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The Role of TRPM2 Channel in Doxorubicin-induced Cell Damage in Laryngeal Squamous Cancer Cells

Tarık Yağcı[a], Ramazan Çınar[b], Halil İbrahim Altıner[c], Rıza Dündar[a], and Kenan Yıldızhan[d,*]

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Abstract—Laryngeal squamous cell carcinoma is a common type of head and neck cancer. This study investigated the role of the TRPM2 channel in doxorubicin (DOX)-induced cell damage in human laryngeal squamous cancer cells (Hep-2). Cells were exposed to various DOX concentrations and the appropriate dose was found. Then, TRPM2 antagonist ACA was treated. At the end of the study, cell viability test, Western blot and oxidative stress and inflammatory markers were examined. The results showed that TRPM2 channel expression increased with DOX administration, and DOX incubation in cells caused an increase in ROS, MDA, IL-1β, IL-6, and TNF-α levels, while GSH and GSH-Px levels decreased. Concurrent treatment with ACA attenuated these effects and reduced oxidative stress and inflammation. In addition, DOX-induced apoptosis markers including Casp-3, Casp-8, Casp-9, p53, and Bax were elevated, while Bcl-2 levels were decreased; ACA treatment reversed these changes. The study demonstrated that DOX treatment significantly enhances TRPM2 channel activation and ROS production in Hep-2 cells, thereby initiating apoptotic pathways that lead to cell death. Consequently, targeting the TRPM2 channel may represent a promising therapeutic strategy for treating laryngeal cancer.

Keywords: Laryngeal squamous cell carcinoma, doxorubicin, TRPM2 channel, oxidative stress, inflammation

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INTRODUCTION

Laryngeal carcinoma comprises approximately one-third of all head and neck malignancies [1]. Although many factors play a role in its development, such as genetics, hormonal factors, alcohol, nutritional habits, air pollution, laryngopharyngeal reflux and viruses, the most critical risk factors for laryngeal carcinoma are smoking and tobacco use [2]. The majority of laryngeal cancers are squamous cell carcinomas (SCC). SCC is a type of cancer originating from epithelial cells and can occur in various organs, including the skin, mouth, throat, lungs, and cervix. SCC can lead to serious health problems if not treated in its early stages. The treatment of SCC involves various methods, such as surgical intervention, radiotherapy, and chemotherapy [3, 4].

[a] *Department of ENT, Faculty of Medicine, Bilecik Seyh Edebali University, Bilecik, Türkiye*
[b] *Department of Biophysics, Faculty of Medicine, Bilecik Seyh Edebali University, Bilecik, Türkiye*
[c] *Department of Otorhinolaryngology, Bilecik Training and Research Hospital, Bilecik, Türkiye*
[d] *Department of Biophysics, Faculty of Medicine, Van Yuzuncu Yil University, Van, Türkiye*
**e-mail: kenanyldzhan@gmail.com*

Doxorubicin (DOX) is an anthracycline-class chemotherapeutic drug widely used in the treatment of various cancer types. This drug inhibits the proliferation of cancer cells through DNA intercalation and topoisomerase II enzyme inhibition [5]. DOX can be used both as a monotherapy and in combination with other chemotherapy agents. In treating SCC, DOX offers an effective strategy to inhibit the growth and spread of tumor cells [6]. However, the side effects and toxicity of DOX necessitate careful evaluation during the treatment process. Cardiotoxicity, bone marrow suppression, and gastrointestinal side effects are common adverse effects associated with DOX treatment [7–9]. Therefore, the use of DOX in treating SCC should be carefully planned, considering the patient's overall health status and other treatment options. The relationship between squamous cell carcinoma and DOX is significant in treating this cancer type [6]. The efficacy and usage strategies of DOX are important focal points in cancer treatment research. For these reasons, it will help us better understand the effects of DOX on SCC and optimize treatment protocols.

