



Effects of Pyrroloquinoline Quinone (PQQ) and Coenzyme Q10 on mitochondrial genes, mitomiRs and cellular properties in HepG2 cell line

Muhammed Ali Nalbant^{1,2*} Onur Eroğlu^{3,4}

¹Department of Molecular Biology and Genetics, Graduate Education Institute, Bilecik Seyh Edebali University, Bilecik, Turkey

²Coordinatorship of Occupational Health And Safety, Bandırma Onyedi Eylül University, Bandırma-Balıkesir, Turkey

³Department of Molecular Biology and Genetics, Faculty of Science, Bilecik Seyh Edebali University, Bilecik, Turkey

⁴Biotechnology Research and Application Center, Bilecik Şeyh Edebali University, Bilecik, Turkey

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ABSTRACT

Our study aimed to reveal the effects and changes, antioxidant metabolism (Oxidative Stress), inflammatory response, mitochondrial biogenesis and mitochondrial dysfunction characteristics in hepatocellular carcinoma cell line HepG2; that occur in genes (NRF-1, NRF-2, NFκB and PGC-1α) and miRNAs (miR15-a, miR16-1, miR181-c) that can control related features. To investigate the effects of Pyrroloquinoline quinone (PQQ) and Coenzyme Q10 (CoQ10) in HepG2, and their effects on cell viability, lateral cell migration, gene expression and miRNA expression levels were investigated. If the data we have obtained are evaluated in terms of anti-cancer effectiveness, the most effective use of CoQ10 can be defined as the use alone rather than the combined use. According to the results of the wound healing experiment, we determined that Pyrroloquinoline quinone and combined drug application increased the wound closure area and cell proliferation compared to the control group, while CoQ10 application decreased it. We found that Pyrroloquinoline quinone and Coenzyme Q10 exposure in the HepG2 cell line increased Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression but not NRF-1 gene expression. We reported only a small increase in expression of the NRF-2 gene in the Pyrroloquinoline quinone application compared to the control group. We found that only Pyrroloquinoline quinone and CoQ10 application caused more expression increase in the Nuclear Factor kappa B (NFκB) gene compared to combined application. Pyrroloquinoline quinone and CoQ10 administration down-regulated the expression levels of miR16-1, miR15a and miR181c. The use of Pyrroloquinoline quinone and CoQ10 is effective on epigenetic factors, miR-15a, miR-16-1 and miR181c are important candidate biomarkers in hepatocellular carcinoma and diseases accompanied by mitochondrial dysfunction.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common tumors seen in the liver. It constitutes a very high rate of 90% of tumors seen in the liver. HCC is the fifth most common type of cancer among the cancer types seen in the world. Lung cancer is the first cause of cancer-related death in men, while the second cause is HCC (1).

As a result of the healthy lifestyle trend and increased daily activities, individuals need more supplements and support foods. As a result of this trend, these food supplements, which are in demand, need scientific research on their effectiveness. Coenzyme Q10 (CoQ10) is an important supplement that is preferred as the third most used supplement and in the treatment of various non-communicable diseases (2).

Coenzyme Q10 (CoQ10) is primarily known for its important role in mitochondrial energy metabolism. As a result of extensive studies, it was determined that it is in cell membranes and blood plasma. These studies have been the turning point in the investigation of the antioxidant role and clinical indication of CoQ10. Recent studies have reported that CoQ10 can affect the expression levels of the genes of cell signaling, migration and metabolism.

Low CoQ10 levels have been associated with cancer, diabetes, neurodegenerative and muscle diseases and fibromyalgia (3).

Pyrroloquinoline quinone (PQQ) affects cellular metabolic processes, mitochondrial production events, reproduction, growth and aging, through environmental exposure and diet. Pyrroloquinoline quinone is specifically targeted by essential genes for fatty acid metabolism and the mitochondrial system. Moreover, it provides a combination of other transcriptional enhancers such as PGC-1α and nuclear respiration factors (NRF-1 and NRF-2) that are essential elements for maintaining energy homeostasis and binding antioxidant response genes (4). Bioactive compounds, which are reported to stimulate mitochondrial biogenesis, contain many benefits as a healthy life span, efficient energy usage and protection from reactive oxygen species (5).

Regulatory factors critical for the expression of many mitochondrial functions of vertebrates are the nuclear respiration factors, NRF-1 and NRF-2. NRF-1 of them has been identified due to its binding to a palindromic sequence in the cytochrome c promoter and has been associated with the expression of many genes essential for the expression and function of the respiratory chain. Also, it is

* Corresponding author. Email: mnalbant@bandirma.edu.tr

a transcription factor that interacts with various coactivators including PGC-1 α , to regulate nuclear mitochondrial genes (6).

PGC-1 α is an important coactivator that plays a central role in a regulatory network that governs transcriptional control of mitochondrial biogenesis and respiratory function (7).

Nuclear factor kappa B (NF- κ B) is a family of dimeric transcription factors that coordinate inflammatory responses such as cellular differentiation, proliferation, survival, and immune response. Misregulation of NF- κ B has been associated with a wide variety of diseases, from cancer to inflammatory and immune disorders. NF- κ B is of critical importance to cancer biology that needs to be focused and elucidated (8).

Recently, some types of miRNAs, termed MitomiR, have been identified to be present specifically in mitochondria. MitomiRs originate from mitochondrial mRNAs or the nucleus but mostly are of nuclear origin. The relationship of MitomiRs with mitochondria varies between the species and cell types in which it is present. MitomiRs are located extensively in the mitochondria of tissues and cells. Aberrant expression of MitomiRs in mitochondria has been associated with the occurrence of cancer disease (9).

MitomiR family members, miR-15a and miR16-1 induce apoptosis through the regulation of mitochondrial functions. They occur as a cluster in the 13q14 chromosomal region and are frequently deleted or downregulated in chronic lymphocytic leukemia. miR-15a and 16-1 coordinate multiple oncogenic activities, containing BCL-2 and MCL-1. Furthermore, miR-15a regulates mitochondrial dysfunction, by cytochrome c release into the cytosol and deterioration of mitochondrial membrane potential (10).

MitomiR family element miR181-c is an important miRNA involved in mitochondrial oxidative metabolism that regulates mitochondrial energy metabolism. It originates from the nuclear genome, is processed in the cytosol and translated into mitochondria. Overexpression of miR181-c causes an imbalance between mitochondrial units, promoting reactive oxygen species (ROS) generation (11).

Our study aims to reveal the effects and changes that occur in genes (NRF-1, NRF-2, NF- κ B and PGC-1 α) and miRNAs (miR15-a, miR16-1, miR181-c) and antioxidant metabolism (Oxidative Stress), inflammatory response, mitochondrial biogenesis and mitochondrial dysfunction characteristics in hepatocellular carcinoma cell line HepG2.

Within scope of our study, it was aimed to reveal the selected genes, miRNAs and cellular properties most clearly in accordance with the hypothesis of the study, by choosing hepatocellular cells with the most intense mitochondrial function.

In our study, the cellular, mitochondrial, molecular genetics and epigenetic effects of antioxidant drugs PQQ and CoQ10 in hepatocellular carcinoma cell line HepG2 are comprehensively demonstrated.

