Quercetin and Mercury *In Vitro* Anti-Proliferative Effect in Human Astrocytoma Cells

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Abstract: Mercury (Hg) is a toxic heavy metal to which we are exposed in everyday life. Exposure to environmental Hg may lead to toxicity in the human body associated with major health issues. Quercetin (QE) on the other hand, is a natural flavonoid widely distributed in higher plants and is part of the human diet. Several studies demonstrated the therapeutic and protective effects of QE against multiple diseases and health problems. The aim of this study is to investigate the effect of QE and Hg on the proliferation of human astrocytoma 1321N1 cell line. This study is a continuation of our previous work in which we investigated cadmium (Cd) instead of Hg. The 1321N1 cells were either treated with Hg alone, or pre- or co-treated with QE. Cell viabilities were determined by MTT assay. Results indicated that simultaneous treatment of the cells with 200 μM and 16 μM Hg for 48 hrs significantly reduced cell viability to 11.7 ± 3.1 % compared to the DMSO vehicle-treated cells. Other experiments of QE pre-treatment followed by exposure to Hg alone or with QE indicated a significant ability to reduce proliferation compared to treatment with Hg alone. In conclusion, our study suggested a synergistic anti-proliferative interaction of Hg and QE in malignantly transformed cells. However, this effect is higher when combining Cd and QE as indicated in our previous work. These data may be beneficial in exploiting the biological effect of QE for treating the malignantly transformed cells.

Keywords Quercetin, Mercury, Cadmium, Astrocytoma, 1321N1 Cell line, MTT, synergistic anti-proliferation.

INTRODUCTION

Flavonoids, including quercetin (3, 3, 4, 5, 7-pentahydroxyflavone) (QE) are naturally occurring substances present virtually in all higher plants [1] and as such, they are present in human dietary components of a plant origin [2]. QE as a typical representative of this group of compounds was shown to possess significant therapeutic and protective biological properties, including antiaging, anti-inflammatory, anti-diabetic, anti-obesity, angio-protective, and cytostatic [3, 4]. QE has the potential to inhibit mTOR hyperactivity involved in cancer progression [5]. Studies involving malignant cell lines of different origins revealed that most sensitive-to-QE malignant lines originate from blood, brain, lung, uterine, salivary gland, and melanoma tissues where QE demonstrates selective cytotoxicity against more aggressive cells compared to slowly growing cells. This suggests that the cells with a higher extent of malignant transformation may be targeted by QE [6]. Additionally, QE possesses the potential of inhibiting kinases that are part of deregulation processes in malignantly transformed cells [7]. The effects of QE were reviewed in our previous work [4].

Recently, QE was shown to enhance apoptosis in glioblastoma cells *in vitro* through a significant protective autophagy decrease accompanied by an increase in membrane blebbing, nuclear fragmentation, chromatin condensation, and an increase of apoptotic processes through decreasing Bcl-2 and increasing Bax, decreasing surviving and changing the concentrations of other pro- and anti-apoptotic molecules [8]. Interestingly, a recent review dealt with anti-malignant glioma effects of QE and several other flavonoids (chrysin, epigallocatechin-3-gallate (EGCG), formononetin, hispidulin, icariin, rutin, and silibinin) in combination with other molecules [9]. The essential limitation for the use of flavonoids, including QE, and of some other potentially-therapeutic substances in the therapy of various brain tumors is based on the limited permeability of these molecules through the blood-brain barrier. This relates to the issue of developing advanced delivery platforms. Various solutions to this problem are currently under investigation, such as the use of QE-loaded platelets [10] or various options provided by modern discoveries in the area of nanotechnology and the use of nanoparticles for targeted drug delivery [11].

Mercury (Hg) is a chemical element that is not part of the natural biochemical process of the human body. But it has some use in our everyday life as it is used in thermometers, fluorescent lamps, and other devices. It may be also used in dentistry for amalgam teeth fillings. However, it is well known for its toxicity to humans [12]. Half of Hg in the atmosphere is of natural origin, i.e. from volcanos [13]. The rest of atmospheric mercury is due to various human activities, such as

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smoking [14], the production of power plants that use coal or gas as an energy source, non-ferrous metal, caustic soda, steel, and cement production [15]. Hg toxicity leads after exposure to damage to the brain, kidney, lungs, and also other organs. Hg exposure leads to sensory impairments and damaged cognitive skills. The severity of these depends on the Hg dose, the type of exposure, and the duration of the exposure [15-17]. In general, significant scientific attention to the issue of environmental Hg and its toxicity in humans is being paid [18-20].

When Hg role in cancer was investigated, it was determined that methylmercury (MeHg) stimulates the proliferation of estrogen-receptor-positive breast cancer cells at the 1nM concentration [21]. However, it induces apoptosis in these cells at the concentration 100-times higher (100 nM) [21]. Zefferino *et al*. [22] work indicates that heavy metals such as Hg affect cellular metabolism *via* induction of a pro-oxidative state. This is a relationship with "a suppression of gap junction-mediated intercellular communication and a production of pro-inflammatory cytokines" [18]. The decrease in the concentration of various cytokines and also a decrease in the gap junction intercellular communication correlate. This may play a role in carcinogenic processes induced by Hg [23].

It was also shown *in vitro* in the MCF-7 cells that these cells proliferate significantly better in the presence of mercury chloride ($HgCl_2$). However, their proliferation is completely stopped by an estrogen antagonist (ICI182.780). More to this, $HgCl_2$ could not prevent the binding of estradiol to the estrogen receptor. Consequently, $HgCl_2$ shows an estrogen-like effect. It binds and stimulates estrogen receptors [24]. Hg was also shown to contribute to the apoptotic process in a cell exposed to it. The Hg exposure was accompanied by increases in caspase-positive cells *in vitro* [25]. Accumulation of Hg in nervous cells was documented in humans [26]. An important finding was reported regarding the ability of superoxide anion radicals being able to transform organic mercury-containing substances into inorganic mercury. This increases Hg toxicity and stimulates the accumulation of Hg in the intracellular environment [27]. It was also documented [28] that MeHg functions as a neurotoxin affecting glutathione homeostasis astrocytoma cells.

