MOLECULAR EVALUATION OF CELL CYCLE INHIBITORS AFTER *Hepatocellular carcinoma* (HCC) TREATMENT *In Vitro*

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Cancer arises from the transformation of normal cells into tumor cells in a multi-steps process that normally progresses from a pre-cancerous lesion to a malignant tumor. Richard Doll and Richard Peto produced a groundbreaking study on the aetiology of cancer in 1981 that was partially based on an analysis of cancer incidence in numerous nations. A World Health Organization expert committee came to the conclusion that frequent deadly cancers are potentially preventable because of lifestyle choices and other environmental factors, such as hormone imbalances, dietary inadequacies, and environmental carcinogens, in 1964 (Colditz *et al*., 2005).

Cancer is neither a single type nor a new disease. According to a recent study by Faguet (2015), more than 200 distinct forms of cancer have been found in humans, depending on the type of tissue. Cancer was described in several ancient texts, including Egyptian "Edwin Smith" and "George Ebers" papyri written between 3000 BC and 1500 BC (Faguet, 2015).

According to estimates from the year 2000, liver cancer is still the eighth most prevalent disease in women and the fifth most common cancer in men worldwide. An estimated 564,000 new cases, including 166,000 women and 398,000 men, are reported per year. Liver cancer can develop before the age of 20 in high-risk nations, although it rarely occurs before the age of 50 in low-risk nations. Male liver cancer rates are typically 2 to 4 times greater than female rates (Bosch *et al*., 2004).

HCC is the most frequent primary liver cancer and the leading cause of cancer-related mortality globally (O'Connor *et al*., 2018). Despite breakthroughs in preventative strategies, screening, and new diagnostic and treatment technologies, incidence and fatality rates continue to climb (Balogh *et al*., 2016). ACS Cancer Facts & Figures, (2022) Conducted a research shows that many variables are known to increase the chance of acquiring cancer, some of which are controlled (such as cigarette smoking and excess body weight), while others are not, even if the mechanics of cancer formation are not completely understood (e.g., inherited genetic mutations). These risk factors may initiate or accelerate the progression of cancer, either simultaneously or sequentially (Cancer Facts & Figures 2022, ACS).

More than 90% of primary liver tumors are hepatocellular carcinomas (HCC), which are primary tumors of the liver. Of patients with cirrhosis, HCC affects about 85% of them (Ioannou *et al*., 2007).

Tumorigenesis is caused by an imbalance between cell growth and cell death (apoptosis). p21, a wellknown cyclindependent kinase (cdk) inhibitor, was shown to be critical in regulating cell cycle progression (Harper *et al*., 1993).

The p21 gene is changed in a number of malignancies and works as a cell cycle inhibitor and anti-proliferative effector in normal cells (Wan *et al*.,1996). Some evidences indicated the link between tumor development and p21 protein alteration (Mousses, S. *et al*., 1995) The role of p21 in phenotypic plasticity and its oncogenic/anti-apoptotic activity, dependent on p21 subcellular localization and p53 status, have lately been thoroughly investigated, despite the fact that the tumor-suppressor function of p21 has gotten the greatest attention in cancer research (Shamloo & Usluer, 2019).

According to a review made by Prochownik (2004), c-MYC is involved in the control of a number of normal cellular functions, which includes differentiation, proliferation, and maintenance of cell size, regulation of the intercellular redox state, angiogenesis and apoptosis. In cancer cells this is frequently dysregulated as many of the c-MYC transcription factor's target genes encode proteins that initiate and sustain the transformed state (Prochownik, 2004).

Given the role of c-Myc in HCC carcinogenesis, it's no surprise that it's an appealing target for creating new therapeutics. The first evidence that c-Myc downregulation can be utilized to treat HCC comes from an inducible c-Myc animal model, in which c-Myc inactivation triggered the regression and differentiation of liver tumors (Lin *et al*., 2010).

