Original Article

Pentoxifylline and Cilostazol Against Rat Heart Injuries Induced by Doxorubicin

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A B S T RAC T

We investigated the possible protective effects of pentoxifylline (PTX) and cilostazol on doxorubicin (Dox)-induced cardiotoxicity in rats. Rats are randomly assigned into: saline, Dox (I.P. 2.5 mg/kg every other day six injections over two weeks), Dox (I.P. 2.5 mg/kg every other day six injections over two weeks then PTX (50 mg/kg/day/oral), Dox (I.P. 2.5 mg/kg) every other day six injections over two weeks then cilostazol (50 mg/kg/day/oral). After 21 days these animals were sacrificed and serum CK-MB and troponin I levels were determined. Malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6), and caspase-3 levels also were determined. Heart sections were examinedhistopathologically. Treatment with PTX and cilostazol decreased troponin I and CK-MB while increased SOD activity with decrements in MDA, IL-6, TNF-α, and caspase-3 levels with attenuation of the changes in cardiac histopathology. PTX and cilostazol exert protective effects against Dox-induced cardiotoxicity.

Key Words: Pentoxifylline, cilostazol, doxorubicin, protection rat heart, CK-MB, troponin I, MDA, SOD.

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1. INTRODUCTION

Doxorubicin (Dox) is a widely used antineoplastic drug (Simbreet al.2005). However, despite its therapeutic efficacy, the occurrence of fatal cardiotoxicity limited its clinical usage (Yilmaz et al.2006). The exact pathogenesis of Dox-induced cardiotoxicity is still not well documented although a diverse set of mechanisms have been proposed, including oxidative stress, cytokine release, myofibrillar degeneration and cardiomyocytes apoptosis (Arola et al.2000; Minotti et al.2001).

Numerous studies indicate that anti-oxidants and free radical scavengers may affect Dox- induced cardiotoxicity (Oliveira et al.2004; Yagmuraca et al.2003). Previous studies have shown that many phosphodiesterase inhibitors (PDEI) induce a powerful cardioprotective effects during cardiac ischemic/reperfusion injury (Kukrejaet al.2003; Salloum et al.2003).

Pentoxifylline (PTX) is a methyl xanthine derivative and non- selective phosphodiesterase inhibitor (PDEI) that is widely used in peripheral arterial diseases (Hankey et al.2006). PTX treatment has been shown to decrease the production of several pro-inflammatory cytokines (Haddad et al .2002) and has been shown to reduce ischemia-reperfusion injuries to many organs such as liver (Iwamoto et al.2002), lungs (Thabut et al.2001), kidney (Kim et al.2001),

spinal cord (Savas et al.2002) and brain (Movassaghi et al.2012). However, studies did not provide a detailed information about the mechanisms underlying the cardio-protective effect of PTX.

Cilostazol is a selective inhibitor of type-3 PDE enzyme (Kimura et al.1985). It is currently used in intermittent claudication (Liu et al.2001). Cilostazol also shown to possess anti-oxidant and anti-inflammatory properties in several experimental models (Lee et al.2010; Hattori et al.2009).

The present study was designed to assess the possible protective effects of the PDE inhibitor drugs PTX and cilostazol in Dox- induced cardiotoxicity in rats.

2. MATERIALS AND METHODS

* Animals:

Forty-eight adult male wistar rats (200-220 g) were purchased from National Research Laboratory, Cairo, Egypt. Animals housed under controlled environmental conditions, fed standard pellet chow (El Nasr Chemical Co., Cairo, Egypt) and permitted free access to tap water. All experimental protocols were approved by the Ethics Committee of Zagazig University.

* Drugs and chemicals:

Pentoxifylline (Trental, Sanofi-Aventis, Egypt). Cilostazol (Pletal, Ostsuka Pharmaceutical Co,Japan). Doxorubicin (Oncodox-50 virals, Cipla Ltd., India).

* Experimental procedure:

Study design:

Forty-eight rats were randomly divided 4 equal groups (n = 12 per group). In the control group: animals were injected with normal saline intraperitoneally (I.P.).

