OTJHS
Online Turkish Journal of Health Sciences

e-ISSN: 2459-1467

OTSBD
Online Türk Sağlık Bilimleri Dergisi

Online Turkish Journal of Health Sciences 2024;9(1):97-102 *Online Türk Sağlık Bilimleri Dergisi 2024;9(1):97-102*

The Effect of Thymoquinone on the Protein Levels of PLA2G7, UCP2, and NEDD4L Genes Associated with Lipid Droplets Formation in Prostate Cancer

Prostat Kanserinde Lipid Damlacık Oluşumu ile İlişkili PLA2G7, UCP2 ve NEDD4L Genlerinin Protein Seviyeleri Üzerine Timokinonun Etkisi

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ABSTRACT

Objective: Prostate cancer (PCa) patients suffer severe side effects of standard treatment beside the resistance to castration. PCa cells shows increased lipogenesis. Thymoquinone (TQ) inhibits cell proliferation, metastasis, and invasion. However, there was no study on the effect of TQ on the levels of NEDDL4, PLA2G7, and UCP2 lipid droplets (LD) related proteins. Hence, the study aims to investigate the impact of TQ on PLA2G7, UCP2, and NEDD4L proteins on DU145 and PC3 cell lines.

Materials and Methods: Cells were cultured and treated with TQ with a IC_{50} of 60 µM and 80 µM for DU145 and PC3, respectively. PLA2G7, UCP2, and NEDD4L levels were measured using the ELISA.

Results: TQ has significantly increased the level of NEDD4L ($p<0.01$ for DU145 and $p<0.001$ for PC3) and decreased the level of UCP2 proteins ($p<0.05$).

Conclusions: Our preliminary findings suggest that TQ may impact NEDD4L and UCP2, indicating a potential role in repressing LD. Further investigations are needed to confirm the efficacy of TQ and explore its potential utility as a therapeutic agent for PCa treatment.

Keywords: NEDD4L, PLA2G7, prostate cancer metabolism, thymoquinone, UCP2

ÖZ

Amaç: Prostat kanseri (PCa) hastalarına yönelik standart tedavide, kastrasyona dirençli tipin gelişmesinin yanı sıra ciddi yan etkiler de yaşanmaktadır. PCa hücrelerinin esas olarak artmış lipogenez ile karakterize olduğu bilinmektedir. Timokinonun (TQ) hücre proliferasyonunu, metastazı ve invazyonu inhibe ettiği gösterilmiştir. Ancak literatürde TQ'nun NEDDL4, PLA2G7 ve UCP2 lipit damlacığı ile ilişkili proteinlerin düzeylerine etkisine ilişkin bir çalışma bulunmamaktadır. Bu çalışmanın temel amacı, TQ'nun PLA2G7, UCP2 ve NEDD4L proteinleri üzerindeki DU145 ve PC3 hücre hatları üzerindeki etkisini araştırmaktır.

Materyal ve Metot: Hücreler çoğaldı ve DU145 ve PC3 için sırasıyla 60 µM ve 80 µM IC_{50} ile TQ ile tedavi edildi. İnkübasyondan sonra, PLA2G7, UCP2 ve NEDD4L seviyeleri ELISA yöntemi kullanılarak ölçüldü.

Bulgular: TQ, NEDD4L düzeyini önemli ölçüde artırmıştır (DU145 için $p<0,01$ ve PC3 için $p<0,001$). Ayrıca, TQ'nun UCP2 protein düzeyini azalttığı da gösterilmiştir ($p<0,05$).

Sonuç: Çalışmamızın başlangıç bulguları, TQ'nun öncelikle NEDD4L ve UCP2 üzerinde etkisi olabileceğini göstermektedir. Bu, TQ'nun LD üzerinde baskılayıcı bir etkiye sahip olabileceğini ve daha fazla araştırma ile PCa tedavisi için faydalı bir molekül olabileceğini öne sürmektedir

Anahtar Kelimeler: NEDD4L, PLA2G7, prostat kanser metabolizması, timokinon, UCP2

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Yayın Bilgisi / Article Info:
Gönderi Tarihi/ Received: 21/01/2024
Kabul Tarihi/ Accepted: 20/02/2024
Online Yayın Tarihi/ Published: 11/03/2024

Atıf / Cited: Hadjmımoune A and et al. Have The Effect of Thymoquinone on the Protein Levels of PLA2G7, UCP2, and NEDD4L Genes Associated with Lipid Droplets Formation in Prostate Cancer. *Online Türk Sağlık Bilimleri Dergisi* 2024;9(1):97-102. doi: 10.26453/otjhs.1422576

INTRODUCTION

The increasing incidence of PCa over the past century has resulted in-substantial alterations in diagnosis and therapy.[1,2] Despite these breakthroughs, total cure remains elusive, and a considerable number of men continue to suffer from castration resistance and metastasis, which often necessitate a combination of therapies.[3] Thus far, PCa therapy, mainly Androgen deprivation therapy (ADT) and Docetaxel (DTX),[4] induced side effects and the development of castration resistance underline the necessity of treatment alternatives to enhance the overall survival as well as patient's quality of life.

