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Gallic Acid Attenuates Cisplatin-Induced Apoptosis, Oxidative Stress, and Inflammation in Cardiomyocytes

Gallik Asit Kardiyomiyositlerde Sisplatin ile İndüklenen Apoptoz, Oksidatif Stres ve İnflamasyonu Azaltıyor

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ABSTRACT

Objective: Cisplatin (CIS) is a powerful chemotherapeutic agent that has long been used alone or in combination in the treatment of various cancers. However, the toxicity of CIS in various tissues limits its use. Gallic acid (GAL) has anti-microbial, anti-inflammatory, and anti-tumor properties. Since GAL has broad biological properties and exhibits antioxidant activity, this study aimed to investigate the effect of GAL on CIS-induced cardiotoxicity in H9c2 cardiomyocyte cell lines.

Materials and Methods: H9c2 cardiomyocyte cells as control (CON), CIS, and GAL25, GAL50 in combination along with CIS were used. In the analyses made, glutathione (GSH) and glutathione peroxidase (GSH-Px) enzyme activity, lipid peroxidation levels, inflammation markers IL1β, IL 6, and TNF α, Total Oxidant/ Antioxidant (TOS and TAS) status, reactive oxygen species (ROS) and caspase (Casp 3-9) activity in the cells were determined.

Results: CIS treatment caused cardiomyocyte cell toxicity and increased Casp 3-9, ROS, IL 1β, TNF α, IL 6, TOS, and MDA levels while decreasing GSH-Px, GSH, and TAS levels. Increased inflammation and impaired oxidant/ antioxidant balance in cardiomyocyte cells after CIS treatment were regulated by GAL treatment.

Conclusions: GAL treatment was found to have a protective effect on CIS-induced cardiotoxicity in cardiomyocyte cells.

Keywords: Cardiotoxicity, cisplatin, gallic acid, H9c2 cardiomyocyte, oxidative stress

ÖZ

Amaç: Sisplatin (CIS), çeşitli kanserlerin tedavisinde uzun süredir tek başına veya kombinasyon halinde kullanılan güçlü bir kemoterapötik ajandır. Bununla birlikte, CIS'in çeşitli dokulardaki toksisitesi kullanımını sınırlamaktadır. Gallik asit (GAL) anti-enflamatuar, anti-mikrobiyal ve anti -tümör gibi özelliklere sahiptir. GAL'in geniş biyolojik özelliklere sahip olması ve antioksidan aktivite sergilemesi sebebiyle bu çalışmada GAL'in H9c2 kardiyomiyosit hücre hatlarında CIS kaynaklı kardiyotoksite üzerindeki etkisinin araştırılması amaçlanmıştır.

Materyal ve Metot: Kontrol (CON) olarak H9c2 kardiyomiyosit hücreleri, CIS ve CIS ile birlikte GAL25, GAL50 kombinasyonları kullanılmıştır. Çalışmada yapılan analizlerde kardiyomiyosit hücrelerinde Total Antioksidan/ Oksidan (TAS ve TOS) durumu, inflamasyon belirteçleri TNF α, IL 1β ve IL 6, lipid peroksidasyon düzeyleri, glutatyon (GSH) ve glutatyon peroksidaz (GSH-Px) enzim aktivitesi, reaktif oksijen türleri (ROS) ve kaspaz aktivitesi (Casp 3-9) belirlenmiştir.

Bulgular: Sonuçlar, CIS tedavisinin kardiyomiyosit hücresinde toksisiteye neden olduğunu ve Casp 3-9, ROS, IL 1β, TNF α, IL 6, MDA ve TOS seviyelerini artırırken GSH-Px, GSH ve TAS seviyelerini azalttığını gösterdi. CIS tedavisi sonrasında kardiyomiyosit hücrelerinde artan inflamasyon ve bozulan oksidan/antioksidan dengesi GAL tedavisi ile düzenlenmiştir.

Sonuç: GAL tedavisinin kardiyomiyosit hücrelerinde CIS kaynaklı kardiyotoksisite üzerinde koruyucu bir etkiye sahip olduğu bulunmuştur.

