ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
El-Emam, 2020, 4 (4), **153-160**

Journal of Advanced Pharmacy Research

Gallic Acid Protects Lung Cells Against Cyclophosphamide-induced Toxicity via Regulating RAGE/NF-κB/autophagy Signaling

Soad Z. El-Emam*

Department of Pharmacology and Toxicology, Faculty of Pharmacy, October 6 University, 6 October City, Giza, Egypt

***Corresponding author:** Soad Z. El-Emam, Department of Pharmacology and Toxicology, Faculty of Pharmacy, October 6 University, 6 October City, Giza, Egypt. Tel. +201140708629*
Email address: soadzakaria@o6u.edu.eg

Submitted on: 16-07-2020; Revised on: 21-08-2020; Accepted on: 30-08-2020

To cite this article: El-Emam, S. Z. Gallic Acid Protects Lung Cells Against Cyclophosphamide-induced Toxicity via Regulating RAGE/NF-κB/autophagy Signaling. *J. Adv. Pharm. Res.* **2020**, *4* (4), 153-160. DOI: 10.21608/aprh.2020.36038.1112

ABSTRACT

Objectives: This study aimed to investigate the protective effect of gallic acid (GA) against cyclophosphamide (CYP)-induced lung toxicity with exploring the mechanism of CYP cytotoxicity using normal human lung cells, WI-38 cell line. **Methods:** WI-38 cells have been categorized into three groups; first group was considered as control, untreated, cells; CYP-group, cells were treated with IC_{50} concentration of CYP for 24 h; GA+CYP group, cells were pretreated with a selected dose of GA for 24 h then followed by CYP for additional 24 h. After incubation times, cells were collected to evaluate cell viability by trypan blue assay, cell morphology and nuclear condensation by DAPI stain and apoptosis was assessed by AO/EB staining. Lipid peroxidation and total antioxidant capacity were determined in cell lysate biochemically. ELISA technique was applied to determine inflammatory markers (TNF-α and IL-1β), total caspase-3 and LC3-B. RAGE, NF-κB, and Beclin-1 mRNA-expressions were estimated by qRT-PCR. **Results**: WI-38 cells treated with CYP showed increased cell death by both necrosis and apoptosis with elevated caspase-3 level. Additionally, RAGE expression was up regulated accompanied with activated inflammatory cascade and autophagy. While, GA pretreatment suppressed oxidative stress, inflammatory and autophagy signaling induced by CYP. **Conclusion:** GA is considered as a promising natural therapeutic option that can halt oxidative damage and RAGE expression activation induced by CYP in lung cells thus preventing lung injury.

Keywords: *Autophagy; Beclin-1; Cyclophosphamide; Gallic acid; Lung injury; RAGE*

INTRODUCTION

Cyclophosphamide (CYP), an alkylating agent, is a widely used chemotherapeutic and immune-suppressive agent in treating different solid tumors and leukemias as well as many autoimmune disorders [1]. CYP is a prodrug that is metabolized in liver to a toxic metabolite, acrolein, which is responsible for different organs toxicities via activating oxidative damage/inflammatory signaling[2,3]. It is reported that CYP induces lung injury due to interference with the antioxidant system of lung tissues and increased reactive oxygen species (ROS) production[4]. Furthermore, the receptor for advanced glycation end-products (RAGE) activation plays an important role in inducing inflammatory signals related to CYP [5]. RAGE is a multiligand receptor belonging to the cell surface molecules of the immunoglobulin superfamily which

acts as a counter-receptor for various molecules such as damage-associated molecular patterns (DAMPs) that are released in response to injured and dying cells due to stressful conditions, playing a crucial role in modulating the lung injury response [6,7]. As a result of Nuclear factor-κB (NF-κB) translocation, excessive inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) can be released in response to RAGE activation causing an extreme tissue damage [8].

Autophagy is a regulating homeostatic process by which various cytoplasmic debris and damaged organelles are removed by lysosomal degradation as well as controlling microorganism infection [9]. However, when autophagy level is disturbed either by excessive reduction or increase, the intracellular environment may be impaired, contributing to excessive cell proliferation or degeneration and death, respectively [10]. RAGE activation has s role in mediating autophagy, since the study of Gao and his colleagues reported that RAGE inhibition reversed autophagy activation by suppressing autophagy related proteins (LC3BII/I and Beclin-1) [11]

Gallic acid (GA) is a natural polyphenolic found abundantly in dietary plants as pomegranate, grapes, nuts, and green tea [12]. GA is a powerful antioxidant and can suppress DAMPs release providing a protection against stress-induced cell death [13]. The effect of GA on the lung toxicity induced by CYP has not yet been studied. Therefore, this study aims at investigating the protective effect on GA against CYP-induced lung toxicity using WI-38 cell line as an in-vitro model to explore the influence of GA on CYP-activated inflammatory and autophagy signaling

MATERIAL AND METHODS

Drugs and chemicals

4′,6-diamidino-2-phenylindole (DAPI), Acridine orange, ethidium bromide, Cyclophosphamide (CYP), and gallic acid (GA) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) and reagents used to maintain cell culture have been purchased from (Biowest).

