Toxicology Reports 14 (2025) 102007

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Chrysin mitigates cyclophosphamide-triggered cardiotoxicity in rats: Insights into cardioprotection via Treg expression modulation and iNOS downregulation

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ARTICLE INFO

Handling Editor: Dr. L.H. Lash

Keywords:
Chrysin
Cyclophosphamide
Cardiotoxicity
ECG changes
Flow cytometry-iNOS

ABSTRACT

Cyclophosphamide (CP) is a potent chemotherapeutic agent widely used in cancer treatment; however, its clinical efficacy is limited by severe cardiotoxic side effects. This study aimed to evaluate the cardioprotective effects of chrysin, a natural flavonoid, against CP-induced cardiotoxicity in rats. To achieve this aim, forty male Wistar rats were randomly divided into five groups (n = 8 per group). Chrysin was administered orally at doses of 25, 50, or 100 mg/kg for 7 days before and 7 days after a single intraperitoneal injection of CP (200 mg/kg). Electrocardiography (ECG) was performed in vivo using the ECG PowerLab module to assess cardiac function, measuring the RR interval, heart rate, and corrected QT (QTc) interval. Serum levels of cardiac injury markers—creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH)—were also determined. Flow cytometry was utilized to evaluate the expression of regulatory T cell markers (CD4, CD25, and Foxp3) and apoptotic marker Annexin V. Histopathological assessment of myocardial tissues was conducted using hematoxylin and eosin (H&E) staining. Immunohistochemical analysis of inducible nitric oxide synthase (iNOS) expression was also performed. CP administration significantly elevated serum levels of cardiac injury markers compared with normal controls. ECG revealed that CP significantly altered cardiac function, as evidenced by a reduced RR interval, an increased heart rate, and an elevated QTc interval. In contrast, chrysin coadministration produced dose-dependent improvements; the highest dose (100 mg/kg) most effectively reduced serum CK-MB and LDH levels, improved the RR interval, decreased the heart rate, and partially restored QTc values. Moreover, CP significantly decreased the cardiac expression of regulatory T cell markers (CD4, CD25, and Foxp3) while markedly increasing Annexin V expression. Chrysin treatment reversed these changes in a dose-dependent manner, with the 100 mg/kg dose eliciting the greatest improvement in Treg expression and reducing Annexin V expression toward normal levels. Histopathological examination confirmed that CP induced myocardial congestion, edema, necrosis, and inflammatory cell infiltration, which were progressively ameliorated by chrysin, with the highest dose restoring near-normal myocardial architecture. Additionally, immunohistochemical analysis demonstrated that CP markedly upregulated iNOS expression in cardiac tissue, whereas chrysin dose-dependently downregulated iNOS, achieving complete normalization at the highest dose. Collectively, these findings suggest that chrysin exerts significant cardioprotective effects against CP-induced cardiotoxicity, likely through the modulation of Treg expression, attenuation of apoptosis, and suppression of iNOS-mediated inflammatory responses, underscoring its potential as an adjunctive therapy in chemotherapy-associated cardiac complications.

1. Introduction

Cyclophosphamide (CP) is an abundantly utilized chemotherapeutic agent known for its efficacy in treating various cancers and autoimmune diseases. However, its clinical utility is hindered by cardiotoxic side effects, ranging from mild arrhythmias to severe congestive heart failure [14,46]. Despite its therapeutic benefits, CP-induced cardiotoxicity remains a significant concern in clinical practice. In recent years,

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https://doi.org/10.1016/j.toxrep.2025.102007
Received 30 November 2024; Received in revised form 25 February 2025; Accepted 19 March 2025
Available online 22 March 2025

considerable attention has been given to identifying pharmacological agents capable of ameliorating CP-induced cardiotoxicity while preserving its therapeutic efficacy [5].

Cyclophosphamide is bioactivated by cytochrome P450 enzymes to generate toxic metabolites, notably acrolein [47]. Acrolein induces cardiotoxicity by generating excessive reactive oxygen species (ROS), which trigger lipid peroxidation, mitochondrial dysfunction, and membrane disruption in cardiomyocytes. Concurrently, acrolein impairs coronary endothelial function and upregulates pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS), leading to peroxynitrite formation [19]. These combined processes promote cardiomyocyte apoptosis and necrosis, culminating in myocardial edema and impaired contractility. This mechanistic cascade underlies CP-induced cardiotoxicity and supports the therapeutic potential of chrysin, whose antioxidant and anti-inflammatory properties may counteract these deleterious effects [4,7].

Furthermore, studies have shown that CP treatment leads to alterations in immune regulatory mechanisms, including the modulation of regulatory T cells (Tregs) expressing the CD4, CD25, and Foxp3 markers [20]. Tregs play a pivotal role in immune homeostasis by suppressing excessive immune responses and autoimmunity, serving as guardians of inflammatory and autoimmune diseases by suppressing the activity of T effector cells, including CD4+ T helper (Th) and CD8+ cytotoxic T (CTL) cells. Tregs employ diverse mechanisms to exert their immunosuppressive functions. CP-induced dysregulation of Tregs can disrupt immune tolerance and exacerbate inflammatory responses [34].

Moreover, CP treatment has been associated with increased expression levels of Annexin V, a sensitive marker of apoptotic cell death, in various pathological conditions, including chemotherapeutic-induced cardiotoxicity [52]. Additionally, CP-induced cardiotoxicity has been linked to the upregulation of inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of nitric oxide (NO) in response to inflammatory stimuli. Elevated iNOS expression contributes to oxidative stress, inflammation, and tissue injury, including the cardiac damage associated with CP treatment [31].

In this context, chrysin (5,7-dihydroxyflavone), a naturally occurring flavonoid abundantly found in various plant extracts, honey, and propolis, has garnered attention as a promising adjunctive therapy. The appeal of chrysin lies in its multifaceted pharmacological properties, including its potent antioxidant, anti-inflammatory, and cytoprotective effects [12,51]. Its pharmacological effects by scavenging ROS, inhibiting lipid peroxidation, and modulating key signaling pathways. Notably, it enhances endogenous antioxidant defense mechanisms, thereby mitigating oxidative stress and inflammation-induced cellular damage [40,44]. Preclinical studies have demonstrated that chrysin exhibits a favorable safety profile with minimal toxicity at therapeutically relevant doses [19]. Although its bioavailability is influenced by metabolic conjugation, emerging formulation strategies, such as nanoencapsulation and bioenhancer co-administration, have shown promise in optimizing its pharmacokinetic properties, supporting its potential therapeutic application [27].

Thus, the exploration of chrysin as a cardioprotective agent represents a pivotal endeavor in enhancing the safety and efficacy of CP-based chemotherapy regimens, ultimately advancing the quality of care for cancer patients. This study aimed to comprehensively investigate the possible cardioprotective ability of chrysin against cardiotoxicity induced by CP in rats, with a focus on multiple aspects of cardiac function and molecular mechanisms. These investigations provide a comprehensive understanding of the cardioprotective mechanisms of chrysin and its potential as a therapeutic agent for mitigating CP-induced cardiotoxicity. To achieve this aim, the effects of chrysin administration on serum markers and cardiac enzymes, electrocardiogram (ECG) parameters, and the protein levels of iNOS in cardiac tissue and regulatory T cells (Tregs), viz. CD4, CD25, and Foxp3 markers were assessed. Additionally, the effects of chrysin on the expression of annexin V, a marker of apoptosis, were investigated, and histopathological changes in cardiac tissue were analysed.

2. Materials and methods

2.1. Chemicals

Cyclophosphamide (CP) and chrysin were purchased from Sigma—Aldrich, USA (CAS numbers: 605-19-2 and 480-40-0, respectively). CP and chrysin were freshly prepared in saline. CP was obtained in its lyophilized form and freshly dissolved in sterile normal saline (0.9 % NaCl) immediately before administration to ensure stability. The CP solution was prepared at a concentration suitable for a single intraperitoneal (i.p.) injection, with each rat receiving 0.5 ml per 100 g of body weight to achieve the required dose. Chrysin was obtained in a high-purity form, suspended in distilled water, and thoroughly dissolved to achieve a suitable concentration for oral gavage administration at the required doses.

2.2. Animals

A total of 40 adult male Wistar rats (140–160 g) were procured from the animal breeding unit of the National Research Centre, Giza, Egypt. To ensure their well-being and reliable experimental outcomes, the rats underwent a one-week acclimatization period under standard laboratory conditions. Rats were housed in groups within appropriately sized cages, allowing for adequate movement and social interaction. The environment was maintained at a controlled temperature of 22 ± 2 °C with a 12-h light/dark cycle and relative humidity of 50–60 %. They were provided with a standard commercial diet meeting all nutritional requirements and had unrestricted access to fresh drinking water.

