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# Gallic Acid Enhances Cisplatin-induced Death of Human Laryngeal Cancer Cells by Activating the TRPM2 Channel

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**Abstract**—Cisplatin (CIS) is widely used in the treatment of laryngeal cancer, one of the most common and lethal cancers. However, it is not a satisfactory chemotherapeutic agent. Therefore, there is a need to identify new agents, such as gallic acid (GAL), that can exert a synergistic effect to elucidate the pathophysiological mechanisms of the chemotherapeutic effects of CIS and to increase the effectiveness of treatment by preventing drug resistance. For this purpose, we investigated the stimulatory role of GAL on CIS-induced human laryngeal cancer (Hep-2) cell death via TRPM2 channel activation. For the study, four groups were formed from human laryngeal cancer (Hep-2) cells as Control, GAL (1OO μM), CIS (25 μM), and GAL + CIS. In the analyses made, cell viability, glutathione (GSH) and glutathione peroxidase (GSH-Px) enzyme activity, lipid peroxidation (LPx) levels, inflammation markers I-1β, IL-6, and TNF-α, Total Oxidant/Antioxidant (TOS and TAS) status, reactive oxygen species (ROS), caspase (Cas-3-9) activity, Transient Receptor Potential Melastatin 2 (TRPM2), and Poly Adp Ribose Polymerase-1, (PARP-1) levels in the cells were determined. CIS treatment caused laryngeal cancer cell cytotoxic and increased Cas-3-9, ROS, IL-1β, TNF-α, IL-6, TOS, LPx, TRPM2, and PARP-1 levels while decreasing cell viability, GSH-Px, GSH, and TAS levels. The combination of GAL and CIS treatment made the treatment even more effective. In conclusion, the increase in ROS and cell death levels mediated by TRPM2 activation in CIS Hep-2 cells was further enhanced by GAL treatment. Thus, CIS chemotherapy in Hep-2 cells may be enhanced by the synergistic effect of the GAL combination, and drug resistance may be reduced.

**Keywords:** cisplatin, gallic acid, HEp-2 cell, oxidative stress, PARP-1, TRPM2

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## INTRODUCTİON

With more than 350 000 deaths per year, head and neck cancer is the sixth most common cancer worldwide [1]. Laryngeal squamous cell carcinoma is the most common malignant cancer of the head and neck, and its incidence is increasing significantly every day [2]. While squamous cell carcinoma of the head and neck is curable if it is diagnosed at an early stage, the prognosis is very poor if it is diagnosed at an advanced stage [3]. Chemotherapeutic drugs are important in the treatment of laryngeal tumours, although they also have some adverse effects on normal cells [4]. Furthermore, drug resistance is an important issue with chemotherapies [5]. Cancer cell lines have made enormous contributions to cancer research and are derived from primary patient tissue [6]. Human laryngeal cancer cells (Hep-2) are the cell lines used for this purpose [2, 7].

Cisplatin (CIS) is a potent chemotherapeutic agent with a long history of use in the treatment of various cancers, including laryngeal squamous cell carcinoma [8, 9]. Several mechanisms have been proposed for the chemotherapeutic efficacy of CIS. Reactive oxygen species (ROS), which increase with the action of CIS, cause oxidative stress (OS) and $Ca^{2+}$ overload [4, 10, 11]. The increase in intracellular $Ca^{2+}$ induced by CIS leads to the activation of cell death pathways and produces an anti-cancer effect [11–13]. In addition, recent reports suggest that an increase in inflammatory markers also plays a role in the sensitivity of CIS to kill tumour cells [14, 15]. It has been observed that CIS-induced $Ca^{2+}$ influx and disruption of the oxidant/antioxidant balance cause resistance to CIS treatment in laryngeal cancer cells [2, 11]. Although the anti-cancer activity of CIS has been demonstrated, resistance to CIS treatment has been observed in approximately 30% of patients with laryngeal squamous cell carcinoma [11, 16]. Some studies have reported that CIS-induced drug resistance can be overcome by increasing ROS production and $Ca^{2+}$ influx in various tumour cells using antioxidant supplements such as selenium and alpha lipoic acid [13, 17, 18]. However, while the anti-tumour activity of

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GAL has been investigated in numerous studies, its effect on CIS-induced drug resistance in Hep-2 cells has not been investigated. Therefore, we suggest that GAL may enhance the effects of CIS by inhibiting drug resistance by increasing ROS production and $Ca^{2+}$ influx in tumour cells.

Many plants, such as sumac, thuja, and green tea, contain GAL, a flavonoid, as part of their structure [19, 20]. GAL has a wide range of biological activities, including antimicrobial, antioxidant, and anti-inflammatory properties, most notably anti-tumour activity [20, 21]. While normal $Ca^{2+}$ levels are required for physiological cell functions such as secretion and contraction, excessive $Ca^{2+}$ entry activates apoptotic pathways [22, 23]. In tumour cells, excessive $Ca^{2+}$ entry is also required for apoptosis [19, 24]. Activation of gated calcium channels increases the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]i$) in the cytosol [25]. The recently discovered transient receptor potential (TRP) superfamily consists of 6 subfamilies in mammals, and one of the subfamilies of the TRP superfamily is TRP melastatin (TRPM) [22, 23]. The TRPM2 cation channel is a member of the TRPM subgroup [24]. TRPM2 is activated by oxidative stress and/or ADPR [26]. Activated TRPM2 may mediate anti-tumour effects by increasing intracellular $Ca^{2+}$ concentration. Some recent studies have shown that some substances such as alpha-lipoic acid, selenium, curcumin enhance the anti-cancer effects of CIS by activating various TRP channels [2, 13, 17]. Accordingly, a similar potentiating effect of GAL may be present in CIS-treated laryngeal cancer cells. In recent years, because GAL can inhibit cancer tumour growth by inducing tumour apoptosis, there has been considerable interest in using GAL to treat cancer [27, 28]. Accumulating evidence suggests that GAL also has a pro-oxidant effect in several types of cancer and that it activates calcium channels [20, 29, 30]. The pro-oxidant effect of GAL may enhance the efficacy of CIS in cancer treatment, given the importance of increased ROS and ROS-activated $Ca^{2+}$ entry (via TRPM2 channel activation) for tumour cell apoptosis [17, 20].

