PEG-HU on the Hela cell line is cytotoxic and induces apoptosis and can be a promising drug for cancer treatment.

**KEY WORDS:** *Hydroxy Urea, Titanium, Hela cells, Anti-cancer drugs, Apoptosis.*

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## Introduction

Cervical cancer is the fourth most common cancer in the world, according to statistics. The incidence of cervical cancer in Iran is lower than in some other countries. However, in some provinces it has the seventh rank (1,2). Researchers are now looking for technologies that offer the least side effects in anti-cancer drugs, and they can increase their therapeutic effects by targeting cancer cells (3). Hydroxyurea is a standard anti-cancer drug used in the treatment of some cancers, including cervical cancer, and so on (4). Its mechanism of action is to prevent the formation of doxycyclohexides and DNA replication by inhibiting the ribonucleotide reductase enzyme (5).

Advantages of drug nanoparticles in drug delivery include high efficiency, targeted administration, reduced dosage, reduced side effects, the possibility of passing through various environmental barriers within the body, regular drug kinetics, and controlled biological distribution (6).

Titanium dioxide nanoparticles (TiO<sub>2</sub>) have been widely used in anticancer studies due to their properties such as optical stability, non-toxicity, high oxidative properties, biological properties and availability, and can be used as nanocomposite drugs. TiO<sub>2</sub> nanoparticles have an anti-inflammatory effect due to the production of ROS under UV irradiation and increase the sensitivity of the tumor cells. ROS causes toxicity and cell death with impaired cellular respiration and membrane damage (7,8). In the design of drug nanocarriers, the use of polymers, such as PEGs, is commonly used to effect and increase the drug entry to the target cell, decrease immunogenesis, and more stability and more solubility of the drug in the bloodstream. Thus, the pegylation of nanocomposites, such as TiO<sub>2</sub>, which have a poor dissolution in physiological pH, increases their solubility in body fluids (9).

In this study, with the aim of increasing the cytotoxicity of hydroxyurea, drug delivery and reducing its adverse effects in cancer chemotherapy, the hydroxyurea nanorods were used based on the biodegradable nano-carriers of pegylated titanium dioxide. Therefore, in order to investigate the anticancer properties of this constructed nanodrug, the toxicity of titanium dioxide-hydroxy urea nanodrug and induction of apoptosis in vitro were investigated on Hela cell line.

## Methods

In this experimental study, the cervical cancer cell line Hela (C155) was prepared from the Institute of Pasteur Bank of Iran. The cells were cultured in a DMEM medium containing 10% FBS at 37 °C and in a humid atmosphere with 5% carbon dioxide in a single layer (10). Cell culture materials and other materials were purchased from the company (Gibco BRL, Scotland) or (Sigma Aldrich, USA). Nanoparticle TiO<sub>2</sub>-PEG-HU was used after confirmation of structure by FTIR spectroscopy and determination of morphology and dimensions by SEM imaging in this study. Hydroxyurea nanosilver was prepared in DMSO solution at concentrations of 200, 400, 800 and 1600 micrograms per milliliter, and after 2 hours ultrasonic was filtered with 0.20-micron filter.

**MTT Assay:** In MTT assay, yellow tetrazolium bromide is synthesized by the mitochondrial succinate dehydrogenase enzyme of resuscitated cells and metamorphic crystals. In this test, 10000 Hela cells were added to each well of 96 plates and after 24 hours of incubation, drug solutions were added at 200, 400, 800, and 1600 µg/ml concentrations to each well. As control, only cells without medication were added. After 48 and 120 hours' incubation, the MTT color with a final concentration of 0.5 mg/ml was added to each cavity and after 4 hours, the formazan crystals were solved in DMSO solvent and finally the adsorption rate was read by the ELISA plate reader (Tecan-USA) at 570 nm. All trials were repeated 3 times, and viability and IC<sub>50</sub> (Inhibitory concentration 50) were reported. The biological power of the cells was calculated according to the following formula (11).

