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Research Article

Gossypin Regulated Doxorubicin-Induced Oxidative Stress and Inflammation in H9c2 Cardiomyocyte Cells

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Abstract

Aim: Doxorubicin (DOX), an anthracycline, is widely used in chemotherapy due to its effectiveness in fighting many cancers. Experimental and clinical studies prove that this drug damages non-targeted tissues (including cardiomyocytes) and reduces patients' quality of life during and after DOX treatment. The discovery of potent compounds as a protective tool to slow cardiomyocyte damage during the use of anti-cancer drugs such as DOX is crucial for both more effective cancer treatment and to improve patient's quality of life. Gossypin (GOS) is a flavonoid with several important properties, such as anti-cancer, analgesic, antioxidant, and anti-inflammatory. GOS shows supportive effects against oxidative stress and inflammation by activating antioxidant defense enzymes.
Material and Method: For the study, four groups were formed from H9c2 embryonic cardiomyocyte cells as Control, DOX (1 µM, 48 h), GOS25 (25 µg/ml, 48 h), and GOS50 (50 µg/ml, 48 h). In the study, Total antioxidant and oxidant status (TAS and TOS), levels of the inflammatory cytokines IL 1 beta and 6, and TNF α, lipid peroxidation levels as malondialdehyde (MDA), glutathione peroxidase (GSHPx), and glutathione (GSH) levels in the H9c2 embryonic cardiomyocyte cells were determined.
Results: The results showed that DOX treatment caused cell toxicity in the embryonic cardiomyocyte cells and increased TOS, IL 1 beta and 6, TNF α, and MDA levels while decreasing TAS, GSH, and GSHPx levels. This situation improved with GOS treatment.
Conclusion: As a result, it was determined that GOS treatment showed a protective effect in the DOX-induced cell toxicity model in H9c2 embryonic cardiomyocyte cell lines.

Keywords: Oxidative stress, inflammation, H9c2 cardiomyocyte cell, gossypin, doxorubicin

INTRODUCTION

Doxorubicin (DOX), an anthracycline and one of the most effective anti-cancer drugs since the 1960s, is widely used in chemotherapy and is effective against many types of cancer, including blood cancer, sarcoma, and cancer (1). However, experimental and clinical studies have shown that DOX is toxic to non-target organs and reduces the quality of life during and after treatment (2). Known harmful side effects such as myelosuppression, cardiotoxicity, brain, kidney, and liver toxicity, and alopecia limit the clinical use of this drug (3-5).

It is unknown which factors are involved in the mechanisms of cardio-toxicity induced by DOX. Reports in the literature suggest that oxidative stress and inflammation may be important mechanisms underlying cardiotoxicity (6,7). Increased reactive oxygen species (ROS) have been reported to cause lipid peroxidation, organelle damage, and cellular signaling imbalances in various cells, including cardiomyocytes (8-11). In addition, some reports that increased oxidative stress with DOX exposure increase the release of pro-inflammatory cytokines such as interleukin 1 beta (IL 1 beta) and tumor necrosis factor alpha (TNF α) (6,12).

The discovery of potent compounds as a protective tool to slow down myocyte damage during the use of anti-cancer drugs such as DOX is crucial both for more effective cancer treatment and for improving patients' quality of life. Gossypin (GOS) is a flavonoid with various essential properties such as anticancer, analgesic, antioxidant, and anti-inflammatory (13,14). GOS shows its supportive

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effects against oxidative stress and inflammation by activating antioxidant defense enzymes (15). Although DOX is an effective chemo-therapeutic agent, cardio-toxicity is known to contribute to patient mortality (11). There are many experimental and clinical studies in the literature investigating how to reduce the cardio-toxic effects of DOX (10,11,16,17). Cardio-protective effects of GOS have been reported in a limited number of in vitro and in vivo experimental studies (13,18). However, whether GOS can ameliorate the cardio-toxic effects of DOX, which is widely used in many cancers, has not been investigated. Therefore, this study was designed to examine whether GOS has protective effects against DOX-induced cardio-toxicity in a dose-dependent manner in the H9c2 cell line. This cell line is a cardio-myoblast derived from embryonic rat heart tissue and is widely used in heart disease research (13,19,20).