Despite significant improvements, the present strategy for treating cancer is fundamentally reductionist. Single molecular aberrations or cancer pathways have been the focus of successful treatment interventions that have marginally improved survival in several cancers. The

"magic bullet" approach of using a single medicine to target a specific characteristic or route, however, is unlikely to result in the cure of cancer (Zugazagoitia *et al*., 2016).

The discovery and implementation of various nanotechnologies for more efficient and safe cancer treatment—hereafter referred to as cancer nanomedicine—was spurred by the inherent limitations of conventional cancer therapies (Shi *et al*., 2016). Engineered nanoscale materials have been created as new prototypes for biomedical applications and improved therapy as a result of recent advancements in nanotechnology and biotechnology. Numerous nanomaterials have been created as a result of their distinctive characteristics, which include a large surface area, structural characteristics, and a longer blood circulation time than small molecules. These materials have the potential to completely change how diseases are detected and treated (Sanna *et al*., 2014).

Nanomaterials from the graphene family, such as graphene oxide and reduced graphene, have been the subject of numerous investigations. These investigations ultimately led to the creation of GQDs by Ponomarenko and Geim in 2008, which signaled the start of a wealth of medicinal applications. Then, researchers concentrated their attention on GQDs and discovered that they are the best quantum dots for biological applications (Xu *et al*., 2013).

GQDs the most recent member of the graphene family, have sparked a lot of attention in recent years due to their excellent physical, chemical, electrical, optical, and biological properties (Iannazzo *et al*., 2020). Being a one-dimensional (0D) object (GQDs) Promising biomedical applications have been discovered due to their ultra-small size, non-toxicity, biocompatibility, high photo stability, tunable fluorescence, water solubility, and so on, garnering substantial interest in the biomedical area (Younis *et al*., 2020).

This work investigates the effect of GQDs on HCC therapy *in vitro*, through observing its effect on two key cell cycle inhibitors, the P21 and c-MYC genes.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Egypt's Central Public Health Laboratories (CPHL). Primers were purchased from (Applied Biosystems), the RNA extraction kit from (Qiagen, Hilden, Germany), and the PCR kit HERA SYBER GREEN/ROX RT-qPCR from (Applied Biosystems) (Applied Biosystems, Foster City, California, USA). All work was done in Egypt's Central Public Health Laboratories (CPHL).

Graphene quantum dots

The graphene Quantum Dots were purchased from Sigma-Aldrich, Egypt.

Cell line and Cell culture

Human hepatocarcinoma cell line (Huh-7), the cell line was obtained from central public health laboratories in Egypt (CPHL). The cells were cultivated in T75 tissue culture flasks in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mM/L-glutamine and incubated in a 95% humidified incubator containing 5% CO2 at 37ºC. Now cells ready for treatment with Graphene quantum dots.

Cytotoxicity

To evaluate the cell viability and the cytotoxicity was assessed using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic antimycotic mixture. After 24 h of cell preparation, the growth medium was aspirated from each well and the cells washed with 1X phosphate buffered saline (PBS). Different concentrations of Graphene Quantum dots were two fold serially diluted in DMEM then added to cultured cells in 96-well plate in triplicate and incubated for 24 h post treatment to determine the cytotoxic concentration 50 (CC50). The medium was then removed and the monolayer of cells washed with 1X PBS three times before adding MTT solution (20 µL/well of 5 mg/ml stock solution) and incubated at 37ºC for 4 h till formulation of formazan crystals. Crystals were dissolved using a volume of 200 µL of of acidified isopropanol and the absorbance measured at λmax 540 nm using an ELISA microplate reader. Finally, the percentage of cytotoxicity compared to the untreated cells was determined. The CC50 of Graphene Quantum dots were determined from a linear exponential equation.