To date, there have been no reports of GAL treatment potentiating CIS-induced apoptosis and oxidative damage in laryngeal cancer cells. We investigated whether GAL synergistically enhances the anti-cancer activity of CIS by activating TRPM2 channels in laryngeal cancer cells. We also investigated the possible molecular signalling pathway underlying this effect.

## MATERIALS AND METHODS

### *Chemicals*

Cisplatin (Cat; PHR1624) and Gallic acid (Cat; G-7384) were purchased from Sigma Aldrich C. (USA). Caspase 3 (REF; DZE201120970), Caspase 9 (REF; DZE201120969), Interleukin-1β (Product no; 201-12-0144K), Interleukin-6 (Product No; 201-12-0091K), Reactive Oxygen Species (REF; DZE201129433), Tumor necrosis factor-α (Product No; 201-12-0083B) ELISA kits was purchased from Sun.Red Biotech Comp (SRB) Ltd (China). Transient receptor potential cation channel subfamily M member 2 (Cat. no; abx551423) ELISA kits and Poly ADP ribose polymerase 1 (Cat. no; abx250333) ELISA kits were purchased from Abbexa Ltd (UK). Total Antioxidant/Oxidant Capacity (TAS-Pro no: RL0017 and TOS-Pro no: RL0024) ELISA kit was purchased from RelAssay (Türkiye).

### *Cell Culture*

The growth medium for the cells used in the study, Dulbecco's modified Eagle's medium (DMEM), was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. The cells were passaged and divided into four groups after reaching 80–85% confluence, and then this process was repeated (6 repetitions for each group). Cells in T25 culture flasks were cultured in an incubator (95% air, 5% $CO_2$, and 37°C). At the end of the incubation period, trypsin-EDTA-(0.25%) was used to detach the cells from the bottom of the flask.

### *Experimental Groups*

The HEp-2 cell line was divided into four groups.

**CON (n; 6),** HEp-2 cells in this group were not treated with any treatment and were incubated (48 h).

**GAL (n; 6)**, HEp-2 cells in this group were kept in cell culture conditions for 24 h without any treatment and then incubated with GAL (100 μM) for 24 h as described in previous studies [31–33].

**CIS (n; 6),** HEp-2 cells in this group were kept in cell culture conditions for 24 h without any treatment and then incubated with CIS (25 μM) for 24 h as described in previous studies [2].

**GAL + CIS (n; 6)**, The cells in this group were kept with GAL (100 μM) in the cell culture medium for 24 h, and then they were treated with CIS (25 μM) further for a period of 24 h.

### *Cell Homogenate Preparation Steps*

Following the kit instructions, the cells for each group were added to separate Eppendorf tubes and centrifuged (1000 rpm, 20 min). The following steps were followed: Using a pipette, the supernatants were removed from the top of the Eppendorf tubes, and the cell pellets underneath were diluted in PBS to a concentration of $1 \times 10^6$ cells/ml. The cell structure was lysed by freeze-thaw repetition, and the mixture was centrifuged at 4°C (3000 rpm, 10 min) after removing the cytoplasmic components. Any supernatants were

removed by automated pipetting and transferred to new sterile tubes for further analysis.

## ANALYSES

### *Cell Viability Assay*

CCK-8 assay (Abbkine, Cat_KTA1020) was a simple and reliable method to determine cell viability. To investigate the anti-tumor effect of gallic acid against CIS-induced cytotoxicity on laryngeal squamous cell carcinoma Hep-2 cells, the cell viability rate was checked; for this purpose, The CCK-8 assay was performed. The cell viability was measured on the BioTek ELx808™ instrument at 450 nm with commercial ELISA kits, following the instructions in the kit procedure. 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).

### *Total Antioxidant/Oxidant Status Levels*

Supernatants of the samples were used for TAS and TOS analyses, and the following steps were followed for TAS and TOS level measurement according to the kit protocol and the manufacturer's instructions. Sample supernatants were mixed with Reagent 1 buffer, and the absorbance was measured by an ELISA reader (TAS 660 nm, TOS 530 nm after incubation). Then, a Reagent-2 buffer was added, and absorbance was measured by an ELISA reader (TAS 660 nm, TOS 530 nm after incubation) (second absorbance value). For TAS analysis, each sample data was calculated using the kit's standard (equivalent to 1 mmol/L of Trolox). For TOS analysis, the assay was calibrated with hydrogen peroxide, and the results are expressed in micromolar hydrogen peroxide equivalents per litre (μmol $H_2O_2$ equivalents/L). The percentage ratio of the TOS to the TAS was accepted as the oxidative stress index (OSI), an indicator of the degree of oxidative stress. For calculations, the resulting unit of TAS, mmol Trolox eq/L, was converted to μmol Trolox eq/L, and the OSI value was calculated using the following formula: OSI = [TOS (μM $H_2O_2$ eq/L)/TAS (μmol Trolox eq /L)] × 100 [34].

