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Functionalization of Carbon Nanotubes Loaded with Tamoxifen and Their Anticancer Potential against Human Breast Cancer Cells Review

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Abstract

Background & Objectives: Carbon nanotubes (CNTs) serve as molecular carriers for in vivo and in vitro delivery. Initial studies have suggested that nanotubes in drug delivery can enhance the therapeutic response to anti-cancer drugs. The present study intended to investigate the effect of CNTs carrying tamoxifen (TAM-CNTs) on the induction of apoptosis in the MDA-MB-231 cell line.

Materials & Methods: The cells were treated with various concentrations of TAM and TAM-CNTs. The IC50 for these compounds was determined using a MTT assay. The cells were then treated with a lower concentration of IC50. The *BAX* and *BCL-2* genes expression were evaluated by Real-Time PCR and Western blot. Flow cytometry was employed for evaluating apoptosis induction by TAM and TAM-CNTs.

Results: The IC50 value of TAM and TAM-CNTs in a 48-hour period was 66.19 mg/mL and 36.59 mg/mL, respectively. The results demonstrated that BAX in the cells treated with TAM and TAM-CNTs was upregulated 3.64 and 7.88 times, respectively ($P < 0.05$). Conversely, BCL-2 was downregulated 3.98 and 5.31 times ($P < 0.05$). Furthermore, Western blot experiments confirmed the expression of BAX and BCL-2 proteins based on their gene expression. Flow cytometry results indicated that the viability of MDA-MB-231 cells in the control group, TAM-treated, and TAM-CNTs-treated cells was 95.3%, 64.9%, and 13.75%, respectively. This suggests that TAM-CNTs significantly diminishes cell viability compared to TAM ($P < 0.001$).

Conclusion: The findings revealed that TAM accompanied by CNTs exhibits a greater cytotoxic and apoptotic effect on MDA-MB-231 cells.

Keywords: Tamoxifen, Carbon Nanotubes, Nano-delivery system, Apoptosis

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Introduction

Tamoxifen is a drug used to reduce or stop the effects of estrogen in the female body. This drug has been manufactured for over 30 years and is used in the treatment of late stage and early stage breast cancer (1). More recently, TAM has been used as an adjunct or additional treatment after primary treatment in the early stages of breast cancer and as an experimental method to prevent breast cancer in women at risk (2). The development of TAM resistance in cancer cells results from various factors such as environmental carcinogens, host-related factors such as kinetics, by-products, and the rate of drug metabolism in the patient's body, and finally,

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intracellular molecular mechanisms involved in cell growth and proliferation. Multiple signaling cascades of cell growth and proliferation, molecules that regulate the cell cycle, and genes involved in cytosolic and nuclear growth pathways are among the most important factors that may play a role in the phenomenon of resistance (3, 4).

Nanotechnology has led to the development of various types of nanomaterials for wide applications in materials science, including biology and nanomedicine (5). Nanotubes, ranging in size from 1 to 1000 nm, are primarily studied for the diagnosis and treatment of human cancers, leading to new areas of nano-oncology (6). Nanotubes possess unique chemical and physical characteristics, including size, shape, and a high surface-area-to-volume ratio, which make them suitable for various medical and biological applications (7). At times, they are smaller than or comparable in size to cellular structures, viruses, proteins, or genes. Upon injection into animals, these substances are quickly distributed throughout most organs and tissues, and their cellular absorption is highly intense (8).

CNTs possess unique electrical, chemical, thermal, and mechanical properties, making them ideal for a variety of multipurpose applications. Their exceptional characteristics have led to their widespread use in biomedicine, where they have been utilized for numerous therapeutic and diagnostic purposes (9). CNTs are highly valuable in both therapeutic and diagnostic applications. CNTs can form new carrier systems that deliver therapeutic agents to specific locations and can also be used in bioassays. Many advanced CNT systems have been reported, which have huge biomedical potential for the future. The aim of this research was to investigate the therapeutic efficacy of carbon nanotubes (CNTs) as carriers of TAM against breast cancer cells, in comparison to the use of TAM alone.

Materials and Methods

Cell culture

The MDA-MB-231 cell line was obtained from the Pasteur Institute of Iran. Morphology and risk of cellular contamination were assessed. The cells were cultured in DMEM medium containing penicillin/streptomycin (Pen/Strep, Biosera, USA) and stored in an incubator at 37°C, 5% CO_2, and 95% humidity. As this work was initiated before the application for the code of ethics, it was not subject to that code.

