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ORIGINAL ARTICLE

Protective Effect of Caffeic Acid Phenethyl Ester against Acute and Subchronic Mice Cardiotoxicity Induced by Cyclophosphamide Alone or Plus Naproxen

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ABSTRACT

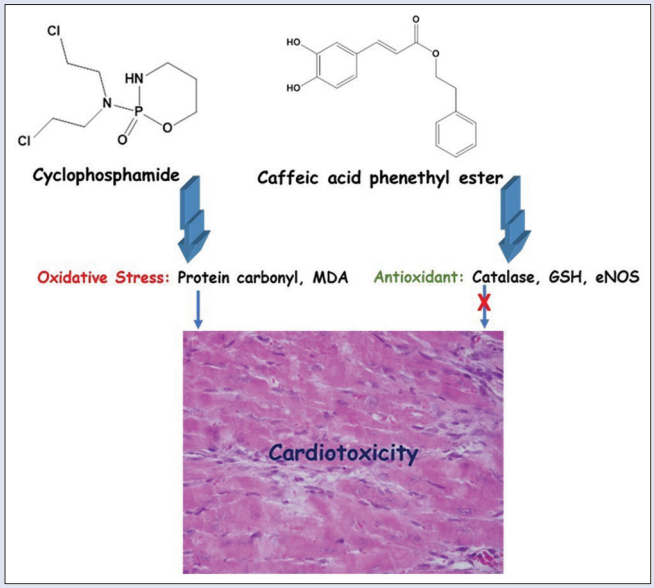
Background: The limiting factor in the use of cyclophosphamide (CYP) in cancer chemotherapy is its induced oxidative cardiotoxicity. **Objectives:** This study aims to investigate the possible protective effect of caffeic acid phenethyl ester (CAPE) in the co-administration of CYP and naproxen (NAP) with acute and subchronic treatments in mice. **Materials and Methods:** Male BALB/c mice were divided into two phases of acute (24 h) and subchronic (30 days) treatments, of which seven groups each were used. Two groups from both acute and subchronic treatments represented untreated controls and CAPE groups, while others were CYP, NAP, CYP+NAP, CYP+CAPE and CYP+NAP+CAPE groups for both treatments. The activity of the cardiac antioxidative enzyme catalase was measured. The levels of cardiac reduced glutathione (GSH), protein carbonyl and malondialdehyde (MDA) were also assayed. In addition, histopathology of the heart tissues and immunohistochemistry of endothelial nitric oxide synthase (eNOS) expression were evaluated. **Results:** Our results showed that catalase and GSH were significantly decreased in all subchronic treatments. Furthermore, protein carbonyl and MDA were increased in both acute and subchronic treatments. Histopathological examination showed hypertrophic cells induced by CYP, NAP, and in combination. Moreover, CYP, NAP and in combination, significantly reduced eNOS levels. However, CAPE significantly prevented changes induced by CYP and NAP in both treatment groups. **Conclusion:** These observations highlight the protective potentials of CAPE in CYP-NAP-induced cardiotoxicity.

Key words: Caffeic acid phenethyl ester, cardiotoxicity, cyclophosphamide, naproxen, oxidative stress

SUMMARY

- Cyclophosphamide exhibited marked oxidative stress and therefore mice cardiotoxicity
- Co-administration with naproxen further exacerbated these effects
- Caffeic acid phenethyl ester is effective in ameliorating these oxidative changes.

Abbreviations used: CAPE: Caffeic acid phenethyl ester; CYP: Cyclophosphamide; eNOS: Endothelial nitric oxide synthase; GSH: Reduced glutathione; MDA: Malondialdehyde; NAP: Naproxen; NSAIDs: Non-steroidal anti-inflammatory drugs; ROS: Reactive oxygen species.



