

RESEARCH / ARAŞTIRMA

Potential Therapeutic Synergy of Thymoquinone and Nivolumab in Experimental Models of Renal Cancer

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ABSTRACT

Objective: We investigated the effects of Thymoquinone in combination with Nivolumab on experimental renal cell cancer models. Thymoquinone, a bioactive component extracted from *Nigella sativa* seeds, exhibits anti-inflammatory, antioxidant, and anti-tumor properties against various cancers.

Material and Methods: The effects of Thymoquinone and Nivolumab were analyzed using the Renca cell line with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide and Annexin V + propidium iodide assays. Renca cells were subcutaneously injected into 8-week-old male C57BL/6 mice. Groups were designed as control, Thymoquinone, Nivolumab, Thymoquinone+Nivolumab. Tumor tissues were analyzed for apoptosis/necrosis rates, expression of immunomarkers, and ribonucleic acid expression at a significance level of $p < 0.05$.

Results: The results showed that Thymoquinone had anti-tumor effects both *in vitro* and *in vivo*. The combination treatment with Nivolumab led to an increase in immune-mediated tumor cell death, resulting in significant necrosis or complete response to therapy in the tumor tissue.

Conclusion: Thymoquinone has been found to have an anti-tumor effect on renal cell carcinoma, and Nivolumab has been shown to enhance the impact of immune cell apoptosis. Additionally, there are no side effects on other organs when used alone or in combination.

Keywords: Immune checkpoint inhibitors, renal cell cancer, thymoquinone.

Deneyisel Renal Kanseri Modelinde Thymoquinone ve Nivolumab'ın Potansiyel Sinerjistik Etkisi

ÖZET

Amaç: Thymoquinone ve Nivolumab kombinasyonunun deneyisel böbrek hücre kanseri modelleri üzerindeki etkilerini araştırdık. Thymoquinone, *Nigella sativa* tohumlarından çıkarılan biyolojik olarak aktif bir bileşiktir, Thymoquinone 'nun çeşitli kanserlere karşı anti-inflamatuar, antioksidan ve anti-tümör özellikleri vardır.

Gereç ve Yöntem: Thymoquinone ve Nivolumab'ın etkisi, Renca hücre hattı kullanılarak 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromür ve Annexin-V + propidium iodide testleri ile analiz edildi. Renca hücreleri 8 haftalık erkek C57BL/6 farelerine subkutan olarak enjekte edildi. Gruplar kontrol, Thymoquinone, Nivolumab, Thymoquinone +Nivolumab olarak tasarlandı. Tümör dokuları, $p < 0,05$ anlamlılık düzeyinde apoptoz/nekroz oranları, immünmarkerlerin ekspresyonu ve ribonükleik asit ekspresyonu açısından analiz edildi.

Bulgular: Bulgularımız, Thymoquinone 'nun hem *in vitro* hem de *in vivo* olarak anti-tümör etkileri olduğunu gösterdi. Nivolumab ile kombinasyon tedavisi, immün aracılı tümör hücre ölümü artışına yol açarak tümör dokusunda önemli nekroz veya tedaviye tam yanıt ile sonuçlandı.

Sonuç: Thymoquinone'un böbrek hücreli karsinom üzerinde anti-tümör etkisi olduğu ve Nivolumab'ın bağışıklık hücresi apoptozunu artırdığı sonucuna varılmıştır. Ayrıca, tek başına veya kombinasyon halinde kullanıldığında diğer organlarda yan etkisi yoktur.

Anahtar Kelimeler: Bağışıklık kontrol noktası inhibitörleri, böbrek hücreli kanseri, thymoquinone.

1. Introduction

Kidney cancer is a prevalent malignancy, with over 430.000 new cases reported globally in 2020, leading to approximately 180.000 deaths (1). The five-year survival rate of patients with kidney cancer has significantly increased over the past 60 years, from 34% in 1954 to 75% from 2009 to 2015 (2). Risk factors for renal cell carcinoma (RCC) include smoking, hypertension, and

obesity, as well as chronic kidney disease and acquired cystic kidney disease (3–5). RCC occurs in approximately 6% of chronic dialysis patients (6,7). The appropriate treatment for RCC is determined by staging, using the American Joint Cancer Committee (AJCC) tumor-node-metastasis (TNM) classification system (8).

