Melatonin combination with perindopril alleviated doxorubicin cardiac toxicity in L-NAME hypertensive rats: comparative study with perindopril

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Introduction: Doxorubicin is a highly effective anticancer agent with serious cardiotoxic effects. Hypertension is considered as a major risk factor for doxorubicin cardiotoxicity. It should be noted that about one-third of cancer patients have hypertension, and melatonin can have cardio-protective effects. The present study aimed to further investigate the possible beneficial effects of melatonin co-administration to perindopril against doxorubicin cardiotoxicity in hypertensive rats. **Method:** Rats were randomly assigned to naïve group and L-NAME group, which was further subdivided into untreated, doxorubicin, doxorubicin/perindopril, doxorubicin/melatonin and doxorubicin/perindopril/melatonin subgroups. Cardiac functions, CK-MB, malondialdehyde, superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and cardiac percentage area of collagen fibers were evaluated. **Results:** Combining melatonin with perindopril to doxorubicin produced significant decreases in left ventricular end diastolic pressure, malondialdehyde, TNF-α, and TGF-β. It resulted in significant increases in left ventricular dP/dt$_{max}$ and SOD, in addition to apparent improvement in cardiac histopathology with a significant decrease in percentage area of collagen deposition compared to perindopril alone. **Conclusion:** Co-administration of melatonin to perindopril in hypertensive rats who received doxorubicin alleviated doxorubicin cardiac toxicity more than using perindopril alone. These effects could be explained by the reported antihypertensive, anti-inflammatory, anti-oxidant, and anti-fibrotic effects of melatonin.

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3   Running title

4   **Melatonin/perindopril alleviated doxorubicin-cardiotoxicity**

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17 **Abstract**

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20 noted that about one-third of cancer patients have hypertension, and melatonin can have cardio-

21 protective effects. The present study aimed to further investigate the possible beneficial effects of

22 melatonin co-administration to perindopril against doxorubicin cardiotoxicity in hypertensive rats.

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24 subdivided into untreated, doxorubicin, doxorubicin/perindopril, doxorubicin/melatonin and

25 doxorubicin/perindopril/melatonin subgroups. Cardiac functions, CK-MB, malondialdehyde,

26 superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α), transforming growth factor-β

27 (TGF-β), and cardiac percentage area of collagen fibers were evaluated.

28 **Results:** Combining melatonin with perindopril to doxorubicin produced significant decreases in

29 left ventricular end diastolic pressure, malondialdehyde, TNF-α, and TGF-β. It resulted in

30 significant increases in left ventricular dP/dt_{max} and SOD, in addition to apparent improvement in

31 cardiac histopathology with a significant decrease in percentage area of collagen deposition

32 compared to perindopril alone.

33 **Conclusion:** Co-administration of melatonin to perindopril in hypertensive rats who received

34 doxorubicin alleviated doxorubicin cardiac toxicity more than using perindopril alone. These

35 effects could be explained by the reported antihypertensive, anti-inflammatory, anti-oxidant, and

36 anti-fibrotic effects of melatonin.

37 **Keywords:** Melatonin, Perindopril, Doxorubicin, L-NAME, Hypertension, Cardiotoxicity.

38

1. Introduction

Hypertension has been identified as the most common comorbid condition reported in cancer registries (Jain and Townsend, 2007). For example, 73% of the patients with diffuse large B-cell lymphoma had hypertension (Hershman et al., 2008). Doxorubicin (DOX) is an anthracycline antibiotic with high anti-neoplastic activity to breast cancer, aggressive lymphomas, childhood solid tumors and soft tissue sarcomas (Volkova and Russell, 2011). Many studies reported a link between hypertension and DOX cardiotoxicity, as patients with hypertension showed a higher probability of developing DOX-induced heart failure (Hequet et al., 2004; Pinder et al., 2007; Szmit et al., 2014). Indeed, studies have also shown hypertension to be a strong predictor for DOX-related congestive heart failure (Swain et al., 2003) with "an acceptable prognostic role"(Lotrionte et al., 2013). In turn, this caused hypertensive patients to have more delays in subsequent treatment, more reductions in DOX doses, and early discontinuation of chemotherapy (Szmit et al., 2014). Accordingly, an aggressive effort to reduce hypertension in patients who are about to receive DOX should be considered (Hershman et al., 2008).

The pathophysiology of cardiomyocyte injuries by hypertension and DOX may be commonly related to oxidative stress and fibrotic and inflammatory processes (Kuriakose et al., 2016). DOX is known to produce hydroxyl radicals, hydrogen peroxide and superoxide anions (Rajagopalan et al., 1988; Lee et al., 1991). Oxygen radical-induced injury of membrane lipids is considered to be the most important factor responsible for the development of DOX-induced cardiotoxicity (Sarvazyan et al., 1995). Overproduction of reactive oxygen species (ROS) damages multiple cellular components such as DNA, membranes and proteins, causes cardiomyocyte contractility impairment, and promotes cell fibrosis or death (Seddon et al., 2007).

The exact mechanisms on how hypertension affects the heart stress remain largely unexplored. Increased systemic vascular resistance stimulates the heart to augment its contractility in order to accommodate a higher afterload. The left ventricle undergoes concentric hypertrophy to compensate such higher mechanical demands. With cardiac remodeling, an increase in left ventricular mass and wall thickening would eventually lead to gradual decrease in contractility and filling properties, largely due to increased tissue fibrosis. Studies have implicated the role of angiotensin II, TGF-β1, and tumor necrosis factor α as the mediators of myocyte damage during the transition from compensated to decompensated heart failure (Hunter

69 and Chien, 1999; Testa et al., 1996). Administration of angiotensin-converting enzyme inhibitor

70 showed significant efficacy and safety in reducing cardiotoxicity in high-risk cancer patients

71 undergoing anthracycline treatment (Cardinale et al., 2006). These drugs primarily reduce blood

72 pressure, which, in turn, decreases the compensatory burden faced by the heart.

73 The pineal hormone melatonin (MT) is known to have both direct and indirect

74 antioxidant actions as it attenuates lipid peroxidation and DNA degradation due to the direct

75 scavenging of ROS and activation of DNA reparation enzymes. In other words, MT indirectly

76 stimulates antioxidant properties. It has anti-inflammatory action as it blocks transcriptional

77 factors that induce pro-inflammatory cytokines (De Talamoni et al., 2014).

78 The present study was designed to elucidate the possible beneficial effects of MT co-

79 administration to perindopril against DOX cardiotoxicity in hypertensive rats.

80 **2.   Material and Method**

81 **2.1.   Chemicals**

82 Perindopril Arginine was purchased from Servier Industries Ltd. (France). MT (N-acetyl-5-

83 methoxytryptamine) supplied as white pills were purchased from Twinlab (USA) and N-Omega-

84 Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) purchased from Glentham, Life

85 sciences, (UK). DOX hydrochloride (Adricin) was purchased from EIMC United

86 Pharmaceuticals (Egypt). Urethane was purchased from Sigma Aldrich Chemicals (Germany).

87 All were dissolved and diluted in distilled water.

88 **2.2.   Animals**

89 Thirty-six male albino rats (weighting 200-250 g) were purchased from the National Research

90 Institute (Cairo, Egypt) and housed in an animal room with 12 h light/dark cycle with free access to

91 food and water. They were fed on rat chow. All animal procedures were carried out in accordance

92 with the National Institute of Health guide for the care and use of laboratory animals (NIH

93 Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics

94 Committee for Ain Shams University, Faculty of Medicine (FWA 00001809).

95 **2.3.   Methods**

96 **Induction of hypertension:**

97   Hypertension was induced with daily oral administration of L-NAME (40 mg/kg/day) for 8

98   weeks by gastric gavage (Raja, 2010). At the end of 5th week of the experiment, the systolic blood

99   pressure (SBP) was measured to ensure hypertension development and enrolment in the study.

