

# Gliclazide prevents irradiation-induced cardiotoxicity and lung injury through inhibiting Oxidative Stress: a biochemical and histopathological study

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## Research Article

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# Abstract

## Background

Cardiotoxicity and lung injury are two of the main side effects of oxidative stress following radiotherapy in patients with thoracic region cancer. Gliclazide (GLZ) as an antihyperglycemic drug has antioxidant, anti-inflammatory, and anti-apoptotic activities. This study aimed to evaluate the effect of GLZ in cardiotoxicity and lung injury induced by irradiation (IR).

## Materials and methods

In this experimental study, Sixty-four mice were divided into eight groups: control, GLZ (5, 10, and 25 mg/kg), IR (6 Gy), and IR + GLZ (in three doses). GLZ was administrated for 8 consecutive successive days and mice were exposed with IR on the 9th day of study. On the 10th day of study, tissue biochemical assay and at 14th day of study, histopathological assay were performed to evaluate for cardiotoxicity and lung injury.

## Results

The findings revealed that IR induces atypical features in heart and lung histostructure, and oxidative stress (an increase of MDA, PC levels, and decrease of GSH content) in these tissues. GLZ administration preserved heart and lung damages and improves oxidative stress markers in mice.

## Conclusion

Data have authenticated that GLZ could protect heart and lung histostructure against oxidative stress-induced injury through inhibiting oxidative stress.

## 1. Introduction

Radiotherapy is one of the main treatments for localized primary malignancies in the chest wall or intrathoracic malignancies [1]. Irradiation (IR) is toxic for tumor cells and normal tissues, too [2]. As the use of radiotherapy is increasing and the overall survival rate of cancer patients is high, the hazards correlated with radiotherapy must be intently considered. Among side effects induced by radiotherapy, cardiovascular diseases and lung injury are always seen in the tumors of the thoracic region [3, 4]. The severity of tissue toxicity in radiotherapy depends on the type, time, source, beam characteristics, fractionation, the volume of normal tissues irradiated, radiosensitizers, dose, and dose rate of radiation [2, 5]. Clinical studies have shown that a radiation dose of 1–4 Gy causes cardiovascular diseases and inflammation, a radiation dose of 5–8 Gy causes myocardial infarction, angina, pericarditis, and a decrease in left ventricular diameter, and a radiation dose of more than 8 Gy induces fibrosis myocardial

[6]. DNA damage, oxidative stress, vascular endothelial cell injury, inflammation, and fibrosis are causes of cardiac toxicity post-radiotherapy [7]. Pneumonitis and pulmonary fibrosis are complications of lung injury caused by radiotherapy [2]. Studies have shown that a total dose of more than 20 Gy and a daily dose fraction of more than 2.67 Gy lead to pneumonitis [2]. Researchers have shown that oxidative stress and inflammation are the main mechanisms in radiation-induced cardiac toxicity. Chemotherapy and radiotherapy increase oxidative stress [8]. Oxidative stress is caused by an imbalance between ROS generation and disruption of the cellular antioxidant defense system [9]. It mediates damage to the structure of cellular macromolecules, such as lipids, proteins, and DNA [10]. Also, IR directly breaks the mitochondrial respiratory chain, leading to dysfunction of the respiratory chain, resulting in decreased ATP production, increased ROS production, decreased antioxidant capacity, and induced apoptosis [11]. Therefore, the use of antioxidant therapies is an important therapeutic goal for radiation-induced cardiotoxicity and lung injury.