TQ, derived from Nigella sativa seeds, is a promising biopharmaceutical with potent anticancer properties, enhancing treatment efficacy while reducing toxicity. Its synergistic effects with existing drugs further underscore its potential in cancer therapy.[6]

Lipid metabolism rewiring enables PCa cells to grow and increase in aggressiveness and metastasise. Therefore, new means of resistance related to lipogenesis reprogramming have recently risen.[7] Subsequently, LD accumulation is positively associated with an increased Gleason score of PCa.[8] In this study, we focused on three proteins related to LD synthesis and regulation.

First, aside from the role of Phospholipase A2 (PLA2s) in LD synthesis, they may act as providers of free fatty acids, generators of metabolite that may control LD formation and direct controllers of LD.[9]

Furthermore, another study confirmed the effect of Uncoupling protein 2 (UCP2) downregulation in Promoting Phospholipid Synthesis and Increasing Expression of Lipogenic Enzymes, thus lipid accumulations and a potential promotion of LD formation.[10]

Thirdly, in PCa, NEDD4L levels decrease with progression toward malignancy, which is shown to correlate with an increased Gleason score.[11] It was also reported that E3 ligase (NEDD4 proteins family) plays a ubiquitination role of adipophilin on LD, which suggests its potential regulatory role.[12]

Targeting specific cancer therapeutics is of great interest in current cancer therapy; therefore, specific molecular targets for TQ should also be investigated. [9, 10, 12,13, 14]

Our study seeks to conduct a preliminary investigation into the impact of TQ on the protein levels of NEDD4L, PLA2G7, and UCP2, which are implicated in the formation of LD. Through this initial exploration, we aim to elucidate any potential modulatory effects of TQ on these key proteins, laying the groundwork for further comprehensive investigations.

MATERIALS AND METHODS

Ethics Committee Approval: Ethics committee approval is not required for studies to be conducted on commercially available human cadavers, cadaver parts, and other biological materials. Ethics committee approval was not needed since a commercially available cell line was used in this study.

Cell Culture: The Human Prostate Carcinoma Cell lines used in the study, PC3 and DU145, were supplied by Mugla Sıtkı Kocman University and Yeditepe University, respectively. DU145 and PC3 cell lines were cultured in a complete growth media containing % 10 FBS (Capricorn, CP17-1756), % 1 Penicillin-Streptomycin (Capricorn Scientific), % 1 L-Glutamine in DMEM (Capricorn Scientific). Our cell lines were incubated at 37 ◦C with 5% CO2.

Thymoquinone Preparation and Treatment: For this study, 14 mg of Thymoquinone powder (BLD pharm, BD233118, 2-Isopropyl-5-methylcyclohexa-2,5-diene-1,4-dione, 99%, China) was dissolved into 1 ml of DMSO to get the stock solution with a final concentration of 85 mM. This stock was later diluted in DMEM to produce concentrations of 60 µM and 80 µM to treat DU145 and PC3 cell lines, respectively.[15] After successive dilutions, the concentration of DMSO was brought down to less than 0.1 %, which is favourable since DMSO concentrations up to 1% are considered safe and non-cytotoxic.[16] To prepare cell lysates, a freeze-thaw procedure was followed by incubating the cells at -80 °C for 5 min, then at 37°C for the same period for three cycles. This process was followed by centrifugation at +4°C at 1000 rpm for 5 min, after which the supernatant was collected into sterile and labelled Eppendorf tubes.

ELISA Test: ELISA (Enzyme-Linked Immunosorbent Assay) was used to determine the levels of NEDD4L (Shanghai Coon Koon Biotech, 20522), UCP2 (Shanghai Coon Koon Biotech, 13901) and PLA2G7 (Shanghai Coon Koon Biotech, 12314) proteins in both cell types. The kits used in this study were based on sandwich ELISA, which has the advantage of high sensitivity and specificity. The measurement was made at 450 nm absorbance in the Epoch Reader Spectrophotometer device.

Statistical Analysis: GraphPad Prism software is used at this step. The testing for normality was done with the Shapiro-Wilk-normality test. Statistical differences were evaluated using a two-tailed Student's t-test. Differences at $p < 0.05$ were considered as significant.