100   **2.4.   Animal groups and drugs administration:**

101   Rats were divided randomly into two main groups: Naive group (n=6) and L-NAME

102   (hypertensive) group in which L-NAME was administered to the rats. The latter group was

103   further subdivided into 5 subgroups (6 animals each): **untreated**, **DOX** that received DOX (2.5

104   mg/kg/twice per week) by intraperitoneal injection; **DOX/Perindopril (PD)** that received

105   perindopril (5 mg/kg/day**;** Louise et al., 2005) by daily oral gavage; **DOX/MT** that received MT

106   (10 mg /kg**;** Simko et al., 2014**)** by daily oral gavage; and **DOX/PD/MT** that received perindopril

107   (5 mg/kg/day) and MT (10 mg /kg) by daily oral gavage. All drugs were administered daily for 3

108   weeks starting from the 6th to 8th week of the experiment. When MT or PD was given in

109   combination with DOX, they were administered 3 h before DOX injection. MT dose was

110   selected from previous works, such that it lowered blood pressure and protected the

111   cardiovascular system against deleterious effect of hemodynamic overload (Mukherjee et al.,

112   2012)[21] with no sedative effects (Simko et al., 2014).

113   **2.5.   Outcome measures:**

114   **2.5.1. Measurement of SBP:**

115   Non-invasive SBP measurement was done using the tail-cuff technique of Power Lab (NIBP,

116   AD Instruments, Australia). Measurement was carried out on conscious rats at three occasions

117   of the experiment (Baseline, end of 5th weeks and end of 8thweek).

118   **2.5.2. Measurement of left ventricular end diastolic pressure (LVEDP) and left**

119   **ventricular dP/dt$_{max}$:**

120   At the end of the study, each rat was anesthetized by urethane (1.2g/kg) i.p. Then the animals

121   were dissected according to a previous study (Wu et al., 2011) as follows. They were fixed and

122   intubated, and an incision was done over the xiphoid process. The chest wall and diaphragm

123   were cut through to expose the apex of the heart. A stab wound was made near the apex of the

124   heart using a 25–30 gauge needle. After the removal of the needle, the catheter tip (Millar Mikro-

125   Tip® pressure catheter) was inserted retrograde into the left ventricle. After stabilization of the

126   signal for 10 min, the LVEDP and LV dP/dt$_{max}$ were recorded. The catheter was connected to the

127   cable of the pressure module of the Power Lab instrument.

2.5.3. Samples preparation

After hemodynamic measurements, blood samples were collected in test tubes from the aorta of each rat. Samples were subsequently centrifuged at 5000 r.p.m for 15 min, and the serum was stored at -80 °C until assayed. Heart homogenate was prepared according to the method described by a previous study (Noori et al., 2012). Briefly, after cutting the samples and checking for weight, the phosphate buffer saline was added in the ratio of 4 g tissue for 16 mL of phosphate buffer (pH = 7.5). The homogenates were centrifuged at 2,000 r.p.m for 20 min at 4 °C, and the supernatant was frozen at -80 °C until analysis.

2.5.4. Creatine Kinase–MB (CK-MB) isoenzyme level in rat serum: CK-MB isoenzyme was determined by using a double-antibody sandwich enzyme-linked immunosorbent corresponding ELISA kit purchased from Shanghai Crystal Day Biotech Co., Ltd. CK-MB isoenzyme was added to monoclonal antibody enzyme well which was pre-coated with rat CK-MB isoenzyme monoclonal antibody.

2.5.5. Malonyldehyde (MDA) level in heart homogenate: The MDA level was determined using diagnostic kits (Jiangchen, Nanjing, China) with a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. Simply, it involved the reaction of one molecule of the malondialdehyde and two molecules of thiobarbituric acid to form a red MDA-thiobarbituric acid complex that can be quantified using the spectrophotometer at 535 nm wave length.

2.5.6. Superoxide dismutase (SOD) level in heart homogenate: The SOD level was determined using a corresponding ELISA kit purchased from Cell Biolabs, Inc. (USA). The absorbance was read at 490 nm on a microplate reader.

2.5.6. Tumor necrosis factor–alpha (TNF-α) level in heart homogenate: The TNF-α was determined using rat TNF-α ELISA kit purchased from Quantikine® (USA). A monoclonal antibody specific for rat TNF-α was pre-coated onto a microplate. Standards control and samples were pipetted into the wells, and any rat TNF-α present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α was added to the wells. The sample values are then read off the standard curve, and the absorbance was measured at 450 nm.

2.5.7. Transforming growth factor-beta (TGF-β) level in heart homogenate: The TGF-β level was determined by using a corresponding ELISA kit purchased from Sigma-Aldrich Co.

159 The heart samples were centrifuged for 20 minutes at 1000×g. The microtiter plate provided in

160 this kit was pre-coated with an antibody specific to TGF-β. The color change is measured

161 spectrophotometrically at a wavelength of 450 nm ± 10 nm.

162 **2.6. Histopathological study of heart samples:**

163 Heart specimens were collected at the end of the experiments. The wall of each heart was cut

164 longitudinally, and the left ventricle was cut into small specimens fixed in 10% formal-saline for

165 one week. This was followed by dehydration, clearing and embedding in paraffin. Serial sections

166 were cut at a thickness of 5μm and subjected to hematoxylin & eosin staining and Masson's

167 triple stain for detecting collagen fibers that appeared green in color.

168 **2.6.1. Morphometric studies of histopathological sections:**

169 Six different stained sections of the wall of the heart from six rats were examined in each group.

170 Five high power fields/sections were chosen to measure the percentage area of collagen fibers

171 in the myocardium. The sections were examined using an image analyzer Leica Q win V.3

172 program installed on a computer in the Histology Department Faculty of Medicine Ain Shams

173 University. The computer was connected to Leica DM2500 microscope (Wetzlar, Germany).

174 **2.7.   Statistical Analysis:**

175 Statistical analysis was carried out using Graph pad prism software program, version 5.0. (2007)

176 (Inc., CA, USA). All values in the results were expressed as means ± SD. Statistical differences

177 among groups were determined using one-way analysis of variance (ANOVA) followed by

178 Tukey's multiple comparison test. But for blood pressure, two-way ANOVA test was used with

179 Bonferroni's post-hoc test. P values < 0.05 were considered statistically significant.

180 **3. Results**

181 **3.1. Effect on SBP:**

182 Table (1) shows that there were insignificant differences in baseline (day 0) SBP recordings

183 among all rat groups. After 5 weeks of L-NAME administration, there was a significant increase

184 in SBP in all named groups before any drug administration when compared to their baseline (day

185 0) recording. L-NAME untreated rats exhibited insignificant increase in the 8th week compared

186 to the 5th week. DOX administration for 3 weeks resulted in a significant decrease in SBP to 5th

187 week. Rats who received PD, MT and PD/MT in concomitant with DOX showed a significant

188 decrease in their SBP by when compared to their 5th week recordings. In addition, there was

189   insignificant difference in SBP in DOX/PD/MT treated rats in comparison to DOX/PD treated

190   rats.