In the Dox group: animals were injected I.P. with Dox 2.5 mg/kg dissolved in normal saline every other day six injections over a 2 weeks' period (Arafa et al.2014). In the PTX treated group: animals were also injected with Dox at a dose of 2.5 mg/kg I.P. every other day six injections over a 2 weeks and then received PTX at a dose of 50 mg/kg/day by gavage through a gastric needle (Zhijunet al.2015). In the cilostazol treated group: animals were injected with Dox at a dose of 2.5 mg/kg/I.P. every other day six injections over a 2 weeks and then after the 2 weeks received cilostazol at a dose of 50 mg/kg/day also by gavage through a gastric needle (Honda etal.2006).

On the day 21 from the beginning of the treatment by both PTX and cilostazol, all rats were sacrificed by cervical dislocation after thiopental 25mg⁄kg, i.p. anesthesia (Urakamiet al.1997).

Collection of blood and heart specimens:

Blood samples were collected and serum was separated by centrifugation to be stored at -80°C. The heart were excised, a part taken of each heart tissue was fixed in 10% formalin for histopathological examination while the other part was kept in -80°C and thawed just before homogenization in phosphate buffered saline for the biochemical assay.

Heart enzymes assays:

The activities of rat cardiac Troponin-I and rat cardiac creatine kinase CK-MB were measured in serum using ELISA method with a commercially available kit (KT-480, KAMIYA Biomedical Company) and (BoehringerMannheimkit, BM/Hitachisystem 911 automated analyzer) for Troponin-I and CK-MB respectively.

Assessment of cardiac lipid peroxidation:

Heart samples which collected from each group were homogenized with cold phosphate buffer (pH 7.4) and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatant was used to assay malondialdehyde (MDA) and superoxide dismutase (SOD). The levels of MDA and SOD were measured using a MDA and SOD assay kits (oxiselect^{TM} TBARS Assay kit USA) and SOD ELISA kit, USA, respectively, following the manufacturer's instructions.

Determination of tumor necrosis factor-alpha (TNF-α) concentration in cardiac homogenates:

This assay measures tissue level of tumor necrosis factor α (TNF-α) by quantitative enzyme-linked immunosorbent assay (Ray Bio® rat TNF-α ELISA kit) according to Bonavida (1991). Appropriate controls and standards were used, as specified by the manufacturer's instructions and the data are expressed as pg/ml of homogenates.

Determination of serum IL-6:

Interleukin -6 (IL-6) was evaluated in the tissue using quantitative enzyme linked immunosorbent assay (Ray Bio® rat IL-6 ELISA kit).

Determination of caspase-3 (Casp. 3) concentrations in cardiac homogenates:

This assay employs the quantitative sandwich enzyme immunoassay technique according to Wei et al. (2011). Briefly, antibody specific for Casp-3 has been precoatedonto a microplate standards and samples are pipette into the wells, and any casp-3 present is bound by the immobilized antibody. After removing any unbound substance, a biotin-conjugated antibody specific for casp-3 is added to the wells. After washing avidin-conjugated horseradish peroxidase is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells, and color develops in proportion to the amount of casp-3 bound in the initial step. The optical density of each well was determined within 5 min, using a microplate reader set to 450 nm.

Histology:

The hearts were isolated immediately after scarifying the animals cleaned and washed in ice-cold physiological saline. Small pieces of the myocardium of the left ventricle fixed in 10% neutral buffered formalin for 24 h, end embedded in paraffin, sectioned at 3 micrometer sections according to the standard procedure. The blocks were sectioned from the ventricular portion, deparaffinized, hydrated gradually then stained with hematoxylin and eosin (H & E) method and were examined by microscopy (Drury and Wallington, 1980; Chennuru and Salem, 2013).

Statistical analysis:

Statistical analysis was performed using Statistical Package for the Social Sciences, version 17 (SPSS software ,SPSS, Inc., Chicago, USA) and data were expressed as the mean±SEM .Statistical comparison of the mean values between multiple groups were performed with one-way analysis of variance(ANOVA) followed by the Tukey post hoc test

for multiple comparison, where P-value <0.05 was considered statistically significant.

