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Research Article

The Effect of Quercetin Application on Desmin and Vimentin Levels in Ovariectomized Rats with Cyclophosphamide-Induced Cardiotoxicity

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Abstract

The aim of our study was to investigate the effect of quercetin on cyclophosphamide-induced cardiotoxicity. A total of 35 female rats were used in the study, divided into five groups of seven rats each. All of the rats, except those in the control group, underwent ovariectomy and had their ovaries removed. This eliminated the potential impact of hormones such as oestrogen and progesterone on the study. All rats were fed ad libitum. While the control and ovariectomy groups received no treatment, 50 mg/kg quercetin (oral) was administered to the quercetin group, 100 mg/kg cyclophosphamide (intraperitoneal) to the cyclophosphamide group, and 50 mg/kg quercetin (oral) and 100 mg/kg cyclophosphamide to the quercetin + cyclophosphamide group was applied. electrocardiography (ECG) measurements were taken before toxicity induction (day 0) and on day 5 after toxicity induction. At the end of the study, the rats were euthanized under anesthesia in accordance with ethical rules, and tissue and blood samples necessary for analysis were taken. Troponin, creatine kinase (CK), and creatinine kinase MB (CK-MB) parameters were measured in the blood samples taken. Histopathological and immunohistochemical analyses (desmin and vimentin) were performed on heart tissue. According to the analysis, an increase in troponin, CK, and CK-MB parameters was observed in the cyclophosphamide group, while a decrease was observed in the quercetin group. In desmin and vimentin immunoreactivity, a decrease was observed in the cyclophosphamide group, while an increase was observed in the quercetin group. In conclusion, in our study, we demonstrated that quercetin has a positive effect against cyclophosphamide-induced cardiotoxicity.

Keywords: Cyclophosphamide, Troponin, Creatine kinase, CK-MB, Desmin, Vimentin

Introduction

The phenomenon of drug-induced cardiotoxicity represents a significant threat to human health. Furthermore, the growing prevalence of cardio-oncology in the context of cardiotoxicity represents a significant contemporary concern [1,2]. Cyclophosphamide is an alkylating anticancer substance that was first discovered in experimental studies on rat tumours. The substance exhibits significant cytotoxic and immunosuppressive properties, classifying it as an oxazaphosphorine-substituted nitrogen mustard [3]. It serves as the foundation for the majority of organ transplant preparatory procedures. In addition to its utilisation in combination chemotherapy for Hodgkin's disease, non-Hodgkin's lymphoma, leukemia, rheumatoid arthritis, Burkitt's lymphoma, lupus erythematosus, multiple sclerosis, neuroblastoma, multiple myeloma, endometrial cancer, breast cancer, and lung cancer, it is also a widely active anticancer and immunosuppressive agent. Cyclophosphamide is administered in high dosages for the treatment of lymphomas and solid tumours. The combination of cyclophosphamide and bone marrow transplantation is a viable therapeutic option [1,4,5]. Cyclophosphamide has been demonstrated to induce cardiac damage when administered in high

doses. Patients who receive doses in excess of 150 mg/kg per day are particularly vulnerable to cardiotoxicity [6]. Cardiotoxicity of an irreversible nature has been observed in conjunction with cyclophosphamide treatment [1]. Fatal cardiomyopathy has been reported in 2-17% of patients treated with cyclophosphamide [7]. Cardiotoxicity, a potential complication of cyclophosphamide treatment, has been observed in 7 to 28% of patients receiving the drug [4].

Quercetin is a polyphenol that is found in abundance in nature. It is a prevalent ingredient in numerous plant-based products. Onions are reported to contain the highest concentration of quercetin. It is hypothesised that this plant component exerts an anti-aging effect in addition to its antioxidant capabilities. It is recognised to exist in both conjugated and free forms. Quercetin has been shown to possess a range of biological activities, including antiviral, antioxidant, anti-inflammatory, anti-proliferative, anti-carcinogenic, and anti-diabetic properties. Quercetin, a bioavailable chemical compound, has been demonstrated to be efficacious in addressing a number of health issues [8]. Quercetin is a potent antioxidant that is currently employed in a variety of pharmaceutical products. Quercetin has been demonstrated to have therapeutic benefits in the treatment of various pathologies, including cancer, allergic reactions, inflammation, arthritis, and cardiovascular disorders [9]. A diet abundant in quercetin has been demonstrated to engender a number of health-promoting advantages. Its function as a coagulation, hyperglycaemia, inflammatory, and hypertension-lowering agent has been demonstrated. The supplementation of quercetin has been demonstrated to be efficacious in the treatment and prevention of a number of chronic conditions, including cardiovascular issues [10].

