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Investigation of immigration in breast cancer cells after following affecting nano-graphene oxide

Setareh Azeri Asgari [1], Saeed Hesami Takallou [2*], Hakimeh Zali [3]

1. Setareh Azeri Asgari [1]
M.Sc. Student in Cellular and Molecular Biology, Biochemistry, Islamic Azad University, Tehran Central Branch, Tehran, Iran.
2. Saeed Hesami Takallou [2*]
Department of Biology, Islamic Azad University, Tehran Central Branch, Tehran, Iran.
*Corresponding author email: s.hesamitakalloo @ iauctb.ac.ir
3. Hakimeh Zali [3]
Assistant professor, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Abstract

The breasts are the protrusions in the upper abdominal area of the male and female primate trunks. In women, the breasts act as mammary glands to produce and store milk for babies. Certain parts of the breast can become cancerous, like other tissues and organs. Breast cancer is very common. One of the parts of the breast where tumors may form is the lobules. These tumors are also called carcinomas. There are many breast cancer treatments, including surgery, radiation therapy, hormone therapy, and chemotherapy, each with its own side effects and disadvantages.
This study investigates the effect of breast cancer cell migration after NGO exposure and also examines the expression and modification of T1MP1 and CYP1B1 proteins. Initially, MDA cancer cells were prepared and cultured. These cells were then treated by the NGO and examined by APOP ICC, SCRATCH, DAPPY, SRB tests.
Studies have shown that in all of these tests, cell survival was reduced due to the NGO's effect on cancer cells. Cell survival was increased only in the SRB test by increasing graphene concentration, which can be attributed to NGO light absorption.

Keywords: Breast cancer, Nano graphene, T1MP1 and CYP1B1 ProteinsIntroduction

Breast cancer is considered to be the most common malignancy in women worldwide. The number of Iranian women with breast cancer is increasing day by day (X). Breast cancer accounts for about 76% of the most common cancers among Iranian women. A total of 41,000 people in Iran have breast cancer, to which more than 7,000 patients have added annually (1).

An increasing number of patients with this disease has made it doubly important to know and find effective treatments. Breast cancer is caused by a combination of lifestyle, hormonal, genetic, and environmental factors, some of which are fixed, some of which are probable, and some of which are not fixed. Genetic factors include age, race, family history, gender, height, early menstruation, late menopause, benign proliferative diseases, and childhood risk factors (2).

Most recently, a genome-wide association study (GWAS) identified genetic diversity (single-nucleotide polymorphism) in familial breast cancer status, and case-control studies of breast cancer identified common, low-penetration genetic variations in at least 120 candidate genes. Among these candidate genes, the major influences associated with breast cancer risk include genes involved in cell cycle control, steroid hormone metabolism, and cellular transmission pathways. Common predisposing alleles, including LSP1 and MAP3K1, TNC9, FGFR2, TGFβ1, CASP8, have the strongest and most consistent evidence of breast cancer (3).

Numerous articles also point to the effectiveness of two other genes in breast cancer treatment and prognosis: tissue inhibitor metalloproteinases-1 (TIMP-1), a member of the TIMP family, identified

two decades ago. It was initially identified as an endogenous matrix metalloproteinase inhibitor (MMPI).

Evidence suggests that TIMP-1 overexpression is common in several types of human cancer, including breast cancer. As a major cytokine and regulator of ECM degradation, TIMP-1 has various functions associated with cancer progression (X). In addition to its inhibitory activity against MMP, TIMP-1 also increases cell proliferation rates in various cell types (X), including breast cancer cells (4).

Numerous studies have reported a clear link between high levels of TIMP-1 and poor prognosis at mRNA and protein levels in breast cancer. Some studies have reported that TIMP-1 is a predictive marker of chemotherapy and hormone therapy in those with high levels of TIMP-1 who show no response.

Breast cancer is one of the most common cancers. There are several treatments for breast cancer, including surgery, radiotherapy, and hormone therapy, each with its own disadvantages and side effects (5).

Another disadvantage of these methods is drug resistance, which leads to non-response to treatment and, consequently, disease progression.

About 70% of patients show a therapeutic response to clinical examination and imaging. However, studies suggest a complete pathological response (PCR) of only 3-30%. Residues of cancer cells in the blood of these patients lead to recurrence (relapse) and, subsequently, their transfer to new areas during the EMT process (6).

Failure to respond to treatment can also be due to residual CSCs in the blood, which can reproduce and metastasize.

The ability to destroy CSCs is considered an effective step in treating this disease, the destruction of existing tumors, and the inactivation of future metastases. An increase in the incidence of cancer and deaths from it requires the development of effective strategies to control it. A new approach, complementary to conventional cancer treatment and diagnostic methods, is the use of nanoparticles as a carrier and protector of biological drugs. This is the first revolution in the field of nanotechnology (7).

Nanotechnology-based cancer treatment eliminates Klein's treatment problems and side effects. Following the functionalization and deployment of drugs used to treat cancer on nanoparticles, drugs can target cancer cells with higher potency, thereby reducing toxicity and side effects on healthy cells (8).

