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The protective effect of apelin-13 on cardiorenal toxicity induced by cyclophosphamide

Özden Kutlay, Arzu Keskin Aktan, and Esra Aslan

Abstract: Cyclophosphamide is a chemotherapeutic drug that is widely used in the clinic and can cause multi-organ toxicity. Apelin-13 is an endogenous adipocytokine with antioxidant properties. Therefore, this study investigated the possibility of apelin-13 being a potential therapeutic agent on cardiac toxicity and nephrotoxicity caused by cyclophosphamide. In this study, a total of four groups were formed with eight rats in each group. Group I: the control group was administered only saline (i.p.). Group II: cyclophosphamide, a single dose of 200 mg/kg (i.p.) on day 7. Group III: apelin-13 (15 μg/kg), for 7 days (i.p.). Group IV: administered apelin-13 (15 μg/kg) (i.p.) for 7 days and a single dose of cyclophosphamide (200 mg/kg) (i.p.) on day 7, the rats were sacrificed on day 8. Lactate dehydrogenase, cardiac troponin I (cTnI), creatine kinase MB (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), malondialdehyde, creatinine, and blood urea nitrogen were found to be high in the cyclophosphamide group; however, these values were reduced with apelin-13 administration. Antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and reduced glutathione (GSH) decreased in the cyclophosphamide group, and apelin-13 increased these enzyme activities. In addition, histopathological examinations also supported the results obtained. The findings of this study showed that apelin-13 has a protective effect against cardiorenal toxicity caused by cyclophosphamide.

Key words: apelin-13, cardiotoxicity, nephrotoxicity, cyclophosphamide toxicity.

Résumé : La cyclophosphamide est un médicament chimiothérapeutique largement utilisé en clinique qui peut entraîner une toxicité dans de nombreux organes. L'apeline-13 est une adipocytokine endogène dotée de propriétés antioxydantes. Par conséquent, cette étude portait sur la possibilité que l'apeline-13 constitue un agent thérapeutique éventuel contre la toxicité cardiaque et la néphrotoxicité entraînées par la cyclophosphamide. Dans cette étude, nous avons formé au total 4 groupes comprenant chacun 8 rats. Groupe 1 : le groupe témoin a reçu une solution saline (i.p.). Groupe 2 : dose unique de cyclophosphamide à 200 mg/kg (i.p.) au jour 7. Groupe 3 : apeline-13 à 15 μg/kg sur 7 jours (i.p.). Groupe 4 : administration d'apeline-13 à 15 μg/kg (i.p.) pendant 7 jours avec une dose unique de cyclophosphamide à 200 mg/kg (i.p.) au jour 7, puis les rats étaient euthanasiés au jour 8. Nous avons observé une hausse de la déshydrogénase lactique, de la cTn1, des CK-MB, de l'AST, de l'ALT, de la phosphatase alcaline, du malondialdéhyde, de la créatinine et de l'azote uréique dans le groupe cyclophosphamide, mais les valeurs diminuaient avec l'administration d'apeline-13. Les enzymes antioxydantes comme la SOD, la GPx, la catalase et le GSH étaient à la baisse dans le groupe cyclophosphamide, tandis que l'apeline-13 entraînait une augmentation de l'activité de ces enzymes. De plus, les examens histopathologiques venaient aussi appuyer les résultats obtenus. Les résultats de cette étude ont montré que l'apeline-13 a un effet protecteur contre la toxicité cardiorénale entraînée par la cyclophosphamide. [Traduit par la Rédaction]

Mots-clés : apeline-13, cardiotoxicité, néphrotoxicité, toxicité de la cyclophosphamide.

Introduction

Cyclophosphamide (CP) is a chemotherapy anticancer drug preferred in malignant diseases such as breast cancer, multiple myeloma, acute and chronic leukemia, as well as kidney diseases including rheumatoid arthritis, bone marrow suppression, and nephrotic syndrome resistant to corticosteroids (Teles et al. 2017), which is commonly used alone or in combination with other agents (El-Naggar et al. 2015). However, the effectiveness of the drug decreases as CP, like other chemotherapy drugs, damages healthy cells as well as cancer cells (El-Sheikh et al. 2017).

It has been shown that reactive oxygen species (ROS) such as superoxide anions are produced during the activation of CP (Ogunsanwo et al. 2017), which requires metabolic activation through the cytochrome P-450 enzyme system (Mansour et al. 2015). The underlying cellular mechanism of CP toxicity is caused by reactive oxygen radicals, which are highly produced by metabolites. Acrolein is a CP metabolite with a toxic side effect and interferes with the tissue antioxidant defense system, causing the production of ROS with mutagenic effects in mammals (Ogunsanwo et al. 2017). The ROS production causes changes in cell redox balance, causing damage to healthy cells along with cancerous cells (Mansour et al. 2015). Thus, CP, like other chemotherapeutic drugs, can also cause serious side effects, although it is the main tool in the treatment of malignancy. This toxic effect of CP can affect different organs such as the heart, kidney, bone marrow, brain, lungs, testicles, ovaries, liver, and spleen (El-Sheikh et al. 2017).

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In nephrotoxicity, which can accompany CP treatment, the primary adverse effects are decrease in glomerular filtration rate, increased serum creatinine and blood urea nitrogen (BUN), and renal failure (El-Sheikh et al. 2017). In addition, CP consumes protective antioxidants such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) (Goudarzi et al. 2017). In CP cardiotoxicity, however, changes in diastolic dysfunction and ejection fraction in the heart are followed by a prognosis that translates into fatal acute heart failure within a month (El-Sheikh et al. 2017). Within 10 days after the administration of a high dose of CP, an acute type of cardiotoxicity, which occurs as a combination of myopericarditis symptoms, lead to fatal complications such as congestive heart failure, arrhythmias, cardiac tamponade, and myocardial depression (Viswanatha-Swamy et al. 2013). Severe morbidity and sometimes mortality are observed because of teh effects on the heart, kidneys, and liver caused by CP. To date, no effective adjuvant has been found to overcome the multi-organ toxicity caused by CP (El-Sheikh et al. 2017).

Apelin, an adipocytokine, was discovered in the stomach tissue by Tatemoto et al. (1998). It was also found to be synthesized in various tissues such as the heart, lungs, kidneys, liver, adipose tissue, gastrointestinal tract, brain, adrenal gland, and endothelium. Apelin receptor is a G protein-coupled receptor found in various tissues such as the brain, heart, blood vessel, adipose tissue, and kidney. This wide distribution of the apelin receptor indicates that it has a wide range of activities in different organs (Aydin et al. 2014). Apelin was first introduced to the world of science as a vasodilator adipocytokine, positive inotropic agent, and free radical scavenger (Seifirad and Masoudkabir 2013). Apelin reduces blood pressure by vasodilatation of blood vessels through endothelium nitric oxide activation. Apelin affects glomerular hemodynamics in the kidney by relaxing the kidney renal arterioles contracted by angiotensin II (Kim et al. 2017). Antioxidant and cell-protective properties of apelin reduce nephrotoxicity and correct kidney perfusion (Seifirad and Masoudkabir 2013). Apelin, however, also contributes to the regulation of antioxidant defense systems and the weakening of lipid peroxidation in myocardial ischemia and reperfusion damage (Pisarenko et al. 2014). Moreover, experimental studies show that apelin prevents the production of ROS. The antioxidant effect of this peptide is accompanied by SOD, CAT, and glutathione peroxidase (GPx) upregulation. These data show that apelin has a role in the control of free oxygen radicals and contributes to the improvement of antioxidant potential during oxidative stress (Pelogeykina et al. 2015). Although some studies have shown the antioxidant properties of various peptides, (Freitas et al. 2013; Chakrabarti et al. 2014), no studies have been found that show the antioxidant effect of apelin in heart and kidney toxicity created by CP.

