

**Kuersetin ve Hiperterminin OVCAR-3 Hücrelerine Etkileri****The Effects of Quercetin and Hyperthermia on OVCAR-3 Cells**Özlem ÖZGÜR GÜRSOY<sup>1</sup>, Canan VEJSELOVA SEZER<sup>2</sup>, Ceren YILDIZ EREN<sup>1</sup>, Hulusi Gökkuğ GÜRER<sup>1</sup><sup>1</sup>Eskisehir Acibadem Hospital, Obstetrics and Gynecology Clinic, Eskisehir, Turkey<sup>2</sup>Eskisehir Technical University, Faculty of Science, Department of Biology, Eskisehir, Turkey**Öz****GİRİŞ ve AMAÇ:** Bu çalışmanın amacı kuersetinin insan epitelyal over kanseri hücreleri (OVCAR-3) üzerindeki büyümeyi baskılayıcı etkisini göstermek ve hiperterminin etkisini irdelemektir.**YÖNTEM ve GEREÇLER:** OVCAR-3 hücreleri farklı konsantrasyonlardaki kuersetine maruz bırakılarak apoptotik değişiklikler konfokal mikroskop ve transmisyon electron mikroskopi ile kaydedildi. Aynı zamanda Annexin-V ve reaktif oksijen türleri (ROS) analiz edildi. Kuersetin ile birlikte hipertermiye maruz kalan OVCAR-3 hücreleri üzerinde sitotoksitesite testi yapıldı.**BULGULAR:** Kuersetinin OVCAR-3 hücreleri üzerindeki antikanserojen etkisi in vitro proliferasyonu baskılayarak oluşur. Kuersetinin 48 saat sonraki kaydedilen IC50 değeri 122 µM. Kuersetine maruz kalan OVCAR-3 hücreleri üzerinde hiperterminin beligin bir etkisi yoktur.**TARTIŞMA ve SONUÇ:** Bulgularımız kuersetinin OVCAR-3 hücreleri üzerindeki apoptotik etkisinin zaman ve konsantrasyon bağımlı olduğunu göstermektedir.**Abstract****INTRODUCTION:** This study aims to exhibit the growth inhibitory effect of quercetin on the human ovarian epithelial carcinoma cells (OVCAR-3) and scrutinize the effect of hyperthermia.**METHODS:** OVCAR-3 cells were exposed to different concentrations of quercetin and apoptotic changes were recorded with confocal microscopy and transmission electron microscopy. Also Annexin-V and reactive oxygen species (ROS) measurement were analyzed. Cytotoxicity was tested on quercetin exposed OVCAR-3 cells with hyperthermia.**RESULTS:** Quercetin has good anticancerous activities in vitro by inhibiting the proliferation of OVCAR-3 cells. The recorded IC50 value of quercetin was 122 µM for 48 hours. Hyperthermia didnot influence significantly on the growth inhibition of OVCAR-3 cells applied with quercetin.**DISCUSSION and CONCLUSION:** Our results demonstrated that quercetin could induce apoptosis in a time and concentration-dependent manner in OVCAR-3 cells.**Anahtar Kelimeler:** kuersetin; OVCAR-3 hücreleri; hipertermi.**Keywords:** quercetin; OVCAR-3 cell; hyperthermia.**INTRODUCTION**

Ovarian cancer (OC) is the eighth most common female malignancy worldwide and the fifth cause of death due to cancer in women (1). Approximately %70 of patients are diagnosed at its advanced stage because of the difficulty in detecting in its early stages (2). The common treatment strategy is cytoreductive surgery followed by systemic platinum–taxane-based chemotherapy (3). However, due to its heterogeneous features, 20-30% of patients showed chemoresistance, and also 70% of chemosensitive tumors relapse within 5 years (4). In addition, almost all drugs for cancer therapy have significant toxicity to normal cells. For these

reasons, new treatment modalities are put forward like phytochemicals and hyperthermia. Phytochemicals preoccupy a good option of therapy because of their safety in usage, non-toxic effects during long-term therapy, and cytotoxicity for cancer cells but not on normal cells (5).

Most studied phytochemicals are flavonoids in cancer cell lines and have been identified to show growth inhibitory effects in ovarian cancer cells (6). Quercetin is one of the most abundant flavonoids found in vegetables and fruits (7). The anti-cancer activity of quercetin on OC cells elucidated as to cause cell cycle arrest in the G0/G1 phase, apoptosis, DNA fragmentation,

increase in p53 protein, and p53 phosphorylation. But the complete mechanisms of it have not been clearly defined (8).