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

Transient Receptor Potential Melastatin 2 (TRPM2), Poly Adp Ribose Polymerase-1 (PARP-1), Caspase 3 and 9, ROS, and inflammation markers (TNF-α, IL 1β, and IL-6) levels in laryngeal squamous cell carcinoma Hep-2 cell supernatants were determined (ELISA kit). According to the kit protocol and the manufacturer's instructions, the following steps were followed: supernatants were incubated (37°C, 60 min) according to the specified protocols, the supernatant and standard samples were transferred into 96-well plates and incubated, washing steps were applied, and staining solutions were added and incubated at 37°C temperature for 15 min. At the end of all these procedures, a stop solution was added, and an ELISA spectrophotometer was used to read the absorbance values (Bio Tek EL808™) [34, 35].

### *Glutathione, Glutathione Peroxidase, and Lipid Peroxidation Levels*

Laryngeal squamous cell carcinoma Hep-2 cell GSH, GSH-Px, and Lipid peroxidation (LPx) levels were measured with a V-730 UV spectrophotometer (Japan).

In the experiment (LPx), cell groups were diluted 1/9 (2.25 ml) with thiobarbituric acid (TBARS) solution. A mixture of 1/9 of TBARS and 0.25 ml phosphate buffer was used as a blind. Samples and blind were placed in boiling water, cooled, and centrifuged at 3500 RPM. The top pink-coloured liquid was taken and read against the blind in a spectrophotometer at 532 nm wavelength in a cuvette with 1 cm light transmission.

The solutions required for GSH determination were 10% trichloroacetic acid (TCA) solution and Tris-II buffer. 0.1 ml of cell homogenate and 0.4 ml of TCA were transferred to an Eppendorf tube, mixed, and centrifuged. Then 0.4 ml supernatant was taken into a glass tube, and 2.0 ml Tris-II and 0.1 ml DTNB were added. It was read at 412 nm wavelength with a spectrophotometer.

Solutions required for GSH-Px determination: Tris-I buffer solution, GSH solution, CHPO (cumene-hydroperoxide) solution, 10% TCA solution, Tris-II buffer, DTNB [5,5 dithiobis (2 nitrobenzoic acid)] solution. 0.5 ml cell homogenate, 0.3 ml Tris-I HCl, 0.1 ml CHPO were mixed, and 0.1 ml GSH was added. It was kept at room temperature for 10 min, and 1.0 ml TCA was added and centrifuged. Then 0.1 ml supernatant was taken into a glass tube, and 2 ml Tris-II and 0.1 ml DTNB were added. It was read with a spectrophotometer at a wavelength of 412 nm [34, 35].

### *Statistical Analysis*

Data analyses were performed with SPSS (ver. 17.0, software, USA) software, and all data were expressed as mean ± standard deviation (SD). A one-way ANOVA, Post-hoc Tukey test was used to evaluate all data showing statistically significant differences between groups. A value of $p \leq 0.05$ was considered statistically significant.