Cell culture and cell viability assay

Human embryonic lung fibroblast, WI-38 cell line, was purchased from the American Type Culture Collection (ATCC, USA). WI-38 cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin at 37 °C in a 5% CO_2 humidified incubator. All the experiments of cell culture were performed in the cell culture unit of October 6 University

The used concentrations of GA, and CYP in this study was determined by MTS assay; performed according to the manufacturer's instructions (Abcam, USA). Briefly, in 96-well culture plates, WI-38 cells (1×10^4 cells/well) were seeded with DMEM supplemented with 10% FBS then treated with various concentrations for each drug. After incubation times (24 or 48 hours), 20 µl of MTS reagent was added to each well and incubated for additional 4 hours at 37°C. The absorbance was measured using the microplate ELISA reader (FLUOstar Omega, BMG, Labtech, Germany) at 490 nm. The percentage of relative cell viability was determined using the following equation: [Absorbance of treated cells/ Absorbance of control cells)] X 100. The IC_{50} was calculated by using a Graph-Pad PRISM version 6.

Treatment protocol of WI-38 cells

WI-38 cells were seeded in six-well culture plates with 10% FBS supplemented DMEM media at 37°C with 5% CO_2 in a humidified incubator. Each three wells were allocated for each group as the following: Control group: WI-38 cells were incubated in complete DMEM media. CYP group: WI-38 cells were treated with CYP using IC_{50} concentration value estimated by MTS assay then incubated for 24 hr. GA+CYL group: cells were pretreated with GA using the most safe dose as evaluated by MTS assay for 24 h followed by CYP then incubated for additional 24 h.

Cell treatment was repeated three times separately for preparing three runs of samples. The first run was used for trypan blue assay and acridine orange/ethidium bromide fluorescent stain, where, the cells were collected by trypsinization, washed twice with PBS, then used immediately. The second run, cells were collected by scraping, and lysed in ice-cold lysis buffer supplemented with protease inhibitor cocktail then passed through a 21-gauge needle, afterwards centrifuged at 14,000 × g for 15 min at 4 °C and the supernatants were used for determining lipid peroxidation, total antioxidant capacity (TAC) and ELISA. The third run, cells were used for mRNA extraction for quantifying gene expression by real time-PCR.

Evaluating the protective effect of GA

In order to evaluate the protective effect of GA against CYP-induced cytotoxicity in WI-38 cells, a trypan blue assay was performed by adding 10 µl of trypan blue 0.4 percent to an equal volume of cell suspension and mixed well then, using a hemocytometer, both dead and viable cells were counted under a light microscope [14].

% of cell viability = (number of viable cells/total cell number) × 100.

Table 1. The list of the gene-specific primers used in qRT-PCR

Gene	Sequence (5′-3′)
RAGE	F: CTGATCCTCCCACAGAGCC R: CAGGACCAGGGAACCTACAG
NF-KB	F: TGGTGCCTCACTGCTAACT R: GGATGCACTTCAGCTTCTGT
Beclin1	F: GGCTGAGAGACTGGATCAGG R: CTGCGTCTGGGCATAACG
GAPDH	F: CTCTGATTTGGTCGTATTGGG R: TGGAAGATGGTGATGGGATT

