

# Ameliorative Effects of Quercetin in Cyclophosphamide-Induced Vascular Toxicity in Rats

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## Research Article

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# Abstract

Cyclophosphamide (CYP) causes vascular toxicity and endothelial damage. In this study aimed the determination of the protective effects of Quercetin (Q) in the CYP-induced vascular toxicity in rats. The rats were randomly divided into the following five groups: Control, CYP, Q50+CYP, Q100+CYP and Q100. The control group was given intragastric (i.g.) corn oil for seven days. The CYP group received i.g. corn oil for seven days and a single dose (200 mg/kg) of CYP via intraperitoneal (i.p.) injection on the seventh day. The rats in the three Q-treated groups received Q for seven days. On the seventh day after the Q treatment, the Q50+CYP, and Q100+CYP groups were injected to single dose (200 mg/kg, i.p.) of CYP. The CYP-treatment both worsen the Phenylephrine (PE)-induced contractions and acetylcholine (ACh)-induced relaxation responses in isolated thoracic aorta of rats, and the application of Q corrected these responses. The malondialdehyde (MDA) levels were significantly higher in the CYP-treated groups. The both dose of Q decreased the MDA level. Superoxide dismutase (SOD) and glutathione (GSH) activities were significantly decreased in the CYP group, whereas the high dose of Q increased SOD and GSH activities. Q treatment attenuated CYP-induced pathologies, and endothelial damage. According to results, Q has protective effects against CYP-induced vascular toxicity in rats.

## Introduction

Cyclophosphamide (CYP) is an alkylating agent, which is widely used for the treatment of lymphomas, acute and chronic leukemia, neuroblastomas, retinoblastomas, and ovarian and breast cancer (Asiri 2010). The administration of low-dose CYP in cancer patients is drop the serum levels of the vascular endothelial growth factor (VEGF) and as a result anti-angiogenic properties of CYP may enhance the anti-tumor effect (Di Lisi et al. 2017). CYP is metabolized into two active compounds (phosphoramidate and acrolein) by the hepatic microsomal P450 enzyme (Jeelani et al. 2017). Phosphoramidate causes cytotoxicity and acrolein has toxic effects on normal cells and it activates reactive oxygen species (ROS), and induces peroxynitrite formation, which is extremely damaging to proteins, lipids, and cellular DNA (Korkmaz et al. 2007). CYP causes the organ toxicity as hepatotoxicity (Gedikli and Sengul 2019), gonadal toxicity (Kenney et al. 2001), lung toxicity (Şengül et al. 2017), nephrotoxicity (Dağ et al. 2018), vascular toxicity (Al-Hashmi et al. 2012), urotoxicity (Gelen et al. 2018a), and cardiotoxicity (Morandi et al. 2005). The cardiovascular pathologies such as cardiotoxicity, heart failure and hypertension have been determined after systemic anticancer treatment (Senkus and Jassem 2011). Injury to the cardiovascular system the induced by anticancer agents like CYP may cause to fatal organ dysfunction consisting of the cardiovascular (Ghobrial et al. 2004) or respiratory systems (Uderzo et al. 2009). The mechanism of cardiovascular injury is thought to be associated with to vascular endothelium and myocytes damage mediated through its toxic metabolites (Kupari et al. 1990). Various antioxidant compounds, including Quercetin (Q), have been used to decrease the possible oxidative stress and side effects of anticancer agents such as CYP (Sengul et al. 2021). Q, many fruits and vegetables contain abundant amounts, which has antioxidant, anti-inflammatory, and anticancer properties (Gelen et al. 2021a; Gelen et al. 2017a; Gibellini et al. 2011) and it scavenges superoxide anion radicals and lipid

peroxides (Ertuğ et al. 2013; Gedikli et al. 2017). Q has attenuated the 5-FU-induced hepatotoxicity, and cardiotoxicity in rats (Gelen et al. 2017a; Sengul et al. 2021) and it has been determined that Q has provided a protective effect by inhibiting ROS and mast cell degranulation in the CYP-induced lung toxicity in rats (Şengül et al. 2017).

In our study was aimed to determination the first time by evaluating in terms of physiological, biochemical, histopathologic and immunohistochemical of the probable protective effects of Q in CYP-induced vascular toxicity.

## Materials And Methods

### Chemicals

CYP (PubChem CID: 2907) and Q (PubChem CID: 5280343) were purchased from Sigma-Aldrich Scientific International, Inc. CYP and Q were dissolved in saline and corn oil immediately before injection, respectively. All other chemicals that were used were of the highest analytic grade.

### Animal housing and experimental design

In this study, 50 male Sprague Dawley rats ( $250\pm 25$  g) were used. The animals were obtained from the Experimental Medicine Research Center in Atatürk University. The experimental studies were conducted according to ethical rules approved by the Local Ethics Committee of Atatürk University for Animal Experiments (Protocol no: 2015/107).