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INTRODUCTION

The treatment of cancer diseases varies in that it can be targeted at killing/eliminating tumor cells or arresting their growth to limit their invasiveness. However, these modes of treatments are not usually selective hence causing multiple damages to normal cells in a variety of ways.[1] One of the mechanisms by which chemotherapeutic agents cause adverse toxicity is through free radicals generation that can trigger oxidative stress.[2,3] Oxidative stress can be described as the disturbance in the oxidant-antioxidant balance in favor of oxidative activity.[4] The association of oxidative stress with cellular injury has attracted intense research because of their roles in regulating cellular functions and metabolic pathways.[5] It is known that the use of chemotherapeutic agents like cyclophosphamide (CYP) in cancer treatments produces cardiotoxic side effects due to the generation of free radicals.[6-9] Studies also show

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that the mechanism of cardiotoxicity of CYP involves the generation of reactive oxygen species (ROS) by its metabolite, chloroacetaldehyde, causing a decrease in antioxidant defense mechanism activities in the heart. This action in itself is capable of also causing apoptosis of the cardiac cells, which could as well produce immunological reactions.[10-12]

CYP, an alkylating agent and immunosuppressive drug, is used in cancer chemotherapy for the management of many malignant conditions.[13,14] However, its use has been implicated in many organ failures and hence is limiting its clinical usefulness.[8,15] Documented evidence has shown that antioxidants can be beneficial in mitigating the cardiotoxic effect of CYP and thereby lower the incidence of morbidity associated this adverse effect.[16,17] Agents that can decrease free radical generation stimulate protective immunity and can be of benefit in enhancing the efficacy and tolerance of chemotherapeutic medications like CYP.

Caffeic acid phenethyl ester (CAPE) is an active propolis constituent and which has been reported to have the potential to have antineoplastic and antioxidants activities.[18] Accumulated evidence shows that CAPE possesses the ability to decrease activities of ROS generated in the tissues and also protect the activities of free radical scavenging enzymes, thereby acting in parallel with and as antioxidants.[19-21] Since ROS are the main culprit implicated in drug-induced cellular injury, a treatment combination with CAPE could attenuate drug-induced cellular injury in normal cells.[22,23] This might provide a treatment strategy for preventing cardiac injury in the use of CYP.

Sequel to cancer is pain, and studies revealed that about 75% of cancer sufferers require pain management as an additional treatment.[24] Opioids or non-opioid medications like non-steroidal anti-inflammatory drugs (NSAIDs) are usually employed in this regard.[25] Moreover, NSAIDs have anticancer activity.[26] Previous studies have shown that the administration of NSAIDs is associated with cardiovascular toxicity.[27] Naproxen (NAP, an NSAID), particularly, has been reported to exacerbate adverse cardiovascular events by inducing oxidative stress.[28,29] The aim, therefore, was to evaluate the cardiac protective effect of CAPE in the acute and subchronic administration of CYP alone and plus NAP in mice.

MATERIALS AND METHODS

Materials

CYP, NAP sodium and CAPE powders as well as catalase, reduced glutathione (GSH), protein carbonyl content and lipid peroxidation (assessed as malondialdehyde [MDA]) assay kits, all were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Animals

Male BALB/c mice with an average weight of 28 g (6–8 weeks old) were obtained from the Department of Biological Sciences, College of Science, King Faisal University, Saudi Arabia. They were maintained as six mice per an individually ventilated cage at a temperature of 22°C–23°C and humidity of 65% with a 12 h light and 12 h dark cycle at the College of Medicine Laboratory Animal Center. The animals were allowed to acclimatize for 2 weeks before the commencement of the experiment. They were allowed food and water *ad libitum*. Animal care and experimental procedures were performed in accordance with the animal research ethical standards of the Research Ethics Committee, King Faisal University and in compliance with the guidelines of the National Committee of Bioethics, KACST, Saudi Arabia.