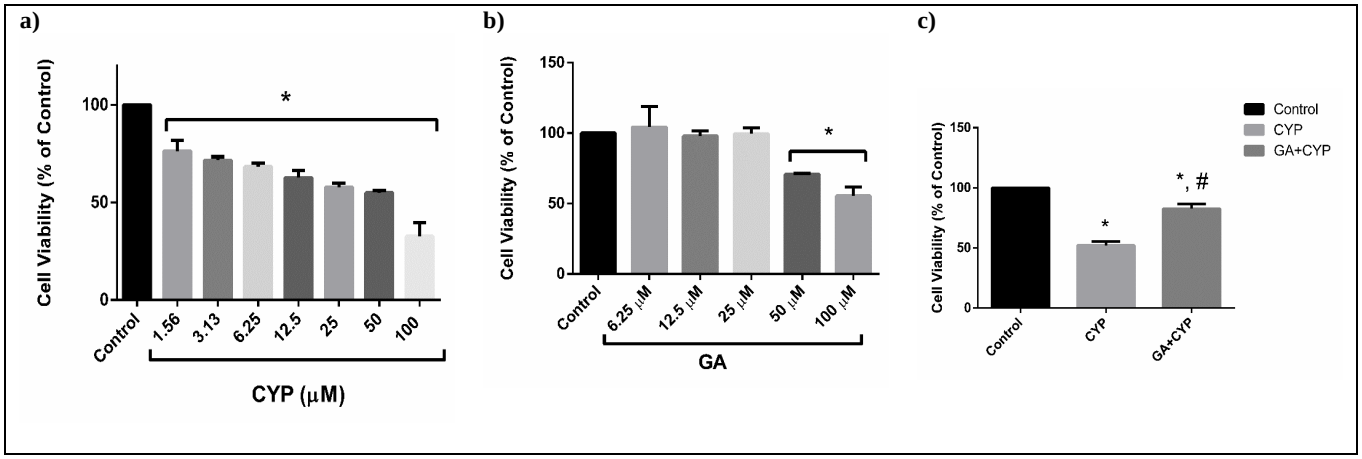

Figure 1: Cell viability assays. **a)** The cytotoxic effect of different concentrations of CYP on WI-38 cells. **b)** The effect of different concentrations of GA on WI-38 cell viability for selecting a safe dose. **c)** The protective effect of GA against CYP-induced cytotoxicity in WI-38 cells. The experiments were carried out in triplicates independently, and the data are expressed as Mean ± S.D. (N = 3), * $p < 0.05$ in comparison with control group. # $p < 0.05$ compared with CYP group.

Evaluating the mode of cell death

To examine nuclear morphology, 4′,6-diamidino-2-phenylindole (DAPI) stain was used [15]. Cells were rinsed with PBS immediately after harvesting cells and incubated at 37°C with DAPI 1 µM for 15 min. Cells were rinsed after incubation, and then examined using a fluorescent microscope (Olympus, Japan).

Acridine orange/ethidium bromide (AO/EB) fluorescent staining was utilized to evaluate cell death mode by determining apoptosis and necrosis ratio. WI-38 cells were collected and stained using an equal dye mixture of AO/EB in PBS, then cells were examined using fluorescence microscopy within 20 min [16]. At least 500 cells were counted for each sample, and the percentage of apoptotic or necrotic cells was calculated as follows: percentage of apoptotic or necrotic cells = (total number of apoptotic or necrotic cells / total counted number) × 100.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to estimate the levels of inflammatory cytokines such as TNF - α and IL-1β in cell supernatant, in addition to estimating the levels of caspase-3 and LC3-B in cell lysate, according to the ELISA kits manufacturer's protocols (Sunlong Biotech, China).

Evaluating antioxidant/oxidative stress markers

Spectrophotometrically lipid peroxidation was detected as the end-product, malondialdehyde (MDA), at 534 nm using a commercial kit (Biodiagnostic Co., Cairo, Egypt). Total antioxidant capacity (TAC) was estimated at 570 nm in Trolox equivalents, as instructed by the manufacturer using the TAC assay kit (Sigma - Aldrich, USA).