Anahtar Kelimeler: Gallik asit, H9c2 kardiyomiyosit, kardiyotoksisite, oksidatif stres, sisplatin

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INTRODUCTION

Cisplatin (CIS) is a powerful chemotherapeutic agent that has long been used in the treatment of various cancers.[1] However, the toxicity of CIS in various tissues limits its use.[2] Although the anticancer effects of CIS are well understood, the mechanisms of toxicity in non-cancerous tissues are poorly understood. There is evidence that oxidative stress (OS)[3] and inflammation[4] are essential factors in CIS toxicity. CIS exerts its anticancer activity by inducing reactive oxygen species (ROS) that trigger cell death and DNA damage.[5] However, in addition to these desired effects on cancer cells, CIS causes toxicity by inducing the same effects on other cells in the body, including the myocardium.[2] Mitochondrial dysfunction due to increased ROS and subsequent activation of the apoptotic pathway has been reported as another essential mechanism involved in the pathogenesis of CIS toxicity.[6] In addition, existing reports indicate that inflammation markers play an essential role in CIS toxicity.[7]

By clarifying the multifactorial physiopathological mechanisms underlying the cardiotoxic effects of CIS, it may be able to reduce its side effects during treatment. Although various mechanisms related to the cardiotoxic effects of CIS, including increased OS and inflammation, have been suggested, no clear conclusion has been reached, and research on this issue continues.[8,9] The effects of adding antioxidant and anti-inflammatory compounds to CIS chemotherapy to slow CIS-induced myocyte damage have been studied, but the results are uncertain. Therefore, in vitro and in vivo preclinical studies should be continued.

Many plants, such as sumac, thuja, and green tea, contain Gallic acid (GAL), a flavinoid, as part of their structure.[5] GAL has a wide range of biological activities, including anti-microbial, antioxidant, and anti-inflammatory properties.[10] Recent studies have shown that GAL is effective in preventing CIS-induced damage in many tissues.[11,12] On the other hand, we were not able to find any studies on the regulatory effect of GAL on the cardiotoxicity induced by CIS.

This study aimed to investigate CIS-induced cardiotoxicity and the regulatory effect of GAL in the H9c2 cell. For this purpose, H9c2 cells, which are widely preferred in experimental myocardial injury in vitro models, were used. Caspase activation (Casp 3 and 9), markers of total antioxidant and oxidant status (TAS and TOS) levels, GSH, GSH-Px, and lipid peroxidation and markers of inflammation levels (TNF α, IL 1β, and IL 6) were analyzed to determine the damage caused by CIS in H9c2 cells.

MATERIALS AND METHODS

Ethics Committee Approval: 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.

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 ºC). 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 H9c2 cell line was divided into four groups.

CON (n;6), H9c2 cells in this group were not treated with any treatment, and were incubated (24 h).

CIS (n;6), H9c2 cells in this group were treated with 40 µM CIS, and were incubated (24 h).[2,9]

GAL25 (n;6), H9c2 cells in this group were pretreated with 25 µM GAL[13] 3 hours before 40 µM CIS treatment, followed by 40 µM CIS treatment, and were incubated (24 h).

GAL50 (n;6), H9c2 cells in this group were pretreated with 50 µM GAL[13] 3 hours before 40 µM CIS treatment, followed by 40 µM CIS treatment, and were incubated (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 $1x10^6$ cells/ml. The cardiomyocyte 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.

Caspase, Reactive Oxygen Species, and Inflammation Markers Levels: Caspase, ROS, and inflammation marker levels in cardiomyocyte 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™).[14,15]

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 Reagent-2 buffer was added, and absorbance was measured by ELISA reader (TAS 660 nm, TOS 530 nm after incubation) (second absorbance value).[14] 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.

Glutathione, Glutathione Peroxidase, and Lipid Peroxidation Levels: Cardiomyocyte cell GSH, GSH-Px, and Lipid peroxidation (malondialdehyde, MDA) levels were measured with a V-730 UV spectrophotometer (Japan).

In the experiment (MDA), 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 blinds 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 minutes, 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.[14,15]

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.

RESULTS

GAL treatment modulated the increase in ROS, and Casp 3-9 levels in CIS-treated cardiomyocyte cells (Figure 1). A significant increase in ROS (Figure 1A), Casp 3 (Figure 1B), and Casp 9 (Figure 1C) levels was observed in the CIS-treated group compared to CON, GAL25, and GAL50 groups ($p \leq 0.001$). Significant reductions were obtained in both H9c2 embryonic cardiomyocytes pre-treated with 25 and 50 µM GAL for ROS, and Casp 3-9 levels. However, the CIS-induced and disturbed oxidant/antioxidant balance was further regulated by 50 µM GAL.