Gliclazide (GLZ) as second generation sulfonylurea is oral antihyperglycemic medicine that is used for diabetic patients [12]. The pharmacological properties of GLZ have been reported to be antioxidant, anti-inflammatory, and anti-apoptotic properties [13]. The protective effect of GLZ was evaluated on 5-FU-induced oral mucositis [14], peripheral neuropathy [15], nephropathy-induced diabetic [16], nephrotoxicity-induced cisplatin [17], cerebral artery occlusion and reperfusion [18], acetic acid-induced colitis [19]. Radioprotective of GLZ evaluated in our previous study on human normal lymphocytes *in vitro* [20]. Also, cardioprotective effect GLZ was assayed in the myocardial injury in the diabetic rats [21]. GLZ enable to improve oxidative stress and lowering blood glucose that prevented apoptosis and fibrosis of myocards [21]. According to the above statements, it is hypothesized that GLZ is able to improve heart and lung injury induced by IR in mice. In the current research, we determined the protective effects of GLZ by evaluating histopathology and oxidative stress examinations.

## 2. Materials And Methods

### 2.1. Materials

GLZ was prepared from Tehran Daru (Iran) at pharmaceutical grade. The biochemical reagents and solvents were purchased from Sigma (USA) and Merck (Germany) companies.

### 2.2. Animals and experimental design

Sixty-four male adult male BALB/c mice with a mean weight of 25–30 g were obtained from the Animal Center of Mazandaran University of Medical Sciences. All stages of this research work were performed according to the protocol of the Institutional Animal Ethics Committee. The animals were under suitable hygiene conditions, at a temperature of  $23 \pm 2^\circ\text{C}$ , light/dark cycle 12-h, and humidity of  $55 \pm 5\%$ . The animals were housed in individual cages and also had free *ad libitum* access to water and food. This study was approved by Ethical committee of Mazandaran University of Medical Sciences (ID#IR.MAZUMS.REC.1400.8619). The animal study were performed according to Health guide for the

care and use of Laboratory animals approved by the Mazandaran University of Medical Sciences, Iran, Sari.

In the present research, animals were randomly distributed into eight groups (8 animals/group) as follows: Control group (I), mice received distilled water via gavage daily for 8 days consecutive; Groups II, III, and IV, mice were received GLZ at three doses of 5, 10 and 25 mg/kg/day; respectively) for 8 successive days via gavage; Group V (IR), lung and cardiac injuries were induced in animals at a single irradiation dose of 6 Gy on the 9th day of study. Group VI, VII, and VIII, mice were treated with GLZ (5, 10, and 15 mg/kg) with IR (6 Gy). The dose of IR was picked out based on the study of others [10, 22] and GLZ based on our previous study [23, 24].

## **2.3. Irradiation of mice**

For animal irradiation, the box was designed in Plexiglas material with 18 separate houses for each mouse separately. On the 9th day of the study, mice without anesthesia were exposed whole body to X-ray at dose 6 Gy with 6 MeV X-ray beam that produced by a clinical linear accelerator (linear accelerator, Siemens, Primus, Germany) at a dose rate of 2 Gy/min. Immediately after irradiation exposure, mice were released from cages and kept under previous standard conditions. An overview of the experimental design is illustrated in Fig. 1.

## **2.4. Samples collection**

One day after irradiation, half of the animals were anesthetized with ketamine (5 mg/kg) and xylazine (50 mg/kg) for biochemical evaluation. The thorax was opened and the lung and heart were removed, were immediately dissected and washed in phosphate buffer saline (PBS), and stored at  $-80^{\circ}\text{C}$  for evaluation of oxidative stress parameters. The other half was anesthetized a week later for histological assay. For histopathological examination, lung and heart were removed, fixed in 10% neutral buffered formalin.

## **2.5. Biochemical assays**

Malondialdehyde (MDA) levels, as lipid peroxidation marker, were determined by the measurement of thiobarbituric acid (TBA) in the heart and lung tissues. Frozen heart and lung samples were homogenized in mannitol buffer at  $4^{\circ}\text{C}$  using a variable-speed homogenizer. After homogenization, samples were blended with 2-thiobarbituric acid, phosphoric acid, and distilled water. The mixture was boiled for 45 min, then was cooled, later n-butanol was added to extract the cold thiobarbituric acid reactants. Then, samples were centrifuged (3500 rpm, 10 min). The n-butanol layer optical density was distinguished by spectrophotometry. A standard curve of MDA was drawn. MDA content was expressed as nmol/gr tissue.