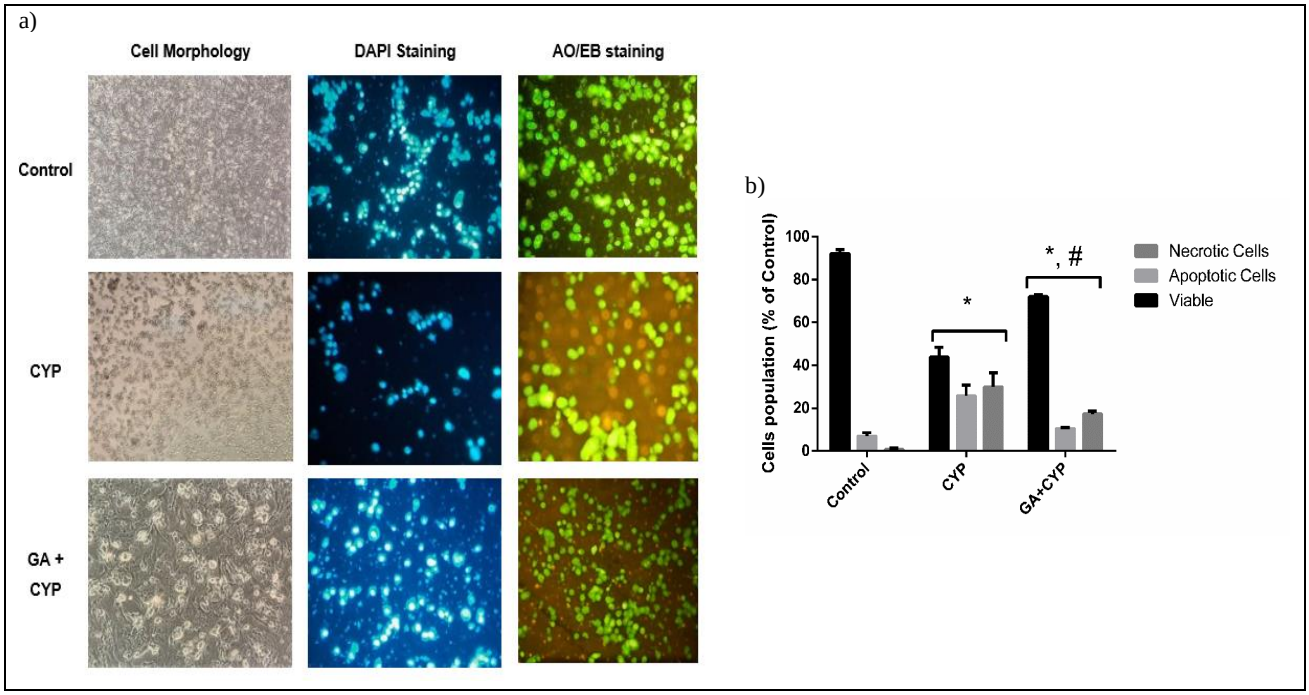

Figure 2. The effect of GA pretreatment on CYP-induced cell morphological change and cell death. a) Cell morphology, DAPI, and AO/EB staining of WI-38 cells. **b)** The percentage of different cell populations (viable, apoptotic and necrotic cells). The experiments were carried out in triplicates independently, and the data are expressed as Mean ± S.D. (N = 3), * $p < 0.05$ in comparison with control group. # $p < 0.05$ compared with group CYP group.

Quantitative real time-PCR (qRT-PCR)

The magnitude of gene expression was quantified using RT-PCR for RAGE, NF-κB, and Beclin-1. In short, total RNA was extracted from the collected cells using RNeasy Mini kit (Qiagen, Germany, GmbH) as directed by the manufacturer. In a 25 µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U / µL) (Thermo Fisher), 8.25 µl of water, and 3 µl of RNA template, the primers (Metabion, Germany), listed in **Table 1** were used. The reaction was carried out in a RT- PCR system with StratageneMX3005P. The ct of each sample was compared with that of the positive control group using the following ratio: ($2^{-\Delta\Delta ct}$). GAPDH had been used as a gene for housekeeping.

Statistical analysis

The statistical analysis the data was implemented using Prism software version 6.01 (GraphPad Software Inc., CA, USA). Data were given as mean ± standard deviation. Statistical significance was revealed using one-way ANOVA following with a Tukey-Kramer post hoc test for estimating the differences between groups. The statistically significant level was recognized at p-value < 0.05.

RESULTS

Effect of GA on CYP-induced cytotoxicity in WI-38 cells

As presented in **Figure 1a**, cell viability of WI-38 cells was decreased by increasing CYP concentration. The concentration of CYP used for treating WI-38 cells was that of IC_{50} which determined by MTS assay, 40 µM. The protective dose of GA used in our treatment protocol was chosen according cell-viability response curve selecting the highest safe dose for WI-38 cells after 24 h incubation time of different serial concentrations.

As presented in **Figure 1b**, the concentration of 25 µM was considered safe to WI-38 cells when compared to control group, therefore, this concentration was selected to investigate the protective effect of GA against CYP-induced lung toxicity. The cytoprotective effect of GA was screened by trypan blue exclusion assay, since CYP treatment reduced the percentage of viable cells to 52.1% ± 3.4 when compared with control group; however, pretreatment of WI-38 cells with GA, 25 µM, for 24 h before CYP significantly reduced CYP-induced cytotoxicity and cell viability was significantly enhanced to 82.6% ± 3.8 when compared with CYP group.