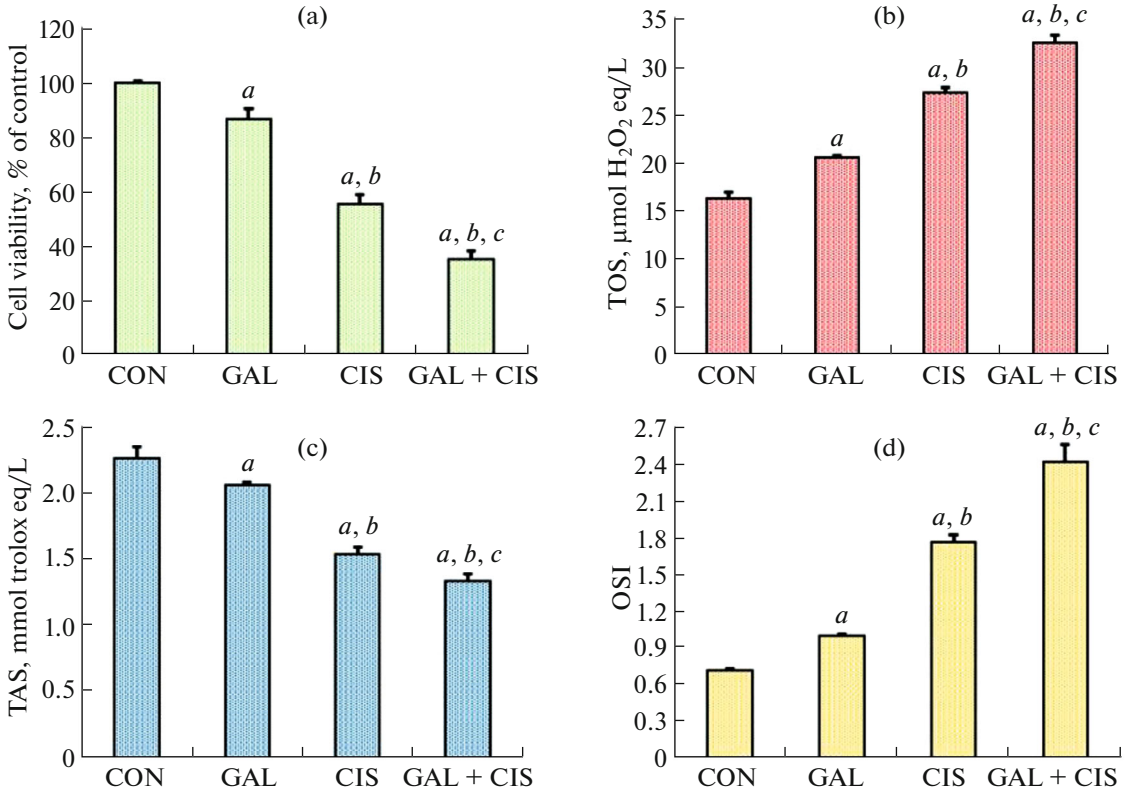


**Fig. 1.** Effect of GAL on Cell viability (a), TOS (b), TAS (c), and OSI (d) levels in laryngeal cancer cells after CIS-induced cytotoxicity (mean ± SD) ($^a p \le 0.001$ vs CON group; $^b p \le 0.001$ vs GAL group; $^c p \le 0.001$ vs CIS group).

## RESULTS

### *Cell Viability, Oxidant, and Antioxidant Levels in Laryngeal Cancer Cells*

We investigated the protective or toxic effects of GAL against CIS treatment in cancer (Hep-2) cells. Cell viability and TAS levels in laryngeal cancer cells decreased significantly with CIS treatment. 24 h after GAL treatment, cell viability and TAS levels decreased further after 24 h of CIS treatment (Fig. 1). A significant decrease in Cell viability (Fig. 1a), and TAS (Fig. 1c) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). A significant increase in TOS (Fig. 1b), and OSI (Fig. 1d) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). CIS treatment decreased cell viability rate (Fig. 1a), and TAS (Fig. 1c) levels compared to CON group ($p \le 0.001$), but decreased further after GAL + CIS treatment compared to CON group. In parallel with the results of cell viability rate and TAS levels, TOS (Fig. 1b), and OSI (Fig. 1d) levels were increased in the CIS group compared to the CON group ($p \le 0.001$), but increased further in the GAL + CIS groups with the effect of GAL treatment.

### *Lipid Peroxidation, Glutathione Peroxidase, and Glutathione Levels in Laryngeal Cancer Cells*

ROS are scavenged by antioxidants. Members of the thiol redox system, such as GSH and GSH-Px, play the main role in the scavenging of ROS in several cells [23, 24]. GAL supports reduced-GSH concentration and GSH-Px activity in normal cells [36]. Thiol groups also play a main role in the activation of TRPM2 channel and reduced-GSH depletion in cells induced by the excessive activation of TRPM2 channel through the increase of oxidative stress [23].

After observing an increase in ROS levels, we have considered that decreased GSH concentration and GSH-Px activity may induce the activation of TRPM2 channel in the laryngeal cancer cells. GSH and GSH-Px levels in laryngeal cancer cells decreased significantly with CIS treatment. 24 h after GAL treatment, GSH and GSH-Px levels decreased further after 24 h of CIS treatment (Fig. 2). A significant decrease in GSH (Fig. 2a), and GSH-Px (Fig. 2b) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). A significant increase in LPx (Fig. 2c) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). CIS treatment decreased GSH (Fig. 2a) and GSH-Px (Fig. 2b) levels compared to CON group ($p \le 0.001$), but decreased further after GAL+CIS treatment com-