Basically, it is aimed to investigate the effects of drugs used due to antioxidant support and therapeutic response in many different diseases, especially cancer. Do the drugs involve really elicit miracle responses, as expected, by acting on genetic and epigenetic factors? How it affects mitochondrial functions is another question mark that our

study tries to resolve. How it affects cell proliferation and cancer development when it is applied to cancer cells in vitro is one of the hypotheses our study tries to reveal.

Materials and Methods

Preparation of Pyrroloquinoline Quinone (PQQ) and Coenzyme Q10 (CoQ10)

Supplied in powder form Pyrroloquinoline quinone (Sigma-Aldrich) and Coenzyme Q10 (Sigma-Aldrich) were prepared in 10 μ M Dulbecco's Modified Eagle Medium (DMEM) cell culture solution and the prepared solutions were stored at -20 $^{\circ}$ C. PQQ was prepared in serially diluted concentrations ranging from 200 μ M to 6.25 μ M. CoQ10 was prepared in serially diluted concentrations between 100 μ M and 3.125 μ M. The serially diluted concentration range of the cell group treated with the PQQ+CoQ10 drug combination is between 100 μ M and 3.125 μ M. Cells without drug administration constitute the control group.

Cell culture

HepG2 cells (ATCC $^{\circ}$ HB-8065 $^{\text{TM}}$) were cultured 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin containing Dulbecco's Modified Eagle Medium (DMEM), in 75 cm 2 flasks, at 37 $^{\circ}$ C, in a 5% CO $_2$ conditions.

Cell viability assays

The effects of PQQ, CoQ10 and combined drug administration on cell viability in the HepG2 cell line were analyzed using the XTT test. In our study, the Biotium XTT kit with catalog number 30007 was used. Experimental steps were carried out in accordance with the kit protocol. Firstly, the stock solutions were prepared by dissolving PQQ and CoQ10 powders in 10mL DMEM (10mM). Doses were prepared by serial dilution as specified in Section 2.1. Briefly, HepG2 cells were seeded into 96-well plates at a density of 10,000 cells per well. After the attachment of the inoculated cells (3 days later) drugs were applied. The absorbances were read at 475 nm with a microplate reader (ThermoFisher Scientific).

IC $_{50}$ concentrations were determined by plotting a linear regression curve. The percentage of cell growth inhibition was calculated as; Relative inhibition rate (%) = $(OD_{\text{control}} - OD_{\text{drug administration}}) / OD_{\text{control}} \times 100$. Relative inhibition rates and linear regression curves were calculated and graphed using the Microsoft Excel program.

Cell count

Cell counting was performed with a cell counter with the RDW branded product code Automated Cell Counter (C100). Since the device can calculate 40,000 cells in 1 mL, cell cultivation was carried out in 96 well plates.

Cell wound healing assay

In order to reveal the effect of applied drugs on lateral cell migration and cell-cell interaction, a cell wound healing experiment was performed. HepG2 (1x10 5) cells were seeded in 6 well-plates. In the 1 $^{\text{st}}$ well, drug-free control, 2 $^{\text{nd}}$ well CoQ10, 4 $^{\text{th}}$ well PQQ and 5 $^{\text{th}}$ well PQQ+CoQ10 drug-treated cells were seeded. Wells 3 and 6 were left blank. When the cells reach a confluency of 75%, a scratch was created in the middle of each well with a 200 μ L mi-

cropipette tip. Then, the cells were washed in 1xPBS. After washing, 2 mL of fresh medium was added to all wells. At 24, 48 and 72 hours, the movements of the cells were monitored and imaged under an inverted microscope. The images obtained from the wells were evaluated according to the dimensions of the scar area. The resulting images were transferred to the Image-Pro Premier 9. 2. 22 programs to evaluate the closure rate of the scar. Measurements and markings were made using Image-Pro Premier 9. 2. 22 to compare wound healing gaps.

Wound closure percentages were calculated according to the formula $(W_{10} - W_{72}) / W_{10} \times 100$. (W_{10} : Wound width of 0 hr, W_{72} : Wound width of 72 hr) (12).

mRNA Products Real-Time Polymerase Chain Reaction (Real-Time PCR/qRT-PCR)

RNA isolation

For the isolation of RNA from mRNA products, GeneAll brand, RiboEx (301-001 catalog no) and Hybrid-R (305-101 catalog no) kits were used. Cell pellets (~1x10⁷) obtained after centrifugation of the cell samples were treated with the buffers contained in the kit in accordance with the kit protocol. In the last step, RNA samples were obtained after centrifuging the columns at 10. 000xg for 1 minute were stored at -80 °C.

cDNA synthesis

After RNA isolation, cDNA synthesis was passed. To obtain cDNA used, A. B. T brand, cDNA Synthesis Kit (High Capacity) (catalog no C03-01-05). After the master mix was prepared for cDNA synthesis, the reverse transcription reaction stage was started. After reverse transcription, the obtained cDNA samples were stored at -80 °C.

Real-Time qPCR phase

After obtaining cDNA, the Real-Time qPCR stage was started. For Real-Time qPCR was used, A. B. T brand, 2X qPCR SYBR-Green Mastermix (Without Rox) (Q03-01-05 catalog no) kit. Primer information used for expression analysis was synthesized as shown in Table 1. The β-ACTIN (ACTB) gene was used as a reference gene in the qRT-PCR step of mRNA products.

miRNA Products Real Time Polymerase Chain Reaction (Real Time PCR/qRT-PCR)

RNA isolation

For miRNA isolation, were used GeneAll brand RiboEx (301-001 catalog no) and Hybrid-R miRNA (325-

150 catalog no) kits. Cell pellets (~1x10⁷) obtained after centrifugation of the cell samples were treated with the buffers contained in the kit in accordance with the kit protocol. In the last step, the RNA samples were obtained after centrifuging the columns at 11. 000 rpm for 2 minutes were stored at -80 °C.

miRNA-cDNA synthesis

After miRNA isolation, the cDNA synthesis phase was started from the miRNAs obtained. Specific "stem-loop primers" were designed for each miRNA to be investigated and these primers were used in cDNA synthesis. For cDNA extraction from miRNAs, used A. B. T.™ brand, cDNA Synthesis Kit with Rnase Inh. A kit named (High Capacity) (catalog no C03-01-05). Followingly; was started the reverse transcription reaction stage and the obtained miRNA-cDNA samples were stored at -80 °C.

miRNA-cDNA products Real-Time qPCR phase

For Real-Time qPCR, used A. B. T™ brand, A. B. T.™ 2X miRqGreen MasterMix (with ROX) (Q04-02-01 catalog no) kit. The same MasterMix conditions were used for all primers. The Real-Time qPCR reaction was performed on the Applied Biosystems™ 7500 Fast Real-Time PCR instrument. The RNU6 gene was used as a reference gene in the qRT-PCR step of miRNA products.

Evaluation of results and statistical analysis

All experiments conducted within the scope of our study were performed with 3 technical repetitions. Statistical analysis of our study was performed using GraphPad Prism 9. 3. 1.

Normality tests of PQQ, CoQ10 and PQQ+CoQ10 XTT results were performed using the Shapiro-Wilk test and Kolmogorov-Smirnov test. Then, the "Normality Graph" of the relevant data was prepared. Whether the PQQ, CoQ10 and PQQ+CoQ10 XTT values were statistically significant was investigated by performing the "One Sample t-test" within the group. Then, statistical analysis between groups was made using "One-Way ANOVA". The significance level was accepted as p<0. 05.