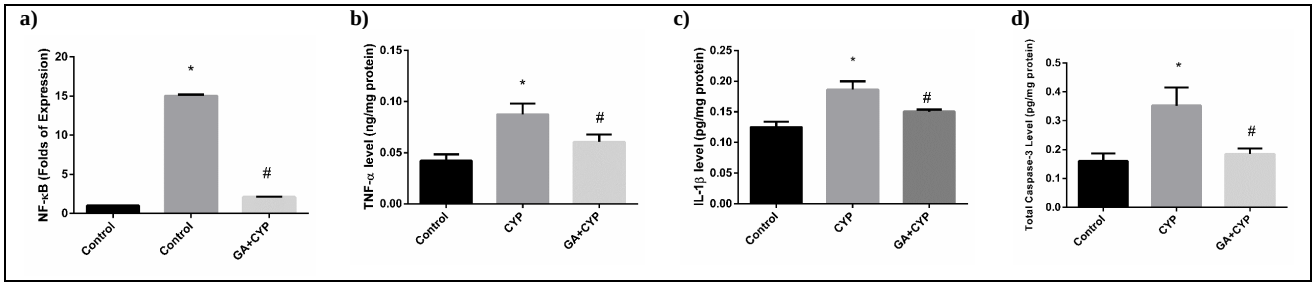

Figure 3. GA pretreatment alleviates the inflammatory response and increased total caspase-3 level induced by CYP treatment in WI-38 cells. a) NF-κB expression, **b)** TNF-α level, **c)** IL-1β level, **d)** Total caspase-3 level. All the results are expressed as Mean ± S.D., n = 3. * $p < 0.05$ in comparison with control group. # $p < 0.05$ compared with group CYP group.

Morphological changes and mode of cell death induced by CYP in WI-38 cells and the effect of GA pretreatment

The morphology of control WI-38 cells as inspected by the innervated microscope were characterized by their smooth-edged spindle-like shape. Treating WI-38 cells with CYP (40.7 μM) for 24 h changed cell morphology and induced cell shrinkage with an abnormal nuclear condensation that was observed upon DAPI staining (**Figure 2a**). Additionally, AO/EB staining showed that CYP induced cell death by both apoptosis and necrosis when compared with control cells. As presented in **Figure 2b** different cell populations were determined as live cells (green), apoptotic cells (yellow and light orange) and necrotic cells (dark orange and red) imaged by a fluorescent microscope. GA pretreatment, however, maintained viable cells and reduced morphological changes and nuclear condensation induced by CYP as well as significantly decreased both apoptotic and necrotic cell populations as compared with the CYP group ($p < 0.05$).

The effect of GA on inflammatory markers and caspase 3 induced by CYP in WI-38 cells

Inflammatory insult was initiated by CYP treatment in lung cells evinced by increased inflammatory mediators as NF-κB, TNF-α and IL-1β. The mRNA expression of NF-κB was significantly elevated in CYP-treated cells. Accordingly, TNF-α and IL-1β levels were elevated by 2 and 1.5 folds respectively when compared with control group ($p < 0.05$). Moreover, total caspase-3 was increased significantly when WI-38 cells were exposed to the IC_{50} of CYP for 24 h. on the other hand, the GA pretreated cells showed significant reduced inflammatory markers and caspase-3 level (**Figure 3**) as compared with CYP group ($p < 0.05$).

GA pretreatment attenuates CYP-induced oxidative stress and RAGE activation

The oxidant/antioxidant balance was disturbed upon exposing WI-38 cells to CYP due to elevated MDA levels reflecting an increase in lipid peroxidation with simultaneous significant reduction in TAC level as illustrated in **Figure 4**. Moreover, RAGE gene expression was upregulated due to CYP treatment 10 times that of the control. In contrast, GA pretreatment restored the antioxidant balance significantly as well as reduced the upregulated RAGE gene expression by 50% when compared with CYP group ($p < 0.05$).

GA halts CYP-induced autophagy activation in WI-38 cells

In the current study autophagy signaling was activated by CYP in lung cells as we observed the over expression of Belin-1 in addition to increased level of LC3-B; both elevated by six and three folds regarding that of the control cells ($p < 0.05$). on the other side GA pretreated cells showing lower levels of Belin-1 expression and LC3-B when compared with CYP treated group as presented in **Figure 5**.

DISCUSSION

CYP is one of the nitrogen mustard alkylating agent oxazaphosphorin chemical class. It is widely used in treating various neoplastic diseases and owing to its immunosuppressive effect, it is used in organ transplantation and managing several autoimmune diseases [17]. However, CYP adversely affects multiple organs including lungs. Lung toxicity can be induced by CYP by different pathological patterns resulting in lung fibrosis on long term [18]. As demonstrated in our results, treating normal human lung, WI-38, cells with CYP reduced cell viability in concentration-dependent manner with concurrent change in cell morphology associated with nuclear condensation. It was reported that CYP-induced cytotoxicity is mediated via acrolein, a drug toxic metabolite, inducing cell death either by apoptosis or necrosis [19] as the toxic metabolites increased in a dose-dependent manner resulting in