Based on these results, high Hg-related toxicity can be expected in any type of cell. The intriguing situation is related to QE which can decrease free radical concentrations and protect the cellular system from Hg and other toxicities or it may possess toxicity to astrocytoma cells as indicated by our previous results [4] and also by some other scientists [6]. The aim of this work was to investigate the effect of QE-Hg interaction in malignant human brain astrocytoma 1321N1 cells and to compare the toxicity of Hg to the reported earlier toxicity of cadmium (Cd) as well as the interaction of both heavy metals with QE in this type of cells [4].

MATERIALS AND METHODS

Unless otherwise specified, all material was purchased from Sigma-Aldrich (St. Louis, MO, US).

Cell Culture

The human brain astrocytoma 1321N1 cell line was purchased from the Health Protection Agency Culture Collection (Porton Down, Salisbury, UK), and it was grown in culture flasks (Corning Life Sciences, Tewksbury, Massachusetts, USA) containing Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 supplemented with 10 % fetal calf serum, 1 % L-glutamine (Gibco, ThermoFischer Scientific, Grand Island, New York, USA) and 1 % penicillin/streptomycin (Gibco). The cell culture was maintained and treated at 37°C in a humidified atmosphere containing 5 % CO_2.

Stock Solutions and Concentrations used for Treating the Cells

Stock solutions of QE and $HgCl_2$ were prepared by dissolving in the vehicle DMSO at concentrations calculated to attain 0.5 % DMSO final concentration in the culture medium. Treatment, pre-treatment, and co-treatment of cells with QE were at 100 [29] or 200 μM, whereas, their exposure to Hg was performed with a concentration of 16 μM [30].

Treatments of the 1321N1 Cells with QE and Mercury

The effects of QE and Hg on cell viability were evaluated in the 1321N1 cells by the commonly used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The MTT assay depends on the mitochondrial dehydrogenase-mediated conversion of water-soluble yellow MTT to a water-insoluble blue formazan product [31]. Incubation of the cells with individual or combined QE and Hg was performed with or without pre-incubation with QE. The experimental conditions of the incubations are summarized in Table **1**.

Table 1: Combined Incubations Scheme of 1321N1 Cells with QE or/and Hg

QE pre-incubation	QE pre-incubation time	Hg incubation	QE and Hg incubation	Hg or/and QE incubation times	Remarks
-	-	-	√	24 hrs / 48 hrs	-
√	24 hrs	-	√	24 hrs / 48 hrs	-
√	48 hrs	-	√	24 hrs / 48 hrs	-
√	48 hrs	√	√	24 hrs / 48 hrs	QE washed off with *HBSS or medium

*Hanks Buffer Saline Solution. -: Not Applicable; √: Applicable.

The 1321N1 cells were seeded overnight in a 96-well plate at the density of 1 X 10^5 cell/ml leaving a lane containing medium only that served as a blank control. On the next day, the cells were incubated in a medium containing the vehicle DMSO or QE or/and Hg. At the end of the incubation times (Table **1**), the cells were incubated for 5 h with 500 µg/ml MTT reagent at 37°C. After which, the media were removed and 150 µl DMSO was added to dissolve formazan crystals and the absorbance values of the colored formazan solutions were measured at 540 nm using a plate reader (BioTek, Highland Lake, VT, USA). Mean absorbance values were calculated across 8 wells for each treatment. Blank's mean absorbance was subtracted from all other absorbance values and the results were expressed as percentages of cell viability related to vehicle-treated cells from independent experiments. Data were analyzed by the two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test using the GraphPad Prism software package (version 7). *p* values of ≤ 0.05 were considered significant. Graphs were presented as bars of mean ± SEM values versus the incubation times.

RESULTS

Evaluation of the Anti-Proliferative Effect of the Vehicle (0.5%) DMSO and QE

Data on the antiproliferative effect of the medium containing DMSO were already published in our previous work [4]. Briefly, the antiproliferative effect of QE on the 1321N1 cell line was evaluated by considering incubation times collected from various experiments. A concentration of 0.5 % DMSO was used as a vehicle at which QE was incubated with the cells at 100 and 200 µM, respectively. After treatment with either vehicle or QE, cells were subjected to an MTT assay. For the vehicle-treated cells, the percentages of cell viability were expressed as mean ± SEM compared to the control untreated cells, and those percentages of QE treated cells were compared to data obtained with the vehicle-treated cells. The two-way ANOVA followed by the Bonferroni test was used for the statistical analysis, where $p \leq 0.05$ was considered significant.

Treatment with 0.5 % DMSO slightly but not significantly affects cell viability at time points 24-, 72- and 96-hrs. In contrast, treatment with the same vehicle for 48 hrs significantly reduced cell viability compared to control untreated cells [4]. This significant effect appeared to be transient since it was recovered by the cells at a 72-time point [4]. Thus, all cell viability data for cells exposed to QE or/and Hg were corrected for the effect of the vehicle 0.5% DMSO throughout the appropriate time points in the present study as in the previous work [4].

Treatment of 1321N1 cells with 100 µM QE significantly reduced cell viability compared to the vehicle-treated cells throughout incubation times of 24, 48, 72, and 96 hours [4]. Such reduction of the cellular viability was even more significant with 200 µM QE compared to 100 µM QE [4]. Therefore, the reduction in percentages of 1321N1 cell viability by QE shows a dose- and time-dependent manner [4].