The normality test of the wound healing experiment data, in which the cell proliferation and migration ability were measured depending on the drug administration was performed with the Shapiro-Wilk test. The results obtained were statistically analyzed, with the measurement values of 4x and 10x images separately. The "One Sample t-test" was performed within the group to determine whether the measurement values of the 4x images were statistically significant within each group (Control-PQQ-CoQ10-

Table 1. Primer information used for gene expression analysis.

NRF1-F	ATCGTCTTGTCTGGGAAAC	Oligomer
NRF1-R	TGTTCCAATGTCACCACCTC	Oligomer
NRF2-F	ATGCCCTCACCTGCTACTTT	Oligomer
NRF2-R	GATGCCACACTGGGACTTG	Oligomer
NFKB-F	GAAGCACGAATGACAGAGGC	Oligomer
NFKB-R	GCTTGCGGATTAGCTCTTTT	Oligomer
Pgc-1α-F	CGAGAGTTTCCGTTCCCTTT	Oligomer
Pgc-1α-R	CCAGGAGGAGCCGTAGTTGT	Oligomer
B-ACTIN-F	AAACTGGAACGGTGAAGGTG	Oligomer
B-ACTIN-R	AGAGAAGTGGGGTGGCTTTT	Oligomer

PQQ+CoQ10). Then, statistical analysis between groups was made using “One-Way ANOVA”. (p<0. 05)

For the analysis of qRT-Pcr results; threshold cycle, Ct value, ΔCt value, ΔΔCt and Fold Change (coefficient change after comparison of samples) values were calculated. While calculating the ΔCt value, the formula (Ct target miRNA – Ct reference miRNA) was used. While calculating the ΔΔCt value, the formula (ΔCt target miRNA – Mean ΔCt control miRNA) was used. While calculating the Fold Change value, the $2^{-\Delta\Delta Ct}$ formula was used. The Fold Change values in the range of 0 to 1 were evaluated to define the downstream regulation. The normality test of the ΔCt value and Fold Change values obtained were performed with the Shapiro-Wilk test. Whether the ΔCt value and Fold Change values were statistically significant was investigated by using the “One Sample t-test” within the group. Statistical comparison of genes and miRNA products between groups was made using “One-Way ANOVA”. (p<0. 05) The standard deviation (SD) was calculated by calculating the population standard deviation. The population SD was calculated according to the formula $SD: \sqrt{\sum (xi-M)^2 / n}$. (Xi: Values in series, M: Median, n: Sample size)

Results

Cell viability assays

XTT test was applied to reveal the effects of PQQ and CoQ10 on cell viability. The IC₅₀ concentration for PQQ was determined as 46,331 μM. (Figure 1) Applied CoQ10 concentrations are given in (Figure 2). The IC₅₀ concentration for CoQ10 was determined as 23,99 μM. The IC₅₀ concentration for the PQQ+CoQ10 drug combination was determined as 32,1992 μM. (Figure 3)

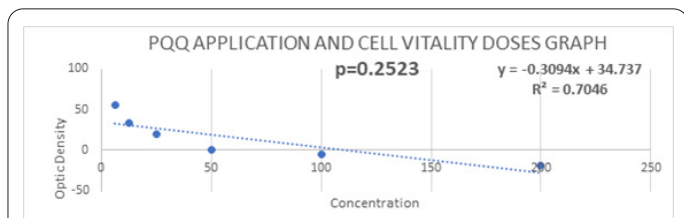


Figure 1. Pyrroloquinoline Quinone (PQQ) administration doses and linear regression curve.

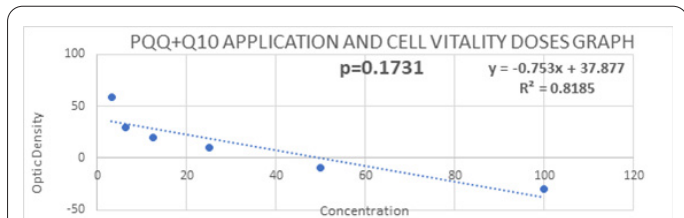


Figure 2. Coenzyme Q10 (CoQ10) administration doses and linear regression curve.

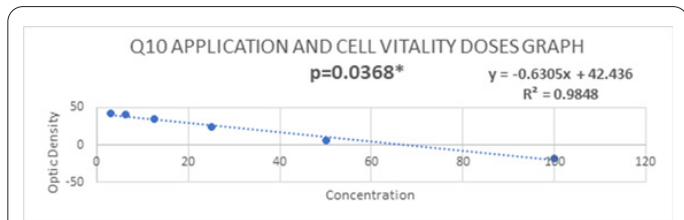


Figure 3. PQQ+CoQ10 combined administration doses and linear regression curve.

According to the results of the One-Sample t Test, which was carried out to statistically evaluate the XTT results within the groups, the p-value of the PQQ administration was found to be 0. 2523, the p-value of the CoQ10 administration was 0. 0368*, and the p-value of the PQQ+CoQ10 administration was 0. 1731. (p<0. 05 significance level) Statistical analysis between groups was performed using “One-Way ANOVA” at p<0. 05 significance level. The P-value was determined as 0. 8888 and it is statistically insignificant, the groups are independent of each other.

Cell wound healing assay

In the wound healing experiment, we performed on the hepatocellular carcinoma cell line HepG2, PQQ, CoQ10, PQQ+CoQ10 combined drug administration and control group was followed in the wells at 0, 24, 48 and 72 hours. Wound closure distances were examined under a light microscope at 4X and 10X enlargement, and the images were recorded (Figure 4 and Figure 5) The images we obtained from the wound healing assay results of our study were transferred to Image-Pro Premier 9. 2. 22 program to evaluate the closure rate. Wound closure percentages in the control and drug administration groups of distances measured from 10X images; Control: %33. 01, PQQ: %36. 79, CoQ10: %18. 09, PQQ+CoQ10: %38. 77. According to

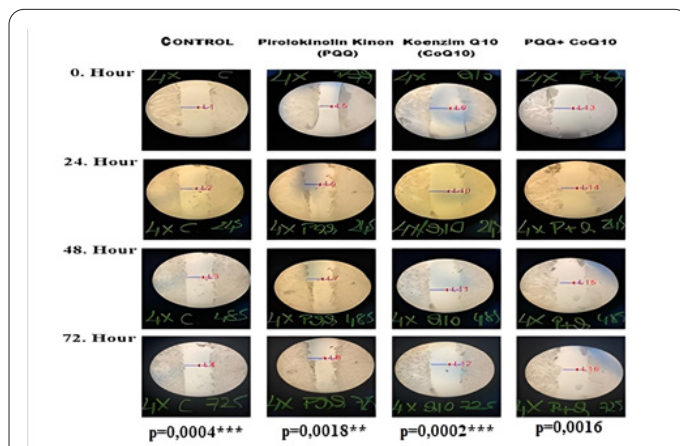


Figure 4. Pyrroloquinoline Quinone (PQQ), Coenzyme Q10 (CoQ10) effects on cell migration and proliferation (The wound area was examined under a light microscope (4X)).