The Transient Receptor Potential Melastatin 2 (TRPM2) is a calcium-permeable ion channel implicated in various physiological and pathological processes [10, 11]. This channel is predominantly triggered by oxidative stress and other cellular stressors, including reactive oxygen species (ROS) [12]. TRPM2

is critical in maintaining calcium homeostasis, regulating cell death pathways, and modulating inflammatory responses. Found in various tissues and organs such as the central nervous system, immune system, and pancreas, TRPM2's broad biological significance is evident. Emerging research suggests that TRPM2 contributes to the development of numerous diseases, including neurodegenerative disorders, diabetes, cardiovascular conditions, and cancer [11, 13–15]. Thus, a comprehensive understanding of TRPM2's function and regulation is essential for devising new therapeutic approaches targeting this channel across different disease states.

In this study, we focused on the role of the TRPM2 channel in the mechanism of DOX-induced damage in the laryngeal squamous cancer cell line. For this purpose, firstly, appropriate dose determination was made for DOX in Hep-2 cells, and the effect of TRPM2 channel inactivation on this damage mechanism and the change in TRPM2 channel expression level in the cells during this process were examined. To measure the oxidative stress parameter changes, which is an important player in the TRPM2 channel activation mechanism, the levels of antioxidant scavengers' glutathione (GSH), glutathione peroxidase (GSH-Px), and oxidative damage markers such as malondialdehyde (MDA), and pro-inflammatory (IL-1β, IL-6, and TNF-a) levels were examined. In addition, ROS, PARP-1 and TRPM2 levels, which are the most important markers in the TRPM2 channel activation pathway, and are well expressed in the literature, were investigated [16–18]. Then, the role of the TRPM2 channel in the DOX-induced damage mechanism in Hep-2 cells was recorded with apoptotic markers.

MATERIALS AND METHODS

Cell Cultures and Treatment

Hep-2 cells, a human laryngeal squamous cancer cell line, were obtained from Şap Institute (Ankara, Turkey) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) enriched with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St.Louis, MO, USA) in 5% CO_2 humidified incubator at 37°C. Hep-2 cells were exposed to DOX at concentrations of untreated, 0.75, 0.5, 1, 1.25, and 1.5 μM for 24 h and subsequently co-treated with TRPM2 channel antagonist ACA at a concentration of 25 μM for the last 30 min of the 24 h of the study [19].

Cell Viability Assay

CCK-8 assay (Abbkine, Cat_KTA1020) was a simple and reliable method to determine cell viability. Hep-2 cells were plated at a 1×10^4 cells/well density in 96-well plates. Following treatment with DOX and ACA for a certain period. The experiment was repeated three times to determine the cell viability rate in the groups, and the data were given as a percentage compared to the control group (% of control).

Western Blotting Analysis

Total protein amounts in supernatants obtained from Hep-2 cells were determined spectrophotometrically according to the procedure using the BCA kit (Thermofisher 23227). Each well was loaded with 50 μg protein. Electrophoresis was performed on the Biorad mini-protean tetra cell gel electrophoresis system. Following execution, gels were prepared for transfer. A sandwich model was created by placing a sponge, filter paper, gel, nitrocellulose membrane, filter paper and sponge into the transfer cassette from cathode to anode. Proteins in the gel were transferred to a nitrocellulose membrane using the Western blotting technique using the Biorad semi-wet transfer system. Membranes TRPM2; 1/1000; Bcl2; 1/1000, Bax; 1/1000, p53; 1/1000, and Beta-actin 1/1000; incubated overnight with antibodies diluted with 5% milk powder. Secondary antibodies suitable for the primer were diluted in 5% milk powder (Anti Mouse Seconder Antibody: 1/5000, Anti-Rabbit Seconder Antibody: 1/10000), and the membranes were incubated with these antibodies for 1 h. Membranes were treated with chemiluminescent conjugate (ECL). Band images were obtained using the SYNGENE G:Box Chemi XRQ imaging device. Band intensities in the obtained images were measured using ImageJ software. Protein expression levels were normalized to beta-actin, which was used as internal control and expressed as a percentage of the control.