Graphene-based nanomaterials, especially GO, have excellent potential for cancer-specific chemotherapy as drug carriers and active agents, thanks to their unique physicochemical properties, including wide surface area, manipulable active groups, and high biocompatibility (54). Due to the anticancer properties of quinoline-derived compounds, such as 8-hydroxy (79, 1), and the increasing use of GO nanocomposites as drug carriers and their anticancer properties (14), this study used GO sheets as 8-hydroxyquinoline compound carriers. The aim was to evaluate the efficacy of this compound in cytotoxicity by induction of apoptosis in breast and colon cancer line cells, assuming synergistic anticancer properties of GO nanosheets with 8-hydroxyquinoline. (9)

In two studies conducted in 2008, functionalized GO with amine-terminated branched polyethylene glycol (PEG) resulted in the production of PEGylated nanographene oxide (NGO-PEG with ultra-small size (10-50 nm) and high stability in physiological solutions. In CNTs, the graphene surface is displaced by π electrons and can be used to effectively load anti-aromatic anticancer drugs, such as doxorubicin and water-insoluble SN38. Very high graphene levels, with all the atoms on its surface, allow very high loading of the drug. The end of PEG chains is considered to be suitable sites for binding to targeting ligands, such as antibodies, which facilitate the targeted drug delivery process to specific types of cancer cells. Remarkable in drug delivery (10).

Using these nanoparticles as a drug to treat malignant cancer cells has no adverse effect on healthy cells and tissues (11).

One of the most promising carbines of nanoparticles can be their use in performing both tumor diagnosis and drug delivery to the tumor. (12)

Following treatment of stem cells with GO solution, the researchers found that the oxide not only disrupted the ability of stem cells to proliferate tumorspheres in all cancers but was also safe for skin cells. GO appears to cause CSCs to become non-cancerous stem cells. In this method, GO prevents the production of future tumors by stem cells. This theory is based on the fact that GO interferes in the signaling pathways of cell membranes and disrupts their proliferative mechanism.

In their study, Imani et al. (2017) investigated the effect of graphene on gene delivery in breast cancer treatment. In most cancers, cancer cells have many folic acid receptors on their surface due to their high metabolism level. They utilize folic acid molecules as targets for cancer cells and increase nanocarrier uptake by the cell. In this case, folic acid binds to the activated nanocarrier. Chloroquine was also added to the structure to increase the delivery efficiency and functionality of the nanocarrier within the cell. The dimensions of this nanosystem are designed to not pass through the wall of normal tissue vessels, while cancerous tissue vessels allow nanoparticles to pass due to their greater permeability. Experiments on two breast cancer cell lines have shown that a designed GO nanocarrier can efficiently enter cancer cells, prevent them from proliferating through the desired gene delivery and cause their gradual death (13).

In their study, Li et al. (2018) investigated the effect of GO application on the inhibition of metastasis in breast cancer. For this purpose, they used heparin-engineered GO engineered with PEG as the doxorubicin carrier, which allowed the drug to be overloaded in cancer cells. GDC0941, phosphatidylinositide 3-kinase/Akt was also used to enhance metastasis inhibition. The results showed that this treatment prevented metastasis up to 76% in vitro and up to 73% in vivo (14).

In a 2019 study, the effect of paclitaxel delivery on breast cancer cells was investigated using folic acid-labeled graphene. This study analyzed the fabrication level of GO-folic acid/paclitaxel nanoparticles using FTIR, XRD, SEM, and TEM techniques. The cytotoxicity of these particles on the MDA-MB-231 cell line was also investigated using the MTT method and the effect of the drug in vitro on rats with DMBA-induced breast cancer. The results showed that these nanoparticles could effectively target cancer cells (15).

This study aimed to investigate the effect of nanographene particles on the prevention of metastasis in breast cancer.

Materials and Methods

Cancer Cell Culture

The 7-MCF cell line was purchased from the Center for Genetic Resources. The cells were then cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin) and stored in a 25 cm3 flask in an incubator at 37°C and 5% CO2.

After reaching the required density, the cells are passaged. As the cells adhere to the bottom of the flask, the previous culture medium is first drained, and the flask is washed twice with PBS buffer. Then, Trypsin 10x is poured into the cells, and they are incubated in the incubator for 5-10 minutes. The process of separating cells from the bottom of the flask is examined under a microscope. FBS is then added to them to inhibit trypsin. Then, the contents of the Falcon are centrifuged for 10 minutes at 12000g. Then, the contents are emptied and poured on the sediment of the new culture medium. The cells are now counted and tested in the next steps.

Solutions required for cell culture: 1.043 g of DMEM (Gibco) culture medium containing L-Glutamine, 0.2 g of NaHco3 (sigma) dissolved in 80 ml of deionized water. The pH was set to 7-7.1. The volume of the solution was brought to 90 ml with deionized water and filtered with a syringe filter. Then, 10 ml of FBS (Gibco) was added to the solution.

Trypsin-EDTA solution: This solution was used to separate cells from the bottom of the cell culture dish.

1% Agar: 1% Agar (Merk) was used as a layer to cover the bottom of the plate for spheroid culture.

SRB solution required: In the SRB method, after treatment, TCA (Trichloroacetic acid) is added and kept for 30 minutes at 4 ° C. It was then washed with water five times. After drying, 4% sulforhodamine B solution dissolved in 1% acetic acid was added. It was then washed with 1% acetic acid five times. After drying and adding 10mM Tris, the light absorption of each well was measured using ELISA (Stat fax-2100, Spain) at 570 nm.

Tris solution: This solution was used for staining and cell counting.

Paraformaldehyde: This solution was used to hold the colonies in place.