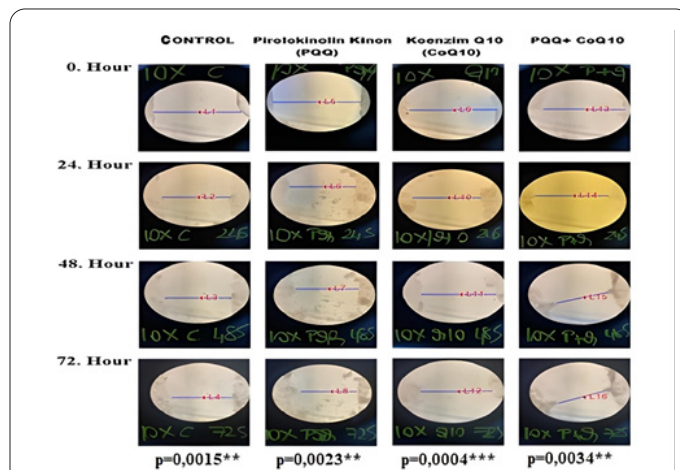


Figure 5. Pyrroloquinoline Quinone (PQQ), Coenzyme Q10 (CoQ10) effects on cell migration and proliferation (The wound area was examined under a light microscope (10X)).

the assay results; PQQ application increases wound closure area and cell proliferation compared to the control group. CoQ10 administration compared to the control group; It has been determined that it prevents the closure of the wound area and cell proliferation. In combined administration, the drugs acted antagonistically to each other, increased wound closure and supported cell proliferation. In the evaluation of the measurement results at the end of 72 hours compared to the control group, the highest cell proliferation and wound closure were observed in the PQQ+CoQ10 combined drug application. A greater proliferation stimulus occurred from the individual administration of the drugs.

When we analyzed the gap closure data of 4X and 10X images statistically, we found that the data of both groups had a normal distribution (Figure 4 and Figure 5). The data of 4X images are at $p < 0.05$ significance level, the p-value for the Control group is 0.0004***, the p-value for PQQ is 0.0018**, the p-value for CoQ10 is 0.0002***, and for PQQ+CoQ10** p-value was determined as 0.0016 and all data obtained are statistically significant. Statistical analysis between groups was performed using “One-Way ANOVA” at $p < 0.05$ significance level. p-value was determined as 0.0781 and it is statistically insignificant, the groups are independent from of other.

The data of 10X images were at $p < 0.05$ significance level, the p-value was determined for the control group 0.0015**, for PQQ 0.0023**, for CoQ10 0.0004***, for PQQ+CoQ10 was 0.0034** and all the data obtained are statistically significant. Statistical analysis between groups was performed using “One-Way ANOVA” at $p < 0.05$ significance level. p-value was determined as 0.6848 and it is statistically insignificant, the groups are independent of each other.

mRNA products Real Time Polymerase Chain Reaction (Real Time PCR/qRT-PCR)

We first identified in the qRT-PCR phase of our study were investigated, expression levels of the genes (PGC-1 α , NRF-1, NRF-2 and NF- κ B). As a result of the applied qRT-PCR, the Ct value, Δ Ct value, $\Delta\Delta$ Ct and Fold Change values of the relevant genes were calculated.

After the mRNA products were determined to have a normal distribution in the qRT-PCR experiment results; according to the results of the One-Sample t Test performed to statistically evaluate qRT-PCR Δ Ct values within groups, the p-value was determined for PGC1- α 0.0057**, for NRF-1 the 0.0031**, for NRF-2 0.0066**, for NF- κ B 0.0036**. ($p < 0.05$ significance level) Statistical analysis between groups was performed using “One-Way ANOVA” at $p < 0.05$ significance level. p-value was determined as 0.0002*** and it is statistically significant.

According to the results of the One-Sample t Test performed to statistically evaluate the qRT-PCR Fold Change values within the groups, the p-value was determined as for PGC1- α 0.1094, for NRF-1 0.0139*, for NRF-2 0.0210*, for NF- κ B 0.1766. ($p < 0.05$ significance level) Statistical analysis between groups was performed using “One-Way ANOVA” at $p < 0.05$ significance level. p-value was determined as 0.1038 and it was statistically insignificant, the groups were independent of other.

According to the qRT-PCR data of our study; PQQ and CoQ10 administration supports the increase of PGC1- α gene expression. While the highest expression increase

was seen in the CoQ10 application, there was almost the same increase in the PQQ application. However, the combined application of drugs, according to their separate applications; expression of the PGC1- α gene was increased to a much lesser extent (Figure 6). Although PGC1- α gene expression increased in all treatments compared to the control group due to drug administration, it was not found to be statistically significant.

In our study; we investigated the effects of NRF-1 and NRF-2 genes. When we examined the effects of PQQ and CoQ10 application on NRF-1 gene expression; we found that in all three applications (PQQ-CoQ10-PQQ+CoQ10) it did not increase gene expression compared to the control group, on the contrary, it caused less expression than the control group (Figure 7). When we evaluated the data statistically within the group, we found it statistically significant. For the NRF-2 gene, we detected a very small increase in expression only in the PQQ application compared to the control group. We found a decrease in expression compared to the control group in CoQ10 and combined drug administration. (Figure 8). When we statistically evaluated the data of the NRF-2 gene within the

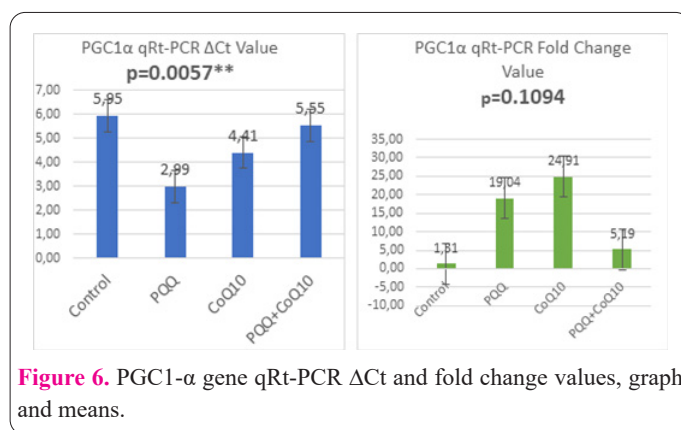


Figure 6. PGC1- α gene qRt-PCR Δ Ct and fold change values, graph and means.

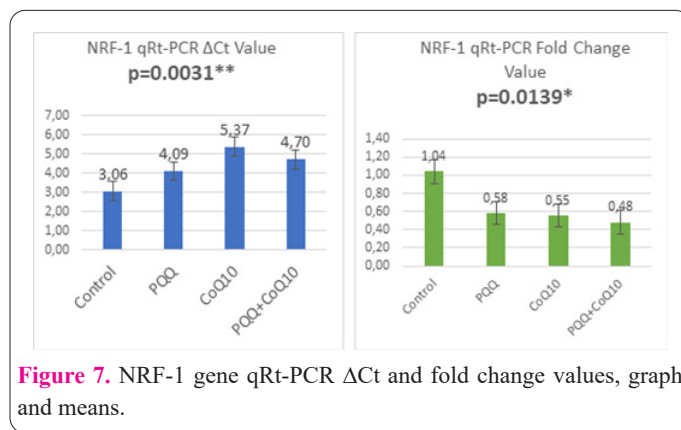


Figure 7. NRF-1 gene qRt-PCR Δ Ct and fold change values, graph and means.