191   **3.2. Effect on LVEDP and Left Ventricular dP/dt$_{max}$:**

192   As depicted in figure (1 A & B), L-NAME-untreated and DOX administered rats showed a

193   significant increase in LVEDP and decrease in LV dP/dt$_{max}$ in comparison to naive group by

194   25.5%, 57.98% and 52.3%, 84.7%, respectively. The hypertensive rats who received DOX

195   exhibited a significant increase in LVEDP by 21.5% and decrease in LV dP/dt$_{max}$ by 36.5%

196   when compared with L-NAME-untreated rats. There was a significant decrease in LVEDP and

197   a significant increase in LV dP/dt$_{max}$ in DOX/PD- and DOX/PD/MT-treated rats by 17.7%,

198   233.1% and 32.3%, 382.9%, respectively, in comparison to DOX rats. Meanwhile, DOX/MT

199   insignificantly decreased LVEDP by 15.6% and significantly increased LV dP/dt$_{max}$ by 354.9%.

200   Furthermore, there was a significant decrease in LVEDP and an increase in LV dP/dt$_{max}$ by

201   17.6% and 44.99%, respectively, in DOX/PD/MT-treated rats when compared to DOX/PD-

202   treated rats.

203   **3.3. Effect on CK-MB isoenzyme:**

204   As shown in figure (!-C), hypertensive untreated rats and DOX rats exhibited a significant

205   increase in CK-MB isoenzyme by 161.5% and 235.9%, respectively, when compared to naive

206   rats. The DOX subgroup showed increased CK-MB isoenzyme level by 28.4% over untreated

207   subgroup. Administration of PD and MT either alone or in combination to DOX significantly

208   decreased CK-MB isoenzyme by 65%, 38.5%, and 36.5%, respectively, in comparison to DOX

209   rats. There was an insignificant difference in CK-MB isoenzyme in DOX/PD/MT treated rats in

210   comparison to DOX/PD rats.

211   **3.4. Effect on MDA and SOD:**

212   As shown in figure (2 A & B), hypertensive untreated and DOX rats exhibited a significant

213   increase in MDA and decrease in SOD by 169.6%, 55.8% and 248%, 71.9%, respectively, as

214   compared to naive group. A notable significant increase in MDA by 29.1% and a decrease in

215   SOD by 57.4% were observed in DOX subgroup when compared to L-NAME untreated rats.

216   Administration of PD and MT either alone or in combination to DOX significantly decreased

217   MDA and increased SOD by 51.4%, 48.9%, 65% and 148.5%, 104.5%, 208.4%, respectively,

218   in comparison to DOX rats. It is worthy to mention that DOX/PD/MT rats showed a significant

219   decrease in MDA by 27.97% and an increase in SOD by 24.1% when compared to DOX/PD

220   rats.

221   **3.5. Effect on TNF- α:**

222   Figure 2-C shows that hypertensive untreated and DOX rats exhibited a significant increase in

223   TNF-α by 123.5% and 203.9%, respectively, as compared to naive group, with significant

224   increase in DOX by 35.96% compared to untreated subgroup. Administration of PD and MT

225   either alone or in combination to DOX significantly decreased TNF-α by 46.97%, 46.77%,

226   61.1%, respectively, in comparison to DOX subgroup. Furthermore, there was a significant

227   decrease in TNF-α in DOX/PD/MT subgroup by 26.6% in comparison to PD subgroup.

228   **3.6. Effect on TGF-β:**

229   As shown in figure 2-D, untreated hypertensive rats and DOX rats exhibited a significant

230   increase in TGF-β by 491% and 666.2%, respectively, as compared to the naive group with a

231   significant increase in DOX by 29.7%. Administration of PD and MT either alone or in

232   combination to DOX significantly decreased TGF β by 63.2%, 65.3%, 81.1%, respectively, in

233   comparison to DOX rats. There was a significant decrease in TGF-β in DOX/PD/MT-treated rats

234   by 48.5% in comparison to DOX/PD subgroup.

235   **3.7. Histopathological changes in heart tissues:**

236   **1**. **Hematoxyline and Eosin stain:**

237   Examination of stained sections by light microscopy of naive group revealed that the

238   myocardium was formed of branching and anastomosing acidophilic cardiac muscle fibers with

239   large, oval and vesicular nuclei. Nuclei of connective tissue cells in between the cardiac muscle

240   fibers were observed to be flat and darkly stained (figure 3-A). The stained sections of L-NAME

241   untreated and DOX subgroups showed extravasated blood cells between cardiac muscle fibers.

242   Some fibers showed pyknotic nuclei while others were disrupted, fragmented and widely

243   separated (figures 3-B & C). The stained sections of DOX/PD subgroup revealed some areas

244   with congested interstitial blood capillaries between muscle fibers (figure 3-D), while those of

245   DOX/MT subgroup showed some cardiac muscle fibers to be disorganized with widening of

246   interstitial spaces (figure 3-E). The stained sections of DOX/PD/MT subgroup showed the

247   cardiac muscle fibers to be slightly disorganized with mild spacing and extravasated blood cells

248   (figure 3-F).

249   **II. Masson's trichrome stain:**

250   Sections of naive normal rats showed few collagenous fibers surrounding cardiac muscle fiber

251   (figure 4-A). The stained sections of untreated and DOX subgroups showed a marked increase in

252   the amount of collagen fibers between disorganized, fragmented muscle fibers (figure 4-B & C)

253   while the sections of DOX/PD subgroup showed a moderate amount of collagen fibers around

254   blood capillaries and between cardiac muscle fibers (figure 4-D). Moreover, stained sections

255   from DOX/MT subgroup showed moderate amount of collagen fibers between disorganized

256   muscle fibers (figure 4-E) while that of DOX/PD/MT subgroup showed mild amount of collagen

257   fibers between regularly arranged muscle fibers (figure 4-F).

258   **III. Morphometric analysis:**

259   Notably, the percentage areas of collagen fibers were significantly increased in hypertensive

260   untreated and DOX animals by 602.5% and 772%, respectively, compared to naive rats with a

261   significant increase by 24.23% in DOX animals compared to untreated subgroup. Meanwhile,

262   the administration of PD and MT either alone or in combination to DOX significantly decreased

263   the percentages area of collagen fibers by 54.02%, 51.62%, 82.96%, respectively, in comparison

264   to DOX rats. Furthermore, there was a significant decrease in percentage areas of collagen fibers

265   in DOX/PD/MT by 62.99% in comparison to DOX/PD (Table 2).

266   **4.   Discussion**

267   In the present study, DOX administration to hypertensive rats showed a decrease in SBP, increase

268   in LVEDP and decrease in LV dP/dt_{max}, denoting a reduction in cardiac functions of the heart,

269   especially myocardial contraction. These findings are in accordance with previous studies (Warpe

270   et al., 2015; Gandhi et al., 2013)**.** The development of oxidative stress evidenced by a decrease in

271   SOD levels and an increase in MDA and myocardial damage was associated with a significant

272   increase in serum CK-MB**.** The heart's unique vulnerability to oxidative stress has given this

273   aspect of DOX-induced cardiomyopathy an overwhelming prominence in the literature (Octavia et

274   al., 2012). The cardiac oxidative stress promotes the development of cardiac fibrosis by

275   upregulating TGF-β expression, which subsequently enhances cardiac collagen synthesis and

276   suppresses collagen degradation in hypertensive rats. This is evidenced by increased cardiac

277   percentage area of collagen fibers deposition in the present study (Zhao et al., 2008; Al-Shabanah et

278   al., 2012), along with an increased level of cardiac TNF-α **(**Pecoraro et al., 2016). All of these

279   findings lead to significant cardiac pathology and increased mortality (Octavia et al., 2012).

280   Agents that can prevent or attenuate fibrotic reactions may prove to be important additions

281   to the therapeutic aims for managing at-risk patients with the goal of early intervention before

282   fibrosis (Feldman et al., 2016).