In this study, we evaluated the protective effects of GOS on rat H9c2 embryonic cardiomyocytes by determining the oxidant/antioxidant status (TAS, TOS, GSHPx activation, GSH, and lipid peroxidation levels). In addition, we aimed to evaluate the inflammation status in myocytes by investigating inflammatory cytokines IL 1 beta and 6 and TNF α levels in H9c2 embryonic cardiomyocyte cells.

MATERIAL AND METHOD

Chemicals and ELISA Kits

GOS (Cat; 652-78-8) was purchased from Sigma Aldrich C. (USA/St Louis). DOX (Cat; T1020) was purchased by TargetMol (Target Molecule Corp., USA). IL 6 (Sunred, Cat; SRB-T-83168), IL 1 beta (Sunred, Cat; SRB-T-83324), and TNF α (Sunred, Cat: SRB-T-82883) were purchased from SunRed Biotechnology Company (SRB) Ltd (Shanghai, China). Total Oxidant and Total Antioxidant Capacity (TOS and TAS) ELISA kit was obtained from Rel Assay (Gaziantep/Türkiye).

Cell Culture and Experimental Groups

A growth medium was prepared for the cells used in the research according to the instructions provided by the seller. FBS (cytiva Cat; SV30160.03) (10%) and penicillin/streptomycin (biosera Cat; LM-A4118) (1%) were added to equivalent volumes of DMEM (biowest Cat; L0064) as growth medium contents. Cells were cultured in 25 cm^2 culture flasks in an incubator at 37°C under a 5% CO_2 atmosphere. H9c2 embryonic cardiomyocyte cell lines were divided into four groups and incubated according to the experimental procedure. DOX and GOS were freshly prepared on the experimental days. The following incubation procedure was applied to the experimental groups. After the incubation period was completed, the cells were washed with fresh 1xPhosphate Buffered Saline (Biochrom/Germany), and 0.25% Trypsin-EDTA (Sigma-Aldrich) was applied to separate the cells from the flask floor. After completing the experimental steps, analyses were performed for all groups.

For the study, H9c2 cardiomyocyte cells in the experiment were divided into four groups as follows.

Control group (n=5), the cardiomyocyte cells were kept in a culture medium for 48 hours without treatment.

DOX group (n=5), the cardiomyocyte cells were incubated with DOX (1 µM for 48 h) (11,21).

GOS25 group (n=5), cardiomyocyte cells in this group were pretreated with GOS (25 µg/ml) 3 hours before DOX treatment and then incubated with DOX (1 µM) for 48 hours (13).

GOS50 group (n=5), cardiomyocyte cells in this group were pretreated with GOS (50 µg/ml) 3 hours before DOX treatment and then incubated with DOX (1 µM) for 48 hours (13).

Preparation of Cells Homogenates

For each group, cells were transferred into separate sterile falcon tubes and centrifuged according to the kit procedure (1000 rpm and 20 min). After centrifugation, the supernatants on the top of the falcon tubes were removed with the help of an automatic pipette, the cells were suspended in PBS, and a cell suspension with a density of approximately 1×10^6 cell/ml was obtained. The cell structure was lysed (PBS) by freeze-thaw repetition, and the mixture was centrifuged (4000 rpm, 10 min) after removal of cytoplasmic components. The supernatant remaining at the top of the falcon tubes was removed with pipettes and taken in Eppendorf tubes for analysis. The Bradford protein assay kit (Merck-Millipore) measured total protein levels in the groups.