3. RESULTS

1- Effect of PTX and Cilostazol on serum levels of CK-MB and troponin I in Dox induced cardiotoxicity:

The serum level of CK-MB was significantly increased from 2.08±0.15 ng/ml in the control group to 10.03±0.55 ng/ml in the Dox group (P<0.05). Treatment with PTX and cilostazol significantly decreased the level of serum CK-MB to 2.93 ± 0.09 ng/ml and 4.28 ±0.17 ng/ml respectively in relation to Dox group (Figure 1A) PTX-treated group showed insignificant statistical difference with the control group. Butcilostazol treated group still significantly (P<0.05) higher levels when compared with the control group (Figure 1A).

The Troponin I serum level in Dox group was significantly increased to 14.78±0.89 ng/ml when compared to the control group 2±0.13 ng/ml. Treatment with PTX significantly decreased the level of troponin I to 2.63±0.17 ng/ml compared with the Dox treated group (Figure 1B). Treatment with cilostazol significantly decreased the level of troponin I to 5.35±0.25 ng/ml compared with the Dox treated group. Cilostazol-treated group showed significant (P<0.05) increased in troponin I levels when compared the control group (Figure 1B).

2- Effect on cardiac oxidative stress parameters:

The MDA level (oxidant system) within the cardiac tissue in Dox group was significantly increased (P<0.05) to 29.75±0.86 umol/g from 5.33±0.36 umol/gm in the control group. Both PTX and cilostazol groups showed significantly decreased (P<0.05) the MDA level to 6.6±0.26 umol/g and 13.38±0.77 umol/gm respectively indicating that both PTX and cilostazol have a reductive effect on the oxidant system.

However, the MDA level still significantly (P<0.05) higher in the cilostazol group compared to the control group (Fig 2A).

The SOD level (antioxidant enzyme) within the cardiac tissue in the Dox group was 1.5±0.04 u/g, this level was significantly lower (P<0.05) than that of the control group (11.2±0.42 u/g). The antioxidant activity of SOD was significantly increased (P<0.05) in the both PTX and cilostazol treated groups to 10.25±0.37 u/g and 5.2±0.32 u/g respectively when compared to the Dox group (Fig 2 B).

3- Effect of PTX and cilostazol treatment on rat IL-6, TNFα and caspase-3 levels:

Dox- induced a significant (P<0.05) increase in IL-6 level from 7.63±0.27 pg/gm tissue in the control group to 29±1.94 pg/gm tissue. PTX treatment produced a significant (P<0.05) decrease in IL-6 level from 29±1.94 pg/gm tissue in Dox group to 6.53±0.33 pg/gm tissue. Cilostazol treatment produce a significant (P<0.05) decrease in IL-6 level from 29±1.94 pg/gm tissue in Dox group to 15.78±0.61 pg/gm tissue. Level of IL-6 in cilostazol group still significantly higher when compared with the control group (Fig. 3A).

The level of TNF-α was significantly (R<0.05) increased by Dox from 5.18±0.54 pg/gm tissue in the control group to 25±1.4 pg/gm tissue. The level of TNF-α was significantly (P<0.05) decreased to 7.15±0.32 pg/gm tissue and 12.08±0.56 pg/gm tissue in PTX and cilostazol treated groups respectively, cilostazol treatment still showed significant (P<0.05) higher level of TNFα compared with the control group (Fig. 3B).

Dox- induced apoptosis of the heart cells evidenced by significant (P<0.05) increase in caspase-3 level to 17.9±1.05 ng/gm tissue from 3.98±0.2 ng/gm tissue in the control group. Treatment with both PTX and cilostazol significantly (P<0.05) decreased caspase-3 level to 4.78±0.19 ng/gm tissue and 9.5±0.38 ng/gm tissue respectively in relation to that in Dox group. Cilostazol treated group showed significantly (P<0.05) higher levels of caspase-3 compared with the control group (Fig. 3C).