Another promising OC therapy is regional hyperthermia. The fundamental of the treatment based on the knowledge of protein denaturation and irreversible DNA damage occurrence by temperature increment (9). Hyperthermic intraperitoneal chemotherapy (HIPEC) also increases the antitumor effectivity of the drugs like cisplatin and mitomycin C (10). Based on this data, most cancer institutes apply HIPEC with the surgery simultaneously. But some studies found out no effect of hyperthermia so this approach remains controversial (11).

In the light of promising anticancer effects of flavonoids, this study aimed to further exhibit the growth inhibitory effect of quercetin on the human ovarian epithelial carcinoma cells (OVCAR-3) and scrutinize the additive effect of hyperthermia.

## METHODS

The human ovarian epithelial carcinoma cell line OVCAR-3 (ATCC® HTB-161™) cells were purchased from the American Type Culture Collection (Manassas, USA). Quercetin (Pure) was purchased from Akcan Kimya (Turkey), fetal bovine serum, penicillin/streptomycin, dimethyl sulfoxide, and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, USA). Roswell Park Memorial Institute medium (RPMI-1640) was from GIBCO (Grand Island, USA). Oxidative Stress and Annexin-V Kits were from (Merck, Millipore, USA).

### Cell culture

OVCAR-3 cells were grown in RPMI-1640 medium containing penicillin-streptomycin (100 units/mL-100 µg/mL) and fetal bovine serum (10%) at 37°C and 5% CO<sub>2</sub> in a humidified incubator. Cells were used in the test at the

confluency level of 85%.

### Cytotoxicity

The stock solution of quercetin (in distilled water) was prepared and further diluted in a fresh complete culture medium. Quercetin concentrations ranging from 6,25-200 µM were applied to OVCAR-3 cells (5×10<sup>3</sup>/well) and incubated for 24 and 48 hours at 37°C and 5% CO<sub>2</sub> in a humidified incubator. After the incubation, MTT solution (5 mg/mL) was added to each well (20 µL) and plates were further incubated for 3 hours. After the incubation, DMSO (200 µL/well) was added to the plates and was read on an ELISA reader (HTX Synergy, BioTek, USA) at a wavelength of 564 nm (n = 3). Viability percentages were obtained from the obtained absorbances. The IC<sub>50</sub> concentrations were found from the viability percentages and used for further tests.

### Hyperthermia

The test was realized to test the influence of temperature on the viability of cells exposed to quercetin. For this manner, to imitate the Hyperthermic Intraperitoneal Chemotherapy (HIPEC) clinical conditions OVCAR-3 cells were incubated at hyperthermia with different concentrations (6,25-200 µM) of quercetin for 1 hour. After the incubation period, media was aspirated and standard culture media for each cell line were added to the plates (200 µL/well) and plates were further incubated at 37°C and 5% CO<sub>2</sub> in a humidified incubator for 24 and 48 hours. At the end of the incubation period, the MTT procedure was applied and viability percentages were calculated and recorded.

### Morphological changes of OVCAR-3 cells

The morphological changes caused by quercetin on OVCAR-3 cells were analyzed under a Leica ICS-SP5 II confocal microscope supplemented with adequate software (Leica Confocal Software Version 2.00, Leica, Germany). Here, OVCAR-3

cells were plated on coverslips (5x10<sup>5</sup> cells/well) and exposed to the IC<sub>50</sub> value of quercetin at 37 °C under adequate culture conditions for 48 hours. After the incubation period, the samples were fixed in glutaraldehyde for 5 minutes at room temperature. Fixed samples were washed with PBS (1x) and stained with Alexa fluor-488 phalloidin and acridine orange for confocal microscopic imaging then imaged under a confocal microscope.

### Ultrastructural changes of OVCAR-3 cells

For detecting the ultrastructural changes, OVCAR-3 cells were exposed to IC<sub>50</sub> value of quercetin for 48 hours. All cell samples were fixed in glutaraldehyde (2.5%) and post-fixed with osmium tetroxide (2%). After the dehydration in ethanol, all samples were embedded in Epon 812 epoxy and polymerized for 48 hours. The blocks were sectioned (100 nm) by an ultramicrotome (Leica EMUC6) and placed in copper grids for staining with uranyl acetate and lead citrate per 10 minutes at room temperature. Stained cells were imaged using a TEM (FEI Tecnai BioTWIN).