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Thymoquinone (TQ), a phytochemical compound found in the plant *Nigella sativa*, has been used as a natural treatment for various diseases for centuries, despite a lack of scientific basis. TQ has been shown to have anti-proliferative effects on lung, breast, pancreatic, and colorectal cancer (9–11).

It can affect the cell cycle and induce apoptosis in both p53-dependent and p53-independent ways, and it has been shown to affect mechanisms of metastasis and angiogenesis (12,13). TQ's efficacy has been demonstrated as a chemopreventive and chemotherapeutic agent *in vivo* (14).

Tyrosine Kinase Inhibitors (TKIs) have not been very effective in the adjuvant treatment of RCC. A phase III study of KEYNOTE-564 showed that using adjuvant pembrolizumab for one year provides a disease-free survival advantage in patients with intermediate-high to high-risk tumor recurrence, leading to its Food and Drug Administration (FDA) approval for the adjuvant treatment of RCC (15,16). Nivolumab (NV) has also shown improvement in overall survival (OS) for patients who progress after first-line anti-angiogenic therapy (17,18). Anti-angiogenic therapies, along with combined immunotherapy, are commonly accepted treatments for advanced or metastatic RCC. Further research is needed to determine the role of anti-Vascular Endothelial Growth Factor (VEGF) /immunotherapy combinations versus other combinations such as NV and Ipilimumab (19).

2. Material and Method

2.1. Cell Culture

The Renca cell line, which models renal cell carcinoma, was used for the cancer cell line. The cells were cultured in RPMI 1640 medium (Cegrogen), along with 20% fetal bovine serum (Cegrogen), 1% penicillin-streptomycin (100 U/mL) (Cegrogen), and 1% L-glutamine (200 mM) (Cegrogen). The cells were kept in a 37°C incubator with 5% CO₂ and 95% air atmosphere. The cells were subcultured every two days.

2.2. MTT Viability Assay

The viability of Renca cells was assessed using MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide) assay (AppliChem). Six replicates of 200 µL of Renca cells (10⁴ cells per well) were seeded in 96-well plates. TQ was tested at concentrations of 5 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM, and NV was tested at concentrations of 1 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM, 160 µM, and 200 µM. After 24, 48, and 72 hours, 10 µL of MTT reagent was added to the cells. The plates were incubated for four hours at 37°C with 5% CO₂. After that, 50 µL of the medium was removed and 50 µL of Dimethylsulfoxide (DMSO) was added to precipitate formazan crystals. The plates were incubated for 15 minutes at 37°C with 5% CO₂, and the absorbance values were measured at 570 nm using an ELISA plate reader (Thermo, Multiskan). The viability was expressed as a proportion of the control.

2.3. In-Vivo Experiments

The study utilized C57/BL6 mice. The Renca cell line (ATCC: CRL-2947) was selected to establish a renal cell cancer model. 1x10⁷ Renca cells were combined with a matrix and injected subcutaneously, resulting in tumor development after roughly 40 days. Twenty-eight mice were randomly assigned to four groups. They were granted unrestricted access to food and water. The mice were maintained at room temperature (20±20°C) and subjected to a 12-hour light/dark cycle throughout the study duration.

The animals received daily observation. Following the onset of tumor formation, the tumor size was assessed regularly. Tumors with a diameter of 8 mm were treated. Agents were administered intraperitoneally (IP) with 0.1 mL of physiological saline (PS),

either singularly or in combination, or assigned to the control group.

The groups were control, TQ (20 mg/kg IP for 1, 4, 7, and 14 days), NV (10 mg/kg IP for 1st, 6th, and 13th days), and a combination of TQ and NV. NV applied IP on the 1st, 6th, 10th, and 13th days. This dose was adequate based on previous reports from the European Medicines Agency (EMA) in April 2015. TQ was dissolved in powdered DMSO (3.5 g in 2 mL DMSO) and subsequently diluted with saline solution (SS). A DMSO concentration of less than 0.01% was used in the TQ applied to the animal. The animals treated with agents were sacrificed at the end of the experimental period. Cardiac blood was collected during the sacrifice. The internal organs and tumor tissue were removed and preserved in 10% formalin. Following tumor excision, a portion of the tissue was fixed in 10% formalin for histopathological evaluation. The remaining tumor samples were preserved at -80°C in a cell culture medium supplemented with 1% DMSO to maintain cellular integrity for apoptotic assessment and further analyses. Molecular analyses were immediately performed.