Cell culture:

DMEM culture medium 10% -FBS10% - 1% penstreptate (penicillin and streptomycin) with these concentrations were suitable for culturing cancer cells.

After 48 hours, cancer cell growth and isolation of grown cancer cells were assessed using Falcon.

Order:

Initially, the culture medium was drained due to cells adhering to the bottom of the plate. Growing does not separate from Falcon. Falcon was then washed with PBS buffer solution to completely remove the medium from the cells. Trypsin was then used to isolate cells from the Falcon surface. The trypsin was then poured into the Falcon and incubated in the incubator for 5-10 minutes. It was then exported, and the accuracy of cell separation was checked again under a microscope. It was observed that the cells were isolated from the surface and suspended. Some FBS was added to trypsin-containing Falcons to neutralize trypsin. The contents of the Falcon (suspended cells and FBS) were then centrifuged for 5 minutes at 1200 rpm. It was observed that the cells precipitated in the bottom of the container and were placed on them as a medium. The cells were then counted under a microscope using neobar lam and 10 lambdas below lam.

Calculation process: The number of cells counted divided by 4 times 10 to the power of 4: 1500000.

Freezing cells

After counting the cells, 2-3 million cells in one ml of a solution containing 95% FBS and 5% DMSO were transferred to a freezer vial. The vial was placed for 30 minutes at 4 ° C, 1 hour at -20°C, and 24 hours at -80°C. They were then transferred to a nitrogen tank at -170°C.

SRB test

The SRB test can be used to obtain IC50, based on sulfate reduction by living cells on plates. After the cells grew and the nanodrug was added at the desired concentrations during the test (i.e., 0 -10-20-40-8 μM), the cells were fixed on a plate. This was done with the TCA solution. This was followed by adding SRB color to the wells. It was then washed using 1% acetic acid.

Scratch method

The scratch method is a suitable method not only to monitor the migration of a homogeneous cell population but also to study single-cell migration by considering cells at the edge of the scratch. It lasts from a few hours to one night. This method uses 6-well plates, in each of which 1×10^5 cells are poured along with 2 ml of DMEM culture medium containing FBS. Previous medium and adding mitomycin to the cell culture medium to prevent cell division. After two hours, scratch is created in the middle of each house. This is followed by draining the perimeter of each house and washing it three times with PBS. Concentrations of 10, 20, and 40 with a control. Then GO was poured on it and placed in the incubator after 48 hours.

Each sample at the scratch site was photographed by a camera attached to a microscope at moments 0 and 24 to detect the rate of migration after scratch. Migratory cells were measured by manual counting. Then the filling of this scratch was checked as a result of adding an NGO.

3-4-1-6-8- DAPPY test

Following the determination of IC50 as an important issue, another test called DAPPY, another type of staining was performed. After treating MCF7 cells, MDA-MB231 with similar nanodrug concentrations were fixed in the SRB test by paraformaldehyde. Then, DAPPY dye was added to the wells. Following staining, cell mortality rates were examined by fluorescent microscopy. Each well was photographed five times at random (Figure 4-4). Then the number of cells was counted, and their mean was calculated.

Cells were cultured on agar. According to the method, they were placed in an incubator for 48 hours to grow. Then, the growth rate of the cells was examined after 48 hours under a microscope. They were then washed with PBS. GO was then treated, and this was followed by centrifugation.

The cells were completely isolated from the culture medium and fixed with 4% paraformaldehyde. The paraformaldehyde was placed on the cell for 30 minutes and then placed in the refrigerator. The cell solution, paraformaldehyde, were then rinsed for 30 minutes. 0/2 normal HCLs were then placed inside the wells to cover the cells. After 30 minutes, it was washed with PBS twice. The Triton was then diluted to a ratio of 38: 9975 and poured into the samples. It was then rinsed after 30 minutes with ps for three times. At this point, the serum was diluted to 100: 900 Lambda with PBS and placed in the refrigerator for 30 minutes. Then, the 1: 100 Lambda primary antibody was diluted with PBS and poured onto the samples. It was then incubated in the refrigerator for 24 hours. The primary antibody was then washed. The secondary antibody was then diluted in the dark at a ratio of 1: 200 Lambda with ps and poured onto the samples. It was then refrigerated for 90 minutes to 2 hours for ICC testing. DAPPY or pi dye was used to dye the cores. It was placed for 10-20 minutes.

Apoptosis and cell cycle: Cultured cells were sent to 6-well plates for flow cytometry.