Study protocol

The study was divided into two phases, acute and subchronic treatments. For both studies, same groups of animals and drugs were used but in different dosages and duration. The acute phase was terminated after 24 h, whereas the subchronic investigation ran for a duration of 30 days. Mice were randomly placed into 14 experimental groups, which consist of 6 mice in each group making a total of 84 mice. Forty-two mice, respectively, were used for acute and subchronic studies consisting of seven groups each. Group 1 represented the control and Group 2 was treated intraperitoneally with 5 mg/kg CAPE.[30] Group 3 intraperitoneally received 200 mg/kg of CYP,[16] while Group 4 was orally given NAP (70 mg/kg).[29] Group 5 was treated with CYP+NAP, whereas Group 6 got CAPE+CYP, and finally, Group 7 was treated with CAPE+CYP+NAP. While in acute study, animals received a single dose of the assigned drug, in subchronic study, animals received assigned drugs (CYP: 100 mg/kg[31] and NAP: 40 mg/kg)[29] but for 30 days repeated drug administration. At the end of the experiments, heart tissues were carefully excised and stored at –85°C.

Catalase activity

Catalase activity was determined spectrophotometrically according to the method of Higgins *et al*.[32] Essentially, the enzymatic activity was measured from the rate of decomposition of hydrogen peroxide (expressed as μmol/min/ml) at the absorbance of 240 nm.

Reduced glutathione level

GSH levels were determined according to the method described by Moron *et al*.[33] Briefly, it is a colorimetric reaction of 5,5'-dithiobis (2-nitrobenzoic acid). Heart tissue levels of GSH were measured at 412 nm using a standard curve plot.

Protein carbonyl level

Protein carbonyl level as a marker of oxidative damage was determined according to the method described by Velmurugan *et al*.[34] Oxidized proteins produce protein carbonyl, which then reacts with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone that were measured spectrophotometrically by reading the absorbance at 375 nm.

Malondialdehyde level

Lipid peroxidation was measured in the heart tissue homogenates by determining the levels of formed thiobarbituric acid-reactive substances, as was described by Ohakawa *et al*.[35] Into each sample homogenate, 1% of phosphoric acid solution was added with a known volume of thiobarbituric acid and then incubated at 95°C for 1 h. The subsequent thiobarbituric acid-reactive substances concentrations were then measured using a spectrophotometer at the absorbance of 535 nm.

Histopathological analysis

The specimens from the heart were removed and immediately placed in phosphate-buffered saline (PBS) for a few minutes and then fixed in 10% formaldehyde. Tissue slides were prepared after the tissues were treated with various percentages of alcohol to dehydrate them. Thereafter, 4-μm tissue thickness were cut and stained with hematoxylin/eosin. Then, histological analysis was performed by examining the slides under the microscope at ×400.

Immunohistochemical analysis

Harvested heart tissues were fixed by immersion in 4% paraformaldehyde in PBS containing 4% sucrose for 10 min. Thereafter, 0.2% Triton X-100 was used to permeabilize the cells for 5 min and was washed with PBS containing 25 mM glycine. Tissues were incubated in the primary antibody against endothelial nitric oxide synthase (eNOS) overnight at 4°C. Then, tissues were washed and incubated for 1 h

in the corresponding secondary antibody and analyzed using the Leica microscope to determine the presence of eNOS stained cells as described by Caviedes *et al.*[36]

Data analysis

Data are presented as mean ± standard deviation. GraphPad Prism 5.00 was used to compute the statistical analysis using one-way analysis of variance. Significant statistical differences were considered at the level of $P < 0.05$.

RESULTS

Biochemical analysis of oxidant/antioxidant markers

Acute administration of 200 mg/kg of CYP to mice showed no significant difference in catalase activity among the CYP and CYP+NAP group [Figure 1a]. However, in the subchronic treatment groups of CYP, NAP and CYP+NAP, catalase activities were decreased significantly compared to the CAPE alone group. Results also showed that treatment with CAPE significantly increased catalase activities when compared with the CYP group in the subchronic treatment groups.

Figure 1b shows the GSH level determined in different groups in both the acute and subchronic treatments. The acute treatments CYP and CYP+NAP groups displayed a significant increase in GSH levels compared to CAPE alone group; however, the subchronic treatments of CYP, NAP and CYP+NAP showed significant decrease instead over time. Moreover, CAPE administration, together with these agents, fully restored GSH level in the acute treatment groups compared to CAPE alone group and significantly increased GSH level in the subchronic treated groups compared with the CYP group.