2.4. Apoptosis Detection

The lethal dose (LD₅₀) values of TQ and NV were determined as 100 µM and 200 µM, respectively, after 72 hours of incubation *in vitro*. Also, these doses were used in the *in vivo* studies. The apoptosis/necrosis ratios of tumor tissues excised from mice were determined. For this, the following steps were briefly followed: the collected tumor tissues were homogenized to form a single cell suspension. The cells were washed twice with 500 mL of Phosphate Buffer Saline (PBS), centrifuged, and the supernatant was discarded. After centrifugation, the cells were resuspended in 100 µL of Annexin Binding Buffer (1X), and 5 µL each of Annexin V- Fluorescein Isocyanate (FITC) and Propidium Iodide (PI) were added to the cells. 15-minute incubation in the dark at 4°C was carried out according to the protocol, and then 400 µL of Annexin-V binding buffer was added to each tube. Flow cytometry (BD Accuri) was used for analysis, with 488 nm excitation and 530 nm emission for Annexin-V, and 488 nm excitation and 650 nm emission for PI, evaluated using BD Accuri C6 software.

2.5. Histopathological Examination

Following histopathological follow-up, tumor samples were fixed in paraffin blocks and sectioned into 5-micron thin slices for histological staining. Hematoxylin-eosin staining was performed to investigate the cellular morphology and to evaluate the tumor grade. Immunohistochemical (IHC) staining was conducted on tumor tissues using primary antibodies Cluster of Differentiation (CD) 4, CD8, C20, CD34, CD68, Vascular Endothelial Growth Factor Receptor (VEGFR), Programmed Death-1 (PD1), Programmed Death Ligand-1 (PDL1), Caspase-3, and Caspase-9 to investigate immune cell activation, the tumor microenvironment, tumor vascularization, and apoptotic markers. The tissue sections were incubated with primary antibodies specific for each marker, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The signal was visualized using a 3,3'-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. The IHC results were evaluated and quantified by an experienced pathologist.

2.6. Real Time-PCR

Ribonucleic acid (RNA) extraction from animal tumor tissues was performed with the spin column technique with the High Pure RNA Isolation Kit (Roche, 11828665001). Following the measurement of the extracted RNA, it was reverse transcribed into complementary deoxyribonucleic acid (cDNA). To evaluate the expression levels of genes associated with angiogenesis and immunity in the tumor, the following genes were investigated: c-

kit, cellular MNNG-HOS Transforming gene (c-met), Intracellular Cell Adhesion Molecule (ICAM), E-selectin, Vascular Cell Adhesion Molecule (VCAM), Tyrosine kinase with Immunoglobulin-like and EGF-like domains 2 (Tie2), Axl, Fms-like tyrosine kinase (Flt3), Fibroblast Growth Factor Receptor (FGFR), Rearranged Transfection (Ret), VEGFR, Platelet Derived Growth Factor Receptor (PDGFR), CD4, CD8, C20, CD34, CD68, PD1, PDL1, Caspase-3, and Caspase-9. Quantitative RT-PCR was performed to determine the impact of the TQ and NV combination on gene expression. Expression levels were normalized to beta-actin, a universal gene, to control for variations in RNA loading and reverse transcription efficiency.

2.7. Statistical Analysis

The data were analyzed with SPSS software version 22.0 (SPSS, Chicago, IL). The expression levels of IHC in tumor cells and surrounding immune system cells were examined in relation to the therapeutic response rates following drug administration. The independent factors analyzed statistically included IHC examination results in tumor cells and T cells, cell viability percentages, age, gender, pathological characteristics of tumor cells, tumor size, and location. The dependent variables comprised the mean of three replicates of the cell culture condition for each drug. The independent variables were analyzed using the Mann-Whitney U test, Kruskal-Wallis test, and Chi-Squared test. Statistical analysis was not conducted for the dependent variables; only their means were calculated. The Wilcoxon test was employed to compare drug groups against their respective controls. A significance level of $p < 0.05$ was utilized in the analyses.

2.8. Ethical Aspect of the Research

The study was conducted in accordance with the ethical principles of the 1964 Helsinki Declaration and complied with these standards. All experimental procedures with animals were carried out in accordance with the Dokuz Eylül University Experimental Animal Care Regulation and were approved by the Dokuz Eylül University Ethics Committee on 16.05.2017 with approval number 22/2017.