Evaluation of the Anti-Proliferative Effect of Hg

The antiproliferative effect of Hg on 1321N1 cells was evaluated at incubation times of 24 and 48 hrs collected from various experiments. Like QE, the salt $HgCl_2$ was dissolved in DMSO at a concentration calculated to attain 16 µM at 0.5 % final concentration of DMSO for treating cultured cells in the 96-well plate.

As shown in Figure **1**, treating the cells with Hg for 24 hrs significantly reduced cell viability (67.1 ± 4.4%) when compared to the cells treated with the vehicle only ($p < 0.0001$). The cell viability values were reduced even more when they were exposed to Hg for

48 hrs (36.7 ± 3.8%) in comparison to cells treated only with the vehicle ($p < 0.0001$) (Figure **1**).

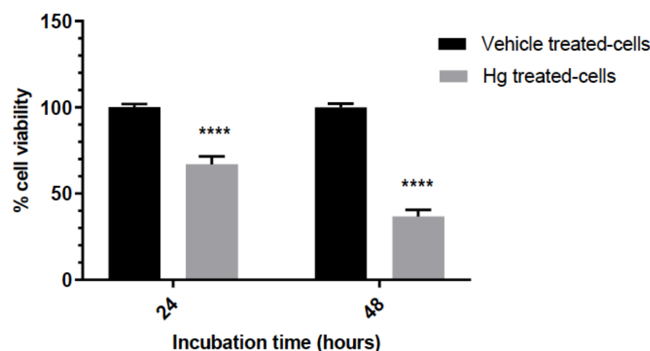


Figure 1: The anti-proliferative effect of Hg on 1321N1 cells. Cells at the density of 1 X 10^5 cell/ml were incubated with 16 µM Hg for up to 48 hrs. Cells were then subjected to an MTT assay and the resulting absorbance values were expressed as percentages of the values obtained with the vehicle-only treated cells. The bars represent mean ± SEM (n = 40). ****$p < 0.0001$.

Evaluation of the Anti-Proliferative Effect of QE and Hg in Co-Treatment

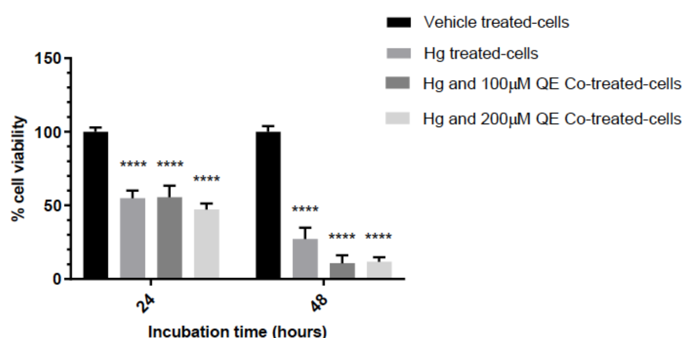
The 1321N1 cells were directly co-treated with QE and Hg or they were pre-treated with QE and then co-treated with QE and Cd (Figure **2**). As shown in Figure **2A**, treatment with Hg and co-treatment with QE and Cd for 24 and 48 hrs significantly reduced cell viability ($p >0.0001$). Exposing the cells to Hg for 24- and 48-hrs resulted in cell viabilities of 54.9 ± 5.2% and 27.2 ± 7.8%, respectively compared to the vehicle-only treated cells (Figure **2A**). Similar results were obtained by co-treating the cells with 100 µM QE and Hg for 24 hrs (55.5 ± 2.8%), however slightly less by co-treatment with 200 µM QE and Hg at the same time point (47.2 ± 4.1) compared to the vehicle-treated cells (Figure **2A**). Higher reduction values were obtained by co-treating cells with QE (100 µM and 200 µM) and Hg for 48 hrs (cell viabilities 10.8 ± 1.9% and 11.7 ± 3.1%, respectively) (Figure **2A**).

Figure **2B** shows the effect on cell viability induced by pre-treating the cells with QE before co-treatment with QE and Hg. The pre-treatment with QE for 24 hrs followed by co-treatment with Hg and 100 µM of QE for 24 hrs resulted in an insignificant reduction in cell viability (87.3 ± 6.5%, $p < 0.9999$) (Figure **2B**), however, resulted in a significant reduction in cells viability (46.7 ± 7.2%, $p > 0.0001$ (at 200 µM of QE for the same incubation time, compared to vehicle-treated cells (Figure **2B**). Further extension of co-treatment time to 48 hrs under the same experimental conditions led to a higher and significant reduction of cell viability with 100 µM of QE (37.6 ± 8.9% cell viability, $p < 0.0001$), and even higher reduction of cell viability at 200 µM QE (10.3 ± 6.0%, $p < 0.0001$) (Figure **2B**). In contrast, treating the cells for 24 hrs with Hg alone resulted in an insignificant reduction in cell viability (85.8 ± 11.2 %, $p < 0.9999$), whereas carrying out the same treatment for 48 hrs significantly reduced cell viability (53.7 ± 5.0, $p = 0.0006$) (Figure **2B**).

