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Original Article

Effects of Ubiquinone on Oxidant and Antioxidant Status in Hepatocellular Carcinoma Cell Line

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A B S T R A C T

Background and Aims: The concomitant use of antioxidants during chemotherapy is controversial. It is unknown whether antioxidants increase or decrease the effectiveness of anticancer drugs. Therefore, the present study aimed to investigate ubiquinone's cytotoxic and antioxidant effects on the HepG2 cell line.

Materials and Methods: The HepG2 cell line was chosen as an experimental model for hepatocellular carcinoma in this study. The cytotoxic effect of ubiquinone was assessed as a function of time and concentration using the colorimetric MTT assay. The half-maximal inhibitory concentration (IC_{50}) was determined to assess the cytotoxic effects of different ubiquinone concentrations. The protective impacts of ubiquinone on HepG2 cells were evaluated by assessing the oxidative stress profile.

Results: The MTT showed that the IC_{50} after treatment with ubiquinone was 350 and 335 µM at 24 and 48 hours, respectively. Evaluation of redox homeostasis in HepG2 cells using three doses of ubiquinone, including IC_{50} and one dose higher and one dose lower than IC_{50}, showed a decrease in oxidative stress and an increase in the antioxidant capacity of HepG2 cells in a dose-dependent manner ($p < 0.01$). An increase in redox hemostasis decreased the viability of HepG2 cells.

Conclusion: Our results showed ubiquinone could reduce cancer cell survival by interfering in redox oxidative-redox status. Therefore, ubiquinone can be used as an antioxidant supplement along with chemotherapy drugs.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide [1]. The prevalence of HCC has increased dramatically worldwide over the last two decades [2]. Currently, the most common treatment strategies for HCC are radiofrequency ablation, surgical resection, and liver transplantation [3]. As with other cancers, HCC is a complex multistep process, mainly influenced by genetic and environmental (epigenetic) factors. Disease results from the accumulation of tissue alterations, inflammation, oxidative stress, hypoxia, and other molecular mechanisms, such as the expression of chemokines, cytokines, growth factors, and pro-angiogenic factors that cause DNA damage and mutation [4].

Free radicals and other reactive oxygen species (ROS) are continuously produced in several biological processes as metabolic by-products or by specific oxidases in the plasma membrane in response to various cytokines and growth factors. These factors are toxic to cells at high concentrations, but they are required as second messengers in regulating gene expression, cell signaling, and maintaining general homeostasis at low concentrations. For this purpose, cells are equipped with antioxidant defense systems, including enzymatic and non-enzymatic systems, to maintain ROS at normal physiological levels by converting free radicals and oxidants into more stable and less harmful molecules [5, 6].

Several pieces of evidence have reported that cancer cells produce more ROS than non-cancerous cells [7, 8]. Although high production of ROS is toxic and is expected to cause cell death, hyperproliferation of cancer cells despite high production of ROS indicates that cancer cells are compatible with the existing situation. In cancer, ROS act in two ways. It can have both tumor-promoting and anti-tumor effects. ROS enhances many molecular signaling pathways, such as mitogen-activated protein kinase/ activator protein 1/ hypoxia-inducible factor (HIF)-1/ nuclear factor-κB (NF-κB), which are associated with cancer metastasis and angiogenesis, as well as by inactivating cell cycle checkpoint-related proteins [9, 10]. Increased ROS can also activate metastasis by inducing matrix metalloproteinases (MMPs) through hypoxia and cathepsin expression, and induce angiogenesis by stabilizing HIFα and vascular endothelial growth factor (VEGF) expression. Therefore, increased ROS below toxic levels for HCC cells is required to improve cell growth, proliferation, survival, and cancer progression [11]. Ubiquinone or coenzyme Q10 is a vitamin-like substance in the respiratory chain located in the inner membrane of mitochondria. Similar to vitamin K, it has a quinone structure. It is a benzoquinone containing a 50-carbon isoprenoid side chain with ten isoprenyl units in its side chain. Its solubility in water is very low (0.7 ng/mL) due to its high molecular weight (863) and high lipophilicity [12]. Ubiquinone is an essential cofactor in the production of ATP during oxidative phosphorylation. In addition, ubiquinone is part of the intracellular antioxidant defense that protects proteins and cell membranes from free radicals and oxidative damage. Many medical conditions associated with ROS's

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increased formation and action lead to decreased ubiquinone levels in the human body. Ubiquinone deficiency leads to the dysfunction of the respiratory chain due to the insufficient production of highly energetic compounds, which decreases the efficiency of cells [13]. *In vivo* studies have demonstrated ubiquinone has therapeutic effects by modulating alpha-fetoprotein expression, inducible nitric oxide synthase, cyclooxygenase-2, and NF-κB in the liver tissue of rats with HCC [14].