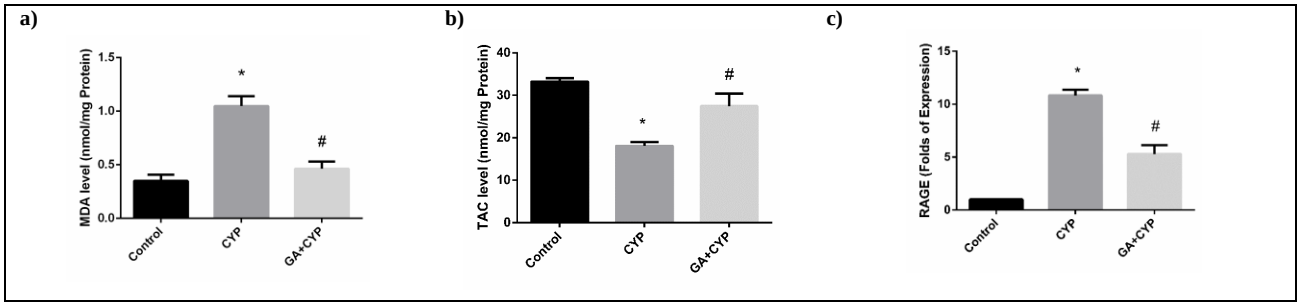

Figure 4. The effect of GA on oxidative stress and RAGE activation induced by CYP in WI-38 cells. a) MDA level, **b)** TAC level, **c)** RAGE expression. All the results are expressed as Mean ± S.D., n = 3. * $p < 0.05$ in comparison with control group. # $p < 0.05$ compared with group CYP group.

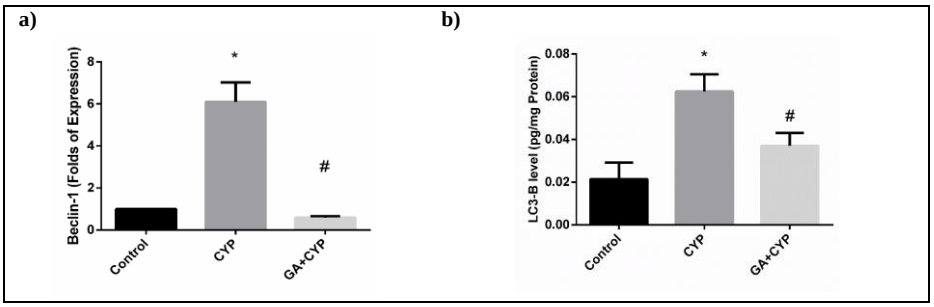

Figure 5. The effect of GA on autophagy activation induced by CYP in WI-38 cells. **a)** Beclin-1 expression, **b)** LC3-B level. All the results are expressed as Mean ± S.D., n = 3. * $p < 0.05$ in comparison with control group. # $p < 0.05$ compared with group CYP group.

higher cell death rate due to ROS generation [20]. Additionally, CYP is dependent on the caspase cascade to induce apoptosis. In harmony with our results, Cengiz et al, reported that CYP increased the proapoptotic protein, Bax, as well as caspase-3 level as a result of oxidative stress induction [21].

In the current study, we used GA to protect against CYP-induced lung cells injury. GA is a well-known natural antioxidant of a plant origin as green tea, grapes, and apples. Several literatures reported the wide range of biological activities exerted by GA in different experimental models such as its anti-inflammatory, antioxidant and antiapoptotic effects [22,23]. The effect of GA against CYP-induced lung toxicity has never been explored before, thus our study aimed to investigate the potential protective effect of GA. From the results, GA mitigated cell death and nuclear condensation induced by CYP in WI-38 cells and improved cell morphology due to its anti-inflammatory and antioxidant properties, thus attenuating the oxidative damage as reported by the study of Nikbakht et al who showed that GA protects lungs against bleomycin-induced lung fibrosis [24].

A recent literature reported that CYP mechanism of toxicity is mediated by the release of DAMPs such as HMGB1, HSP60 and glucose-regulated protein 94 (Grp94) that play a critical role in initiating inflammatory signals [5]. RAGE is one of the scavenger receptors that interacts with DAMPs inducing various signaling pathways as that involved in inflammation and autophagy [11,25]. In the same vein, our results showed the increased expression of RAGE in CYP-treated WI-38 cells with concurrent increase in inflammatory signaling evidenced by increased NF-κB expression with elevated TNF-α and IL-1β levels. Additionally, Beclin-1 and LC3-B, autophagy markers, were elevated. Autophagy signal activation is regarded as a defense system, since ROS production and oxidative stress signaling activation are the main intracellular signal transducers for autophagy [26,27]. ROS production was correlated with CYP treatment due to increased lipid peroxidation and repressed TAC level, antioxidant defense system. On the other side, GA pretreatment hindered RAGE expression and oxidative stress augmentation induced by CYP, in addition to suppressing inflammatory and autophagy signaling pathways. Previously it was reported that down-regulation of RAGE expression or blocking RAGE signaling could be considered as a promising