### Apoptotic profile of OVCAR-3 cells

OVCAR-3 cell's death mode was tested by using the annexin V test. Briefly, OVCAR-3 cells were grown (5x10<sup>5</sup>cells/well) in six-well plates. OVCAR-3 cells were incubated with IC<sub>50</sub> concentration of quercetin for 48 hours at the same culture conditions. Following the incubation period, all cell groups were trypsinized and washed in PBS. Washed cells were resuspended and 100µL of annexin-V reagent was added to the test tubes then cells were incubated for 20 minutes in the dark at room temperature (Muse® Annexin-V and Dead Cell Assay Kit). The analysis of all cell samples was performed by using Muse™ Cell Analyzer (Merck, Millipore, Hayward, California, USA).

### Reactive Oxygen Species of OVCAR-3 cells

OVCAR-3 cells were treated with quercetin for 48 hours then harvested using trypsin. Harvested cells were resuspended with 1X assay buffer. All samples were exposed to Muse® Oxidative Stress working solution at 37°C for 30 minutes. After the incubation cell samples were analyzed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, California, USA).

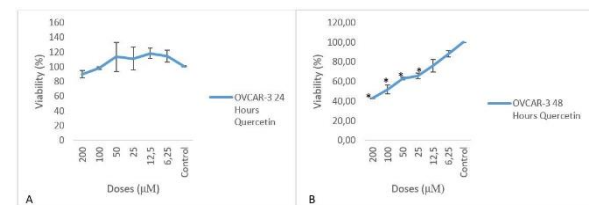
### Statistics

Statistical analysis of the data obtained from the experiments was performed on one-way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows. The results of apoptotic studies were given as mean SD values and were analyzed by ANOVA repeated measures test. The  $p < 0.05$  value of differences was considered as statistically significant.

## RESULTS

### Cytotoxicity findings

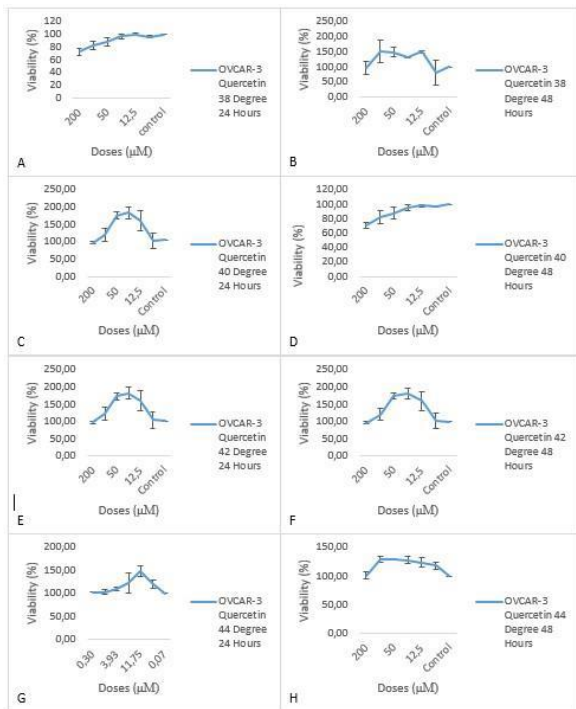
Based on MTT findings we conclude that quercetin has a cytotoxic effect on OVCAR-3 cells for the incubation period of 48 hours. IC<sub>50</sub> concentration for 48 hours on OVCAR-3 cells was calculated as 122 µM.



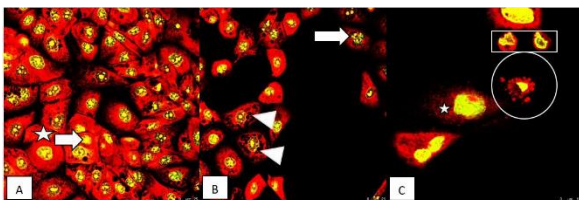
**Figure 1.** The detected significant decrease in the viability of OVCAR-3 cells is dose and time depended (Figure 1A and B).

Figure 2 displays the influence of hyperthermia on cell growth inhibition of OVCAR-3 cells. Temperature values applied to OVCAR-3 cells together with quercetin were realized to imitate original HIPEC conditions in the clinics. Based on our findings the viability of OVCAR-3 cells at the 200 µM quercetin concentrations was found to be decreased to 72% at 38°C for an incubation period of 24 hours. Moreover, at 40°C of temperature, the viability was decreased to 80%

for an incubation period of 48 hours. Other applied temperatures do not directly and effectively contribute to the cytotoxicity of quercetin on OVCAR-3 cells.