RESULTS

The analysis of PLA2G7 protein expression revealed notable differences between the experimental groups. In PC3 cells, the expression level was measured at 2.52 ng/mL in the absence of TQ (PC3(TQ-)) and reduced to 1.98 ng/mL in the presence of TQ (PC3(TQ+)). In DU145 cells, the expression level was 2.40 ng/mL without TQ (DU145(TQ-)) and slightly lower at 2.35 ng/mL with TQ (DU145 (TQ+)). In DU145 cells, there was no significant alteration in PLA2G7 protein expression between the treated and control groups, as depicted in (Figure 1a). Conversely, in PC3 cells, a subtle yet statistically insignificant reduction in PLA2G7 protein expression was observed with TQ treatment, as illustrated in (Figure 1b). These findings underscore the differential effects of TQ on PLA2G7 protein expression in the two cell lines studied.

As illustrated in Figure 2, the expression levels of the UCP2 protein were notably distinct between the DU145 (TQ-) and DU145 (TQ+) cells, measuring 98.1 pg/mL and 16.7 pg/mL, respectively. In the PC3 cell lines, the expression levels of UCP2 were similarly varied, with 159 pg/mL in the TQ- group and 62.4 pg/mL in the TQ+ group. The analysis presented in Figure 2 reveals a significant reduction in UCP2 protein levels upon treatment with TQ in both DU145 ($p < 0.05$) (Figure 2a) and PC3 cells ($p < 0.05$) (Figure 2b). This suggests an inhibitory effect of TQ on the level of UCP2 protein, underscoring its potential as a modulator of UCP2 activity in these cell lines.

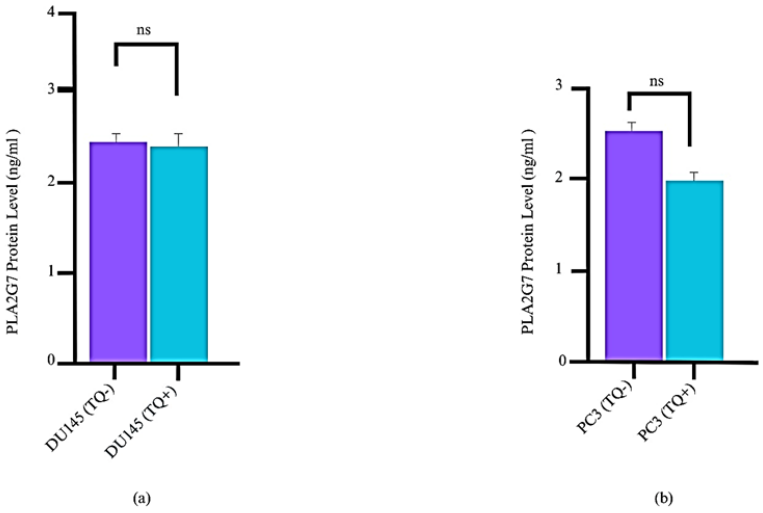

Figure 1. The effect of TQ on PLA2G7 levels in DU145 and PC3. (a) and (b) compared to the control group; ns: $p > 0.05$; Error bars indicate standard ±mean error.