Cytotoxicity (%)=

(Absorbance of cell without treatment – Absorbance of cell with treatment) / Absorbance of cell without treatment X100

Real-Time RT PCR Analysis

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Five hundred nanograms of purified mRNA was used to generate cDNA with random hexamer primers (Thermo Scientific) and with Reverse Transcriptase according to the manufacturer's protocol (HERA SYBR® green RT-qPCR kit). The quantitative real-time PCR (qRT-PCR) reaction mixture (25 μL) consisted of the following: 12.5 μL of Maxima SYBR green PCR master mix (Thermo Scientific), 0.5 μL of cDNA template, and 1 μL of each primer (100 μM forward and reverse primers). Reactions were run in duplicate on Applied Biosystems 7500 real-time PCR system. The cycling conditions were as follows: 2min at 50ºC, 2min at 95ºC, and 50 cycles, with 1 cycle consisting of 15 s at 95ºC and 30s at 60ºC. Threshold cycle (Ct) values were normalized to the values for β-actin house-

keeping transcripts and log fold change was calculated according to the equation of 2^-ΔΔCT (Rao *et al.*, 2013).

Gene	Primers
c-Myc	5'-CCTGGTGCTCCATGAGGAGAC-3' (forward)
	5'-CAGACTCTGACCTTTT-GCCAGG-3' (reverse)
P21	5'-GTGGCTCTGATTGGCTTTCTG-3' (forward)
	5'-CTGAAAACAGGCAGCCCAAG-3'(reverse)
β actin	5'- CACCATTGG-CAATGAGCGGTTC -3'(forward)
	5'-AGGTCTTTGCG-GATGTCCACGT -3'(reverse)

The primers of p21, C-MYC and β actin

RESULTS AND DISCUSSION

- **Results**

The effect of (graphene quantum dots) on HUH7 cell lines as models of human liver cancer cell lines was examined in this work. P21, c-MYC, and B.Actin as housekeeping gene (positive control).

1-Cytotoxicity of graphene quantum dots against HUH-7 Cell Lines Using MTT assay.

Cytotoxicity assays are normally based on assessing damage to cellular membranes or cell viability or cell apoptosis or cell proliferation. Creative Biolabs has explored a variety of assays for your flexible choice to best fit current results. To evaluate the cytotoxic activity of two different concentrations of the GQDs against human Liver cancer cells (HUH-7), were incubated with different concentrations (0.5% to 1%) of GQDs. After 24 hours of incubation, cell viability was determined by the MTT assay. The results of cytotoxicity assay are presented in (Fig .1).

Cytotoxicity assays are typically designed to evaluate damage to cellular membranes, cell viability, cell apoptosis, or cell proliferation. Creative Biolabs has investigated a number of assays for your flexible selection to best match my results. To assess the cytotoxic efficacy of two distinct doses of Graphene quantum dots against human liver cancer cells (HUH-7), the cells were treated with Graphene quantum dots at varying concentrations (0.5 percent to 1 percent). The MTT test was used to measure cell viability after 24 hours of incubation. The cytotoxicity assay results are shown in (Fig.1)

The cytotoxicity of the graphene quantum dots extract was evaluated in HUH7 cells using MTT assay. Graphene quantum dots were almost not toxic for studied cells up to a dose of 4.2 0r 4.3 µg/ml for graphene quantum dots. The toxic effect of tested graphene quantum dots was dose dependent. The result showed that the cytotoxic concentration 50 (CC50) value of graphene quantum dots was 4.2 OR 4.3 µg. Therefore, for further studies we selected the safe concentrations of 1 -0.5 µg/ml for subsequent cellular signal studies.

Evaluation of P21 and c-MYC gene expression after treatment with different concertation of Graphene quantum dots.

To investigate the effects of Graphene quantum dots on c-MYC and P21 expression in Hepatocellular carcinoma, reverse-transcription PCR was done after treatment with 1 -0.5 µg /ml Graphene quantum dots for various time periods (0 h, 8h, 16h, 24h, 32h, 40h, 48h, 56h, 64h and 72h). In comparison to untreated controls, gene expression of c-MYC was considerably down regulated (decreased) with 1 -0.5 µg /ml Graphene quantum dots treatment. Furthermore, when 1 -0.5 µg/ml Graphene quantum dots were used, gene expression of P21 was considerably upregulated (raised) compared to untreated controls.