Results

SRB test

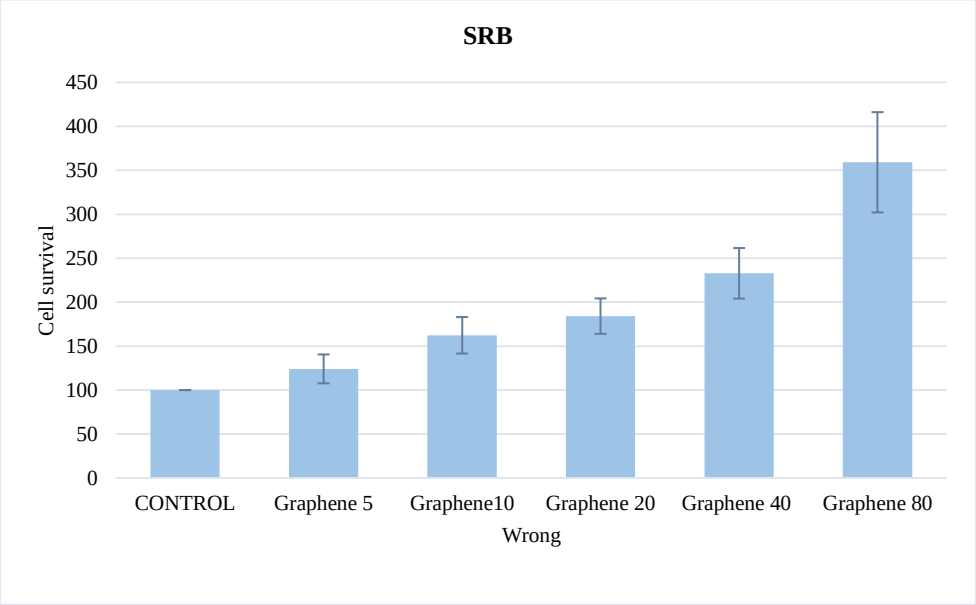


Diagram 1: SRB test

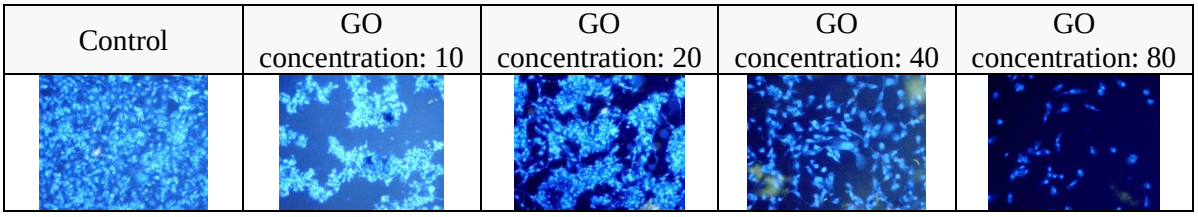
As with other tests, in this test, cell survival decreased with increasing nanodrug concentration. However, the increase shown in Figure 1 can be attributed to the fact that the NGO itself absorbs light.

DAPPY test

According to the protocol, the DAPPY test was performed with specific concentrations on the samples, with the results shown in Table 1. The results showed decreased cell survival with increasing nanodrug concentration.

Table 1: DAPPY test results

Control	Concentration: 10	Concentration: 20	Concentration: 40	Concentration: 80
712	574	578	369	123
596	235	677	307	50
704	367	756	201	68
735	251	646	185	134



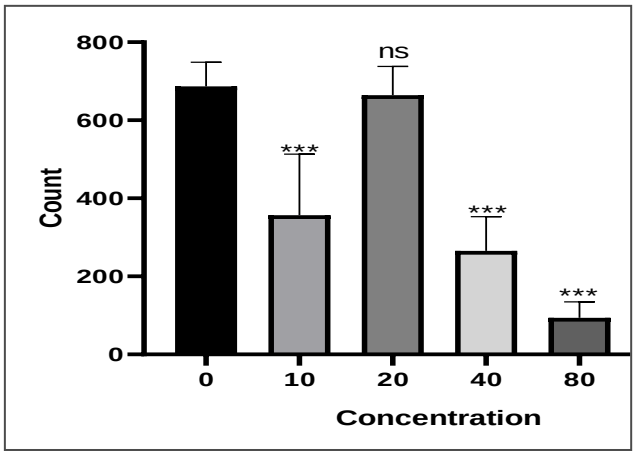


Figure 1: Sample DAPPY test images

Cell cycle after GO treatment

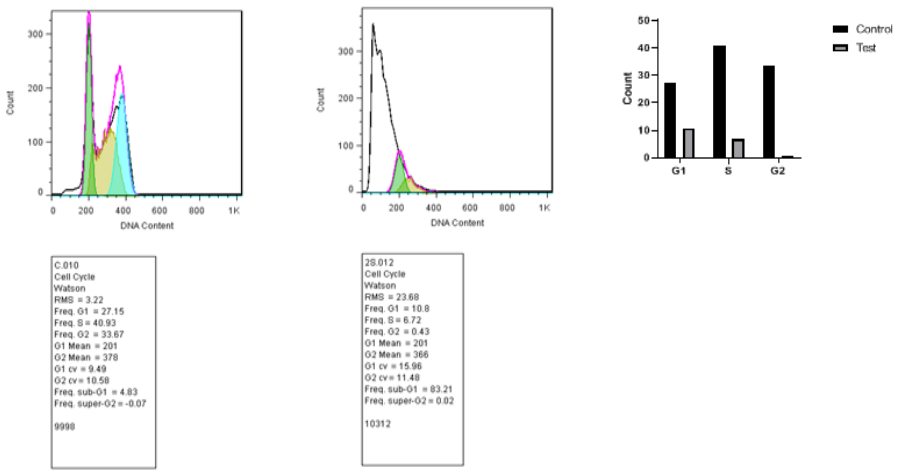


Figure 2: Cell cycle in the control group and test group

Examination of Figure 2 shows that the NGO significantly increased the rate of breast cancer cell death.

Evaluating apoptosis using flow cytometry

Following the effect of GO on the expression of TIMP1 and CYP1B1 proteins, the effect of this treatment on the level of apoptosis in the cells of the control and treatment groups was investigated.