2.3. Ethical statement

All experimental procedures were conducted in accordance with ethical guidelines and approved by the Medical Research Ethics Committee (MREC) of the National Research Centre, Egypt (Permit no. 01410124). Animal welfare was rigorously maintained, with humane handling techniques and continuous monitoring to minimize distress. This study was designed and reported following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, ensuring transparency, reproducibility, and ethical integrity. All procedures adhered to the 3Rs principles (Replacement, Reduction, and Refinement) to minimize animal suffering while obtaining scientifically robust and reliable data.

2.4. Experimental design

Forty male Wistar rats were randomly divided into five groups, each consisting of eight rats, to ensure an unbiased distribution for the experiment, as illustrated in Fig. (1). The study followed a controlled, randomized design, with animals assigned to experimental groups in a manner that minimized bias and ensured reproducibility. Group I served as the control group and received an intraperitoneal (i.p.) injection of 0.2 ml saline daily for 15 days to establish baseline conditions and account for any effects of the injection procedure itself. Group II followed a slightly more complex regimen: these rats were initially injected with 0.2 ml of saline i.p. daily for the first 7 days. On day 8, they received a single intraperitoneal dose of CP at 200 mg/kg, a chemotherapeutic agent known to induce physiological changes. Saline injections (0.2 ml i.p.) were subsequently given for another 7 days. This group setup was designed to assess the effects of CP after saline pretreatment and to compare these effects against those of the other groups treated with both saline and CP [28,50]. Groups III, IV, and V were designed to investigate the potential protective effects of chrysin, a natural flavonoid, against CP-induced damage. Each group received different doses of chrysin to evaluate the dose-dependent effects. Specifically, Group III rats received

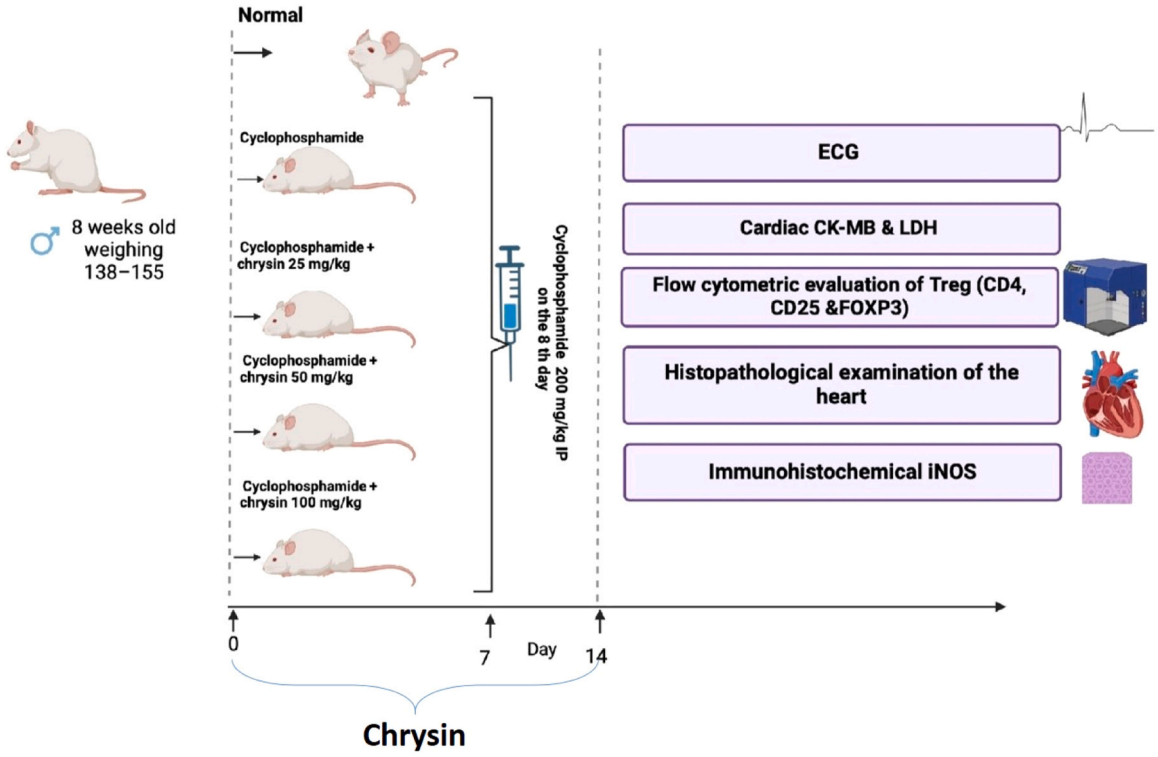

Fig. 1. A schematic representation illustrating the experimental timeline, including the acclimatization period, chrysin pretreatment, cyclophosphamide administration, post treatment duration, and endpoints for sample collection and analysis.

chrysin at 25 mg/kg body weight (b.w.) orally for the first 7 days. The Group IV rats were given 50 mg/kg b.w. chrysin, and the Group V rats received 100 mg/kg b.w. chrysin orally for the same duration.

On day eight, all three groups were administered a single intraperitoneal dose of CP at 200 mg/kg to induce toxicity. Following this, the administration of chrysin continued for another 7 days at the same respective doses (25, 50, and 100 mg/kg b.w.) for Groups III, IV, and V. This setup aimed to determine whether chrysin could mitigate the adverse effects of CP and whether its efficacy was dose dependent [40]. Following the completion of the treatment regimen, which included the last dose of saline, CP, or chrysin, the rats were anaesthetized via a mixture of ketamine and xylazine at dosages of 60 mg/kg and 5 mg/kg, respectively, administered intraperitoneally (i.p.). This anaesthesia was necessary to ensure that the rats remained immobile and pain free during the subsequent procedures. Once anaesthetized, cardiac function was assessed through electrocardiography (ECG) recordings. The use of ECG provided detailed insights into the cardiac health of the rats, ensuring a thorough examination of how the different treatments influenced heart function.

The serum levels of cardiac damage indices, including lactate dehydrogenase (LDH) and cardiac creatinine kinase (CK)-MB, were evaluated twenty-four hours after the electrocardiogram (ECG) was performed. Blood samples were obtained from the tail vein, allowed to clot, and then centrifuged at a speed of four thousand revolutions per minute for ten minutes. After that, the rats were allowed to sleep and scarify, their hearts were removed and cleansed in cold phosphate-buffered saline (PBS), and portions of the left ventricle were stored in liquid nitrogen until they were frozen. To evaluate the histopathological picture of the heart and the immune-histochemical evaluation of the cardiac values of inducible iNOS, additional samples from the heart were fixed in neutral buffered formalin at a concentration of 10 %. The remaining cells were homogenized in 10 mM ice-cold Tris–HCl buffer (pH 7.4) and centrifuged at 6000 rpm for ten minutes, and the clear supernatant was used to determine the levels of Tregs (CD4, CD25, and Foxp3) expressed.

2.5. Evaluation of CK-MB and LDH levels

Spinreact (Girona, Spain) provided the reagent kits that were used to measure serum CK-MB and LDH. The analysis was carried out in accordance with the instructions provided by the manufacturer.

2.6. Evaluation of electrocardiography alterations in rats

Electrocardiograms were taken one day before the scarification procedure, and the rats were anaesthetized with a combination of ketamine and xylazine [1,2,18]. Additionally, electrocardiograms were recorded with the assistance of an “ECG Powerlab module” that included an animal bioamplifier from Australia as well as a PowerLab/8 sp. Additionally, Lab Chart 7 software was utilized, which included an ECG analyser module which is able to detect the R-R interval, heart rate, QTc, R-amplitude, QRS interval, ST height for rodents and PR interval. Rats were placed in a supine position, and electrodes were attached to the limbs to record standard lead II ECGs [17,21,38].

2.7. Flow cytometric evaluation of Tregs

Conjugation of rat anti-CD4 (catalogue number 554837), anti-CD25 (catalogue number 550616), and FOXP3 (catalogue number 71577549) was performed on the cells that were produced from cardiac specimens. A BD-Accuri-C6 flow cytometer (BD Biosciences, San Jose, California, United States) was used to evaluate the level of cell damage, and the data obtained from the flow cytometer were analysed with Cell Quest v3.3 software [36,43].