Protein carbonyl analysis indicated that in CYP and CYP+NAP groups, significant increases were observed for acute treatments, whereas CYP, NAP and CYP+NAP groups produced a significant increase in subchronic treatments compared with CAPE alone group. Co-administration with CAPE in the aforementioned groups restored protein carbonyl level in acute treatments compared to CAPE alone group and significantly attenuated its elevation in the subchronic treated groups compared with the CYP group [Figure 1c].

In addition, MDA estimation indicated that in the acute treatment groups, there were significant increases in the CYP and CYP+NAP groups. However, treatment with CAPE restored the MDA level when

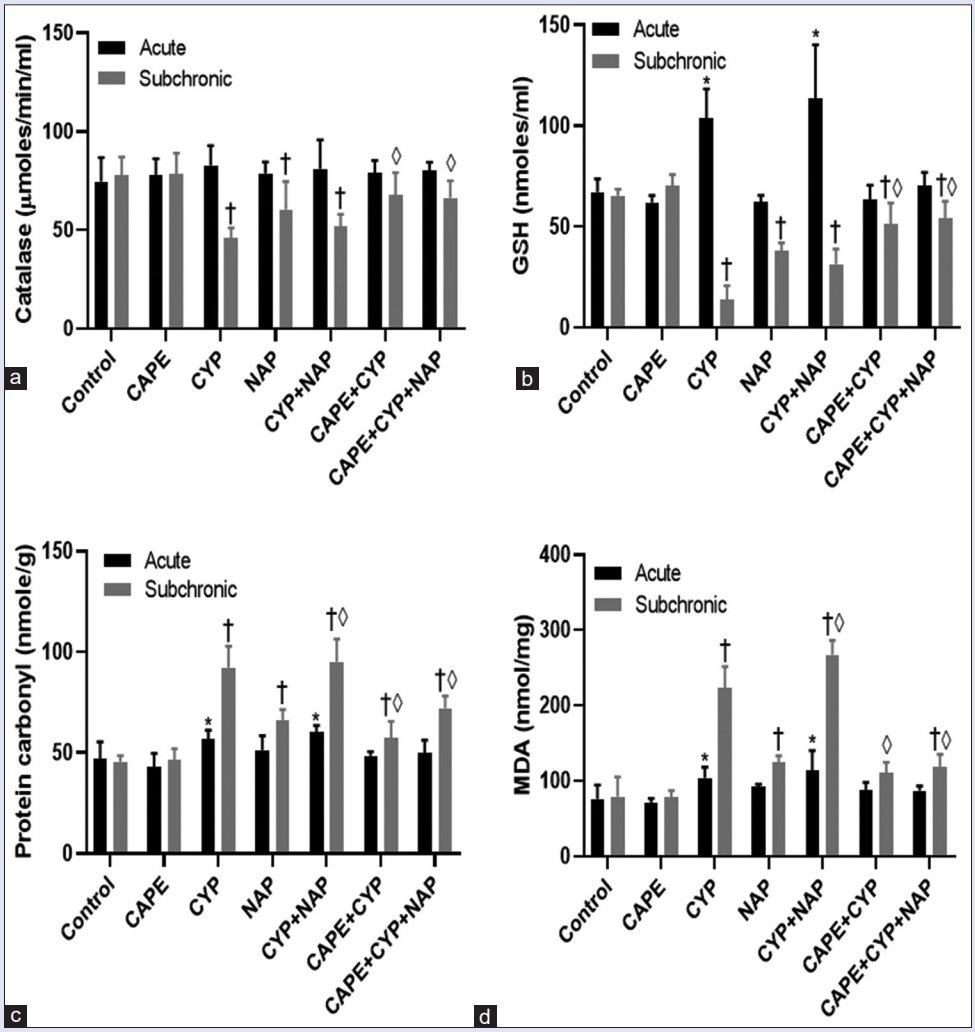

Figure 1: Effect of acute (1 day) and subchronic (30 days) administration of caffeic acid phenethyl ester on cardiac catalase (a), reduced glutathione (b), protein carbonyl (c) and malondialdehyde (d) levels of cyclophosphamide and/or naproxen-induced cardiotoxicity in mice. Data are mean ± standard deviation of 6 rats. *Significantly different from acute caffeic acid phenethyl ester alone group at $P > 0.05$, †, ◊Significantly different from subchronic caffeic acid phenethyl ester alone and cyclophosphamide groups, respectively, at $P > 0.05$.

compared with CAPE alone group. Furthermore, the increased levels of MDA were found to be significant in the subchronic treatment groups administered with CYP, NAP and CYP+NAP in relation with the CAPE alone group. Results also showed that the inclusion of CAPE in these groups significantly reduced the levels of MDA compared with the CYP group [Figure 1d].

