

The Effects of Quercetin on Cyclophosphamide-Induced Cardiotoxicity in Rats

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Abstract

Cyclophosphamide (CYP), an anticarcinogenic agent, is widely used in the chemotherapy. At high doses of the CYP causes fatal cardiomyopathy with acute cardiotoxicity. Our aim in this study investigations effects of quercetin (Q) on CYP-induced cardiotoxicity. In the present study used fifty piece Sprague Dawley male (250 ± 50 gr) rats. Rats were divided randomly to five group ($n = 10$). The control group was given intragastric (ig) corn oil (1 ml) for seven days. The CYP group rats were applicate ig corn oil for seven days and injected intraperitoneal (ip) a single dose of CYP 200 mg/kg on the seventh day. The groups Q50 + CYP and Q100 + CYP, respectively, were given Q in doses of 50 and 100 mg/kg dissolved in corn oil and administered ig for seven days. In addition, these groups were given single dose of CYP (200 mg/kg, ip) on the seventh days of application Q. The Q100 group was given Q (100 mg/kg-i.g) for seven days. In the end experimental applications, the blood was collected from anesthetized rats and rats were sacrificed. Serum was separated by centrifugation and utilized for the evaluation of various cardiac enzymes (CK, CK-MB, LDH, AST, ALT). The cardiac tissues used for biochemical and histopathological analysis. The data were analyzed by Tukey test in the one-way ANOVA. When data are showed compared among groups from the point of view aortic and vascular tissues, MDA level was significantly higher in the CYP group compared with control group, and determined to be decreased in CYP + Q100 group. SOD and GSH levels were significantly decreased in the CYP group compared to the control and CYP + Q100 groups. AST, CK, CK-MB, ALT and LDH levels in the serum were significantly increased in the CYP group compared with the other groups. The histopathological examination of cardiac tissue determined in the CYP group had significantly degenerated cells and cardiac myofibril. Intensity of terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) and beta-myosin heavy chain (β -MHC) positivity was higher in the CYP group sections compared to the control and CYP + Q100 groups sections. Both biochemical results and immunohistochemical evidence showed that Q has protective effects on CYP-induced cardiotoxicity.

Introduction

Cyclophosphamide (CYP) is alkylating agent which is widely used for the treatment of lymphoma, acute and chronic leukemia, neuroblastoma, retinoblastoma and cancers of the ovary and breast (Asiri 2010; Al-Yahya et al. 2009). But at the same time, it is used in the treatment of scleroderma, chronic hepatitis, glomerulonephritis, arthritis, chronic interstitial pneumonia, systemic lupus erythematosus, multiple sclerosis and organ transplantation (Anderson et al., 1995). CYP is metabolized the two active compound as phosphoramidate and acrolein metabolite by hepatic microsomal P450 enzyme (Ludeman 1999; Ren, 1997; J.P. Kehrer et al., 2000). Phosphoramidate causes to cytotoxicity and acrolein have toxic effects on normal cells (Gelen et al. 2018). Acrolein activates reactive oxygen species (ROS) and induces peroxynitrite formation which extremely damages on the proteins, lipids and DNA in the cell (Korkmaz et al., 2007). CYP causes to hepatotoxicity (Zhu et al., 2015; Gedikli and Sengul., 2019), gonadal toxicity (Kenney et al., 2001), genotoxicity (Khodeer et al. 2020), lung toxicity (Ahmed et al., 2015; Şengül et al., 2017), nephrotoxicity (Rehman et al., 2012) and cardiotoxicity (Gunes et al., 2016). Cardiotoxicity is one