2.8. Assessment of apoptotic cells

Cardiac levels of Annexin V were assessed via propidium iodide (PI) staining and flow cytometry to determine the number of apoptotic cells [49]. The harvested tissue was placed in an appropriate amount of diluted enzyme(s) in PBS, incubated before being gently pipetted and

filtered through a cell strainer to remove clumps and debris. The cells were subsequently centrifuged for 4–5 min at 2–8 °C at 400 × *g* to remove the supernatant. Finally, the cells were resuspended in the appropriate amount of flow cytometry stain to conduct a cell count and viability test via flow cytometry with an Annexin V/PI apoptosis detection kit [42].

2.9. Histopathological analysis

Heart specimens from all of the experimental groups were collected and then fixed in neutral buffered formalin at a concentration of 10 %. After that, the samples were dehydrated with increasing concentrations of ethyl alcohol, cleaned in xylene, and then embedded in molten paraffin. For the purpose of histopathological examination, sections with thicknesses of 4–5 μm were prepared and stained with hematoxylin and eosin (H&E) [10]. These sections were then examined by a pathologist in a blinded manner via a light microscope (BX43, Olympus). Additionally, the sections were photographed via CellSens dimension software (Olympus) connected to an Olympus DP27 image capture device. Histopathological changes in the cardiac tissues were quantitatively assessed on a scale from 0 to 3 in five randomly investigated microscopic fields for each animal (n = 6–8). The following is how the criteria were applied: the value (0) indicated that there were no changes, whereas the values (1), (2), and (3) indicated that there were mild, moderate, and severe changes [24].

2.10. Immunohistochemical analysis of inducible nitric oxide synthase (iNOS)

With reference to Saleh et al. [40] and Sedik et al. [43], an investigation of the expression of iNOS in cardiac sections was carried out. The sections were incubated with primary antibodies against iNOS (sc-7271) at a dilution of one hundred (Santa Cruz Biotechnology Inc., Dallas, Texas, United States of America). Diaminobenzidine tetrachloride (DAB, Sigma Chemical Company, St. Louis, Missouri, United States of America) was utilized to visualize the immunological reaction. Brown staining was observed in the cytoplasm and/or nucleus of the immunoreactive cells, with positive results. The staining intensity and its distribution were rated as negative (no staining), weak, moderate, or strong intensity, depending on the individual. For the purpose of estimating the amount of iNOS, the percentage of area expressed in five randomly selected fields within each section was measured and then averaged via the ImageJ image analysis software, version 1.46a, National Institutes of Health, Bethesda, Maryland, United States.

3. Statistical analysis

The results are presented as the means plus or minus the standard deviation (n = 8). We used one-way analysis of variance (ANOVA) with Tukey's post hoc test between the groups. To assess significant differences between the groups in terms of the histopathological lesion score, the nonparametric K independent samples Kruskal—Wallis test and Dunn's multiple comparison test were utilized during the analysis. Before analysis of variance (ANOVA), the Shapiro—Wilk test was used to determine whether all the samples were normally distributed. A statistically significant result was defined as a P value of less than 0.05. To analyse the data, GraphPad Prism version 9.0 (GraphPad Software, Inc., California, USA) was used. When the p value was less than or equal to 0.05, the difference was considered statistically significant.

4. Results

4.1. Effect of chrysin on the serum levels of CK-MB and LDH in cyclophosphamide-induced cardiotoxicity in rats

The results presented in Table 1 illustrate the serum levels of LDH and CK-MB in the various experimental groups. Compared with the normal control, a single intraperitoneal injection of CP at a dose of 200 mg/kg led to notable increases in both the serum LDH and CK-MB levels. Concurrent administration of chrysin at doses of 25, 50, and 100 mg/kg alongside CP resulted in a dose-dependent reduction in the serum LDH and CK-MB levels compared with those in the CP-injected group. In particular, coadministration of chrysin at the highest dose (100 mg/kg) with CP resulted in the most pronounced reductions in the serum LDH and CK-MB levels. These findings suggest a potential protective effect of chrysin against CP-induced cardiotoxicity.

Table1
Effect of chrysin on the serum levels of CK-MB, and LDH against cyclophosphamide-induced cardiotoxicity in rats.

	Serum LDH (ng/ml)	Serum CK-MB (pg/ml)
Normal control group	2.13 ± 0.03	28.56 ± 0.74
CP (200 mg/kg)	7.14 ± 0.07*	95.35 ± 1.73*
CP + chrysin (25 mg/kg)	5.01 ± 0.05*@	58.01 ± 1.09*@
CP + chrysin (50 mg/kg)	4.03 ± 0.07*@	46.63 ± 0.95*@
CP + chrysin (100 mg/kg)	2.83 ± 0.07*@	33.85 ± 0.58*@

Each bar represents the mean ± SE of 5–6 rats.
* Vs. Normal control group at $p < 0.05$.
@ Vs. Cyclophosphamide (200 mg/kg) control group at $p < 0.05$.

4.2. Effect of chrysin on the electrocardiography of rats with cyclophosphamide-induced cardiotoxicity

Compared with the normal control, CP (200 mg/kg) significantly altered cardiac contractility, as evidenced by a 16 % decrease in the RR interval ($p < 0.05$) and a 19 % increase in the heart rate ($p < 0.05$). Treatment with chrysin dose-dependently mitigated these effects. Compared with CP alone, chrysin at the highest dose of 100 mg/kg improved the RR interval by 18 % and reduced the heart rate by 15 % ($p < 0.05$ for both), although neither parameter fully normalized. In terms of conductivity, the CP and chrysin groups presented significantly shorter QRS intervals than the normal group did ($p < 0.05$), with no effect of chrysin. In terms of rhythmicity, compared with normal conditions, CP increased the QTc by 26 % ($p < 0.05$), and chrysin again improved the QTc in a dose-dependent manner, reducing the QTc by 18 % and 15 % at doses of 50 and 100 mg/kg, respectively ($p < 0.05$). However, chrysin does not fully normalize the QTc. Overall, chrysin ameliorated CP-induced impairment of cardiac contractility markers in a dose-dependent manner but did not improve conductivity or fully restore normal rhythmicity. The chrysin dose of 100 mg/kg provided the most substantial protective effects on cardiac function (Fig. 2 and 3).

4.3. Effect of chrysin on the cardiac expression levels of Tregs (CD4, CD25, and Foxp3) in cyclophosphamide-induced cardiotoxicity in rats

The cardiac expression levels of CD4 (Fig. 4), CD25 (Fig. 5), and Foxp3 (Fig. 6) in the rats that received CP were significantly decreased by 23 %, 16 %, and 47 %, respectively, compared with those in the normal control group. Compared with those in the CP group, the expression of these markers significantly increased by 40 %, 23 %, and 10 %, respectively, in the chrysin (25 mg/kg)-treated group. In addition, compared with those in the CP group, the preceding markers in the chrysin (50 mg/kg) group were significantly increased by 54 %, 35 %, and 14 %, respectively. Moreover, the aforementioned parameters significantly increased by 68 %, 47 %, and 18 %, respectively, in the chrysin-treated (100 mg/kg) group compared with those in the CP-treated group.

