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Enhancing Cisplatin Sensitivity In Skov-3 Through The Antiproliferative Effects Of Thymoquinone

Shivani S. Tendulkar[1], Aishwarya Hattiholi[2], Mehul A. Shah[3], Suneel Dodamani[4]*

[1, 4]*Dr. Prabhakar Kore Basic Science Research Centre, KLE Academy of Higher Education and Research, Belagavi, India, PhD Scholar, M.Sc - ORCID ID: https://orcid.org/0000-0002-3936-6873[1], https://orcid.org/0000-0002-7463-8037[2]
[2]Maratha Mandal's Central Research Laboratory NGH, Bauxite Road, Belagavi, India, ORCID ID: https://orcid.org/0000-0003-0533-0350
[3]Department of Public Health Dentistry, KLE VK Institute of Dental Sciences, KLE Academy of Higher Education and Research, Belagavi, India, ORCID ID: https://orcid.org/0000-0003-3291-8716

***Corresponding Author: -** Dr. Suneel Dodamani
Basic Science Research Centre, KLE Academy of Higher Education and Research, Belagavi 590010, India.
Contact number: +919901943638, E-mail ID: suneelddmn18@gmail.co

ABSTRACT

Background: The challenges posed by ovarian cancer (OvCa) malignancy are compounded by the unfavourable prognosis linked to resistance against platinum-based drugs. The use of phytochemicals has emerged as an innovative strategy for treating OvCa. Thymoquinone (TQ), recognized for its ability to inhibit cell proliferation, has shown promising potential in targeting multiple pathways that can potentially trigger apoptosis. This study seeks to enhance the effectiveness of TQ in overcoming drug resistance induced by Cisplatin (CDDP) in SKOV-3 cells.

Methods: The cells were subjected to a time-dependent treatment with progressively higher doses of TQ. After obtaining the IC_{50} value, the gene expression for PI3KCA/B, RAD51 and BRCA1/2 was analyzed by reverse transcription polymerase chain reaction (RT-PCR).

Results: The study evaluated the effectiveness of TQ in overcoming CDDP-induced drug resistance in SKOV-3 cell lines. The minimum inhibitory concentration for CDDP in SKOV-3 cells was found to be 3 µM, while for TQ, it was 14 µM. In the case of CDDP-resistant SKOV-3 cells, the minimum inhibitory concentration was 6 µM for CDDP and 14 µM for TQ. The results revealed that TQ treatment reduces the expression of PIK3CA/B, RAD51 and BRCA1/2 in the cell lines.

Conclusion: TQ exhibits an enhanced apoptotic effect in SKOV-3 cells and demonstrates efficacy against CDDP-resistant cells. These findings highlight the significant potential of TQ in the treatment of recurrent OvCa cases that have developed resistance to CDDP.

INTRODUCTION

One of the primary causes of mortality worldwide is cancer, estimating 18.1 million deaths and 9.6 million new cases. OvCa is the second most common and lethal gynaecological malignancy, often diagnosed at progressive stages. Signs and symptoms generally occur at advanced stages such as III and IV (Bray, Ferlay, and Soerjomataram 2018). There are different types of reported OvCa, with epithelial OvCa being the most prevalent one with a poor prognosis (Stewart, Ralyea, and Lockwood 2019). The symptoms and signs of OvCa are indistinct, unclear, and vague. The risk factors include age, menstrual period, hormonal and infertility treatment, family history, genetic mutations, obesity, and socioeconomic status (Momenimovahed et al. 2019). After a sequence of platinum-based treatment or radiation, the majority of women experience a relapse of tumour or resistance to the drug and hence it is essential to develop innovative therapeutic approaches (Kumar, Kushwaha, and Gupta 2019).