**$100 \times (\text{mean optical absorbance of control} / \text{average optical absorption of test}) = \text{bioavailability}$**

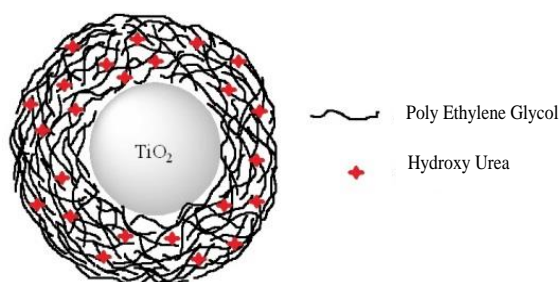
**Apoptosis assessment by flow cytometry:** During apoptosis, phosphatidylserine is transferred from the internal membrane to the outer surface of the cell membrane and attached to the Annexin V-FITC conjugates during staining. In the necrotic cell population or delayed apoptosis, the membrane of the cell is degraded and the cell is permeated to the PI color. The color of PI was detected in the fragmented DNA of the nucleus of dead cells and was detected by flow cytometry (FACS Calibur BD-American). To

investigate cell death and determine the percentage of apoptotic cells, Hela cells were treated for 24h with a concentration of IC<sub>50</sub> of nano-hydroxyurea and then the cellular deposition were stained according to the instructions of the Annexin/ propidium iodide kit (Affymetrix) using a 488nm stimulation wavelength tube and a 515nm readout filter for fluorosine isothiocyanate (FITC) and a filter of 600nm for color (PI) were evaluated and the percentages of each of the quadrants were recorded as total (12).

**Statistical analysis:** Data and IC<sub>50</sub> were analyzed using Graph Pad Prism software version 6.1. The results were analyzed using one-way ANOVA and independent T-test. Efficiency analysis and type of cell death by flow cytometric method were also performed by Flowjo software version 1.07 and  $p < 0.05$  was considered significant.

## Results

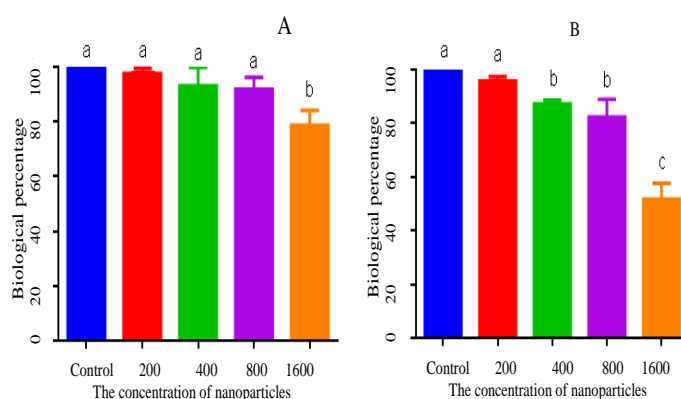
**Titanium Dioxide-Polyethylene Glycol-Hydroxyurea structure and size Verification:** By studying the FTIR spectra of nanoparticles synthesized, the chemical structure of nanoparticles was confirmed. The absorption frequency of the tensile vibrations at 2920, 3420, 1660, 1120, 664 and 540  $\text{cm}^{-1}$  corresponds to CH<sub>2</sub> groups of polyethylene glycol, OH and NH in polyethylene glycol and hydroxyurea, C=O hydroxyurea, COC in Polyethylene glycol and Ti-O-Ti bonds of TiO<sub>2</sub> nanoparticles are in the structure of the nanoparticle. Increasing the adsorption of the CH<sub>2</sub> group confirms the loading of polyethylene glycol on the TiO<sub>2</sub> core. Spherical surface morphology and nanoparticle diameter were confirmed with a SEM image between 30 and 60 nm (Fig 1).



**Figure 1. Structural properties of titanium dioxide-polyethylene glycol nanoparticles containing hydroxypropylene**

**Reduced biological power of Hela cells treated with Nano drug:** The bioavailability of treated cells during the 48-hour exposure period with high concentration of hydroxyurea nanodrug was 1600  $\mu\text{g/ml}$ , equivalent to  $78.66 \pm 5.52\%$  that had a significant biological loss than control group ( $p < 0.0001$ ). Lower concentrations of nanodrug in the same effect period, despite reducing cell survival, were not statistically significant (Fig 2 a). While increasing the time of nanomaterial exposure to 120 hours, the bioavailability of the treated cells at concentrations of 400, 800 and 1600  $\mu\text{g/ml}$  was  $87.15 \pm 1.41$  ( $p < 0.05$ ),  $30.6 \pm 6.5$  ( $p < 0.001$ ) and  $51.73 \pm 5.83$  ( $p < 0.0001$ ), respectively, which showed statistically significant differences (Fig 2 b) ( $p < 0.001$ ). Therefore, increasing the concentration of nanodrugs caused an increase in cell death.