Measurement of Biochemical Parameters in Hep-2 Cells by ELISA Kits

The cells for each group were placed in separate sterile tubes. The tubes were centrifuged at 1000 rpm for 20 min by the kit's procedure. After removing the supernatants, the cell pellets were suspended in PBS (pH 7.4) to produce a cell suspension at approximately 1×10^6/ml concentration. Cells were lysed through repeated freeze-thaw cycles to allow internal components to escape. It was centrifuged for 10 min at 4000 rpm at 4°C. The supernatants were collected for biochemical analysis. The Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determine the total protein levels in the samples. The levels of GSH, MDA, GSH-Px, IL-1β, IL-6, TNF-α, ROS, PARP1, TRPM2, Casp-3, Cas-8, and Casp-9 levels in the obtained supernatants of the cells were determined using ELISA commercial kits (SunRed Biological Technology, Shanghai, China). These analyzes were performed considering the protocols determined by the companies for commercial kits. The

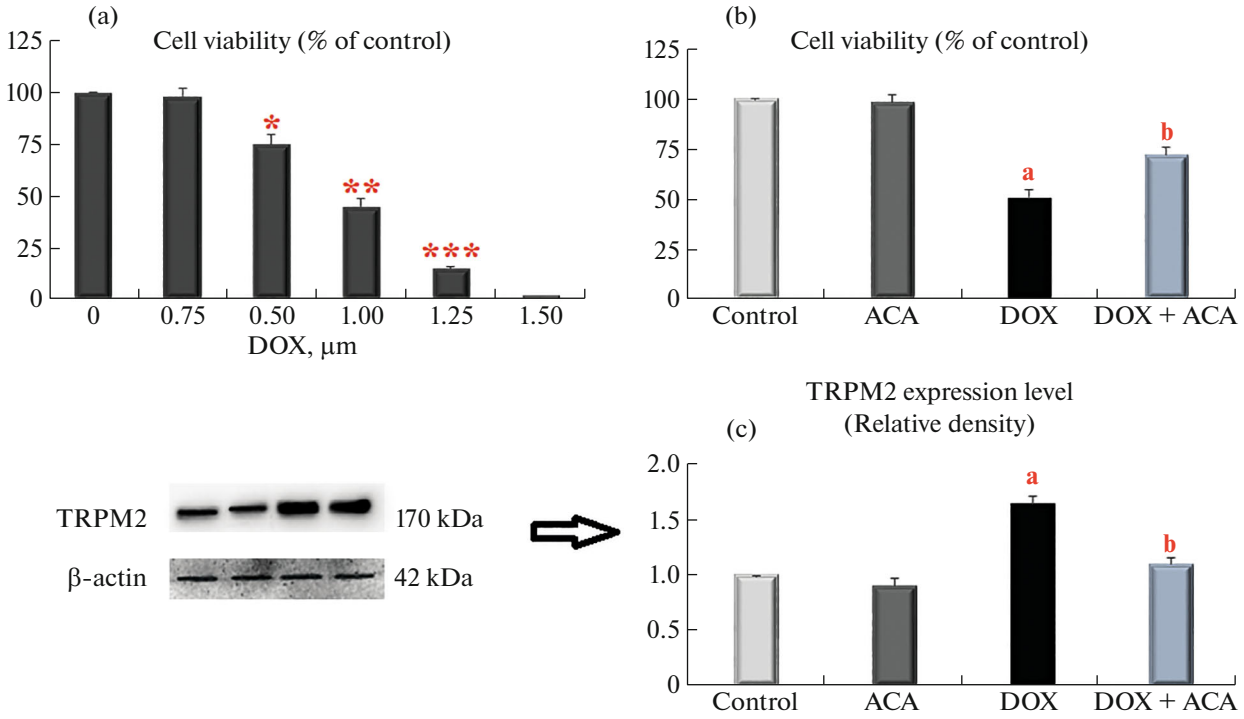

Fig. 1. The role of TRPM2 channel in cell viability in DOX-induced Hep-2 cells. (a) Exposure to DOX-induced concentration-dependent cell viability in Hep-2 cells. (b) Effect of treatment with TRPM2 channel antagonist ACA (25 μM for 30 min) on DOX (1μM for 24 h)-induced cell viability in Hep-2 cells. (c) Western blots showed expression of TRPM2 between the groups, and TRPM2 channel expression increased despite a DOX-induced decrease in cell viability in Hep-2 cells. (Data represented as means ± SD). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ vs. untreated control group. [a] $p < 0.05$ vs. control and ACA groups. [b] $p < 0.05$ vs. DOX group.

obtained mean values in the microplate reader were indicated as fold increases.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) and were analyzed by one-way univariate analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Statistical analysis was performed using the SPSS (version 23.0, SPSS Inc, Chicago) program. Each experiment was conducted at least in triplicate. Statistical significance was set at $p < 0.05$.