Histopathological and clinical features determine the stages of ovarian cancer (OvCa) and are associated with mutations and gene expression patterns that contribute to its malignant transformation. Genes like PI3KCA/B, RAD51, and BRCA1/2 play a role in malignancy across various cancer types. Mutation in BRCA1/2 tumour suppressor genes can lead to resistance to cisplatin (CDDP) in OvCa cells. Overexpression of RAD51, involved in DNA repair, contributes to acquired drug resistance in malignancies. RAD51 levels affect responsiveness to CDDP, carboplatin, paclitaxel, docetaxel, and PARP inhibitors. BRCA1/2 and RAD51 mutations in OvCa disrupt genomic stability. The PI3K/AKT/PTEN/mTOR pathway is pivotal in OvCa, promoting cell proliferation, tumorigenesis, and drug resistance(Feng et al. 2021; Ghoneum and Said 2019a).

Phytochemicals, plant-based compounds, have shown anti-carcinogenic effects in various cancers, including OvCa, cervical, and endometrial cancer. They inhibit early-stage carcinogenesis and have chemo-preventive properties(Woźniak et al. 2021). TQ, abundantly present in black cumin seeds, exhibits various beneficial properties such as anti-cancer, anti-oxidant, anti-inflammatory, anti-microbial, anti-histaminic, and analgesic effects. It exerts its anti-cancer effects by reducing anti-apoptotic genes and increasing the production of reactive oxygen species (ROS), leading to apoptosis induction. TQ also inhibits cell migration and metastasis by targeting multiple phosphorylated pathways, increasing the expression of tumor suppressor genes, and preventing DNA methylation. When combined with CDDP, TQ enhances the inhibition of SKOV-3 cell proliferation by up-regulating Bax and down-regulating Bcl-2(Liu et al. 2017). TQ possesses diverse pharmacological properties that make it a promising intervention for cancer treatment. It directly targets phosphorylated pathways and interferes with up-regulated pathways involved in cell survival, such as PI3K, mTOR, PTEN, and AKT. TQ has demonstrated effectiveness in combination therapy with chemotherapeutic agents, counteracting proliferation, angiogenesis, migration, and metastasis. As a novel drug for OvCa, TQ offers improved safety and efficacy by providing cryoprotection in healthy cells(Mostofa et al. 2017).

MATERIALS AND METHODS

Chemicals and reagents

Cisplatin and Thymoquinone (Sigma Aldrich), Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat. No.-11965092), Antibiotic –Antimycotic (100X) solution (Thermofisher Scientific, Cat. No.-15240062), and Fetal bovine serum (FBS) (Gibco, Cat No.-10270106), MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich). Dimethyl sulfoxide (DMSO) and paraformaldehyde were procured from Qualigens, India.

Methodology

Cell culture

The human ovarian cell line SKOV-3 was acquired from NCCS, Pune, India. SKOV-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. The cells were maintained in a CO_2 incubator at 37°C (New Brunswick Galaxy 170R, Eppendorf India Private Ltd., India).

Cell viability assay

The cells were grown in the culture medium until they reached 80% confluency for cell viability. After washing the cells with phosphate-buffered saline (PBS), they were detached using trypsin-EDTA and counted using a hemocytometer. Next, 1X102 cells were seeded in a 96-well plate and allowed to incubate for 24 hours. Subsequently, the cells were treated with varying concentrations of CDDP and TQ for 24 and 48 hours. After the incubation period, the cells were suspended in 100µl of MTT solution and incubated at 37°C for 3 hours. The formazan crystals formed were dissolved in 100µl of dimethyl sulfoxide (DMSO). The optical density (OD) of the solution was measured at 490nm using a Lisa plus Microtitre Reader. Untreated cells were used as a control group. The cell viability percentage for each group was calculated by comparing

it with the control group. The inhibitory concentration (IC50), which represents the concentration at which 50% of cell death occurred, was determined for each drug. Statistical analysis was performed using GraphPad Prism 5.1 software. We also developed CDDP resistant cell line and used it for MTT and RT-PCR (referredto as R_SKOV_3)

Formula:

Surviving cells (%) = Mean OD of sample /Mean OD of Negative control ×100

RNA extraction

The cells were seeded in a 6-well plate at a density of $1X10^6$ cells and incubated at 37°C with 5% CO_2. After 24 hours, the cells were treated with different concentrations of TQ. Following the treatment, the cells were collected by trypsinization and resuspended in 200-300µl of TRIzol reagent (Invitrogen). The cell suspension was incubated for 5 minutes at room temperature for complete dissociation and then transferred to 1.5 ml Eppendorf tubes. The tubes were centrifuged at 12,000 rpm, and the upper layer was carefully collected using an Eppendorf centrifuge 5810 R. The collected RNA pellet was subjected to quantification using a Biophotometer (Eppendorf BioPhotometer plus)(Rio et al. 2010).