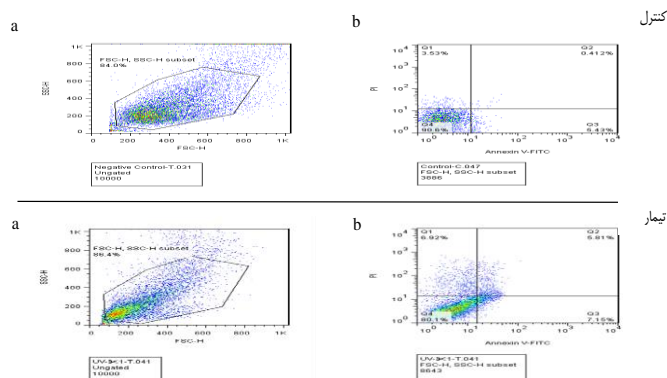
The IC<sub>50</sub> concentration of nano-titanium dioxide-hydroxybutyric acid was calculated for Hela cells treated with 3876  $\mu\text{g/ml}$  for 48h and 1724  $\mu\text{g/ml}$  for 120h, which is an average of 2800  $\mu\text{g/ml}$ . Comparison of bioavailability at 48 and 120 hours showed that time dependent nanomaterial significantly decreased Hela cells death in each of the concentrations of 800 ( $p < 0.015$ ) and 1600 ( $p < 0.0001$ ) (Fig 2).



**Figure 2. Effect of different concentrations of TiO<sub>2</sub>-PEG-HU nanoparticles in 48 hours of effect (A) and within 120 hours of effect (B)** The data is expressed as the mean  $\pm$  sd deviation. Different English letters above each column represent a significant difference between the two groups. Also, the presence of the same letters on top of each column indicates that there is no significant difference between the two.

**Induction of apoptosis in Hela cells treated with TiO<sub>2</sub>-PEG-HU nanodrugs:** Flow cytometry analysis by using propidium/iodide staining showed the type and

percentage of cell death as apoptosis and necrosis of the cells. (Fig 3) (Table 1). The mean percentage of Hela apoptotic and necrotized cells in both control and treated groups with mean IC<sub>50</sub> (2800 µg/ml) of hydroxyurea nanoparticles after two replicates was shown in Table 1.



**Figure 3. Results of Annexin/PI histogram in the flow cytometry test for apoptosis in nano-drug treatment groups**

Histogram a is the investigated area (Gating). Area 1Q Histogram b is indicator of necrosis (Annexin/PI+), area 2Q indicates delayed apoptosis (Annexin +/PI+), Q3 region represents primary apoptosis (Annexin+/PI-) and Q4 region is indicator of living cells (Annexin/PI-). (In the figure only one repeat is displayed)

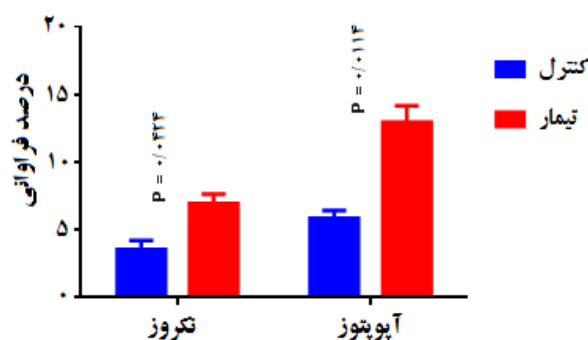
**Table 1. The mean of apoptosis and necrosis in the control group (without drug treatment) and treated with mean concentration of IC<sub>50</sub> nanosilver TiO<sub>2</sub>-PEG-HU during the effect of 24 hours**

Group	Treated	Control	P-value
<b>Cell death</b>			
Primary apoptosis(Q3)	7.86±1.00	5.03±0.57	0.0739
(Q2) delayed apoptosis	5.9±0.12	0.01±0.405	0.0003
apoptosis (Q2+Q3)	13.76±1.13	5.435±0.57	0.0114
Necrosis(Q1)	7.82±1.27	3.07±0.65	0.0424
Alive (Q4)	2.82±1.78	91.49±1.26	0.0257

The values obtained were based on mean ± SD and mean differences at  $p < 0.05$ . The type of test used is independent t test.