All the animals were housed under standard environment conditions at  $21\pm 2$  °C and a 12-h light/12-h dark cycle and were allowed access ad libitum to a standard diet and drinking water. The rats were randomly divided into five groups, as described below, with 10 rats in each group:

- I. The control group was given corn oil via the intragastric (i.g.) route for seven days.
- II. The CYP group was given corn oil for seven days and injected to a single dose (200 mg/kg) of CYP via the intraperitoneal (i.p.) route on the seventh day.
- III. The Q50+CYP group was given Q (50 mg/kg) dissolved in corn oil for seven days and injected to a single dose of CYP (200 mg/kg, i.p.) on the seventh day.
- IV. The Q100+CYP group was given Q (100 mg/kg) dissolved in corn oil for seven days and injected with a single dose of CYP (200 mg/kg, i.p.) on the seventh day.
- V. The Q100 group was given Q (100 mg/kg, i.g.) dissolved in corn oil for seven days.

Twenty-four hours after the CYP treatment according to a previously published protocols (Mansour et al. 2015), the rats were anesthetized with ketamine hydrochloride (ip, 75 mg/kg) (Ketalar, Pfizer, Turkey) and xylazine (15 mg/kg) (Rompun, Bayer, Turkey), and the rats were euthanized by cervical dislocation.

### Isolated organ bath analysis

After euthanize, thoracic aortas were quickly removed into 4°C Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM Na<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 1.12 mM CaCl<sub>2</sub>, 11 mM glucose-pH 7.4). The connective and fat tissues surrounding the thoracic aorta were dissected and then was cut into approximately 5 mm rings. The endothelium of the aortic rings was kept intact, and the aortic rings were prepared and suspended horizontally in a 10 ml organ bath (EMKA) containing Krebs-Henseleit solution. The aortic rings were mounted on stainless steel triangles and connected to an isometric force transducer. The bath was continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37 °C. Then, the rings were applied the tension (1g) and equilibration period of 1 h was used. During the equilibration period, the Krebs solution was changed every 15 min. At the end of the equilibration period, phenylephrine (PE) (10<sup>-9</sup>-10<sup>-5</sup> M cumulative) and the submaximal dose (10<sup>-6</sup> M) of ACh determined in our previous studies were applied. Dose-response curves to cumulative of PE were obtained. To demonstrate that CYP decreases the relaxation responses by causing to vascular endothelial damage, the relaxation effects of ACh (10<sup>-6</sup> M) on PE-induced (10<sup>-5</sup> M) contractions were compared among the groups.

### **Biochemical analysis**

Thoracic aortic tissues were disintegrated by using liquid nitrogen. The homogenates from these tissue samples were prepared for analysis of malondialdehyde (MDA) and glutathione (GSH) levels and superoxide dismutase (SOD) activity. MDA and GSH levels and SOD activity were analyzed by using commercial ELISA kits (YL Biotech, Shanghai, China) according to the manufacturer's instructions. The well plates were read at 450 nm via an ELISA plate reader (BioTekEpoch 2 Microplate Spectrophotometer).

### **Histopathologic and immunohistochemical analysis**

At the end of the study, after the rats had been sacrificed under anesthesia, the thoracic aortas were removed and fixed in 10% buffered neutral formalin solution for 72 h. The tissue samples were embedded to paraffin following exposure to a graded series of xylene and ethanol washes. The paraffin blocks were cut into 5-µm thick sections by using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). The thoracic aortic sections were stained with Verhoeff's elastic tissue stain, and changes in the elastin content based on the structural arrangement of the vessel walls were evaluated. The stained specimens were examined under a light microscope (Nikon eclipse i50, Tokyo, Japan), and photographic images were taken for histopathological evaluation.

For immunohistochemical analysis, from the tissues embedded in paraffin blocks, cross-sections were put on to adhesive-containing slides. The sections were passed through gradients of xylol and alcohol, and deparaffinization and dehydration were performed. The tissues were washed with phosphate-buffered saline (PBS), kept for 10 min in the 3% H<sub>2</sub>O<sub>2</sub> solution. To prevent the antigens in the tissues from being masked, the samples were microwave-treated for 2x5 min with an antigen retrieval solution. After this process, rabbit polyclonal to eNOS primary antibody (catalog no: ab5589, dilution 1:100; Abcam, UK) was added. Afterwards, 3- 3'Diaminobensidine was used as chromogen. The sections that were

counterstained by haematoxylin were observed under a light microscope. The pathologists counted the number of positive cells in each high-power field and calculated the average number of positive cells to reflect the intensity of positive expression. The sections were evaluated as none (-), mild (+), moderate (++) , and severe (+++) according to their immunopositivity.

### **Statistical analysis**

The statistical evaluation was performed using SPSS, version 20.0 (IBM, Armonk, NY, USA). Tukey's test and a one-way ANOVA were applied to all the data. The data are expressed as mean±standard deviation (SD).  $p < 0.05$  was considered statistically significant.