It is generally accepted that myocardial contusion is suspected due to high creatinine kinase MB (CK-MB) activity, whether it is expressed as a fraction of electrocardiography (ECG) findings and creatine kinase (CK)-total activity [11,12]. The presence of artefacts in the blood, such as creatine phosphokinase and CK macroenzymes, can complicate the measurement of CK-MB activity. It has been demonstrated that measurements of CK-MB mass remain unaltered by these effects. However, it is important to note that elevations in CK-MB mass and CK-MB activity have also been observed in cases of severe skeletal muscle damage. Another typical diagnostic method is a 12-lead ECG. However, in the initial hours following a significant injury, an aberrant ECG reading may simply be indicative of metabolic alterations [13].

Vimentin and desmin, the primary components of fibroblastic intermediate filaments, are present in the majority of mesenchymal cells. The distribution and localization of vimentin and desmin have been investigated in several neuromuscular diseases using monoclonal antibodies. Vimentin has been reported to be expressed in the fibres that regenerate in several neuromuscular diseases, despite being rarely found in normal human muscle fibres. Furthermore, it is mentioned that these fibers exhibit a high level of desmin antibody positivity. In standard muscle fibres, desmin is localised exclusively at the level of the Z line. It has been hypothesised that desmin and vimentin may be overexpressed during muscle regeneration processes due to their importance in the structural organisation of the sarcomere [14]. Concurrently, a mutation in the desmin gene has been demonstrated to be a causative agent for an inherited heart and skeletal muscle disease [15].

The objective of this study was to demonstrate quercetin's protective effect on desmin and vimentin levels against cyclophosphamide-induced cardiotoxicity.

Materials and Methods

Ethical Approval

The study was initiated subsequent to the acquisition of the permission number KAÜ-HADYEK, 2022-018 from the Ethics Committee of Kafkas University for Animal Experiments.

Animals

In the present study, a total of 35 female Wistar albino rats, with a weight range of 250-350 g, were utilised, with seven rats assigned to each group. Prior to the commencement of the experiment, the rats underwent ovariectomy. The rats selected for inclusion in the study were accommodated in standard cages, with an ambient temperature maintained at 22±2°C, a photoperiod of 12 h of light and 12 h of darkness, and access to ad-libitum tap water.

Ovariectomy

The study involved the performance of ovariectomy operations on a total of 28 rats, which were divided into four groups. The rats were anaesthetized using a combination of ketamine HCl (75 mg/kg) (Ketalar®, Pfizer) and xylazine HCl (15 mg/kg) (Rompun®, Bayer) for the ovariectomy procedure. Subsequent to the administration of anaesthesia, the rats were positioned in a supine position, and the operative area was prepared by means of shaving. The operation was performed with a median incision; the skin, muscle layers, and peritoneum were cut, the abdominal cavity was reached, and the suspensory ligaments and veins of the right and left ovaries were ligatized and removed using 3.0 polyglactin 910 (Vicryl® Ethicon). Subsequent to this procedure, the peritoneum and muscles were sutured using simple continuous stitches, while the skin was sutured using horizontal U stitches. In order to prevent postoperative complications, the administration of antibiotics (Iespor®, MENARINI)