of the most life-threatening complications of cancer therapy. CYP-induced cardiotoxicity is a well-known adverse effect (Morandi et al., 2005). Symptoms reveals mostly from 1 to 3 weeks and mortality rate can be as high as 43% (Goldberg et al., 1986; Yeh et al., 2004; Slordal and Spigset., 2006). The side effects of CYP are generally seen after a single high dose administration. The some antioxidant compounds were used that to decrease the possible oxidative stress and side effects of the anticancer agents (Bülbül et al., 2018; Dağ et al., 2018; Gelen and Sengul 2020; Gelen et al., 2018; Gelen et al., 2017). Quercetin (Q) is one of the compounds that used for this purpose. The various fruits and vegetables contains abundantly Q which is a compound antioxidant, anti-inflammatory and anti-cancer effective (Gibellini et al., 2011). Q scavenges the superoxide anion radicals and lipid peroxides (Morales et al., 2012; Ertuğ et al., 2013; Gelen et al., 2017, Şengül et al., 2021; Gedikli et al. 2017; Abdel-Diam et al. 2019). Besides, Q protects the cardiomyocytes against to oxidative stress that induced by H₂O₂ (Angeloni et al., 2007). Q attenuates cardiotoxicity, which induced by other anticancer agents (Dong et al., 2014). However, it is not known whether Q's have a protective effect on CYP-induced cardiotoxicity in rats. Our purpose in this study investigation that Q's protective effects on CYP-induced cardiotoxicity.

Material And Methods

Drugs and chemicals

CYP and Q were purchased from Sigma-Aldrich Scientific International. Inc. (Hampton. NH. USA). CYP and Q were dissolved normal saline solution and corn oil immediately before injection, respectively. All other chemicals were used of the highest analytic grade.

Animal housing and experimental design

In this study, fifty male Sprague Dawley rats (250±50 gr) were used. The animals obtained from Experimental Medicine Research Center in Atatürk University. The experimental studies were conducted according to ethical rules approved by the Local Ethics Committee of Ataturk University for Animal Experiments (107/HADYEK, 27th May 2015). All the animals were housed under the standard environment condition at 21±2°C, 12 h light/12 h dark cycle and were allowed ad libitum to a standard diet and drinking water. All rats were divided into 5 groups as described below and there were 10 rats in each group:

I: The control group was given corn oil (1ml) intragastric (ig) per days for seven days.

II: The CYP group done corn oil (1ml-ig) per days for seven days and seventh day was injected intraperitoneal (ip) to single dose (200 mg/kg) of CYP.

III: The Q50+CYP group was given Q (50 mg/kg-ig) in the corn oil dissolved per days for seven days and seventh day was injected to single dose (200 mg/kg-ip) of CYP.

IV: The rats in Q100+CYP group were given Q (100 mg/kg-ig) in the corn oil dissolved per days for seven days and seventh day was injected to single dose (100 mg/kg-ip) of CYP.

V: The Q100 group was given Q (100 mg/kg-ig) in the corn oil dissolved per days for seven days.

Blood Sample Collection

Twenty four hours after from the CYP treatment or in other words 8th day of the experiment, rats were anesthetized with ketamine hydrochloride (ip, 75 mg/kg) (Ketalar, Pfizer, Turkey) and xylazine (15 mg/kg) (Rompun, Bayer, Turkey) and blood samples were separately collected from the heart of each rat. After rats were euthanized with cervical dislocation. The blood samples were centrifuged at 1500 g for 12 min within 1 h after collection to obtain sera samples. The blood and sera samples were immediately studied with a blood counter (Abacus Junior Vet5, Diatron, Austria).

Histopathologic Analysis

At the end of the study, after all rats were sacrificed by under anaesthesia, the hearts of rats were removed and fixed in 10% buffered neutral formalin solution for 72 hours. Tissue samples were embedded in paraffin after xylene and ascendent ethanol series. The paraffin blocks were cut 5- μ m thick using a Leica RM2125RT microtome (*Leica Microsystems, Wetzlar, Germany*). The heart sections of all groups were stained with Mallory's triple stain modified by Crossman. The stained specimens were examined under a light microscope (*Nikon eclipse i50, Tokyo, Japan*) and photo images were taken for histopathological evaluation.

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% H₂O₂ 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, polyclonal anti- β -myosin heavy chain (β -MHC) primary antibody (catalog no: sc-53089, dilution 1:50; Santa Cruz Biotechnology Inc., USA) 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.