Histopathological effects on the cardiac tissue by hematoxylin and eosin staining: (Table 1 & Fig. 4)

The heart sections from the control group revealed the normal preserved cardiomyocytes without any signs of degeneration and necrosis. Also no vaculation or inflammation score (-) damage (Fig. 4- A).

Whereas the heart sections from the Doxorubicin group revealed severe loss of striation and inflammation (++) (Fig. 4-B) myofibril loss (+++), cytoplasm vacuolization (++), patchy necrosis and inflammatory cells (Fig 4-C). Lack of cross striations and that most of the cardiomyocytes show increased sarcoplasmic eosinophilia, score (+++). These negative effects were detected to be decreased in both PTX and cilostazol treated groups.

The heart sections from the group of animals treated with PTX revealed a marked reduction in the severity of muscle injury, mild myofibril loss (+) no cytoplasm vacuolization (-) and no inflammation (-) as compared to Dox treated group (Fig.4 –D, E).

The heart sections from the group of animals treated with cilostazol revealed a moderate reduction in the severity of the muscle injury than Dox group, moderate histopathologic alterations and moderate degenerative changes occurred in the cardiac muscle, mild myofibril loss (+), moderate vacuolization of the cytoplasm (++) and mild inflammation (+) as compared to the Dox treated group (Fig. 4-F, G).

Table 1: Light microscopy assessment of the histopathological changes of Dox, PTX and Cilostazol on the cardiac tissue by hematoxylin and eosin staining.

Groups	inflammation	Myofibril loss	Interstitial fibrosis	Cytoplasmic vacuolization
Control	(-)	(-)	(-)	(-)
Dox	(++)	(+++)	(+++)	(++)
PTX	(-)	(+)	(-)	(-)
Cilostazol	(+)	(+)	(+)	(++)

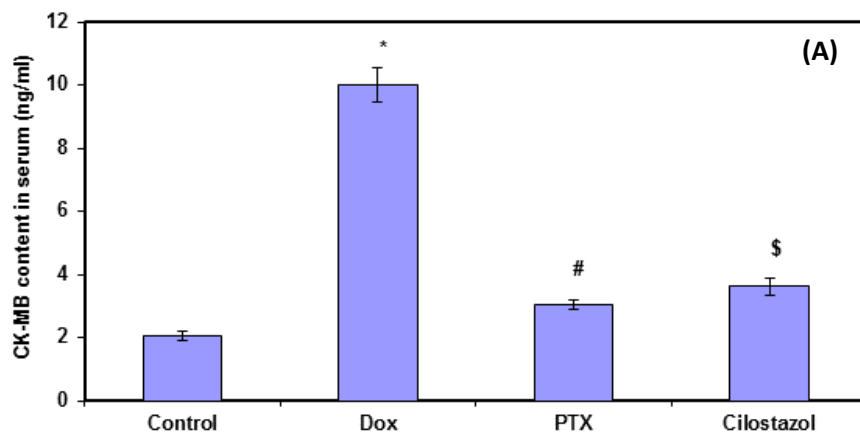(Dox: Doxorubicin; PTX: Pentoxifylline).

Fig. 1: Effect of Dox, PTX and cilostazol on serum levels of CK-MB and Troponin I in rats.
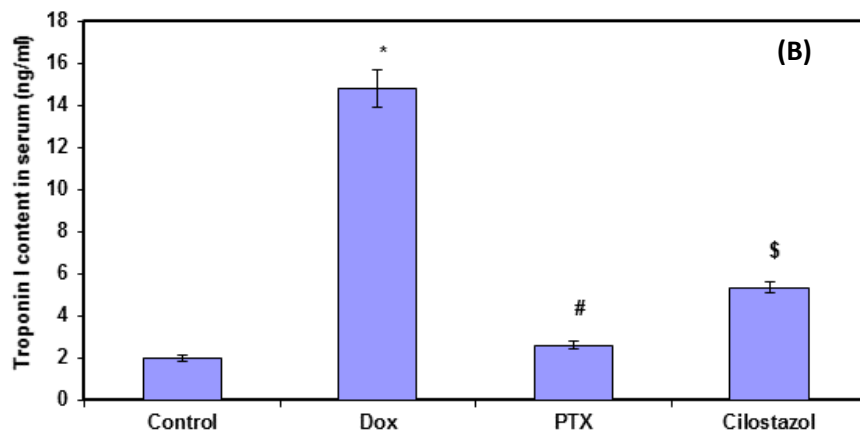