Analyses

Measurement of Total Oxidant-Antioxidant and Inflammatory Cytokines Levels in the H9c2 Embryonic Cardiomyocyte Cells

TOS and TAS and inflammatory cytokines (IL 6 and 1 beta and TNF α) levels in H9c2 embryonic cardiomyocyte supernatants were determined using ELISA kits. For the analyses, supernatants were first incubated (37°C, 60 min) by the protocols specified by the companies for commercial kits and then placed in 96-well plates with automatic pipettes. The supernatant and standard samples placed on the plate were incubated for 60 minutes, followed by washing steps, and then staining solutions were added and incubated (15 min). A stop solution was added at the end of all these procedures, and absorbance values were read on an ELISA (BioTek Epoch™) microplate spectrophotometer (22).

Measurement of Glutathione/Glutathione Peroxidase and Lipid Peroxidation Levels in the H9c2 Embryonic Cardiomyocyte Cell

Lipid peroxidation activity, which is known as malondialdehyde (MDA) release in DOX-induced cell toxicity in H9c2 embryonic cardiomyocyte cells, was determined by thiobarbituric acid (TBARS) reaction in a highly sensitive spectrophotometer (V-730 UV-Visible

Spectrophotometer, Japan) according to the method of Placer et al. All cell groups were reconstituted with 1/9 (2.25 ml) TBARS solution. The experiment used a mixture of 0.25 ml phosphate buffer and 1/9 of TBARS as a blind. Samples and blind were kept in 100 °C water for 20 minutes (23,24). It was then cooled on ice and centrifuged (1000 g, 5 min). The upper pink liquid was taken with an automatic pipette and read against the blind in a spectrophotometer at 532 nm wavelength in a 1 cm light transmission cuvette. The standard was standard: 1, 1, 1, 3, 3 tetraethoxy propane solution prepared in the same proportions. Values were determined as µmol/g protein.

GSH levels of H9c2 embryonic cardiomyocyte cells were determined spectrophotometrically (412 nm) using the Sedlak and Lindsay method (25). H9c2 embryonic cardiomyocyte cells (10^6 cells per mL) were transferred to sterile falcon tubes with the help of an automatic pipette and centrifuged to separate the proteins after mixing with 10% trichloroacetic acid. After centrifugation, 0.1 ml of the supernatant remaining on the falcon tube was taken and placed in a glass tube, 0.5 mL 5.5-dithiobis (2-nitrobenzoic acid), 2 mL phosphate buffer (pH 8.4), and 0.4 mL distilled water were added. The resulting sample was read (412 nm) in a spectrophotometer. Values were determined as µmol/g protein.

GSHPx levels of H9c2 embryonic cardiomyocyte cells were determined spectrophotometrically at 412 nm by the method of Lawrence and Burk (26). GSHPx activity was expressed as international units (IU) oxidized glutathione/g protein.

Statistical Analysis

All data are expressed as mean±standard deviation in this study, and data analysis was performed by one-way ANOVA using SPSS. For all data with a statistically significant difference, the post-hoc Tukey test was used. p≤0.05 was considered to be statistically significant.

RESULTS

Effect of Gossypin on TOS and TAS Levels in H9c2 Embryonic Cardiomyocyte Cells

It is shown in Figure 1 that GOS treatment modulated the decrease in TAS and increase in TOS levels as a result of DOX treatment in H9c2 embryonic cardiomyocyte cells. A significant increase in TOS levels (Figure 1A) was observed in the DOX-treated group was compared to Control, GOS25, and GOS50 groups (p≤0.05), and in parallel, a significant decrease in TAS levels (Figure 1B) was observed in the DOX-treated group was compared to Control, GOS25, and GOS50 groups (p≤0.05). The decrease in TAS and increase in TOS levels after DOX treatment in H9c2 embryonic cardiomyocyte cells were regulated by GOS treatment. Significant results were obtained in TAS and TOS levels in H9c2 embryonic cardiomyocyte cells pretreated with 25 and 50 µg/ml of GOS. However, the 50 µg/ml of GOS further regulated the DOX-induced and disrupted oxidant/antioxidant balance.