TUNEL Assay

TUNEL assay was carried out according to the manufacturer's instructions (In Situ Cell Death Detection Kit, POD, Roche, Germany,). After deparaffinization and rehydration, the sections were treated with 10 mmol/L protease K for 15 min. The slides were immersed in TUNEL reaction mixture for 60 min at 37°C in

a humidified atmosphere in the dark. A converter POD was used to incubate the slides for 30 min. The slides were then analyzed using an light microscope (*Nikon eclipse i50, Tokyo, Japan*) and photo images were taken for TUNEL-positive cells number. Sections examined under light microscope were evaluated according to TUNEL-positive cell type as none (-), mild (+), moderate (++) and severe (+++).

Biochemical Analysis

Heart tissues were disintegrated using liquid nitrogen. Afterwards, in the homogenates prepared from these tissue samples were worked in accordance with the directives of the kits to MDA levels (Tbars Assay Kit, Item No: 10009055-Cayman), GSH (Glutathione Assay Kit, Item No: 703002-Cayman) and SOD activities (Superoxide Dismutase Assay Kit Item No: 706002-Cayman). Absorbance measurements were read in the Elisa device the Biotek brand.

Statistical analysis

The statistical evaluation was performed by using the SPSS Version 20.0 (IBM, Armonk, NY, USA). All values were analyzed by Tukey test in the one-way ANOVA. Data are expressed as mean \pm standard deviation. $P < 0.05$ was considered as statistically significant.

Results

Cardiac function tests

The control compared with CYP-treated rats observed that there were a significant increase in the serum activities of LDH, AST, ALT, CK and CK-MB (Table 1, **= $p < 0.01$, *= $p < 0.05$, $n = 10$). In treatment with both doses (50 and 100 mg/kg) of the Q for seven days prior to CYP significantly decreased the serum activities of LDH, AST, ALT, CK and CK-MB compared with CYP-treated rats (Table 1, **= $p < 0.01$, *= $p < 0.05$, $n = 10$).

Oxidative Stress Paramameters

According to the control in the CYP, there was significantly decrease the SOD and GSH activities and significantly enhance the MDA levels in heart tissue (Fig. 1, $p < 0.05$, $n = 6$). The cardiac SOD and GSH activities and MDA levels were reversed to the control values by treatment with especially Q high dose prior to CYP application (Fig. 1, $p < 0.05$, $n = 6$).

Table 1

The values of the some cardiac enzymes in the experimental groups (**=p < 0.01, *=p < 0.05, n = 10)

Groups	LDH	AST	ALT	CK-MB	CK
Control	1579 ± 102	263 ± 56	81 ± 11	649 ± 124	556 ± 53
CYP	3402 ± 51**	287 ± 42*	138 ± 8**	1740 ± 227**	1167 ± 299**
Q50 + CYP	1495 ± 118	223 ± 35	42 ± 12	839 ± 304	680 ± 270
Q100 + CYP	1134 ± 169	200 ± 45	46 ± 4	643 ± 210	585 ± 246
Q100	2421 ± 154	234 ± 53	75 ± 11	627 ± 231	540 ± 163

Histopathological Findings

Histopathological observations indicate that heart tissue sections of control group (Fig. 2A) exhibit normal histological structure. Similar histological appearance have been seen in Q100 group (Fig. 2E). In the CYP group heart tissue sections were seen myofiber degeneration/necrosis that characterized by a fragmented, hypereosinophilic myofibril cytoplasm (Fig. 2B). There was a significant reduction in the number of degenerative cells in the Q50 + CYP and Q100 + CYP group, respectively (Fig. 2C, D).

Immuno-histochemical Findings

β -MHC positive cells were strongly detected in the CYP group (Fig. 3B). The number of β -MHC positive cells were lower in the control and Q100 groups than in the CYP group (Fig. 3A, E). There was a decrease in the expression of β -MHC in heart tissues of the Q treated groups (Fig. 3C, D). The β -MHC positive cells were significantly decreased in the Q100 + CYP group compared with the CYP group (Fig. 3D). Semi-quantitative staining scores for β -MHC for all groups are shown in the Table 2.