TAM loading on CNTs

To load the TAM inside the CNTs, we used a method based on the use of a dialysis membrane. We filled the dialysis bag, prepared according to the manufacturer's protocol, with 1 g of CNTs and added 10 mL of water before closing it firmly. The dialysis bag was immersed in a beaker with 500 mL of deionized water on a magnetic stirrer for 4 hours. This allowed the nanotube to reach its maximum swelling limit and, consequently, provided more space for establishing hydrogen bonds with the drug. Next, 500 mL of ethanol saturated with TAM was prepared, and the dialysis bag containing nanotubes was placed in this solution for 6 hours. The nanotubes loaded with TAM were separated from the TAM-saturated ethanol through centrifugation. After freeze-drying, TAM-CNTs were prepared. Then, various concentrations of TAM/CNTs were arranged, including 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, and 1/640.

Cytotoxicity of TAM and TAM-CNTs

To investigate the toxicity of TAM and TAM-CNTs against the MDA-MB-231 cell line, we incubated approximately 10^4 cells in a final volume of 200 µL DMEM+10% FBS (Bio idea, USA) in each well of a 96-well plate at 37°C for 24 h. After draining the culture medium, we treated the cells with concentrations of 1, 5, 10, 20, 50, and 100 mg/mL of TAM and TAM-CNTs in the culture medium containing 2% FBS. The plates were incubated for 48 hours, and cell viability was assessed using the MTT assay. To achieve this, the medium in each well was removed and the plate was incubated with 20 µL of MTT solution (5 mg/mL) for 4 hours. Afterward, the supernatant

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was slowly removed to prevent the crystals from detaching from the bottom of the plate. Then, 100 µL of DMSO was added to each well, and the plates were placed on a low-speed shaker for 15 minutes until the formazan precipitates were fully dissolved. Finally, the optical density (OD) of each well was measured using an ELISA reader at a wavelength of 570 nm. The relative cell viability for each concentration of TAM and TAM-CNTs was calculated by comparing the number of viable treated cells to the number of untreated control cells.

The expression of the genes involved in apoptosis

The study evaluated the expression of apoptosis-related genes *BCL2* and *BAX* in the MDA-MB-231 cell line treated with 60 mg/mL of TAM and 30 mg/mL of TAM-CNTs. RNA was extracted from the cells using the Trizol kit (Yekta Tajhiz Azma, Iran) following the manufacturer's protocol. The extracted RNA was checked for quantity and quality using spectrophotometry and agarose gel electrophoresis. The cDNA synthesis kit from Yekta Tajhiz Azma in Iran was used along with oligo dT primers for cDNA synthesis. The reverse transcription reaction was performed at 37˚C for 15 minutes. Real-time PCR was conducted using the SYBR™ Green PCR Master Mix and primers listed in Table 1. *GAPDH* was used as a housekeeping gene.

Table 1. The primer sequences for Real Time PCR

Primer name	Primer Sequence (5'→3')	Amplicon size (bp)
GAPDH Forward	TGCCTCCTGCACCACCAAC	131
GAPDH Reverse	CGGAGGGGCCATCCACAG	
BAX Forward	TTGCTTCAGGGTTTCATCCAGG	171
BAX Reverse	GGCGGCAATCATCCTCTGC	
BCL-2 Forward	GAACAGGGTACGATAACCGGGAG	162
BCL-2 Reverse	CTGGATGGGGCGTGTGCC	

The expression of proteins involved in apoptosis

To investigate the impact of TAM and TAM-CNTs on the expression of BCL2 and BAX proteins in the MDA-MB-231 cell line, we utilized the western blot assay. First, we extracted the protein from the cell lysate by centrifuging the microtube containing the cells at 1300 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µL of RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS, and 0.01% (w/v) sodium azide at a pH of 7.4). The mixture was then incubated at -20˚C for 1 hour, followed by centrifugation at 1300 rpm for 10 minutes at 4˚C. The resulting supernatant, which contained the

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protein, was transferred to a new microtube. SDS-PAGE was performed using a 28% resolving gel and a 22% stacking gel. The separated protein was then transferred onto a PVDF membrane and blocked with a solution containing 5% skim milk in a 0.05% PBS-Tween-20 solution at 4°C for 12 hours. The membrane was subsequently washed with PBS-Tween-20 for 5 minutes (Merck, Germany). After incubation with the primary antibody, the membrane was washed three times with PBS-Tween-20 for 5 minutes. Subsequently, it was incubated for 1 hour at 25°C with 0.4 µg/mL labeled secondary antibody (Sheep anti-mouse-peroxidase conjugate, 1:5000 with 3% skim milk in 0.05% PBS/Tween-20). Following this, the membrane was washed again and the bands were checked and verified using a gel documentation device.

Assessment of apoptosis induction by TAM and TAM-CNTs

The evaluation of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (Agilent, USA). 5×10^5 treated cells were added to each tube and raised to a volume of 500 µL with binding buffer. The cells were first washed with PBS to remove the medium. Four tubes were used: one without FITC, one with Annexin V-FITC, one with PI, and one with both FITC and PI. Annexin V-FITC (5 µL) was added to the 2nd and 4th tubes and incubated for 15 min at 4°C in the dark. After incubation, the tubes were washed with 1 ml of 1X binding buffer and centrifugedat 1500 rpm for 5 min. The supernatant was removed, and 500 µL of 1X binding buffer was added. At the time of sample reading, PI (3 µL) was added to the 3rd and 4th tubes.