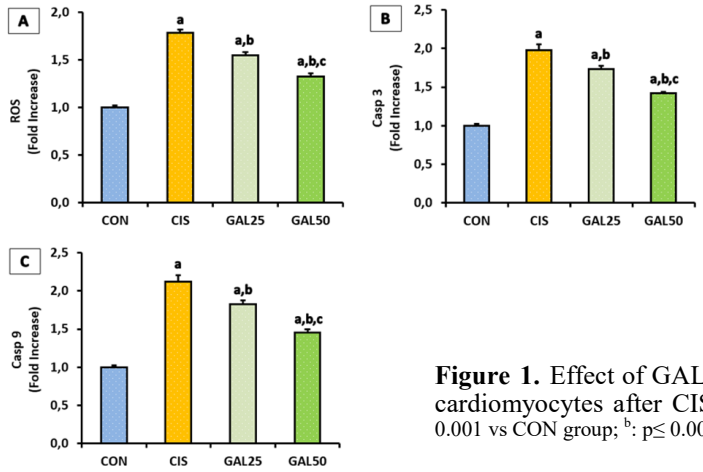

Figure 1. Effect of GAL on ROS (A), Casp 3 (B), and 9 (C) levels in cardiomyocytes after CIS-induced cardiomyotoxicity. (mean ± SD); ([a]: $p \leq 0.001$ vs CON group; [b]: $p \leq 0.001$ vs CIS group; [c]: $p \leq 0.001$ vs GAL25 group).

GAL treatment modulated the increase in inflammation marker levels in CIS-treated cardiomyocyte cells (Figure 2). A significant increase in IL 1β (Figure 2A), IL 6 (Figure 2B), and TNF α (Figure 2C) levels was observed in the CIS-treated group compared to CON, GAL25, and GAL50 groups (p≤0.001). Significant reductions were obtained in both H9c2 embryonic cardiomyocytes pre-treated with 25 and 50 µM GAL for TNF α, IL 1β, and IL 6 levels. However, the CIS-induced and disturbed inflammatory cytokines balance was further regulated by 50 µM GAL.

GAL therapy regulated the increase in lipid peroxidation (MDA) and disturbance of the antioxidant balance (GSH, GSH-Px, and TAS) levels in CIS-treated cardiomyocyte cells (Figure 3 and 4). A significant decrease in GSH (Figure 3A), GSH-Px (Figure 3B), and TAS (Figure 4B) levels was observed in the CIS-treated group compared to CON, GAL25, and GAL50 groups (p≤ 0.001). A significant increase in MDA (Figure 3C), TOS (Figure 4A), and OSI (Figure 4C) levels was observed in the CIS-treated group compared to CON, GAL25, and GAL50 groups (p≤0.001). Elevated MDA, TOS, and OSI levels and reduced GSH, GSH-Px, and TAS levels following CIS therapy in cardiomyocytes were regulated by GAL therapy. However, the CIS-induced and disturbed oxidant/antioxidant balance was further regulated by 50 µM GAL.

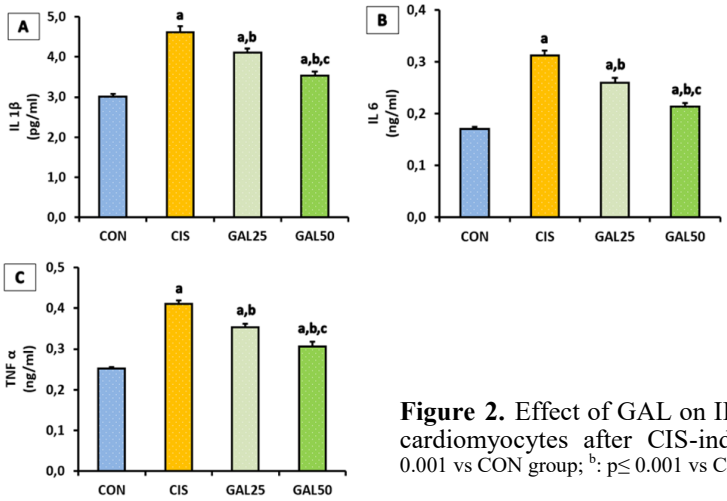

Figure 2. Effect of GAL on IL 1β (A), IL 6 (B), and TNF α (C) levels in cardiomyocytes after CIS-induced cardiomyotoxicity. (mean ± SD); ([a]: p≤ 0.001 vs CON group; [b]: p≤ 0.001 vs CIS group; [c]: p≤ 0.001 vs GAL25 group).