therapeutic targeting to protect from tissue injuries [28]. Accordingly, the study of Umadevi et al. reported the protective effect of GA against RAGE activation-mediated cardiac fibrosis which in turn decreased NF-κB nuclear translocation thus suppressing inflammatory cascade [29]. Besides, down-regulating RAGE expression could suppresses autophagy which contributes in the pathogenesis of various lung diseases including acute lung injury [30]. Therefore, using GA as a potent free radical scavenger can decrease RAGE activation, accordingly, which can halt the inflammatory cascade and decrease cell death ,as well as autophagy, is a possible treatment strategy for CYP-induced lung injury.

CONCLUSION

We conclude that GA protects against oxidative damage induced by CYP in lung cells via regulating RAGE, NF-κB and Beclin-1 expression thus suppressing inflammatory and autophagy signaling owing to its antioxidant and anti-inflammatory properties.

Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. Emadi, A.; Jones, R. J.; Brodsky, R. A. Cyclophosphamide and cancer: Golden anniversary. *Nat. Rev. Clin. Oncol.* **2009**, *6* (11), 638-47.
2. Jnaneshwari, S.; Hemshekhar, M.; Santhosh, M. S.; Sunitha, K.; Thushara, R.; Thirunavukkarasu, C.; Kemparaju, K.; Girish, K. S. Crocin, a dietary colorant mitigates cyclophosphamide-induced organ toxicity by modulating antioxidant status and inflammatory cytokines. *J. Pharm. Pharmacol.* **2013**; *65* (4), 604–614.
3. Lau, S.; Rangarajan, R.; Philidet, C.; Krüger-Genge, A.; Braune, S.; Kammerer, S.; Küpper, J.H.; Lendlein, A.; Jung, F. Effects of acrolein in comparison to its prodrug cyclophosphamide on human primary endothelial cells in vitro. *Toxicol. Vitr.* **2020**, *62*, 104685.
4. El-kashef, D.H. Role of venlafaxine in prevention of cyclophosphamide-induced lung toxicity and airway hyperactivity in rats. *Environ. Toxicol. Pharmacol.* **2018**, *58*, 70–76.
5. Chen, M.; Zhang, C.; Zhang, J.; Kai, G.; Lu, B.; Huang, Z.; Ji, L. The involvement of DAMPs-mediated inflammation in cyclophosphamide-induced liver injury and the protection of liquiritigenin and liquiritin. *Eur. J. Pharmacol.* **2019**, *856*, 172421.
6. Tolle, L. B.; Standiford, T. J. Danger-associated molecular patterns (DAMPs) in acute lung injury. *J. Pathol.* **2013**, *229* (2), 145–156.
7. Tobon-Velasco, J.; Cuevas, E.; Torres-Ramos, M. Receptor for AGEs (RAGE) as Mediator of NF-kB Pathway Activation in Neuroinflammation and Oxidative Stress. CNS Neurol. Disord.- *Drug Targets.* **2014**, *13* (9), 1615–1626.
8. Jin, X.; Yao, T.; Zhou, Z.; Zhu, J.; Zhang, S.; Hu, W.; Shen, C. Advanced Glycation End Products Enhance Macrophages Polarization into M1 Phenotype through Activating RAGE/NF- κ B Pathway. *Biomed Res. Int*. **2015**, *2015*, 1–12.
9. Gomes, L.R.; Menck, C.F.M.; Leandro, G.S. Autophagy roles in the modulation of DNA repair pathways. Int. J. Mol. Sci. **2017**, *18* (11), 2351.
10. Zhang, Q.; Kang, R.; Zeh, H.J.; Lotze, M.T.; Tang, D. DAMPs and autophagy: Cellular adaptation to injury and unscheduled cell death. *Autophagy*, **2013**, *9* (4), 451–458.
11. Gao, W.; Zhou, Z.; Liang, B.; Huang, Y.; Yang, Z.; Chen, Y.; Zhang, L.; Yan, C.; Wang, J.; Lu, L.; et al. Inhibiting Receptor of Advanced Glycation End Products Attenuates Pressure Overload-Induced Cardiac Dysfunction by Preventing Excessive Autophagy. *Front. Physiol.* **2018**, *9*, 1333.
12. Samuel, K.G.; Wang, J.; Yue, H.Y.; Wu, S.G.; Zhang, H.J.; Duan, Z.Y.; Qi, G.H. Effects of dietary gallic acid supplementation on performance, antioxidant status, and jejunum intestinal morphology in broiler chicks. *Poult. Sci.* **2017**, *96* (8), 2768–2775.
13. Zhou, Y.; Jin, H.; Wu, Y.; Chen, L.; Bao, X.; Lu, C.. Toxicol. Vitr. **2019**, *57*, 226–232.
14. Strober, W. Trypan Blue Exclusion Test of Cell Viability. In Current Protocols in Immunology (John Wiley & Sons, Inc.) **2001**.
15. Chazotte, B. Labeling nuclear DNA using DAPI. Cold Spring Harb. Protoc. **2011**, *6*. doi: 10.1101/pdb.prot5556.
16. Liu, K.; Liu, P, C; Liu, R.; Wu, X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Med. Sci. Monit. Basic Res.* **2015**, *21*, 15–20.
17. Rehman, M.U.; Tahir, M.; Ali, F.; Qamar, W.; Lateef, A.; Khan, R.; Quaiyoom, A., Oday-O-Hamiza, and Sultana, S. Cyclophosphamide-induced nephrotoxicity, genotoxicity, and damage in kidney genomic DNA of Swiss albino mice: The protective effect of Ellagic acid. Mol. *Cell. Biochem.* **2012**, *365*, (1-2), 119–127.
18. El-Kholy, A.A.; Elkablawy, M.A.; El-Agamy, D.S. Lutein mitigates cyclophosphamide induced lung and liver injury via NF-κB/MAPK dependent