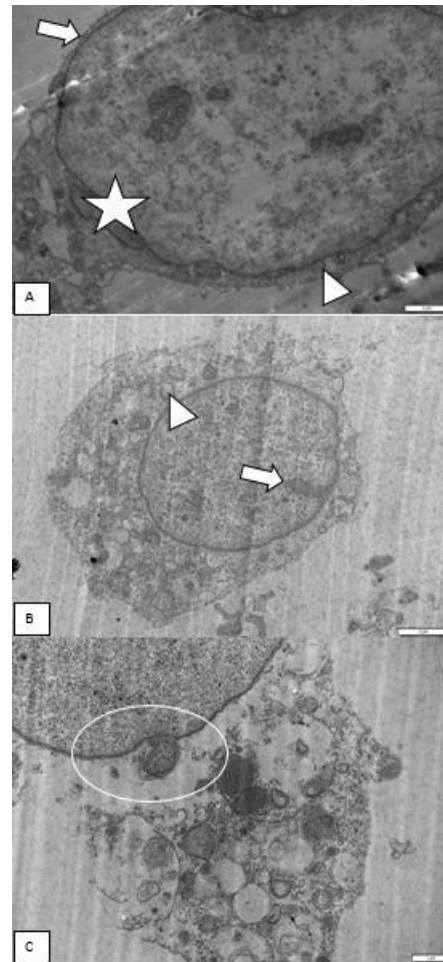


**Figure 2.** Displays the influence of hyperthermia on cell growth inhibition of OVCAR-3 cells.



**Figure 3.** Displays the morphological changes of OVCAR-3 cells.

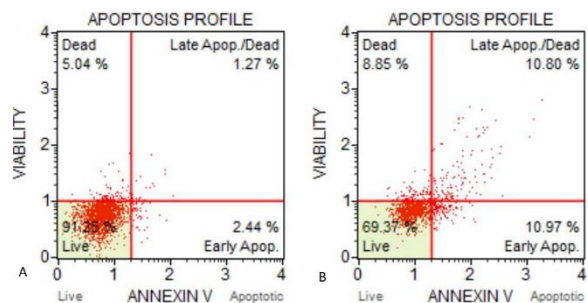
Figure 4 displays the ultrastructural changes of OVCAR-3 cells



**Figure 4.** Displays the ultrastructural changes of OVCAR-3 cells

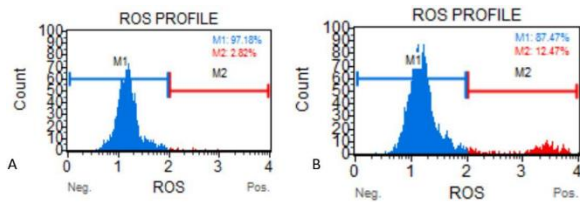
TEM findings imply the excessive mitochondrial dysfunction that is taken as a sign of promoted apoptosis by quercetin application to OVCAR-3 cells. Based on the ultrastructural changes detected on TEM as membrane blebbing, loss of cristae, and chromatin condensation it can be seen that apoptotic cell death is triggered that is irreversible.

Figure 5 displays the apoptotic profile of OVCAR-3 cells.



**Figure 5.** displays the apoptotic profile of OVCAR-3 cells

Figure 6 displays the reactive oxygen species levels of OVCAR-3 cells.



**Figure 6.** Displays the reactive oxygen species levels of OVCAR-3 cells

## DISCUSSION

Ovarian cancer is the most leading cause of death due to gynecological cancers (1). The absence of early symptoms causes the disease diagnosed at an advanced stage (2). High chemoresistance and recurrence rates resulted in low survival rates (4). Pursuit of alternative treatments leads to the use of flavonoids in combination with chemotherapeutics. Flavonoids are capable of inducing selective signaling pathways related to carcinogenesis, but the exact mechanisms of anticancer effects are controversial due to different researches (12).

Quercetin is one of the most studied flavonoids found in diverse foods and drinks of the human diet. It is a natural antioxidant with anti-inflammatory, anti-apoptotic, antioxidant, and anticancer activities (13). It is used in a variety of diseases like cancer, circulatory, and neurodegenerative disorders and also in chronic inflammatory diseases (14). American Cancer Society Guidelines declared that the key lifestyle factor for the prevention of cancer is a diet rich in plant origin compounds, so quercetin could be an attractive candidate for this (15). Its role in the treatment of breast, colon, prostate, ovary, endometrium, liver, brain, colon, and lung cancer has been shown in multiple studies (15). In the promise of this knowledge, we studied the effect of quercetin alone and also with the combination of heat on the OVCAR-3 cell line.