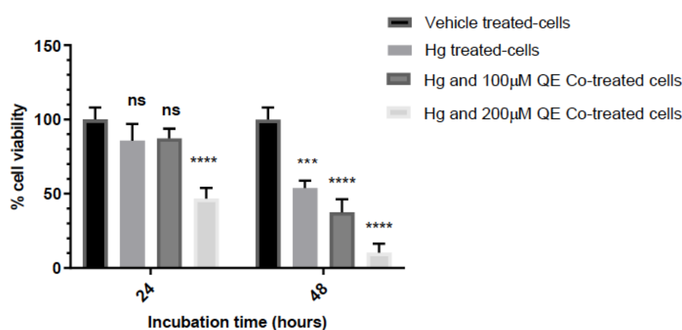
Compared to Figure **2B** which shows the cell viability when cells were pre-treated with QE for 24 hrs prior to co-treatment with QE and Hg, Figure **2C** shows results of carrying out the same pre-treatment using 48 hrs pretreatment followed by the co-treatment. A higher and more significant reduction in cell viability was observed when the cells were pre-treated with QE for 48 hrs and co-treated with QE and Hg for 24 hrs with concentrations of QE being 100 µM (47.9 ± 2.8% cell viability, $p < 0.0001$) or 200 µM (15.0 ± 3.0% cell viability, $p < 0.0001$) (Figure **2C**). As shown in Figure **2C**, the extension of co-treatment with QE and Hg to 48 hrs (under the otherwise same experimental conditions), resulted in more reduced cell viabilities at 100 µM QE (26.1 ± 2.7%, $p < 0.0001$) and 200 µM QE (7.3 ± 2.4%, $p < 0.0001$) (Figure **2C**). Under the same experimental conditions, exposing the cells to Hg alone for 24 hrs resulted also in a significant reduction in cell viability (80.4 ± 8.5%, $p = 0.0058$), and even more when exposing the cells to Hg for 48 hrs (63.2 ± 2.6% cell viability, $p < 0.0001$) (Figure **2C**). However, the viability decrease was much lower compared to the combination of Hg with quercetin.

In consequent experiments, the antiproliferative effect of QE – Hg interaction was evaluated after the removal of QE from the tissue culture. The experiments were carried out by washing off QE after pre-treating cells for 48 hrs. Pre-treated cells were then exposed to Hg or Hg and QE, and cell viability was compared to that of cells exposed to Hg without pre-treatment with QE. As shown in Figure **3**, despite washing off QE from the cells, the antiproliferative effect of QE remained for 24 and up to 48 hrs when the cells were treated with Hg alone. Exposing cells for 24 hrs to Hg alone significantly reduced cell viability (45.0 ± 2.6 %, $p > 0.0001$), whereas exposing cells to Hg after removing 100 and 200 µM QE from the cell culture resulted in a significantly higher reduction of cell viability (31.6 ± 4.4 and 25.6 ± 2.8 %, respectively, $p < 0.0001$) (Figure **3**). In contrast to this, exposing cells for 48 hrs to Hg alone had significantly higher reduction effect on cell viability compared to only 24 hrs exposure (19.1 ± 6.4 % cell viability, $p < 0.0001$) (Figure **3**). However, the exposure to Hg for 48 hrs after washing off the cells pre-treated

A. Lack of pre-treatment with QE



B. Pre-treatment with QE for 24 hrs



C. Pre-treatment with QE for 48 hrs

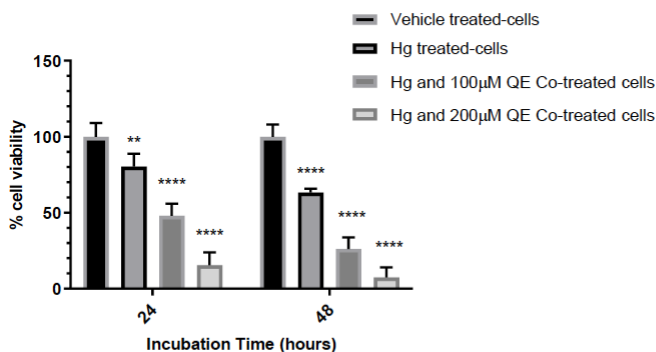


Figure 2: The anti-proliferative effect of co-treating 1321N1 cells with QE and Hg. Cells seeding density at the start of the experiments was $1X10^5$ cell/ml. At the end of incubation times, cell cultures were evaluated for their viability by MTT assay and the resulting absorbance values were expressed as percentages in comparison to vehicle-treated cells. The bars represent mean ± SEM (n = 8). ***p* = 0.0058, ****p* = 0.0006, *****p* > 0.0001, ns - not significant. [x]The pre-treatment with QE was only for cells later co-treated with QE and Hg together.

with 100 μM QE resulted in a higher and significant effect on cell viability (10.1 ± 1.8 %, p < 0.0001), and even higher significant effect on cell viability after washing off the cells pre-treated with 200 μM QE (8.6 ± 1.8 % cell viability, *p* < 0.0001) (Figure **3**).

To ensure whether the reduction in the cell viability after washing off QE was due to QE or the washing-out process itself, similar experiments were carried out in which QE was washed off but re-added to co-incubated with Hg (Figure **4**). The obtained cells viability did not differ significantly from values shown in Figure **3**. Pre-treating cells with QE for 48 hrs and then treating them with Hg and QE (100 μM and 200 μM) for 24 hrs, after washing off QE, resulted in significant and similar reduction in cell viability (22.7 ± 4.9% and 23.4 ± 4.5%, respectively, *p* < 0.0001) (Figure **4**). Under the same experimental condition, co-treating the cells with Hg and QE (100 μM and 200 μM) for 48 hrs resulted in even more reduction of cell viability (12.7 ± 4.8% and 3.6 ± 0.8%, respectively, *p* < 0.0001) (Figure **4**). In