Fig. 1 (A): The Changes of serum level of CK-MB: PTX and cilostazol suppressed the CK-MB level with a significant reduction as compared to Dox group. (B): The changes of serum level of troponin I: PTX and cilostazol suppressed the troponin I level with a significant reduction as compared with Dox group.
(Dox: Doxorubicin; PTX: Pentoxifylline).
Data represented as mean± standard error.
* Significant when compared with control group.
 # Significant when compared with Dox group.
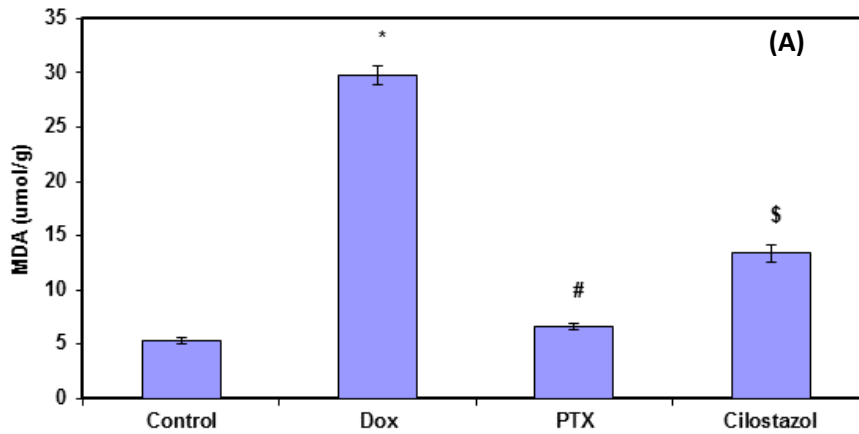**$ Significant when compared with control and Dox group.**

Fig. (2): Effect of Dox, PTX and cilostazol on SOD activity (u/g tissue) and MDA level (umol/g) in heart tissue of rat
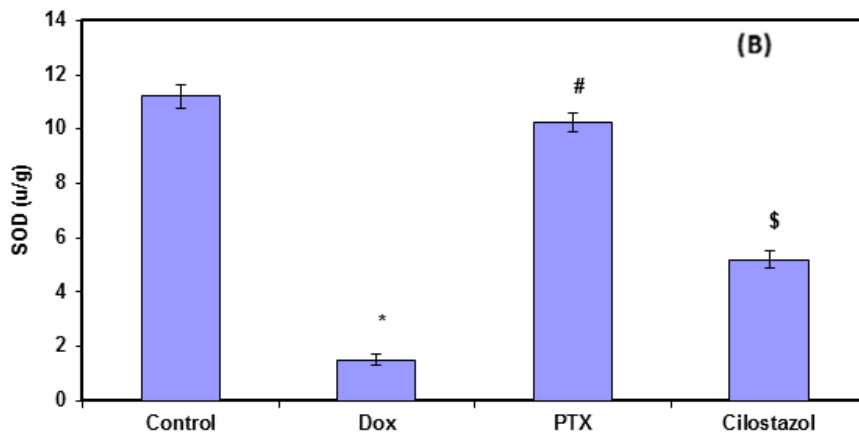