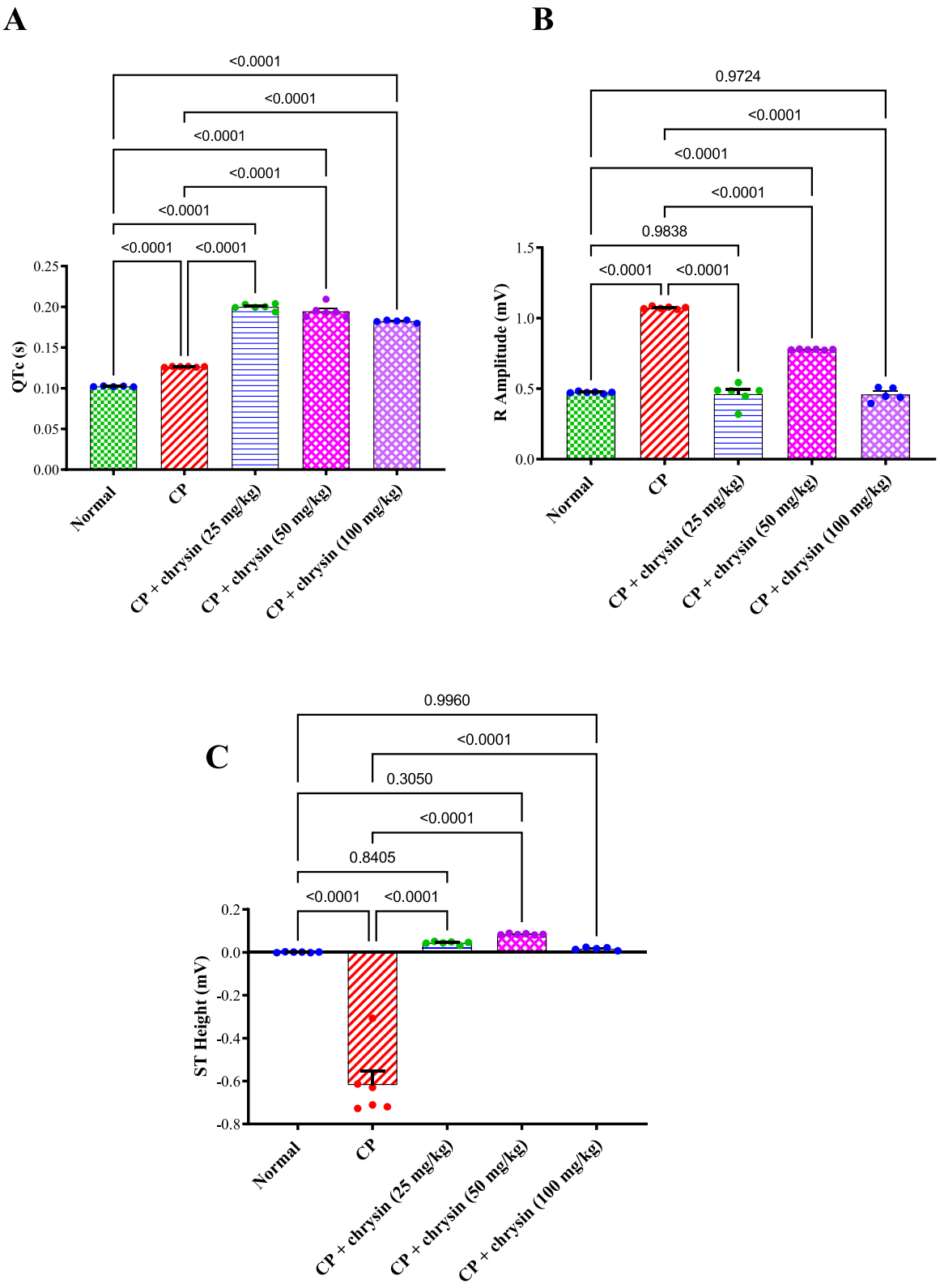

Fig. 2. Effect of chrysin on Cardiac Conductivity and Rhythmicity measurements QTc Interval [F = 607.2 (DFn = 4, DFd = 23 P < 0.0001] (A), R Amplitude [F = 268.7 (DFn = 4, DFd = 25 P < 0.0001] (B), and ST Height [F = 96.68 (DFn = 4, DFd = 24 P < 0.0001] (D) against Cyclophosphamide-induced cardiac derangements in Rats. Each bar represents the mean ± SE of 4–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

4.4. Effect of chrysin on the cardiac expression levels of Annexin V in cyclophosphamide-induced cardiotoxicity in rats

Cardiac expression levels of annexin V were evaluated by flow cytometry. We observed a significant increase ($p < 0.05$) in annexin V expression to 39 % in the CP group compared with the normal control group. However, compared with CP alone, chrysin at 25, 50, and 100 mg/kg significantly decreased ($p < 0.05$) annexin V expression to

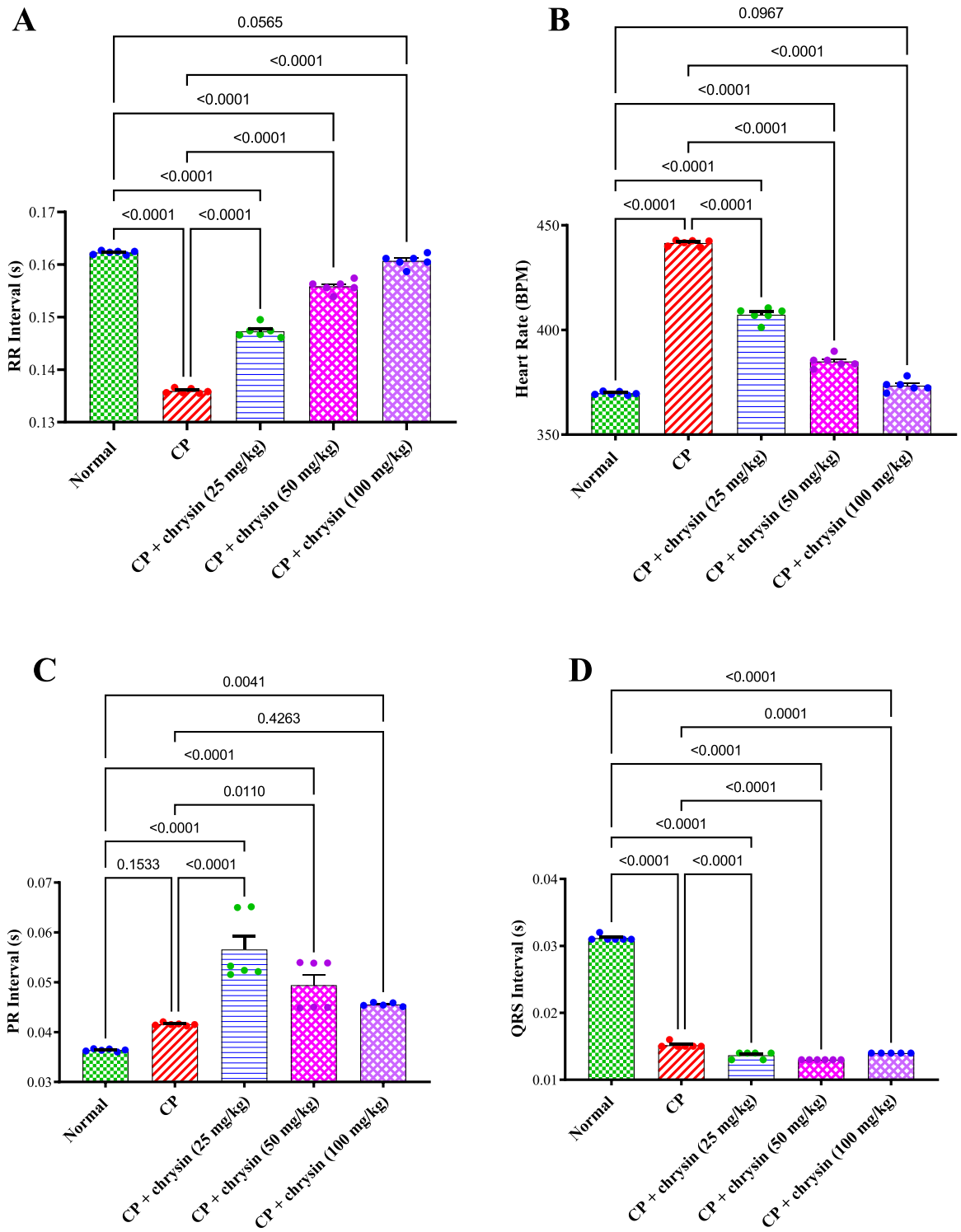

Fig. 3. Effect of chrysin on Cardiac Contractility measurements; RR Interval [F = 787.5 (DFn = 4, DFd = 25 P < 0.0001] (A), Heart Rate [F = 898.4 (DFn = 4, DFd = 25 P < 0.0001] (B), and PR interval [F = 25.01 (DFn = 4, DFd = 24 P < 0.0001] (C) QRS interval [F = 2848 (DFn = 4, DFd = 24 P < 0.0001] (D) against Cyclophosphamide-induced cardiac derangements in Rats. Each bar represents the mean ± SE of 5–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

19 %, 14 %, and 12 %, respectively (Fig. 7).