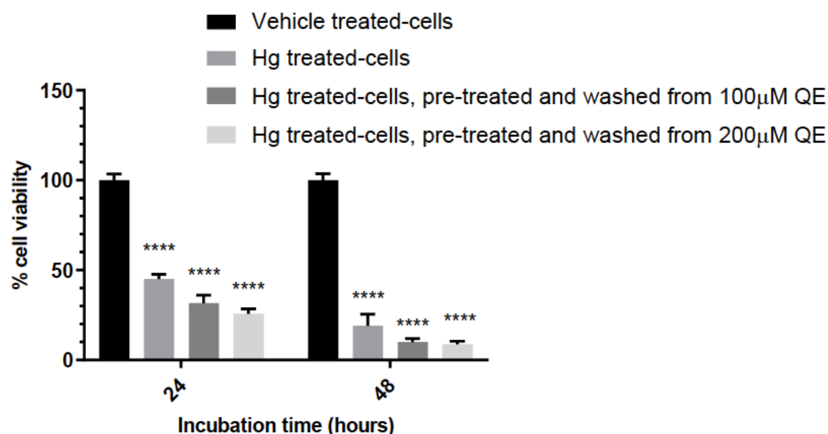


Figure 3: The anti-proliferate effect of Hg on 1321N1 after pre-treatment and washing off of QE. Cells at the density of $1x10^5$ cells/ml were pre-treated with 100 or 200 µM of QE for 48 hrs. QE was washed off and cells were subsequently treated with Hg alone for 24 or 48 hrs. Cell cultures were then evaluated using an MTT assay and the resulting absorbance values were expressed as percentages of the values obtained with the vehicle-treated cells. The bars represent mean ± SEM (n = 8). ****$p < 0.0001$.

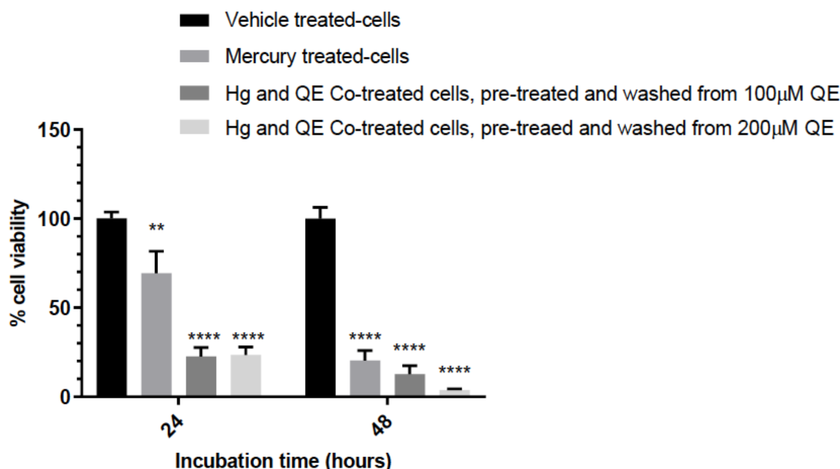


Figure 4: The anti-proliferate effect of co-treating 1321N1 cells with QE and Hg after pre-treatment and washing off of QE. Cells at the density of $1x10^5$ cells/ml were pre-treated with 100 or 200 µM of QE for 48 hrs. QE was washed off and cells were subsequently co-treated with Hg and QE for 24 or 48 hrs. Cell cultures were then evaluated using an MTT assay and the resulting absorbance values were expressed as percentages of the values obtained with the vehicle-treated cells. The bars represent mean ± SEM (n= 8). **$p = 0.0055$ and ****$p < 0.0001$.

contrast, exposing the cells to Hg alone for 24 hrs and 48 hrs without pre-treatment with QE resulted in cell viability 69.3 ± 13.4%, $p = 0.0055$ and 20.3 ± 5.6%, $p < 0.0001$, respectively (Figure **4**). This confirmed that the remaining anti-proliferative effect of QE for up to 48 hrs is due to the intracellular QE as extracellular QE was washed off. The data confirm that the washing-off of QE does not affect its anti-proliferative effect.

DISCUSSION

QE is a flavonoid that is widely distributed in higher plants. The therapeutic and protective utilization of QE has been published in several reports. It was shown that QE has anti-aging, anti-inflammatory, anti-obesity, anti-diabetes, anti-cancer, angio-protective, and cytostatic effects. Cells originating from the brain are one of the most sensitive malignant cell lines to QE. However, the limited permeability through the blood-brain barrier is an essential challenge in using QE in the treatment of various brain tumors. To overcome this challenge, various options to deliver QE are currently under investigation.

Hg is a toxic metal element to which we are exposed in our everyday life. Half of the atmospheric Hg is from natural sources such as volcanos, whereas the rest is from various human activities such as smoking. The most widely existing mercuric compounds are $HgCl_2$ and MeHg. Throughout studying the toxic effect of Hg on various organs, sensory impairments and damaged cognitive skills were

indicated because of brain toxicity. This toxic effect of Hg depends on the exposure dose and duration. The most demonstrated mechanism of Hg toxicity is correlated to glutathione homeostasis largely associated with neutralizing the reactive oxygen species (ROS).

In our previous work [4] we studied the cytotoxic effect of Cd, a carcinogenic industrial metal pollutant, in a model of malignant brain astrocytoma human 1321N1 cell line. It is well-known that exposure to Cd induces the production of ROS resulting in lipid peroxidation and DNA damage. Because QE is a component of the human diet, we exploited the well-known attenuating effect of QE on free radicals produced that may reduce Cd toxicity. Thus, we aimed to investigate the effect of co-treating 1321N1 malignant cells with QE and Cd. Results showed that Cd itself is less cytotoxic on 1321N1. In contrast, QE alone induced more toxicity but did not exert a protective effect on the astrocytoma cells when combined with Cd. Instead, a significant synergistic anti-proliferative interaction was indicated in 1321N1 cells when co-treated with QE and Cd [4]. In the present study, we explored the effect of the flavonoid QE and Hg in the same model of 1321N1 cells. Treatment with QE in our study was prior to or simultaneous to Hg exposure where the effects on cell viabilities were compared to those of cells exposed to Hg alone. Results of the present study were compared to results obtained previously from exposing the same cells to Cd under the same experimental conditions [4].