Ubiquinone is available both endogenously via the mevalonate pathway and exogenously via food. Oral ubiquinone acts as an antioxidant in the body after being converted to ubiquinol by nicotinamide adenine dinucleotide phosphate reductase. As a powerful lipophilic antioxidant, ubiquinol can recycle and regenerate other antioxidants, including vitamins E and C [15, 16]. There are concerns about the use of antioxidants as dietary supplements due to conflicting reports on the effects of antioxidants on cancer. Therefore, new studies should be conducted to examine the effects of antioxidants on cancer [17-19].

Materials and Methods

Ubiquinone (Cad.no: 303-98-0, 99%); Dimethyl-formamide (DMF, Cad.no: 68-12-2); Cell culture media (DMEM F-12, Cad.no BI-1011); fetal bovine serum (FBS, Cad.no: 12483020); HepG2 cells (National center of genetic and biological resources of Iran) were the materials used in this study.

Cell culture and treatment

Cells were cultured in DMEM F-12 containing 2 mM glutamine and 200 mM streptomycin/ penicillin, supplemented with 10% heat-inactivated FBS. Cell cultures were incubated at 37 °C., 98% relative humidity, and 25% CO2. The cell culture medium was changed when the cell density reached 75-80%. The medium was renewed daily.

Cell viability

Cells were seeded in 96-well plates at a density of 25,000 cells per well and incubated in the mentioned medium at 37 °C for 24 hours to reach the log phase. After 80 minutes, cells were treated with various doses of ubiquinone (80–440) and placed in the incubator (37 °C) for 24 and 48 hours, respectively.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetra-zolium Bromide (MTT) was determined by the change of the water-soluble yellow dye of "3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide" to the water-insoluble violet formazan by mitochondrial reductase. Dimethyl sulfoxide (DMSO) then solubilized formazan, and the concentration was estimated at 570 nm using a spectrophotometer (BioTek, Winooski, USA). Results were expressed as percent absorbance (sample absorbance/control absorbance) x 100 relatives to the control value for each ubiquinone concentration. The IC_{50} was estimated by plotting x-y curves and fitting the data to a regression line. IC_{50} concentrations and one dose higher and one lower than the IC_{50} were selected for biochemical studies. Cells were tested for oxidative stress in 6-well plates after exposure to three doses of ubiquinone for 24 hours.

Measurement of the intracellular levels of total oxidant status (TOS)

TOS levels of HepG2 cells were evaluated colorimetrically using the TOS kit (Natos™ TOS

Assay Kit). Active forms of oxygen and nitrogen are created in metabolic and physiological processes. TOS refers to the total oxidant content in a sample and may include free oxygen species (ROS) or nitrogen (RNS). In this experiment, iron(II) (Fe^{2+}) is oxidized to iron(III) (Fe^{3+}) in the presence of an oxidant, producing a color in the presence of a chromogen. This dye absorbs at the wavelength of 530 nm and is readable. The amount of absorption has a direct relationship with the amount of oxidant. Hydrogen peroxide (H_2O_2) was used for calibration, and results are expressed as µmol H_2O_2 Eq/L (µmol H_2O_2 equals/L).

Measurement of the intracellular levels of malondialdehyde (MDA)

Changes in MDA values were assessed using a commercially available assay kit (Nalondi™ Lipid Peroxidation (MDA) Assay Kit). MDA is a compound formed when unsaturated fatty acids are peroxidized due to oxidative stress. Measuring the amount of MDA in a biological sample indicates the extent of damage caused by oxidative stress. The basis of this assay is the combination of MDA and 2-thiobarbituric acid (TBA) to form a pink product with maximum absorption at 532 nm. Samples containing MDA and MDA standards first react with He-TBA at 95 °C. After a few minutes of incubation, samples and standards can be measured in a spectrophotometer. MDA values for unknown samples can be determined by comparison to the MDA standard curve.

Measurement of the intracellular levels of total antioxidant capacity (TAC)

TAC was measured through the reduced antioxidant capacity of iron based on the manufacturer's instructions (NaxiferTM TAC assay kit). The reduction of Fe^{3+} to Fe^{2+} through the samples was taken as an antioxidant capacity index. In this method, a complex of Fe^{2+} and tripyridyltriazine (Fe^{2+}-TPTZ) produces a blue color with a maximum absorbance at 593 nm. It was compared with a standard curve obtained from 1 ml of ferric reducing ability of plasma reagent (300 mM acetate buffer, 10 mM 2,4,6-tripyridly-1,3,5-triazine (TPTZ) /HCL solution, 20 mM ferric chloride). Values were expressed as µM Fe^{2+}. The research was approved by the Ethical Committee of Iran University of Medical Sciences, Tehran, Iran (Code No: IR.IUMS.REC.29879).