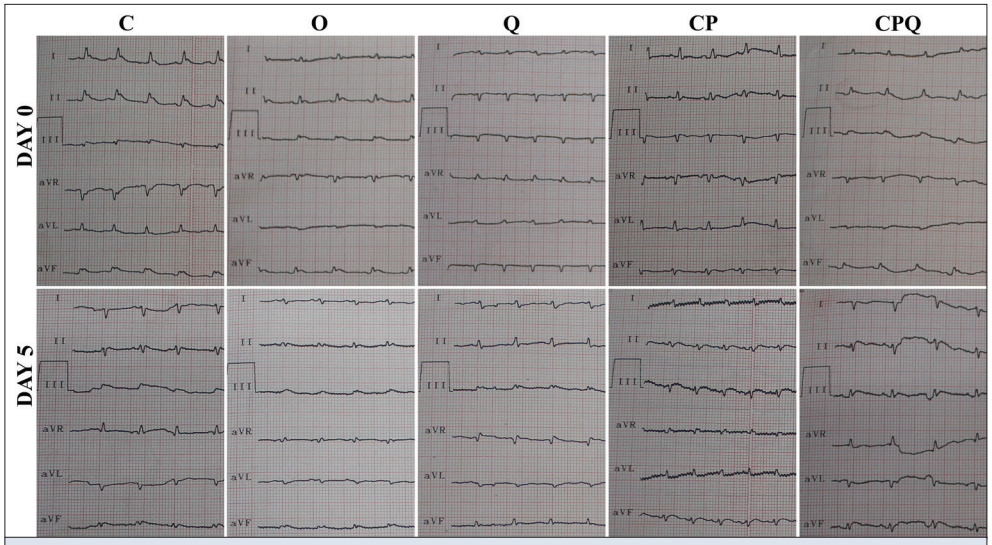

Fig 1. All groups electrocardiogram by days. C: Control group, O: Ovariectomy group, Q: Quercetin group, CP: Cyclophosphamide group, CPQ: Quercetin + cyclophosphamide group

was initiated for a period of four days. Thereafter, the incision site of the rats was examined on a daily basis for the presence of peritonitis and inflammation.

The commencement of the experimental study was scheduled 10 days subsequent to the ovariectomy procedure.

Creation of the Experimental Model

Control group (C, n = 7): Rats in this group will be fed standard feed and water.

Ovariectomy group (O, n = 7): Rats in this group were fed standard feed and water and then underwent ovariectomy.

Quercetin group (Q, n = 7): Rats in this group underwent ovariectomy, and 50 mg/kg quercetin was administered by oral gavage once a day for a period of 5 days [16].

Cyclophosphamide group (CP, n = 7): Rats in this group underwent ovariectomy, and 100 mg/kg cyclophosphamide was administered by intraperitoneally once daily for a period of 5 days [17].

Quercetin + cyclophosphamide group (CPQ, n = 7): Rats in this group underwent ovariectomy and started to administer quercetin 50 mg/kg by oral gavage 72 hours before cyclophosphamide administration, followed by 100 mg/kg cyclophosphamide [17] by intraperitoneally thread and 50 mg/kg quercetin oral gavage once a day for a period of 5 days [16].

At the conclusion of the experiment, the rats were euthanized under anesthesia by cervical dislocation. Blood (intracardiac) and tissue sampling were then performed on the rats. Following the centrifugation of the blood samples at 3000 RPM, the extracted sera were stored at -20°C until analysis. The presence of heart tissues was detected in a 10% formol solution for the purposes of histological and immunohistochemical analysis.

Electrocardiogram Measurement

Electrocardiography *(Fig. 1)* measurements were obtained from each animal in the experiment while it was under anesthesia. ECG measurements were taken prior to the induction of toxicity (day 0) and on day 5 following the induction of toxicity. The Nihon Kohden Cardiofax S ECG-1250 device was utilised for this purpose. Digital ECG records were obtained using the following leads: I, II, III, aVR, aVL, and aVF. The velocity of the ECG was set at 50 mm/s, and the calibration was set at 1 mV=10 mm. A 50-Hz filter was also applied *(Fig. 1)*. It is evident that no supplementary computations were conducted, as the device automatically calculates the QTc data.

Biochemical Analysis

In serum samples, troponin and CK-MB parameters were measured spectrophotometrically with a Beckman DXI 800 autoanalyzer, while the CK parameter was measured spectrophotometrically with a Beckman-Coulter AU5800 autoanalyzer.

Histological Examinations

Following the completion of the tissue follow-up procedure, the tissues are to be processed for paraffin embedding. Subsequently, hematoxylin-eosin staining will be performed on 5-µm sections taken from the paraffin blocks. A histological evaluation of the heart tissues will then be conducted.

Immunohistochemical Examinations

The streptavidin-biotin peroxidase method was applied to the sections taken on slides coated with chromium alumine gelatin. PBS (0.1 M, pH 7.2) buffer was utilised for the purpose of washing throughout the procedure. Sections: In order to inhibit endogenous peroxidase activity, the sample