3. Results

3.1. Cell Viability

Different doses of TQ and NV were applied to Renca cells. The cells were treated with the agents for 24, 48, and 72 hours. Figure 1 shows the effects of TQ and NV at various concentrations on the viability of Renca cells after 24, 48, and 72 hours, assessed via the MTT assay. TQ showed its strongest and most substantial cytotoxic effects at 72 hours, exhibiting a distinct dose-dependent reduction. Elevated doses (80–100 μM) lead to cell viability decreasing below 50%, indicating that the LD_{50} is attained within this interval. Lower doses (5–40 μM) sustain cell viability at approximately 60–70%. NV-treated cells showed a slight decrease in viability at elevated concentrations (160–200 μM); however, overall cytotoxicity remained moderate, with the majority of values still over 60%. This indicates that NV does not reach an LD_{50} within the evaluated time period or concentration spectrum.

3.2. In vivo Experiments

In the experiment, 1×10^7 Renca cells mixed with 0.2 mL of matrix were injected subcutaneously on the left side of C57/BL6 mice. Tumor development was observed after the second or third successive injection. All mice were divided into four groups: TQ, NV, TQ+NV, and a control group ($n=7$). Each drug was administered using the intraperitoneal (IP) treatment. Sacrifice was performed 30 days after tumor development. Accordingly, as shown in Figure 2, the tumor sizes in the TQ+NV group significantly decreased compared to the control group ($p < 0.05$).

In the group where TQ was administered alone, no significant reduction was detected in comparison to the control. A relative decrease in tumor sizes was observed in the NV group alone in comparison to the TQ group.

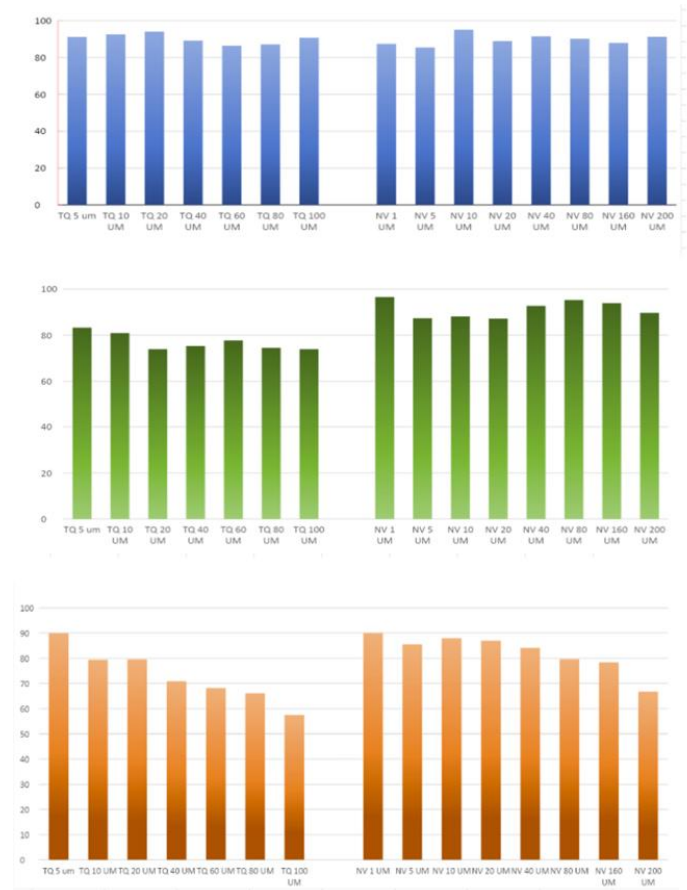


Figure 1. TQ and NV were applied at different dose ranges in the Renca cell line. Blue bars represent 24 hours, green bars 48 hours, orange bars 72 hours. The highest cytotoxic effect was seen at 72 hours. All viability tests were performed using the MTT assay.