Fig. 2(A): The changes of MDA level: PTX and cilostazol suppressed the MDA level with a significant reduction as compared to Dox group. Fig. 2(B): The changes of SOD activity: PTX and cilostazol significantly increased the SOD activity as compared with Dox group.
(Dox: Doxorubicin; PTX: Pentoxifylline).
Data represented as mean±standard error.
* Significant when compared with control group.
 # Significant when compared with Dox group.
$ Significant when compared with control and Dox groups
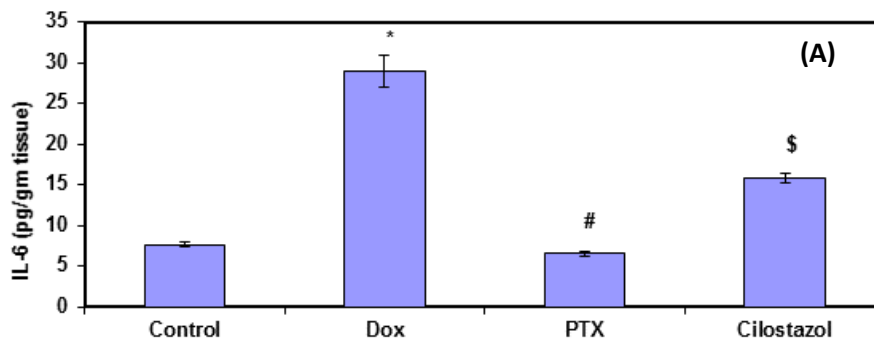

Fig. (3): Effect of Dox, PTX and cilostazol on IL-6, TNF-α and caspase-3 levels in rat heart:
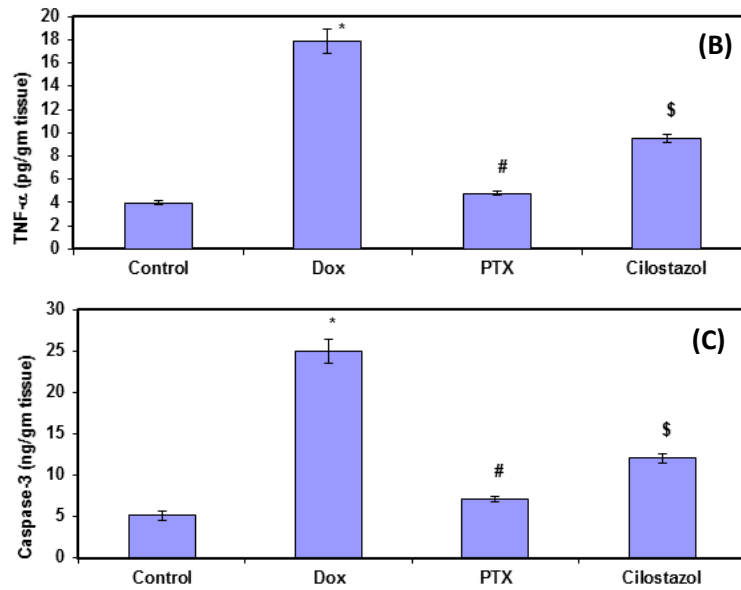
Fig. (3) (A): The Changes of IL-6 level: PTX and cilostazol suppressed the IL-6 level with a significant reduction as compared to Dox group. (B): The changes of TNF-α level: PTX and cilostazol suppressed the TNF-α level with a significant reduction as compared with Dox group. (C): The Changes of caspase-3 level: PTX and cilostazol suppressed the caspase-3 level with a significant reduction as compared to Dox group.

Data represent mean ± standard error.

* Significant when compared with control group.   # Significant when compared with Dox group.