The present study focused on assessing the potential protective efficacy of apelin-13, an endogenous adipocytokine, against cardiotoxicity and nephrotoxicity caused by CP. To this end, oxidative stress and antioxidative markers were performed in both tissues, as well as histopathological examinations in both heart and kidney tissues.

Materials and methods

Chemicals and kits

CP (Endoxan) was purchased from Baxter (Halle, Germany); apelin-13 from Santa Cruz Biotechnology (Heidelberg, Germany); sodium thiopental from Bioveta (Komenskeho, Czech Republic); SOD, CAT, malondialdehyde (MDA), GSH, GPx, and cardiac Troponin I (cTnI) Elisa kits from Bioassay Technology Laboratory (Shanghai, China); and primary antibodies factor-1 alpha (Hif1-α) and interleukin-6 (IL-6) from the Woburn, United States.

Animals and experimental design

The study included 32 male Sprague–Dawley rats weighing 250 ± 20 g, which were about 3–4 months old and healthy. All rats in the study were obtained from the Afyonkarahisar University, Faculty of Veterinary Medicine, Experimental Animals Application and Research Center (Afyonkarahisar, Turkey) and fed with normal tap water and pellet feed in a standard environmental life in the same center. Ethical Committee Approval Animals were treated following the guidelines of the European Convention ETS 123 and all the methods implemented in this study were approved by the Animal Experiments Ethics Committee of the Afyonkocatepe University (AKÜHADYEK-68-19). During the study period, the rats were cared for in rooms with 12 h of daytime and 12 h of nighttime conditions, with automated temperatures (about 22 ± 2 °C) and humidity (about 45%–50%). As shown in Fig. 1, rats were randomly divided into four groups, and the groups were organized with eight animals in each. G*Power version 3.1.9.7 was used to calculate the required sample size for observing a large effect (Cohen's f = 0.65) at $p < 0.05$. Calculations revealed that 8 subjects in each of the four groups (32 in total) were sufficient to reach statistical power of 0.80.

CP was dissolved as 200 mg/kg (in saline) and apelin as 15 μg/kg (in phosphate-buffered saline) and the solutions were prepared for injection. Saline (i.p.) was administered to the control group for 7 days, and apelin (15 μg/kg per day, i.p.) to the apelin group for 7 days. The CP group was administered saline (i.p.) for 6 days and a single dose of CP (200 mg/kg) on day 7. Apelin and a single dose of CP (200 mg/kg) on day 7 were administered to the apelin+CP group for 7 days (15 μg/kg per day, i.p.). On day 8, when the injection was not performed, all rats were sacrificed (Fig. 1). The dose for CP toxication in kidney and heart tissue (Bhatt et al. 2017; Chakraborty et al. 2017; Mansour et al. 2015; Alhumaidha et al. 2016; ALHaithloul et al. 2019; Sharma et al. 2017; Goudarzi et al. 2017) and the prescribed dose of apelin-13 to prevent CP toxication were decided according to the in vivo studies similar to our study (Bircan et al. 2016; Chen et al. 2015; Kim et al. 2017; Gunes et al. 2018; Yamaleyeva et al. 2016). All animals were weighed on the first day of the experimental protocol and before the first injection. In addition, body weights (BM) were measured just before the animals were sacrificed and after they were sacrificed, heart (HM) and kidney (RM) weights were also measured. Organ indices as heart mass:body mass ratio (HM:BM (%)) and renal mass:body mass ratio (RM:BM (%)) were calculated.

Samples collection and tissue preparation

At the end of the study, the rats were anesthetized with 50 mg/kg of sodium thiopental (i.p.) and their thoraces were opened. For biochemical evaluations, heart blood was taken into hemogram tubes through an intracardial application. Each rat's heart and kidney were excised, washed with saline, and then weighed. HM and RM were proportioned to total BM and presented in the form of HM:BM (%) or RM:BM (%). Each heart and kidney were sectioned, then fixed in 10% formalin and separated for histopathological examinations. For biochemical analyses, heart and kidney tissues were stored in 1 mL of ice-cold PBS (0.01 M, pH 7.4), in the tissue homogenizer (Ika, T25, Deutschland, Germany) for 15 min, homogenized at 3000 rpm, and the resulting supernatant was stored at −80 °C until it was used.

Plasma biochemical analysis

Intracardial blood samples were centrifuged for 10 min at 1008g (3000 rpm) to obtain plasmas. Plasma samples transferred to polyethylene tubes were stored in a freezer at −80 °C for biochemical analyses. From plasma samples, lactate dehydrogenase (LDH), creatine kinase (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) enzymes, creatinine, and BUN were studied according to the manufacturer's instructions of commercial kits in a fully automatic autoanalyzer (Roche, cobas integra 400 plus).

Fig. 1. Study groups and experiment modeling process.
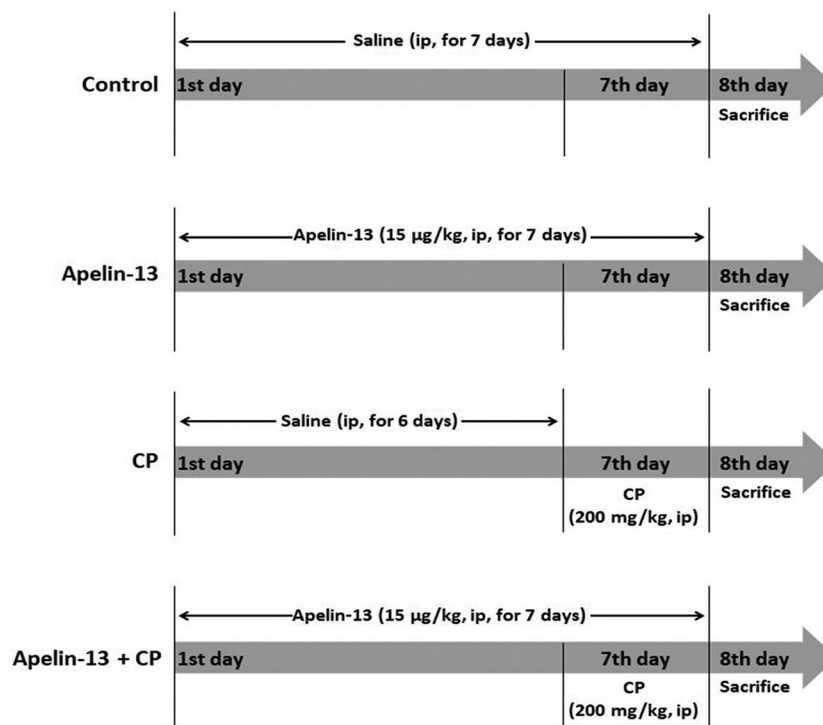
Determination of tissue lipid peroxidation product and antioxidant enzymes