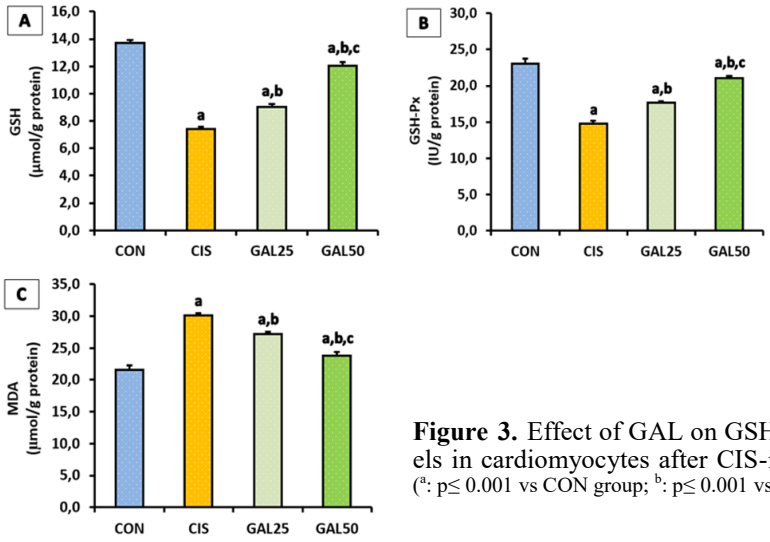

Figure 3. Effect of GAL on GSH (A), GSH-Px (B), and MDA (C) levels in cardiomyocytes after CIS-induced cardiomyotoxicity. (mean ± SD); ([a]: p≤ 0.001 vs CON group; [b]: p≤ 0.001 vs CIS group; [c]: p≤ 0.001 vs GAL25 group).

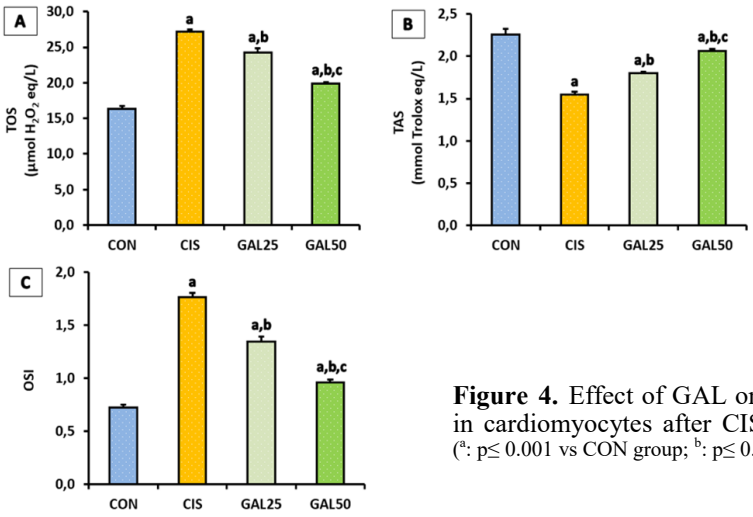

Figure 4. Effect of GAL on TOS (A), TAS (B), and OSI (C) levels in cardiomyocytes after CIS-induced cardiomyotoxicity. (mean ± SD); ([a]: p≤ 0.001 vs CON group; [b]: p≤ 0.001 vs CIS group; [c]: p≤ 0.001 vs GAL25 group).

DISCUSSION AND CONCLUSION

In this study, we planned to understand the mechanisms underlying CIS-induced cardiotoxicity and to determine new therapeutic strategies using GAL to prevent cardiotoxicity, we treated H9c2 cells with CIS. To investigate the efficacy of GAL in the prevention of cardiotoxicity, we applied GAL at two different doses. Our results showed that CIS treatment caused a substantial increase in inflammation indices, apoptosis, and OS, as well as a decrease in antioxidant capacity. However, different doses of GAL pre-treatment decreased cardiomyocytes damage.