Table (1) shows that, there was significant statistical increase in Graphene quantum dots 1% compared to Graphene quantum dots 0.5% at 8, 16, 32, 48, and 64 hours, (p=0.007, 0.034, 0.003, 0.038, and 0.000, respectively.

Table (2) shows that there was significant statistical increase in P21 in Graphene quantum dots 1% compared to Graphene quantum dots 0.5% at 16, 24, 32, 40, 48, 56, 64, and 72 hours, (p=0.014, 0.001, 0.000, 0.047, 0.000, 0.000, 0.000 and 0.000, respectively.

• DISCUSSION

Hepatocellular carcinoma is one of the most common causes of cancer-related death globally. The recent study discovered that Graphene dots made from spies had the ability to prevent several cancer cell types from proliferating and migrating. More cancer cells were suppressed by a combination of Graphene dots and a traditional chemotherapy medication than by either therapy by itself. Together, these results imply that Graphene dots may be a potent complementary and alternative medicine for the treatment of cancer (Xia *et al*., 2019). c-Myc is among the most frequently overexpressed genes in human cancers. Overexpression of c-Myc in hepatic cells leads to Progression of liver cancer. c-Myc can currently regulate up to 15%–20% of human genes either directly or indirectly. These genes are involved in the regulation of the cell cycle, protein synthesis, the cytoskeleton and cell motility, cell metabolism, and microRNA, which are tiny regulatory molecules that influence the stability and translation of target mRNA (Lin *et al*., 2010). Studies have found that c-Myc interacts with Miz-1 and recruit DNA methyltransferase DNMT3 to p21 promoter to silence p21 transcription, a critical step during tumorigenesis (Brenner *et al*., 2004). Results means, when we used different concentrations of curcumine(0.5-1 µg/ml) for different duration time (0 h, 8h, 16h, 24h, 32h, 40h, 48h, 56h, 64h and 72h) on different genes related to liver cancer (c-Myc and p21), this is lead to down regulation c-Myc and up regulation of P21.Given the importance of c-Myc in HCC carcinogenesis, it is not surprising that c-Myc is an attractive target for developing novel therapies. The

first evidence that down-regulation of c-Myc can be used as a strategy to treat HCC comes from an inducible c-Myc animal model, in which inactivation of c-Myc induced the regression and differentiation of liver tumors (Shachaf *et al*., 2004).

SUMMARY

Hepatocellular carcinoma (HCC) is one of the most prevalent types of cancer. HCC is the sixth most popular cancer in the world and the fourth most common cancer in Egypt, respectively. Egypt is the third and fifteenth most populated countries in Africa and the globe, respectively. The goal of this study is to examine the effect of graphene quantum dots (GQDs) on the expression of P21 & c-MYC genes on a cell line in liver cancer namely "HuH-7 cell line." The area of studying the anticancer effect of GQDs is attracting growing attention because of its valuable properties. Especially due to its nano-sized sheets, it tends to infiltrate the cell nucleus and interfere with DNA function due to its ultra-small size. The results emphasize the validity of using GQDs as anticancer agent, with varied concentrations of GQDs inhibiting the development of cancer cells (HuH-7) *via* gene up regulation.

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Table (1): Effect of concentration of Graphene quantum dots 0.5% and 1% on c.MYC expression on Cell line: HUH7.

concentration of Graphene quantum dots 0.5% and 1% on Cell line: HUH7 on C-MYC expression				
Hours	Graphene quantum dots 1% on Cell line: HUH7	Graphene quantum dots 0.5% on Cell line: HUH7	T test	P value
0	22.41±0.025	22.43±0.230	0.125	0.907
8	26.87±0.321	25.24±0.122	8.210	0.007*
16	30.23±0.306	29.31±0.020	5.224	0.034*
24	31.267±0.586	31.23±0.045	0.098	0.926
32	33.00±0.173	32.103±0.108	7.612	0.003*
40	35.43±0.611	34.62±0.153	2.246	0.140
48	37.08±0.473	36.23±0.047	3.039	0.038*
56	38.37±0.666	37.94±0.049	1.098	0.334
64	39.99±0.100	39.02±0.072	12.363	0.000*
72	39.97±0.058	39.07±0.025	0.183	0.867