SOD, CAT, MDA, GSH, GPx, and cTnI were measured in line with the recommendations of the manufacturer of bioassay technology laboratory ELISA measurement kit (Bioassay Technology Laboratory, Shanghai, China) to determine the antioxidant status and lipid peroxidation product (i.e., MDA) in heart and kidney tissues. The absorbency reading was done on a Chromate 4300 ELISA reader device (Awareness Technology Inc., Palm, USA).

Histopathological analyses

Kidney and heart samples were subjected to histological analyses after being fixated in 10% neutral formalin. A total of 4–5 μm sections of paraffin blocks were taken to classic slides with polylysine. Hematoxylin and eosin (H&E) staining was performed to examine cellular structures in kidney tissue, Periodic acid–Schiff staining to evaluate changes in basal membrane structure and glycogen accumulation, and Masson's trichrome staining to show collagen matrix (Bradbury 1982). Heart samples were stained with H&E and Masson's trichrome techniques. They were then evaluated under the light microscope (Eclipse E-600, Nikon, Japan) using an image analysis system (NIS Elements Nikon). Evaluation of the amount of glomerular and tubulointerstitial damage to the kidney was made using a semiquantitative method. The glomerular injury was evaluated to include mesangial matrix expansion and (or) hyalinosis together with focal adhesions, capillary dilatation, glomerular occlusion, and sclerosis. Tubular dilatation and interstitial fibrosis parameters were used in the evaluation of tubulointerstitial damage. Lymphocyte infiltration and fibrosis were also evaluated in heart tissue.

Immunohistochemical analyses

Sections taken from both kidney and heart tissues were evaluated by staining with Hif-1α primary antibodies, which are induced by IL and hypoxia. The sections deparaffinized in xylene were rehydrated with rated ethanol and then subjected to 20-min antigen retrieval with citrate buffer (pH = 6.0) in a microwave. Then, 3% hydrogen peroxide was used to block endogenous peroxidase activity. Tissues were treated with blocking solution to prevent background staining and were incubated at 4 °C for one night with primary antibodies of IL-6 (ABclonal, A16873, 1/100 dilution) and Hif-1α (ABclonal, A11115, 1/200 dilution). The next day, staining was completed using the horseradish peroxidase secondary antibody kit (Anti-polyvalent HRP, Labvision Corp, Fremont, CA). AEC was used for coloring, and Mayer's hematoxylin for contrasting staining, and it was sealed using a water-based concealer.

All sections were evaluated under the light microscope (Eclipse E-600) and photographed and analyzed with the image analysis system (NIS Elements). In 400× magnification in different, randomly selected areas, 500 cells were counted per section. In the sections, the scoring was performed using a semiquantitative method based on the percentage of stained cells and the degree of staining. The degree of staining was evaluated as follows: 0 (no staining), 1 (poor staining), 2 (medium staining), and 3 (strong staining). Immunohistochemical staining scoring for each section was statistically analyzed after using a scoring formula called H-score and calculated with the formula: H-score = S (I + 1) × PC, where S is the total, I is the degree of staining, and PC is the percentage of cells stained at all degrees (Sahin et al. 2011).

Statistical analysis

The results were presented as average ± standard deviations. All data were tested for normal distribution using the Shapiro–Wilk Test and Kolmogorov–Smirnov. One-way ANOVA (post hoc least significant difference test) was performed to examine the differences between the study groups. The statistical analysis was performed using the SPSS software version 20 and a p value of <0.05 was accepted as statistically significant.

Table 1. Ratios of heart (HM) and renal mass (RM) to body mass (BM) in groups treated with apelin and cyclophosphamide (CP).

	Body mass (g)	Heart mass (g)	Renal mass (g)	HM:BM (%)	RM:BM (%)
Control	259.14 ± 35.24	0.79 ± 0.07	1.93 ± 0.2	0.31 ± 0.02	0.74 ± 0.06
Apelin (15 μg/kg)	275 ± 38.01	0.82 ± 0.04	1.76 ± 0.15	0.3 ± 0.04	0.65 ± 0.09
CP (200 mg/kg)	256.71 ± 11.62	0.81 ± 0.04	2.32 ± 0.29*	0.36 ± 0.02*	0.97 ± 0.14*
Apelin+CP	240 ± 23.5	0.84 ± 0.18	1.96 ± 0.32†	0.31 ± 0.01†	0.82 ± 0.15†

*Compared with the control group ($p < 0.05$).

†Compared with the CP group ($p < 0.05$).