The glutathione, as an antioxidant, levels in the heart and lung were calculated using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent. The reaction between thiol and DTNAB produces yellow color that absorbance was read at 412 nm and introduced as  $\mu\text{M}$  [17].

The protein carbonyl (PC), as a biomarker of severe oxidative protein damage, levels in heart and lung supernatants were evaluated by using 2,4-dinitrophenylhydrazine (DNPH) reagent. In this assay, 200  $\mu\text{L}$  of sample supernatant was mixed with 300  $\mu\text{L}$  of DNPH, and the mixture was heated in the bath at 37  $^{\circ}\text{C}$  for 30 min, then was centrifuged (10 min at 10000  $\times g$ ). After washing with ethanol-ethyl acetate, the mixture was centrifuged again. The precipitated proteins were dissolved in 600  $\mu\text{L}$  guanidine hydrochloride, and then was heated at 37  $^{\circ}\text{C}$  for 15 min, and centrifuged. The absorbance of samples was read at wavelength 375 nm. Lung and heart protein carbonyl content was introduced as mM [25].

## 2.6. Histopathological evaluation

The fixed heart and lung were dehydrated in the alcohol series, clarified in the xylene, and embedded in the paraffin. The sections of 5  $\mu\text{m}$  thickness were stained with hematoxylin and eosin (H&E) and evaluated under a microscope (Nikon; Tokyo, Japan) with the magnification of  $\times 40$  in a blinded fashion. For the semi-quantitative assessment of sections, slides were then evaluated for heart and lung injury using a scoring system. Given the extent and severity of lung injury in each field, inflammatory cell infiltration (neutrophils, erythrocytes, macrophages, lymphocytes) in the alveolar sac and perivascular region, alveolar sac exudation, alveolar sac collapse, hyaline arteriosclerosis, thickened alveolar walls, edema, congestion and hemorrhage, lung damage was scored on a scale from 0 (normal lung), 1 (slight), 2 (mild), 3 (moderate), and 4 (severe) [26]. The heart injury based on the extent and severity of inflammation, interstitial proliferation, myocyte necrosis, myocyte vacuolization, edema, congestion, and hemorrhage in a field was scored on a 0–4 scale. Score 0 was assigned to normal heart, 1 to the lesion was minimal, 2 (mild), 3 (moderate), and 4 (severe) [27]. In the semi-quantitative evaluation, eight slides from each sample and five fields of each slide were evaluated with the light microscope and then, the average scores were considered for each lung and heart sample in the groups.

## 2.7. Statistical analysis

Data were analyzed by GraphPad Prism (version 6.07, USA) software. All the data are expressed as the mean  $\pm$  SD. Different groups were compared using one-way ANOVA and Tukey tests to determine differences among the groups. The Kruskal–Wallis test, one of the nonparametric tests, was used for the analysis of the data obtained semiquantitatively in histopathological examinations. A  $p$ -value of  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Effects of GLZ and IR on oxidative stress markers of lung and heart

Figures 2 and 3 introduce the oxidative stress markers in the lung and heart of mice. MDA increased significantly in the IR exposed mice compared to the control and GLZ groups ( $p < 0.0001$  and  $p < 0.01$ , respectively for heart and lung) which is connotative of lipid peroxidation and induction of oxidative stress. GLZ administration in all three doses in IR-exposed mice decreased significantly MDA when

compared to the IR alone group. Meanwhile, the 25 mg/kg dose was more effective than the other doses ( $p < 0.0001$ ) (Fig. 2A and 3A).