Evaluation Of Tunel Assay

Apoptotic cells in the heart tissues of all groups were identified by TUNEL assay. Only a few TUNEL-positive cells were observed in the control group (Fig. 4A) and Q100 group (Fig. 4E). However, the number and signal density of TUNEL-positive germ cells significantly increased in the CYP group (Fig. 4B). Q treatment reduced the reactivity and the number of apoptotic germ cells (Fig. 4C, D). The the number of apoptotic germ cells were significantly decreased in the Q100 + CYP group compared with the diabetic group (Fig. 4D). Semi-quantitative staining scores for TUNEL assay and for all groups are shown in the Table 2.

Table 2

The positive cell intensity was scored as follows: none: (-), mild (+), moderate (++) and severe (+++)

Experimental Groups	TUNEL	β -MHC
Control	-	-/+
CYP	+++	+++
Q50 + CYP	++	+
Q100 + CYP	+	+
Q100	-	-/+

Discussion

CYP is anticancer agent which commonly used in the chemotherapy (Asiri 2010). This agent causes to toxicity of the many organs in the organism. Some biologically active compounds isolated from the structure of plants are widely used in researches for to prevent or treat possible side effects of anticancer agents. One of the compounds used for this purpose is Q flavonoid which is found in the structure of many fruits and plants. In this study, CYP-induced cardiotoxicity rat model was determined the protective effects of Q.

The LDH, AST and ALT are well known diagnostic parameters of cardiac injury, which heart failure, cardiotoxicity, myocarditis and myocardial infarction (Shanmugarajan, 2008). The LDH is a specific enzyme released into the blood and cytoplasm during cardiotoxic dysfunction. AST and ALT enzymes are critical transaminases that are released as a result of cardiac metabolism. CK and CK-MB enzymes are specific biomarkers that are determined in the heart failure (Viswanatha Swamy, 2013; Wei B, 2013; Benstoem 2015; Xu 2015; Alhumaidhaa, 2015). It has determined that CYP injection caused cardiac damage and increased serum LDH, CK-MB and AST enzyme levels (Liu, 2015). In the doxorubicin-administered rats has detected that have increased the LDH and CK-MB levels and this enzyme levels have decreased to normal levels by Q treatment (Matouk, 2013). In our study, CYP enhanced the levels of CK, CK-BM, LDH, AST and ALT enzymes and Q treatment reduced the CYP-induced elevated. So, the protective effect of Q on CYP-induced heart failure was consistent with the literature.

In the CYP-induced cardiotoxicity increases the free radicals production. This free radicals causes to deterioration the integrity and function of myocardial membrane. Besides, this is accompanied by vascular and endothelial damage in the myocardium. CYP is cause oxidative stress that decrease the myocardial GSH and SOD activities as well as increase the MDA level (Gunes, 2016; Asiri, 2010; Mythili, 2004; Gado, 2013; Shrivastava, 2011; Kumar, 2011). On the other hand, Q has normalized the increase in MDA levels and the decrease in SOD and GSH activities induced by anticancer agents or oxidative stress

(Matouk, 2013; Cao, 1997; Muthukumaran, 2008; Haleagrahara, 2009). Consistent with the literature, our study has showed that CYP treatment was increased MDA levels and decreased the SOD and GSH activities and the Q application corrected the these disorders.

The hemorrhagic myocarditis, cardiac degeneration, edema and lymphocyte infiltration in the myocardium are some pathologies in the CYP-induced cardiotoxicity (Song, 2016). Additionally, in the CYP-injected rats have determined myocardial hyalinization (Tarek, 2010). Also, the myocardial effusion, thickening of the left ventricular wall, fibrillation in cardiac myocytes and interstitium are histopathologic changes in cardiotoxicity (Dhesi, 2013). CYP causes histopathologic changes in vascular tissue as well as myocardium in cardiovascular system. CYP is metabolized the phosphoramidate and acrolein that these compounds could be cause damage in the vascular endothelium (Kupari, 1990). At the same time, CYP causes to deterioration of the integrity of endothelial layer in the tunica intima, a slight increase in aortic wall thickness and the cytoplasmic vacuolation in vascular smooth muscle cells in the tunica media (Moirangthem, 2016). In accordance with the literature, in our study has determined that CYP was caused to endothelial dysregulation, degenerative areas, thickening in vascular wall and breaking away and breaks in the elastic fibers in the especially medial layer in the cardiac tissue sections. It was determined that Q was normalized these changes with the protective effect.