## **Results**

### **Effects of CYP and Q on thoracic aorta contractility**

PE-induced ( $10^{-9}$  and  $10^{-8}$  M) contractions among the experimental groups were not significantly different (Fig. 1,  $p > 0.05$ ). The contraction responses to  $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  M doses of PE were lower than those of the other groups in the CYP group (Fig. 1,  $p < 0.05$ ). The relaxation responses of ACh ( $10^{-6}$  M) on PE ( $10^{-5}$  M)-induced contractions in the CYP group were significantly decreased as compared to the other groups (Fig. 2,  $p < 0.05$ ). The PE-induced contraction and ACh-induced ( $10^{-6}$  M) relaxation responses in the Q-applied groups were different from CYP group (Figs. 1 and 2).

### **Effects Of Q On Cyp-induced Vascular Oxidative Stress**

Vascular MDA levels of rats increased significantly as compared to the control in CYP group. Both doses of Q prevented the CYP-induced vascular lipid peroxidation (Fig. 3A,  $p < 0.05$ ). CYP were also significantly decreased to SOD activity (Fig. 3B,  $p < 0.05$ ) and GSH level (Fig. 3C,  $p < 0.05$ ). The high dose of Q significantly prevented to the decrease in the activities and levels of antioxidant enzymes (Fig. 3B and 3C,  $p < 0.05$ ).

### **Histopathological And Immunohistochemical Findings**

The thoracic aortic tissues of the control group displayed a normal structure and morphology, as shown by staining of the sections with Verhoeff's elastic tissue stain (Fig. 4). However, the aortic tissues of the CYP group displayed endothelial irregularities, degenerative areas, breaks, ruptures, and thickening in the aortic wall, especially in the medial layers of the elastic fibers of the thoracic aorta (Fig. 4). These degenerative changes were less severe in the Q50 + CYP and Q100 + CYP groups than CYP group (Fig. 4).

As determined by the immunohistochemically evaluations, eNOS-positive cell counts were significantly higher in the CYP group than in the control group (Fig. 5). However, as compared with the CYP group, the

other groups showed a significant decrease in eNOS-positive cell counts (Table 1). Furthermore, immunopositivity of eNOS was significantly decreased in the especially Q100 + CYP group compared with the CYP groups (Fig. 5). Semi-quantitative staining scores for eNOS immunopositivity for all the groups are shown in the Table 1.

Table 1  
Semi-quantitative staining scores for eNOS immunopositivity.

Experimental Groups	eNOS
Control	-
CYP	+++
Q50 + CYP	++
Q100 + CYP	+
Q100	-
The positive cell intensity was scored as follows: none (-), mild (+), moderate (++), and severe (+++)	

## Discussion

CYP, an anticancer agent, is commonly used in chemotherapy (Asiri 2010) and this agent causes to toxicity of the many organs in the organism (Al-Hashmi et al. 2012; Çelebi et al. 2020; Kenney et al. 2001; Morandi et al. 2005). To prevent or treat possible side effects of anticancer agents, the probiotics (Gelen et al. 2019; Sengul et al. 2019), chemical compounds or biologically active compounds isolated from the structure of plants are widely used in researches (Abdel-Daim et al. 2020; Abdel-Daim et al. 2019; Gelen et al. 2021b; Gelen and Sengul 2020; Gelen et al. 2018b). One of the compounds used for this purpose is Q flavonoid which is found in the structure of many fruits and plants (Dong et al. 2014). In this study firstly determined that Q has protective effects in the CYP-induced vascular toxicity.

Anticancer agents have adverse effects on the vascular system (Al-Hashmi et al. 2012; Olukman et al. 2009) and CYP, a chemotherapeutic, causes the some histopathological changes in vascular tissue. Previous research showed that the CYP metabolites (phosphoramidate and acrolein) causes vascular endothelium damage by inducing the free radical production (Soultati et al. 2012). In accordance with the literature, in the present study, CYP caused endothelial dysregulation, degenerative areas, thickening in the vascular wall, and breaking away and breaks in elastic fibers, especially in the medial layer of the thoracic aortic sections. Elastic fibers are responsible for vessel elasticity, and fragmentation of these fibers reduces distensibility and elasticity (Chung et al. 2007). Chemotherapeutics causes stiffness by deforming the elasticity of thoracic aorta. As a result of the deterioration of vascular elasticity, the contractility of the thoracic aorta is impaired (Olukman et al. 2009). According to one study in contrast to this data, noradrenaline-induced contractions in thoracic aortic strips of CYP administration mice were not different from control group (Al-Hashmi et al. 2012). But in our study was determined that the CYP