Fig. 2. Evaluation of (A) blood urea nitrogen (BUN), (B) creatinine, (C) aspartate aminotransferase (AST), (D) alanine aminotransferase (ALT), (E) alkaline phosphatase (ALP), (F) lactate dehydrogenase (LDH), (G) creatine kinase (CK-MB), and (H) cardiac troponin I (cTnI) serum levels of rats treated with saline (control), apelin, cyclophosphamide (CP) or apelin+CP. Results are presented as mean ± standard deviation (SD). [a]$p < 0.05$ compared with the control group, [b]$p < 0.05$ compared with the CP group. Mean ± SD values of each group (control, apelin, CP, apelin+CP): BUN (21 ± 3.06, 20.14 ± 3.72, 50 ± 13.49, 38 ± 6.75); creatinine (0.3 ± 0.02, 0.24 ± 0.03, 0.48 ± 0.11, 0.37 ± 0.03); AST (66.5 ± 16.83, 82.63 ± 21.11, 106.83 ± 4.55, 83.29 ± 1.73); ALT (32 ± 7.81, 35.65 ± 8.5, 56.8 ± 4.32, 23 ± 4.97); ALP (107.36 ± 15.15, 118 ± 29.29, 152 ± 5.94, 99.14 ± 12.29); LDH (104.69 ± 35.93, 115.06 ± 42.5, 174.56 ± 19.02, 110.71 ± 11.05); CK-MB (30.8 ± 6.3, 36.31 ± 4.17, 48.8 ± 4.75, 24.12 ± 3.1); cTnI (1.75 ± 0.16, 2.02 ± 0.2, 3.22 ± 0.34, 2.24 ± 0.2).
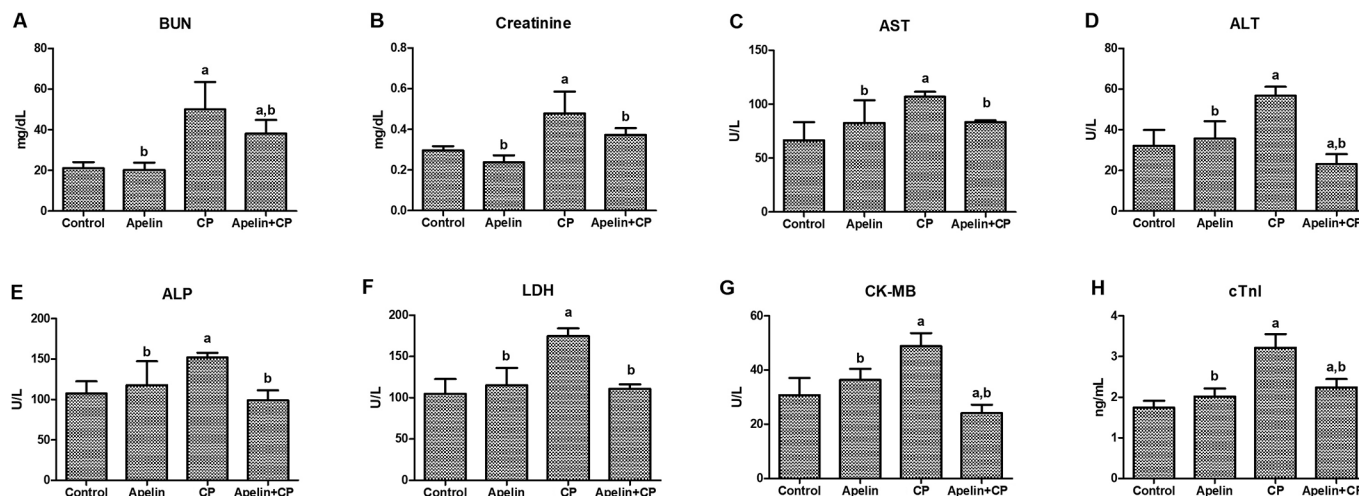
Results

Effect of apelin-13 on CP-induced cardiotoxicity and nephrotoxicity

Effect on HM:BM and RM:BM

This study showed that the ratio HM:BM (%) in CP-induced cardiotoxicity increased compared with the control group. The 7-day administration of apelin normalized the HM:BM of the rats treated with CP (Table 1). In renal toxicity, CP increased both RM and RM:BM (%) compared with the control group. The 7-day administration of apelin normalized the RM:BM of the rats treated with CP (Table 1).

Blood biochemistry variables related to heart and kidney function

Compared with both control and apelin groups, the serum BUN ($p < 0.001$, $p < 0.001$), creatinine ($p < 0.001$, $p < 0.001$), AST ($p = 0.001$, $p = 0.03$), ALT ($p < 0.001$, $p < 0.001$), ALP ($p = 0.001$, $p = 0.009$), LDH ($p = 0.006$, $p = 0.016$), CK-MB ($p < 0.001$, $p < 0.001$), and cTnI ($p < 0.001$, $p < 0.001$) levels were found to be significantly higher in the CP group. In the apelin+CP group, all biochemical parameters decreased significantly compared with the CP group (respectively, $p = 0.016$, $p = 0.017$, $p = 0.034$, $p = 0.027$, $p < 0.001$, $p = 0.011$, $p = 0.029$, and $p < 0.001$) (Fig. 2). In addition, while BUN and cTnI were higher ($p < 0.001$, $p = 0.011$) in the apelin+CP group compared with the control group, the ALT and CK-MB levels were found to be lower ($p = 0.027$, $p = 0.029$).

Oxidative stress levels in heart and kidney tissues

SOD, CAT, and GSH levels in heart and kidney tissue decreased in the CP group compared with the control group ($p = 0.029$, $p = 0.002$, and $p = 0.007$ for heart; $p = 0.015$, $p = 0.032$, and $p = 0.02$ for kidney), while they increased in the apelin+CP group compared with CP group ($p = 0.007$, $p = 0.038$, and $p = 0.003$ for heart; $p < 0.001$, $p = 0.008$, and $p = 0.001$ for kidney). GPx levels decreased in kidney tissue ($p < 0.001$) in the CP group compared with the control group, and there was no significant difference between these two groups in terms of GPx levels in heart tissue ($p = 0.858$). GPx increased in both the heart and kidneys in the apelin+CP group compared with the CP group ($p = 0.006$, $p = 0.019$). Besides, the application of apelin alone was found to be effective in increasing cardiac SOD and GPx compared with the control group ($p = 0.035$, $p = 0.022$), while similar effects were not observed in the kidney ($p = 0.957$, $p = 0.168$) (Figs. 3A–3D). MDA, a lipid peroxidation product in both heart and kidney tissue, increased in the CP group compared with control and apelin groups ($p < 0.001$ and $p < 0.001$ for the heart; $p = 0.004$ and $p = 0.013$ for kidney) and decreased in the apelin+CP group compared with the CP group ($p < 0.001$ for heart, $p = 0.16$ for kidney) (Fig. 3E). In addition, the application of apelin alone was found to be effective in reducing MDA in the heart compared with the control group ($p = 0.037$), but this effect was not observed in the kidney ($p = 0.789$).

Histopathological results

Kidney and heart tissues appeared normal in the control and apelin groups. Edema in tubules, partly necrotic areas in tubular