Data Analysis

Data analysis was performed using SPSS software version V.26. Due to the normality of the data distribution, the comparison between groups was analyzed by one-way ANOVA, and the p value was calculated. $P<0.05$ was considered significantly different. The Tukey method was used as a post hoc test.

Results

Evaluation of synthesized TAM-CNTs

After synthesizing the CNTs in the laboratory, we conducted thermogravimetric analysis on them (see Figure 1a). The analysis revealed two stages of weight loss. The first stage occurred from ambient temperature up to approximately 150°C, while the second stage occurred from 150°C to 400°C. The synthesis of CNTs involves two phases: evaporation of moisture and solvents in the first phase, and destruction of carbon chains in the second phase, resulting in shortened and torn chains. TAM binding to the CNTs is indicated by weight reduction. The size of the synthesized CNTs was evaluated using electron microscope imaging (Figure 1b), which confirms successful synthesis with an average size of approximately 100 nm.

MTT assay

The toxicity of TAM and TAM-CNTs against the MDA-MB-231 cell line was measured using the MTT assay. The results showed that both TAM and TAM-CNTs exhibit dose-dependent toxicity. As the concentration of TAM and TAM-CNTs increased, the percentage of cell viability decreased. Furthermore, the toxicity of TAM-CNTs was greater than that of TAM alone at all concentrations tested (1, 5, 10, 20, 50, 100 mg/mL) (Chart 1). The IC50 was calculated for a 48-hour period. The IC50 value of TAM and TAM-CNTs was 66.19 mg/mL and 36.59 mg/mL, respectively. This indicates that the toxicity of TAM-CNTs against MDA-MB-231 cancer cells was significantly higher than that of TAM alone ($P<0.05$).

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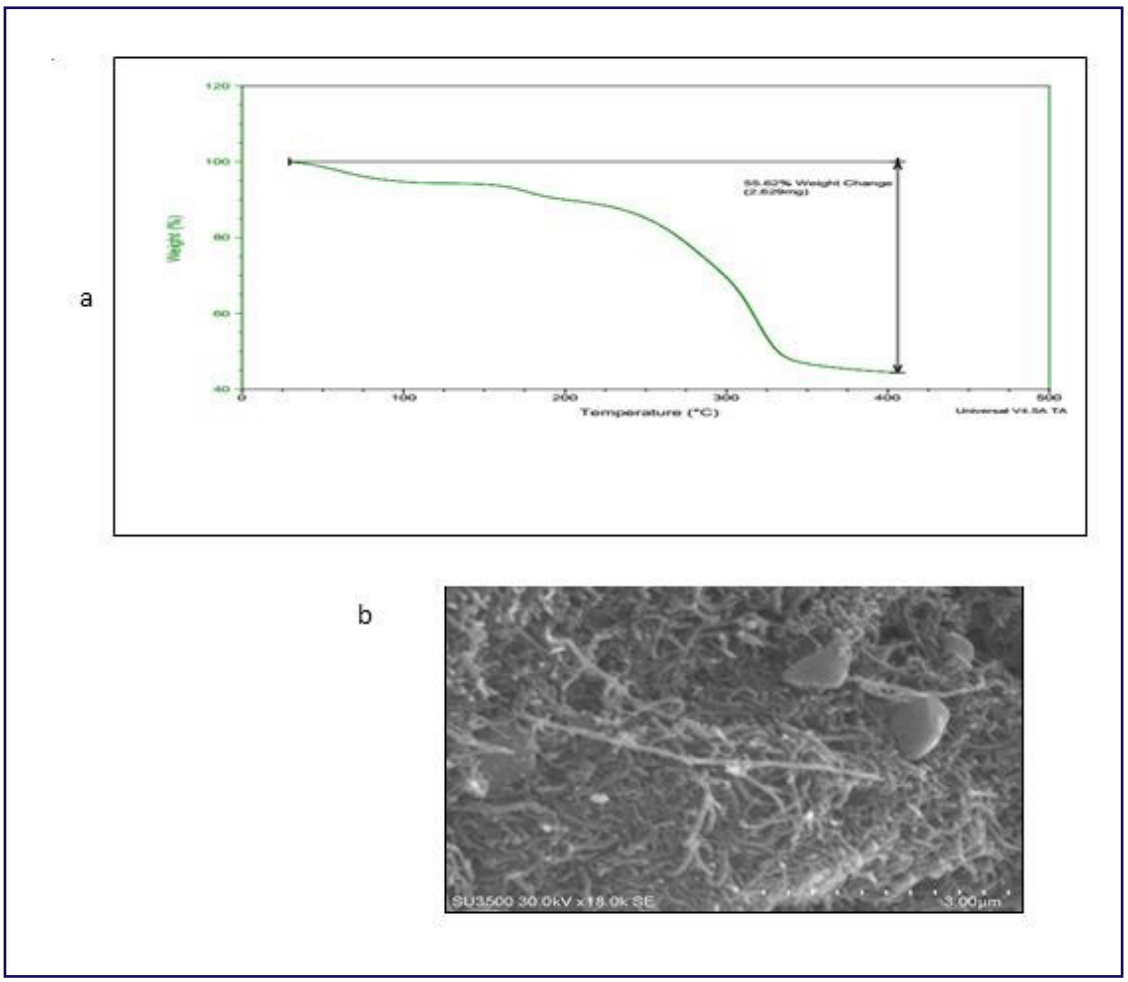