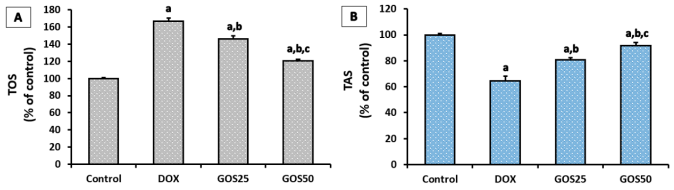

Figure 1. Effect of GOS on TOS (**1A**) and TAS (**1B**) levels in H9c2 embryonic cardiomyocyte cells after DOX-induced cell toxicity (mean±SD). ([a]p≤0.05 vs Control group, [b]p≤0.05 vs DOX group, [c]p≤0.05 vs GOS25 group)

Effect of Gossypin on Inflammatory Cytokines Levels in H9c2 Embryonic Cardiomyocyte Cells After Doxorubicin-induced Cell Toxicity

GOS treatment modulated DOX-induced IL 6 and 1-beta, and TNF α levels in the H9c2 embryonic cardiomyocyte cells are shown in Figure 2. When the DOX-induced treated group was compared to the Control, GOS25, and GOS50 groups between the groups, it was observed that the IL 1 beta, IL 6 (Figure 2A-2B), and TNF α (Figure 2C) levels increased considerably (p≤0.05). The increase in IL 1 beta and 6 and TNF α levels after DOX treatment in H9c2 embryonic cardiomyocyte cells was regulated by GOS treatment. Significant results were obtained in IL 6 and 1 beta, and TNF α levels in H9c2 embryonic cardiomyocyte cells pretreated with 25 and 50 µg/ml doses of GOS; however, the 50 µg/ml dose of GOS further regulated the DOX-induced and disrupted inflammation balance.

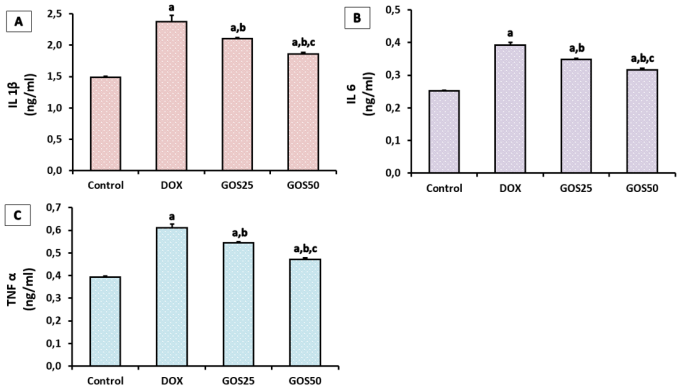

Figure 2. Effect of GOS on IL 1β (**2A**), IL 6 (**2B**), and TNF α (**2C**) levels in H9c2 embryonic cardiomyocyte cells after DOX-induced cell toxicity (mean±SD). ([a]p≤0.05 vs Control group, [b]p≤0.05 vs DOX group, [c]p≤0.05 vs GOS25 group)

The Gossypin Treatment Attenuated the Doxorubicin-induced Changes in GSH, GSHPx, and MDA Levels

The changes in GSH, GSHPx, and MDA levels in cells against DOX-induced cell toxicity of GOS in the groups formed were measured with the spectrophotometrically (V-730 UV-Visible Spectrophotometer, Japan). It is shown in Figure 3 that GOS treatment modulated the increase in oxidative stress (MDA) and decrease in antioxidant (GSH and GSHPx) levels as a result of DOX treatment in H9c2 embryonic cardiomyocyte cells. When the DOX-induced treated group was compared to the Control, GOS25, and GOS50 groups, it was observed that the GSH levels (Figure 3A) and GSHPx levels (Figure 3B) decreased considerably (p≤0.05). MDA levels (Figure 3C) were significantly

increased between the groups when the DOX-induced treated group was compared to the Control, GOS25, and GOS50 groups (p≤0.05). The decrease in GSHPx and GSH levels and increase in MDA levels after DOX treatment in H9c2 embryonic cardiomyocyte cells were regulated by GOS treatment. Significant results were obtained in GSH, GSHPx, and MDA levels in H9c2 embryonic cardiomyocyte cells pretreated with 25 and 50 µg/ml doses of GOS. However, the 50 µg/ml dose of GOS further regulated the DOX-induced and disrupted oxidant/antioxidant balance.