Evaluation of GSH as endogenous antioxidant showed a significant reduction in GSH content of IR-exposed mice ( $p < 0.0001$  and  $p < 0.01$ , respectively for heart and lung) when compared to the control and GLZ alone groups. Treatment with GLZ in the IR-treated mice was able to increase GSH content compared to the IR alone group ( $P < 0.001$  and  $P < 0.01$ , respectively for heart and lung) (Fig. 2B and 3B).

For PC, a significant increase was observed in the IR-exposed mice compared to the control and GLZ groups ( $P < 0.0001$ ). This finding elucidates that GLZ at three doses can reform endogenous antioxidants such as GSH. GLZ administration at a dose of 25 mg/kg had more efficient compared with at doses of 5 and 10 mg/kg. Analysis of PC levels in the control and GLZ groups were significantly lower than the IR group ( $P < 0.0001$ ) (Fig. 2C and 3C).

### **3.2. Effect of GLZ on the histological appearance of lung and heart in IR-exposed mice**

Photomicrographs of lungs showed no difference for their histological appearance amongst control with GLZ at three tested doses. IR-exposed mice showed infiltration of leukocytes (mainly macrophages, lymphocytes, and neutrophils), presence of erythrocytes, edema, severe congestion, alveolar damages with alveolar interval thickness, and alveolar sac collapse in lung tissue. Administration of GLZ was able to reduce the severity of inflammation, the thickness of the air sac walls, and the degree of congestion to some extent (Fig. 4).

In the control group was no significant change in the myocardial histopathology of mice compared with the GLZ-treated groups. Cardiotoxicity was induced with IR exposure with a dose of 6 Gy and revealed with histological features such as hemorrhage area, infiltration of leukocytes (mainly lymphocytes and macrophages) between myocytes, congestion, edema, vacuolization of the cytoplasm, degeneration, and necrosis of myocytes. GLZ administration in IR exposed mice showed improvement in the heart muscle when compared with IR alone group. Mild edema, mild mononuclear cell infiltrations, and small degeneration were seen (Fig. 5).

The heart and lung injury mean scores of all groups were revealed in Fig. 6. IR at a dose of 6 Gy increased heart and lung injury scores ( $P < 0.0001$ ). The score of heart and lung injury in the GLZ administration at three doses in the IR-exposed mice decreased compared to IR alone groups ( $P < 0.001$  and  $P < 0.05$ , respectively for heart and lung).

## **4. Discussion**

In this study, the radioprotective effect of GLZ was evaluated *in vivo* study. GLZ at low dose, with its antioxidant property, was able to counteract the oxidative stress damage caused by IR to the heart and lung tissues. IR increased MDA and PC levels in both lung and heart tissues and decreased GSH levels.

GLZ was able to improve oxidative stress markers. The histopathological evaluation also confirmed the effect of GLZ on oxidative stress.

The level of increased lipid peroxidation is correlated to oxidative stress. In this study, IR exposure significantly elevated the MDA levels of heart and lung when compared to the control and GLZ-treated alone groups. Intense cellular infiltration and severe myocardial injury in the IR-exposed mice can be attributed to oxidative stress. These findings of IR-increased oxidative stress biomarkers are consistent with other studies [25, 28]. The elevated oxidative stress were diminished by GLZ treatment. There was a significant reduction in GSH content of heart and lung tissues in irradiated mice as compared to the control group. GLZ pretreatment in the irradiated mice elevated significantly this biomarker compared to the irradiated alone group. Other researchers reported the same results [25, 29].