was incubated in 3% H_2O_2 prepared in 0.1 M PBS for a period of 15 min. Subsequently, the citrate buffer solution was subjected to microwave heating for a period of 10 minutes at its maximum temperature setting, with the objective of releasing antigens. Subsequently, the specimen was subjected to an incubation with Large Volume Ultra V Block solution for a period of 10 min. Subsequently, the anti-desmin (SC-271677, 1/500 dilution) and anti-vimentin (SC-6260, 1/100 dilution) primary antibodies were applied to the sections at room temperature and in a humid environment for a period of 1 h. Subsequently, the samples were subjected to an incubation process. This involved the application of a biotin-conjugated solution comprising goat anti-B polyvalent antibodies, along with a streptavidin-streptavidin peroxidase complex, to the samples at ambient temperature. The duration of this incubation was set at 15 min for each solution. The dihydrochloric acid-hydrogen peroxide (DAB-H_2O_2) chromogen substrate solution was administered for the purpose of staining. A modified Gill III hematoxylin solution was utilised for the purpose of contrasting staining. In order to ascertain whether the immunoreactivity of desmin and vimentin was specific, all procedures were applied to the sections stored in PBS without the addition of primary antibodies. For the purpose of immunohistochemical evaluation, the target cells were evaluated by two independent observers according to the characteristics of non-staining (0), weak staining (1), moderate staining (2), or severe staining (3). This evaluation was conducted with consideration for the staining characteristics and density, and values ranging from 0 to 3 were assigned. Sections that had been prepared for histological and immunohistochemical studies were evaluated and photographed under a light microscope [18].

A total of three preparations were selected from each group for the purpose of counting immunopositive cells. The experimental units were then subdivided into four equal segments, and cell counts were made from a total of 12 areas in each group. The ImageJ software was utilised for the purpose of cell counts.

Statistical Analyses

Prior to the commencement of the study, power analysis was conducted using G-Power 3.1.9.7. The analysis indicates that the sample size was determined in accordance with the test power of 0.95 and the significance level of 0.05. A two-way analysis of variance (ANOVA) was conducted on ECG parameters in order to ascertain whether there was a difference between the groups under consideration. A one-way analysis of variance (ANOVA) was conducted on the biochemical and immunohistochemical parameters in order to ascertain whether there was a difference between the groups. A P-value of less than 0.05 was considered to be statistically significant. The statistical analyses were conducted using GraphPad 8.1 (San Diego, CA, USA).

Results

ECG Results

A comparison of the heart rate *(Fig. 2)* on day 5 reveals a significant increase in the CP group compared to the other groups. Concurrently, a statistical increase is observed in the CP group on day 5 in comparison with day 0.

With regard to the results of the PR analysis *(Fig. 2)*, a significant decrease was observed in the CP group on day 5 in comparison to the O and CPQ groups. Furthermore, a statistically significant decrease was observed in the CP group on day 5 in comparison with day 0.

An analysis of the QRS data *(Fig. 2)* revealed a statistically significant increase in the O group on day 5 compared to day 0.

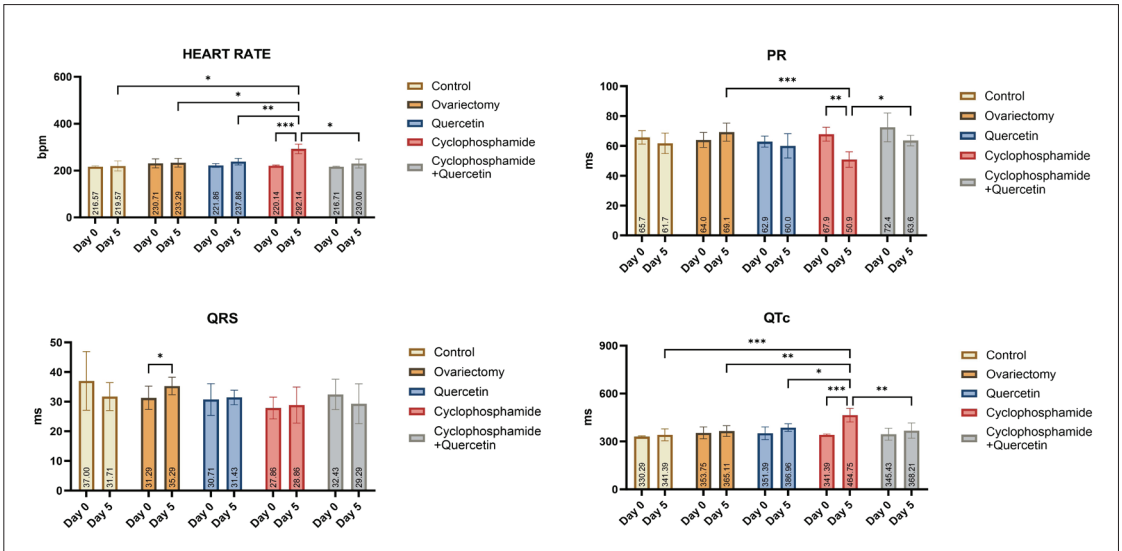

Fig 2. Means and standard deviation of the four groups for electrocardiography. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001