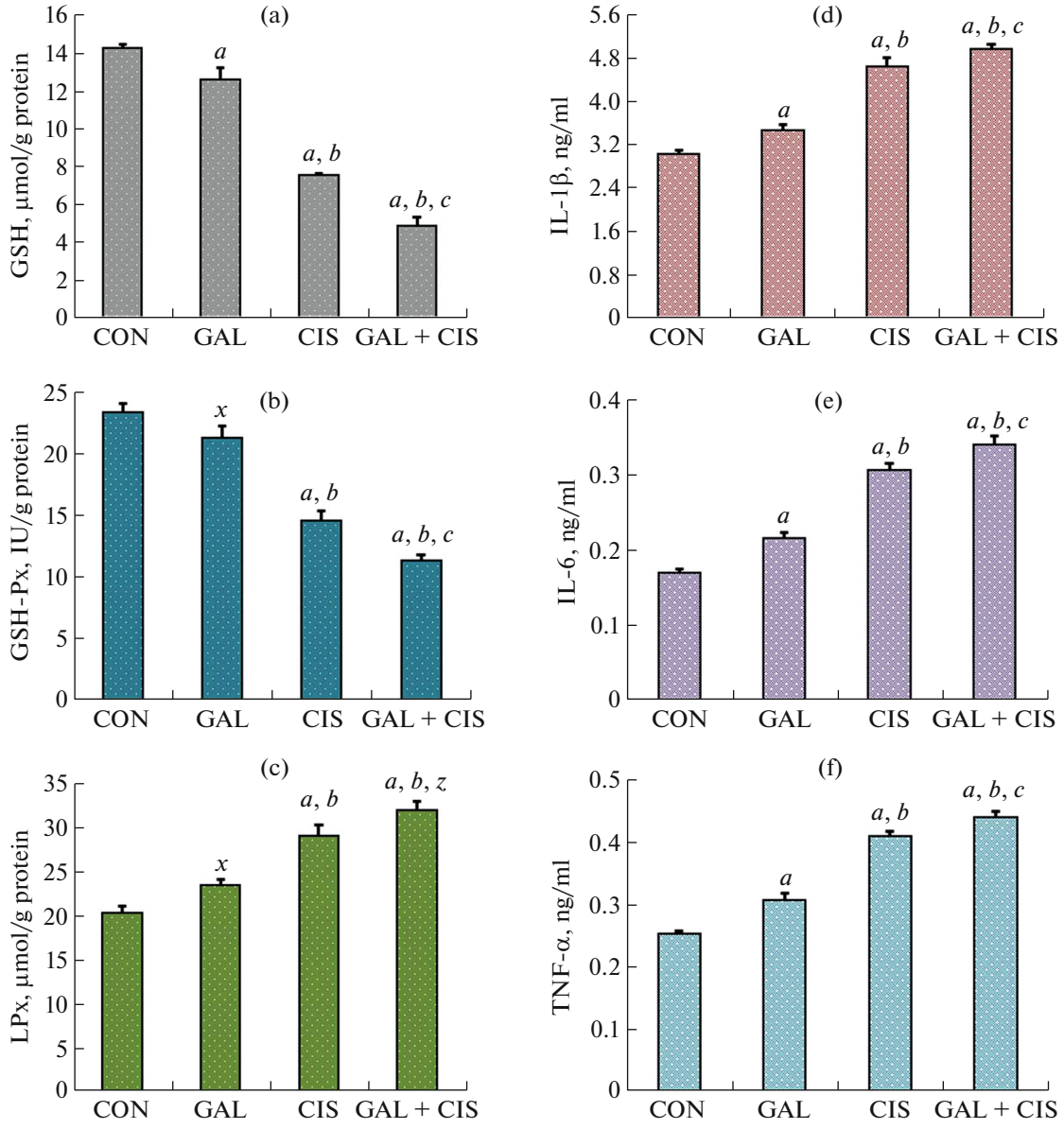


**Fig. 2.** Effect of GAL on GSH (a), GSH-Px (b), LPx (c), IL-1β (d), IL-6 (e), and TNF-α (f) levels in laryngeal cancer cells after CIS-induced cytotoxicity (mean ± SD) ($^{a}p \le 0.001$, $^{x}p \le 0.01$ vs CON group; $^{b}p \le 0.001$, $^{y}p \le 0.01$ vs GAL group; $^{c}p \le 0.001$, $^{z}p \le 0.01$ vs CIS group).

pared to CON group. In parallel with the results of GSH and GSH-Px levels, LPx (Fig. 2c) levels were increased in the CIS group compared to the CON group ($p \le 0.001$), but increased further in the GAL + CIS groups with the effect of GAL treatment. Our data showed that the effect of CIS-induced cell death could be further enhanced by GAL treatment. These reduced-GSH and GSH-Px results confirmed the glutathione depletion effect through excessive oxidative stress mediated by TRPM2 channel activation in the laryngeal cancer cells.

*Inflammation Levels in Laryngeal Cancer Cells*

Inflammation marker levels in laryngeal cancer cells increased significantly with CIS treatment. 24 h after GAL treatment, inflammation marker levels increased further after 24 h of CIS treatment (Fig. 2). A significant increase in IL-1β (Fig. 2D), IL-6 (Fig. 2e), and TNF-α (Fig. 2f) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). The combination of GAL and CIS treatment made the treatment even more effective. This data showed that the effect of CIS-induced cell death and