mechanism. *Biomed. Pharmacother.* **2017**, *92*, 519–527.
19. Kern, J.C.; Kehrer, J.P. Acrolein-induced cell death: A caspase-influenced decision between apoptosis and oncosis/necrosis. *Chem. Biol. Interact.* **2002**, *139* (1), 79–95.
20. Mills, K. A.; Chess-Williams, R; McDermott, C. Novel insights into the mechanism of cyclophosphamide-induced bladder toxicity: chloroacetaldehyde's contribution to urothelial dysfunction in vitro. *Arch. Toxicol.* **2019**, *93* (11), 3291–3303.
21. Cengiz, M.; Sahinturk, V.; Yildiz, S.C.; Şahin, İ.K.; Bilici, N.; Yaman, S.O.; Altuner, Y.; Appak-Baskoy, S.; Ayhanci, A. Cyclophosphamide induced oxidative stress, lipid per oxidation, apoptosis and histopathological changes in rats: Protective role of boron. *J. Trace Elem. Med. Biol.* **2020**, *62*, 126574.
22. Saygin, M.; Ozturk, O.; Ozmen, O.; Ilhan, I., Gonca, T.; Gumral, N.; Orhan, H.; Aslankoc, R. The impact of methotrexate on lung inflammatory and apoptotic pathway biomarkers—The role of gallic acid. *Biomed. Pharmacother.* **2016**, *84*, 1689–1696.
23. Shree, A.; Islam, J.; Vafa, A.; Mohammad Afzal, S.; Sultana, S. Gallic acid prevents 1, 2-Dimethylhydrazine induced colon inflammation, toxicity, mucin depletion, and goblet cell disintegration. *Environ. Toxicol.* **2020**, *35* (6), 652-664
24. Nikbakht, J.; Hemmati, A. A.; Arzi, A.; Mansouri, M.T.; Rezaie, A.; Ghafourian, M.. Protective effect of gallic acid against bleomycin-induced pulmonary fibrosis in rats. *Pharmacol. Reports* **2015,** (6) 67, 1061–1067.
25. Roh, J.S.; Sohn, D. H. Damage-associated molecular patterns in inflammatory diseases. *Immune Netw*. **2018**, 18 (4), 27.
26. Filomeni, G.; De Zio, D.; Cecconi, F. Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death Differ*. **2015**, (3)*22*, 377–388.
27. Lin, P. Homeostasis : Cells Special Issue. **2019**, 10–13.
28. Yamagishi, S. ichi; Nakamura, K.; Matsui, T. Regulation of advanced glycation end product (AGE)-receptor (RAGE) system by PPAR-gamma agonists and its implication in cardiovascular disease. *Pharmacol. Res.* **2009**, *60* (3), 174–178.
29. Umadevi, S.; Gopi, V.; Elangovan, V. Regulatory mechanism of gallic acid against advanced glycation end products induced cardiac remodeling in experimental rats. *Chem. Biol. Interact*. **2014**, *208*, 28–36.
30. Qu, L.; Chen, C.; Chen, Y.; Li, Y.; Tang, F.; Huang, H.; He, W.; Zhang, R.; Shen, L. High-mobility group box 1 (HMGB1) and autophagy in acute lung injury (ALI): A review. *Med. Sci. Monit.* **2019**, 25, 1828–1837.