Statistical analysis

The obtained data from the groups were analyzed with statistical prism software using repeated measures analysis of variance (ANOVA) and compared among the groups. Means were considered significant at P value < 0.05. The data were expressed as means and standard deviations (SD).

Results

Cytotoxicity and cell viability evaluation

The effect of ubiquinone on the survival of HepG2 cells was evaluated by the MTT method at treatment times of 24 and 48 hours. Figure 1 shows the results of the toxic effect of ubiquinone on HepG2 cells. Results showed a dose-dependent decrease in the percent cell viability of HepG2 exposed to concentrations of 80-440 µM ubiquinone for 24 and 48 hours. The inhibitory activity of ubiquinone on HepG2 cells was found to be $IC_{50} = 350 \pm 3$ at 24 hours and 335 ± 2.5 at 48 hours. The 24-hour treatment period was

chosen for biochemical assays because there was no significant difference between his IC_{50} values at 24 and 48 hours. The doses selected for subsequent treatment were the IC_{50} (350 μM) and one dose above and below the IC_{50} (250 and 450 μM). Cell viability at doses of 250 μM and 450 μM was 70% and 30%, respectively, compared to Neg-C.

Effects of ubiquinone on oxidant and antioxidant capacities: Figure 2 compares cellular levels of oxidative stress markers in ubiquinone-treated HepG2 cells and untreated cells (Neg-C).

Figure 2A shows the TOS in HepG2 cells after treatment with different doses of ubiquinone and compares them with the negative control sample. Comparison of groups by one-way ANOVA shows significant differences in total oxidant concentrations between treatments ($p < 0.001$). Treating with ubiquinone caused a dose-dependent significant decrease in TOS levels in HepG2 cells, and this decrease was statistically significant for all treatments compared with the negative control ($p < 0.05$).

Figure 2B shows MDA levels after treatment with various doses of ubiquinone and compared to the negative control. Comparison of groups with one-way ANOVA shows a significant alteration in MDA production following treatment with different doses of ubiquinone ($p < 0.001$). The treatment decreased MDA concentration in HepG2 cells dose-dependently, and this decrease was significant at all concentrations compared with untreated His-HepG cells ($p < 0.05$). Figure 2C shows the total antioxidant capacity of groups treated with different doses of ubiquinone compared to the negative control. Comparison of groups with one-way ANOVA shows a significant difference in total antioxidant levels ($p < 0.0001$). In a dose-dependent manner, treatment with ubiquinone significantly decreased TAS levels in HepG2 cells ($p < 0.05$).
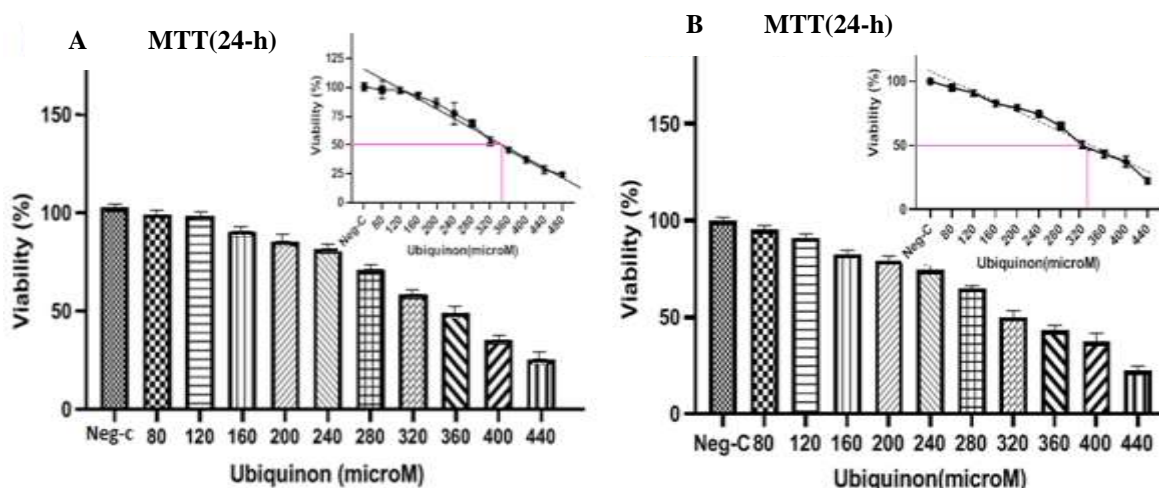