Table (2): Effect of concentration of Graphene quantum dots 0.5% and 1% on P21 gene expression.

concentration of Graphene quantum dots 0.5% and 1% on Cell line: HUH7 on P21 Expression				
Hours	Graphene quantum dots 1% on Cell line: HUH7	Graphene quantum dots 0.5% on Cell line: HUH7	T test	P value
0	30.00±0.092	29.94±0.046	1.014	0.387
8	28.20±0.269	28.56±0.081	2.237	0.135
16	26.40±0.252	26.99±0.101	3.757	0.041*
24	24.32±0.095	25.557±0.031	21.384	0.001*
32	23.71±0.035	27.03±0.104	52.152	0.000*
40	23.08±0.101	26.063±1.178	4.369	0.047*
48	21.20±0.095	23.973±0.021	49.311	0.000*
56	19.62±0.046	21.97±0.015	84.144	0.000*
64	19.04±0.061	21.697±0.100	39.169	0.000*
72	18.64±0.076	20.92±0.03	48.610	0.000*

Table (3): Effect of graphene quantum dots concentration 1% on gene expression of C.Myc in cell line HuH7.

Hours	c.MYC	B. Actin	ΔCt	ΔΔCt	2^-ΔΔCt	log (2^-ΔΔCt)
0 hr	22.4	22	0.4	0	1	0
8 hr	26.9	22.2	4.7	4.3	0.051	-1.294
16 hr	30.2	22.4	7.8	7.4	0.006	-2.228
24 hr	31.3	22.1	9.2	8.8	0.002	-2.649
32 hr	33	22.6	10.4	10	1E-03	-3.01
40 hr	35.4	23	12.4	12	2E-04	-3.612
48 hr	37	22.8	14.2	13.8	7E-05	-4.154
56 hr	38.4	22.9	15.5	15.1	3E-05	-4.546
64 hr	40	23.1	16.9	16.5	1E-05	-4.967
72hr	40	23.6	16.4	16	2E-05	-4.816

Table (4): Effect of graphene quantum dots concentration 1% on gene expression of P21 in cell line HuH7 for 72 hours.

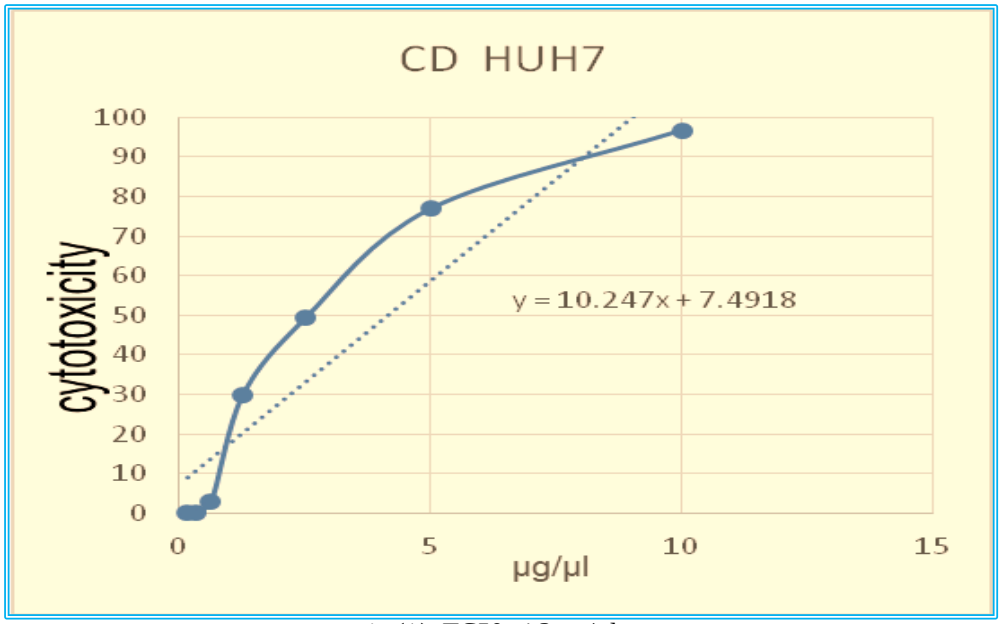
	P21	B. Actin	ΔCt	ΔΔCt	2^-ΔΔCt	log (2^ΔΔCt)
0 hr	30	22	8	0	1	0
8 hr	28.2	22.2	6	-2	4	0.602
16 hr	26.4	22.4	4	-4	16	1.204
24 hr	24.3	22.1	2.2	-5.8	55.72	1.746
32 hr	23.7	22.6	1.1	-6.9	119.4	2.077
40 hr	23	23	0	-8	256	2.408
48 hr	21.2	22.8	-1.6	-9.6	776	2.89
56 hr	19.6	22.9	-3.3	-11.3	2521	3.402
64 hr	19	23.1	-4.1	-12.1	4390	3.642
72hr	18.6	23.6	-5	-13	8192	3.913