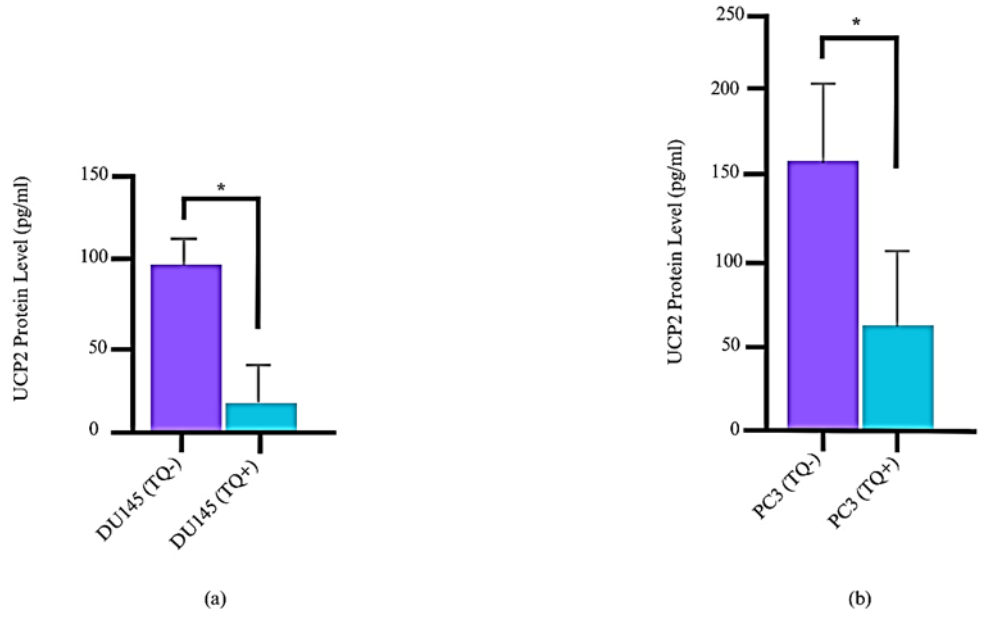

Figure 2. The effect of TQ on UCP2 levels in DU145 and PC 3. (a) and (b) compared to the control group; *: $p < 0.05$; Error bars indicate standard ±mean error.

The expression levels of NEDD4L protein exhibited notable variations across different cell lines and treatment conditions. In PC3 cells, the expression level of NEDD4L protein was 1.30 ng/mL in the untreated group (TQ-), which increased to 2.11 ng/mL in the TQ-treated group (TQ+). Similarly, in DU145 cells, the expression level of NEDD4L protein was 0.52 ng/mL in the untreated group, rising to 1.65 ng/mL in the TQ-treated group. Interestingly, the impact of TQ on NEDD4L protein expression was particularly pronounced in DU145 cells. TQ led to a significant increase in the NEDD4L protein level compared to the untreated group ($p < 0.01$) (Figure 3a). Remarkably, in PC3 cells, this effect was even more substantial, with TQ resulting in a highly statistically significant elevation of NEDD4L protein levels ($p < 0.001$) (Figure 3b), as illustrated in the provided figure.

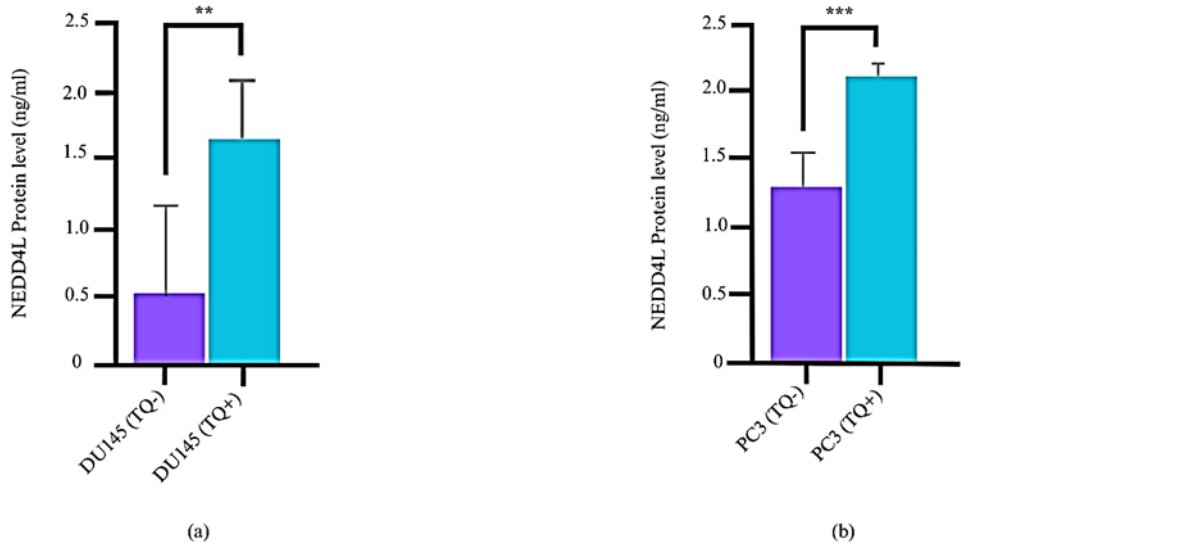

Figure .3 The effect of TQ on NEDD4L levels in DU145 and PC3. (a) and (b) compared to the control group; **: $p < 0.01$; ***: $p < 0.001$; Error bars indicate standard ±mean error.

DISCUSSION AND CONCLUSION

Cancer cells exhibit metabolic alterations that promote tumor growth. And facilitate coping with new growth rate demands during malignancy.[17,18] Intensive research is being conducted on methods to modify the metabolic reprogramming of cancer cells in PCa.[17] Consistently, an expanding body of research has demonstrated a strong positive correlation between upregulated lipogenesis and PCa pathogenesis.[19] An increased number of LD is now considered a hallmark of aggressive PCa.[20] Therefore, studies indicate that an increase in LD is positively linked with a higher Gleason Score of PCa. This may justify the role of LD in the progression of PCa to a more aggressive and malignant form.[8]

These findings give rise to new possibilities for using lipid metabolomics to find therapeutic targets. The use of natural and synthetic drugs that modify the lipogenic phenotype in cancer is a fast-expanding topic of study.[21] Following that, in an unprecedented attempt, we have selected TQ to investigate possible effects on LD in PCa.