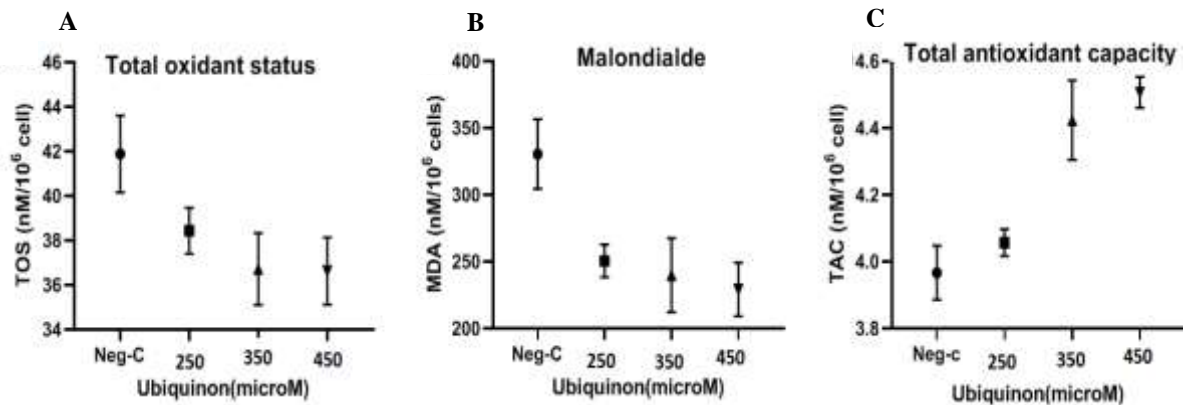**Fig. 1.** MTT test: ubiquinone toxicity test graphs for 24-hour (A) and 48-hour (B) treatments

Fig. 2. Comparison of total oxidant status (A), Malondialdehyde (B), and total antioxidant capacity (C) levels in the treatment with different doses of ubiquinone

Discussion

This study demonstrated that ubiquinone increases total antioxidants and decreases oxidative stress as well as oxidative markers of MDA in HepG2 cells in a dose-dependent manner. Liu et al., in their study on hepatocellular carcinoma patients after surgery, demonstrated that a dose of 300 mg/d of ubiquinone supplementation significantly increased the antioxidant capacity and reduced the oxidative stress and inflammation levels in HCC patients after surgery [14]. Decreasing ubiquinone levels in cancer cells increase superoxide anion production in mitochondria and entry into the cytoplasm. ROS induces endoplasmic reticulum stress and increases calcium release from endoplasmic reticulum stores, which induces ER stress. After treating HepG2 cells with ubiquinone to reduce free radicals and oxidative stress, ER stress and calcium release from the ER are expected to decrease. Fouad et al. also reported a decrease in the expression of genes of hepPar-1, alpha-fetoprotein, inducible nitric oxide synthase, cyclooxygenase-2, and nuclear factor-κB in

liver tissue of rats with hepatocellular carcinoma after treatment with ubiquinone supplements [20].

Despite these ameliorative effects, treatment with high doses of ubiquinone reduced the viability of HepG2 cells. There was a negative correlation between the dose of ubiquinone and the viability of HepG2 cells. AL-Megrin et al reported that ubiquinone reduces oxidants' deleterious cellular side effects due to its antioxidant, anti-inflammatory and anti-apoptotic effects against experimentally-induced hepatocellular carcinoma in rats [21]. However, there are controversial and conflicting reports regarding the use of antioxidant supplements in cancer [17, 22, 23]. Increased ROS can activate metastasis by inducing MMPs, activate angiogenesis by stabilizing HIFα, and activate hypoxia-induced VEGF expression. Cancer cells must always maintain high ROS levels in their environment for proliferation, growth, and survival. On the other hand, it keeps ROS levels below cytotoxic levels by increasing inhibitory mechanisms and

limiting ROS production. Excess ROS levels cause oxidative stress-induced cancer cell death by stimulating anti-tumor signaling; thus, reducing ROS decreases cancer cells' proliferation and metastatic potential [7, 11, 24]. However, a balance of oxidative states is essential for maintaining cancer cell homeostasis [25]. Alimohammdi et al. [26] and Ragip Pala et al. [27] demonstrate that ubiquinone supplementation enhances mitochondrial phosphorylation pathways, reduces oxidative stress as an antioxidant, and protects cells from oxidative damage. Hodge et al. reported that ubiquinone supplementation significantly increased the antioxidant capacity and reduced oxidative stress and inflammation levels in HCC patients after surgery [28]. Thus, treatment with ubiquinone may impair the redox state of cancer cells and decrease viability in a dose-dependent manner.

Conclusions

Cancer cells must keep ROS levels sufficiently high while limiting ROS production below cytotoxic levels for proliferation, growth, and survival. A dose higher than the IC_{50} determined in this study showed that ubiquinone can significantly inhibit cancer cell proliferation and progression by reducing reactive oxygen species. In conclusion, this *in vitro* study showed that ubiquinone supplementation could be considered a complementary treatment strategy for patients with HCC, particularly those under higher oxidative stress and inflammation levels.

Conflict of Interest
The authors declare no conflicts of interest.

Acknowledgments

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