Table (5): Effect of graphene quantum dots concentration 0.5% on gene expression of C.Myc in cell line HuH7.

	c.MYC	B.Actin	ΔCt	ΔΔCt	2^-ΔΔCt	log (2^-ΔΔCt)
0 hr	22.4	23	-0.6	0	1	0
8 hr	25.2	23.6	1.6	2.2	0.218	-0.662
16 hr	29.3	23.4	5.9	6.5	0.011	-1.957
24 hr	31.2	24.1	7.1	7.7	0.005	-2.318
32 hr	32.1	23.9	8.2	8.8	0.002	-2.649
40 hr	34.6	23.7	10.9	11.5	3E-04	-3.462
48 hr	36.2	24.2	12	12.6	2E-04	-3.793
56 hr	37.9	24.6	13.3	13.9	7E-05	-4.184
64 hr	39	24.6	14.4	15	3E-05	-4.515
72hr	40	24.8	15.2	15.8	2E-05	-4.756

Table (6): Effect of graphene quantum dots concentration 0.5% on gene expression of P21 in cell line HuH7.

	P21	B.Actin	ΔCt	ΔΔCt	2^-ΔΔCt	log (2^-ΔΔCt)
0 hr	30	23	7	0	1	0
8 hr	28.6	23.6	5	-2	4	0.602
16 hr	27	23.4	3.6	-3.4	10.56	1.024
24 hr	25.6	24.1	1.5	-5.5	45.25	1.656
32 hr	27	23.9	3.1	-3.9	14.93	1.174
40 hr	26.1	23.7	2.4	-4.6	24.25	1.385
48 hr	24	24.2	-0.2	-7.2	147	2.167
56 hr	22	24.6	-2.6	-9.6	776	2.89
64 hr	21.7	24.6	-2.9	-9.9	955.4	2.98
72hr	21	24.8	-3.8	-10.8	1783	3.251