RESULTS

The Role of TRPM2 Channel in DOX-Induced Cell Death

To investigate the role of the TRPM2 channel in the toxic effect of DOX on Hep-2 cells, in addition to the literature review, the DOX dose to be used in the study was determined, and cells treated with 1 μM DOX for 24 h were used as a cellular model ($p < 0.05$, Fig. 1a). To investigate the role of the TRPM2 channel in DOX-induced cell damage, incubations with ACA, a TRPM2 channel antagonist, showed that DOX-induced damage in Hep-2 cells decreased with TRPM2 channel inactivation ($p < 0.05$, Fig. 1b). Additionally, when TRPM2 channel expression levels were examined between the groups, it was observed that the TRPM2 channel expression level in Hep-2 cells increased in parallel with the decrease in cell viability ($p < 0.05$, Fig. 1c).

Antioxidant, Oxidant and Cytokine Levels in TRPM2 Channel Inactivation

After determining that DOX-induced cell damage increases in Hep-2 cells and that the TRPM2 channel is both activated and expressed at an increased level in this process, we wanted to examine some biomarker levels in the cells. With DOX incubation, a decrease in GSH and GSH-Px levels was observed in Hep-2 cells, while an increase in MDA, IL-1β, IL-6, and TNF-α levels was observed. When ACA treatment was examined to reduce TRPM2 channel activation, it was observed that GSH and GSH-Px levels increased, while MDA, IL-1β, IL-6, and TNF-α levels decreased in the DOX+ACA group compared to the DOX group ($p < 0.05$, Fig. 2).

ROS, PARP-1, and TRPM2 Channel Levels in TRPM2 Channel Inactivation

TRPM2 is a Ca^{2+} permeable cationic channel that is activated in response to oxidative stress and acts as a

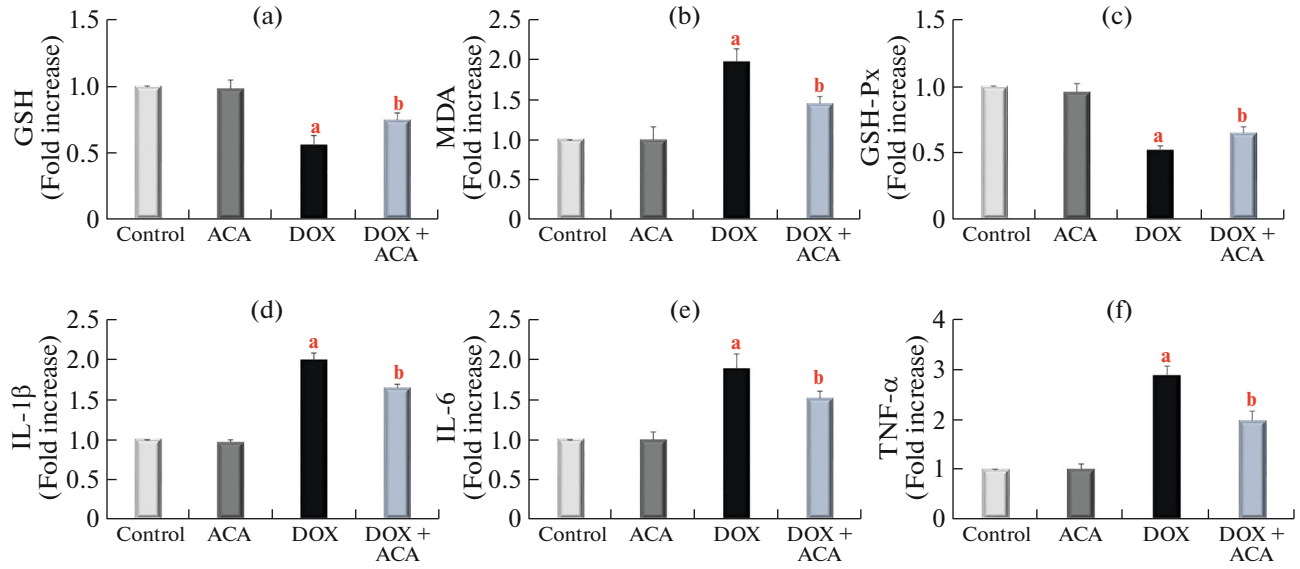

Fig. 2. Effect of treatment with TRPM2 channel antagonist ACA on changes in (a) GSH, (b) MDA, (c) GSH-Px, (d) IL-1β, (e) IL-6 and (f) TNF-α levels in DOX-induced Hep-2 cells. (Data represented as means ± SD). [a] $p < 0.05$ vs. control and ACA groups. [b] $p < 0.05$ vs. DOX group.