283   In accordance with Mukherjee and his colleagues (2012), MT administration produced

284   significant antihypertensive effect with increased cardiac contractility. Co-administration of MT

285   to PD in rats who received DOX produced a significant decrease in SBP and better control of

286   cardiac hemodynamics in comparison to PD alone. The antihypertensive effect of MT could be

287   explained in part by the activation of MT receptors on endothelial cells. This could also trigger

288   the formation of nitric oxide (NO), whose vasodilatory potential could overwhelm the MT-

289   dependent contraction of smooth muscle cells (Paulis and Simko, 2007). As a result, the

290   antihypertensive effect of MT is less pronounced in L-NAME-induced NO-deficient hypertension

291   compared with other studies on spontaneously hypertensive rats (Pechanova et al., 2007; Simko et

292   al., 2009). This could be related to the L-NAME-induced blockade of eNOS activity that might

293   have disabled MT from improving the NO pathway. Thus, some data support the idea that

294   augmentation of NO-signaling plays an important role in the antihypertensive action of MT

295   (Paulis and Simko, 2007). However, the antihypertensive effect of MT is mediated by other

296   mechanisms including a direct hypothalamic effect, a lowering of catecholamine levels, and most

297   importantly, its antioxidant properties, which contribute to better vascular functions and blood

298   pressure regulation (Tengattini et al., 2008; Pechanova et al., 2014). Besides, MT could apparently

299   potentiate acetylcholine effects by central MT receptor stimulation (Weekley, 1995). Furthermore,

300   chronic administration of MT decreased the blood pressure, inhibited the sympathetic activity,

301   restored cardiac β-adrenoceptor function, and improved baroreflex (Girouard et al., 2004). In

302   healthy young men, MT reduced the SBP along with norepinephrine levels and reduced blood

303   pressure and pulse wave velocity in association with the attenuation of sympathetic tone (Yildiz et

304   al., 2006).

305   MT is a powerful antioxidant which can limit damage in cardiovascular disease (Yang et

306   al., 2014), and a lot of studies have demonstrated that MT influences antioxidant enzyme

307   activities after the administration of free radical generating substances (Rodriguez et al., 2004). It

308   has direct and indirect antioxidant actions as it attenuates lipid peroxidation with a decrease in

309   MDA level and DNA degradation due to direct scavenging of both ROS and activation of DNA

310   reparation enzymes. Indirectly, MT has antioxidant actions through stimulation of enzyme

311 activation like SOD (Şehirli et al., 2013; De Talamoni et al., 2014). In a model of pulmonary

312 hypertension (Maarman et al., 2015), it was found that plasma SOD activity was increased by

313 chronic daily administration of MT either as a preventive or a curative treatment.

314 In agreement with the current study, a previous study (Patel et al., 2010) reported that

315 pretreatment of MT resulted in reduced activity of CK-MB enzyme in serum in a model of

316 isoproterenol-induced heart failure. Thus, MT could maintain membrane integrity, thereby,

317 restricting the leakage of these enzymes.

318 Co-administration of MT and PD to DOX produced better control of inflammatory and

319 fibrotic reactions than PD alone by significant decreases in TNF-α, TGF-β and percentage area

320 of cardiac fibrosis.   The anti-inflammatory and antifibrotic effects of PD have been reported

321 previously (Madej et al., 2009; Toblli et al., 2005). The anti-inflammatory mechanism of MT could

322 decrease the NF-κB expression in endothelial cells of spontaneously hypertensive rats (Tamura et

323 al., 2009). It mediated this effect through the regulation of gene transcriptional and translational

324 levels in human vascular smooth muscle cell (Shi et al., 2012). MT significantly attenuated the

325 cardiac fibrosis by lowering the mRNA expression of genes associated with cardiac hypertrophy,

326 especially TGF-β in the hypoxic rats (Yeung et al., 2015). The antifibrotic effect of MT was also

327 demonstrated in a model of L-NAME-induced hypertension and in a model of combined

328 continuous light exposure and L-NAME administration (Paulis et al., 2009; Simko et al., 2010).

329 As mentioned before, the antifibrotic effect of MT could be attributed to not only its ability to

330 decrease TGF-β but also its powerful antioxidant properties (Al-Shabanah et al., 2012).

331 **In Conclusion,** co-administration of MT and PD in hypertensive rats who received DOX

332 alleviated DOX cardiac toxicity more than using PD alone. These effects could be explained by

333 the reported antihypertensive, anti-inflammatory, anti-oxidant, and anti-fibrotic effects of MT.

334 Accordingly, it would be valuable to test MT in clinical trials for prevention of possible heart

335 damage associated with DOX in hypertensives.

336 **Declarations:**

337 • Competing interests: The authors declare that they have no competing interests.

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5. References

341 Al-Shabanah OA, Aleisa AM, Hafez MM, Al-Rejaie SS, Al-Yahya AA, Bakheet SA and Sayed-
342 Ahmed MM (2012). Desferrioxamine Attenuates Doxorubicin-Induced Acute Cardiotoxicity through
343 TFG-β/Smad Pathway in Rat Model. Oxidative medicine and cellular longevity, 53.

344 Cardinale D, Colombo A, Sandri MT, et al. (2006). Prevention of high-dose chemotherapy-induced
345 cardiotoxicity in high risk patients by angiotensin-converting enzyme inhibition. Circulation, 114
346 (23), 2474–2481.

347 De Talamoni NT, Areco VA, Lopez MEP, de Barboza, GD, Rivoira MA, and Carpentieri AR (2014).
348 Past, Present, and Future of Melatonin's Clinical Uses. In Melatonin and Melatonergic Drugs in
349 Clinical Practice, 27-42.

350 Feldman AM, Haller JA and DeKosky ST (2016). Valsartan/Sacubitril for Heart Failure: Reconciling
351 Disparities between Preclinical and Clinical Investigations. JAMA, 315(1), 25-26.

352 Gandhi H, Patel VB, Mistry N, Patni N and Nandania JR (2013). Doxorubicin mediated cardiotoxicity
353 in rats: Protective role of felodipine on cardiac indices. Environmental toxicology and
354 pharmacology, 36(3), 787-795.

355 Girouard H, Denault C, Chulak C and de Champlain J (2004). Treatment by N-acetylcysteine and
356 melatonin increases cardiac baroreflex and improves antioxidant reserve. Am. J.Hypertens; 17, 947–
357 954.

358 Hequet O, Le QH., Moullet I. et al., (2004). Subclinical late cardiomyopathy after doxorubicin
359 therapy for lymphoma in adults,"Journal of Clinical Oncology, 22 (10), 1864–1871.

360 Hershman DL, McBride RB, Eisenberger A, Tsai WY, Grann VR, and Jacobson JS (2008).
361 Doxorubicin, cardiac risk factors, and cardiac toxicity in elderly patients with diffuse B-cell non-
362 Hodgkin's lymphoma, Journal of Clinical Oncology, 26(19), 3159–3165.

363 Hunter JJ. and Chien KR (1999). "Signaling pathways for cardiac hypertrophy and failure.The New
364 England Journal of Medicine. 341 (17), 1276–1283.

365 Jain M and Townsend RR (2007). Chemotherapy agents and hypertension: a focus on angiogenesis
366 blockade. Current Hypertension Reports, 9(4), 320–328.

367 Kuriakose RK, Kukreja RC, Xi L, (2016). Potential Therapeutic Strategies for Hypertension-
368 Exacerbated Cardiotoxicity of Anticancer Drugs. Oxid Med Cell Longev, 2016, 8139861.