Figure 1. Characterization of CNTs a: The result of thermogravimetric analysis of the CNTs, b: Scanning electron microscopy of the CNTs, Diameter of CNT (100 nm) is shown by Yellow line

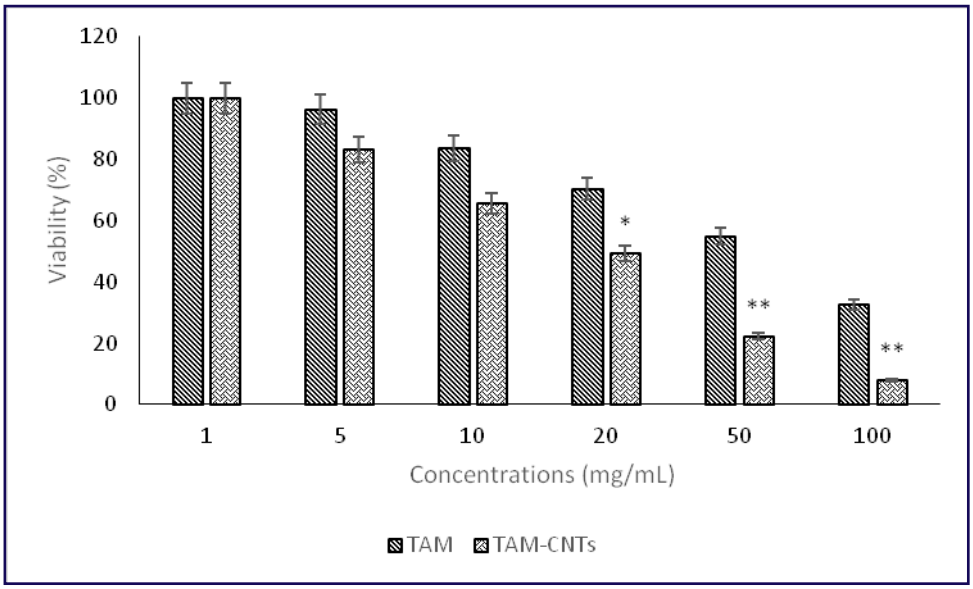

Chart 1. Viability of MDA-MB-231 cells treated with TAM and TAM-CNTs. * $p<0.05$, ** $p< 0.01$

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Real Time PCR

Real-time PCR was used to analyze the expression of *BCL-2* and *BAX* genes in the MDA-MB-231 cell line treated with TAM and TAM-CNTs (Chart 2). The *BAX* gene was overexpressed compared to the reference gene (*GAPDH*) by 3.64 and 7.88 times in the treatment with TAM and TAM-CNTs, respectively ($P<0.05$). Additionally, the expression level of the *BCL-2* gene decreased by 3.98 and 5.31 times in the treatment with TAM and TAM-CNTs, respectively, compared to the reference gene ($P<0.05$).