The results showed an increase in the induction of apoptosis in cancer cells by GO treatment. It can be hoped that this compound will succeed in destroying these cells (Figure 3).

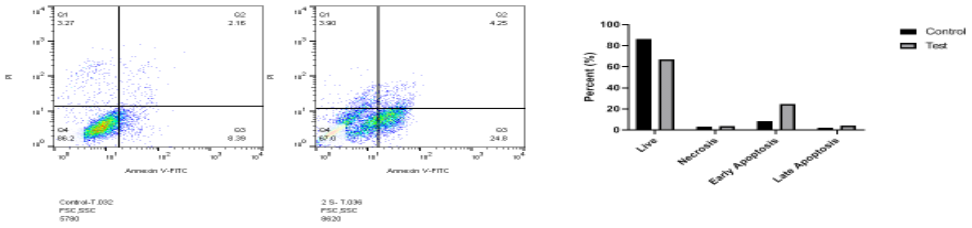


Figure 3: Results of GO treatment on apoptosis in control and experimental groups

Evaluating the scratch test in the control group and treatment group in the presence of GO

In this study, we first try to scratch the cell culture with a sampler tip and then examine the growth rate of the cells together at different times after treatment. The results are shown in Figure 4 and Table 2. The results showed that the cell growth rate was much slower in GO-treated samples than in the control group. Also, it was found that the growth rate of cells decreases sharply with increasing GO concentration, and the distance between two scratched cell lines is much greater.

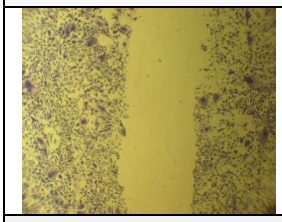
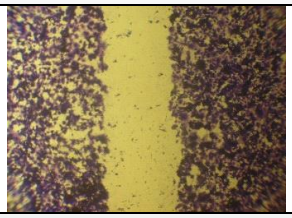
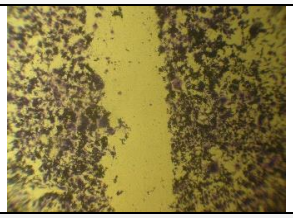
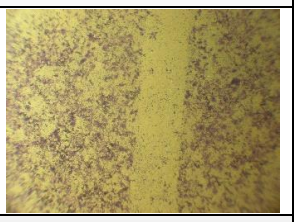
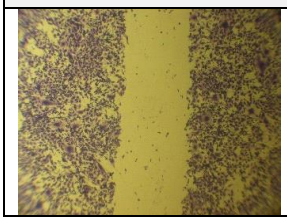
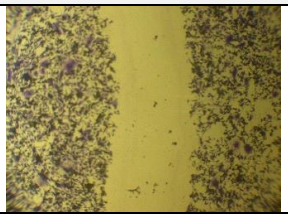
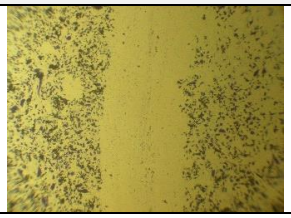
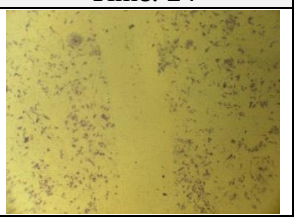
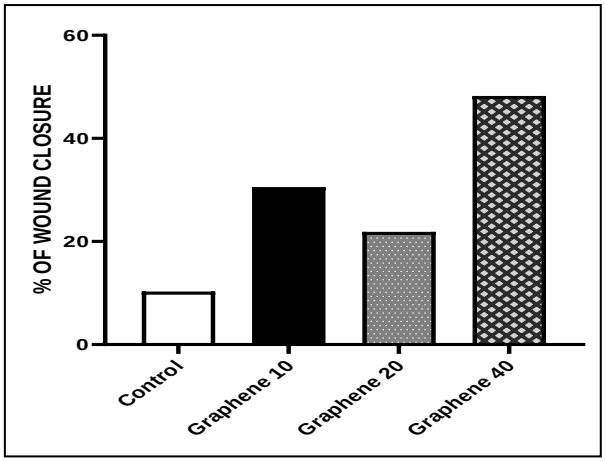
Control, Time: 0	GO concentration: 10; Time: 0	GO concentration: 20; Time: 0	GO concentration: 40; Time: 0
			
Control, Time: 24	GO concentration: 10; Time: 24	GO concentration: 20; Time: 24	GO concentration: 40; Time: 24
			

Figure 4: Scratch test cell migration images

Table 2: Scratch test at different concentrations of GO

AG10 0-AG10 24	AG20 0-AG20 24	AG40 0-AG40 24	%WOUND CLOSURE C	%WOUND CLOSURE G10	%WOUND CLOSURE G20	%WOUND CLOSURE G40
1.98	1.32	3.8	10.30150754	30.55555556	21.85430464	48.22335025