ig.(1*)*: TC50=4.3 µg/µl

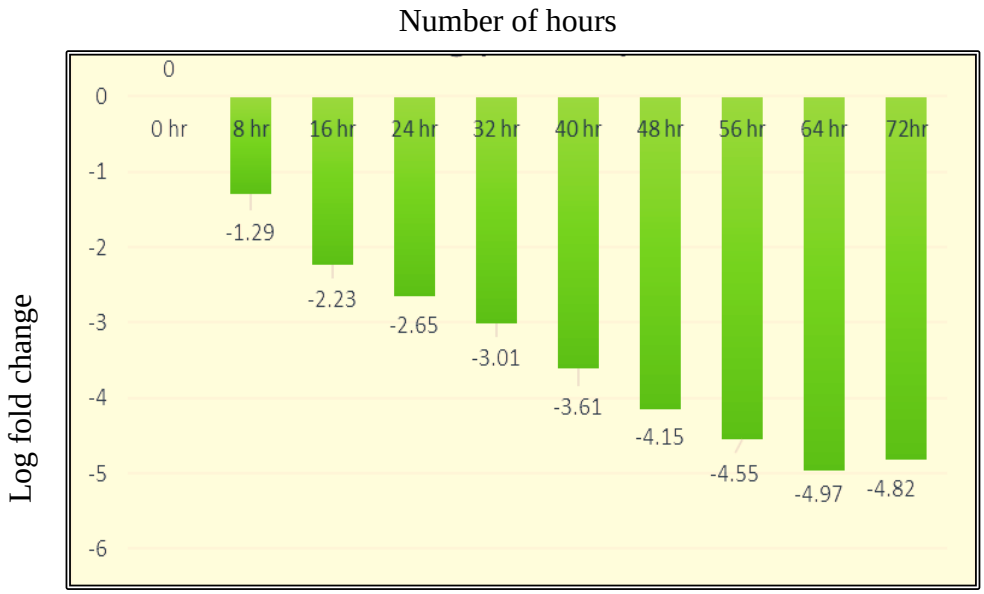


Fig. (2): the impact of concentration of Graphene quantum dots 1% on the gene expression of the C-Myc Gene in HUH7 cell line. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene C-MYC was increase.

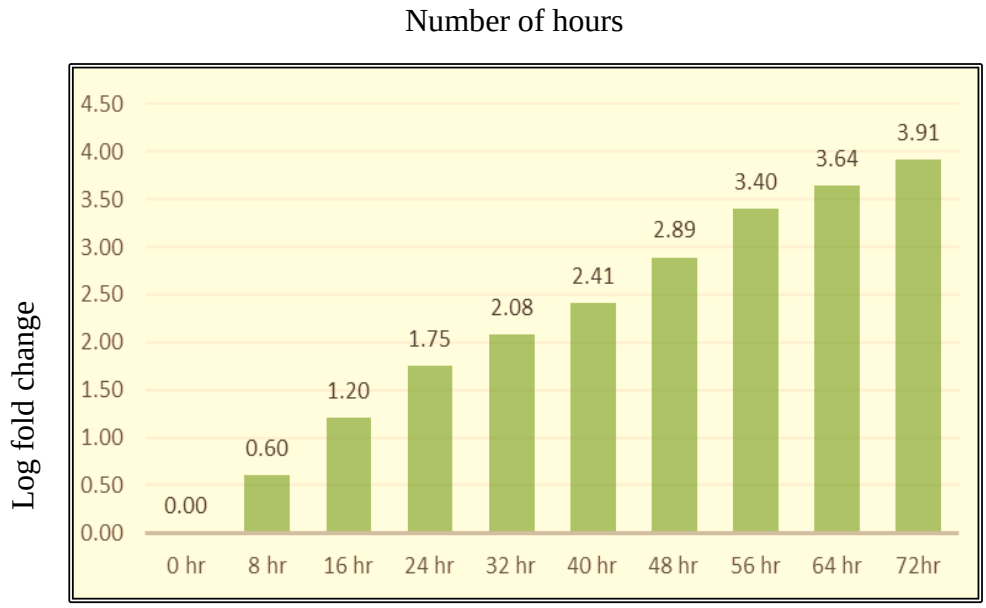


Fig. (3): the impact of concentration of Graphene quantum dots (1%) on the gene expression of the P21 Gene in HUH7 Cell Line. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as the time increase the Up regulation of the P21 gene was increase.