When compared to vehicle-treated cells, the antiproliferative effect of Hg on 1321N1 cells was evaluated at incubation times of 24 and 48 hrs collected from various experiments. Exposing the cells to 16 μM Hg for 24 hrs significantly ($p > 0.0001$) reduce cell viability by approximately 1.5- fold. However, exposing the cells to Hg for 48 hrs reduced cell viability by approximately three-fold. These data were processed, and the final values are based on data from several individual experiments (n = 40) (Figure **1**). However, by going through each of these experiments, some inconsistencies were determined by exposing the cells to Hg for 24 hrs. Reduction in cell viability was insignificant in one experiment (Figure **2B**), and less significant in two experiments (Figure **2C**; $p = 0.0058$ and Figure **4**; $p = 0.0055$). The insignificant and less significant reducing effect of Hg on cell viability could be explained by the recovery response mechanism of 1321N1 cells exposed to Hg for 24 hrs. A similar observation was made in our previous work by exposing the same 1321N1 cells to Cd for 24 hrs where there was an insignificant reduction in cell viability apart from one experiment [4]. In contrast to exposing the cells to Hg for 24 hrs, carrying out the exposure for 48 hrs significantly reduced cell viability throughout all experiments ($p > 0.0001$).

Overall, our study showed that cell viabilities of 1321N1 cells exposed to Hg alone are lower than those of cells exposed to QE alone [4] when compared to the vehicle-treated cells. Exposing the 132N1 cells to Hg for 24 and 48 hrs resulted in lower cell viability by 1.5-fold and three-fold, respectively [4], whereas exposing the same cells to 100 and 200 μM QE for the same time period, resulted in lower cell viability by 1.3-fold and 2.2-fold, respectively [4]. Thus, it was suggested that the cytotoxic effect of Hg on the astrocytoma 1321N1 cells is higher than that of QE. Similarly, exposing the same cells to Cd alone for 24 and 48 hrs resulted in lower cell viability by 1.1-fold and 1.2-fold, respectively [4]. These results indicated that Hg has a higher toxic effect on 1321N1 cells than Cd.

Co-treating 1321N1 cells exposed to Hg with 100 and 200 μM QE significantly reduced cell viability under different experimental conditions (Figure **2A** $p > 0.0001$). Cells were simultaneously exposed and treated with Hg and QE for 24 and 48 hrs. Compared to the vehicle-treated cells, the cell viability was reduced by approximately two folds after 24 hrs of co-treatment with Hg and QE at both QE concentrations. By extending the co-treatment time to 48 hrs, the cell viability further decreased by nine-folds at both QE concentrations. Under the same experimental conditions, the viability of cells exposed to Hg alone for 24 hrs reduced by two-folds, however by Three-folds after 48 hrs of exposure. These results indicated the high cytotoxic effect of co-treating 1321N1 cells with QE and Hg for 48 hrs compared to treating the same cells with Hg alone. In our previous study [4], treating the same cells with Cd and QE for 24 hrs resulted in reduced cell viabilities by 1.6 folds at both QE concentrations, whereas, carrying out the same treatment for 48 hrs resulted in cell viability further decreased by approximately five-folds at 100 μM QE, however by 14.5- folds at 200 μM QE [4]. Thus, the higher cytotoxic effect is exerted by co-treating the 1321N1 by 200 μM QE and Cd than carrying out the same co-treating with Hg and the same concentration of QE for 48 hrs.

Pre-treating the cells with QE before co-exposure to Hg had variable effects on cell viability (Figure **2B**).

Incubating the 1321N1 cells with 100 µM QE for 24 hrs followed by co-treatment with Hg and the same concentration of QE, insignificantly reduced cellular viability by 1.1-fold compared to vehicle-treated cells. This result could be explained by the cellular repair mechanism that is stimulated at a lower QE concentration and takes a longer period of time. In contrast, pre-treating and co-treating the cells with 200 µM QE for the same period of time, significantly reduced cell viability by two folds ($p > 0.0001$). Similar cell viability was obtained by co-treatment with Hg and QE at both concentrations for 24 hrs without pre-treatment with QE (Figure **2A**). Thus, it suggested that co-treatment with Hg and QE significantly decreased 1321N1 cell viability whether exposed to 24 hrs pre-treatment with QE or not.

Extending the co-treatment time with QE and Hg to 48 hrs after 24 hrs pre-treatment with QE resulted in a significant decrease in cellular viability by approximately three-folds at 100 µM QE and by 10-folds at 200 µM QE. Under the same experimental conditions, when exposing the cells to Hg alone (without pre-treatment with QE), cell viability was insignificantly reduced by 1.2-folds after 24 hrs and significantly reduced by two-fold after 48 hrs ($p = 0.0006$). In our previous work [4], we showed that the pre-treatment with 100 µM QE for 24 hrs followed by co-treatment for 48 hrs with Cd and QE at the same concentration resulted in an 8-folds reduction in cell viability. Furthermore, under the same pre- and co-treatment conditions, 200 µM of QE resulted in a negative cell viability value [4]. These results confirm the higher cytotoxic effect on 1321N1cells by co-treatment with Cd and QE than Hg and QE after pre-treatment with QE for 48 hrs.

By extending the pre-treatment time with QE to 48 hrs, the co-exposure to Hg and 100 µM QE for 24 hrs decreased cell viability by two folds ($p > 0.0001$), and approximately 6.5-folds by treatment with 200 µM QE under the same experimental conditions (Figure **2C**). Furthermore, after the 48 hrs of pre-treatment with QE, co-treatment with Hg and QE for 48 hrs resulted in an approximately four-fold reduction in cell viability at 100 µM QE, and 14 fold at 200 µM QE. Thus, extending the pre-treatment of 1321N1 cells with 200 µM QE for 48 hrs resulted in less cell viability when co-treated with Hg and QE compared to lack of QE pre-treatment. In contrast to our previous work (4), the pre-treatment with QE for 48 hrs followed by co-treatment for 48 hrs with Cd and QE resulted in negligible cell viability at 100 µM QE and a negative value at 200 µM QE [4]. Under the same experimental conditions, exposing the cells to Hg alone for 24 hrs significantly ($p = 0.0058$) reduced cell viability by 1.2-fold, whereas extending the same treatment to 48 hrs reduced the cell viability by 1.6-fold ($p > 0.0001$).