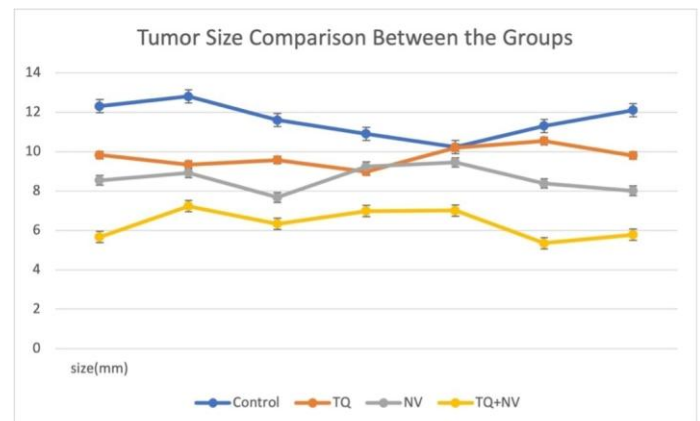


Figure 2. Tumor size comparison between the treatment groups and a control group.

3.3. Apoptotic Cell Death

Tumor samples collected from animal tissues were used to investigate apoptotic cell death. The tissues were converted into a cell suspension. The cells were stained with Annexin V dye to identify the initial phases of apoptosis. To determine necrotic cell death, cells were stained with propidium iodide (PI) dye. Table 1 shows that the TQ+NV combination group showed the highest rate of apoptotic cell death ($p < 0.05$). The TQ group had the minimal apoptotic effect. The apoptotic effect of TQ alone was not preferable to the necrotic impact found in the cells. Compared to the control group, the TQ+NV combination showed

statistical significance in inducing apoptosis in the cells. However, no statistical difference was observed in necrotic death ($p=0.8$).

Table 1. Effective dose ranges of TQ and NV, and a combination of both drugs, were used *in vivo*. Accordingly, the combination group showed the highest apoptotic effect.

	Apoptosis	Necrosis
Control	5.3%	9.6%
TQ	10.4%	12.25%
NV	18.4%	5.7%
TQ+NV	25%	14%

*Compared to the control group, the TQ+NV combination showed statistical significance with $p=0.034$.

3.4. Gene Expression Analyses

Following the conversion of RNA samples collected from *in vivo* tumor samples into cDNA, specific gene expressions were investigated using PCR. Table 2 shows the data resulting from the comparative gene analysis performed between the treatment groups. The Tie2 gene, responsible for encoding the angiopoietin receptor protein, exhibited a markedly reduced gene expression level in the TQ+NV treatment group compared to the control group ($p<0.05$). In the Axl gene, the group administered TQ+NV had a markedly reduced gene expression level relative to the control ($p<0.05$). VEGFR gene expression levels exhibited a reduction solely in the TQ-treated group relative to the control ($p<0.05$). The expression levels of the Flt-3 gene exhibited a substantial reduction solely in the group administered TQ ($p<0.05$). Likewise, FGFR levels markedly diminished in the TQ group relative to the control ($p<0.05$).

Table 2. Specific gene expression analyses were conducted on samples obtained from *in vivo* tumor tissues.

	TQ	NV	TQ+NV Combination
c-kit*	0.144	0.670	0.439
c-met*	0.386	0.465	0.606
ICAM*	0.109	0.873	0.337
E-Selectin*	0.157	0.831	0.462
VCAM*	0.439	0.337	0.631
Tie2*	0.055	0.631	0.025
Axl*	0.631	0.873	0.028
Flt3*	0.025	0.749	0.337
FGFR*	0.025	0.631	0.200
Ret*	0.109	0.200	0.273
VEGFR*	0.016	0.522	0.150
PDGFR*	0.631	0.337	0.522

3.5. Immunohistochemical Staining

The expression of CD4, CD8, CD68, and CD20 proteins was analyzed in the tumor tissues of mice treated with TQ and NV to evaluate T and B cell infiltration in the tumor microenvironment. Additionally, immunohistochemistry (IHC) staining for VEGFR was conducted to determine tumor angiogenesis. PD-1 is an inhibitory receptor that decreases T cell immunological responses. NV enhances T cell activation by inhibiting PD-1. To investigate the effects of the treatment on the immune response and demonstrate the impact of PD-1 expression on tumor growth, staining was performed using PD-1 antibodies. PD-L1 is a mechanism expressed by tumor cells and specific immune cells that inhibits T cell activity. Tumors with elevated PD-L1 expression may exhibit increased responsiveness to immunotherapies. Consequently, IHC staining was conducted to investigate the protein expression of PD-L1. To quantify the percentage of tumor cells undergoing apoptosis as a result of the treatment, staining with Caspase 3 and Caspase 9 was performed (Table 3).