Fig. 3. The levels of (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), (C) catalase (CAT), (D) reduced glutathione (GSH), and (E) malondialdehyde (MDA) in heart and kidney tissue of rats treated with saline (control), apelin, cyclophosphamide (CP) or apelin+CP. Results are presented as mean $\pm$ standard deviation (SD). [a]$p < 0.05$ compared with the control group; [b]$p < 0.05$ compared with the CP group. Mean $\pm$ SD values of each group (control, apelin, CP, apelin+CP) for heart tissue: SOD (1.6 $\pm$ 0.29, 1.95 $\pm$ 0.16, 1.23 $\pm$ 0.19, 1.7 $\pm$ 0.25); GPx (23.1 $\pm$ 1.31, 28.22 $\pm$ 3.23, 22.7 $\pm$ 3.78, 29.31 $\pm$ 3.33); CAT (49.03 $\pm$ 1.94, 50.5 $\pm$ 2.98, 41.46 $\pm$ 4.02, 45.93 $\pm$ 0.86); GSH (216.04 $\pm$ 11.78, 213.83 $\pm$ 7.19, 180.93 $\pm$ 24.33, 220.83 $\pm$ 11.75); MDA (1.38 $\pm$ 0.17, 1.17 $\pm$ 0.15, 1.8 $\pm$ 0.11, 1.25 $\pm$ 0.09). For kidney tissue SOD (2 $\pm$ 0.05, 2.01 $\pm$ 0.17, 1.71 $\pm$ 0.13, 2.35 $\pm$ 0.19); GPx (28.04 $\pm$ 2.44, 25.66 $\pm$ 2.94, 19.34 $\pm$ 2.18, 23.72 $\pm$ 1.29); CAT (36.93 $\pm$ 3.17, 41.2 $\pm$ 1.4, 30.23 $\pm$ 3.28, 38.75 $\pm$ 6.85); GSH (185.7 $\pm$ 14.98, 189.65 $\pm$ 17.72, 154.8 $\pm$ 16.64, 202.46 $\pm$ 16.09); MDA (0.99 $\pm$ 0.08, 1.01 $\pm$ 0.17, 1.32 $\pm$ 0.23, 1.03 $\pm$ 0.11).
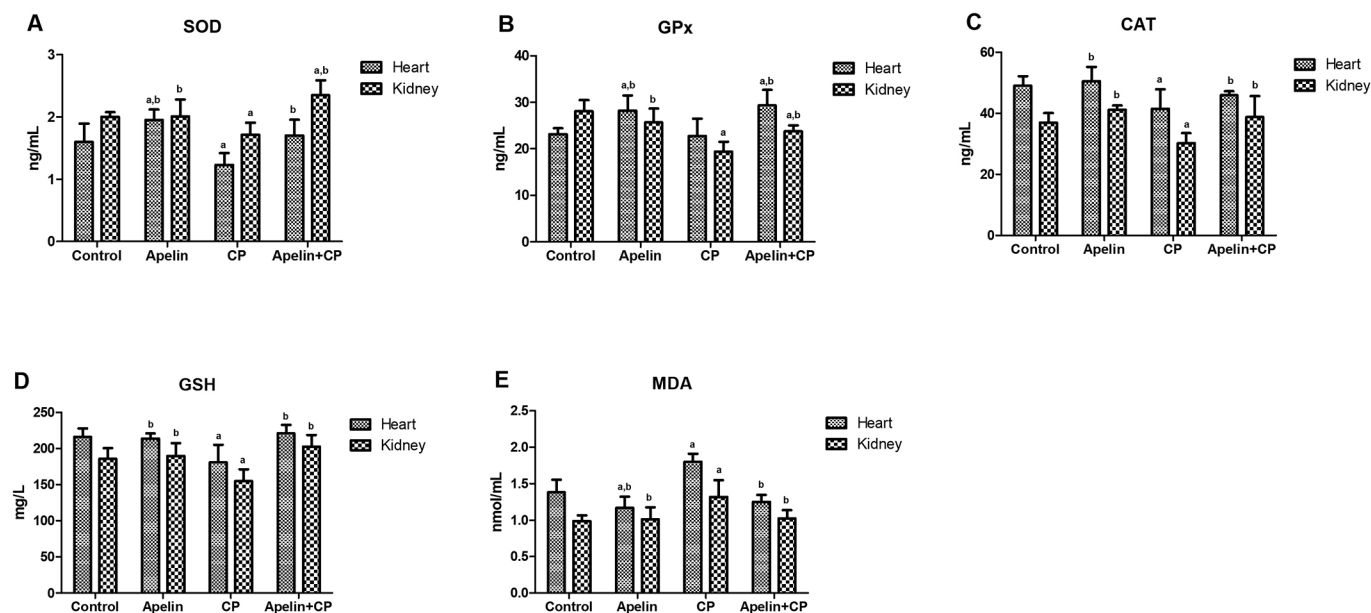
cells, hyalinized material between tubules, and mild tubular dilatation, as well as mild fibrosis, were detected in kidney tissues in the CP group. Mesangial matrix expansion, hyalinosis, glomerular occlusion, and mild fibrosis were observed in glomerules. Lymphocyte infiltration and fibrosis were detected in the heart tissues in this group. These symptoms were found to decline in both tissues in the apelin-protected CP group (Figs. 4 and 5).

Immunohistochemical results

Hif-1α and IL-6 H-score results found no difference between control and apelin groups in both kidney and heart tissue ($p > 0.05$); however, there was a statistically significant increase in both stainings in both tissues in the CP group compared with the control group ($p < 0.05$). In the apelin-protected group, a statistically significant decrease was detected in the scoring of two antibodies in both tissues compared with the CP group ($p < 0.05$), (Figs. 6 and 7).

Discussion

CP is an alkylating agent commonly used in most cancer chemotherapy and immunosuppressive protocols (Alhumaidha et al. 2016); however, the optimal clinical benefit of CP is limited by the high incidence of multi-organ toxicities, including cardiotoxicity, nephrotoxicity, and hepatotoxicity (Jiang et al. 2020). Clinical and experimental studies have reported that high therapeutic CP doses are associated with fatal cardiotoxicity, which is associated with symptoms such as myopericarditis, which can lead to congestive heart failure, arrhythmia, or myocardial diseases (Shanholtz 2001). Although, in the process of renal pathological damage induced by CP, apoptosis and necrosis of renal tubular epithelial cells involve the release of inflammatory factors and the mediation of the inflammatory response (Jiang et al. 2020). Some natural products have been shown to reduce CP-induced nephrotoxicity during CP chemotherapy (Mansour et al. 2017; Kocahan et al. 2017), however, no active ingredients to protect against cardiotoxicity have been found to

the best of our knowledge. The main purpose of this study was to investigate the protective effect of apelin-13, an endogenous adipocytokine, against cardiorenal toxicity caused by CP.

In this study, the CP application caused an increase in both HM:BM and RM:BM ratios compared with the control group. This was considered a sign that the general metabolic function of animals deteriorated and CP toxicity developed (El-Sheikh et al. 2017; Ogunsanwo et al. 2017; Mansour et al. 2015; Goudarzi et al. 2017; Alhumaidha et al. 2016). Increased HM:BM ratio may be due to increased edema or excessive fibrosis of heart muscle fibers, followed by an infestation of damaged tissues by inflammatory cells (Baky et al. 2009). In rats in the apelin+CP group, the HM:BM and RM:BM ratios decreased, so it can be predicted that apelin-13 has a cardioprotective effect (Foussal et al. 2010). We also supported this point with a study with the methodology as our study; Tesfaye et al. (2021) showed an increase in HM:BM ratio in rats 24 h after administration of CP.