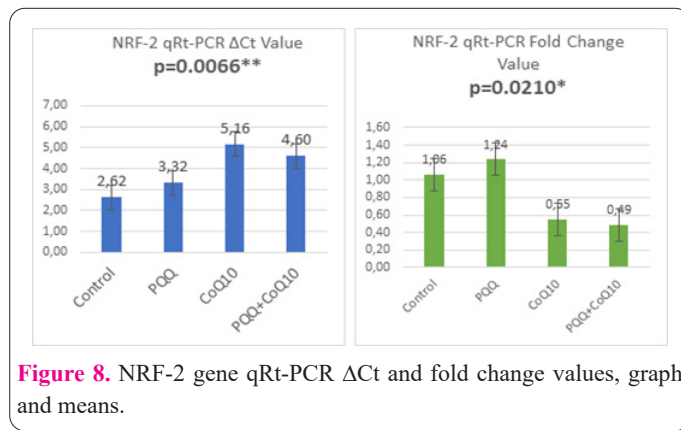


Figure 8. NRF-2 gene qRt-PCR Δ Ct and fold change values, graph and means.

group; We found it statistically significant.

According to the data we obtained; In NFκB gene expression level, PQQ causes the highest increase, followed by CoQ10 application, and finally PQQ+CoQ10 combined application. (Figure 9) While PQQ application caused the highest increase in NFκB gene expression level among the administered drugs, the combined application of PQQ+CoQ10 caused the lowest expression increase. When we evaluate the obtained data statistically; we did not find it statistically significant.

miRNA products Real Time Polymerase Chain Reaction (Real Time PCR/qRT-PCR)

In the qRT-PCR phase of our study, we investigated the expression levels of miRNAs (miR15-a/miR16-1/miR181-c), members of the MitomiR family.

After the miRNA products were determined to have a normal distribution in the qRT-PCR experiment results; according to the results of the One-Sample t Test performed to statistically evaluate qRT-PCR ΔCt values within groups, the p-value was determined for hsa miR 16-1 0.0008***, for hsa miR-15a 0.0014**, and for hsa miR 181-c 0.0001****. (p<0.05 significance level) Statistical analysis between groups was performed using “One-Way ANOVA” at p<0.05 significance level. p-value was determined as 0.0118*, which is statistically significant.

According to the results of the One-Sample t Test performed to statistically evaluate the qRT-PCR Fold Change values within the groups, the p-value was determined for hsa miR16-1 0.3192, for hsa miR-15a 0.2515, for hsa miR181-c 0.0028**. Statistical analysis between groups was performed using “One-Way ANOVA” at p<0.05 significance level. p-value was determined as 0.3075 and it is statistically insignificant, the groups are independent of each other.

According to the qRT-PCR results of our study, miR16-1 was more expressed in all three drug administration groups (PQQ, CoQ10, PQQ+CoQ10) compared to the control group (Figure 10). Combined application had a positive effect on the effectiveness of the drugs. Similar results were also obtained in miR15a, and all three treatment groups (PQQ, CoQ10, PQQ+CoQ10) expressed higher levels of miR15a compared to the control group. (Figure 11). Combined administration had a positive effect on the effectiveness of the drugs. According to miR181c results, although all three application groups (PQQ, CoQ10, PQQ+CoQ10) showed similar results due to down-regulation, the greatest effect was in the PQQ application (Figure 12).

Discussion

Within the scope of our study; we investigated the effects of two antioxidant agents, which are widely used today and recommended as supplements in hepatocellular carcinoma HepG2 cells. We revealed the effects of PQQ and CoQ10 on mitochondrial biogenesis, mitochondrial dysfunction, anti-cancer activity, cell proliferation and migration, gene expression levels, and finally on the expression levels of miRNAs, which are members of the mitoMir family. We conducted the trials of our drug administrations separately and in combination. We performed all our experiments within the scope of our study with 3 technical repetitions.

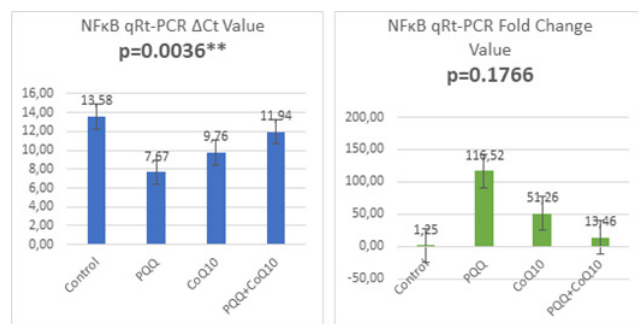


Figure 9. NFκB gene qRt-PCR ΔCt and fold change values, graph and means.

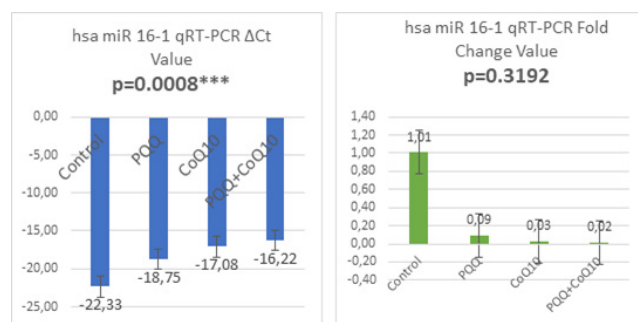


Figure 10. hsa miR 16-1 qRt-PCR ΔCt and fold change values, graph and means.

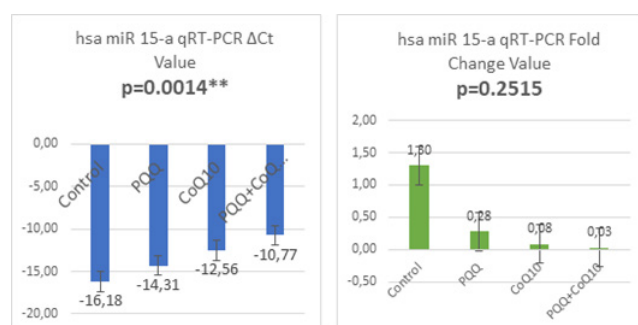


Figure 11. hsa miR 15-a qRt-PCR ΔCt and fold change values, graph and means

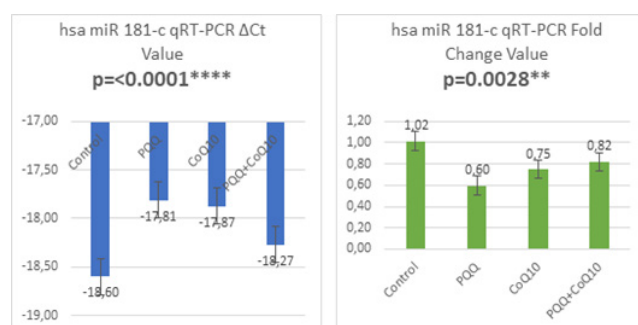


Figure 12. hsa miR 181-c qRt-PCR ΔCt and fold change values, graph and means.

As a result of the experimental research we have done within the scope of our study, the first data we have obtained are the effects on cell viability and the IC₅₀ doses of drugs. In order for the drug administered according to these doses to be effective in the experimental group, PQQ should be used in higher amounts than CoQ10. According to the data obtained in the combined drug administration; alone CoQ10 application may be more effective with a

smaller amount of drug. In studies investigating anti-cancer efficiency, minimum drug use and maximum effect are aimed. When our study is evaluated in this context, only CoQ10 reveals a more effective use. PQQ and CoQ10 are put on the market together or by adding various supplements by drug manufacturers. If the data we have obtained are evaluated in terms of anti-cancer effectiveness, the most effective use of CoQ10 can be defined as the use alone rather than the combined use.