Comparison of the results with the control group showed that the percentage of apoptotic cells increased in nano-treated hydroxy-treated cells compared to the control group. The percentage of total apoptosis in treated cells (primary and secondary apoptosis)

increased by about 2.53 times, and this increase was significant at 0.1114 (Fig 4).



**Figure 4. Comparison of mean apoptosis and necrosis in two groups treated with nano-drug hydroxyurea**

## Discussion

The results of this study showed that TiO<sub>2</sub>-PEG-HU nano-drug has dose-dependent and time-dependent cytotoxic effect on Hela cell line, and by increasing the nanodrug exposure time from 48 to 120 hours, the level of cytotoxicity of the nano-drug increased by 25.2 times so that in the time of further exposure, the lower concentrations of nano-drug also significantly reduced the metabolic activity of Hela cells compared to the control group. These results are consistent with the findings of Lotfian et al., which showed that TiO<sub>2</sub> nanoparticles have a weak inhibitory effect on the MCF-7 cell line after 24 hours of incubation. However, after 48 and 72 hours of incubation, it significantly inhibited MCF-7 cell growth, especially at higher doses (13). The results can indicate the effect of nanoparticles on the plasma membrane of cells. It is also seen that during the 24-hour period, about 7.8% of the cells had primary apoptosis, but they are still alive, but with increasing incubation time, these cells also enter the secondary apoptosis phase and dead cells are observed more at 48 and 120 hours, indicating high cell membrane changes and possibly the effect of nanoparticles on DNA and its failure. Other studies have shown that TiO<sub>2</sub> nanoparticles with nitrogen (N-TiO<sub>2</sub>) doped after activation with visible light can inhibit the growth of A-375 melanoma cells and chronic human myeloid leukemia (K562) cells, depending on the concentration and time, and cause cell death as apoptosis (15, 14). Studies have shown that TiO<sub>2</sub> in the epidermal cell line of JB6 motility induces apoptosis after 72 hours by

activating Caspase 8, Bid, Bax, Caspase 3, and Bcl-2 (16). The results of this study showed that the amount of IC<sub>50</sub> of the nano-hydroxyurea group was 4876 and 1724 µg / ml, respectively, at 48 and 120 hours, which, according to the amount of 10% hydroxyurea loading on the pegilated titanium dioxide, the amount of hydroxyurea in the content of the nanostructure was calculated at an average IC<sub>50</sub> concentration (2800 µg / ml) on the Hela cell line, 280 µg / ml, while the IC<sub>50</sub> reported on the standard hydroxyurea on the Hela cell line is 428 microgram per milliliters (17). Thus, the cytotoxicity of the nanoparticles studied in this study on the Hela cell line was 1.53 times higher than that of the standard hydroxyurea, which can reduce the side effects of hydroxyurea. In a study concurred with the present study, the effect of nano-liposomal hepatotoxic hydroxyurea on the breast cancer cell line was reported to be higher than standard hydroxyurea (18). These reports, as well as the results of this study, suggest that the use of suitable nanoparticles for hydroxy urea can increase the cellular absorption and cytotoxicity of hydroxyurea in cancer cells. The results obtained in this study showed that TiO<sub>2</sub>-PEG-HU nanoparticles compared to the control group induced a 2.5-fold increase in cell death as apoptosis in the Hela cell line. Previous reports by Gui (19) and Vesela (20) also referred to the role of hydroxyurea in inducing apoptosis in the Hela cell line. In these studies, the induced

response was influenced by hydroxyurea, regular chromatin concentration on the nucleus cell wall, and the formation of apoptotic membrane bodies. The researchers investigated fragmented of DNA based on clonogenic data, showing that DNA fragments are regular oligonucleosome patches, indicating the planned cell death. Yeo et al. also found in their studies that hydroxyurea can lead to cellular aging by induction of P53, p21 and Waf1 genes (21), which are in line with the results of this study. According to the findings of this study, the TiO<sub>2</sub>-PEG-HU nanoparticle impairs the survival of Hela cells. It also significantly induces apoptosis in the cells under study. These results indicate that the hydroxyurea nano-drug with lower doses than standard hydroxyurea is an anticancer drug, but there are some limitations because this study is experimental. Therefore, it is recommended that in-vitro studies be performed on animal models to evaluate and reduce the side effects of the drug. Maybe it can be used as a substitute for the standard anti-cancer drug hydroxyurea.

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