ROS accumulation is the main stimulus for activating the inflammatory mechanism, which in turn increases caspase expression and subsequently increases proinflammatory cytokines such as IL-1 $\beta$  [30]. Pneumonitis and pulmonary fibrosis are early and delayed side effects of IR-induced lung injury that occur after radiation exposure [31]. In irradiation with damage to both pneumocyte cells (type I and II) are loss surfactant and leakage serum in the alveolar sac [32]. Also, damage to pneumocytes and secretions of mucus by goblet cells increase the thickness of the basement membrane [32]. When pneumocytes are damaged, cytokines are released and attract inflammatory cells to the alveolar interval septum. Then inflammatory, macrophages, and resident lung cells release cytokines, growth factors, and ROS, which stimulate collagen production by fibroblasts. This process leads to pulmonary fibrosis in the late phase [33]. Collagen deposition decreases elasticity in the lung [34]. Because we histologically evaluated the animals one week after irradiation, only the inflammatory phase was seen in irradiated lung tissue. It should take at least a month to see the fibrosis of the lung tissue. Therefore, pulmonary fibrosis was not seen in the tissue structure.

The biochemical findings of heart induced by IR in mice approved the histopathological results which are represented by leucocytes infiltration, edema, necrosis, and hemorrhagia. Treatment with GLZ at three doses contributed to decreasing of these criteria when compared to the IR alone group. GLZ significantly protected the myocardium cells with a decrease in oxidative stress and slight inflammation are seen in heart histological structure.

In this study, GLZ directly inhibited histological damage induced by IR. Similar findings were showed by Mafra *et al.*, who found GLZ markedly alleviated the oral mucositis induced by 5-FU. They showed GLZ decreased the inflammation of oral mucosa in animals [14]. We reported in previous research, that GLZ protected the normal human lymphocytes against IR by free radical scavenging and supporting endogenous antioxidant enzymes [20]. Therefore, the radioprotection mechanism of GLZ can be attributed to mitigated lipid peroxidation of the cell membrane and scavenging of free radicals, and inhibition of inflammation. Pan *et al.* showed in a study cardiac protection of GLZ on heart damage in diabetic rats [21]. They described the mechanism of action of GLZ in improving oxidative stress, lowering

blood sugar, and regulating the RhoA / ROCK1 / eNOS signaling pathway. While Loubani *et al.* was abolished the cardioprotective effect of GLZ on the human myocardium [35].

## Conclusion

This study indicated that GLZ via antioxidant activity led to a reduction of the deleterious effects of cardiotoxicity and lung injuries induced by irradiation such as increased oxidative stress and histopathological changes. The radioprotective effect of GLZ was clearly stated on human lymphocytes against IR in the prior research. Based on of data of the previous study and present findings, pre-treatment of GLZ prior to radiation therapy can actually help to persuade the clinical use of GLZ as an antioxidant. So GLZ could use as a safe medicine in the clinic.

## Declarations

### Ethics approval and consent to participate

This experimental animal study was confirmed by the Research and Ethics Committee of Mazandaran University of Medical Sciences (ID#IR.MAZUMS.REC.1400.8619). All methods were carried out in accordance with relevant guidelines and regulations of University.

### Consent for publication

Not applicable.

### Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

S.J.H. designed this study. S.A., F.T.A, S.F., and S.J.H. contributed to experiments. F.T.A. and S.J.H. designed and wrote the manuscript. All authors read and approved the final manuscript.