administration decreased the contraction responses induced by  $10^{-7}$ - $10^{-5}$  M doses of PE and contractions in Q administration groups were different from CYP group. Q application prevented the formation of degenerative changes in thoracic aorta and therefore the PE-induced contraction responses differed from the CYP group. Anticancer agents have negative effects on relaxation mechanisms as well as contractions of vessels. Endothelial cells (EC) have an important role in the physiological maintenance of vascular homeostasis as vascular tone, angiogenesis, vascular remodeling and permeability (Chaosuwannakit et al. 2010; Park et al. 2015). Nitric oxide (NO) released from the vascular endothelium increases cyclic guanosine monophosphate (cGMP) production by inducing guanylyl cyclase activity and thus produces vasodilatation (Di Lisi et al. 2017). ACh, a vasodilator agent, increases intracellular  $Ca^{+2}$  in EC which releases of NO from vascular endothelium, and so induces vasorelaxation. Previous research demonstrated that CYP metabolites (acrolein, chloroacetaldehyde) may be causes to endothelial damage and increases the endothelial eNOS expression by inducing the production of reactive oxygen species (ROS) (Al-Hashmi et al. 2012; Duarte et al. 1993a). As a result of decreases in plasma NO levels due to endothelial damage declines the vessel relaxation (Al-Hashmi et al. 2012). In other study has been determined that eNOS activity in the doxorubicin-induced vascular dysfunction has increased and the decrease of ACh-induced relaxation responses is likely due to the decrease in the formation of NO with concomitant increase in oxygen radicals. In the present study, CYP administration resulted with endothelial irregularities, increased eNOS levels, and decreased ACh-induced relaxation responses in endothelium-intact thoracic aortas. In other words, decreased in the ACh responses is more likely due to increasing eNOS activity and decreasing NO level rather than an abnormality in ACh receptor/signal transduction. In addition, the treatment with Q prior to CYP administration normalized the ACh-induced relaxation responses. In a study has been reported in supportive direction the our findings that Q has direct (endothelium-independent) vasodilator effect at in vitro arteries (Duarte et al. 1993a; Duarte et al. 1993b; Fitzpatrick et al. 1993) and this effect might be associated with protein kinases as myosin light-chain kinase and protein kinase C (Ibarra et al. 2002). The normalized effect the ACh-induced relaxation responses of Q may thought to be dependent on both prevention the endothelial damage and have the vasorelaxant effect. It is may thought that ACh-induced relaxation responses were normalized depending upon prevention of endothelial damage through antioxidant properties of Q.

Free radicals are produced not only physiological conditions but also pathological conditions. The overproduced of free radicals may cause injuries by reacting to the organelles as DNA and cell membrane (Kara et al. 2016). In the CYP-induced vascular toxicity increases the production of free radicals. Acrolein, a CYP metabolite, activates the reactive oxygen species (ROS) production and induces peroxy nitrite formation, which is tremendously damaging the cell selection as lipids, proteins and DNA. These free radicals causes also deterioration in the integrity and function the accompanied by vascular wall and endothelial damage (Korkmaz et al. 2007; Yeh et al. 2004). The oxidative stress induced by CYP metabolites results also with vascular endothelium damage (Al-Hashmi et al. 2012). In the present study determined that CYP increased MDA levels, decreased SOD and GSH activities. Besides, the especially high dose of Q provided protection against the CYP-induced vascular toxicity by increasing the SOD and GSH activities and by decreasing the MDA levels in thoracic aorta. Q significantly alleviated endothelial

damage by reducing the oxidative stress induced by CYP metabolites. Q is effective scavengers of several ROS (O'Reilly et al. 2000; Ozgova et al. 2003) and the glucuronized metabolites of Q have also antioxidant properties (Moon et al. 2001). Furthermore, Q can consumed the intracellular thiols like glutathione both in vitro and in vivo (Perez-Vizcaino et al. 2006). Thanks to this property of Q inhibits the lipid peroxidation (Hollman et al. 1997; Sakanashi et al. 2008), which is the process to converted the free radicals by means of the abstraction of hydrogen of the unsaturated fatty acids (Young and McEneny. 2001). The protective effect of Q may be due to the inhibiting of lipid peroxidation as well as acts like scavenger ROS.

## Conclusion

In conclusion, the findings provided evidence that Q has protective effects against CYP-induced vascular toxicity in rats. CYP metabolites induced vascular injury and resulted in histopathological alterations. Especially, high-dose of Q had a protective effect against vascular toxicity induced by CYP in rats.

## Declarations

**Author's contribution** ES, VG, AA: experiment design, experiment application, samples collection. ES, VG, AA: isolated organ bath analysis, serum markers and tissue antioxidant estimation, data curation and analysis, final reviewing. SG, EE: histopathological and immunohistochemical investigation. All the authors contributed to the writing and editing, and they read and approved the final manuscript.

**Availability of data and materials** The authors confirm that the data and materials supporting the findings of this study are available within the article.

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### Compliance with ethical standards

**Ethical approval** The study was designed and conducted according to ethical norms approved by the Atatürk University Rectorate Animal Experiments Local Ethics Committee (Erzurum, Turkey) (Protocol no: 2015/107).