CP is a cardiotoxic agent that causes direct myocardial endothelial damage and destruction of myocardial cells due to its debilitating effects. The cardiotoxic effect of CP is caused by the destruction of myocardial cells by ROS and endothelium damage (Refaie et al. 2020). As a result, enzymes such as LDH, cTn1, and CK-MB are transferred to serum due to leakage caused by the damage to the cells and act as diagnostic markers of myocardial tissue damage (Refaie et al. 2020; Omole et al. 2018; Viswanatha-Swamy et al. 2013). High levels of these enzymes are associated with certain types of heart damage, such as myocardial infarction, myocarditis, and heart failure (Viswanatha-Swamy et al. 2013). In this study, excessive ROS production from CP may be the reason for the elevation of cTn1 and CK-MB levels in the CP group, which are enzymatic indexes of cardiotoxicity. ROS causes membrane damage as well as loss of function and integrity of myocardial membranes (Refaie et al. 2020; Omole et al. 2018; Temel et al. 2020). In accordance with the current results in our study, apelin-13 has been shown to

Fig. 4. Histological features of kidney sections from control, apelin, cyclophosphamide (CP), and apelin+CP groups. Kidney tissues showed normal histological appearance in control and apelin groups. In the CP group, mesangial matrix enlargement in the glomeruli (white arrow), glomerular occlusion (black arrows), necrotic areas in the tubular cells (pink arrows), mild fibrosis (blue arrows) are seen. Histopathological morphology of the kidney was improved in animals pretreated with apelin (×200, scale bar: 50 μm). H-E, hematoxylin and eosin; PAS, Periodic acid–Schiff; MT, Masson's trichrome.
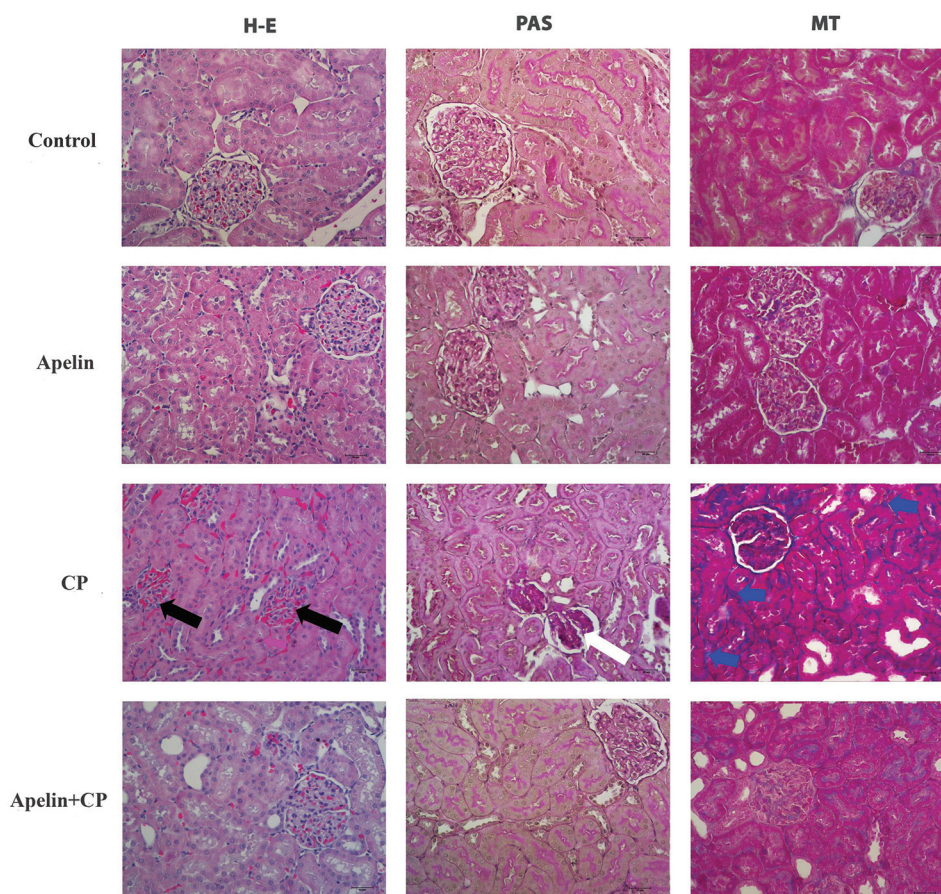
improve CP-induced cardiotoxicity through a decrease in cardiac enzymes; however, some researchers (Oyagbemi et al. 2016; Temel et al. 2020) also state that CP increases the levels of liver enzymes (AST, ALT, and ALP). These statements are consistent with our findings. Compared with the values obtained from the control group, there was an increase in the AST, ALT, and ALP values in the CP groups. These findings actually overlap with the general toxic effects of CP (Oyagbemi et al. 2016; Temel et al. 2020). In the group given apelin together with CP, enzyme levels decreased significantly compared with the values given only CP.

In a different study, apelin-12, which is a member of the apelin family, limited and reduced infarction size in myocardial ischemia and reperfusion damage, and reduced blood plasma levels of LDH and CK-MB. These effects were accompanied by a complete recovery of SOD, CAT, and GPx activities, and at the end of the reperfusion, the MDA content in the at-risk area decreased. The same study also shows that apelin is involved in regulating the cardiac antioxidant defense system and debilitating lipid peroxidation in myocardial ischemia and reperfusion damage (Pisarenko et al. 2014). Based on the abovementioned study and the findings from this study, it is confirmed that apelin may be responsible for restricting the leakage of biochemical markers thanks to membrane stabilization.

Nephrotoxicity and renal damage are characterized by a significant increase in serum levels of BUN and creatinine. To the best of our knowledge, changes in the serum level of BUN may reflect the functional state of the kidney and its discharge function. Creatinine is a small molecule that can be filtered through glomerules, and an increase in serum creatinine levels indicates a decrease in filtration rate, which can help evaluate kidney function (Fouad et al. 2021). The results of this study showed that the administration of CP at a dose of 200 mg/kg increased serum creatinine and BUN levels, indicating abnormal kidney function and kidney toxicity. Increased BUN and creatinine levels after the CP administration may be due to alteration in membrane permeability after kidney damage and penetration into the systemic circulation (Jiang et al. 2020). Various studies have consistently shown that CP induces nephrotoxicity by increasing oxidative stress, especially ROS (Ogunsanwo et al. 2017; Goudarzi et al. 2017; Kim et al. 2017; ALHaithloul et al. 2019; Sharma et al. 2017; Bircan et al. 2016; Chen et al. 2015). These reports suggest that excessive ROS production impairs kidney function, which is accompanied by an increase in serum creatinine and BUN levels. In this study, we observed that pre-application with apelin (15 μg/kg) for 7 consecutive days reduced serum creatinine and BUN levels in the CP group. In the group where apelin and CP were administered together, the BUN and creatinine levels returned to normal, which supported the idea that the pre-application of apelin-13 effectively alleviated CP-induced nephrotoxicity.