β -MHC is isoform of the MHC predominantly expressed in the ventricle (Morkin, 2000). β -MHC levels are either elevated in left ventricular dysfunction patients, or elevated in patients with heart failure (Pingitore, 2006; Taneja, 2014). In the CYP-induced cardiotoxicity in rats has obtained that CYP increases to β -MHC mRNA expression in cardiac tissue (Liu, 2015). In our study has detected that β -MHC levels were higher CYP group from other groups and Q100 + CYP group decreased significantly.

The TUNEL method, which detects fragmentation of DNA in the nucleus during apoptotic cell death *in situ* TUNEL assay is method, which detects fragmentation of DNA in the nucleus during apoptotic cell death *in situ* (Alpsoy, 2013). CYP causes excessive production of free radicals (McCarroll, 2008; Senthilkumar, 2006). These free radicals bring about modification and peroxidation by oxidising carbohydrates, proteins, lipids and DNA in the cell (Halliwell, 1984). Avci et al. has determined that in the CYP intoxication has increased the tissues' DNA fragmentation levels in liver and renal tissue (Avci, 2016). Conklin DJ et al has obtained that CYP has enhanced to TUNEL positive cells in myocardium (Conklin, 2015). Besides, it has determined that Q reduced the count of TUNEL positive cells in the cardiomyocytes induced with doxorubicin treatment (Dong, 2014). In our study has defined that CYP treatment increased the number of TUNEL positive cells and Q decreased significantly the TUNEL positive cell counts.

In conclusion, our findings are supportive of the opinion that Q may have cardioprotective effects in CYP-induced cardiotoxicity. The CYP metabolites induces cardiac injury and the resultant histopathological alterations are consistent with increased of the cardiac enzyme levels. Especially the high dose of Q have protective effect against the cardiac oxidative stress with CYP-induced. Although our data are consistent with previous studies, we believe that there is need further studies on this topic.

Declarations

Compliance with ethical standards

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. 107/HADYEK, 27th May 2015).

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

Author contributions E.Ş. V.G. S.G. S.Ö. A.K C.G F.Ç. A.Ç. : experiment design, experiment application, samples collection. E.Ş. V.G. S. Ö. C.G.: serum markers and tissue antioxidant estimation, data curation and analysis, final reviewing. S.G. and A.K.: histopathological and immunohistochemical investigation. All authors contributed to the writing and editing, and they read and approved the final manuscript.

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

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

Consent to publish All authors have consent for the publication of the manuscript.