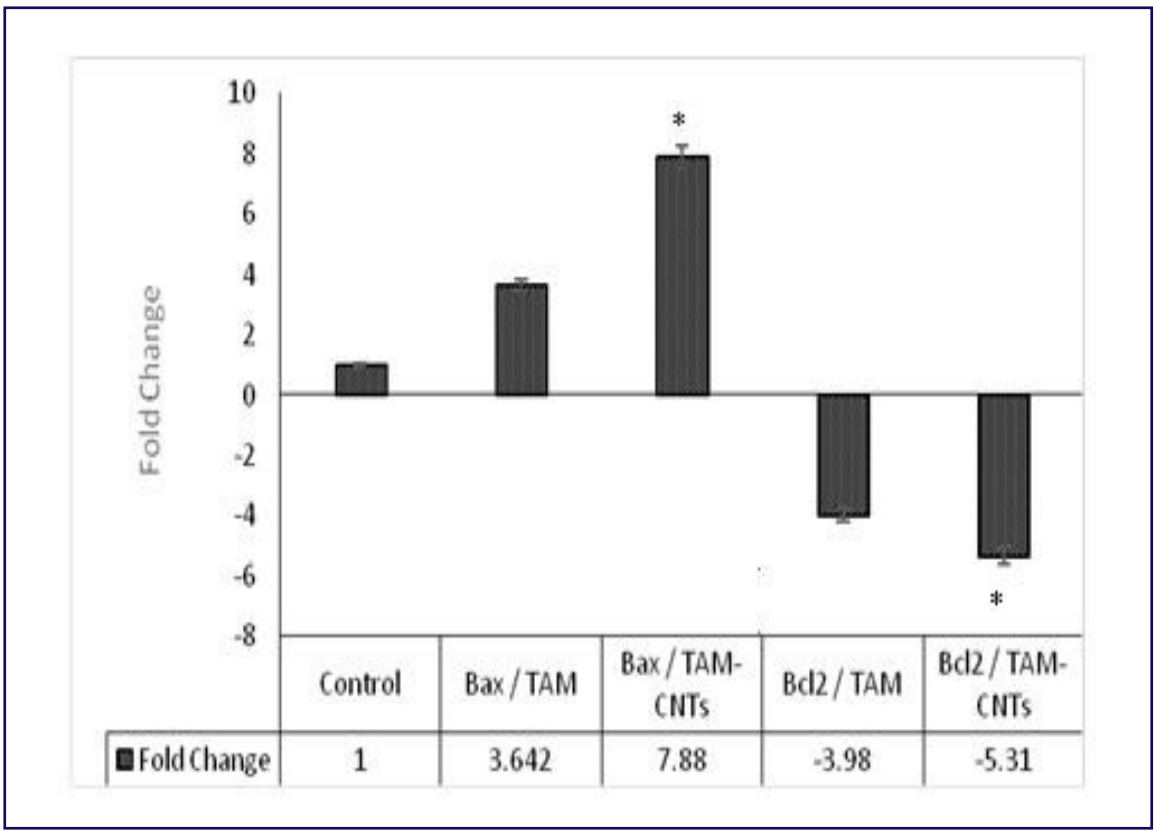

Chart 2. Expression changes of *Bax* and *Bcl2* genes for MDA-MB-231 cell in TAM and TAM-CNTs (*$P<0.05$)

Western blot

The study utilized Western Blot to examine the expression of BAX and BCL-2 proteins, which play a role in the apoptosis process. Figure 2a displays the expression of the 27 kDa BCl-2 protein, while Figure 2b shows the expression of the 23 kDa BAX protein. The intensity of the bands formed indicated a decrease in BCl-2 protein expression in cells treated with TAM and TAM-CNTs compared to the control (Chart 3). The MDA-MB-231 cells showed a greater decrease in protein expression when treated with TAM-CNTs compared to TAM alone. Additionally, BAX protein expression increased in cells treated with both TAM and TAM-CNTs compared to control cells, with a higher increase observed in cells treated with TAM-CNTs.

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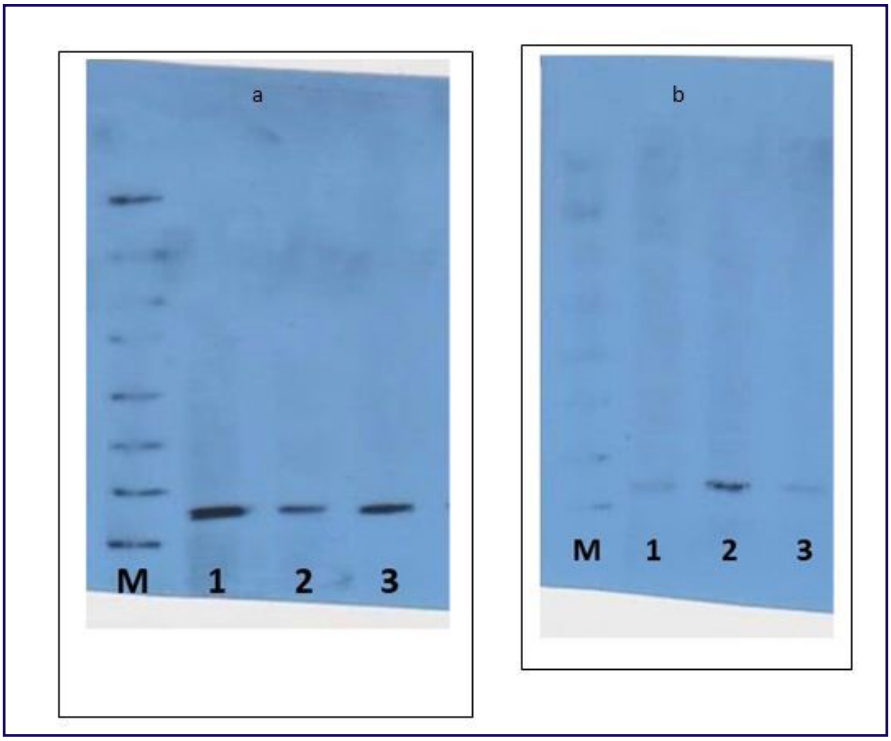