Overall. The obtained data indicated the benefit of longer QE pre-treatment. This, when followed by QE-Hg co-treatment of 132N1 cells resulted in a highly significant effect on cell viability. There is a need for malignant cells to be exposed to QE for a reasonable time to achieve its accumulation within the 132N1 cells and to produce a significant reduction of viability. Then the action of QE and Hg together may lead to a significant decrease in cell viability that is higher than the effect of Hg alone. This was indicated by either directly co-exposing the cells to Hg and 100 or 200 µM QE for 48 hrs, or pre-exposing the cells to 200 µM QE for 24 hrs or 48 hrs followed by co-exposure to the same QE concentration for 24 hrs or even 48 hrs. Thus, QE-Hg anti-proliferative effect on 132N1 cells could be a QE's dose- and time-dependent manner. On the other hand, co-exposing the cells to Hg with either QE concentrations for 24 hrs or even pre-treatment with 100 µM QE for 24 hrs followed by co-treatment with the same QE concentration and Hg for 24 hrs did not exert a different reducing effect on cell viability compared to treatment with Hg alone. This could be due to QE's counteracting effect at lower concentration and shorter exposure period. This effect could mitigate the sole effect of Hg.

In line with our results, a study was conducted by Martins and co-workers [32] on intact *Swiss Albino* female mice (less sensitive to Hg than males) which were exposed on daily basis to 5.4 ± 0.5 mg MeHg/kg alone or in combination with 5 or 50 mg QE/kg. After three weeks of treatment time, it was determined that exposure to MeHg or QE alone did not affect the mice's locomotor activity and motor performance. In contrast, the combination of MeHg and QE resulted in significant synergistic deteriorative effects on animal behavior (locomotor deficits and motor impairment) in QE's dose-dependent manner. Investigation of the underlying mechanism showed that treatment with MeHg or QE alone or their combination significantly induced cerebral lipid peroxidation in an additive rather than synergistic manner. It is shown that such induction of lipid peroxidation by MeHg was related in part to the decrease in cerebral glutathione peroxidase activity, whereas that induction by QE could be a hermetic-like effect which likely to be related to the pro-oxidative properties of QE or its quinone metabolite [32].

Compared to the above described *in vivo* deteriorative effects, Franco and coauthors [33] showed that the *in vitro* treatment with QE had a protective effect against the oxidative stress of MeHg (or $HgCl_2$). The team conducted their study on mitochondrial-enriched fractions prepared from male *Swiss* mice. It was demonstrated that the mercuric compounds induced neurotoxicity through disturbances of mitochondrial integrity, which may initiate cellular death. That was determined to be through the significant induction the glutathione oxidation in a dose-dependent manner of the mercuric compounds [33]. This result is consistent with the above *in vivo* results [32], in which the activity of glutathione peroxidase is reduced when the required glutathione (GSH) is reduced, resulting in increasing the level of the harmful hydrogen peroxide. Such deteriorative effect of glutathione homeostasis by the mercuric compounds was prevented in presence of 100 μM QE and ultimately prevented the mouse brain mitochondrial dysfunction. It was demonstrated that QE did not interfere with the oxidative capability of the mercuric compounds. Therefore, it was hypothesized that QE is involved in detoxifying the reactive hydrogen peroxide generated in presence of the mercuric compounds in brain mitochondria. These data confirmed the *in vitro* protective effect of QE against lipid peroxidation induced through the production of hydrogen peroxide by mercuric compounds [33].

Similarly, a study was conducted on a human-derived liver cell line (HepG2) by Barcelos and co-authors [34]. HepG2 was chosen because it simulates the human detoxifying system in possessing antioxidant and inducible xenobiotic-metabolizing enzymes. Similarly, experimental conditions were designed in a way to simulate the human exposure environment. Treatment of HepG2 cells for 24 hrs with $HgCl_2$ or MeHg at concentrations < 5.0 μM decreased cell viability to below 70%, whereas carrying out treatment with QE at 200 μM decreased cell viability to 40%. Comet formation level indicated the damaging effect of both mercuric compounds on DNA in a dose-dependent manner. The comet formation induced by the mercuric compounds was reduced by pre- and simultaneous treatment with 200 μM QE to 49% and 71%, respectively, but not by post-treatment with QE after exposing and washing off the cells from the mercurials. That could be to the lack of involvement of QE in alterations of DNA-repair processes. In addition, the significant reduction in the levels of reduced glutathione by the mercurials was restored by QE. The significant increase in malondialdehyde, protein carbonyl, and reactive oxygen species by the metal compounds was also reduced in presence of QE. Such biochemical measurements reflect the redox status of the cells which are believed to be responsible for the toxic effect of Hg [34].

Overall, in our work, the action of QE and Hg together may lead to a significant decrease in cell viability that is higher than the effect of these two agents individually. Our experiments show that the QE and Hg may act synergistically on the 1321N1 cells rather than in an additive manner (Table **2**). The combined effect of QE and Hg on the cells was calculated as described in our previous study [4].