In cell culture studies, drug effects are investigated alone or in combination. When combined drug administration is applied, it is tried to obtain maximum therapeutic efficiency with less drug use. For this effect to occur, drugs must act synergistically. The use of combination chemotherapy offers potential advantages such as increased drug efficacy and protection, as well as the chance of dose reduction, reduced toxicity, and reduced or delayed drug resistance development. However, in the opposite case, the same effect may occur with the use of higher concentrations of drugs (13).

When the reading values of the PQQ, CoQ10 and PQQ+CoQ10 XTT relative inhibition rates were evaluated statistically; only the CoQ10 application was found to be statistically significant. CoQ10 will provide effective use at the determined IC_{50} concentration and application intervals. The combination of PQQ and CoQ10, its effectiveness can be investigated by making trials at different concentrations in a wider range of use. When the relative inhibition rates of the PQQ, CoQ10 and PQQ+CoQ10 XTT experiment were evaluated statistically between the groups, no statistical difference was found between the groups. ($p < 0.05$ significance level) According to this result, the drugs show efficacy independently in the applications. This data, as seen in our IC_{50} doses, show higher efficacy with the use of CoQ10 alone than with the combined use of less amount of the drug. Thus, the independence of drug administration groups is supported.

Zhihui Min et al. investigated the anti-cancer activity of PQQ in their study with three different tumor cell lines. In their study, they determined the IC_{50} concentration for PQQ as 50.16 μ M and 56.21 μ M in 24 hours, respectively. It has been reported that PQQ triggers the apoptotic process at low concentrations (30-75 μ M), and apoptotic effects become significant at higher concentrations (120-300 μ M) (14). The IC_{50} (46,331 μ M) value that we determined in our study is compatible with this study. (Figure 1) In the concentration range we determined, the apoptotic process was induced and the survival of cancer cells was adversely affected.

The benefits of CoQ10 for cancer are through immune system enhancement and antioxidant activity. Supplemental CoQ10 (100-200 mg daily) can prevent cardiac damage and adverse effects of chemotherapeutic drugs on the gastrointestinal system. In a systematic review, it was reported that CoQ10 protected against cardiotoxicity and liver toxicity in patients receiving chemotherapeutic drugs such as doxorubicin in studies conducted with cancer patients (15). Because of this feature, the investigation of the anti-cancer activity of CoQ10 and PQQ in our study was carried out in the HepG2 hepatocellular cell line. In this way, we have clearly observed the changes in mitochondrial properties and biogenesis.

Up-regulation of CoQ7, CoQ4 and CoQ8 gene expressions in cancer cell lines of chemotherapeutic drugs such

as camptothecin, etoposide, doxorubicin and methotrexate results in an increase in CoQ10 levels as an antioxidant response to free radical formation. In one study, decreased CoQ10 levels were detected in women with breast cancer and in cancerous breast tissues, suggesting that reduced CoQ10 levels are associated with poor prognosis. Some case reports have reported that a daily intake of 390 mg. of CoQ10 resulted in tumor regression and disappearance in patients with previously identified metastases. Plasma CoQ10 levels were found to be significantly lower in 117 melanoma patients without metastases compared to the control group. In addition, lower CoQ10 levels were detected in patients who developed metastases compared to those who did not. Low plasma levels of CoQ10 have been shown in cervical intraepithelial neoplasia and cervical cancer (15). In our study, the effect of CoQ10 on cell viability was investigated, and the anti-cancer efficacy of externally administered doses to cells with cancer activity (HepG2 Cell Line) was demonstrated. The results of the experiments in which the IC_{50} concentration and cell viability we determined both in practice and statistically for CoQ10 application and support the relevant studies (Figure 2). With the correct dosage of CoQ10; it shows anti-cancer activity and adversely affects the proliferation and survival of cancer cells.

The effect of PQQ and CoQ10 on cell viability as a combined administration has not been investigated before in the literature. In the HepG2 cell line, the efficacy and anti-cancer properties of PQQ and CoQ10 have not been demonstrated before. We demonstrated for the first time the cellular properties of redox factors PQQ and CoQ10 in HepG2, as they prevent mitochondrial dysfunction and play an active role in mitochondrial biogenesis. In the wound healing assay, we performed on the hepatocellular carcinoma cell line HepG2; according to the experimental results, PQQ application increases cell proliferation compared to the control group. CoQ10 application compared to the control group; it has been determined that it prevents cell proliferation. In the combined administration, we found that the drugs increased cell proliferation at the highest rate.

Nagwan Shanan et al., while investigating the effects of PQQ and resveratrol on the survival and regeneration of cerebellar granular neurons in their study, performed a wound healing experiment in cerebellar granular neuron culture at the stage of investigating apoptosis and cell proliferation. According to the results they obtained; PQQ (0.5 μ M) and resveratrol (5 μ M) tended to promote neurite regrowth at the wound area; however, they reported that this effect was statistically insignificant. Combination administration of both drugs showed similar degrees of neurite regrowth to the effect observed with resveratrol alone, and they reported that no synergistic effect was observed between the two drugs (16). In this respect, the study shows parallelism with our study. In our study, we reported that PQQ increases wound healing and cell proliferation.

B. Wan et al., in a study they conducted with P3H4 (Coenzyme Q10/ubiquinone analogue), a type of quinone compound, performed a wound healing experiment to investigate the effects of CoQ10 on proliferation and migration on renal cancer cells (Renca). In the wound healing assay, they reported that the cell migration ability was significantly inhibited in the test group compared to

the control group (17). These results are consistent with the results we obtained in the wound healing assay in our study; this is consistent with our findings that the cell migration ability is inhibited by CoQ10 administration compared to the control group.

The amount of mitochondria is regulated to respond to the energy needs of the cell and to compensate for cellular damage. Increased mitochondrial amounts are supported by PGC1- α , a key regulator of mitochondrial biogenesis. Mart Dela Cruz et al., in their study, found an increase in PGC1- α and mitochondrial numbers in patients with precancerous lesions. Oxidative stress is known to increase mitochondrial mass. They suggest that the increase in mitochondrial mass may be driven by oxidative stress in the early stages of carcinogenesis (18).

PGC1- α activity is regulated by the amount of nutrients and energy balance in the cells. Cells increase mitochondrial production when there is mitochondrial damage to cells. Mitochondrial production events depend on the upregulation of PGC1- α gene expression. By deacetylating PGC1- α by SIRT1, a PGC1- α dependent energy and redox balance is established. PGC1- α is activated together with ERR α (Estrogen-related receptor α) and Sirtuin 3 (SIRT3) expression is provided. In this way, ROS in the cell is cleared by increased superoxide dismutase activity. In telomere dysfunction, p53 suppresses PGC1- α expression. Mitochondrial production decreases during the aging process. In cancers in which p53 is mutated, PGC1- α is downregulated and mitochondrial dysfunction and ROS are increased (19).