369 Lee V, Randhawa AK, Singal PK (1991). Adriamycin-induced myocardial dysfunction in vitro is
370 mediated by free radicals. Am J Physiol., 261: H989–995.

371 Lotrionte M, Biondi-Zoccai G, Abbate A, et al. (2013). Review and meta-analysis of incidence and
372 clinical predictors of anthracycline cardiotoxicity. American Journal of Cardiology, 112 (12), 1980–
373 1984.

374   Louise M. Burrell, John Risvanis, Eiji Kubota and Rachael G. Dean (2005). Myocardial infarction
375   increases ACE2 expression in rat and humans: European Heart Journal, 26, 369-375.

376   Maarman G, Blackhurst D, Thienemann F, Blauwet L, Butrous G, Davies N, Sliwa K. and Lecour S
377   (2015). Melatonin as a preventive and curative therapy against pulmonary hypertension. Journal of
378   Pineal Research, 59, 343–353.

379   Madej A, Buldak L, Basiak M, Szkrobka W, Dulawa A, and Okopien B (2009).The effects of 1 month
380   antihypertensive treatment with perindopril, bisoprolol or both on the ex vivo ability of monocytes to
381   secrete inflammatory cytokines. International journal of clinical pharmacology and therapeutics,
382   47(11), 686-694.

383   Mukherjee D, Ghosh AK, Bandyopadhyay A, Basu A, Datta S, Pattari S. K and Bandyopadhyay D
384   (2012). Melatonin protects against isoproterenol-induced alterations in cardiac mitochondrial
385   energy-metabolizing enzymes, apoptotic proteins, and assists in complete recovery from myocardial
386   injury in rats. Journal of pineal research, 53(2), 166-179.

387   Noori S; Azmat M and Mahboob T (2012). Study on antioxidant effects of cinnamon and garlic
388   extract in liver, kidney and heart tissue of rat. Biosci. Res; 9, 17–22.

389   Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ and Moens AL (2012). Doxorubicin-
390   induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. Journal of molecular
391   and cellular cardiology, 52(6), 1213-1225.

392   Patel V, Upaganlawar A, Zalawadia R and Balaraman R (2010). Cardioprotective effect of melatonin
393   against isoproterenol induced myocardial infarction in rats: a biochemical, electrocardiographic and
394   histoarchitectural evaluation. European journal of pharmacology, 644(1), 160-168.

395   Paulis L and Simko F (2007). Blood pressure modulation and cardiovascular protection by melatonin:
396   Potential mechanisms behind. Physiol Res, 56, 671–684.

397   Paulis L, Pechanova O, Zicha J, Krajcirovicova K, Barta A, Pelouch V and Simko F (2009).
398   Melatonin prevents fibrosis but not hypertrophy development in the left ventricle of NG-nitro-L-
399   arginine-methyl ester hypertensive rats. Journal of Hypertension, 27, S11-S16.

400   Pechanova O, Paulis L and Simko F (2014). Peripheral and central effects of melatonin on blood
401   pressure regulation. International journal of molecular sciences, 15(10), 17920-17937.

402   Pechanova O, Zicha J, Paulis L, Zenebe W, Dobesova Z, Kojsova S, Jendekova L, Sladkova M,
403   Dovinova I and Simko F (2007). The effect of N-acetylcysteine and melatonin in adult spontaneously
404   hypertensive rats with established hypertension. Eur. J. Pharmacol, 561, 129–136.

405   Pecoraro M, Del Pizzo M, Marzocco S, Sorrentino R, Ciccarelli M, Iaccarino G and Popolo A (2016).
406   Inflammatory mediators in a short-time mouse model of doxorubicin-induced cardiotoxicity.
407   Toxicology and applied pharmacology, 293, 44-52.

408 Pinder MC, Duan Z, Goodwin JS, Hortobagyi GN, and Giordano SH (2007). Congestive heart failure
409 in older women treated with adjuvant anthracycline chemotherapy for breast cancer, Journal of
410 Clinical Oncology, 25 (25). 3808–3815.

411 Raja, B (2010). Antihypertensive and Antioxidant Potential of Borneol-A Natural Terpene in L-
412 NAME–Induced Hypertensive Rats. International Journal of Pharmaceutical & Biological
413 Archive, 1(3).

414 Rajagopalan S, Politi PM, Sinha BK, Myers E (1988). Adriamycin-induced free radical formation in
415 the perfused rat heart: implications for cardiotoxicity. Cancer Res, 48 (17), 4766–4769.

416 Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V and Reiter RJ (2004). Regulation of
417 antioxidant enzymes: a significant role for melatonin. Journal of pineal research, 36(1), 1-9.

418 Sarvazyan NA, Askari A, Huang WH, (1995). Effects of doxorubicin on cardiomyocytes with
419 reduced level of superoxide dismutase. Life Sci., 57: 1003–1010.

420 Seddon M, Looi YH., and Shah AM (2007). Oxidative stress and redox signalling in cardiac
421 hypertrophy and heart failure. Heart, 93 (8), 903–907.

422 Şehirli AÖ, Koyun D, Tetik Ş, Özsavcı D, Yiğiner Ö, Çetinel Ş, Tok OE, Kaya Z, Akkiprik M, Kılıç
423 E. and Şener G (2013). Melatonin protects against ischemic heart failure in rats. Journal of Pineal
424 Research, 55, 138–148.

425 Shi D, Xiao X, Wang J, Liu L, Chen W, Fu L and Deng W (2012). Melatonin suppresses
426 proinflammatory mediators in lipopolysaccharide-stimulated CRL1999 cells via targeting MAPK,
427 NF-κB, c/EBPβ, and p300 signaling. Journal of pineal research, 53(2), 154-165.

428 Simko F, Pechanova O, Pelouch V, Krajcirovicova K, Mullerova M, Bednarova K and Paulis L
429 (2009). Effect of melatonin, captopril, spironolactone and simvastatin on blood pressure and left
430 ventricular remodelling in spontaneously hypertensive rats. Journal of Hypertension, 27, S5-S10.

431 Simko F, Pechanova O, Repova Bednarova, K, Krajcirovicova K, Celec P, Kamodyova, N and Paulis
432 L (2014). Hypertension and cardiovascular remodelling in rats exposed to continuous light: Protection
433 by ACE-inhibition and melatonin. Mediators of inflammation, 2014 (703175).

434 Simko F. Pechanova O. Pelouch V, Krajcirovicova K, Celec P, Palffy R and Paulis L (2010).
435 Continuous light and L-NAME-induced left ventricular remodelling: different protection with
436 melatonin and captopril, "Journal of Hypertension, 28(1), pp. S13–S18.

437 Swain SM., Whaley FS and Ewer MS (2003). Congestive heart failure in patients treated with
438 doxorubicin: a retrospective analysis of three trials. Cancer, 97 (11), 2869–2879.

439 Szmit S, Jurczak W, Zaucha JM. et al. (2014). Pre-existing arterial hypertension as a risk factor for
440 early left ventricular systolic dysfunction following (R)-CHOP chemotherapy in patients with
441 lymphoma. Journal of the American Society of Hypertension, 8 (11), 791–799.

442   Tamura EK. Cecon E. Monteiro A.W. Silva C.L and Markus R.P (2009). Melatonin inhibits LPS-
443   induced NO production in rat endothelial cells. J. Pineal Res; 46, 268–274.

444   Tengattini S, Reiter RJ, Tan DX, Terron MP, Rodella LF and Rezzani R (2008). Cardiovascular
445   diseases: protective effects of melatonin. J Pineal Res; 44, 16–25.