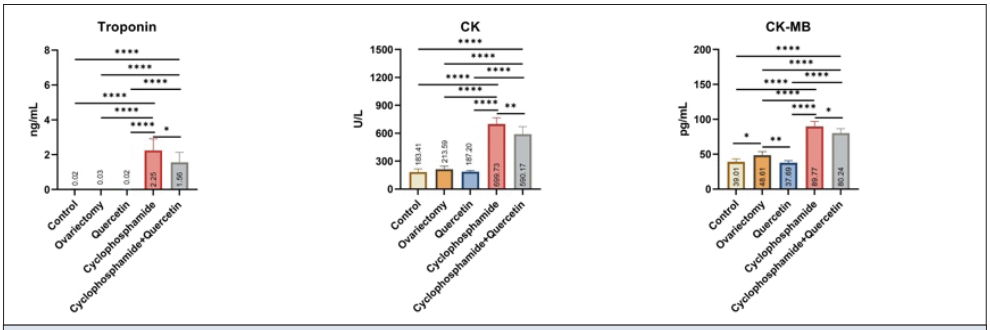

Fig 3. Means and standard deviation of the four groups for biochemical parameters. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. CK: Creatinine kinase (CK), CK-MB: Creatinine kinase MB

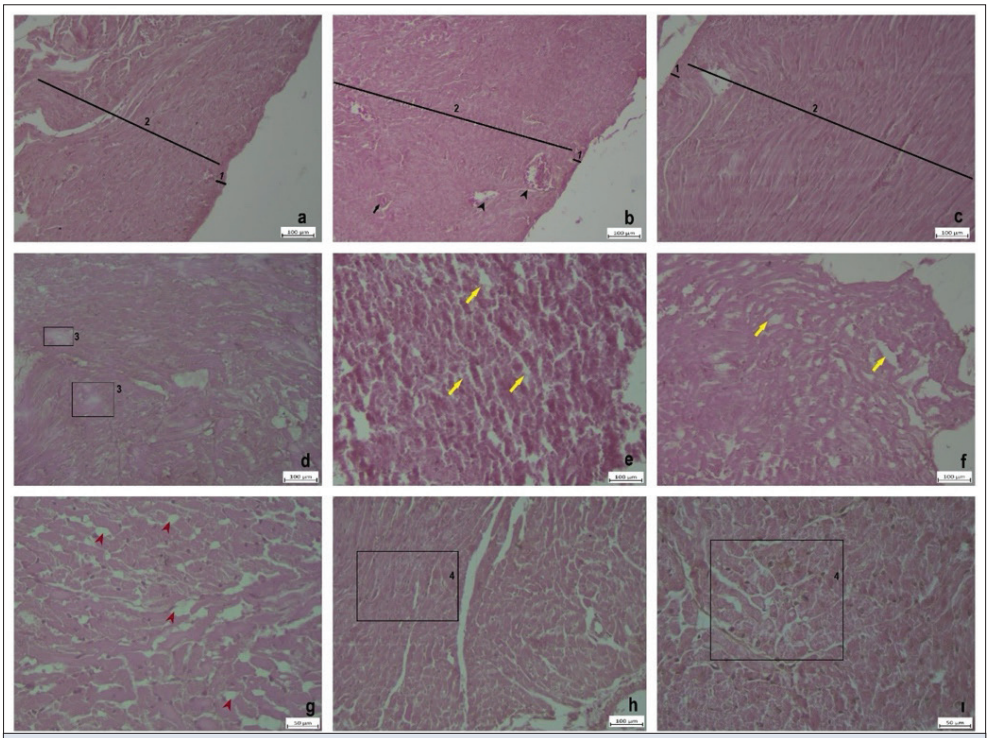

Fig 4. H&E staining of heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d, e, f, g), quercetin + cyclophosphamide group (h, i). Epicardium (1), myocardium (2), artery *(arrow),* vein *(arrowhead),* necrotic areas in heart tissue (3), fragmentation of myofibrils *(yellow arrow),* increased intercellular spaces (red arrow), histological appearance close to the control group (4)

In the QTc data *(Fig. 2),* a significant increase was determined in the CP group compared to the other groups when the day 5 data was analysed. However, a statistical increase was observed in the CP group on day 5 in comparison with day 0.