Histological analysis

Results showed that in the control and CAPE treated groups, for both acute and subchronic treatments, the cardiac architecture appeared normal with an oval nucleus centrally placed. In the acute CYP treatment, photomicrography of mouse heart tissue showed that red blood cells infiltrated the interstitial spaces. After subchronic CYP treatments, the tissues displayed leukocytes infiltration as well as tissue hypertrophy of the cardiac fibers. Both acute and subchronic NAP treatments produced similar effects in terms of red blood cells and leukocytes infiltration as well as tissue hypertrophy of the cardiac fibers. A combination of CYP and NAP in acute treatment showed more red blood cells and leukocytes infiltration. In addition, the subchronic administration of these two agents produced marked cardiac hypertrophy. Co-administration of CYP+CAPE in acute treatment did not completely restore the effect caused by CYP on the cardiac cells as the infiltration of red blood cells was observed but was reduced and not as prominent. In the subchronic treatment, a combination of CYP and CAPE still showed parenchymal infiltration of the leukocytes. CAPE administration with CYP+NAP in acute treatments still revealed infiltration of red blood cells in the interstitial space along with parenchymal infiltration of leukocytes as well, but in both acute and subchronic treatments, hypertrophy of the cardiac fibers was abolished [Figure 2].

Immunohistochemical analysis of endothelial nitric oxide synthase

The evaluation of eNOS protein expression in the different treatment groups was graded and scored according to the level of expression (–) as absence, (+) as present in low levels barely detectable, (++) visibly detectable and (+++) very clearly detectable. Results showed very low expressions of eNOS in CYP and NAP administered groups, but negative (–) expressions in CYP+NAP administered group in the acute treatment. In the subchronic treatment group, negative (–) eNOS expressions were observed with CYP, NAP and CYP+NAP administered groups. However, eNOS expressions were detectable in control (+++) and CAPE (++) treatment groups [Table 1].

Table 1: Endothelial nitric oxide synthase expression scores in acute and subchronic treatment groups

Groups	Scores	
	Acute treatment	Subchronic treatment
Control	+++	+++
CAPE	+++	+++
CYP	+	-
NAP	+	-
CYP+NAP	-	-
CAPE+CYP	++	++
CAPE+CYP+NAP	++	++

CAPE: Caffeic acid phenethyl ester; CYP: Cyclophosphamide; NAP: Naproxen. Levels of expressions are indicated as follows: (-) as absence, (+) present in low levels barely detectable, (++) visibly detectable, and (+++) very clearly detectable.