Real-time PCR (RT-PCR)

The cells were plated in a 6-well plate at a density of 1X106 cells and maintained in a 37°C incubator with 5% CO_2. After 24 hours, the cells were treated with varying concentrations of TQ. Following the treatment, the cells were harvested by trypsinization and suspended in 200-300µl of TRIzol reagent (Invitrogen). The cell suspension was incubated at room temperature for 5 minutes to ensure complete dissociation and then transferred to 1.5 ml Eppendorf tubes. The tubes were centrifuged at 12,000 rpm using an Eppendorf centrifuge 5810 R, and the supernatant was carefully collected. The collected RNA pellet was quantified using a Biophotometer (Eppendorf BioPhotometer plus)(Kugaji et al. 2019a).

No.	Primer name	Sequence 5' to 3'	Length
1.	PI3KCA-F	GGTTGTCTGTCAATCGGTGACTGT	24
	PI3KCA-R	GAACTGCAGTGCACCTTTCAAGC	23
2.	PI3KCB-F	TTGTCTGTCACACTTCTGTAGTT	23
	PI3KCB-R	AACAGTTCCCATTGGATTCAACA	23
3.	RAD51-F	TCTCTTCCCATTGCACACCTT	21
	RAD51-R	ACCTGGAAGCTTTCCTAACTAGAG	24
4.	BRCA1-F	TGAATGACTGCCTTGGGTCC	20
	BRCA1-R	AGGTGATTTCAATTCCTGTGCT	22
5.	BRCA2-F	CCCTTCTTTGGGTGTTTTATGCT	23
	BRCA2-R	CCTTCCTGTGATGGCCAGAG	20
6.	β-actin- F	GCCCTGGCACCCAGCACAAT	20
	β-actin- R	GGAGGGGCCGGACTCGTCAT	20

Table 1: Primer sequence of PI3KCA/B, RAD51, BRCA1/2 and β-actin.

Statistical analysis

Statistical analysis was performed to evaluate the significance of cell viability changes over time. The results indicated a significant difference in viability between 24 hours and 48 hours ($p < 0.0001$). However, no significant changes were observed when comparing data between 48 hours and 72 hours. All experiments mentioned above were conducted in triplicates, and statistical analysis was performed using GraphPad Prism 5.1.

RESULTS

TQ stimulates anti-proliferative effects in SKOV-3 and R_SKOV-3 cells

The objective of this study was to investigate the effects of CDDP and TQ on SKOV-3 and R_SKOV-3 cells(Almosa et al. 2020). The MTT assay was performed in a time and concentration-dependent manner in both cell lines (Fig. 1). The IC50 values for CDDP were determined to be 2 µM and 3 µM for 24 hours and 48 hours, respectively. Similarly, the IC50 values for TQ were found to be 16 µM and 14 µM. For further experiments, the IC50 values obtained at 48 hours were used. CDDP resistance was induced in SKOV-3 cells by exposing them to increasing concentrations of CDDP (Fig. 2). The IC50 value of CDDP after resistance development was found to be 6 µM, while TQ at a concentration of 14 µM effectively inhibited 50% of the R_SKOV-3 cells. Subsequent assays were conducted in both SKOV-3 and R_SKOV-3 cell lines to evaluate the gene expressions.