\$ Significant when compared with control and Dox groups. (Dox: Doxorubicin; PTX: Pentoxifylline).
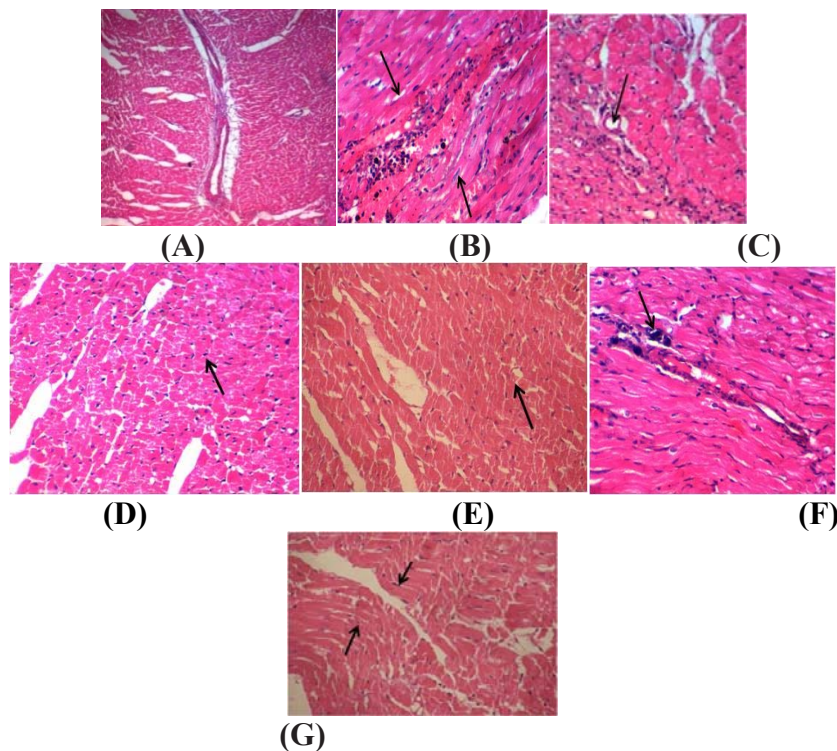

Fig. 4: Representative cardiac tissue samples stained with hematoxylin and eosin (the original magnification was ×400). (A) Control group, showing normal architecture of the muscle fibers with abundant wavy cytoplasm and small nuclei without any remarkable pathological changes; (B, C) Dox group, showing loss of striation and inflammation, myofibrilar loss with sarcoplasmic eosinophilia (arrow, B) cytoplasmic vacuolization, patchy necrosis and inflammatory cells (arrow, C). (D,E) PTX group, showing marked reduction in the severity of muscle injury, minimal histopathologic alterations and only mild degenerative changes in the cardiomyocytes. (F, G) cilostazol group, showing improved histopathologic features, few degenerative changes in cardiomyocytes with less myofibrilar loss and vacuolization.

4. DISCUSSION

Dox is an important therapeutically effective anticancer drug with wide spectrum of activity (Simbreet al.2005), however its clinical application is hampered by cardiotoxicity on chronic treatment (Fadillioglu et al.2003). There are many studies that demonstrated the cardiotoxic effects of Dox mediated by many mechanisms including oxidative stress (Geethaet al.1990; Mohan et al .2006). In this study, the efficiency of PTX and cilostazolwas investigated in the prevention of cardiotoxic effect of Dox.

The results of this study showed that the activity of CK-MB and troponin I as the most characteristic markers for cardiac damage, was significantly increased in the animal group treated with Dox compared to the control rats and these results are in agreement with Luo et al .1997; Milic et al .2010.

The elevation in the activity of these enzymes indicates injury or damage to cardiac cells by Dox which may be due to inhibition of nucleic acid and protein synthesis (Olson and Mushlin.1990).

The increased in CK-MB and troponin I may also attributed to the excessive production of free radicals and lipid peroxides that might have caused leakage of cytosolic enzymes and damage to cell membranes (Oliveira et al .2004).

In this study we found that, oral administration of PTX and cilostazol attenuated the elevations of troponin I and CK-MB induced by Dox. These results are in agreement with (El Shazlyet al.2016) that demonstrated a decrease in CK-MB serum level by prophylactic treatment of PTX against DOX induced cardiotoxicity.