The formation of free radicals has been reported to play an important role in mediating heart and kidney damage (Alhumaidha et al. 2016). Myocardial tissue has endogenous antioxidant enzymes that act as a defense mechanism against oxidative damage. Antioxidant enzymes GPx, CAT, and SOD act in coordination to fight the

Fig. 5. Histological features of heart sections from control, apelin, cyclophosphamide (CP), and apelin+CP groups. A normal histological appearance was seen in control and apelin groups. In the CP group, lymphocyte infiltration (white arrow) and fibrosis (black arrows) were seen in the heart tissues. Histopathological morphology of the heart was improved in animals pretreated with apelin (×200, scale bar: 50 µm). H-E, hematoxylin and eosin; MT, Masson's trichrome.
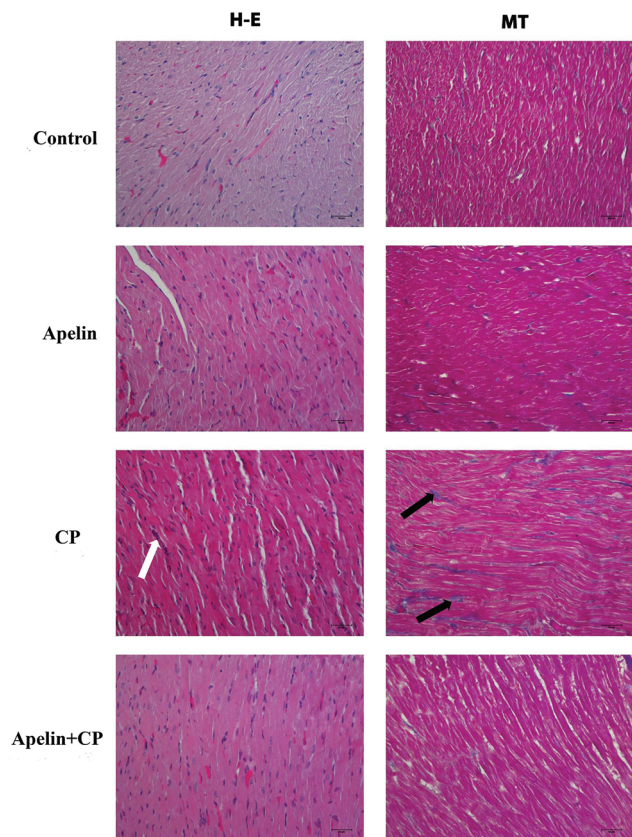
resulting ROS (Ogunsanwo et al. 2017). Free radicals created by CP can attack lipids and cause serious changes in membrane structure and function. Additionally, CP exposure disrupts redox balance and consumes antioxidant defenses in the heart and kidneys, causing oxidative damage to tissue (Elrashidy and Hasan 2021; Goto et al. 2020).

The increase in heart and kidney MDA content in rats administered with CP alone suggests lipid peroxidation (Ogunsanwo et al. 2017). MDA, a lipid peroxidation product, is considered the best indicator of the interaction of ROS with cell membranes. Acrolein increases the formation of MDA with the depletion of antioxidants such as SOD after the formation of ROS (Moghe et al. 2015), and can support lipid peroxidation by combining with GSH (Otunctemur et al. 2015). SOD, CAT, and GPx are essentially the most common antioxidants that inhibit or prevent in vivo free radicals and ROS formation. They are also key indicators that play important roles in the elimination of MDA (Jiang et al. 2020). As in similar toxication studies induced with CP, CP was an important source of cardiorenal oxidative stress, manifested by reduced cardiorenal antioxidant enzyme activity and increased MDA levels when compared with the control group. Nephrotoxicity is associated with the depletion of renal antioxidant enzymes such as CAT, SOD, GPx, and GSH (Alhumaidha et al. 2016; Jiang et al. 2020). We measured the renal tissue GSH level, SOD, GPx, and CAT protein amounts to evaluate the antioxidant

activity in the kidney tissue. We also showed that pretreatment with apelin-13 at a dose of 15 µg/kg for 7 consecutive days increased antioxidative defenses such as SOD, GSH, GPx, and CAT.

Than et al. (2014) have shown that apelin can eliminate the release of pro-oxidant enzymes induced by oxidative stress in mitochondrial biogenesis as well as pro- and anti-inflammatory adipocytokines. In addition, the same research suggests that apelin is a new potential therapeutic target for metabolic diseases due to its antioxidant properties (Than et al. 2014). Bircan et al. (2016) have shown that apelin-13 administered after kidney ischemia and reperfusion dose-dependently increases antioxidant enzyme activity which prevents lipid oxidation, thereby improving renal function. Another study shows that apelin-13 is a protective mechanism in cerebral ischemia due to its important role in suppressing oxidative stress in focal brain ischemia and reperfusion damage (Zhang et al. 2019). In our study, apelin-13 was applied in the pretreatment of CP-treated rats, and it corrected both cardiac and renal antioxidant enzyme activity while improving MDA levels, indicating a protective effect against oxidative stress. These findings support that apelin exerts antioxidant effects by scavenging free radicals directly or increasing antioxidant enzymes indirectly. Therefore, apelin can exhibit its protective effects in severe cell and tissue damage in the heart and kidneys through its antioxidant system.

In this study the heart and kidney tissues of rats in all groups were histopathologically examined, and it was found that the most severe effects were only in the CP group. Cardiorenal histopathological results showed structural changes consistent with the biochemical evaluation. Toxic metabolites induced by CP contribute to the disintegration of endothelium cells and directly damage the myocardium and capillaries, resulting in the formation of edema, interstitial hemorrhage, and microthrombosis (Ayza et al. 2020). In this study, the damage observed in the histological structure of the heart can be explained by the direct or indirect effect of CP metabolites (Avci et al. 2017). Heart sections of the rats in the control group revealed normal heart structures, while lymphocyte infiltration and fibrosis were shown as a confirmation of cardiotoxicity in the heart sections of the rats in the group with CP toxication (Avci et al. 2017). Pretreatment of apelin-13, however, reduced these abnormal pathological findings of the heart tissue and protected the heart tissue from oxidative damage. Histopathological examinations showed that apelin-13 was able to protect heart tissue. Edema in tubules, partly necrotic areas in tubular cells, hyalinized material between tubules and mild tubular dilatation, as well as mild fibrosis, were detected in kidney tissues in the CP group. Mesangial matrix expansion, hyalinosis, glomerular occlusion, and mild fibrosis were observed in glomerules. These histopathological lesions in the heart and kidneys were found to be consistent with previous studies (Temel et al. 2020; Tesfaye et al. 2021; Avci et al. 2017). Interestingly, pretreatment of apelin-13 clearly debilitated the histological changes in kidney tissues caused by CP.

Another topic of interest in this study was researching the beneficial effects of apelin against hypoxia-related inflammation induced by CP, as well as potential cellular and biochemical mechanisms of action. To this end, we determined that Hif-1α and IL-6 (Peng et al. 2020; Temel et al. 2020), proinflammatory cytokines induced by hypoxia, were suppressed in cardiorenal tissue as a result of the immunohistochemical evaluation. In addition to this process, activated inflammatory cascades, nephrocyte necrosis, and apoptosis led to irregularity of kidney tissue and renal dysfunction (Jiang et al. 2020). Therefore, by alleviating the oxidative stress, inflammation, and the state of apoptosis in kidney tissue, apelin-13 can serve as a therapeutic strategy for nephrotoxicity induced by CP.