**Conflict of Interest** The authors declare that there are no conflicts of interest.

**Consent to participate** All authors voluntarily participated in this research study.

**Consent to publish** Not applicable

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## Figures

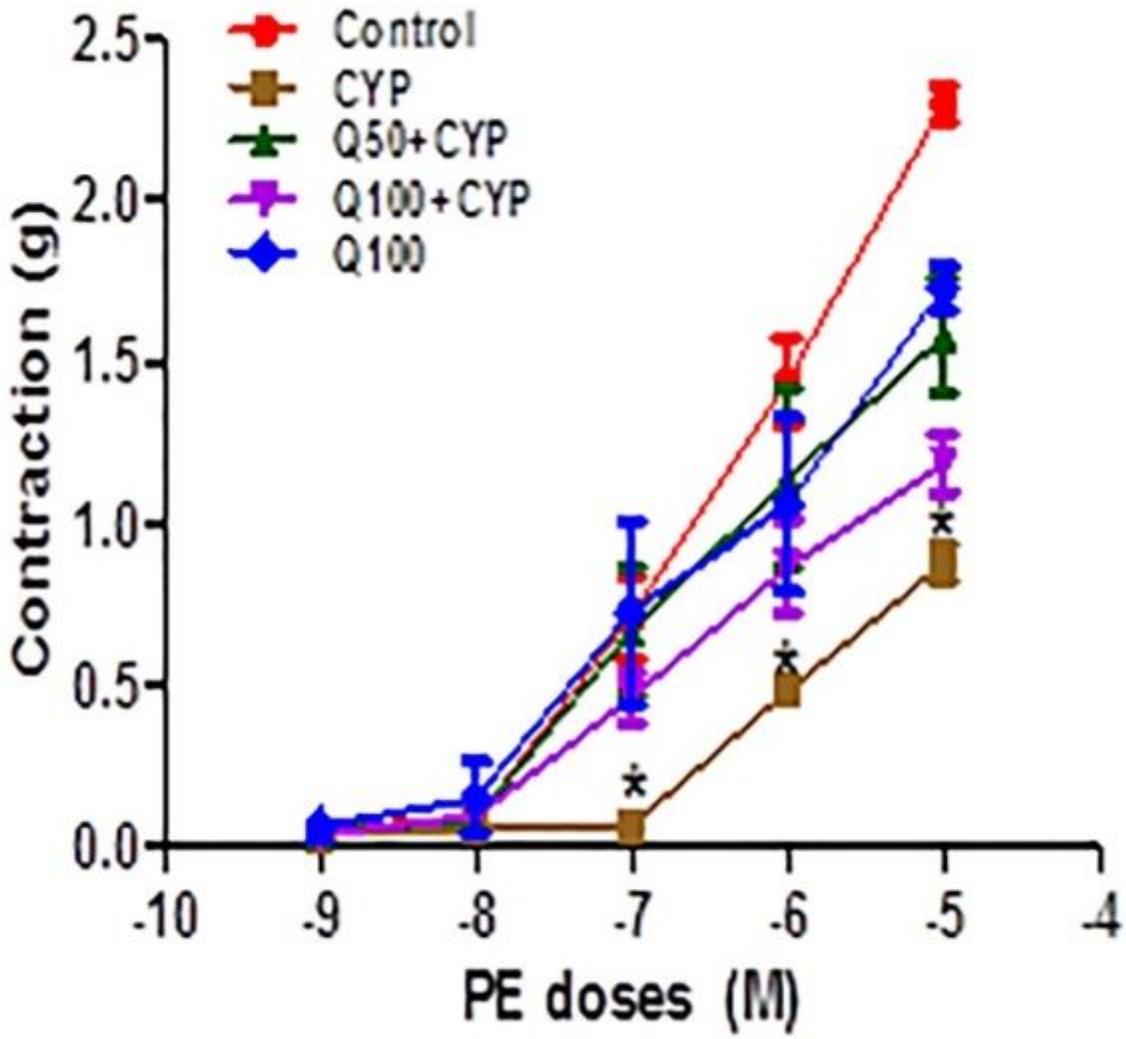


Figure 1

The dose response curves of PE-induced contraction in the thoracic aortas of the experimental groups (\*=p<0.05).