The anticancer effect of quercetin has been classified as long-term and short-term. The short-

term effect acts through the antioxidative and anti-apoptotic pathway by scavenging the free radicals whereby the long-term effect decreases the level of glutamate-stimulating hormones (GSH) (16).

In a study, the anti-tumor activity of quercetin was substantiated by the induction of apoptosis with activation of proteases which are essential in programmed cell death development-caspase-3 and caspase-9 (17).

Quercetin also inhibited the growth of human ovarian carcinoma OVCAR-5 cells (IC<sub>50</sub> for growth inhibition=63  $\mu$ M; LC<sub>50</sub> for cytotoxicity=17  $\mu$ M) and A2780S ovarian cancer cells in a dose-dependent manner (18,19). We found the IC<sub>50</sub> concentration on OVCAR-3 cells was 122  $\mu$ M and exhibited morphological changes on cells after 48 hours of application. The effectiveness of quercetin was also proved by TEM findings of apoptosis with ROS levels analysis and Annexin V test results. Our TEM findings that imply apoptosis in quercetin-treated OVCAR-3 cells are also supported with our ROS levels analysis. Moreover, annexin V results strongly supported our confocal and transmission electron microscopy findings that morphologically and ultrastructurally exert apoptotic sparks.

Our results showed that quercetin induced apoptosis significantly in OVCAR-3 cells by applied IC<sub>50</sub> concentration (Figure 5). Similarly Yi et al found the proapoptotic activity of quercetin on OVCAR-3 cells to be  $11.99 \pm 2.33\%$  by application of IC<sub>50</sub> value of  $153 \mu$ M (20). Another study reported statistically significant early apoptotic cell population in wild type 2008 and C13 cells by exposure to  $0.5 \mu$ M of curcumin and platinum drugs for 24 and 48 h (21).

The role of quercetin in inducing apoptosis in ovarian cancer cells has been revealed by several studies (22). Gao et al. indicated that quercetin induced apoptosis in ovarian cancer cells via the mitochondrial pathway. It is also reported that it

can inhibit the proliferation of human ovarian cancer cells in a dose-dependent manner (23). Wang et al. showed that quercetin had cytotoxic/anti-proliferative properties against ovarian cancer cells (24).

Another impact of quercetin on ovarian cancer cells is to amplify the efficacy of chemotherapeutic agents (25). Chemotherapeutic drugs, especially cisplatin, play a critical role in the treatment of ovarian cancer. However, resistance to these drugs comes out as the main obstacle during convalesce period. Cytotoxic potential of quercetin in ovarian cancer cells: SCOV-3, EFO27, OVCAR-3, A2780P was wrought and determined a significant increase in the sensitivity to cisplatin and paclitaxel (with IC50 values for OVCAR-3 cells=42 µM; for EFO27 cells=59 µM; for A2780P cells=70 µM; SCOV-3 cells=90 µM). Hence, results from this study revealed that flavonoids could overcome the chemoresistance of ovarian cancer cells to some cytostatics (18).

Cytotoxic effects of quercetin include arresting the cancer cell cycle and promoting the apoptosis pathway in the A431 cell line and cisplatin (CDDP)-resistant (C13 and A431Pt) cells. Moreover, ROS production was also increased in both cell lines (26).

Regional hyperthermia, used for the treatment of OC, caused protein denaturation and irreversible DNA damage. It also increases the antitumor effectivity of the drugs like cisplatin and mitomycin C. Most cancer institutes apply HIPEC with the surgery simultaneously (27).

Despite this, some studies found out no effect of hyperthermia as we did (9). According to our findings, the effect of temperature on the viability of cancer cells is not significant and may vary between applied chemotherapeutics or anticancer agents as well as cell lie characteristics.

We found out that the effect of temperature

increment was insignificant statistically.

As a result, to minimize the side effects and resistance to chemotherapeutics, there is a need to develop innovative drugs which are more effective and tolerable to patients. Apoptosis induction is regarded as a new and promising biochemical target for cancer treatment so there is a need for further and more accurate researches to be confirmed for the usage of quercetin in the treatment of OC. Based on the all experiment results of the manuscript, we can conclude that quercetin has good potential to be a candidate for an alternative agent in cancer therapy and can be offered for further investigations on its deeper mechanisms of action and theranostic capability.

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**Informed Consent:** Written consent was obtained from the participants.

**Conflict of Interest:** Authors declared no conflict of interest.

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