446   Testa M, Yeh M, Lee P, et al. (1996). Circulating levels of cytokines and their endogenous
447   modulators in patients with mild to severe congestive heart failure due to coronary artery disease or
448   hypertension. Journal of the American College of Cardiology. 28 (4), 964–971.

449   Toblli JE, Cao G, DeRosa G and Forcada P (2005). Reduced cardiac expression of plasminogen
450   activator inhibitor 1 and transforming growth factor β1 in obese Zucker rats by
451   perindopril. Heart, 91(1), 80-86.

452   Volkova M and Russell R (2011). Anthracycline cardiotoxicity: prevalence, pathogenesis and
453   treatment. Curr.Cardiol. Rev; 7, 214-220.

454   Warpe VS, Mali VR, Arulmozhi S, Bodhankar SL and Mahadik KR (2015). Cardioprotective effect of
455   ellagic acid on doxorubicin induced cardiotoxicity in wistar rats. Journal of Acute Medicine, 5(1), 1-8.

456   Weekley LB (1995): Pharmacologic studies on the mechanism of melatonin-induced vasorelaxation in
457   rat aorta. J Pineal Res; 19(3), 133–8.

458   Wu Y, Yin X, Wijaya C, Huang MH, and. McConnell BK (2011). Acute Myocardial Infarction in
459   Rats. J Vis Exp.; (48): 2464.

460   Yang Y, Sun Y, Yi W, Li Y, Fan C, Xin Z and Yi D (2014). A review of melatonin as a suitable
461   antioxidant against myocardial ischemia–reperfusion injury and clinical heart diseases. Journal of
462   pineal research, 57(4), 357-366.

463   Yeung HM, Hung MW, Lau CF and Fung ML (2015). Cardioprotective effects of melatonin against
464   myocardial injuries induced by chronic intermittent hypoxia in rats. Journal of Pineal Research, 58,
465   12–25.

466   Yildiz M, Sahin B and Sahin A (2006). Acute effects of oral melatonin administration on arterial
467   distensibility, as determined by carotid-femoral pulse wave velocity, in healthy young men. Exp. Clin.
468   Cardiol, 11, 311–313.

469   Zhao W, Zhao T, Chen Y, Ahokas RA and Sun Y (2008). Oxidative stress mediates cardiac fibrosis
470   by enhancing transforming growth factor-beta1 in hypertensive rats. Mol Cell Biochem; 317, 43–50.

Figure 1

Effect on (A) LVEDP, (B) LV dp/dtmax and (C) cardiac CK-MB isoenzyme in hypertensive rats.

Figure-1 (A-C): Effect on (A) LVEDP, (B) LV dp/dtmax and (C) cardiac CK-MB isoenzyme in hypertensive rats. Values are mean ± SD; n = 6 per group, One-way ANOVA followed by Tukey's multiple comparison test. [a]$P<0.05$ compared to naïve group. [b]$P<0.05$ compared to L-NAME untreated subgroup. [c]$P<0.05$ compared to DOX subgroup. [d]$P<0.05$ compared to PD subgroup. LVEDP = left ventricular end diastolic pressure, LV dp/dt_{max} = left ventricular delta pressure/delta time maximum MT = melatonin, PD = perindopril, DOX = doxorubicin.
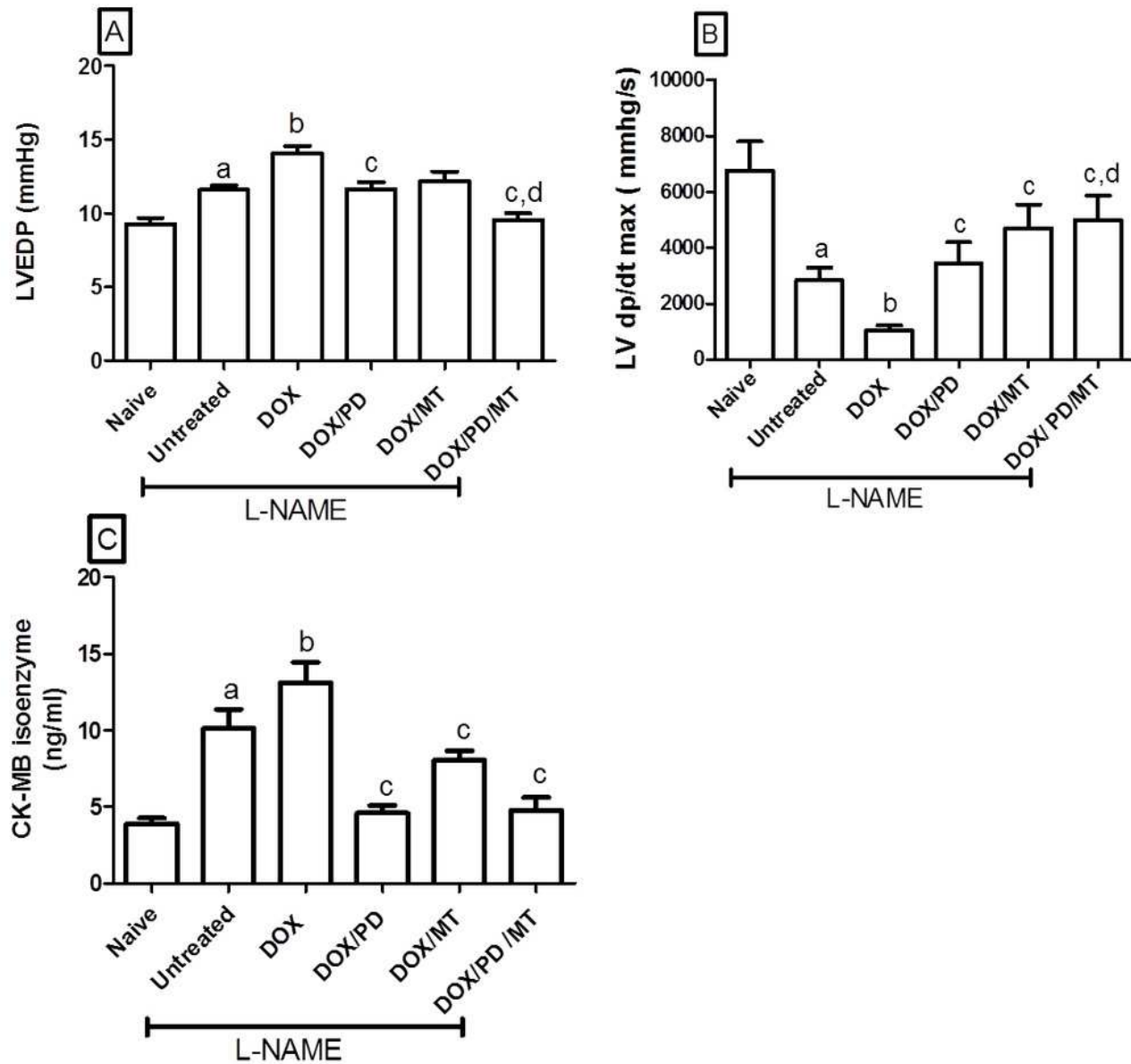
Figure 2

Effect on cardiac (A) Malondialdehyde, (B) superoxide dismutase , (C) cardiac TNF-α and (D) cardiac TGF-β in hypertensive rats.