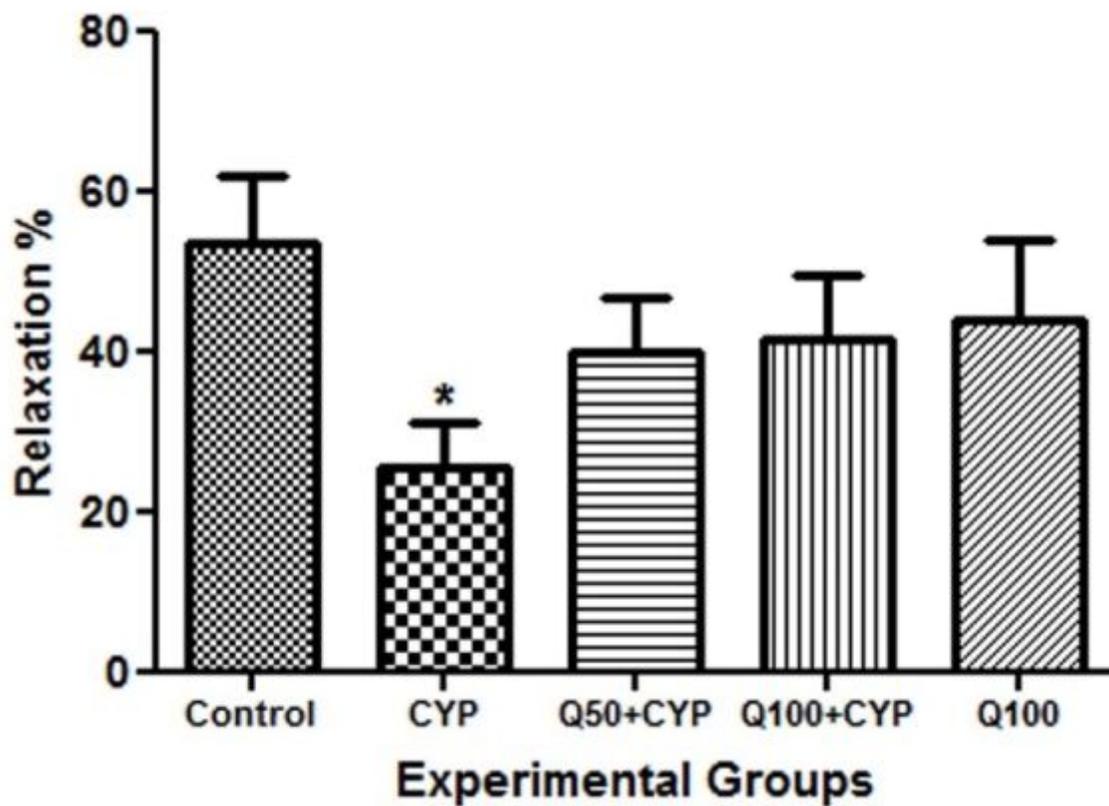
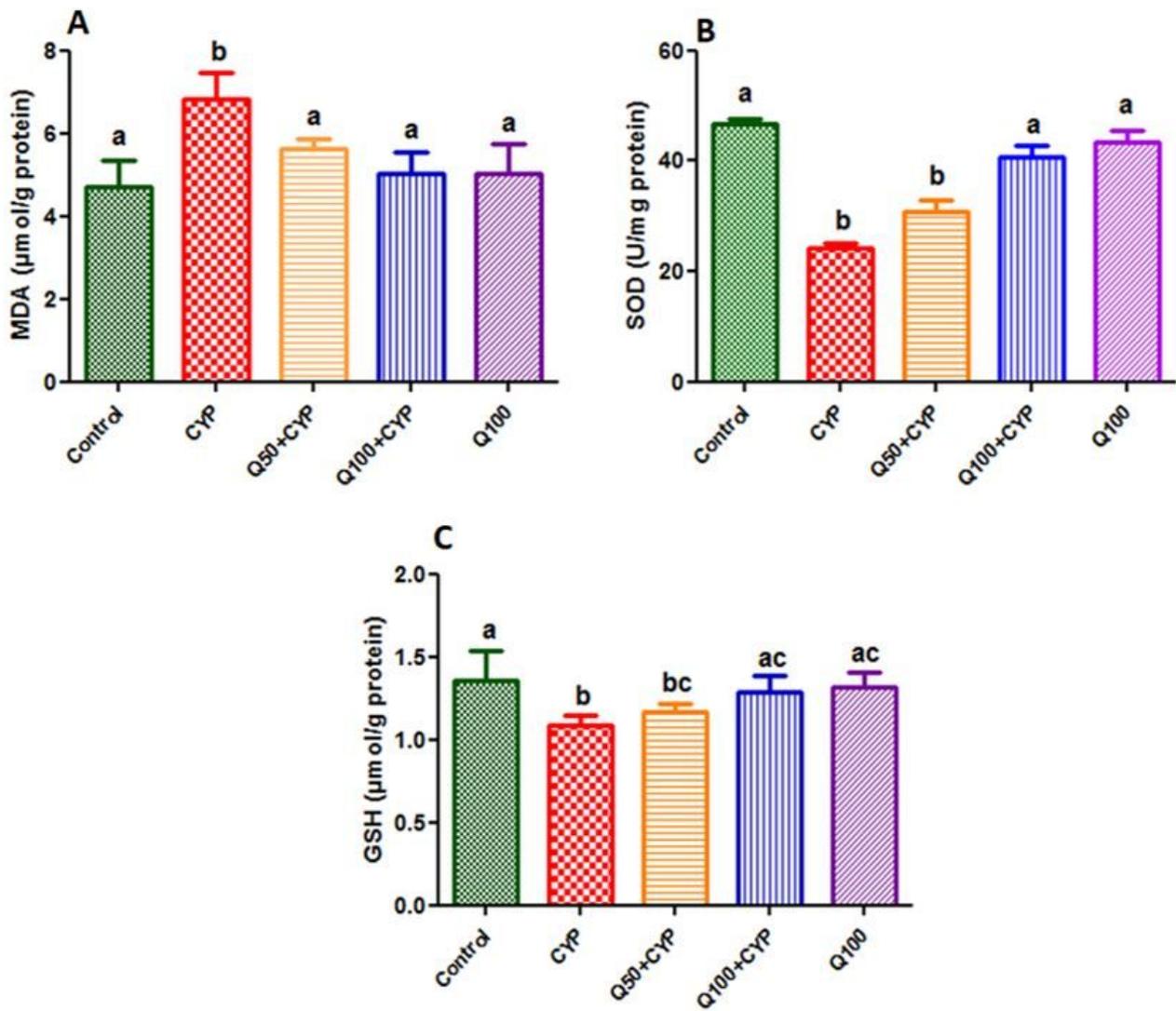