Biochemical Results

Biochemical analysis included the measurement of troponin, CK and CK-MB parameters *(Fig. 3).* In this context, a statistical increase in troponin, CK and CK-MB is observed in the CP group when the CP group is compared with other groups. When the CPQ group was compared with the control and O, as well as Q, groups, a significant increase in troponin, CK and CK-MB was determined in the CPQ group. Furthermore, a substantial increase in CK-MB levels was observed in group O in comparison to groups C and Q.

Histopathological Results

In groups C, O and Q, the heart tissue was observed to have a normal histology *(Fig. 4-a, b, c).* In the CP group, the presence of fragmentation of myofibrils, an increase in the intracellular space, and an increase in the spaces between cardiomyocytes and necrotic areas was detected *(Fig. 4-d, e, f, g).* In the CPQ group, the heart tissue exhibited a histological appearance analogous to that of group C. Myofibrillar fragmentation, intercardiomyocyte gaps, and necrotic areas were found to be present, though at low levels *(Fig. 4-h, i).*

Immunohistochemical Results

Strong desmin immunoreactivity was detected in the cytoplasm of cardiomyocytes in groups C, O and Q *(Fig. 5-a, b, c).* Weak immunoreactivity was detected in the CP

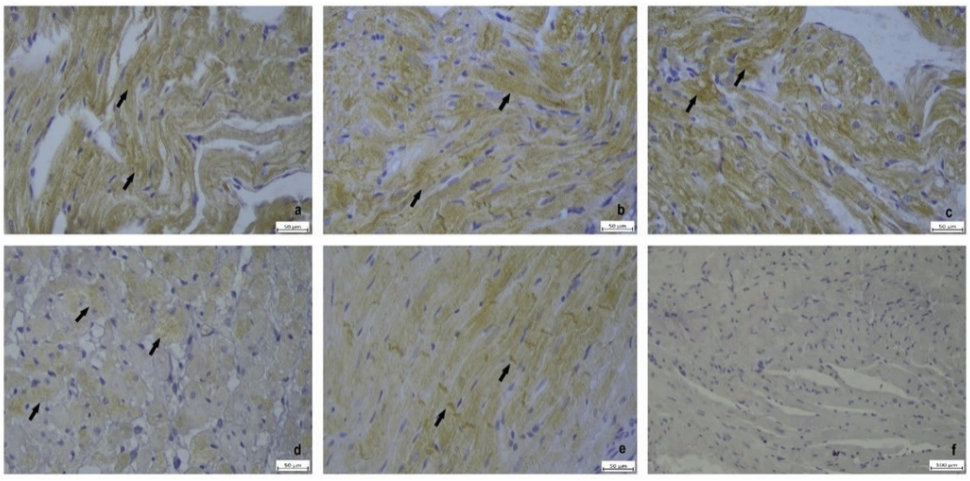

Fig 5. Desmin immunoreactivity in heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d), quercetin + cyclophosphamide group (e), Negative control (f). Cardiomyocytes *(arrows)*

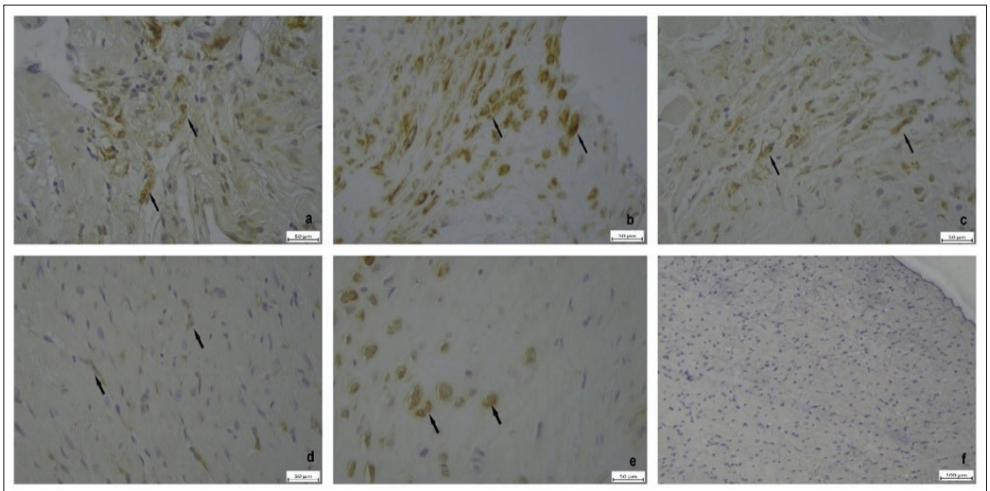

Fig 6. Vimentin immunoreactivity in heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d), quercetin + cyclophosphamide group (e), Negative control (f). Cardiomyocytes *(arrows)*