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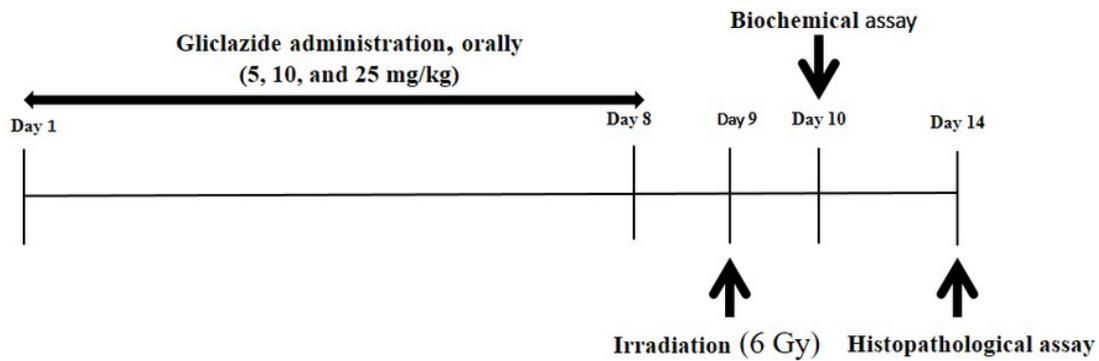
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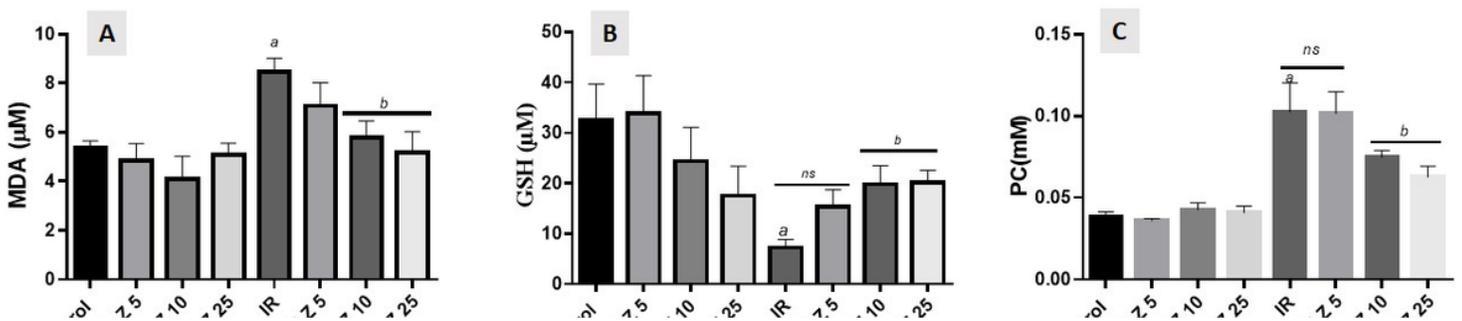
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## Figures



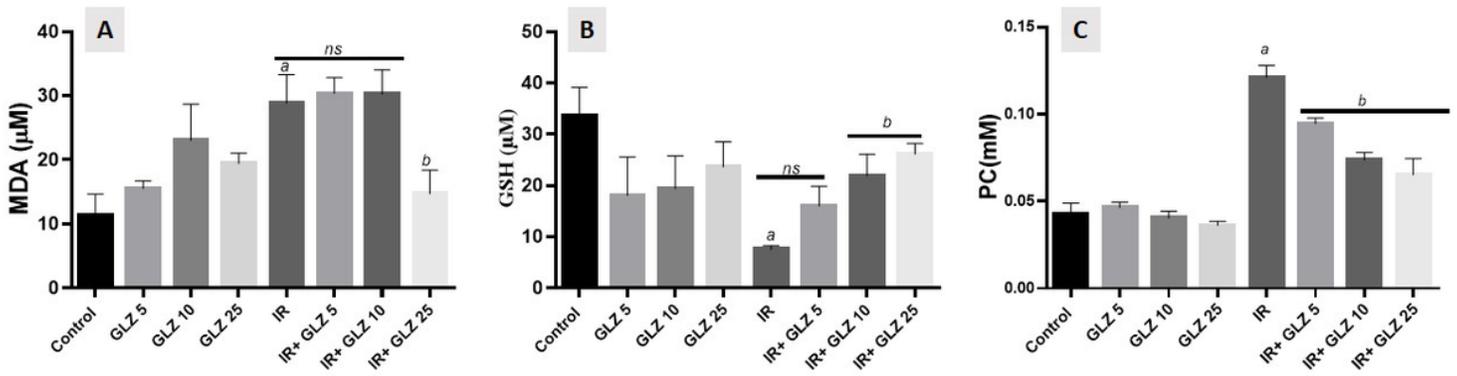
**Figure 1**

Study design diagram for induction of cardiac toxicity and lung injury due to radiation and gliclazide administration



**Figure 2**

Oxidative stress parameter analysis in the heart tissue. Data are displayed as mean ± SD. *a*  $P < 0.0001$  vs control, *b*  $P < 0.0001$  vs IR for MDA, *a*  $P < 0.0001$  vs control, *b*  $P < 0.001$  vs IR for GSH, *a*  $P < 0.0001$  vs control, *b*  $P < 0.0001$  vs IR for PC. IR, Irradiation (6 Gy), GLZ, Gliclazide.