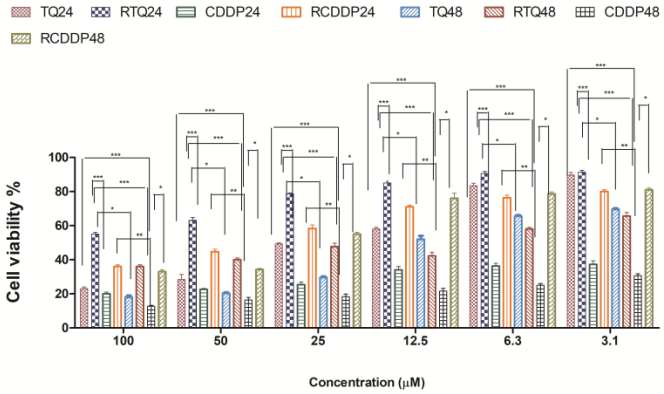

Fig. 1: Illustration of various concentrations of CDDP and TQ treatment for 24 and 48h of cytotoxicity in SKOV-3 and R_SKOV-3.

(TQ24 = Treatment of SKOV-3 cells with TQ for 24h; CIS24 = Treatment of SKOV-3 cells with CDDP for 24h; TQ48 = Treatment of SKOV-3 cells with TQ for 48h; CIS48 = Treatment of SKOV-3 cells with CDDP for 48h; RTQ24 = Treatment of CDDP-R_SKOV-3 cells with TQ for 24h; RCIS24 = Treatment of CDDP-R_SKOV-3 cells with CDDP for 24h; RTQ48 = Treatment of CDDP- R_SKOV-3 cells with TQ for 48h; RCIS48 = Treatment of CDDP- R_SKOV-3 cells with CDDP for 48h)

TQ-mediated gene expression changes in SKOV-3

After treatment with TQ, alterations in the expression levels of PI3KCA/B, RAD51, and BRCA1/2 were observed. In PIK3CA, the mean fold after TQ treatment decreased when compared to the control andCDDP. Similar effects were observed in PIK3CB, RAD51 and BRCA1. Correspondingly, for BRCA2 the mean fold changereduced the highest in SKOV_3 as compared to control and R_SKOV-3.

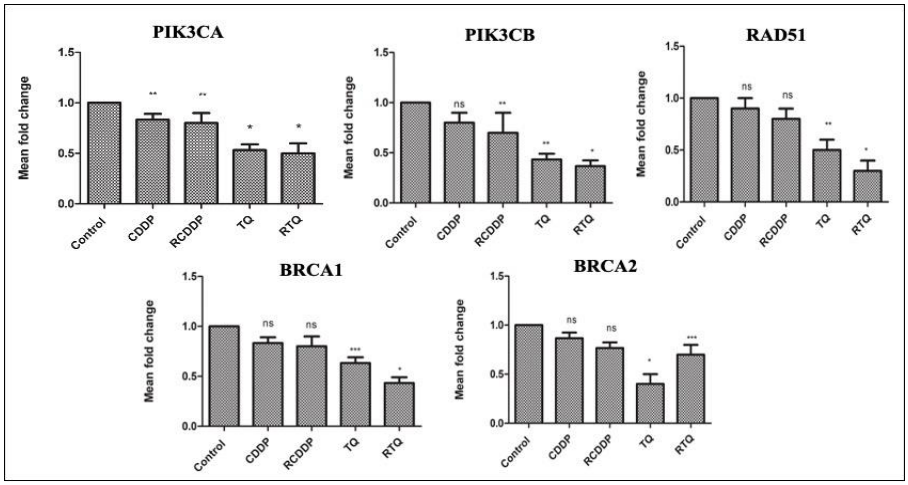

Fig. 2: RT-PCR was used to analyze the expression levels of PI3KCA/B, RAD51, and BRCA1/2 genes in two cell lines, SKOV-3 and R_SKOV-3. The results are presented as mean ± standard deviation (S.D.) of the fold change in gene expression. The data represent the average ± SD of three independent experiments. Statistical significance $p < 0.05$, and non-significant differences were observed between control cells and treated cells.