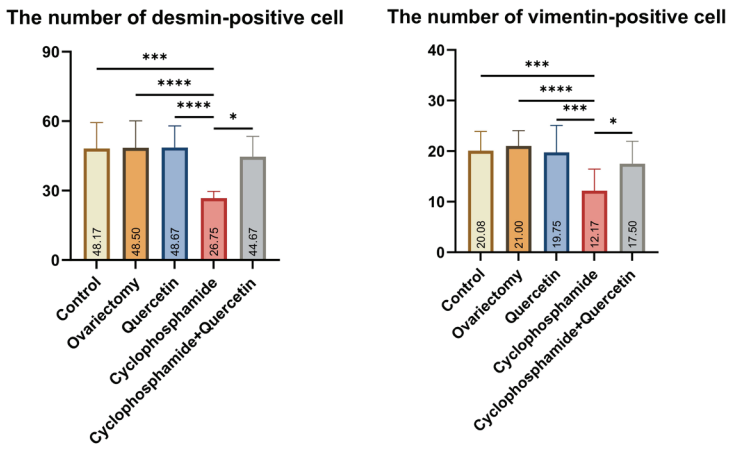

Fig 7. Means and standard deviation of the four groups for desmin and vimentin immunoreactivity parameters. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

group and moderate immunoreactivity was detected in the CPQ group *(Fig. 5-d, e)*.

Diffuse cytoplasmic and strong vimentin immunoreactivity was detected in cardiomyocytes in groups C, O and Q *(Fig. 6 a, b, c)*. Weak immunoreactivity was detected in the CP group and moderate immunoreactivity was detected in the CPQ group *(Fig. 6 d, e)*. In addition, moderate immunoreactivity was observed in the artery and vein walls of the heart tissue in all groups. In addition, when the number of vimentin positive cells was examined, a significant decrease was observed in the CP group compared to the other groups *(Fig. 7)*.

It was noted that desmin immunoreactivity was diffusely cytoplasmic and banded. Additionally, when the number of desmin positive cells was examined, a significant decrease was observed in the CP group compared to the other groups *(Fig. 7)*.

Discussion

Cyclophosphamide is a widely employed anticancer pharmaceutical agent. The medication has been employed in a wide range of clinical settings; however, it has been observed that it can result in cardiotoxicity, which is a significant cause for concern. The observed cardiotoxic properties were reported at the therapeutic dose. Consequently, this condition frequently results in a high mortality rate and suboptimal clinical outcomes [19] Patients receiving cancer chemotherapy can generally be considered to be at high risk of heart failure or in the stage A heart failure group [1].

The primary objective of this study was to investigate the potential protective role of quercetin in modulating the expression of desmin and vimentin in cardiac tissue subjected to cyclophosphamide-induced cardiotoxicity.

The pathophysiology of cardiac damage caused by cyclophosphamide is not fully known [20]. It is hypothesised that proteins, blood cells and hazardous metabolites may extravasate as a result of oxidative stress and direct endothelial capillary damage caused by its byproducts. The presence of toxic metabolites has been demonstrated to induce the degradation of endothelial cells, resulting in the impairment of the myocardium and the formation of capillaries. This process is associated with the occurrence of oedema, interstitial bleeding and microthrombosis [1,21]. Endothelial cells exhibit a heightened sensitivity to damage induced by cyclophosphamide in comparison to other cell types [22]. It has been hypothesised that this phenomenon may be associated with the elevated proliferation rate [23]. It has been reported that the formation of reactive oxygen species (ROS) caused by cyclophosphamide may lead to a decrease in nitric oxide bioavailability, leading to deterioration of endothelial function [24]. As demonstrated in the extant literature, there is evidence to suggest that cyclophosphamide can induce endothelial damage through the process of direct or oxidant damage.

Quercetin is a flavonoid found in various plants such as *Polygonum cuspidatum* Sieb. et Zucc. In addition to having strong antioxidant properties, it also has an anti-inflammatory effect. It is reported to have a therapeutic effect on cardiovascular diseases, metabolic disorders, neurodegenerative diseases, diabetes, cancer and obesity [25,26].