According to the results we obtained; PQQ and CoQ10 exposure increases PGC1- α expression in the HepG2 cell line. Increased PGC1- α expression is associated with increased mitochondrial biogenesis, mitochondrial function and lower ROS levels. Exposure to both antioxidant agents has a positive effect on mitochondrial function. We reported that combined use reduces this effect.

Nuclear respiration factors are vital to the sustainability of mitochondrial function. Mitochondrial biogenesis is regulated by the activity of nuclear transcription factors such as Peroxisome proliferator-activated receptor gamma (PPAR α - γ), NRF-1, NRF-2, Estrogen Related Receptors (ERR α , β , γ).

The expression of NRF-1 and NRF-2 regulates the expression of antioxidant genes and respiratory chain components such as cytochrome and Co subunits. ERR factors regulate the expression of genes related to fatty acid oxidation, the Krebs cycle and oxidative phosphorylation. As a result of increased mitochondrial biogenesis, mitochondrial mass increases in response to nutritional stress (19).

Winyoo Chohanadisai et al., in their study, investigated the effect of PQQ on mitochondrial biogenesis in Hepa1-6 cells. The effects of PQQ were investigated on PGC1- α mRNA and protein expression, NRF-1, NRF-2, CREB and PGC1- α promoter activation. Cells were exposed to PQQ after being transfected with a reporter plasmid containing response elements for NRF-1 or NRF-2. In their study, they reported that PQQ exposure caused an increase in the activation of NRF-1 and NRF-2 promoters, and the activation of PGC1- α promoter, mRNA and protein expression (20).

Low expression of NRF-1 and NRF-2 causes conditions such as decreased oxidative phosphorylation capacity, weak antioxidant capacity, and low cellular metabo-

lism. As a result of exposure to antioxidant agents such as PQQ and CoQ10, an increase in the expression levels of nuclear respiratory factors is expected. In our study, although PQQ exposure was parallel to the study of Winyoo Chohanadisai et al., due to the increase in the expression levels of the PGC1- α gene, there were no similar results in terms of the expression levels of NRF-1 and NRF-2 genes. The reporting of different results for the NRF-1 and NRF-2 genes may be due to the difference in techniques for measuring gene expression.

Xing Li et al., in their study, measured the levels of Nrf-2 and NQO-1 proteins by Western blot technique to investigate the effect of CoQ10 on the protection of the Nrf-2/ARE signaling pathway against oxidative stress and apoptosis. They reported that CoQ10 treatment increased Nrf-2 and NQO-1 protein levels (21). Depending on the application of PQQ and CoQ10 in our study, the decrease we detected in NRF-1 and NRF-2 gene expression levels is at the gene level, and different interactions may occur at protein levels.

PQQ and CoQ10 are two important components known for being powerful antioxidants. Due to these features, we investigated its effect on NF- κ B gene expression levels in our study. NF- κ B is found in the cytoplasm and complexes with I κ B in normal cells. When NF- κ B is stimulated by stress, free radicals and ROS, it becomes activated, leading to phosphorylation of p65. Phosphorylated p65 migrates from the cytosol to the nucleus and activates ROS, eventually resulting in oxidative stress (22).

Lixia Liu et al., in their study on the C2C12 mouse skeletal myoblast cell line, reported that PQQ significantly down-regulated ROS accumulation, increased the activities of important antioxidant enzymes, and decreased NF- κ B phosphorylation. Exposure to an antioxidant agent in the cancer cell line is expected to reduce the oxidative stress of the cells, so the expression of the NF- κ B gene is also expected to decrease. Our studies do not show parallelism in this aspect. Since the cells they used are not cancer cells, the cells may reveal different expression levels in terms of antioxidant properties with hepatocellular carcinoma cells HepG2 cells (23). In our study, although the effects of PQQ and CoQ10 application on NF κ B and antioxidant properties were not statistically significant, this should be investigated in different cell lines.

There was no statistically significant difference between the drug groups whose effects on gene expression levels were investigated by applying them during the qRT-PCR stage. The application of PQQ, CoQ10 and PQQ+CoQ10 was independent between the groups in terms of the effect on the expression levels of PGC1- α , NRF-1, NRF-2, NF- κ B genes. It has been determined that the related genes and drugs are not related to each other due to their applications.

From the MitomiR family; miR16-1, miR-15a and miR181c are the miRNAs whose expression levels we investigated in our study. miR-15a and miR-16-1 induce apoptosis through the regulation of mitochondrial function. miR-15a promotes mitochondrial dysfunction, indicated by cytochrome c release into the cytosol and disruption of mitochondrial membrane potential (24). miR181-c is an important miRNA involved in mitochondrial oxidative metabolism and regulating mitochondrial energy metabolism. Overexpression of miR181c causes an imbalance between mitochondrial units and promotes the formation of ROS (25). Due to these features, miR-

NAs are expected to be more activated than normal cell functions in case of mitochondrial damage, carcinogenesis and impaired membrane potentials. In our study, higher expression of MitomiR expression in hepatocellular cell line HepG2 due to PQQ and CoQ10 applied compared to the control group was considered an advantage for mitochondrial function and antioxidant metabolism. Combined administration adversely affected the expression level of miR181c. When the data obtained were compared within all drug groups, only the values obtained for miR181-c were found to be statistically significant. When the drug administrations were evaluated statistically between the groups, no statistically significant difference was found. It has been determined that the related drug applications do not show a common effect for the miRNAs investigated in the study.

Some miRNAs (miR-192, miR-146a, miR-148a, miR-15a and miR-21) have been reported to be upregulated in drug-induced liver injury. Sharon Lt et al., investigated the effect of CoQ10 on different biochemical biomarkers and miRNA (including miR15-a) expression levels in their study. According to the results they obtained, they detected the expression levels of miR-15a with high expression levels contrary to the downstream expectations. They suggested that the source of miR-15a circulating in patients may be from other organs (26,27). However, as expected in our study, downregulation was detected for miR15-a in all three applications.

Since there is no available research in the literature on investigating the effects of PQQ and CoQ10 administration on miR-16-1 and miR181c expression levels, these effects were revealed for the first time within the scope of our study.

Within the scope of our study, we investigated the effects of PQQ and CoQ10, which are widely used due to their roles in antioxidant and energy metabolism, on the cell line in HepG2, where mitochondrial activities are intense. We reported, the effects of PQQ and CoQ10 on cell survival, proliferation, anti-cancer activity, effects on cell migration, expression levels of PGC1- α , NRF-1 and NRF-2 genes, which are vital for mitochondria, effects on the expression level of the NF- κ B gene, which is an important apoptosis marker and is critical for antioxidant metabolism and finally effects on the changes in expression levels of 3 important miRNAs (miR-16-1, miR-15a and miR181c) that we have chosen for their importance for cancer and being mitochondrial miRNA (MitomiR). We demonstrated the effect of PQQ and CoQ10 on cellular properties as well as their effect on important and relevant mRNA and miRNA expression levels.

As a result, if the data we obtained are evaluated in terms of anti-cancer effectiveness, CoQ10 and PQQ are two different supplements that can reveal very different effects when used separately or together. These interactions must be taken into account when products and supplements containing CoQ10 and PQQ are developed. Indications of the target audience is another concept that should be considered.

We have reported that PQQ supports cancer cell proliferation in cell wound healing assay, so more careful consideration should be given to its use in cancer patients. In addition, detailed effects on many different cancer types and their cell lines should be investigated. In this respect, our study sheds light on new studies. CoQ10 effectively

reduces cancer cell proliferation when used alone, concomitant supplements and drugs should be used considering this feature.