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Figures

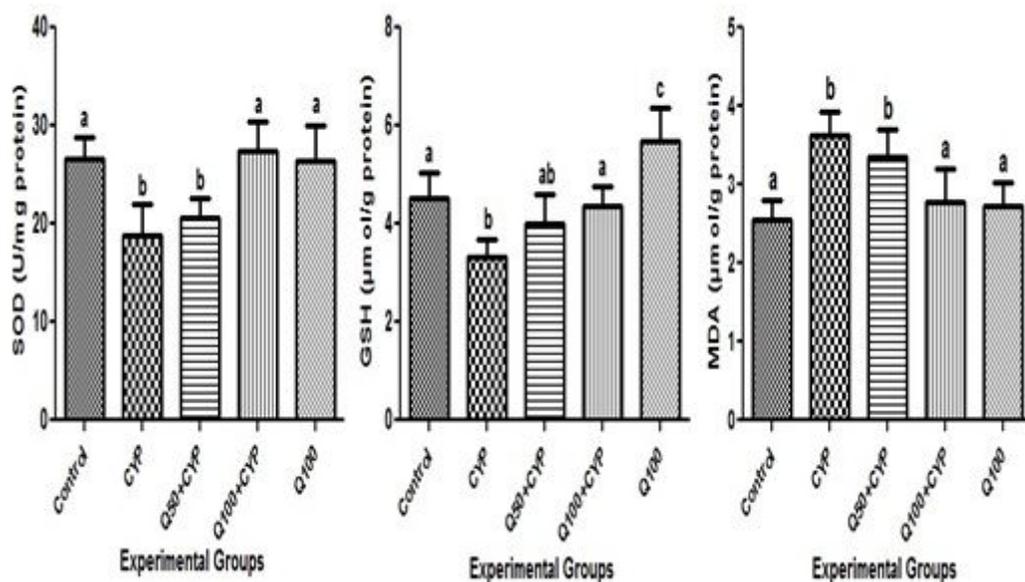


Figure 1

The cardiac SOD and GSH activities and MDA levels in experimental groups (There is no statistical difference between the groups expressed the same letters).

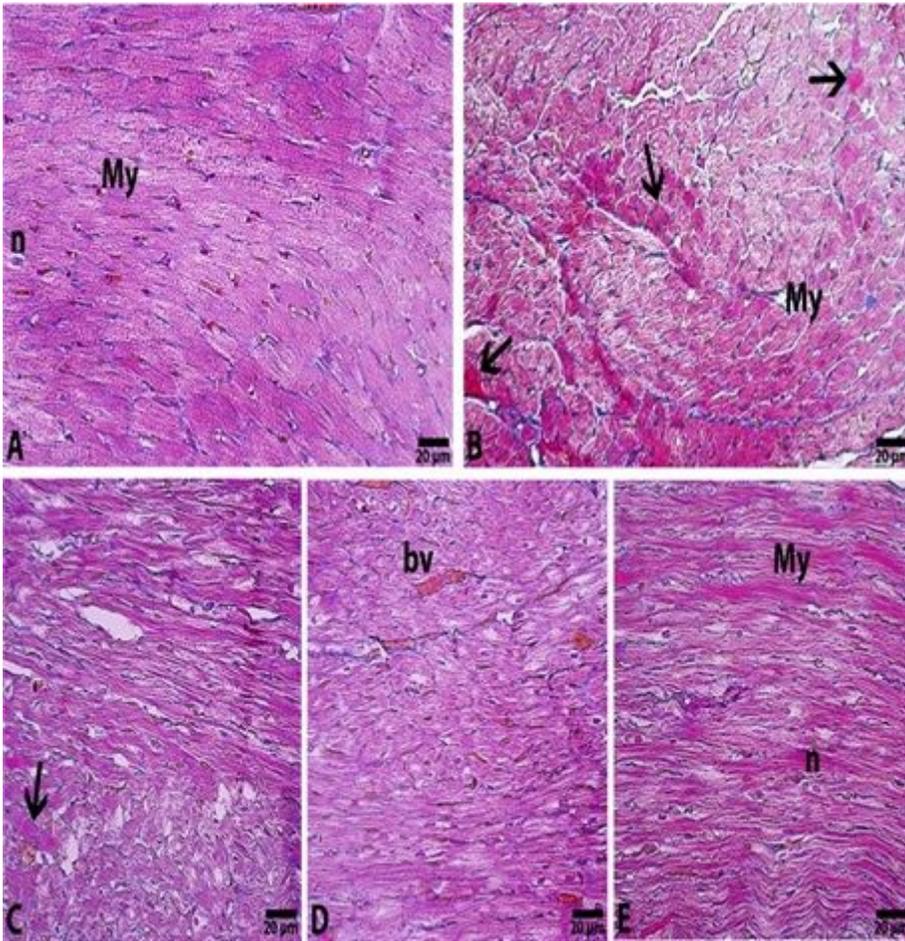


Figure 2

Histopathologic examinations of heart sections. Control (A), CYP (B), Q50+CYP (C), Q100+CYP (D) and Q100 (E) groups. My: cardiac myocyte; n: myocyte nucleus; bv: blood vessel; arrows: necrotic myocardial cells with dense eosinophilic cytoplasm and dark nucleus. Crossman's modified Mallory triple staining.