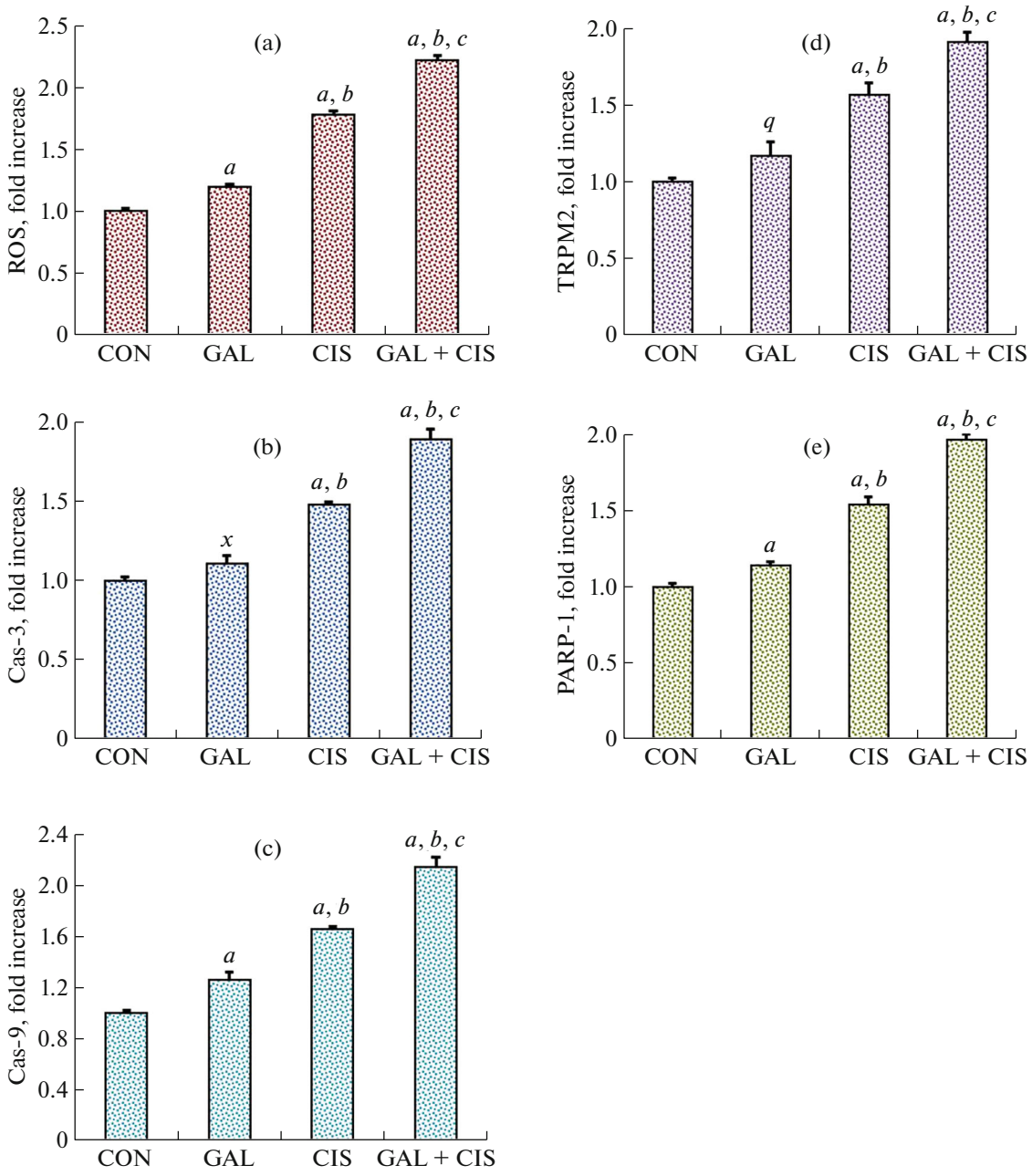


**Fig. 3.** Effect of GAL on ROS (a), Cas-3 (b), Cas-9 (c), TRPM2 (d), and PARP-1 (e) levels in laryngeal cancer cells after CIS-induced cytotoxicity (mean ± SD) ($^{a}p \le 0.001$, $^{x}p \le 0.01$, $^{q}p \le 0.05$ vs CON group; $^{b}p \le 0.001$, $^{y}p \le 0.01$ vs GAL group; $^{c}p \le 0.001$, $^{z}p \le 0.01$ vs CIS group).

inflammation could be further enhanced by GAL treatment in human laryngeal cancer (Hep-2) cells.

### *Caspase and Reactive Oxygen Species Levels in Laryngeal Cancer Cells*

It is well known that increased mitochondrial ROS activation due to the activation of TRPM2 channels in cancer cells results in cell death and apoptosis [2]. In this context, we evaluated ROS and caspase status in cancer cells. ROS and caspase levels in laryngeal cancer cells increased significantly with CIS treatment. 24 h after GAL treatment, ROS and caspase levels increased further after 24 h of CIS treatment (Fig. 3). A significant increase in ROS (Fig. 3a), Cas-3 (Fig. 3b), and Cas-9 (Fig. 3c) levels was observed in the CIS-treated groups compared to CON group ($p \le 0.001$). The combination of GAL and CIS treatment made the treatment even more effective. This data showed that the effect of CIS-induced ROS and caspase could be

further enhanced by GAL treatment in human laryngeal cancer (Hep-2) cells.

### *TRPM2 and PARP-1 Levels in Laryngeal Cancer Cells*

It is well known that cell death is induced by several factors. Two of the important ones among these well-known factors are excessive $Ca^{2+}$ entry and oxidative stress. $Ca^{2+}$ passes the cell membrane through several cation channels. One of these cation channels is the TRP superfamily. Some of the subgroup members of the TRP superfamily, such as TRPA1, TRPM2, and TRPV1, are activated by oxidative stress [23]. Kütük et al. reported that the TRPM2 cation channel has a high expression level in the Hep-2 cell line [2]. Also, the DNA damage-induced ADPR in the nucleus of cells is produced by the activation of PARP-1 and responds when ADPR binds to the TRPM2 channel's enzymatic nudix C domain [23]. In this context, TRPM2 and PARP-1 expression levels were analyzed, and TRPM2 channel and PARP-1 expression levels are shown in Fig. 3. Expression levels of TRPM2 cation (Fig. 3e) channel and PARP-1 (Fig. 3f) in the cells were increased by CIS treatment ($p \leq 0.001$). The expression level of TRPM2 and PARP-1 was further increased in the GAL + CIS group by CIS plus GAL treatment ($p \leq 0.001$).

CIS can lead to cell death in human laryngeal cancer (Hep-2) cells. Accumulating evidence indicates that the generations of ROS and DNA damage (via the activation of PARP-1)-induced ADP-ribose stimulate TRPM2. The nudix box domain of the TRPM2 channel is sensitive to ROS. ROS-induced TRPM2 activation stimulates excessive $Ca^{2+}$ influx. The increase of cytosolic $Ca^{2+}$ via the activation of TRPM2 causes the increase of mitochondrial membrane depolarization ($\Delta\Psi m$). In turn, the increase of $\Delta\Psi m$ causes the excessive generations of cytosolic ROS, mitochondrial oxidative stress, cytokines, and the decrease of cytosolic GSH and GSH-Px levels. The main mechanism in the cell death effect of GAL is mediated by stimulation of ROS-mediated Cas-3 and Cas-9 activations. In response, CIS and GAL in the Hep-2 cells initiate a pro-oxidant response that stimulates the ROS and LPx by inhibiting GSH and GSH-Px. The CIS and GAL, TRPM2 activation-mediated excessive $Ca^{2+}$ influx cause apoptosis and cell death via the activations of caspase pathways such as Cas-3 and Cas-9 in the Hep-2 cell (Fig. 4).