Figure-2 (A-D): Effect on cardiac (A) Malondialdehyde, (B) superoxide dismutase , (C) cardiac TNF-α and (D) cardiac TGF-β in hypertensive rats. Values are mean ± SD; n = 6 per group, One-way ANOVA followed by Tukey's multiple comparison test. [a]P<0.05 compared to naive group. [b]P<0.05 compared to L-NAME untreated subgroup. [c]P<0.05 compared to DOX subgroup. [d]P<0.05 compared to PD subgroup. MT = melatonin, PD = perindopril, DOX = doxorubicin.
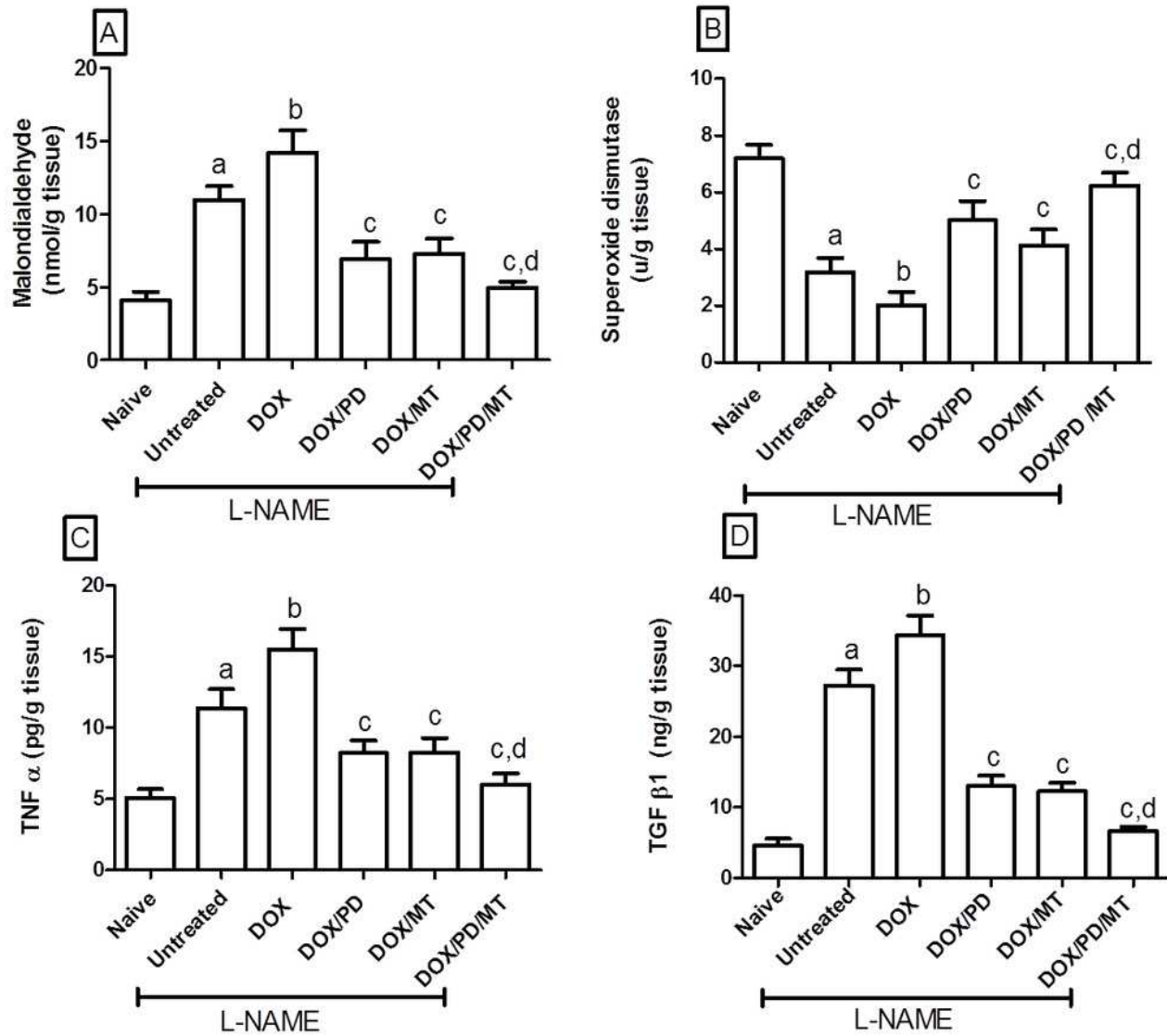
Figure 3

Photomicrographs of myocardium of rats from different groups (H&E X540)

Figure (3 A-F): Photomicrographs of myocardium of rats from different groups **(H&E X540): (A) Naive group**, showing acidophilic cardiac muscle fibers (C) with large vesicular (↑) nuclei. Note darkly stained nuclei of connective tissue cells between the muscle fibers (↑↑). **(B) L-NAME untreated subgroup**, (C) **(B) DOX subgroup** showing extravasated blood cells (R) between fibers. Notice some fibers with pyknotic nuclei (↑) while others disrupted, fragmented and widely separated (↑↑). **(D) DOX/PD subgroup,** showing some areas with congested interstitial blood capillaries (*) between muscle fibers. **(E) DOX/MT subgroup,** showing some cardiac muscle fibers disorganized with widening of interstitial spaces (↑). Note extravasated blood cells (R) between fibers. **(F) DOX/PD/MT subgroup,** showing nearly similar to the control group. The cardiac muscle fibers are slightly disorganized with mild spacing (*) having vesicular nuclei (↑). Notice mildly extravasated blood cells (↑↑).

Figure 4

Photomicrographs of myocardium of rats from different groups (Masson X540):