Figure 2. Western blot to evaluate the expression of BCL-2 (a) and BAX (b) proteins (M: marker, 1: control, 2: TAM-CNTs, 3: TAM)

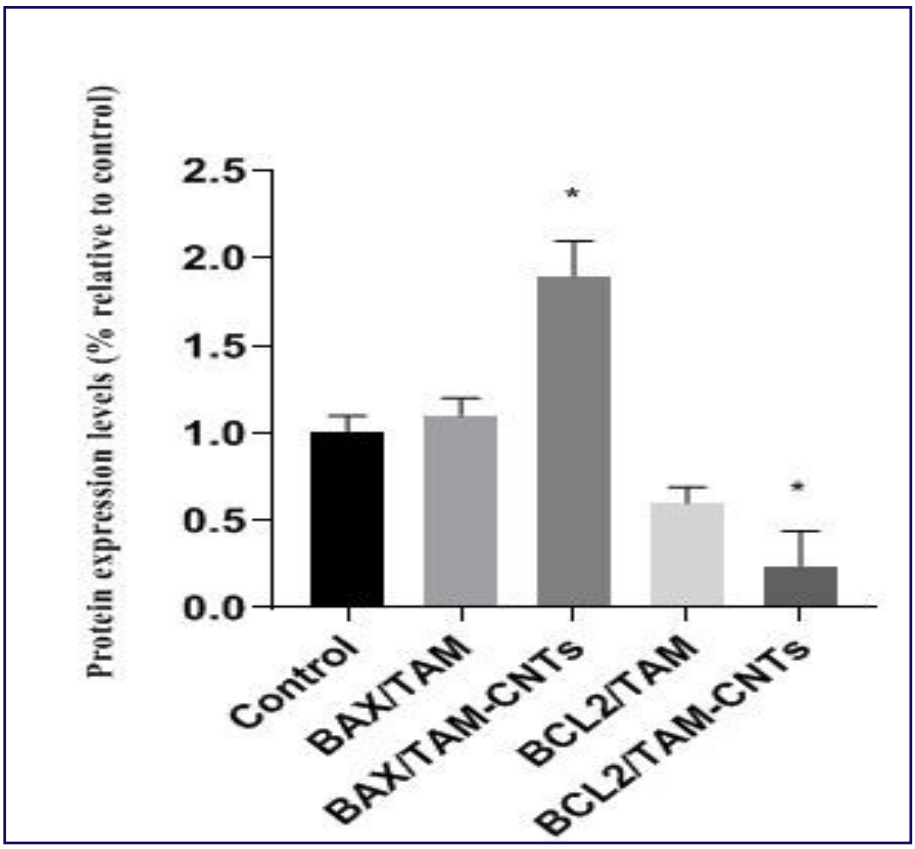

Chart 3. Protein expression of *Bax* and *Bcl2 genes* for MDA-MB-231 cell in TAM and TAM-CNTs (*P<0.05)

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Flow Cytometry

The cells were treated with TAM and TAM-CNTs separately, and the rate of apoptosis induction was assessed (Figure 3). The survival rate of MDA-MB-231 cells in the control group, TAM treatment group, and TAM-CNTs treatment group were 95.3%, 64.9%, and 13.75%, respectively. This indicates that both TAM and TAM-CNTs were capable of inducing cell death, and the difference between the treatment groups and the control group was statistically significant ($P < 0.05$). The study found a significant difference ($P < 0.05$) in survival rates between the group treated with TAM-CNTs and the group treated with TAM. Additionally, the number of early apoptotic cells was significantly higher in the treated cells compared to the control cells. The values for early apoptotic cells in the control, TAM-treated, and TAM-CNTs-treated groups were 2.66%, 4.95%, and 84.92%, respectively. Treatment with TAM-CNTs induced significantly higher apoptosis than TAM alone ($P < 0.05$).