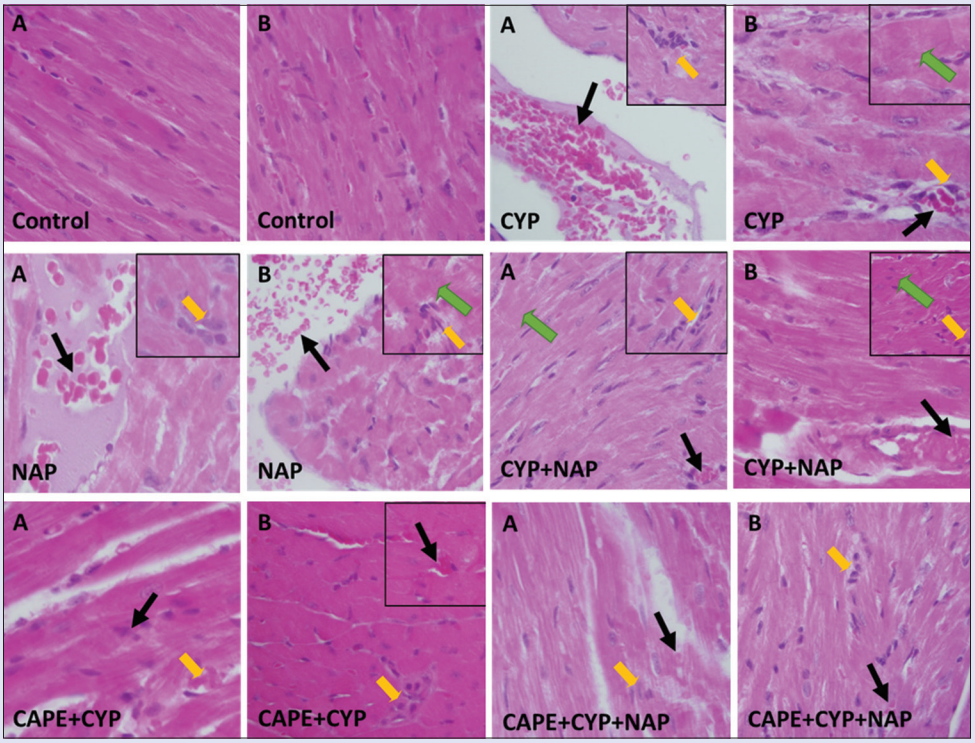

Figure 2: Effect of caffeic acid phenethyl ester on histopathological changes in acute (A) and subchronic (B) mice cardiotoxicity induced by cyclophosphamide alone or plus naproxen (H and E, ×400). Concomitant administration of caffeic acid phenethyl ester with cyclophosphamide and cyclophosphamide/naproxen attenuated their destructive effects and resulted in an improvement of the histological structures, which became comparable to that in the control group. Black arrow: red blood cells infiltration; yellow arrow: leukocytes infiltration; and green arrow: hypertrophy of cardiac fibers. Caffeic acid phenethyl ester alone had no effect on cardiac histopathology (data are not shown)

DISCUSSION

CYP is an anticancer drug used in the treatment of many cancer types, and documented evidence shows that it is cardiotoxic.[10] In addition, the use of NSAIDs in cancer management is well documented.[25,26,37] The present investigation examined the possible protective effects of CAPE against acute, and subchronic CYP alone and plus NAP induced oxidative stress and the extent of cardiotoxic injury in mice. The generation of ROS that is often associated with cardiac oxidative stress has been linked with CYP use. These generated free radicals attack the cell membrane containing polyunsaturated fatty acids, resulting in lipid peroxidation.[10] Several reports have indicated that CYP can induce cardiotoxicity by decreasing the cellular antioxidant activity of the cardiomyocytes.[17,38] Furthermore, reports show that the use of NAP causes cardiovascular injury by the generation of ROS, which induces oxidative cellular stress.[39,40] According to literature, CAPE is equally well documented to exhibit a powerful antioxidant activity in the cardiomyocytes and other tissues by scavenging ROS, thereby attenuating cellular apoptosis by activating the eNOS pathway.[18,19] In agreement with the present investigation, several previous studies noted similar findings regarding the ability of CYP and NAP to decrease cardiac catalase activity.[28,38] Adding CAPE to these groups markedly increased catalase activity because it is reported to reduce the creation of oxidative stress; therefore, the present study is line with the findings of Fadillioglu *et al.* who documented that CAPE increases catalase activity on doxorubicin-induced cardiotoxicity in rats.[41]

Our findings also showed that GSH levels were significantly increased by the administration of CYP and CYP+NAP in acute treatment. These results could be due to the cells reacting in response to drug-induced oxidative stress; however, consistent with the work of Deng *et al.*[42] elevated GSH was not sufficient to protect against CYP-induced cardiac toxicity. On the other hand, in line with the work of Omole *et al.*[38] on subchronic treatments with CYP, NAP and in combination, GSH levels were reduced. Again, administration with CAPE, together with these agents, increased GSH levels significantly. GSH is known to protect cardiac cells against injury from ROS, which creates oxidative cellular stress. This finding is corroborated by the study of Motawi *et al.*[43]