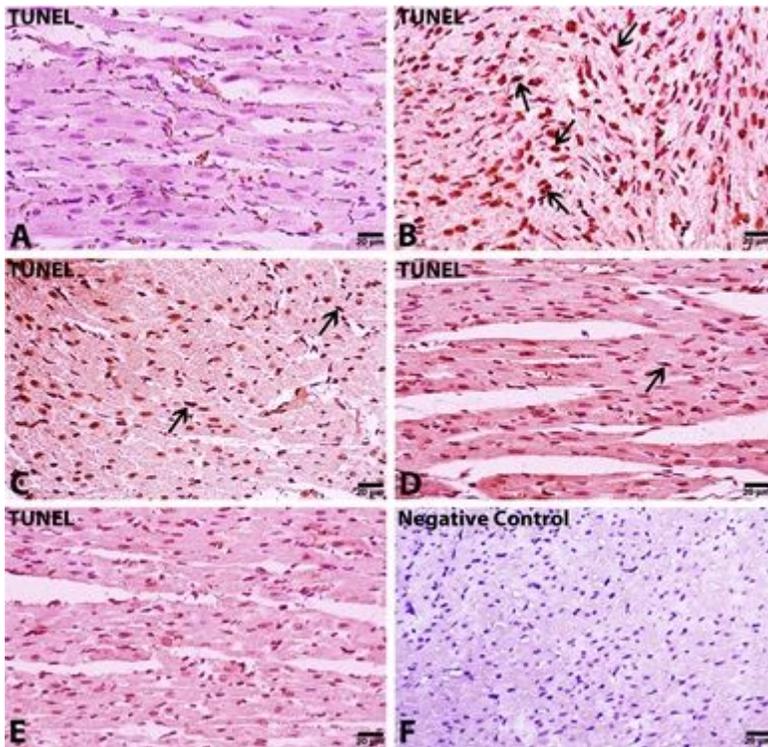


Figure 3

Immunohistochemical staining for the β -MHC for the Control (A), CYP (B), Q50+CYP (C), Q100+CYP (D) and Q100 (E) groups and Negative Control (F). Arrows show β -MHC positivity in cardiac myofibrils.

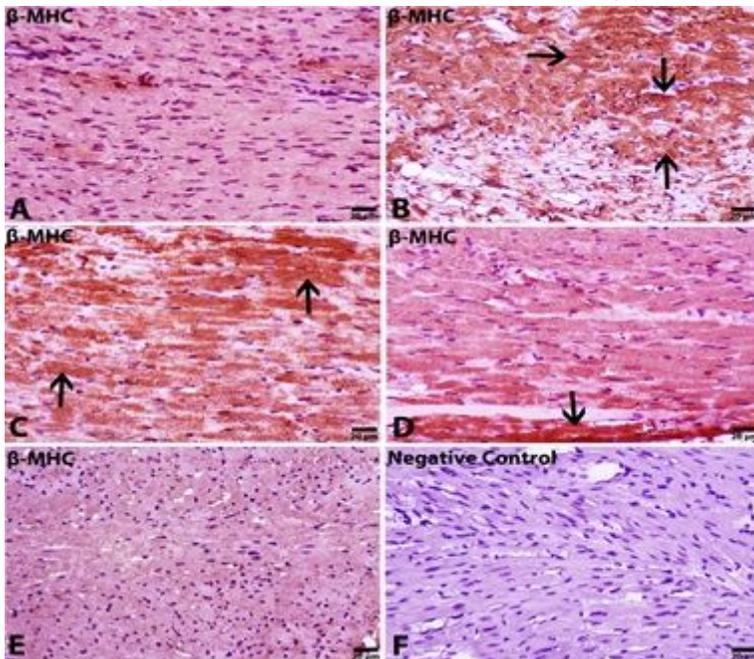


Figure 4

Representative photographs of TUNEL staining in Control group (A), CYP group (B), Q50+CYP group (C), Q100+CYP group (D), Q100 group (E) and Negative Control (F). Arrows indicate positive cells.