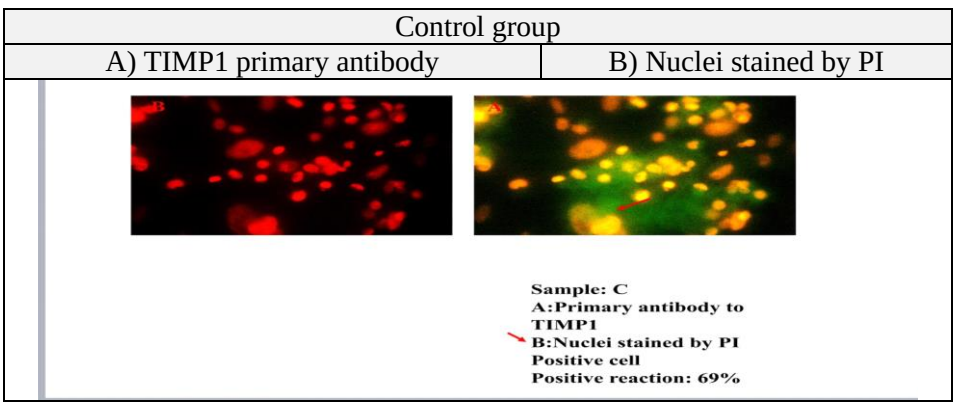


Gene expression

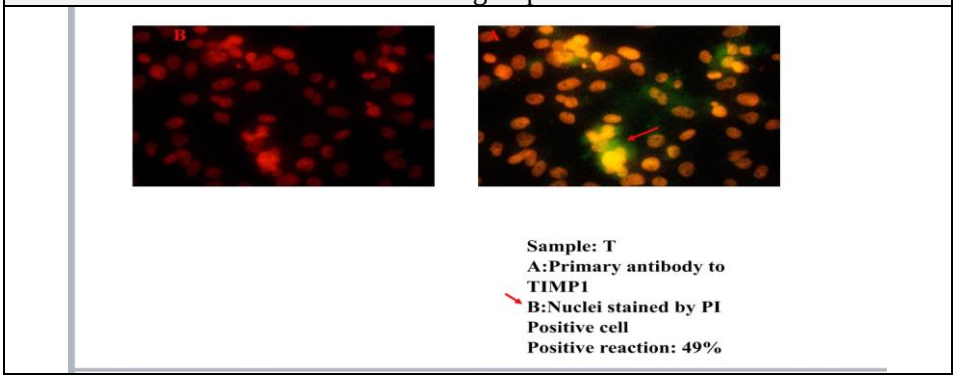
In cultured cells, the expression level of TIMP and CYP1B1 proteins was evaluated according to the mentioned protocol after treatment with GO. Then, the relevant statistical analyzes were performed, the results of which are shown in the graphs, Table 3, and Figure 5. The data showed a decrease in TIMP1 protein expression in the experimental group compared to the control group, a significant difference because P-value = 0.0005. A significant decrease in CYP1B1 protein expression was also observed in the experimental group compared to the treatment group. The significance of the observed difference was confirmed by statistical analysis and P-value = 0.003.

Table 3: Evaluating TIMP1 and CYP1B1 protein expression in control and test groups

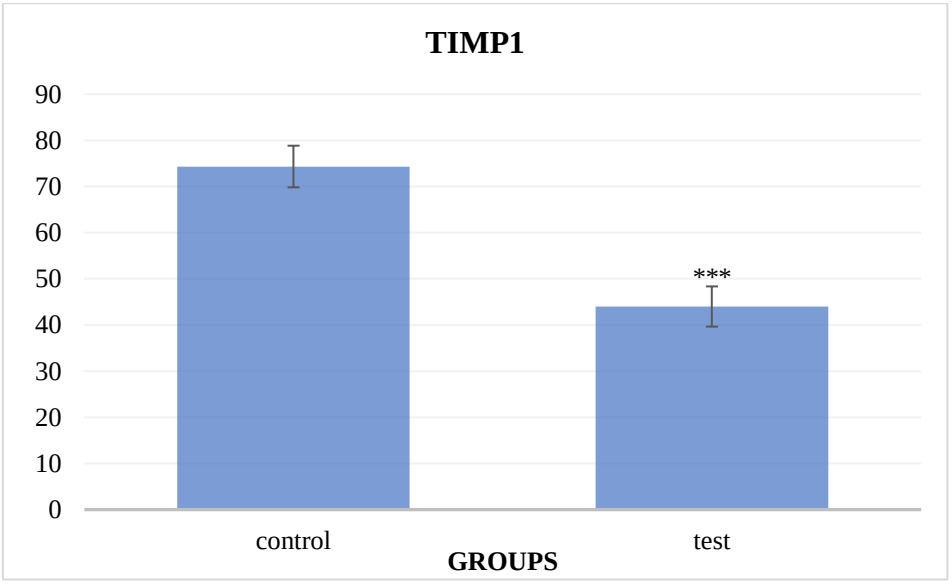
	C	t
TIMP1		
	79	49
	74	42
	70	41
mean	74.33333333	44
sd	4.509249753	4.358898944
t-test	0.000558117	p-value***
	CYP1B1	
	70	6
	66	9
	68	23
mean	68	12.66667
SD	2	9.073772
	0.003355	p-value**