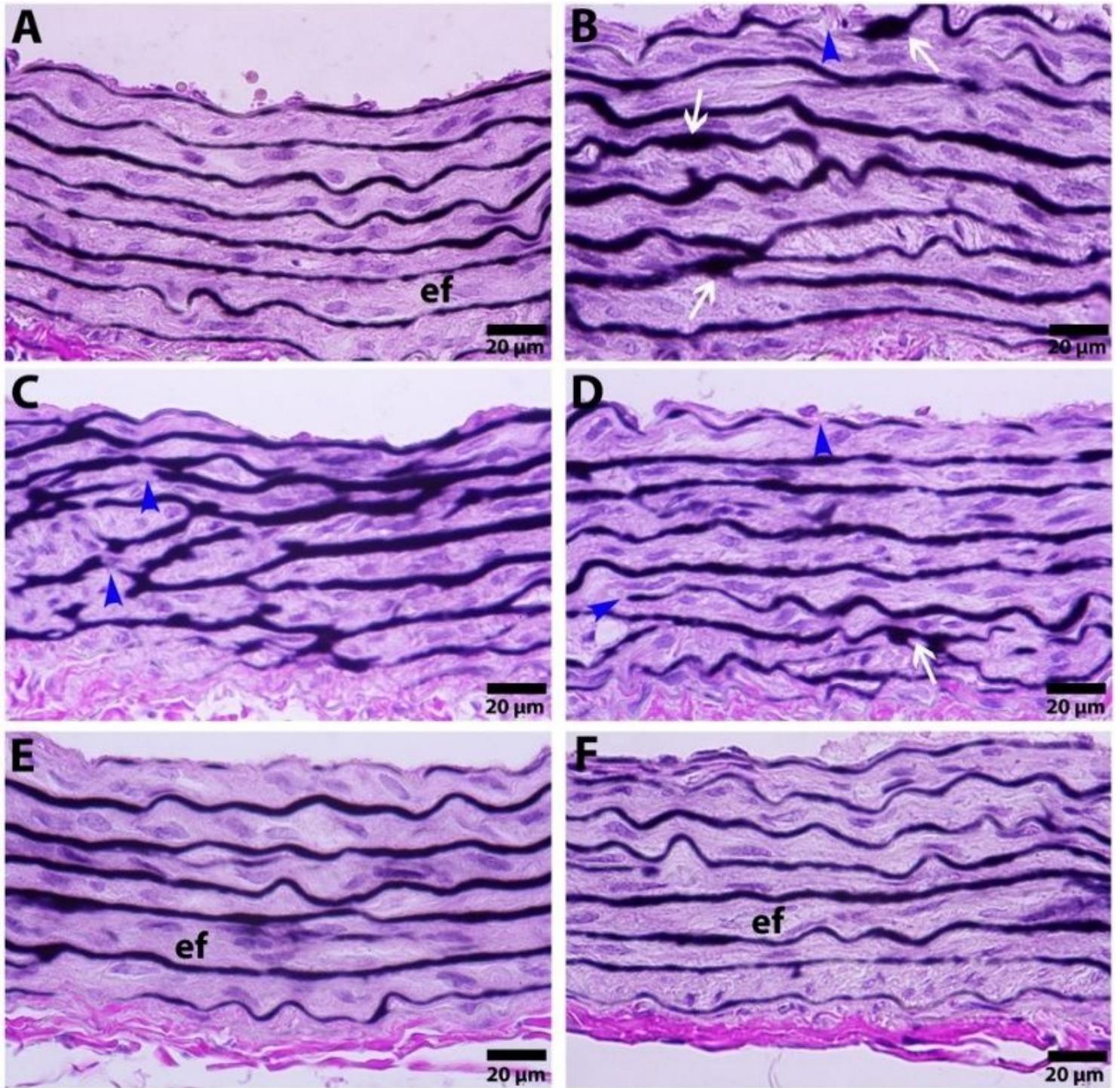
Figure 2

The relaxation effects of the ACh ( $10^{-6}$  M) on the PE-induced ( $10^{-5}$  M) contractions in the thoracic aortas of the experimental groups (\*= $p < 0.05$ ,  $n = 6$ ).