4.5. Effect of chrysin on the cardiac histopathology of rats with cyclophosphamide-induced cardiotoxicity

Fig. 8 and Table 2 elucidate the impact of chrysin on the histopathological alterations induced by CP. Microscopically, the cardiac tissue of normal rats displayed the typical histoarchitecture of heart myocytes (Fig. 8A). Conversely, CP administration led to histopathological damage in cardiac tissue, characterized by myocardial blood vessel congestion, Zenker's necrosis of myocytes, intermyocardial edema, and focal mononuclear cell infiltration (Fig. 8B & C). In contrast, hearts from rats treated with chrysin (25 mg/kg) presented an improved appearance with lesion regression, manifested by reduced myocardial blood vessel

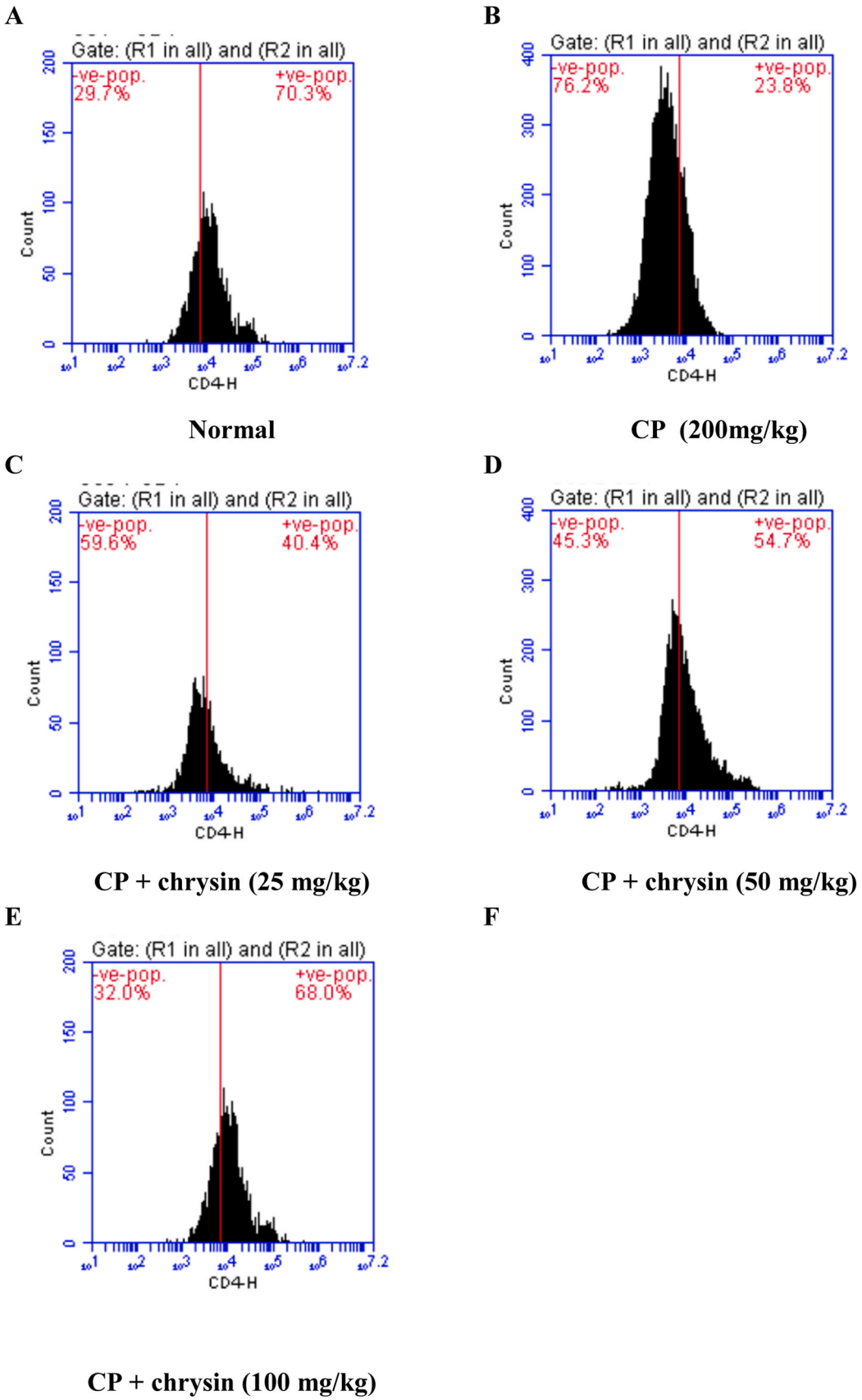

Fig. 4. Effect of chrysin administration on the cardiac percentage of CD4 in rats received cyclophosphamide-induced- cardiac injury [F = 1126 (DFn = 4, DFd = 25 P < 0.0001] Each bar represents the mean ± SE of 5–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

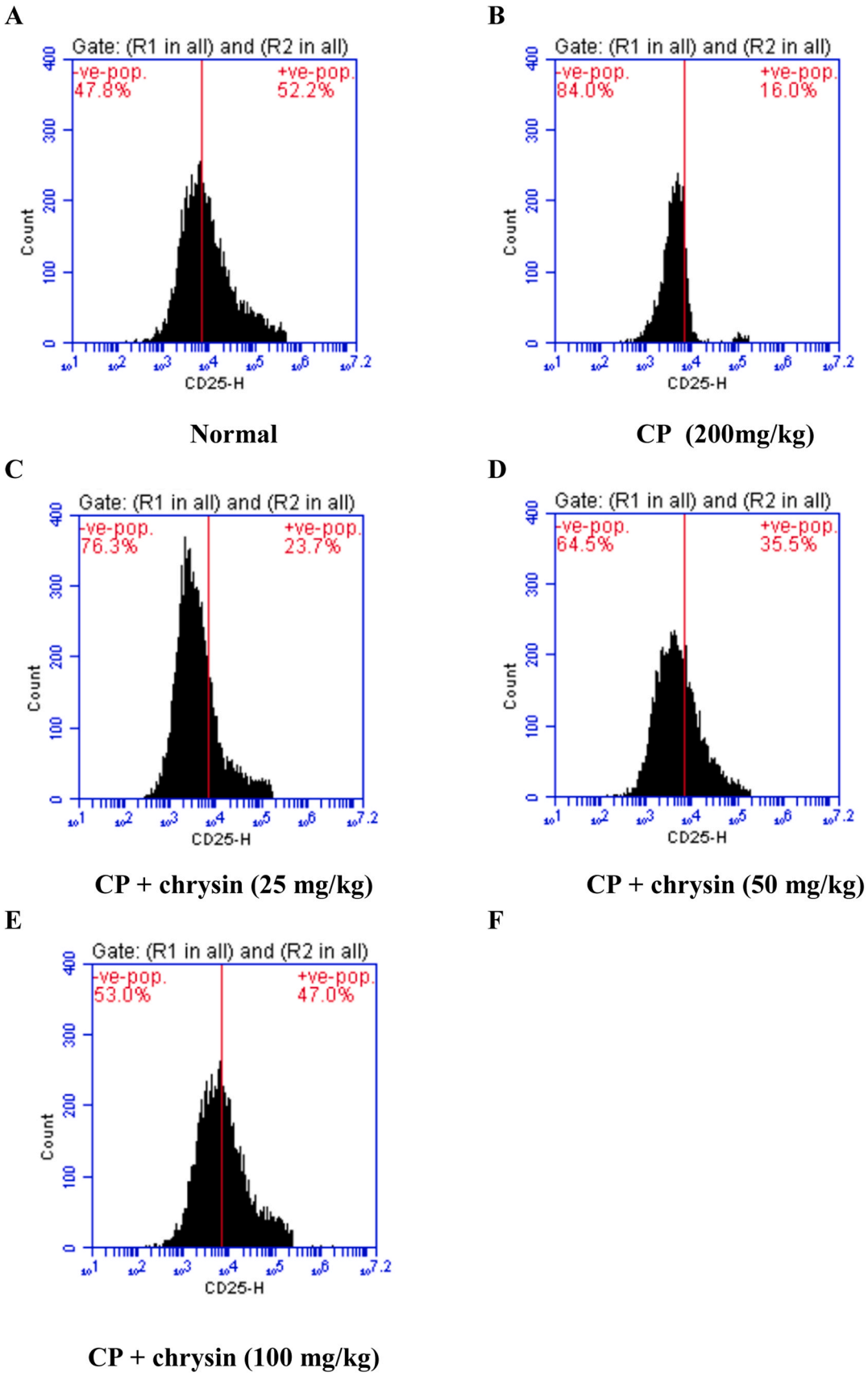

Fig. 5. Effect of chrysin administration on the cardiac percentage of CD25 in rats received cyclophosphamide-induced- cardiac injury [F =568.9 (DFn = 4, DFd = 25 P < 0.0001] Each bar represents the mean ± SE of 5–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