The present study has shown that Dox induced a significant increase in MDA level (oxidant system) with decreased SOD activity (antioxidant enzyme) as compared to the control group. MDA content considered to be an index of excessive formation of free radicals largely arising from oxidative products of Dox, causing injury to the heart (Yin et al.1998). Free oxygen radicals can react with unsaturated lipids, leading to lipid peroxidation. These radicals may induce DNA damage (Iqbal et al.2008). SOD constitutes a mutually supportive enzyme system of the first line cellular defense against oxidative injury by decomposing O_2 and H_2O_2 prior to their interaction to form the more harmful hydroxyl, alkoxyl radicals (Lil et al.1988). The impairment of oxidant-antioxidant systems, initiates peroxidation of membrane bound polyunsaturated fatty acids and protein oxidation, leads to alteration in permeability of myocytes causing finally damage to cardiac tissues (Fadillioglu et al. 2004)

MDA level was significantly decreased after PTX and cilostazol treatment. Moreover, we found that PTX and cilostazol treated rats showed significantly increased SOD activity. Therefore, these results suggested that both cilostazol and PTX could prevent oxidative stress induced by Dox, possibly via their free radical scavenging property and/or by increasing the activity of the endogenous antioxidants. These findings are in agreement with earlier studies on which heart lesions induced by mechanisms other than DOX (Siripornet al.2014; Sridharan et al.2013).

TNF-α and IL-6 are considered to be inflammatory biomarkers which usually lead to tissue destruction (Krishnadasan et al.2003). IL-6 concentration correlates well with the degree of systemic inflammation and the severity of tissue injury, and reported to be associated with delayed apoptosis (Hidirogluet al.2014).

Both TNF-α and IL-6 were included in phagocytic stimulation, chemokine production and variable effects on cell growth and death (Pararajasingarnet al.2000; Gaines et al.1999).

The present study showed that both PTX and cilostazol significantly attenuated Dox-induced elevation of these pro-inflammatory cytokines (TNF-α and IL-6). These effects may be related to their phosphodiesterase enzyme inhibitor effect (Yoshikawa et al.1999).

These results are in agreement with that obtained with Mete et al., 2014 who suggested that PTX and cilostazol reduced the production of cytokines, including TNF-αmay be related to their ability to increase c-AMP. However Show et al.2009, suggested that PTX did not influence the TNF-α pathway and suggested also that PTX exerts anti-inflammatory effects independently of the cytokine levels .

Dox induces activation of the mitochondrial apoptosis in cardiomyocytes (Childs et al.2002). The caspase cascade induction is considered one of the mechanisms of Dox- induced apoptosis (Reeve et al.2007).

In this study we also confirmed Dox- induced myocardium apoptosis and increased caspase-3 activity. We found that PTX and cilostazol treated rats significantly decreased caspase-3 activity and consequently decreased apoptosis.

The results regarding cilostazol are in agreement with Diaa et al. (2014) who explained that cilostazolrenoprotective effect in part by decreased caspase-3 cascade.

In this work, PTX treatment downregulates caspase-3 mediated myocardial apoptosis and this in

agreement with Zhijun et al. (2015). However, Minicucciet al.2016 suggested that PTX did not affect the increased caspase-3 activity.

Dox has been reported to cause clear myocardial histopathological lesions myocyte necrosis, degeneration and inflammatory cell infiltration (Breitbart et al.2001). In agreement with these effects, we found that significant cytoplasmic vacuolization, myofibrillar disorganization, myofibrillar loss, patchy necrosis and inflammatory cellular infiltration observed in the histopathological examination of the cardiac tissues in the Dox administered rats compared to the control group.

This study showed that both PTX and cilostazol treated rats underwent significantly less histological changes of myocardial tissues as compared with DOX treated rats. With marked reduction in the severity of muscle injury, no cytoplasmic vacuolization and no inflammation reported in PTX treated rats.

While moderate reduction in the severity of the muscle injury, moderate vacuolization of the cytoplasm and mild inflammation reported in cilostazol treated rats.

Conclusion, the present study demonstrated that administration of PTX and cilostazolhave been shown to decrease DOX- induced cardiotoxicity may be due to the block in oxidative stress, the expression of pro-inflammatory cytokines, and decrement in serum troponin I and CK-MB associated with a decrease in caspase-3 activity. We found that PTX was more successful at improving all these parameters than cilostazol. This experimental work need further investigations to know the exact pathways of PTX and cilostazol attenuated Dox induced cardiac injury.

Compliance with ethical standards: All experimental protocols were approved by the Ethics Committee of Zagazig University.

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