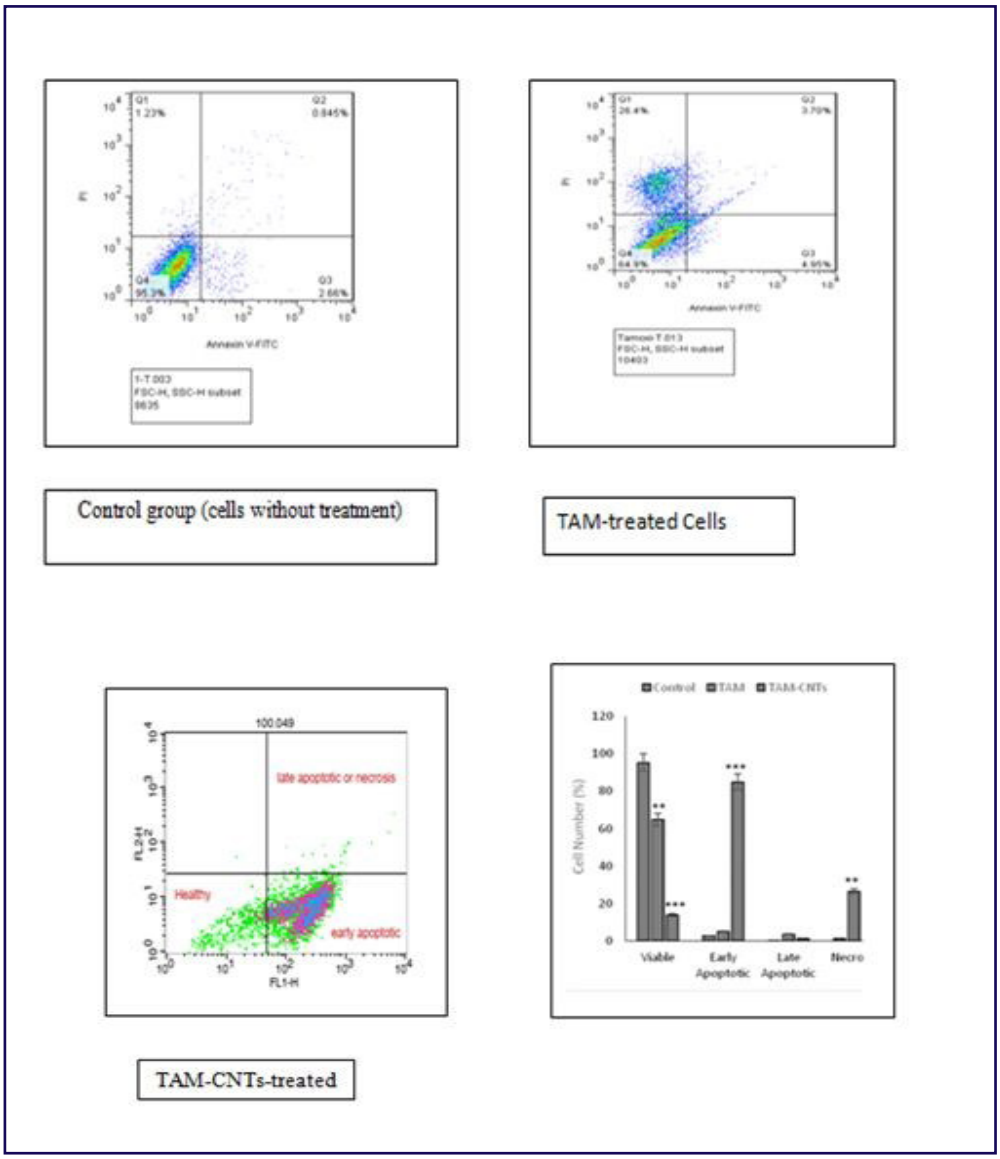

Figure 3. Assessment of apoptosis in MDA-MB 231 cells treated with TAM and TAM-CNTs (**P<0.01, ***P<0.001)

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Discussion

In this study, TAM-CNTs exhibited a cytotoxic concentration against breast cancer cells that was up to two times higher than that of TAM alone. Treatment with TAM and TAM-CNTs upregulated the proapoptotic *BAX* gene in MDA-MB-231 cells by 3.64 and 7.88 times, respectively, compared to the control group. Additionally, the anti-apoptotic *BCL-2* gene was downregulated by 3.98 and 5.31 times in these two groups, respectively, relative to the control group. The protein levels were confirmed by Western blot analysis. *Bcl2* is an oncogen-derived protein that provides negative control in the apoptosis pathway, while *BAX* promotes cell suicide machinery (13). The results indicate that both TAM and TAM-CNTs induced apoptosis. Flow cytometry measurements showed that the amount of apoptosis induction was over four times higher in MDA-MB-231 cells treated with TAM-CNTs than in TAM-treated cells. All observations from this research confirm that the use of TAM-CNTs can regulate the expression of genes involved in apoptosis and induce cell death in MDA-MB-231 breast cancer cells more effectively than TAM.

Although there have been significant improvements in cancer treatment, successful treatment is still a major challenge due to off-target side effects, drug or gene instability, and multi-drug resistance. Nanotechnologies have been applied to biomedical research to address some of these issues. Carbon nanotubes (CNTs) have been used as nanometric drug carriers for cancer treatment over the past decade due to their potential. CNTs' unique physical properties have made them useful in various modern cancer treatment methods (14).