PQQ and CoQ10 administration has important effects on the PGC1- α gene and therefore on mitochondrial biogenesis. We report data inconsistent with the literature for PQQ and CoQ10 administration NRF-1 and NRF-2. The effects of PQQ and CoQ10 administration on NRF-1 and NRF-2 gene expression should be investigated on different cell lines. According to the results of the investigation of PQQ and CoQ10 NF κ B gene expression levels in the HepG2 cell line, especially PQQ should be used with clinical caution in cancer patients. In addition, this activity can be investigated in vivo, opening the door to new research. Our study has created new focus areas where ERR α , β , γ and PPAR γ , Sirtuin (SIRT1 and SIRT3) combined with NRF-1, NRF-2, PGC-1 α and NF κ B genes can be investigated in studies investigating mitochondrial function, mitochondrial biogenesis and free radicals in mitochondria.

In our study, we reported the changes in expression levels as a result of exposure to PQQ and CoQ10 for the first time, as expected for miR-15a, and for the first time in the literature for miR-16-1 and miR181c. The use of PQQ and CoQ10 is effective on epigenetic factors, miR-15a, miR-16-1 and miR181c are important candidate biomarkers in hepatocellular carcinoma and diseases accompanied by mitochondrial dysfunction.

Conflicts of interest

The authors declare that no conflict of interest exists.

References

1. Asafo-Agyei KO, Samant H. Hepatocellular Carcinoma. [Updated 2023 Feb 12]. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2023 Jan. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK559177/>
2. Marta AJ, Suñé-Negre JM, García-Montoya E. Coenzyme Q10 supplementation: Efficacy, safety, and formulation challenges. *Comprehensive Reviews In Food Science And Food Safety*. 2020;19(2):574-594.
3. Garrido-Maraver J, Cordero MD, Oropesa-Avila M, et al. Clinical applications of coenzyme Q10. *Front. Biosci. (Landmark Ed)*. 2014;19(4):619-633.
4. Jonscher KR, Chowanadisai W, Rucker RB. Pyrroloquinoline-Quinone Is More Than an Antioxidant: A Vitamin-like Accessory Factor Important in Health and Disease Prevention. *Biomol* 2021;11(10):1441.
5. Chowanadisai W, Bauerly KA, Tchapanian E, et al. Pyrroloquinoline Quinone Stimulates Mitochondrial Biogenesis through cAMP Response Element-binding Protein Phosphorylation and Increased PGC-1 α Expression. *J Biol Chem* 2010;285(1):142-152.
6. Klinge CM. Estrogenic control of mitochondrial function. *Redox Biol* 2020;31:101435.
7. Scarpulla RC. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* 2011;1813(7):1269-1278.
8. Mitchell S, Vargas J, Hoffmann A. Signaling via the NF κ B system. *Wiley Interdiscip Rev Syst Biol Med* 2016;8(3):227-41.
9. Rencelj A, Gvozdenovic N, Cemazar M. MitomiRs: Their Roles in Mitochondria and Importance in Cancer Cell Metabolism. *Radiol Oncol*. 2021;55(4):379-392.

10. Li P, Jiao J, Gao G, Prabhakar BS. Control of mitochondrial activity by miRNAs. *J Cell Biochem.* 2012;113(4):1104–1110.
11. Das S, Ferlito M, Kent OA, et al. Nuclear MicroRNA Regulates the Mitochondrial Genome in the Heart. *Circ Res.* 2012;110(12):1596–1603.
12. Kaya Çakir H, Eroglu O. In vitro anti-proliferative effect of capecitabine (Xeloda) combined with mocetinostat (MGCD0103) in 4T1 breast cancer cell line by immunoblotting. *Iran J Basic Med Sci.* 2021;24(11):1515–1522.
13. Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. *Cell.* 2017;171(7):1678–1691.
14. Min Z, Wang L, Jin J, et al. Pyrroloquinoline Quinone Induces Cancer Cell Apoptosis via Mitochondrial-Dependent Pathway and Down-Regulating Cellular Bcl-2 Protein Expression. *J Cancer.* 2014;5(7):609–624.
15. Garrido-Maraver J, Cordero MD, Oropesa-Avila M, et al. Clinical applications of coenzyme Q10. *Front Biosci (Landmark Ed).* 2014;19(4):619-33.
16. Shanan N, GhasemiGharagoz A, Abdel-Kader R, Breitingner HG. The effect of Pyrroloquinoline quinone and Resveratrol on the Survival and Regeneration of Cerebellar Granular Neurons. *Neurosci Lett.* 2019;694:192-197.
17. Wan B, Zeng Q, Tang XZ, Tang YX. P3H4 affects renal carcinoma through up-regulating miR-1/133a. *Eur Rev Med Pharmacol Sci.* 2018;22(16):5180-5186.
18. Cruz MD, Ledbetter S, Chowdhury S, et al. Metabolic reprogramming of the premalignant colonic mucosa is an early event in carcinogenesis. *Oncotarget* 2017;8(13):20543-20557.
19. Boland ML, Chourasia AH, Macleod KF. Mitochondrial dysfunction in cancer. *Front Oncol.* 2013;3:292.
20. Chohanadisai W, Bauerly KA, Tchapanian E, et al. Pyrroloquinoline Quinone Stimulates Mitochondrial Biogenesis through cAMP Response Element-binding Protein Phosphorylation and Increased PGC-1 α Expression. *J Biol Chem.* 2010;285(1):142–152.
21. Li X, Zhan J, Hou Y, et al. Coenzyme Q10 Regulation of Apoptosis and Oxidative Stress in H₂O₂ Induced BMSC Death by Modulating the Nrf-2/NQO-1 Signaling Pathway and Its Application in a Model of Spinal Cord Injury. *Oxid Med Cell Longev.* 2019;2019:6493081.
22. Li X, Zhan J, Hou Y, et al. Coenzyme Q10 suppresses oxidative stress and apoptosis via activating the Nrf-2/NQO-1 and NF- κ B signaling pathway after spinal cord injury in rats. *Am J Transl Res.* 2019;11(10):6544-6552.
23. Alkahtani S, Alarifi S, Alkahtane AA, et al. Pyrroloquinoline quinone alleviates oxidative damage induced by high glucose in HepG2 cells. *Saudi J Biol Sci.* 2021;28(11):6127-6132.
24. Li P, Jiao J, Gao G, Prabhakar BS. Control of mitochondrial activity by miRNAs. *J Cell Biochem.* 2012;113(4):1104-10.
25. Das S, Ferlito M, Kent OA, et al. Nuclear miRNA regulates the mitochondrial genome in the heart. *Circ Res.* 2012;110(12):1596-603.
26. Aziziaran Z, Bilal I, Zhong Y, Mahmud A, Roshandel MR. Protective effects of curcumin against naproxen-induced mitochondrial dysfunction in rat kidney tissue. *Cell Mol Biomed Rep* 2021; 1(1): 23-32. doi: 10.55705/cmbr.2021.138879.1001.
27. Pek SL, Tavintharan S, Woon K, et al. MicroRNAs as biomarkers of hepatotoxicity in a randomized placebo-controlled study of simvastatin and ubiquinol supplementation. *Exp Biol Med (Maywood).* 2016;241(3):317-30.