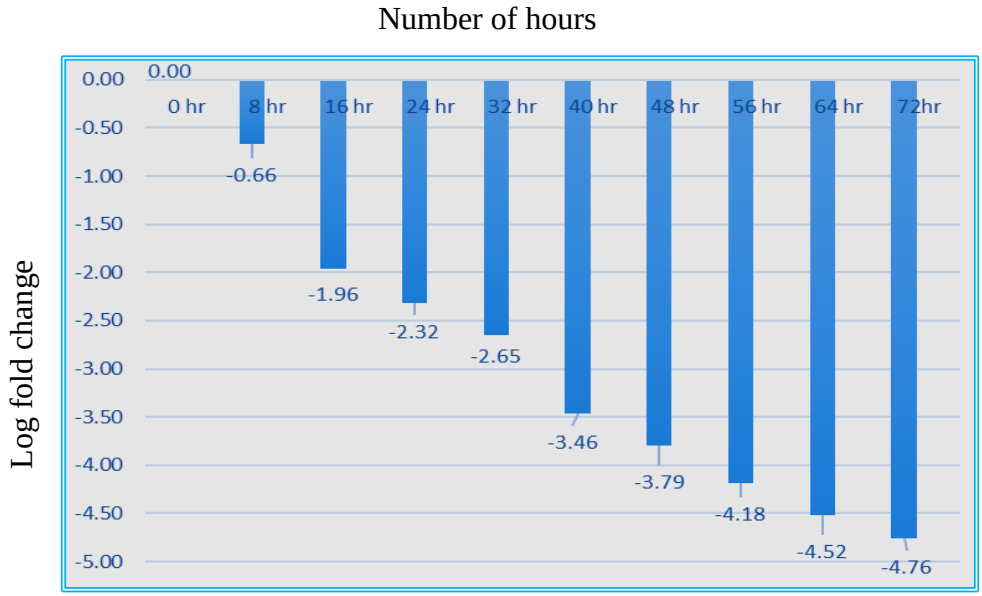


Fig. (4): the impact of concentration of Graphene quantum dots 0.5% on the gene expression of the c.MYC Gene in HUH7 cell line. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene C-MYC was increase.

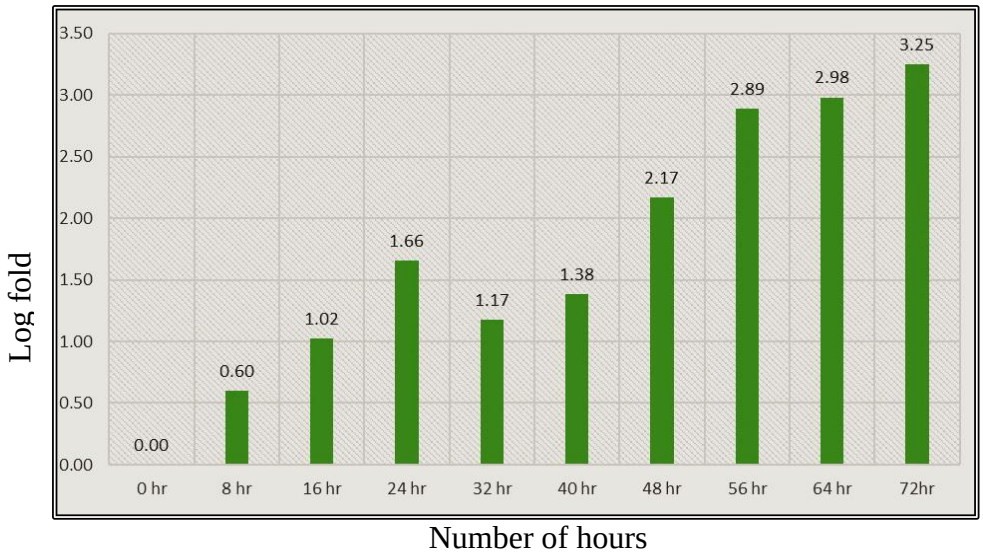


Fig. (5): the impact of concentration of Graphene quantum dots 0.5% on the gene expression of the P21 Gene in HUH7 cell line. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as the time increase the Up regulation of the P21 gene was increase.