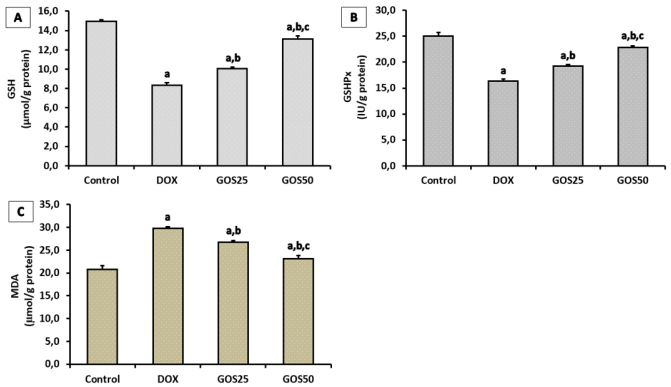

Figure 3. Effect of GOS on GSH (**3A**), GSHPx (**3B**), and MDA (**3C**) levels in H9c2 embryonic cardiomyocyte cells after DOX-induced cell toxicity (mean±SD). ([a]p≤0.05 vs Control group, [b]p≤0.05 vs DOX group, [c]p≤ 0.05 vs GOS25 group)

DISCUSSION

DOX is a vital drug widely used in cancer treatment since the 1960s (1,7). However, its cardiotoxic effects limit its use. It has been emphasized that the cardio-toxic effects of DOX are multifactorial and cause functional disorders in cardiomyocyte cells (27). Based on the physiopathological mechanisms of DOX-induced cardio-toxicity, it may be possible to reduce the side effects of the drug. In this study, we investigated whether GOS administration is protective against the cardio-toxic effects of DOX. For this purpose, we applied GOS at two different doses (25 µg/ml and 50 µg/ml) in H9c2 cells. Our results showed that DOX treatment-induced cardiotoxicity in H9c2 cardiomyocytes and that GOS treatment ameliorated the cardiac damage at both doses, but to a greater extent at the 50 µg/ml dose.

One of the most widely accepted damage mechanisms for DOX-induced cardio-toxicity is the activation of damage mechanisms due to increased oxidative stress (5,6,28). Shaker et al. reported a significant increase in lipid peroxidation and a reduction in TAS levels in the rat heart after DOX administration (6). Similarly, it has been reported that there is a decrease in GSH and GSHPx levels in rats administered DOX and that limonin, known for its antioxidant properties, is beneficial in preventing cardiac damage (29). In another study, Yıldızhan et al. found that DOX therapy caused a significant decrease in the levels of GSHPx and GSH in the serum and samples of heart tissue. In contrast, selenium administration with antioxidant effects caused a considerable increase in GSH and GSHPx levels (7). DOX treatment was shown to increase lipid peroxidation levels, particularly in heart tissue, in another experimental study. However, it decreased GSH and GSHPx levels (30). In another in vivo and in vitro study, heart tissue from mice exposed to oxidative stress and an H9c2 embryonic cardiomyocyte cell line showed a significant reduction in superoxide dismutase (SOD) and GSH activity (13). Our data showed that DOX treatment significantly increased lipid peroxidation and TOS levels and significantly decreased TAS levels in H9c2 embryonic cardiomyocyte cells, consistent with previous studies. Similar to previous studies, the reduction in DOX-induced GSH and GSHPx activity demonstrated the depletion of cardiac antioxidant enzymes. Cinar and colleagues reported an increase in SOD activity and GSH levels in mice and H9c2 embryonic cardiomyocyte cells and a reduction in lipid peroxidation levels when GOS was administered in various dosages (13).