Endothelial dysfunction is a broad term and refers to impairment of nitric oxide (NO) production and/or imbalance of relaxation and contraction factors such as endothelium-derived endothelin-1 (ET-1), angiotensin and oxidants [27]. Endothelial dysfunction is one of the basic mechanisms in the atherosclerotic process. Classical and newly identified risk factors create chronic damage to the endothelium, leading to a decrease in the vasodilator response. Thus, events such as vasoconstriction in the endothelium, accumulation of inflammatory cells, migration of smooth muscle cells, and an increase in cytokine production cause atherosclerotic plaque formation [28,29]. As is known, cardiac muscle consists of smooth muscle cells. In this context, in our study, troponin, CK and CK-MB parameters were evaluated to determine cardiac muscle damage to support endothelial muscle damage caused by cyclophosphamide. The increase in these parameters in the CP group indirectly supports endothelial damage, according to the above literature information. In addition, the decrease in these parameters in the Q group compared to the CP group indicates ($P<0.05$) that quercetin reduces endothelial damage. It is thought that the reason for this is that quercetin reduces endothelial damage by preventing oxidant damage with its antioxidant properties.

A significant constituent of the cytoskeleton is constituted by desmin and vimentin filaments, which are classified as either homopolymers or heteropolymers, and are formed by smooth muscle filaments. Vimentin and desmin proteins are classified as type III filaments due to their structural and sequence similarities. The protein desmin, unique to muscles, is expressed by skeletal muscle, cardiac muscle and smooth muscle [30].

The oxidant effect of cyclophosphamide, particularly on the heart muscle, should be given due consideration. The absence of desmin in cells has been demonstrated to result in mitochondrial dysfunction. This is due to the fact that mitochondria play a pivotal role in the execution of necrotic and apoptotic mechanisms, as well as the synthesis of macroergic chemicals in oxidative muscle cells. There is also mounting evidence that certain types of myopathies and heart failure may be attributable to mitochondrial

dysfunction associated with dysregulation of desmin or vimentin cytoskeletal structures [31]. It is also reported that desmin and vimentin proteins directly participate in signal transduction [32]. A perusal of the study reveals that, on the day 5, QTc is prolonged in the CP group in comparison to the other groups. The rationale behind this phenomenon can be elucidated by referencing the deceleration in signal transmission, particularly during the transition from the atrioventricular nodule to the bundles of His. This decline is attributable to the diminution in desmin and vimentin cells, whose concentrations were reduced in the CP group within the ambit of our study. As demonstrated in our study, a number of cardiotoxicity studies demonstrate that QTc is prolonged during toxicity [33,34]. Conversely, the decline in the PR interval can be attributed to the robust signal emanating from the sinoatrial node, which rapidly reaches the atrioventricular nodule, yet subsequently decelerates due to a deficiency of desmin and vimentin. However, the results of this study suggest that the antioxidant effect of quercetin, which was utilised in the present study, mitigates the mitochondrial dysfunction induced by reactive oxygen species generated by cyclophosphamide. This observation resulted in an enhancement in the parameters observed in the QCP group compared to the CP group ($P<0.05$).

Quercetin has antioxidant, anti-inflammatory, and anticancer properties. It especially scavenges superoxide anion, singlet oxygen, and lipid peroxy radicals [35]. Within ROS-mediated cardiomyopathy, QCT has been reported to scavenge ROS and prevent the activation of mitogen-activated protein kinase and extracellular signal-regulated kinase [36,37]. In the present study, the positive effect on troponin, CK and CK-MB parameters is particularly noteworthy, and the findings are highly effective in light of the existing literature on cardiotoxicity. Consequently, it was determined that quercetin exhibited a therapeutic effect on cyclophosphamide-induced cardiotoxicity.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (MM). The data are not publicly available due to privacy or ethical restrictions.

Ethical Statement: The study has been approved by the Institutional Animal Care and Use Committee of Kafkas University (KAÜ-HADYEK, 2022-018).

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Conflict of Interest: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): Authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: SYA, MM, MK, EKS, AG and SEY analysed and interpreted the data. SYA, MM, MK, and EKS was a major contributor in writing the manuscript. MM analysed and interpreted the ECG data. MO analysed and interpreted the biochemistry data. SYA, EKS, AG, SEY analysed and interpreted the histopathology data. All authors read and approved the final manuscript.

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