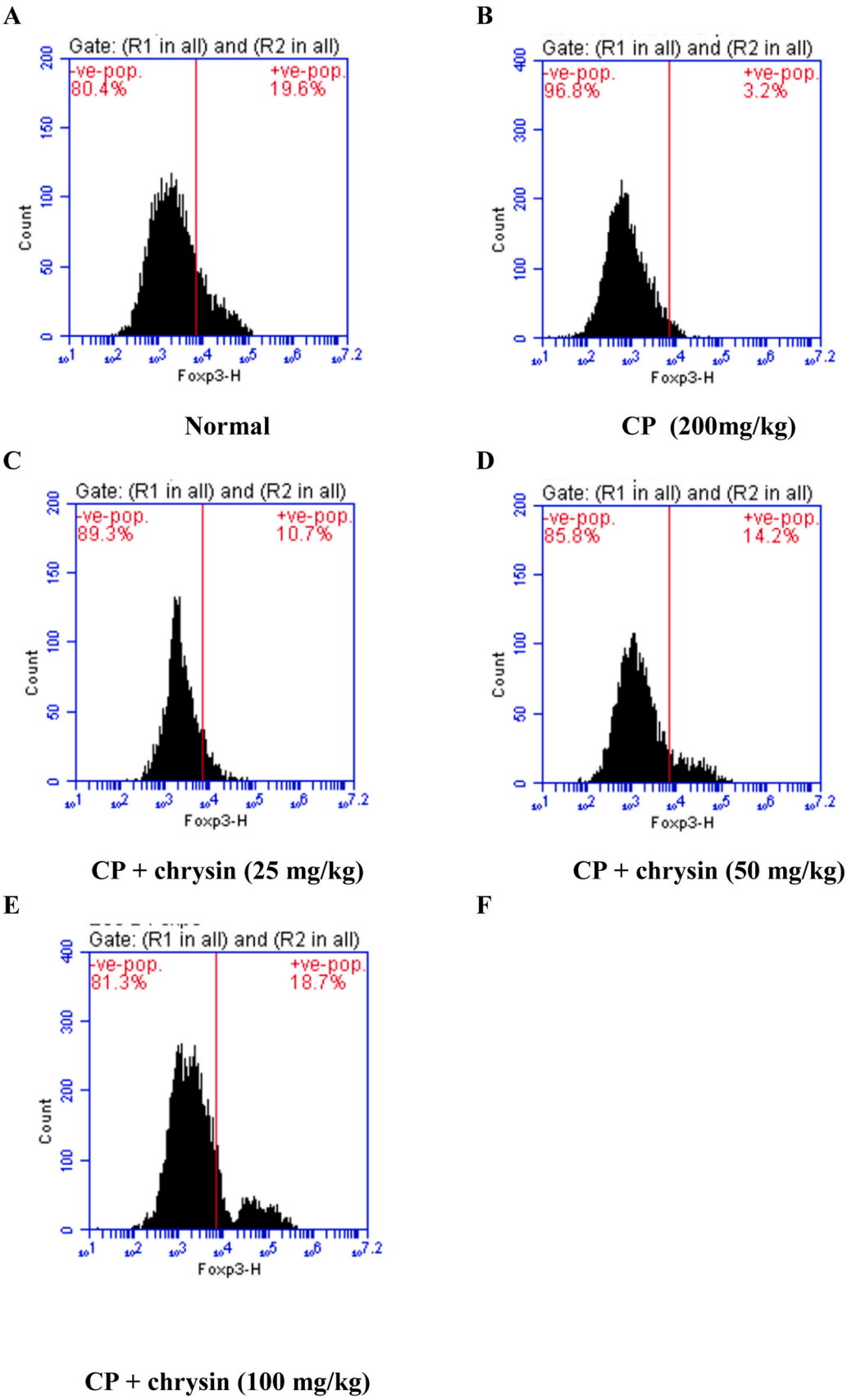

Fig. 6. Effect of chrysin administration on the cardiac percentage of FOXP3 in rats received cyclophosphamide-induced- cardiac injury [F =116.2 (DFn = 4, DFd = 25 P < 0.0001] Each bar represents the mean ± SE of 5–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

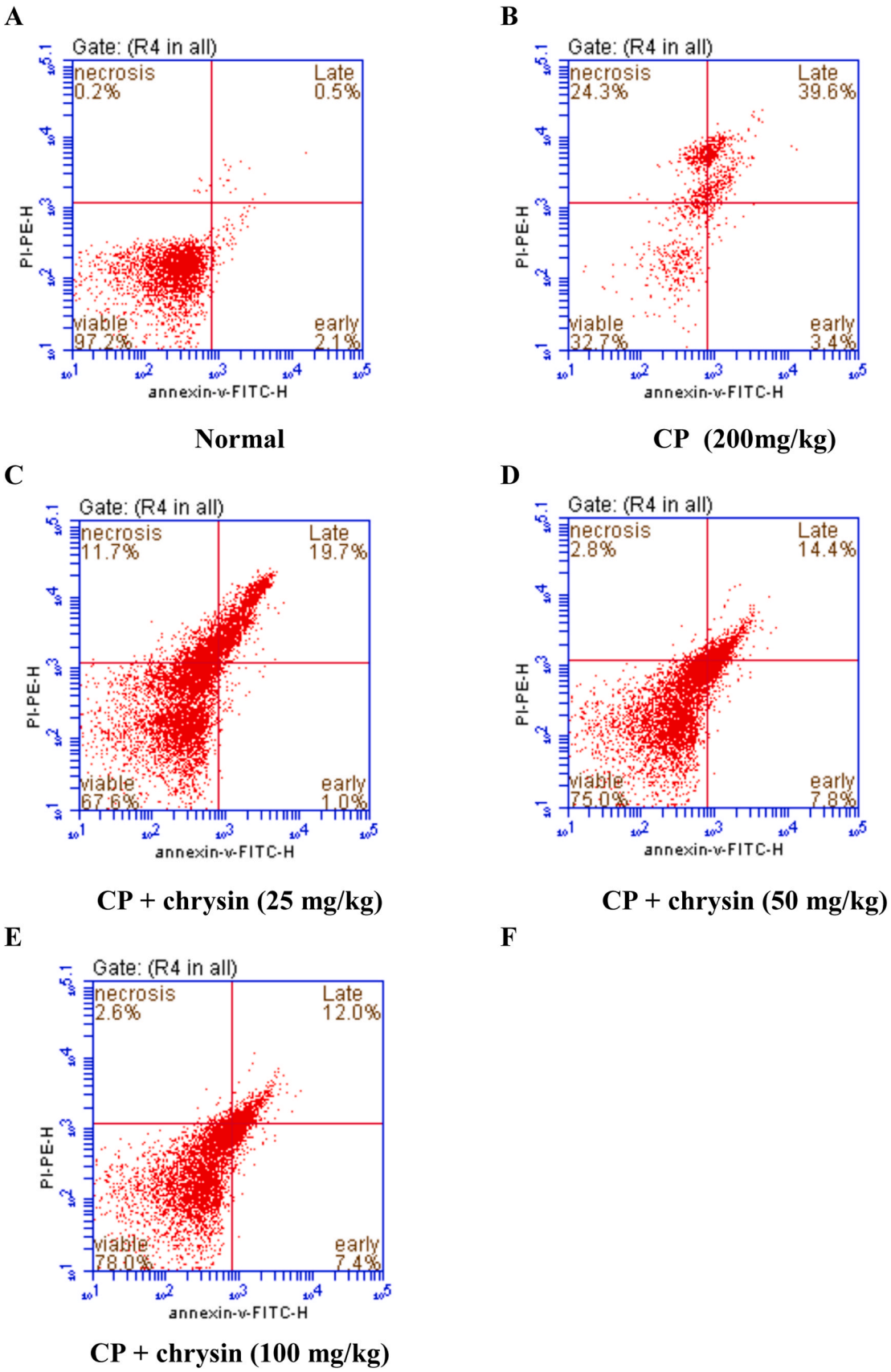

Fig. 7. Effect of chrysin administration on the cardiac content of Annexin V in rats received cyclophosphamide induced- cardiac injury [F = 492.2 (DFn = 4, DFd = 25 P < 0.0001] Each bar represents the mean ± SE of 5–6 rats. The exact P-value of Tukey's multiple comparison is stated above the pairwise comparison.