Test group

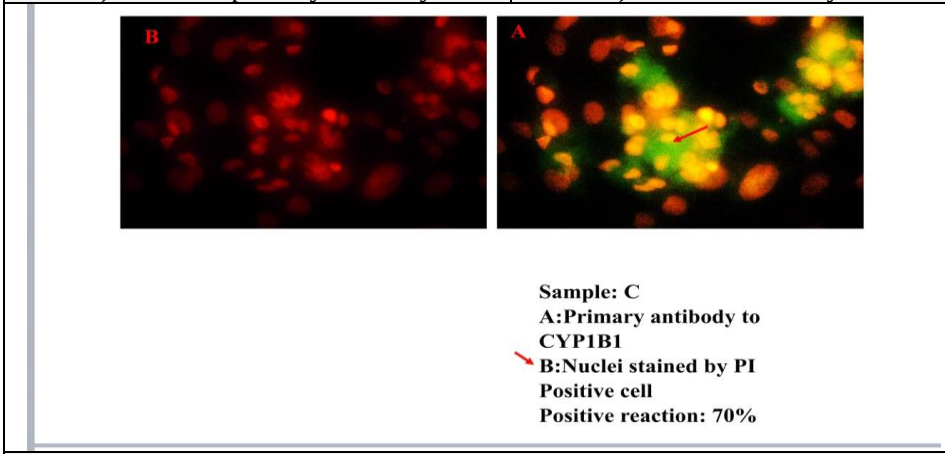


(A)

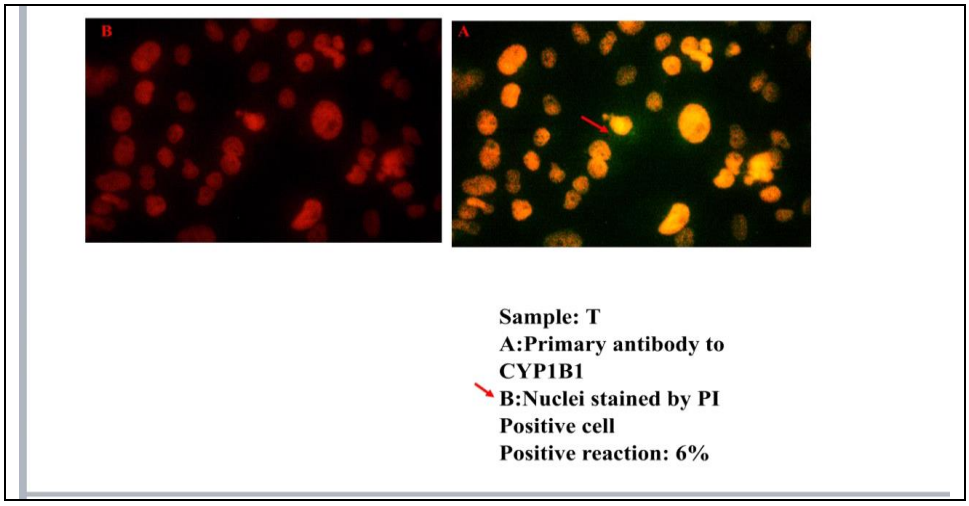


Control line

A) CYP1B1 primary antibody	B) Nuclei stained by PI



Test group



(B)

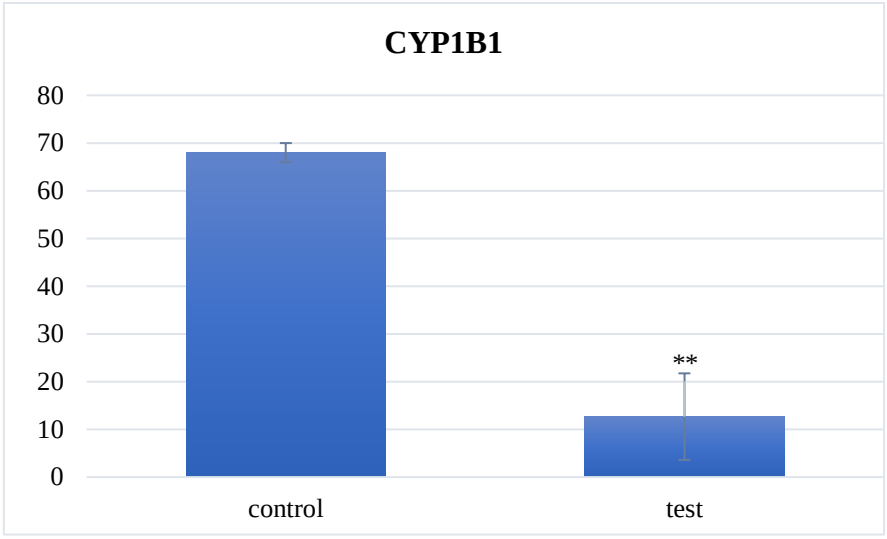


Figure 5: a) ICC test for TIMP1 antibody expression, b) ICC test for CYP1B1 antibody expression

Discussion

Cancer is considered a vital medical issue. Cancer cells are metamorphosed by invading tumor cells from their original location into the bloodstream and creating new colonies in other parts of the body. These invasive cancer cells in the circulatory system are known as mobile tumor cells. Identification and analysis of such cells can provide us with the information needed to manage the spread of cancer (metastasis) and monitor the effectiveness of treatments.

Knee-based cancer therapy eliminates therapeutic problems and side effects. Due to the functionalization and deployment of drugs used in cancer treatment on nanoparticles, the drug can target cancer cells with higher ability. In this case, toxicity and its side effects on healthy cells are reduced (34).

There are several drug delivery systems, including systems designed using nanostructured carriers, as one of the newest and most state-of-the-art systems.