At first, and as the ultimate purpose is to find new treatments for men with hormone-refractory PCa, cell lines were chosen to address a broad spectrum of PCa with distinct hormonal dependency and aggressiveness.[22]

Our research is the first attempt to look at the effect of TQ on PLA2s, yet very few studies have addressed the PLA2s using natural products.[23] Contrary to expectations, our study showed no significant differences between the treated and control groups. One possible explanation for such a result is that TQ may have a time-dependent effect, and the incubation period may have been insufficient to cause an inhibitory effect. As such, further investigations should be held to address these limitations. Despite that, Patel et al. have reported increased levels of PLA2 in DU145 and PC3 cell lines, and that siRNA knockdown or specific inhibition of PLA2 by Wyeth -1 resulted in a decrease in cell growth and proliferation both in-vitro and in-vivo.[24]

Our study's originality lies in preliminarily describing the potential effect of TQ on UCP2, which has yet to be previously reported, providing new insights into the underlying mechanism. Our findings match those of a recent study where Genipin, a plant chemical extract, by decreasing UCP2 expression in PC-3 cells, reduced cell migration and growth in contrast to control,[25] which, as hypothesized, supports the idea that TQ could also be a potential inhibitor of UCP2. Additionally, a growing body of literature

shows that cells expressing elevated amounts of UCP2 are more chemoresistant;[26] thus, lowering the UCP2 levels as an attempt to increase chemosensitivity sounds valid, as depicted by Hua et al.[27] TQ may exert its effects on LD through modulation of oxidative stress pathways acting on UCP2 protein levels. Oxidative stress can influence lipid metabolism by altering enzyme activities and lipid oxidation rates. Additionally, TQ might influence mitochondrial function, which plays a crucial role in lipid metabolism. By affecting mitochondrial activity, TQ could indirectly impact LD dynamics.

Moreover, a discrepancy in the expression level of NEDD4L was reported as it decreases in well-differentiated PCa[11] and increases in poor-differentiated PCa.[28] This indicated that NEDD4L protein level regulation may play a role in PCa and give insights into novel treatment strategies.[29] Therefore, given the fact that the cell lines we used are of an advanced aggressive phenotype, we projected a downregulation of NEDD4L before treatment and that TQ to increase the levels of NEDD4L, which is a potential effect on the ubiquitination of LD.

As expected, our preliminary results by ELISA might suggest that TQ has significantly increased the levels of NEDD4L in both cell lines. Remarkably, TQ exhibited a notably more significant impact on NEDD4L in the PC3 cell line, suggesting a promising efficacy against a potentially more aggressive form of PCa. In the same fashion, Wogonin, a non-synthetic compound, has been shown to increase the expression of NEDD4L, the ubiquitin E3 ligase of Pik3ca, inducing the degradation of Pik3ca which in turn hinders PI3K/Akt pathway.[30] Indeed, inferring from the findings described above, we suggest that TQ is more likely to induce LD degradation similarly. TQ might directly interact with LD formation, stabilization, or degradation proteins. For instance, it could bind to proteins that coat the surface of LD and regulate lipolysis, thereby influencing their stability and turnover.

The integration of these studies with our findings suggests that NEDD4L and UCP2 could emerge as promising targets in drug discovery endeavours aimed at combating PCa, given their implication in tumorigenesis. However, further investigations, using western blot method for instance, are imperative to confirm the efficacy of TQ on the aforementioned proteins and to elucidate its precise mechanism of action. Such studies should encompass a comprehensive set of in vivo and in vitro investigations, advancing our understanding of TQ's potential therapeutic utility in PCa treatment.

Ethics Committee Approval: An ethical approval for the study is not required. In this study, cell culture was used.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept – MEE, EŞ; Supervision – EŞ; Materials – AH, HY; Data Collection and/or Processing – AH, HY; Analysis and/or Interpretation – AH, BEÖB; Writing – AH.

Peer-review: Externally peer-reviewed.

Other information: This study was presented as an oral presentation at the 2nd International Graduate Studies Congress (IGSCONG'22, 08-11 June 2022).

Financial Support: This research is supported by Ankara Yildirim Beyazit University Scientific Research Projects Unit (project number 2310).

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