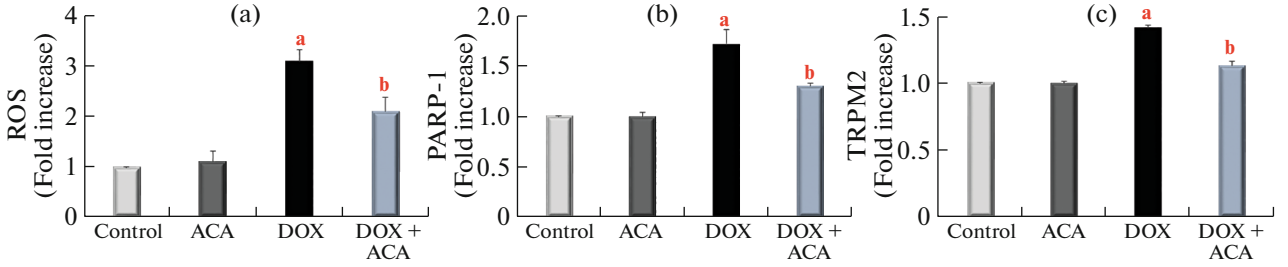

Fig. 3. Effect of treatment with TRPM2 channel antagonist ACA on changes in (a) ROS, (b) PARP-1 and (c) TRPM2 channel levels in DOX-induced Hep-2 cells. (Data represented as means ± SD). [a] $p < 0.05$ vs. control and ACA groups. [b] $p < 0.05$ vs. DOX group.

cellular redox sensor. Disruption of the oxidant/antioxidant balance within the cell and an increase in oxidative stress increase this channel activation [15]. For these reasons, we examined the ROS, PARP-1, and TRPM2 channel levels in Hep-2 cells to determine the role of TRPM2 channel activation in the damage mechanism in DOX-induced Hep-2 cells. DOX increased ROS, PARP-1, and TRPM2 channel levels in Hep-2 cells. We determined that ACA treatment reduces DOX-induced damage by reducing TRPM2 channel activation ($p < 0.05$, Fig. 3).

The Role of TRPM2 Channel Inactivation on DOX-Induced Apoptosis in Hep-2 Cells

Casp-3 (Fig. 4a), Casp-8 (Fig. 4b), and Casp-9 (Fig. 4c) levels between the groups were measured with ELISA kits. The DOX group's Casp-3 and Casp-9 levels were higher than other groups ($p < 0.05$). In the DOX+ACA group, Casp-3 and Casp-9 levels were lower compared to the DOX group ($p < 0.05$). However, compared to the control and ACA groups, the Casp-3 and Casp-9 levels of the DOX+ACA group were higher ($p < 0.05$). Additionally, no significant difference was observed in Casp-8 levels between groups ($p > 0.05$).

When p53 (Fig. 4d), Bax (Fig. 4e) and Bcl-2 (Fig. 4f) expression levels were examined between the groups, the DOX group's p53 and Bax expression levels were the highest compared to the other groups. In contrast, the Bcl-2 level was the lowest ($p < 0.05$). However, in the DOX+ACA group that received ACA treatment, it was observed that the DOX-induced increased expression levels of p53, Bax and Bcl-2 decreased ($p < 0.05$). Compared to the control and ACA groups, it was determined that the p53 and Bax expression levels of the DOX + ACA group were higher, while Bcl-2 levels were lower ($p < 0.05$).