An additional experiment was performed to determine the place of QE action – inside or outside of the 132N1 cells. A pre-treatment with QE for 48 hrs was followed by a washing-off of QE and then treatment with Hg alone. The obtained significant decrease in cellular viability reflected the antiproliferative effect of the intracellular QE in the 1321N1 cells when the removal of extracellular QE was performed. It was also determined that washing off QE from the cells did not eliminate its significant anti-proliferative effect when exposing these cells to Hg (Figure **3** $p > 0.0001$). Treating the cells for 24 hrs with Hg after pre-treatment and washing-off of 100 μM QE

Table 2: Evaluation of the QE and Hg Combined Effect on the Viability of 1321N1 Astrocytoma Cells *In Vitro*

Concentration of QE and/or Hg	Viability		Combined effect calculated	
	24 hrs	48 hrs	24 hrs	48 hrs
QE 100 μM	74.9 ± 4.0*	68.3 ± 3.2*		
QE 200 μM	77.9 ± 4.6*	45.8 ± 4.2*		
Hg 16 μM	67.1 ± 4.4	36.7 ± 3.8		
QE 100 μM + Hg 16 μM	**55.5 ± 2.8****	**10.8 ± 1.9****	42.0	5.0
QE 200 μM + Hg 16 μM	**47.2 ± 4.1****	**11.7 ± 3.1****	45.0	-17.5

*values obtained from reference [4].
**significant synergistic effect.

reduced cell viability by 3.2-folds, whereas carrying out the same treatment at 200 μM QE exerted a reduction effect of four-folds. By extending the duration of treatment with Hg for 48 hrs, 100 μM QE reduced cell viability by ten folds, and 200 μM QE reduced cell viability by 12-folds, under the same experimental conditions. These effects were compared to treatment with Hg without pre-treatment with QE, where cell viability was reduced by approximately two-folds and five-folds by treatment with Hg for 24 hrs and 48 hrs, respectively (Figure **3**).

The described above findings were further confirmed under the same experimental conditions. The washing-off procedure was performed but QE was re-added to the cell culture together with Hg. Cell viability of cells pre-treated for 48 hrs with QE and washed-off and co-treated with QE and Hg for 24 hrs (Figure **4**) were similar to those of cells pre-treated for 48 hrs with QE however treated with Hg alone for 24 hrs after washing-off (Figure **3**). That is, co-treatment with Hg and QE after pre-treatment and washing-off of QE resulted in less cell viability by 4.4-folds at 100 μM QE and 4.3-fold at 200 μM QE.

In contrast, pre-treating the 1321N1 cells with 100 μM QE for 48 hrs followed by washing-off of QE and co-treatment with Hg and same concentration of QE for 48 hrs resulted in 8-folds less cell viability. Compared to the higher concentration of QE, pre-treating the cells with 200 μM QE for 48 hrs, followed by washing-off of QE and co-treatment with Hg and same concentration of QE for 48 hrs resulted in approximately 28-folds less cell viability (Figure **4**). This effect at 200 μM QE was compared to 12-folds less cell viability when treated with Hg alone after washing-off of the same concentration of QE (Figure **3**). Thus, QE exerts a significant anti-proliferative by accumulating the intracellular dose which enhances the cytotoxic action by Hg. This intracellular effect of QE was higher in combination with Hg (Figure **3**) than Cd for 24 hrs, however higher with Cd than Hg for 48 hrs (4). That may be explained by the efficient repair mechanism in response to exposure to Cd for a shorter time [4]. The co-treatment with QE and Cd after washing off the pre-treatment with QE for 48 hrs resulted in a negligible cell viability value at 100 μM QE and a negative cell viability value at 200 μM QE [4]. These results showed a higher cytotoxic effect of Cd in combination with the intracellular QE than Hg in the same combination.

A recent study was carried out by Alazoumi and co-workers [35]. They demonstrated the significant disruption effect of 3mM $HgCl_2$ on the α-helical secondary structure of two heme-proteins: myoglobin and cytochrome c, whereas this effect was little and minute by 120 mM $CdCl_2$ on the proteins, respectively. In contrast, both metal compounds deteriorated the tertiary structures of both heme-proteins [35]. In line with this study, Wang and co-workers [36] recently showed that ferritin from Kuruma prawns has a binding capacity to Hg three times larger than that of Cd. The scientific team aimed at exploring the ability of ferritin, a representative member of protein nanocages to remove heavy metal ions from contaminated food systems. Since prawns' living marine environment is usually rich in different metal ions, ferritin of that specie was utilized. The 24 subunits per ferritin capture three Cd ions spontaneously (ΔG -23.94 kJ mol^{-1}), compared to capturing nine Hg ions with a higher spontaneous reaction (ΔG -25.91 kJ mol^{-1}). It was determined that such coordination mode of Kuruma prawns' ferritin is due to the position of cysteine residues that are buried within the protein shell [36].

These results are consistent with our study where the binding capacity to cellular proteins may be in part responsible for the higher toxicity of $HgCl_2$ than $CdCl_2$. This was indicated by the higher reduction in cell viability by treatment with the mercury(II) salt alone compared to cadmium chloride. However, the longer exposure to a higher concentration of QE may be responsible for the higher synergistic anti-proliferative effect of CdCl2 compared to $HgCl_2$.

CONCLUSIONS

In conclusion, we explored the *in vitro* biological effect of QE in a model of malignantly transformed astrocytoma 1321N1 cells exposed to Hg. This work is a continuation of our previous study on the effect of treating the same cells with QE and Cd. Our present study demonstrated the higher anti-proliferative effect of Hg than Cd on the 1321N1 cells. Despite the documented data on the *in vitro* protective effect of QE in healthy cells exposed to Hg, our results demonstrated a synergistic anti-proliferative interaction of Hg and QE in the astrocytoma cells. Due to the accumulation of the intracellular QE, this effect is concentration- and time-dependent. However, compared to our previous work, the synergistic anti-proliferative effect of Cd and QE is higher than that of Hg and QE in the astrocytoma cells. These data will be useful in further investigations of the biological effects of QE in the treatment of malignantly transformed cells.

CONFLICTS OF INTEREST

The Authors declare no conflicts of interest regarding this study.

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