An increase in the incidence of cancer and deaths from it requires the development of effective strategies to control it. A new approach, complementary to conventional cancer treatment and diagnostic methods, is the use of nanoparticles as a carrier and protector of biological drugs. This is the first revolution in the field of nanotechnology (16). Graphene-based nanomaterials, especially GO, have excellent potential for cancer-specific chemotherapy as drug carriers and active agents, thanks to their unique

physicochemical properties, including wide surface area, manipulable active groups, and high biocompatibility (17).

In general, graphene can be considered as a single-layer sheet of carbon atoms. In graphene, carbon atoms are placed next to each other in a hexagonal structure that closely resembles a beehive. In this structure, a covalent bond is established between each carbon atom and the other three carbon atoms.

GO is the same two-dimensional sheets of graphene that have oxygen bands on them. Due to the basic structure of graphene and the presence of oxygen groups, GO lacks the good thermal-electrical properties of graphene. However, due to the presence of oxygen groups, it has a better ability to interact with materials and can be used in a variety of carbines (17).

This study investigated the effect of nano GO on breast cancer cells as well as the expression of TIMP1 and CYP1B1 proteins. The ICC test results showed a significant decrease in the expression of these proteins in treated cells compared to the control group.

Many studies have been performed on, such as the studies by Chang et al. (2011) and Sasidharan et al. (2011). They reported the toxicity of carbon nanostructures, such as graphene and GO, and carbon nanotubes on different lines of normal and cancer cells (18-19). In our study, we also examined this toxicity by examining apoptosis and the DAPPY test. The results showed an increase in the induction of apoptosis in cancer cells due to the use of GO. On the other hand, in their study, Parivar et al. (2019) showed that the nanocomposite used has high toxicity for breast cancer cell lines (MCF-7) and colorectal cancer (SW-742), while it has much less toxicity for cell lines. Normal breast (MCF-10). This partly indicates relative specificity to cancer cell lines (20). Their results showed that the toxicity effects of nanocomposites are twofold: dose-dependent and time-dependent. In our study, we also found that the GO growth inhibitory effect increased over time. This is quite evident in the scratch test. Also, the toxicity effect of graphene was significantly increased by increasing the treatment dose.

In their study, Parivar et al. Observed that the expression of proapoptotic genes P53, P21, and Bax in the nanocomposite-treated MCF-7 cell line showed the highest increase compared to the control group. This indicates the effects of nanocomposites on the induction of apoptosis in this cancer cell line.

The present study investigated the induction of apoptosis using the Annexin-V/PI method, yielding results similar to those of Parivar et al.

The study also looked at another factor, the cell cycle. Application of nanocomposites caused cell cycle arrest compared to the control group. Various other studies, such as the study of Bianco et al. (1997) (21), Chen et al. (2017) (22), and Sasidharan et al. (2011) (18), acknowledged that one of the methods of cell-killing of GO and quinoline derivatives is cell cycle arrest and induction of apoptosis.

Lie et al. (2016) showed that the binding of GO to other nanoparticles or chemicals increases its efficiency. They also noted that binding of insoluble drug SN38 to GO-bound polyethylene glycol (PEG) and binding of doxorubicin (DOX) to GO activated by chitosan and sodium alginate increased solubility, biocompatibility, drug distribution in tumor tissue, and anti-tumor effects. These drugs have been. This result can confirm the effectiveness of GO cell treatment compared to the control state (22).

Fiorillo et al. (2015) showed that GO specifically inhibits the proliferation and proliferation of cancer stem cells (CSCs) in various cancers. They also noted that GO effectively prevents the formation of tumor-spheres in different lines of breast cancer (MCF-7), ovarian cancer (SKOV-3), prostate cancer (PC3), lung cancer (A549), pancreatic cancer (MIA). -PaCa-2) and glioblastoma (U87MG) (42).

This study investigated the effect of GO nanocomposites on cell culture, but their effect in vivo was not investigated. Future studies are suggested to examine this issue. In his study in mice, Singh (2011) showed that the use of GO led to platelet aggregation (clumping), pulmonary thromboembolism, granulomatous, and pulmonary edema in rats. The most important concern when using nanomaterials is pulmonary toxicity, which may lead to the distribution of charge on their surface and accumulation. This issue should be addressed, and the action should be taken to reduce their likelihood of accumulation in various ways, including binding to and activating other substances (24).

Conclusion

Various nanomaterials have been used to date as carriers of anticancer drugs, including gold, selenium, and iron nanoparticles. Meanwhile, GO has more advantages in drug loading and chemical modifications. It has led to the use of various drugs, including doxorubicin, curcumin, paclitaxel, methotrexate, and other drugs, to treat cancers and various cancers, such as colon cancer, breast cancer, lung cancer, liver carcinoma, and so on. Also, MTT and similar methods have shown increased specificity and cytotoxicity of GO-bound doxorubicin relative to doxorubicin alone on different cells. In many of these studies, the effects of GO on normal cells have been reported to be very low, indicating the relative specificity and greater effect of this substance on cancer cells. The exact mechanism of this difference in effect is not yet known. Based on the results of previous studies and this study, it can be concluded that the use of GO can be an effective way to fight cancer. One of the main mechanisms in this process is probably the induction of apoptosis in cancer cells.

Suggestions for Future Research

Conducting studies in vivo
Investigating the effects of nanocomposites on immunological mechanisms involved in cancer in vivo
Investigating the effects of nanocomposites on immunological mechanisms involved in cancer in vitro.

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