Nanotubes have been extensively studied for drug delivery in biomedicine (15, 16). Carbon nanotubes (CNTs) are particularly noteworthy due to their large surface area, biocompatibility, and ability to deliver large cargos of medicines and biomolecules. Recently, functionalized CNTs have been developed to address some of the limitations of conventional CNTs and improve their effectiveness as a nano-delivery system (17). Various types of nanotubes have been utilized for cancer cell treatment and drug delivery. Gurunathan et al. (2015) reported that biologically synthesized silver nanotubes have a cytotoxic effect on MDA-MB-231 human breast cancer cells. This effect was further increased by inhibiting cell growth, increasing ROS production levels, and activating caspase 3 activity (18). The research also employed CNTs. CNTs are useful in non-cytotoxic drug delivery because they are degradable in the body. This property is advantageous for our research.

Chen et al. (2013) presented a system based on single-walled carbon nanotubes (SWCNTs) modified with an asparagine-glycine-arginine (NGR) peptide. The modification was achieved through a simple non-covalent approach and the resulting system was able to load TAM. The TAM-loaded NGR-modified SWCNTs (TAM/NGR-SWCNTs) retained both the optical properties of SWCNTs and the cytotoxicity of TAM. Furthermore, they were able to enter and accumulate in 4T1 cells. The study investigated the increased cell uptake, antitumor effect, and cell apoptosis of TAM/NGR-SWCNTs compared to TAM, TAM/SWCNTs, and photothermal treatment alone against 4T1 cells in vitro. The results confirmed the superior performance of TAM/NGR-SWCNTs. In vivo investigation of TAM/NGR-SWCNT in tumor-bearing mice further confirmed its significantly higher tumor-targeting capacity and anti-tumor efficacy compared to the control group. Additionally, histopathological analysis demonstrated negligible systemic toxicity (19).

Yi et al. (2018) developed a multi-walled lentinan carbon nanotube delivery system using TAM as an anti-cancer

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agent through a simple non-covalent approach. The system, named MWCNTs-TAM-LEN, demonstrated good stability, water dispersibility, and extraordinary photothermal properties. Practical experiments showed that MWCNTs-TAM-LEN increased cell absorption, antitumor activity, and cell apoptosis in MCF-7 cells compared to TAM and MWCNTs-TAM. The study found that MWCNTs-TAM-LEN treatment resulted in a 67.1% increase in cell growth inhibition and a 66.5% increase in apoptosis rate compared to TAM treatment alone (20).

In a study conducted by Oskoueian et al. (2018), they synthesized and evaluated a TAM-conjugated SWCNT for its anticancer potential against MCF-7 human breast cancer cells. The findings showed that the combination of TAM and functionalized SWCNT increased the cytotoxic effect of TAM against breast cancer cells by 2.3-fold. The higher cytotoxic effect of SWCNT-PEG-TAM than TAM was confirmed by the results of the morphological evaluation and caspase-3 activity. The study results indicate that the delivery system enhances the therapeutic effects and anticancer potential of TAM against human breast cancer cells (21).

This study demonstrated that loading TAM onto CNTs increases its cytotoxicity and may induce apoptosis in MDA-MB-231 cells more effectively than TAM alone. This suggests that CNTs are a suitable carrier for TAM, enhancing its cellular penetration and therapeutic efficacy against cancer cells.

Conclusion

This study aimed to investigate the effect of TAM-CNTs on cytotoxicity and apoptosis induction in the MDA-MB-231 cell line. The findings suggest that the use of CNTs increases the uptake of TAM by cells and enhances its effect on the cancer cell line. This was demonstrated by the upregulation of genes involved in apoptosis at both the mRNA and protein levels, as well as the evaluation of live and dead cells using flow cytometry. The study confirmed the effectiveness of the TAM combined with CNTs in inducing cytotoxicity against cancer cells. These findings suggest that the TAM combined with CNTs may be a more suitable treatment option than the TAM alone. Furthermore, this study utilized carbon due to its biodegradability and biocompatibility in comparison to other compounds used in the nanotube preparation process. Additionally, the use of nanotubes allows for lower doses of TAM to be administered, potentially reducing the drug's side effects and preventing resistance. Therefore, it is hoped that the clinical use of CNTs in cancer drug delivery will receive more attention.

Acknowledegments

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Conflict of interest

The authors declare no competing interest.

Funding

Not applicable.

Ethical considerations

This study was conducted in accordance with ethical considerations.

Code of Ethics

This study was approved by Islamic Azad University, Central Tehran Branch (IR.IAU.TMU.REC.1401.162).

Authors' Contributions

This study was designed and supervised by M.R. and A.M. S. Y. and N.S. participated in sample collection and doing experiments. Statistical analysis, interpretation and drafting the paper were conducted by M.R. and A.M. M.R. revised the paper. All authors have read the manuscript and approved it for publication.

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Data Availability Statement

The data that support the findings of this study are available via corresponding author, [Mina Ramezani], upon reasonable request.

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