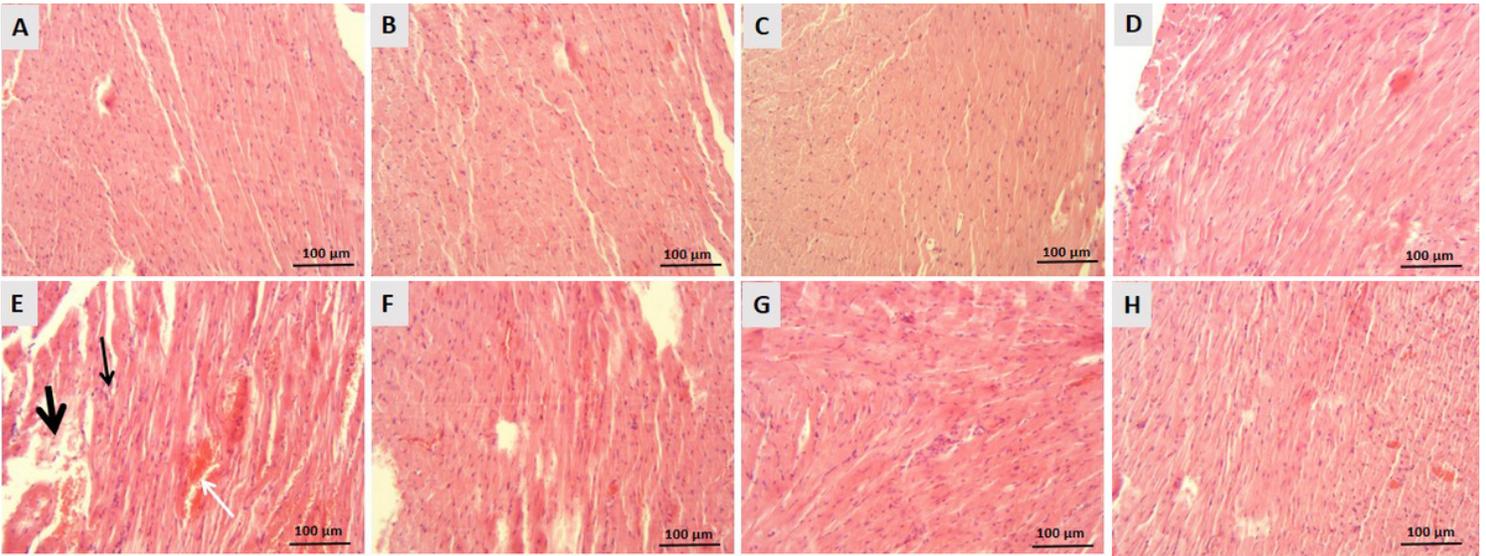


**Figure 3**

Oxidative stress parameter analysis in the lung tissue. Data are displayed as mean  $\pm$  SD. *a*  $P < 0.01$  vs control, *b*  $P < 0.0001$  vs IR, ns: non-significant for MDA, *a*  $P < 0.01$  vs control, *b*  $P < 0.01$  vs IR, ns: non-significant for GSH, *a*  $P < 0.0001$  vs control, *b*  $P < 0.0001$  vs IR for PC. IR, Irradiation, GLZ, Gliclazide.