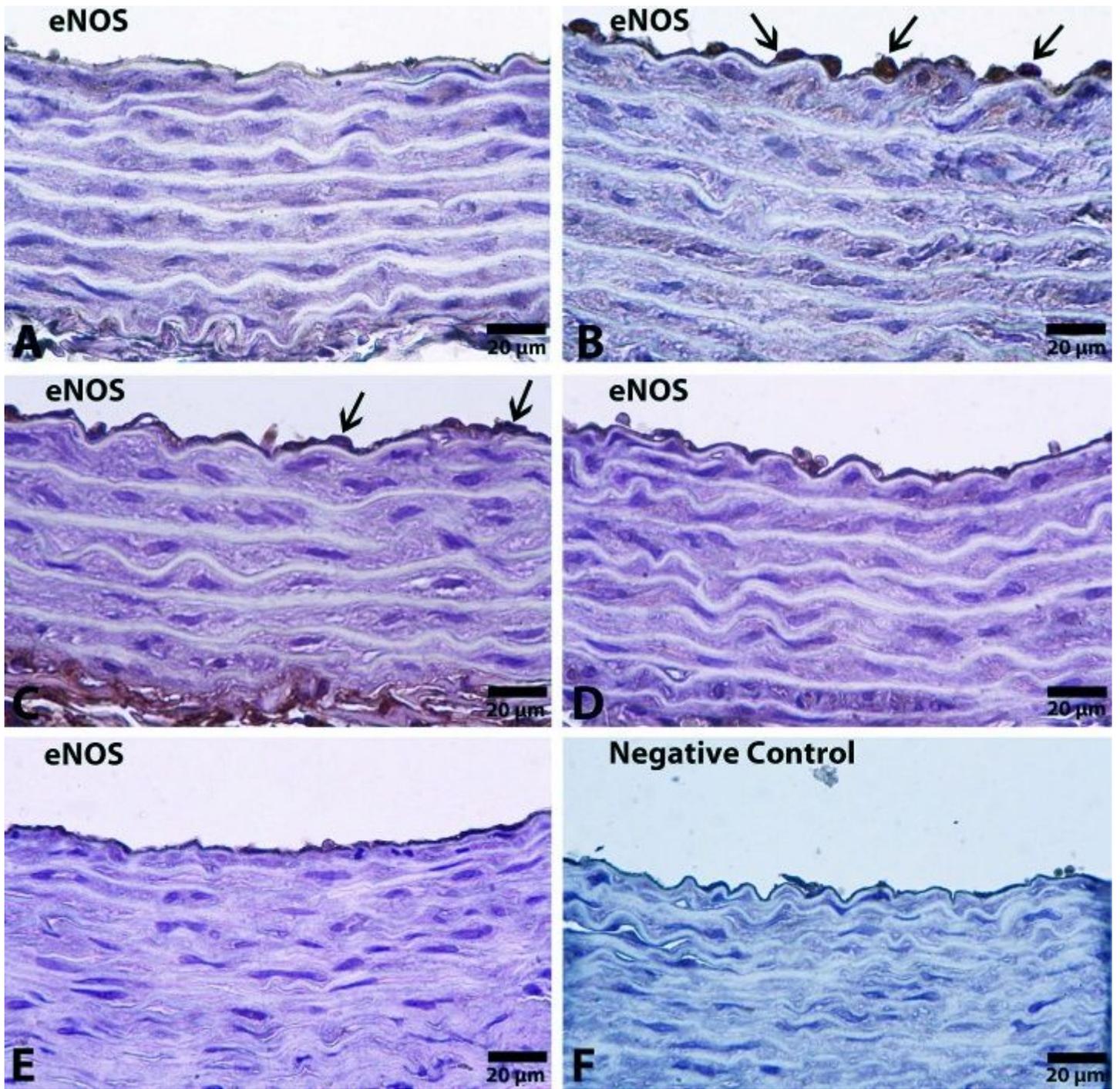
**Figure 3**

The MDA (A) and GSH (C) levels and SOD (B) activities in thoracic aortas of rats in experimental groups (There is no statistical difference among the groups expressed with same letters,  $p < 0.05$ ).



**Figure 4**

Micrograph of aortic section of the Control (A), CYP (B-C), Q50+CYP (D), Q100+CYP (E) and Q100 (F) groups. ef: elastic fibers; blue arrowheads: breaks and ruptures in the elastic fibers; white arrow: thickness and degeneration in the elastic fibers. Stain: Verhoeff's Elastic Stain.



**Figure 5**

Immunohistochemical staining for the eNOS in the Control (A), CYP (B), Q50+CYP (C), Q100+CYP (D) and Q100 (E) and Negative control (F) groups. Arrows show eNOS positivity in the endothelial cells. Negative control; not primer antibody used sections of control and other groups. Streptavidin–biotin peroxidase staining.