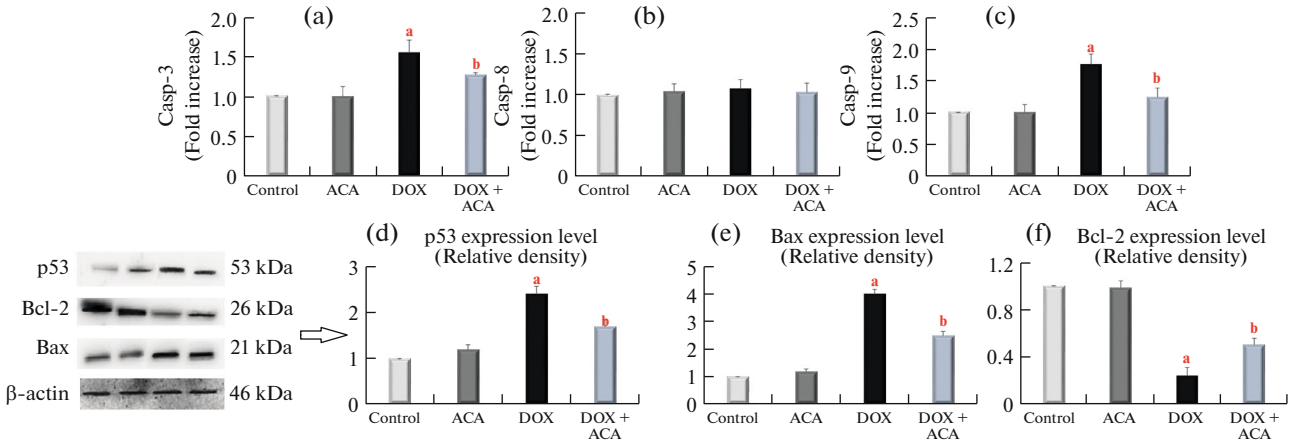

Fig. 4. Effect of treatment with TRPM2 channel antagonist ACA on changes in (a) Casp-3, (b) Casp-8 and (c) Caps-9 levels in DOX-induced Hep-2 cells. Western blots showed (d) p53, (e) Bax, and (f) Bcl-2 expressions between the groups. (Data represented as means ± SD). [a] $p < 0.05$ vs. control and ACA groups. [b] $p < 0.05$ vs. DOX group.

DISCUSSION

Laryngeal squamous cell carcinoma is the second most common type of cancer among head and neck cancers. It is usually associated with factors such as smoking and alcohol use and is mainly seen in individuals between the ages of 55–65. While the disease is highly treatable in its early stages, the prognosis is generally poor in advanced stages Therapeutic methods for treating laryngeal cancer remain very limited due to its complex background [1, 2, 20]. For these reasons, understanding the molecular pathways of damage mechanisms in these laryngeal cancer cells will help treatment approaches. DOX is a drug commonly used to treat many types of cancer [11]. Although doxorubicin is an excellent anticancer treatment, different resistances are encountered. For this reason, it is essential to understand the mechanism of action of DOX in various cell lines. In this study, we elucidated the role of the TRPM2 channel in the mechanism of oxidative stress and inflammation-mediated damage in DOX-induced Hep-2 cells for the first time.

In their study investigating the effect of curcumin on cisplatin-induced human Hep-2 cell death through activation of the TRPM2 channel and mitochondrial oxidative stress, Kütük et al. first confirmed the expression of the TRPM2 channel in them. Moreover, in parallel with the increases in the levels of mitochondrial ROS and cell death in Hep-2 cells, the TRPM2 channel showed that both expression and activation increased [21]. Another study determined that the anticancer effect of Paclitaxel increased with the stimulation of TRPM2 in Hep-2 cells treated with Paclitaxel. Moreover, this study reported that these changes were caused by TRPM2-mediated overload Ca^{2+} entry, Zn^{2+} entry, and mitochondria ROS pathways [19]. Combining different chemotherapy drugs or activating molecular pathways can reduce the dose, toxicity and multidrug resistance of drugs, thus leading to fewer side effects and better treatment methods. In their study on Hep-2 laryngeal cancer cells, Wang et al. determined that 5-fluorouracil and curcumin synergistically induced apoptosis and cell cycle arrest in Hep-2 cells [22]. In this study, incubations with ACA, a TRPM2 channel antagonist, we found that DOX-induced damage in Hep-2 cells was reduced by TRPM2 channel inactivation. Additionally, when TRPM2 channel expression levels were examined between the groups, we determined that the TRPM2 channel expression level in Hep-2 cells was inversely proportional to the cell viability rate, and TRPM2 channel expression increased with the increase in the damage rate (Fig. 1). Kütük et al. summarized the pathways that play a role in tumour cell death through cisplatin incubation Hep-2 cells, TRPM2 channel activation and excess ROS. They reported that an excessive increase in intracellular ROS increased TRPM2 channel activation, causing more Ca^{2+} to enter the cell, and they emphasized that this triggered an increase in ROS and MDA levels by inhibiting GSH and GSH-Px levels in the cells [21]. In our study, a decrease in GSH and GSH-Px levels was observed in Hep-2 cells with DOX incubation, while an increase in MDA, IL-1β, IL-6 and TNF-α levels was observed. When ACA treatment was examined to reduce TRPM2 channel activation, we observed that GSH and GSH-Px levels increased, while MDA, IL-1β, IL-6, and TNF-α levels decreased in the DOX+ACA group compared to the DOX group (Fig. 2).