Our results showed that apelin alleviates free radical damage and oxidative stress, strengthens the antioxidant systems such as SOD, CAT, GPx, and GSH, and protects against oxidative stress and lipid peroxidation product (i.e., MDA) caused by CP in the

Fig. 6. Photomicrographs of immunohistochemical staining of kidney and heart tissues (factor-1 alpha (Hif-1α) and interleukin-6 (IL-6) primary antibodies, ×200, scale bar: 50 μm). Black arrows (Hif-1α) and white arrows (IL-6) indicate positive staining of cells. In the control and apelin groups few cells showing light staining. In the cyclophosphamide (CP) group, most tubular/glomerular cells in kidney and heart cells showing medium/strong staining. In the apelin+CP group, the number of staining cells and staining intensity is smaller than that of the CP group.
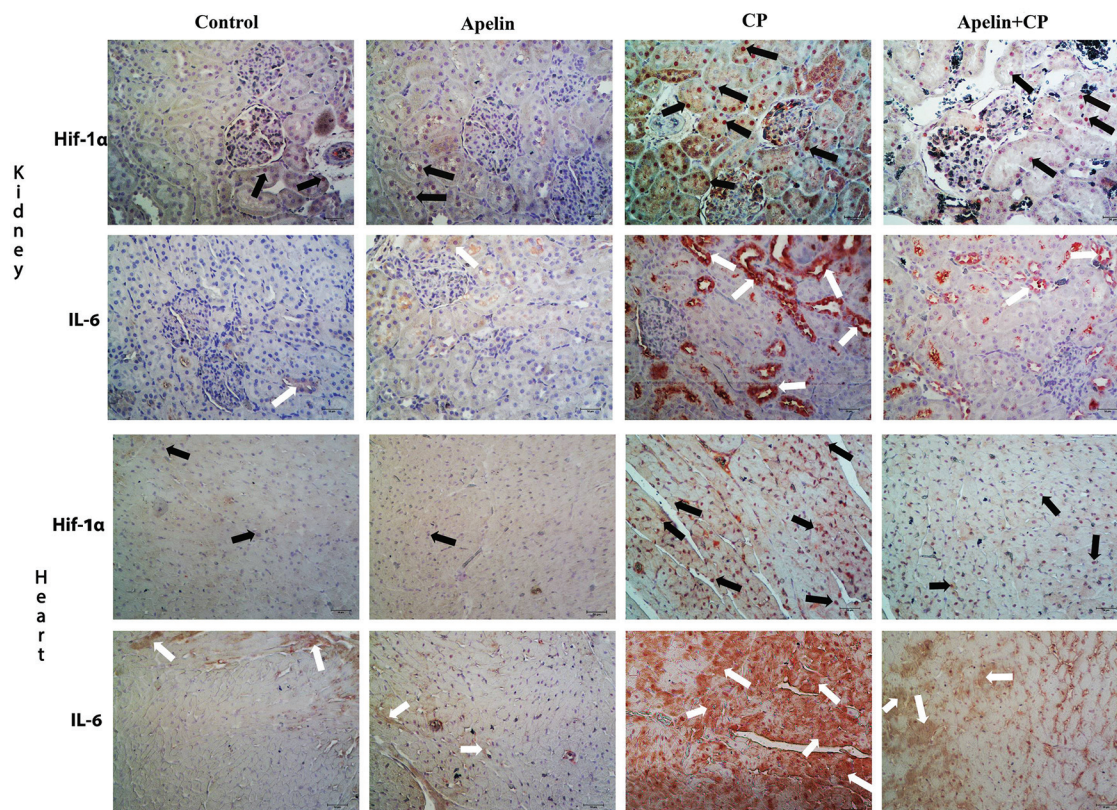
Fig. 7. H-score results obtained as a result of factor-1 alpha (Hif-1α) and interleukin-6 (IL-6) immunohistochemical stainings. *$p < 0.05$, significantly different from the control; #$p < 0.05$, significantly different from the cyclophosphamide (CP) group. Mean ± standard deviation values of each group (control, apelin, CP, apelin+CP): kidney Hif-1α (109.00 ± 3.65, 110.00 ± 2.70, 177.14 ± 24.88, 131.42 ± 4.79); kidney IL-6 (111.42 ± 5.12, 120.14 ± 4.09, 197.14 ± 37.25, 149.71 ± 17.91); heart Hif-1α (104.00 ± 2.44, 108.14 ± 5.98, 216.57 ± 18,39, 139.28 ± 18.28); heart IL-6 (117.14 ± 8.59, 117.57 ± 8.54, 192.14 ± 42.99, 138.57 ± 12.48).
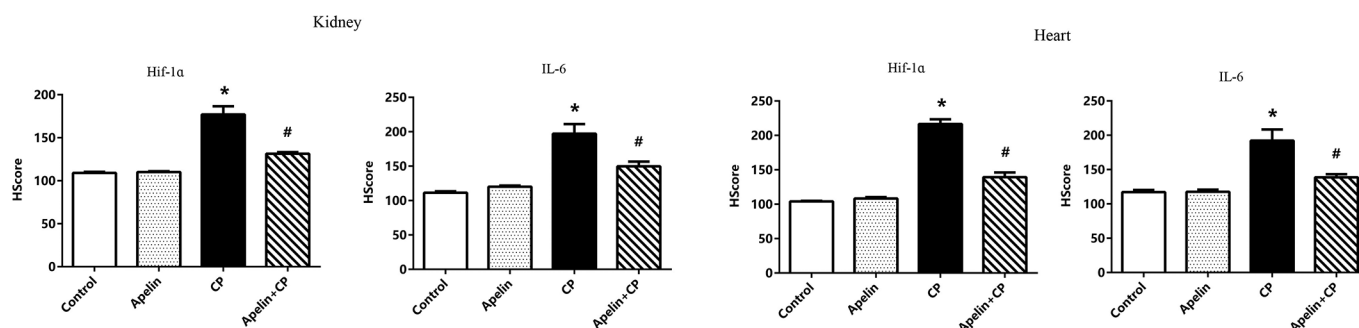
heart and kidney tissues. When the literature was scanned, no study was found showing the possible protective antioxidant effect of apelin-13 in heart and kidney toxicity induced with CP, although the antioxidant properties of different adipocytokines were shown along with apelin. Therefore, it will be of great clinical importance to develop alternative strategies that can protect against cardiotoxicity and nephrotoxicity caused by CP. Apelin-13 may be a new potential therapeutic target for heart and kidney

toxicity induced by CP, or a new antioxidant treatment protocol to alleviate side effects that may occur along with CP treatment. Different clinical studies have shown that the use of antioxidants in combination with chemotherapy and radiotherapy improves patient's life expectancy and quality of life compared with the expected outcome without antioxidant supplements. In this context, it reinforces the idea that apelin-13 can be useful in CP treatment protocol with its antioxidant protective properties.

Limitations of the study

In this study, too many variables were studied to show CP toxicity and how apelin-13 is effective on this toxicity. It was not possible to conduct controlled experiments using the activator or inhibitor of each variable. Apart from the direct effects of apelin-13 and CP on these variables, the correlation findings between the studied variables were discussed without establishing a cause and effect relationship.

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Author contributions

ÖK, designed the study, and ÖK, AKA, and EA constructed the models. All authors wrote the manuscript and commented on the manuscript.

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