GOS is an essential flavonoid with potent antioxidant and anti-inflammatory properties (14). GOS shows its supportive effects against oxidative stress and inflammation by activating antioxidant defense enzymes (15). Cinar and colleagues reported that SOD activity and GSH levels increased and lipid peroxidation levels decreased in mice and H9c2 embryonic cardiomyocyte cells in a study testing the antioxidant effects of various doses of GOS (13). In the same study in which they applied GOS at doses of 25, 50, and 100 µg/ml, they found that the most effective amount of GOS in H9c2 cells was 50 and 100 µg/ml. In our study, we found that GSH, GSHPx, and TAS were significantly increased in the GOS25 and GOS50 groups compared to the DOX group. Furthermore, GOS25 and GOS50 significantly reduced lipid peroxidation and TOS levels compared to the DOX group. Thus, we found that GOS pre-treatment reduced DOX toxicity in myocytes at both doses (25 and 50 µg/ml).

We predict the activation of pro-inflammatory cytokines in cardiac tissue in the physiopathological process of DOX-induced cardio-toxicity. It has been demonstrated in clinical and experimental studies that DOX therapy causes myocardial inflammation (6,31-33). There have been important reports showing that DOX therapy induces the release of various pro-inflammatory cytokines, including TNF α, and causes several inflammatory responses in the myocardium (6,34). We demonstrated significant increases in cardiac IL 1 beta and 6 and TNF α in the DOX group compared to the control group, in line with these reports. The results of this study support the critical role of inflammation in the pathogenesis of DOX-induced cardio-toxicity. When these results are evaluated with the oxidative parameters obtained in this study, they suggest that increased inflammatory markers may be associated with impaired antioxidant capacity (low TAS, high TOS levels) and lipid peroxidation. Yıldızhan et al. reported increased IL 1 beta, TNF α, and ROS levels with significantly reduced GSH and GSHPx levels in the DOX group, consistent with the results of this study (7).

The anti-inflammatory effects of GOS have been reported in the literature (14,18). In a study investigating the

curative impact of GOS against gentamicin-induced nephron-toxicity in rats, inflammatory cytokines in renal tissues were reduced by (TNF α and IL 6) GOS therapy (15). In another study, IL 1 beta and TNF α mRNA expression induced by H_2O_2 in L929 fibroblast cells was significantly reduced with different doses of GOS treatment (25 and 50 µg/mL) (35). Our data showed that DOX treatment in an embryonic cardiomyocyte cell line caused a significant increase in IL 1 beta and 6 and TNF α. Against DOX toxicity, we found that GOS pre-treatment reduced these inflammatory cytokines. In our study, in the GOS25 and GOS50 groups, IL 1 beta and 6 and TNF α levels decreased significantly in a dose-dependent manner, more effectively in the GOS50 group than in the DOX group. This suggests that GOS treatment may have a beneficial effect on inflammation in heart muscle tissue.

Thus, we demonstrated the protective effect of GOS on heart tissue using an H9c2 embryonic cardiomyocyte cell line. In conclusion, the findings in the literature and the results of this study emphasize that GOS, a potent antioxidant and anti-inflammatory agent, can be used as a cardio-protective agent against DOX toxicity.

CONCLUSION

It is known that the cardio-toxic effects of DOX, which is widely used in cancer treatment, are multifactorial and cause functional disorders in cardiac cells. Based on the physiopathologic mechanisms of DOX-induced cardio-toxicity, it is vital to find therapies to reduce the side effects of the drug.

This study demonstrated DOX-induced cellular toxicity in H9c2 embryonic cardiomyocyte cells by disrupting the oxidant/antioxidant balance and increasing the release of inflammatory cytokines. In addition, critical preclinical data were provided to the literature by determining the curative efficacy of GOS. While these data indicate the possibility of using natural substances such as GOS to reduce damage during cancer treatment, more preclinical research is needed.

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Conflict of interest: *The authors have no conflicts of interest to declare.*

Ethical approval: *The current study has no study with human and human participants. The study is not subject to ethics committee approval. Ethics Committee Approval is not required for cell culture studies.*

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