## DISCUSSION

In this study, we planned to determine the effect of the TRPM2-mediated PARP-1 pathway on CIS chemotherapy in Hep-2 cells and whether it would create a synergy in anti-tumour activity when combined with GAL. Accumulating evidence indicates that the generations of ROS and DNA damage (via the activation of PARP-1)-induced ADP-ribose stimulate TRPM2 [2, 23]. The increase of cytosolic $Ca^{2+}$ via the activation of TRPM2 in the cytosol of Hep-2 cell causes the increase of mitochondrial membrane depolarization ($\Delta\Psi m$). In turn, the increase of $\Delta\Psi m$ causes excessive generations of cytosolic ROS, oxidative stress, an increase in the level of cytokines, and the decrease of cytosolic GSH and GSH-Px levels. The CIS and GAL, TRPM2 activation-mediated excessive $Ca^{2+}$ influx cause apoptosis and cell death via the activations of caspase pathways such as Cas-3 and Cas-9 in the Hep-2 cell (Fig. 4).

The results of the present study showed that CIS-induced decrease in cell viability of Hep-2 cells was decreased further by CIS and GAL treatments. CIS induced an increase in TRPM2, PARP-1 expression levels, lipid peroxidation, caspase activity, and ROS production, whereas GSH levels and GSH-Px activity decreased in Hep-2 cells. Thus, CIS treatment was characterized by a decrease in tumour cell viability via increased TRPM2 channel expression, caspase activation, and excessive ROS production in Hep-2 cells. However, TRPM2 activation and ROS production were further increased by GAL treatment, while cell viability and antioxidant capacity were further decreased by combination treatment (GAL + CIS). The present results are the first to compare the effect of GAL on CIS-induced TRPM2 channel activation and cell death via excessive ROS production in Hep-2 tumour cells.

In our study, we first examined cell viability in Hep-2 tumour cells treated with CIS and GAL. We observed that GAL potentiated the decreasing effect of CIS on cell viability in tumour cells (Fig. 1a). Several molecular mechanisms have been proposed for tumour cell death, including increased intracellular $Ca^{2+}$ and excessive ROS production [4, 24, 35]. We therefore decided to investigate the levels of TRPM2 channels, which may mediate the pathological increase in intracellular $Ca^{2+}$ and ROS in Hep-2 cells. Kütük et al. reported that the TRPM2 cation channel is highly expressed in the Hep-2 cell line [2]. TRPM2 expression in cancer cells is activated by PARP-1 and oxidative stress [4, 37]. Our results showed that TRPM2 and PARP-1 expressions were significantly increased in the CIS and GAL + CIS groups, and this increase was much higher in the combined treatment group (Figs. 3d, 3e). Thus, we found that cell viability decreased with increasing levels of TRPM2 and PARP-1 in the CIS and GAL+CIS groups, demonstrating the presence of this pathway in tumour cell death (Figs. 3d, 3e, and 1a). Another important activator of TRPM2 channels is the increase in ROS [2, 23]. Because of the importance of ROS-activated TRPM2 channels in tumour cell death, we investigated the effect of GAL on CIS-induced TRPM2 expression levels through increased oxidative stress in Hep-2 cells. We observed that the increase in ROS in

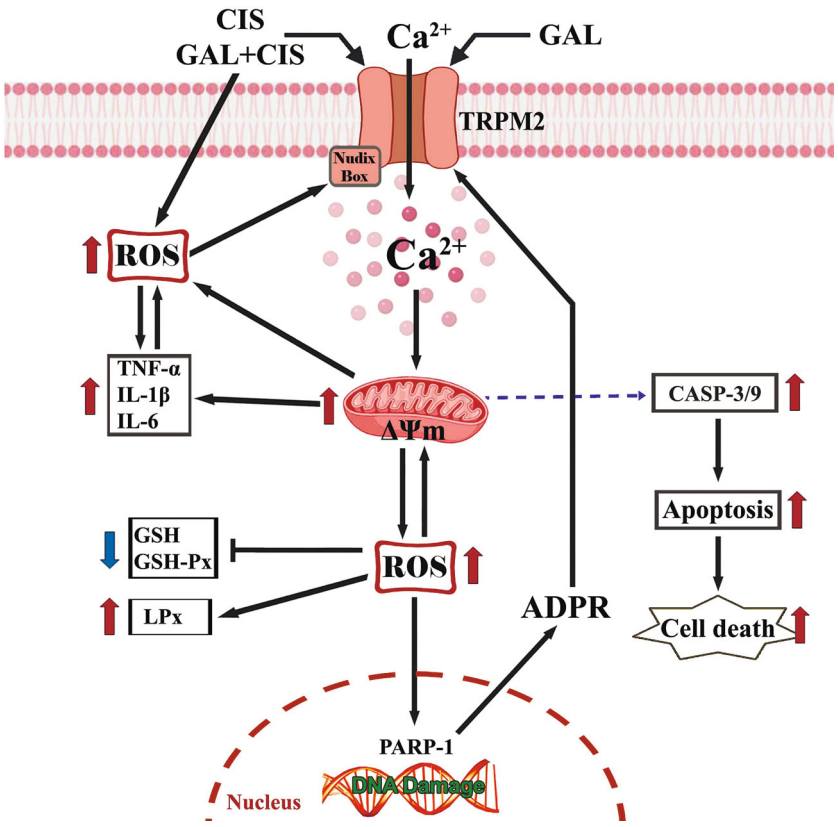


**Fig. 4.** Summary of pathways involved in CIS and GAL-induced TRPM2 channel activation and tumor cell death through excessive ROS.