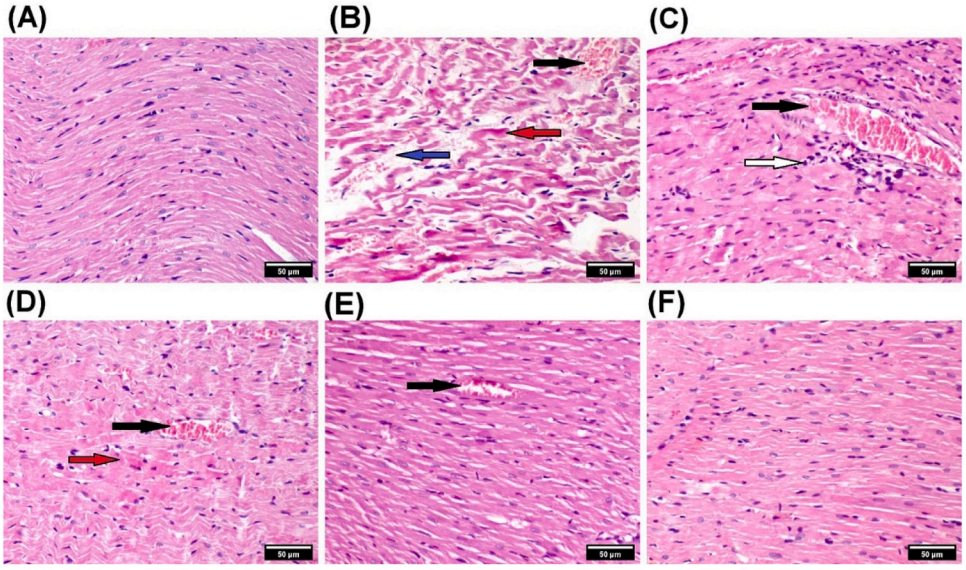

Fig. 8. Effect of chrysin administration on the histopathological picture in rats received cyclophosphamide-induced cardiotoxicity. Photomicrographs of H & E-stained heart sections of rats **(A)** control; showing the normal histoarchitecture of cardiac myocytes. **(B) and (C)** Cyclophoshamide group, showing congestion of myocardial blood vessel (black arrow), Zenker's necrosis of myocytes (red arrow), intramyocardial edema (blue arrow), and focal mononuclear cell infiltration (white arrow). **(D)The** Cyclophosphamide group was treated with chrysin (25 mg/kg), showing congestion of myocardial blood vessels (black arrow) and sparse necrosis of myocytes (red arrow). **(E)** The cyclophosphamide group was treated with chrysin (50 mg/kg), and slight congestion of myocardial blood vessels was observed (black arrow). **(F)** Cyclophosphamide group treated with chrysin (100 mg/kg), showing no histopathological lesions (scale bar, 50 μm).

Table 2
Effect of chrysin on cardiac histopathological lesion score of cyclophosphamide-induced cardiotoxicity in rats.

	Normal control group	CP (200 mg/kg)	CP + chrysin (25 mg/kg)	CP + chrysin (50 mg/kg)	CP + chrysin (100 mg/kg)
Congestion of cardiac blood vessels	0.0 ± 0.0	2.6 ± 0.22*	1.6 ± 0.22*@	0.8 ± 0.18*@	0.4 ± 0.22*@
Zenker's necrosis of myocytes	0.0 ± 0.0	1.8 ± 0.18*	1.2 ± 0.18*@	0.4 ± 0.22*@	0.0 ± 0.0@
Intermyocardial edema	0.0 ± 0.0	2.4 ± 0.22*	0.6 ± 0.22*@	0.4 ± 0.22*@	0.2 ± 0.18*@
Inflammatory cells infiltration	0.0 ± 0.0	1.6 ± 0.22*	0.6 ± 0.22*@	0.2 ± 0.18*@	0.0 ± 0.0@

Each bar represents the mean ± SE of 5–6 rats.
* Vs. Normal control group at $p < 0.05$.
@ Vs. Cyclophosphamide (200 mg/kg) control group at $p < 0.05$.

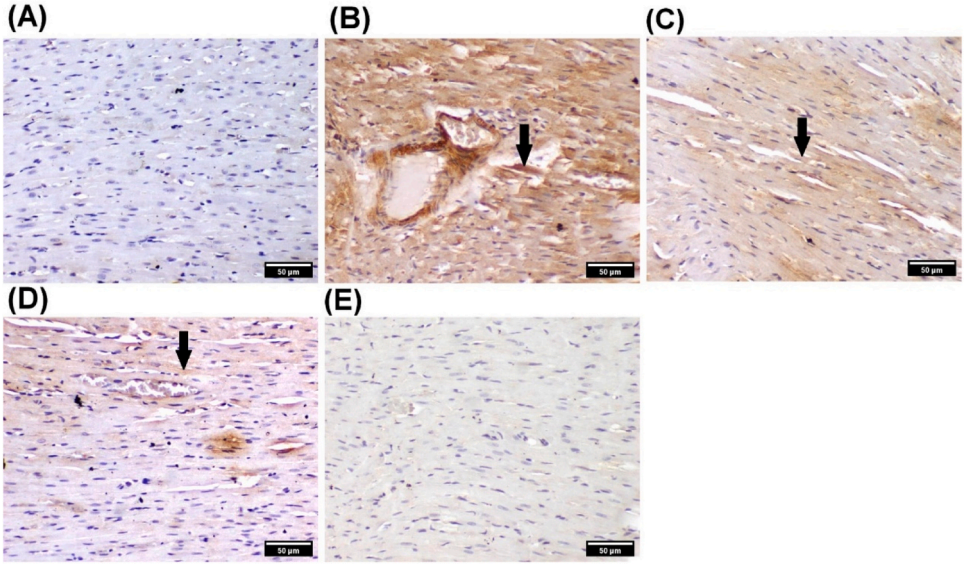

Fig. 9. Effect of chrysin administration on the cardiac expression levels of iNOS in rats received cyclophosphamide-induced cardiotoxicity. Representative photomicrographs demonstrated the brown iNOS immunostaining in the cardiac tissue (scale bar, 50μm); **(A)** Normal control, showing negative iNOS immune expression. **(B)** The cyclophosphamide group showed a significant increase in positive immunostaining cells. **(C)** Cyclophosphamide group treated with chrysin (25 mg/kg) showed moderate iNOS expression. **(D)** The cyclophosphamide group was treated with chrysin (50 mg/kg) and showed a weak immune reaction. **(E)** The cyclophosphamide group was treated with chrysin (100 mg/kg), and no iNOS expression was observed.

congestion and sparse myocyte necrosis (Fig. 8D). Moreover, cardiac sections from rats treated with chrysin (50 mg/kg) significantly improved, with only slight myocardial blood vessel congestion observed (Fig. 8E). Similarly, the hearts of the rats treated with chrysin (100 mg/kg) displayed no histopathological lesions (Fig. 8F) and presented a histologically normal appearance.

4.6. *Effect of chrysin administration on the cardiac expression levels of iNOS in rats subjected to cyclophosphamide-induced cardiotoxicity*

As demonstrated in Fig. 9, immunohistochemical expression of iNOS in the cardiac tissues of the normal control rats revealed negative immune expression (Fig. 9A). In contrast, a significant increase in strongly positive immunostained cells was detected in the cardiomyocytes of the rats that received CP (Fig. 9B). Moreover, treatment with chrysin (25 mg/kg) markedly downregulated the expression of iNOS. Moderate immune reactions were detected in the hearts of rats treated with chrysin (50 mg/kg) (Fig. 9C). Furthermore, weak immune reactions were investigated in the hearts of rats treated with D2 (Fig. 9D). Moreover, the hearts of the rats treated with chrysin (100 mg/kg) exhibited no iNOS immune reactions (Fig. 9E).

5. Discussion

Cyclophosphamide stands as a cornerstone in cancer treatment and is renowned for its potent anticancer efficacy across a spectrum of malignancies. However, despite its therapeutic value, the clinical utility of CP is frequently hampered by the emergence of cardiotoxic side effects. These adverse cardiac effects, ranging from mild arrhythmias to severe cardiomyopathy, pose significant challenges in the management of cancer patients receiving CP-based chemotherapy regimens. Consequently, in recent years, there has been a concerted effort within the scientific community to identify supplementary therapies capable of alleviating CP-induced cardiotoxicity without compromising its antineoplastic efficacy [6–9,11,25]. This study provides evidence that the natural flavonoid chrysin can protect against cardiotoxicity caused by CP in rats via the modulation of immune responses and oxidative stress. CP significantly increased blood levels of heart damage markers (CK-MB and LDH), altered heart rhythm (reduced RR interval, increased heart rate and QTc), and reduced levels of regulatory T cell markers (CD4, CD25, Foxp3) in heart tissue while increasing Annexin V, a marker of cell death. Chrysin treatment, especially at the highest dose, largely reversed these CP-induced changes, improving heart function, reducing injury markers, and restoring T cell marker and Annexin V levels. Furthermore, chrysin reduced CP-induced heart tissue damage (congestion, edema, necrosis, inflammation) and normalized elevated iNOS expression. The findings presented in this study shed light on the potential therapeutic implications of chrysin in mitigating the cardiotoxic effects induced by CP in rats. Understanding the mechanisms underlying CP-induced cardiotoxicity and exploring novel therapeutic interventions to alleviate its adverse effects are crucial for improving cancer treatment outcomes.