Our results showed that NV, once administered alone, markedly elevated CD8+ cytotoxic T cells in the experimental group

relative to the control group (Figure 3). The identical effect was noted in the TQ+NV combination group relative to the control group ($p<0.05$). A notable reduction in the expression levels of the CD68 tumor-associated macrophage marker was detected in both the NV treatment group and the TQ+NV combination treatment group relative to the control ($p<0.05$). The expression levels of Caspase-3 and Caspase-9, indicators of apoptotic cell death, were found to be significantly elevated in the NV group compared to the control ($p<0.05$).

Table 3. Statistical significance values of IHC results of treatment groups compared to control after *in vivo* drug applications.

Biomarker	Group	Value
CD20	TQ	0.439
	NV	0.373
	TQ+NV	0.515
CD4	TQ	0.211
	NV	0.232
	TQ+NV	0.375
CD8	TQ	0.823
	NV	0.019
	TQ+NV	0.024
CD68	TQ	0.776
	NV	0.034
	TQ+NV	0.022
VEGFR	TQ	0.242
	NV	0.464
	TQ+NV	0.533
PD-1	TQ	0.356
	NV	0.794
	TQ+NV	0.372
PD-L1	TQ	0.329
	NV	0.696
	TQ+NV	0.767
CASP3	TQ	0.334
	NV	0.049
	TQ+NV	0.527
CASP9	TQ	0.432
	NV	0.018
	TQ+NV	0.286

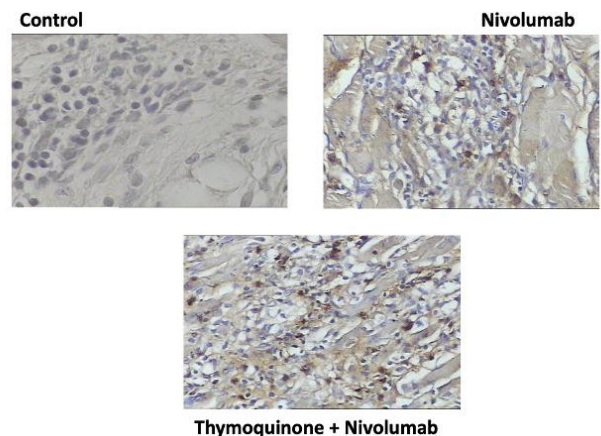


Figure 3. According to the results of immunohistochemistry staining, expression levels of CD8 cytotoxic T lymphocytes were enhanced in the NV group and the combination group.

4. Discussion

TQ's anti-inflammatory effects, regulation of apoptosis signals, inhibition of angiogenesis, and ability to halt the cell cycle have been demonstrated multiple times in both *in vitro* and *in vivo* studies to stop tumor growth (20). Chemosensitization with TQ is mostly limited to *in vitro* studies and has potential as a therapeutic strategy for cancer (21,22). The results of TQ combination studies with chemotherapy generally favor increased efficacy and therapeutic effectiveness (23). However, combination experiments of TQ with anti-PD-1 or anti-PD-L1 agents are not found in the literature. So far, TQ has been shown

to inhibit cell proliferation in many cancer cell lines, including RCC (24).

In our study, we used the Renca cell line to create the RCC model. Looking at the literature, Renca cell lines had not been previously studied with TQ. In our study, the effective dose of TQ was found to be 100 µM, while the effective dose of NV was determined to be 200 µM. In apoptosis, the highest apoptotic death rate was observed under the condition where TQ and NV were given in combination. The necrosis rate is also highest in the combination group. In the *in vivo* phase, while the tumor diameter in the control group increased by an average of 11 mm³, this rate decreased to 6.5 mm³ in the group given TQ, and to 5.1 mm³ in the TQ and NV combination group (p=0.042). Based on these data, there is a direct correlation between the highest effectiveness of apoptosis in the combination group and the reduction in tumor volumes. It has been shown that combination therapy is cytotoxic under *in vivo* conditions, particularly in accordance with cytotoxicity and apoptosis results (25). The results of our study showed that TQ has anti-cancer effects, in line with the literature (26,27). Additionally, the combination of TQ and NV resulted in an increased intra-tumor inflammatory response, a decreased tumor diameter, and an increased number of CD8 cytotoxic T lymphocytes. Moreover, the combination significantly reduced the expression of Tie2 and Axl genes, which are essential in angiogenesis in metastatic RCC.