CIS and GAL + CIS groups paralleled the increase in TRPM2 levels in the same groups (Fig. 3a). We also found that CIS and GAL treatments produced anti-tumour activity with a pro-oxidant effect. While TOS increased in the CIS and GAL+CIS groups, TAS decreased and the OSI index increased, and in addition cell viability decreased in the same groups (Fig. 1). Recent studies show that GAL has pro-oxidant and calcium channel activating effects [21, 28, 29].

Some studies have reported that CIS increases ROS levels and lipid peroxidation while decreasing the activity of antioxidant enzymes [38–41]. Therefore, we investigated the changes in GSH and GSH-Px levels in Hep-2 cells. It has been reported that GSH levels and GSH-Px activity are decreased in laryngeal cancer patients and that these levels are further decreased by CIS treatment [42, 43]. We found that GSH and GSH-Px levels decreased in Hep-2 cells treated with CIS and GAL (Figs. 2a, 2b). This decrease may be due to ROS scavenging. The decrease in GSH levels and GSH-Px activity caused by CIS was further stimulated by GAL treatment. Similarly, LPx levels were significantly increased in the CIS and combined treatment groups (Fig. 2c). Thus, our results demonstrated that lipid peroxidation also contributes to the anti-tumour efficacy of CIS and GAL treatments. GSH depletion has been reported to be important for the activation of the TRPM2 channel [44]. Similarly, we found that TRPM2 channel expression levels and ROS levels increased in treatment groups where we found decreases in GSH levels and GSH-Px activity (more so in the combined treatment group). Thus, we found that increased ROS in Hep-2 cells, together with antioxidant depletion, caused TRPM2 channel activation, and GAL treatment further increased this.

Increased ROS caused by CIS and GAL treatments in Hep-2 cells may lead to mitochondrial depolarisation and subsequent caspase activation (Fig. 4). To prove this, we investigated the effects of CIS and GAL on caspase activation due to increased ROS production in Hep-2 cells. It is known that the pathological increase in intracellular $Ca^{2+}$ concentration mediated by TRPM2 causes an increase in mitochondrial membrane depolarisation in normal and tumour cells, which in turn causes an increase in ROS [45]. CIS treatment resulted in increased intracellular ROS production, mitochondrial depolarisation-induced caspase activation, and increased cell death in Hep-2 cells (Figs. 3a, 3b, 3c). In addition, GAL+CIS treatment further increased these levels. A direct correlation between increased ROS and increased mitochondrial membrane depolarisation and cell death has been reported [23, 45]. Previous reports have shown that the

level of apoptosis increases in tumour lines with CIS-induced mitochondrial membrane depolarisation [4, 9, 13]. CIS treatment activates pro-apoptotic proteins (Cas-3 and -9) in Hep-2 cells by causing an excessive increase in ROS production due to mitochondrial depolarisation [38, 42, 46]. Therefore, CIS treatment may further increase cell death in tumour cells. Our results also showed that CIS treatment increased Cas-3 and Cas-9 levels (Figs. 3b, 3c), and this increase was more pronounced in the GAL+CIS group. In addition, cell viability was found to decrease in these groups, demonstrating that the anti-tumour activity of the combined treatment was higher.

CIS has been reported to increase the expression of pro-inflammatory signalling molecules through stimulation of certain signalling pathways [47]. Most previous publications have found that the increase in CIS is significantly paralleled by an increase in TNF-α, IL-1β, and IL-6 values [48, 49]. Increased ROS in Hep-2 cells by CIS and GAL treatment may lead to overproduction of inflammatory proteins. To determine this, we examined the effects of CIS and GAL on changes in the levels of these markers due to increased ROS production. A significant increase in the levels of the inflammatory markers IL-1β (Fig. 2d), IL-6 (Fig. 2e), and TNF-α (Fig. 2f) was observed in laryngeal cancer cells. The increase was greater in the GAL+CIS group. These data indicated that the effect of CIS-induced cell death and inflammation could be further enhanced by GAL treatment in Hep-2 cells. Thus, it proves that the overproduction of inflammatory proteins contributes to the anti-tumour activity of the combined CIS and GAL treatment.

## CONCLUSIONS

In conclusion, the findings of this study demonstrate that GAL enhances the chemotherapeutic effect of CIS against Hep-2 cells by inducing oxidative stress and inflammation and suppressing cell viability. Importantly, these changes were mediated by activation of the TRPM2/PARP-1 pathway. Collectively, our results suggest that GAL and CIS combination therapy may provide an improved strategy for laryngeal cancer chemotherapy by targeting cellular pathways associated with excessive oxidative stress and TRPM2-related intracellular $Ca^{2+}$ increases.

## AUTHOR CONTRIBUTION

Supervision: YY. Study Design: YY, RÇ. Literature Search: YY, RÇ. Data collection: YY, RÇ. Data interpretation: YY, RÇ. Statistical analysis: YY. Manuscript preparation: YY. All authors reviewed the manuscript.

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research was carried out using cells propagated through commercially available cell culture. Ethics committee approval is not required in this study. The study was conducted following the international declaration, guidelines, etc.

## CONFLICT OF INTEREST

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

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