TRPM2 channel is an important target of oxidative stress and plays a critical role in maintaining cellular homeostasis. Activation of TRPM2 leads to an increase in intracellular calcium levels, which can affect various cellular processes [15, 23]. Under oxidative stress conditions, ROS production increases, causing activation of TRPM2. This activation may occur through metabolites such as ADP-ribose or

NAD^+ [24]. Excessive activation of PARP1 may increase the severity of cellular stress by also increasing the activation of the TRPM2 channel. Additionally, there is thought to be a synergistic interaction between PARP1 and TRPM2; this interaction may contribute to increased cellular damage triggered by oxidative stress [25]. This study examined ROS, PARP-1, and TRPM2 channel levels in Hep-2 cells. DOX increased ROS, PARP-1, and TRPM2 channel levels in Hep-2 cells. We determined that ACA treatment reduced DOX-induced damage by reducing TRPM2 channel activation (Fig. 3).

It has been reported that activation of TRPM2 leads to an increase in Bax, Casp-3, and Casp-9 activity and an increase in oxidative stress. This supports the mediating role of TRPM2 in the apoptosis pathway [26]. In a study involving Hep-2 cells, Ahamed et al. demonstrated the depletion of glutathione and the induction of ROS and lipid peroxidation during cellular damage. They also determined that the chemical they used caused oxidative stress in a concentration-dependent manner. Quantitative real-time PCR data also reported that the mRNA level of the tumor suppressor gene p53 and apoptotic genes (Bax, Casp-3, and Casp-9) was upregulated. In contrast, dolomite's anti-apoptotic gene Bcl-2 was downregulated [27]. In this study, we observed an increase in the levels of Casp-3 and Casp-9 in Hep-2 cells incubated with DOX, but we did not observe a significant difference in the levels of Casp-8 between the groups. We also observed a decrease in the expression levels of p53 and Bax and a decrease in the expression level of Bcl-2. These parameters were modulated in TRPM2 channel inactivation (Fig. 4).

CONCLUSIONS

This study examined the role of the TRPM2 channel in oxidative stress and inflammation-mediated damage mechanisms in Hep-2 cells. It has been shown that TRPM2 channel activation and ROS production increase in Hep-2 cells with DOX application, which triggers apoptotic pathways that lead to cell death. It was concluded that by inactivating the TRPM2 channel, the cellular damage caused by DOX can be reduced, and the levels of oxidative stress and inflammation can be reduced. These findings suggest that targeting the TRPM2 channel may be a potential therapeutic strategy in treating laryngeal cancer.

LIMITATIONS OF THE STUDY

In this study, where the role of the TRPM2 channel in DOX-induced cell damage in the Hep-2 cell line was investigated as a first, the limitations of the study were not using dose ranges or different antagonists for TRPM2 channel in Hep-2 cells due to limited budget and also not supporting different molecular techniques.

AUTHOR CONTRIBUTION

Study Design: T.Y., R.Ç. and R.D., Literature Search: T.Y., R.Ç., H.İ.A., and K.Y., Data collection: All authors., Data interpretation: T.Y., R.Ç., H.İ.A., and K.Y., Statistical analysis: T.Y., R.Ç. and K.Y., Manuscript preparation: K.Y., and R.Ç., All authors reviewed the manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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