The implication of increased protein carbonyl level, which is related to the extent of protein damage is that it is one of the oxidative products, which clearly indicate cardiac oxidative stress due to ROS.[44] Protein carbonyl level in acute treatment groups of CYP and CYP+NAP increased significantly along with subchronic treatment groups of NAP in addition to the aforementioned groups of CYP and CYP+NAP. The observation that CYP increased protein carbonyl level is a confirmation of the report by Mythili *et al.*[45] In agreement with our study, the addition of CAPE to these groups significantly attenuated these effects in all groups.[41]

To further assess the effect of CYP and CYP+NAP in inducing oxidative stress, lipid peroxidation was investigated by quantifying the levels of MDA in all treatment groups. Our findings show significant increases in MDA levels with CYP and CYP+NAP groups for both acute and subchronic treatments, thus, presenting a high magnitude of oxidative stress generation. This observation is in agreement with the reports of many other studies, indicating that CYP actually does increase MDA in cardiomyocytes.[17,38] Furthermore, the administration of CAPE abolished the effects of CYP and CYP+NAP in the induction of lipid peroxidation for both acute and subchronic treatment groups. This finding confirms the results obtained by Mansour and Tawfik.[46]

Histological examination of the mouse heart tissues in the present study exhibited similarities with other studies with respect to the effect of CYP.[38,47] Pathological changes observed include red blood cells and leukocytes infiltration, vacuolation and cardiac hypertrophy. These histological observations were consistent with changes in antioxidant activities measured during the various treatment with CYP and NAP. The current study also showed that CAPE attenuated most of these changes, perhaps by increasing the antioxidant activities. Similar findings were reported [23,43] indicating that CAPE is capable of boosting the antioxidant status by scavenging generated free radicals.

The use of NAP, according to several reported studies, causes increased free radical production and, therefore, oxidative cellular stress.[29,48] Therefore, NAP combination with CYP will potentially exacerbate the oxidative damage observed with CYP alone. This case was observed in the present study as their combination significantly increased the oxidative parameters (protein carbonyl and MDA) compared with CYP alone in the subchronic treatment groups. In addition, this was reflected in the histology examinations. Their combination enhanced oxidative damage, thereby increasing the pathological changes observed, such as cardiac hypertrophy, which was, however, ameliorated by the administration of CAPE. Our finding is in agreement with the report of Pathan *et al.*[28] who observed NAP was cardiotoxic in rats and furthermore exacerbated doxorubicin-induced cardiac adverse events.

To further confirm the role of CYP and CYP+NAP in generating free radical that is detrimental to the functions of the cardiac cells, the current study also investigated the eNOS expressions, through immunohistochemistry examination. eNOS is reported to be a predominant cardiac NOS isoenzyme and it is known to play a key role in cardioprotection.[49] Therefore, eNOS-induced NO production protects the heart against ROS. In the present study, we report that eNOS expression levels were reduced in CYP, NAP and CYP+NAP in the subchronic treatment groups. Our results were corroborated by the work of Refaie *et al.*[50] Documented evidence shows that oxidative stress insults induced by ROS impair the NOS-NO pathway.[51] Daiber *et al.*[52] also reported that eNOS can be uncoupled and instead of producing NO, it will be switched to superoxide generation leading to cardiotoxicity. Moreover, decreased NO production in response to NAP can be explained by a loss of eNOS activity.[39] In the present study, we report that CAPE restored the expressions of eNOS abolished by CYP, NAP and CYP+NAP during the subchronic treatment particularly. The ability of CAPE to restore eNOS in cardiac and other tissues has been corroborated by numerous studies.[19,53]

CONCLUSION

The current study has demonstrated that CYP administration exhibited pronounced oxidative stress and therefore, damage to mice heart tissue. In addition, co-administration with NAP further exacerbated these effects. However, the addition of CAPE to the regimen was effective in ameliorating these oxidative changes by enhancing antioxidant levels, including eNOS. We hereby suggest that CAPE might be beneficial in mitigating CYP-NAP induced oxidative cardiotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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