The significant elevation in the serum levels of LDH and CK-MB after CP administration underscores the cardiotoxicity associated with this chemical. These biomarkers function as key indicators of cardiac injury, reflecting cellular damage and the leakage of enzymes from myocardial cells into the bloodstream [7]. Notably, CP undergoes metabolism via the cytochrome P450 system, resulting in the generation of 4-hydroxycyclophosphamide, which subsequently decomposes into the active compound phosphoramide mustard. Phosphoramide mustard exerts its cytotoxic effects by alkylating DNA at the N7 position of guanine, thereby forming DNA interstrand crosslinks, which are considered the primary cytotoxic lesions [13]. The observed dose-dependent reduction in LDH and CK-MB levels concurrent with chrysin administration suggests a potential protective effect against CP-induced cardiotoxicity, which is consistent with the findings of a previous study [7,51]. The capacity of chrysin to alleviate the elevation of these biomarkers may indicate its role in safeguarding cardiac function and mitigating myocardial damage.

The alterations in electrocardiographic parameters, such as the RR interval, heart rate, QRS interval, and QTc interval, provide insights into CP-induced changes in cardiac function. Specifically, CP significantly increased the heart rate, decreased the RR interval, increased the QTc interval, and caused ST segment depression compared with those of normal control rats. The observed decrease in the RR interval and increase in heart rate following CP administration suggest impaired cardiac contractility and altered cardiac rhythm. The current study revealed that pretreatment and continuous administration of chrysin dose-dependently mitigated these ECG changes triggered by CP, indicating the potential of chrysin to restore cardiac function and rhythm. However, the incomplete normalization of conductivity parameters suggests that chrysin may not fully mitigate all aspects of CP-induced cardiotoxicity.

CP-induced tachycardia and a shortened RR interval indicate the development of sinus tachyarrhythmia, which aligns with clinical data showing an increased resting heart rate in cancer patients receiving CP chemotherapy [14]. Chrysin's lowering of heart rate and partial normalization of the RR interval demonstrate its antiarrhythmic effects. The prolongation of the QTc interval is a biomarker for ventricular arrhythmias, which are a major concern with cardiotoxic chemotherapies [7,15,30,45]. By reducing the QTc closer to normal levels, chrysin likely lowers the risk of lethal ventricular arrhythmias. Similar ECG protection was reported for chrysin against doxorubicin-induced cardiotoxicity in rats [32]. Additionally, chrysin has been reported to attenuate myocardial hypertrophy and prevent fibrosis in the context of isoproterenol cardiotoxicity [33]. The prominent ST segment depression caused by CP reveals myocardial ischemia and ventricular strain, which can precipitate heart failure [14,16,29]. Chrysin significantly ameliorated changes in the ST, indicating its ability to reduce CP-induced ischemic insult and preserve ventricular function. Similar electrocardiographic effects were observed in a prior investigation involving kaempferol, another natural flavonoid assessed for its protective effects against doxorubicin toxicity in rats [22]. Notably, kaempferol and chrysin are structurally similar to flavonoids, as both possess the same backbone. However, they differ in their distinct functional groups. Overall, the ECG data provide physiological evidence that chrysin alleviates key arrhythmogenic triggers and myocardial stress caused by CP. The dose-dependent improvements in ECG parameters related to rate, rhythm, conduction, and ischemia highlight the potential of chrysin to address multiple mechanisms of CP cardiotoxicity.

At the molecular level, the impact of CP on Tregs and lymphocyte populations was investigated in the present study to correlate cardiotoxicity with the levels of CD4, CD25, and Foxp3. CP leads to a reduction in Tregs, and unambiguous data indicate that this is caused by an increase in cell mortality along with a decrease in proliferative capacity. Current research indicates that inhibiting CP inhibits the activation and proliferation of Tregs, leading to improved cytolytic T-cell activity [48]. This is attributed to heightened cell death and diminished proliferative potential, with consistent findings across various research laboratories utilizing doses of approximately 100 mg/kg in rodents. A decrease in Tregs leads to increased cytolytic T-cell activity and increased CD4+ T-cell function, suggesting that CP has a role in inhibiting Treg activation and growth [23]. Notably, the effects of CP on lymphocyte populations appear to be dose dependent, with higher doses being more likely to induce lymphopenia and influence immune activation. These findings align with those of previous studies, suggesting a coherent understanding of the impact of CP on Tregs and lymphocyte subsets, offering valuable insights into the immunomodulatory effects of CP in preclinical models [23,41].

On the other hand, the evaluation of the effects of cardiac expression levels of Annexin V on CP-induced cardiotoxicity in rats in the present study provides crucial insights into the apoptotic processes occurring in

myocardial cells following CP administration. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine, a phospholipid component that is translocated from the inner to the outer leaflet of the plasma membrane during early apoptosis [37]. The observed significant increase in annexin V expression, reaching 39 % in the CP group compared with the normal control group, indicates an increase in apoptosis within cardiac tissue subsequent to CP treatment. This upregulation of annexin V suggests an enhanced apoptotic cascade, potentially resulting from the cytotoxic effects of CP on cardiac cells. These findings underscore the importance of annexin V as a sensitive marker for apoptosis and provide valuable evidence that CP-induced cardiotoxicity is mediated through apoptotic mechanisms. Further elucidation of the molecular pathways involved in CP-induced apoptosis in the heart may lead to the development of novel therapeutic strategies aimed at mitigating cardiotoxic side effects associated with CP chemotherapy.

Understanding the intricate relationship between CP and these molecular pathways, including Treg modulation and Annexin V expression, is crucial for deciphering the mechanisms underlying CP-induced cardiotoxicity. Chrysin shows promise as a potential adjunctive therapy to mitigate the adverse effects of CP on the cardiovascular system via the modulation of Tregs and Annexin V expression in cardiac tissue. These findings suggest that it has immunomodulatory and antiapoptotic effects. CP-induced cardiotoxicity is associated with dysregulation of immune responses and increased apoptosis in cardiac cells. The ability of chrysin to increase Treg numbers and decrease Annexin V expression may contribute to its cardioprotective effects by attenuating inflammation and apoptosis in the heart.

Another biomarker implicated in CP-induced cardiotoxicity is iNOS. While NO typically exerts anti-inflammatory effects physiologically, it assumes a pivotal role in fueling inflammation and oxidative stress under pathological conditions [35]. Consistent with previous findings [25], our study revealed a significant increase in iNOS levels following a single CP administration compared with those in the control group. This elevation can be attributed to acrolein, a CP metabolite, which prompts an increase in iNOS monomers and, subsequently, an increase in nitric oxide and nitrative stress [26].

Compared with CP alone, the concurrent administration of the flavonoid chrysin with CP resulted in a marked reduction in cardiac iNOS levels. The downregulation of iNOS expression in cardiac tissue following chrysin treatment suggests that chrysin has anti-inflammatory and antioxidant properties [39,40]. iNOS is known to play a role in inflammation and oxidative stress, both of which contribute to CP-induced cardiotoxicity. The ability of chrysin to suppress iNOS expression may mitigate inflammation and oxidative damage in the heart, thereby protecting against CP-induced cardiotoxicity. This finding is consistent with a prior investigation that demonstrated the ability of flavonoids to ameliorate NO levels in mice with ovalbumin-induced asthma [3].

Histopathological assessment vividly confirmed the biochemical alterations revealed by myocardial congestion, necrosis, edema, and inflammatory cell infiltration. Chrysin treatment attenuated these histopathological alterations, indicating the ability of chrysin to preserve myocardial structure and integrity. The dose-dependent improvement in cardiac histology suggested that higher doses of chrysin may offer greater protection against CP-induced cardiotoxicity. These results were in accordance with those of Ye et al. [51], who demonstrated that chrysin at doses of 25 and 50 mg/kg improved histopathological alterations in cardiac tissues compared with those in CP-induced rats [51].

In conclusion, chrysin exhibits significant cardioprotective effects against CP-induced cardiotoxicity by modulating Treg expression and downregulating iNOS. Its potential as an adjunctive therapy in CP-based chemotherapy regimens warrants further investigation. While this study highlights its therapeutic promise, limitations such as the use of an animal model and the need for long-term assessments should be considered. Further research is essential to confirm its clinical applicability and long-term safety.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

Dalia O. Saleh: Conceived and designed the study, performed the experiments, analysed the data, wrote the manuscript and revised the manuscript for scientific accuracy. Marawan A. Elbaset and Ahmed A. Sedik: Contributed to the data analysis and interpretation and revised the manuscript critically for important intellectual content. Kawkab A. Ahmed: Provided expertise in pathological assessment and revised the manuscript for scientific accuracy.

CRediT authorship contribution statement

Saleh Dalia: Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Sedik Ahmed:** Writing – original draft, Methodology. **Ahmed Kawkab:** Writing – original draft, Methodology. **Elbaset Marawan:** Writing – original draft, Supervision, Methodology.

Conflict of interest disclosure

The authors declare that they have no conflicts of interest relevant to this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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