Historically, treatment of metastatic RCC has evolved from non-specific immunotherapy treatments such as interleukin and interferon to TKI-targeted therapies (anti-VEGF), and finally to immune checkpoint inhibitors (anti-PD-1, anti-PD-L1, and anti-CTLA-4). Despite these therapeutic advances, the five-year survival rate of patients with metastatic RCC remains poor, particularly in patients with adverse prognostic factors (19). Thus, there is a need for new treatment options for metastatic RCC that have lower toxicity and are less expensive than modern chemotherapeutic agents.

Herbal-based drugs have become an attractive area of research due to their potential to provide such options. TQ has been shown to have anti-cancer and anti-inflammatory properties and to affect cell proliferation by regulating apoptosis signals, inhibiting angiogenesis, and stopping the cell cycle (28). The results of combination studies of TQ with chemotherapy have generally shown increased efficacy and therapeutic efficacy (29). In our study, we used the Renca cell line to create the RCC model, as it was necessary for the model to be immunogenic to study the combination of TQ with NV, an immunotherapy agent. Our *in vitro* results showed that TQ dose-dependently inhibited the proliferation of renal carcinoma cells and led to apoptotic death in a dose-dependent manner. The *in vivo* results showed that the tumor diameter in the combination group was the greatest among all groups. There is a correlation between the highest level of apoptosis in the combination group and the reduction of tumor volume.

While conducting *in vivo* studies of immunotherapy drugs, it is essential to select cell lines whose immunotherapy response has been defined and studied in murine solid tumor models (29). In our study, the anti-angiogenic effect of TQ potentiated the anti-tumor effect of NV, which was reflected in the significant decrease in anti-angiogenic gene expression levels in the combination therapy group. TQ has been shown to interfere with all key steps of neovascularization, from pro-angiogenic signaling to endothelial cell migration and tube formation (30, 31). Overall, our study highlights the potential of the combination of TQ and NV as a treatment option for metastatic RCC. Further studies are needed to validate these findings and investigate the potential of this combination therapy in a larger and more diverse patient population.

4.1. Limitation

While our findings support the therapeutic potential of Thymoquinone and Nivolumab in renal cell carcinoma, the study has some limitations. The use of a single cell line limits the diversity of tumor biology addressed. Additionally, although no side effects were observed, detailed long-term toxicity evaluations and comprehensive immune profiling were beyond the scope of this study. Further studies may help to expand on these promising results.

5. Conclusion and Recommendations

In conclusion, our study demonstrated the anti-cancer potential of TQ in treating renal cell carcinoma (RCC). The results showed that the combination of TQ and NV led to a significant reduction in tumor diameter, an increase in intra-tumor inflammation, and a rise in the number of CD8 cytotoxic T lymphocytes. Additionally, our findings revealed a decrease in gene expression levels of Tie2 and Axl, key pathways in angiogenesis.

Our study is the first to report the use of TQ and NV in combination in the treatment of metastatic RCC, and our results suggest that this combination has the potential to be a safe and effective therapeutic option. These findings suggest that the combination of TQ and NV may provide a promising new approach to treating RCC. This study represents the first investigation of the effects of TQ and NV combination in the treatment of RCC and offers important insights for future research. Further studies are necessary to support and elaborate on these findings, as well as to explore other possible mechanisms of action.

6. Contribution to the Field

The TQ+NV combination treatment in RCC is an experimental *in vivo* study that contributes to the current literature. The BALB/C tumor model was used to investigate the relationships at the gene and protein levels resulting from the combination of TQ and NV, aiming at bringing a more efficient and reliable substance for immunotherapy to the literature.

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None.

Conflict of Interest

The authors declare that there is no conflict of interest regarding this project.

Authorship Contribution

Concept: AD, SA; Design: AD, SA, IS; Supervision: SA, IS; Funding: BAP; Materials: BAP; Data Collection/Processing: ÖG, AE, TA, ES, EK, OY; Analysis/Interpretation: ÖG, AE, ES, EK, OY, ZSA, OY; Literature Review: IS, AD, OY, SA, ZSA; Manuscript Writing: ÖG, AD; Critical Review: SA, ZSA, IS.

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