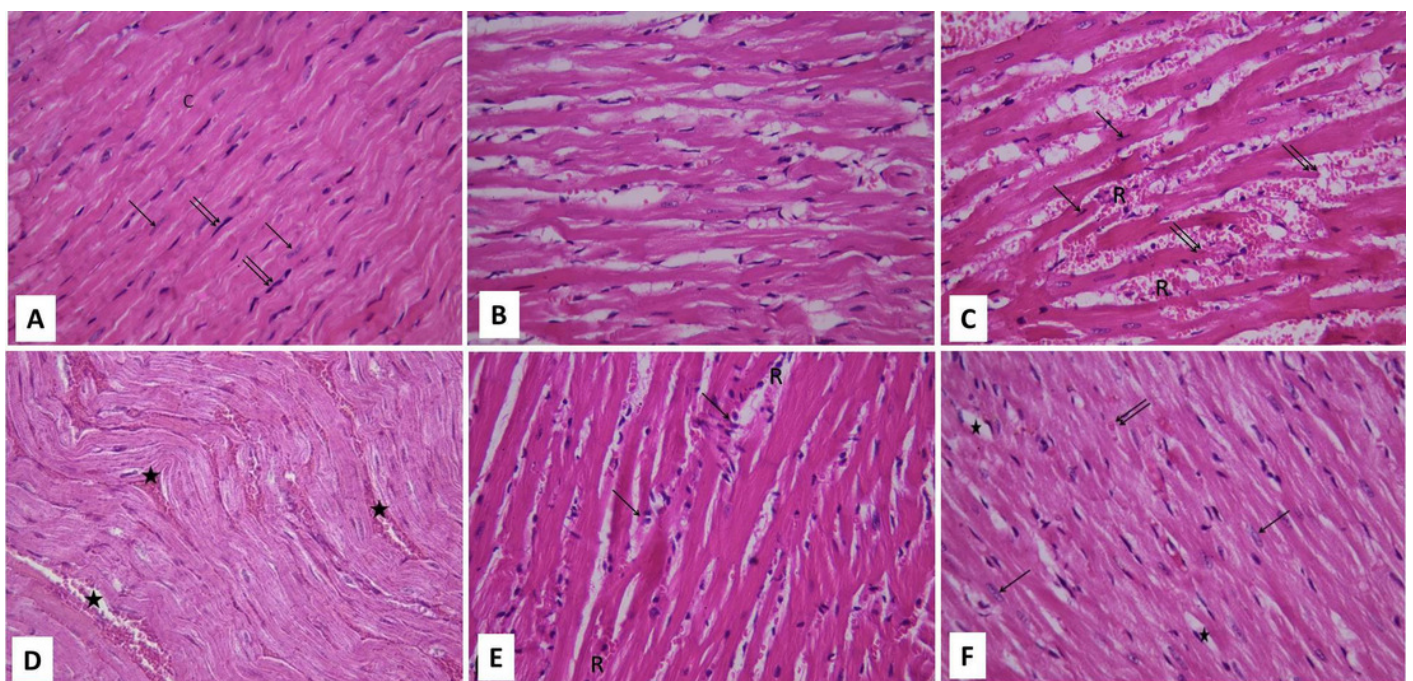**Figure (4 A-F):** <!--[if !vml]--> <!--[endif]--><!--[if !mso]--> <!--[endif]--> **B** <!--[if !mso]--> <!--[endif]--><!--[if !mso & !vml]--><!--[endif]--><!--[if !vml]--> <!--[endif]-->
Photomicrographs of myocardium of rats from different groups **(Masson X540): (A) Naive group**, showing mild amount of collagen fibers (green) (↑) between cardiac muscle fibers. **(B) L-NAME untreated subgroup**, showing moderate increase in the amount of collagen fibers. **(C) DOX** subgroup showing marked increase in the amount of collagen fibers (↑) between disorganized, fragmented muscle fibers **(D) DOX/PD subgroup,** showing moderate amount of collagen fibers (↑) around blood capillaries and between cardiac muscle fibers. **(E) DOX/MT subgroup,** showing moderate amount of collagen fibers (↑) between disorganized muscle fibers. **(F) DOX/PD/MT subgroup,** showing few amount of collagen fibers between (↑) between regularly arranged cardiac muscle fibers.
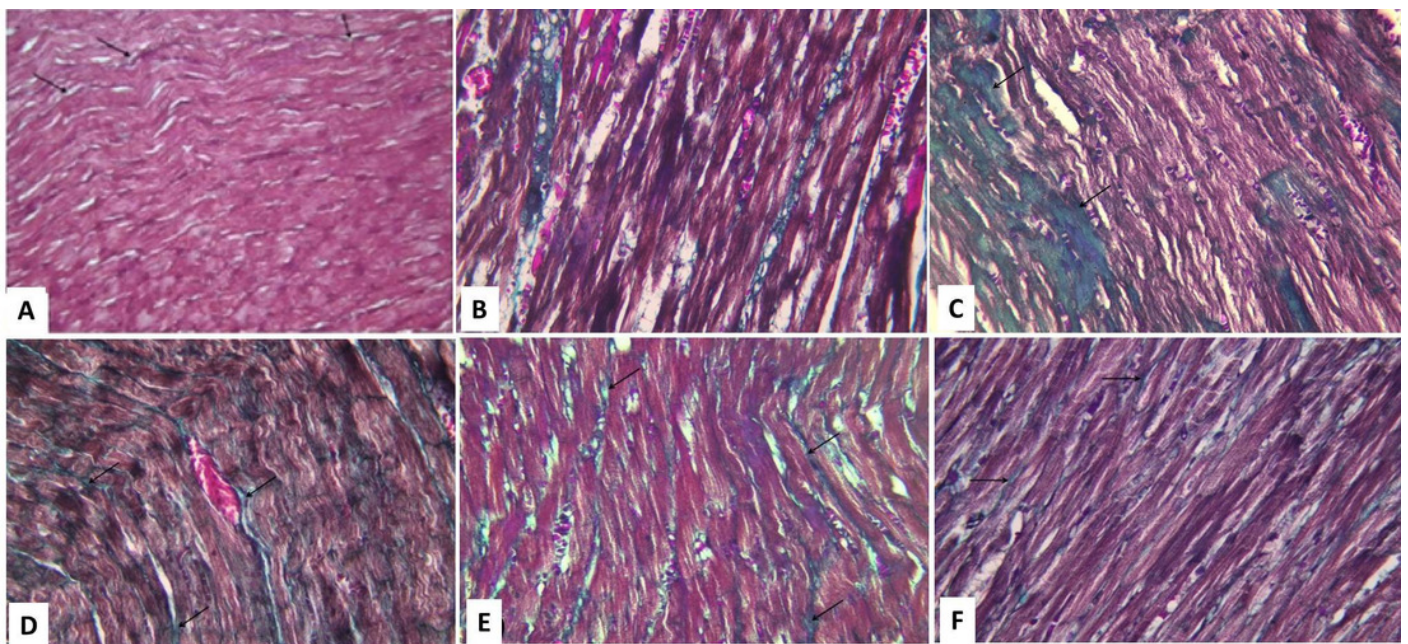
Table 1(on next page)

Effect on systolic blood pressure (SBP)

Values are mean ± SD; n=6 per group. Two-ways ANOVA followed by Bonferroni's post-hoc test for comparison to the corresponding group. [a]$P < 0.05$ compared to corresponding baseline SBP , [b]$P < 0.05$ compared to corresponding 5th week SBP. [c]$P < 0.05$ compared to naive group, one way ANOVA followed by Tukey's multiple comparison test. SBP = Systolic blood pressure, PD = perindopril, MT = melatonin, DOX = doxorubicin.

Table 1: Effect on systolic blood pressure (SBP)

Rat groups	Naive	L-NAME				
		Untreated	DOX	DOX/PD	DOX/MT	DOX/PD/MT
SBP (mmhg) Day 0	119± 1.97	120± 3.52	118± 3.08	123± 3.08	123± 5.01	119± 6.91
SBP (mmhg) 5th week	121± 3.54	161± 10.2[a]	162± 14.8[a]	162± 9.36[a]	157± 7.48[a]	154± 9.81[a]
SBP (mmhg) 8th week	120± 2.79	163± 9.70[c]	111± 2.61[b]	105± 2.86[b]	125± 5.29[b]	106± 3.73[b]

Values are mean ± SD; n=6 per group. Two-ways ANOVA followed by Bonferroni's post-hoc
test for comparison to the corresponding group. [a]$P < 0.05$ compared to corresponding baseline
SBP, [b]$P < 0.05$ compared to corresponding 5th week SBP. [c]$P < 0.05$ compared to naive group,
one way ANOVA followed by Tukey's multiple comparison test. SBP = Systolic blood pressure,
PD = perindopril, MT = melatonin, DOX = doxorubicin.

Table 2(on next page)

Effect on cardiac percentage area of collagen fibers

Values are mean ± SD; n=6 per group. One-way ANOVA followed by Tukey's multiple comparison test. [a]P<0.05 compared to naive group, [b]P<0.05 compared to untreated subgroup, [c]P<0.05 compared to DOX subgroup, [d]P<0.05 compared to PD subgroup. MT = melatonin, PD = perindopril, DOX = doxorubicin

Table 2: Effect on cardiac percentage area of collagen fibers

Rat Groups	Naïve	L-NAME				
		Untreated	DOX	DOX/PD	DOX/MT	DOX/PD/MT
Percentage area of collagen fibers	2.21± 0.91	16.4± 4.35[a]	20.9± 4.95[b]	9.38± 2.51[c]	9.93± 2.70[c]	3.54± 1.14[c,d]

Values are mean ± SD; n=6 per group. One-way ANOVA followed by Tukey's multiple comparison test. [a]$P<0.05$ compared to naive group, [b]$P<0.05$ compared to untreated subgroup, [c]$P<0.05$ compared to DOX subgroup, [d]$P<0.05$ compared to PD subgroup. MT = melatonin, PD = perindopril, DOX = doxorubicin.