Many studies have suggested that CIS increases ROS levels and lipid peroxidation while decreasing the activity of antioxidant enzymes.[16-18] In this context, several antioxidant molecules have been tested in studies predicting that they may prevent toxicity by reducing CIS-induced OS.[19,20] It has been reported that CIS causes OS, inflammation and mitochondrial dysfunction in rat kidney and testis tissues, while GAL has a protective role against CIS toxicity.[5,11,12] On the other hand, there are no reports on the effect of GAL on CIS cardiotoxicity. This study demonstrated that in myocyte cells, MDA levels increased in the CIS-treated groups compared to the CON group. Simultaneously, GSH and GSH-Px enzyme activities decreased. With the observed decrease in cardiac GSH values and increase in MDA values, the reduction in GSH-Px enzyme activity may be evidence of OS caused by CIS therapy. Indeed, similar studies have reported a marked decrease in antioxidant capacity with a marked increase in MDA levels in the heart and other tissues following CIS therapy.[16,19] In our study, we observed a significant improvement in the CIS groups with two different doses of GAL pre-treatment. The ROS, MDA, TOS, and OSI increase with CIS treatment was significantly reduced in both the GAL25/GAL50 groups. This significant improvement was more pronounced in the GAL50 group. Furthermore, we found that although TAS, GSH levels, and GSH-Px activities decreased in the CIS group compared to the CON group, these values increased substantially in the GAL groups compared to the CIS group.

CIS has been reported to increase the expression of pro-inflammatory signalling molecules through stimulation of certain signalling pathways.[21] Most previous publications have found that the increase in CIS toxicity is significantly paralleled by an increase in TNF α, IL 1β, and IL 6 values.[22,23] Kim et al. found that $STAT6^{-/-}$ mice, which produce much less TNF α, IL 1β, and IL 6 are protected from CIS-induced ototoxicity.[23] The literature suggests that activation of pro-inflammatory cytokines in cardiomyocytes may be involved in the physiopathological process of CIS-induced cardiotoxicity. To this end, we analysed the levels of inflammatory markers after the administration of CIS to cardiomyocytes, and we found a significant increase in TNF α, IL 1β, and IL6 values in the CIS treatment groups compared to the CON group. A role for GAL in reducing inflammation-related damage in various tissues has been reported in some studies.[24] There are also studies reporting the therapeutic effects of GAL on cardiotoxicity by suppressing inflammatory signalling pathways.[25] In this study, we have shown that GAL can protect cardiomyocytes from inflammatory damage. We observed that TNF α, IL 1β, and IL6 values were markedly decreased in pretreated GAL25 and GAL50 groups compared to the CIS group. We also found that a dose of 50 µM GAL was more effective. These results suggest that the cardiotoxic effects of CIS chemotherapy may be reduced by GAL treatment.

It has been reported in the literature that it supports cell apoptosis through activation of various pro-apoptotic pathways.[18] Previous studies have shown

that mitochondrial dysfunction due to increased ROS and consequent activation of the apoptotic pathway is involved in the pathogenesis of CIS toxicity.[6] Casp 3-9 are frequently used in studies to evaluate apoptosis, as activation of these caspases is an irreversible step that induces apoptosis.[15] Inflammatory responses, OS and apoptosis were observed after CIS treatment in this study. We examined Casp 3-9 levels after CIS treatment of cardiomyocyte cells. We found a substantial upregulation in Casp 3-9 in the CIS groups compared to the CON group. Previous studies have suggested that CIS causes upregulation of Casp 3 and down-regulation of Bcl-2.[5,21] Qian et al. determined that the activity of Casp 9 and Casp 3 increased significantly after CIS application in H9c2 cells.[26] GAL has been shown to have pharmacological potential in the regulation of various cellular and molecular processes, such as apoptosis and autophagy.[27] Tanaka et al. found that GAL suppressed Casp 3 activity and apoptosis-related gene expression and increased cell viability in human and mouse hepatoma cells.[28] Ahlatci reported that GAL treatment against glutamate-induced cytotoxicity in C6 cells increased cell viability and decreased Casp 3 activity.[29] In our study, we observed an essential improvement in the CIS groups with GAL pre-treatment. Casp 3-9 levels were substantially reduced in the GAL25 and GAL50 groups compared to the CIS group. The improvement was more pronounced in the GAL50 group. Thus, using the H9c2 cell, we showed that GAL prevented a CIS -induced increase in OS and inflammation in cardiac tissue and also prevented the induction of caspases.

In conclusion, the results of this in vitro study suggest that CIS is cardiotoxic, and GAL may be a potential candidate to ameliorate CIS-induced cardiotoxicity. Based on the physio-pathological mechanisms of CIS cardiotoxicity, it is essential to find treatments that reduce the side effects of the drug. Therefore, it would be beneficial to investigate further the effects of GAL treatment in cancer patients treated with CIS in the hope of reducing CIS-induced cardiotoxicity.

Ethics Committee Approval: 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: No conflict of interest was declared by the authors.

Author Contributions: Supervision-BY; Materials-BY, YY; Data Collection and/orna Processing- BY, YY; Analysis and/or Interpretation- BY, YY; Writing- BY, YY .

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