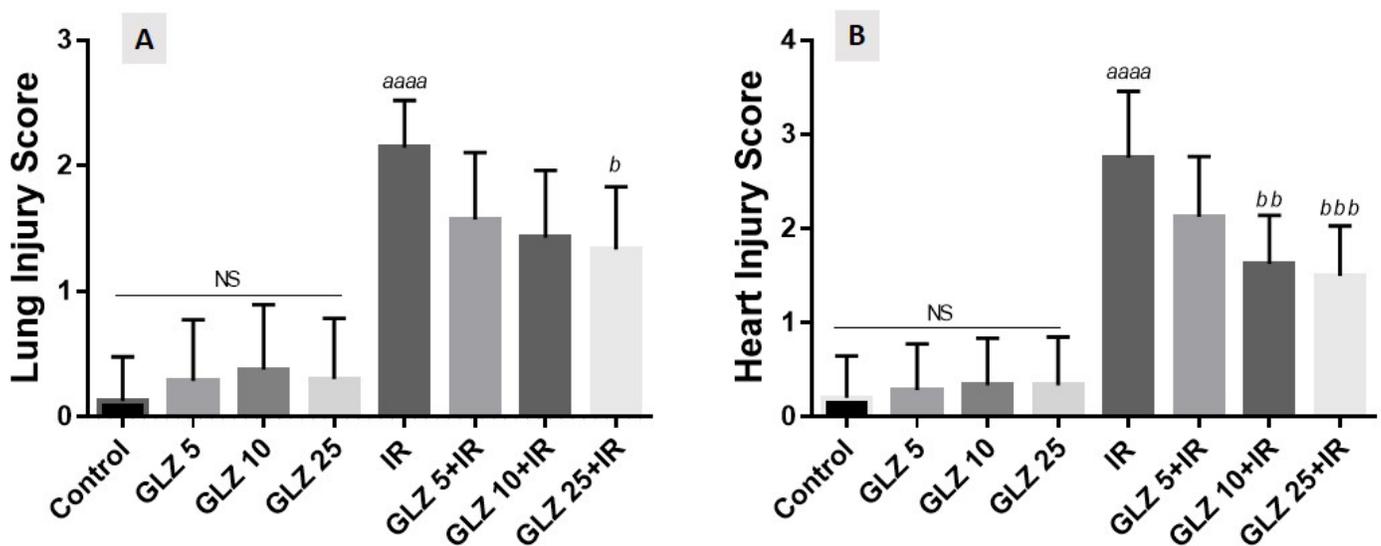
**Figure 4**

Photomicrographs of the effect of GLZ and IR on the histological architecture of lungs in mice. A: Control, B, C, and D: GLZ with 5, 10, and 25 mg/kg, respectively, E: IR, F, G, and H: IR+GLZ with 5, 10, and 25 mg/kg. Inflammatory cell infiltration and acute inflammation in the alveolar space with thickening of the alveolar interval (black arrow) show in irradiated treated mice. GLZ could reduce this damage and mild inflammation was observed in GLZ+IR groups. (H&E staining, Mag:  $\times 40$ , scale bare: 100  $\mu\text{m}$ ), IR, Irradiation, GLZ, Gliclazide.



**Figure 5**

Photomicrographs show the effect of GLZ and IR on the histopathological aspects of the heart. A, Control with normal structure. B, C, and D, GLZ with three doses of GLZ show similar to the control group with normal tissue structure. E, IR with intense inflammatory infiltration (narrow black arrow), hemorrhage (thick black arrow), congestion (white arrow). F, G, and H, IR+GLZ that show moderate inflammatory infiltration. (H&E staining, Mag:  $\times 40$ , scale bare: 100  $\mu\text{m}$ ), IR, Irradiation, GLZ, Gliclazide.



**Figure 6**

The heart and lung injury scores. Data are seen as mean  $\pm$  SD. The most score was for the IR group. *a* significant vs control, *b* significant versus IR group. IR, Irradiation, GLZ, Gliclazide.