DISCUSSION

There has been limited progress in improving the survival rates of OvCa patients, and the outcomes of targeted therapies have been unfavourable. Conventional treatments often fail due to the development of drug resistance, and the recurrence of tumours is common in women with OvCa. During recurrence, the focus of treatment shifts towards managing patient symptoms, controlling disease progression, and maintaining the quality of life(Lheureux, Braunstein, and Oza 2019). Phytochemicals (PCs) have demonstrated chemo-protective effects in various types of cancer, and they can be administered at lower doses with moderate toxicity. These compounds sensitize cancer cells to chemotherapy by limiting proliferation and inhibiting metastasis. Among the PCs, TQ has exhibited anti-cancer activity through multiple mechanisms, such as promoting apoptosis by regulating the balance between Bax and Bcl-2 proteins, increasing interferon levels, inhibiting phosphorylated STAT3, and reducing JAK2 activity(Choudhari et al. 2020).

To determine the anti-cancer effects of potential compounds, it is important to evaluate their ability to inhibit cell proliferation. The MTT assay is commonly used for this purpose, as it allows viable cells to convert the MTT reagent into a formazan product, which can be detected at a specific wavelength, usually 570nm. In this study, a time-dependent MTT assay was conducted to examine the cytotoxic effects of TQ at two-time points: 24 hours and 48 hours.

Furthermore, we investigated the expression levels of key genes that have been implicated in the development and advancement of OvCa. The impact of TQ on the expression of these genes was observed, providing valuable insights into potentially significant pathways. These findings warrant further investigation to gain a deeper understanding of their significance and potential implications in OvCa progression. The genes PI3KCA/B play a crucial role in the initiation and progression of diverse types of cancer, including OvCa(Ghoneum and Said 2019a; Zhao et al. 2018a). Increased copy numbers of PIK3CA/B have been observed in ovarian cancer, contributing to the activation of cancer-promoting pathways. These genes aid in the proliferation of cancer cells and enable them to evade apoptosis. Interestingly, in this study, it was noted that TQ induces apoptosis in SKOV-3 and R_SKOV-3 cells without downregulating PIK3CA/B expression. This suggests that TQ induces apoptosis in a manner independent of PI3K signalling. The expression levels of RAD51 provide insights into the development of resistance mechanisms(Ghoneum and Said 2019b; Zhao et al. 2018b).

RAD51 is upregulated in OvCa and serves as a marker for malignant behaviour. Given its significance as an oncogene in ovarian cancer, it has been targeted for therapeutic interventions, with ongoing studies investigating various inhibitors. Additionally, RAD51's involvement in DNA repair mechanisms contributes to drug resistance in cancer cells, making it a valuable indicator of drug responsiveness. Notably, RAD51 directly interacts with the BRCA1/2 genes to facilitate DNA replication and repair processes(Golmard et al. 2017; Wang et al. 2015).Despite previous suggestions that TQ indirectly downregulates the expression of RAD51, our study did not find substantial evidence to support this claim. However, our findings demonstrate that RAD51 expression is indeed decreased in ovarian cancer cells following treatment with TQ. This highlights the potential sensitizing effect of TQ on ovarian cancer cells, as reported in other studies, by diminishing the repair and replication capabilities of these cancer cells. Nevertheless, it is important to note that certain studies have indicated a suppressive impact of TQ on the regulation of BRCA1/2 genes(Kugaji et al. 2019b; Linjawi et al. 2015).

In this particular study, the cells treated with TQ exhibited a decrease in the expression levels of BRCA1/2. This observation suggests that TQ plays a role in effectively targeting and exhibiting anti-cancer activities in both ovarian cancer cells and their resistant counterparts. Further investigations will be conducted to explore the specific expressions induced by TQ in resistant cells, thus expanding our understanding of its potential mechanisms of action in overcoming resistance.

CONCLUSION

The identification and characterization of various types of OvCa have provided opportunities for innovative therapies and their translation into clinical practice. In rare forms of ovarian cancer, establishing a robust network that bridges the gap between research and medical care is crucial for enhancing patient outcomes and quality of life. Plant-based compounds (PCs) have shown promise in this regard, as they exhibit dual roles at optimal doses with minimal side effects. However, they may also present certain limitations, including inconsistent metabolism, challenges in targeted delivery, and shortcomings in pharmacokinetics and pharmacodynamics.

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Conflict of Interests

The authors declare no conflicts of interest.

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