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Cardioprotective activity of melatonin and its novel synthesized derivatives on doxorubicin-induced cardiotoxicity

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Abstract—This study aimed at evaluation of melatonin and some of its novel synthesized derivatives **3**, **4**, **9** and **10b** as cardioprotective agents against doxorubicin-induced acute cardiac toxicity in rats. Also, this work was extended to study the potential role of each synthesized derivative in comparison with the parent compound melatonin. Intraperitoneal injection of a single dose (15 mg/kg B.W.) of doxorubicin resulted in significant increase in serum troponin I, leptin, triglycerides, cholesterol and LDL-cholesterol levels with concomitant decrease in serum T_3, T_4 and IL-1α levels. In contrast, intraperitoneal injection of melatonin or its synthesized derivatives especially compounds **3** and **10b** in a dose of 5 mg/kg B.W./day for 10 days reversed doxorubicin-induced changes in the above mentioned parameters towards the normal values. The present data revealed that doxorubicin exerts its action mainly through the oxidative stress. This appeared from the significant elevation in serum nitric oxide level and cardiac lipid peroxidation level with concomitant decrease in cardiac antioxidative enzymes activity. Treatment with melatonin and its derivatives **3** and **10b** could reduce the markers of oxidative stress and restore the activity of the antioxidative enzymes in the heart tissue. In conclusion, the cardioprotective effect of melatonin and its derivatives may be mediated through the antioxidant and free radical scavenging activity of these compounds.

1. Introduction

Doxorubicin (Dox), an anthracycline antibiotic, is a potent broad-spectrum chemotherapeutic agent that is effective against a wide range of human neoplasm.[1] However, the clinical uses of Dox have been restricted owing to its serious cardiotoxic effects.[2] A number of different mechanisms have been proposed for cardiotoxic effect of Dox, including mitochondrial dysfunction,[3] calcium overload,[4] inhibition of several membrane-bound molecules,[5] interaction with nucleic acid and nuclear components,[6] alteration of fatty acid oxidation that leads to the depression of energy metabolism in the cardiac tissue[7] and induction of apoptosis.[8] Moreover, increased oxidative stress and release of reactive oxygen radicals as well as antioxidative deficits have been suggested to play a major role in Dox-induced heart damage.[9,10]

Melatonin (*N*-acetyl-5-methoxytryptamine), the major hormone of the pineal gland, acts primarily via the hypothalamus to modulate the hypothalamic pituitary axis.[11] Recently, melatonin has shown to be a potent antioxidant and free radical scavenger.[12] It could control the peroxidative breakdown of lipids and reduce the oxidative damage.[13] Also, some of melatonin derivatives were studied for their free radical scavenging and antioxidant activity.[14] Regarding the effect of melatonin and its novel synthesized analogs on the antitumour efficacy of Dox, it was reported that melatonin itself does not interfere with the antitumour effect of Dox,[15] however, parallel studies have not been carried out with its new analogs.

In this study we synthesized new compounds using melatonin as a starting material and the antioxidative potential of melatonin and some of its novel synthesized derivatives against Dox-induced cardiac toxicity in rats was investigated.

2. Results and discussion

2.1. Chemistry

The synthesis of several melatonin analogs and melatonin derivatives, which exhibit marked medicinal

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activities has attracted the attention of many scientists.[14,16] The authors previously conducted work for the synthesis of 2-hydrazinomelatonin (**1**) by the reaction of 2-bromomelatonin with hydrazine hydrate in refluxing ethanolic triethylamine solution.[16] Compound (**1**) seemed to be a promising candidate for further chemical transformations. Thus, the condensation of compound (**1**) with equimolar amount of pentan-2,4-dione (acetyl acetone) **2** in boiling absolute ethanol yielded the corresponding 2-pyrazolylmelatonin derivative **3** (Scheme 1).

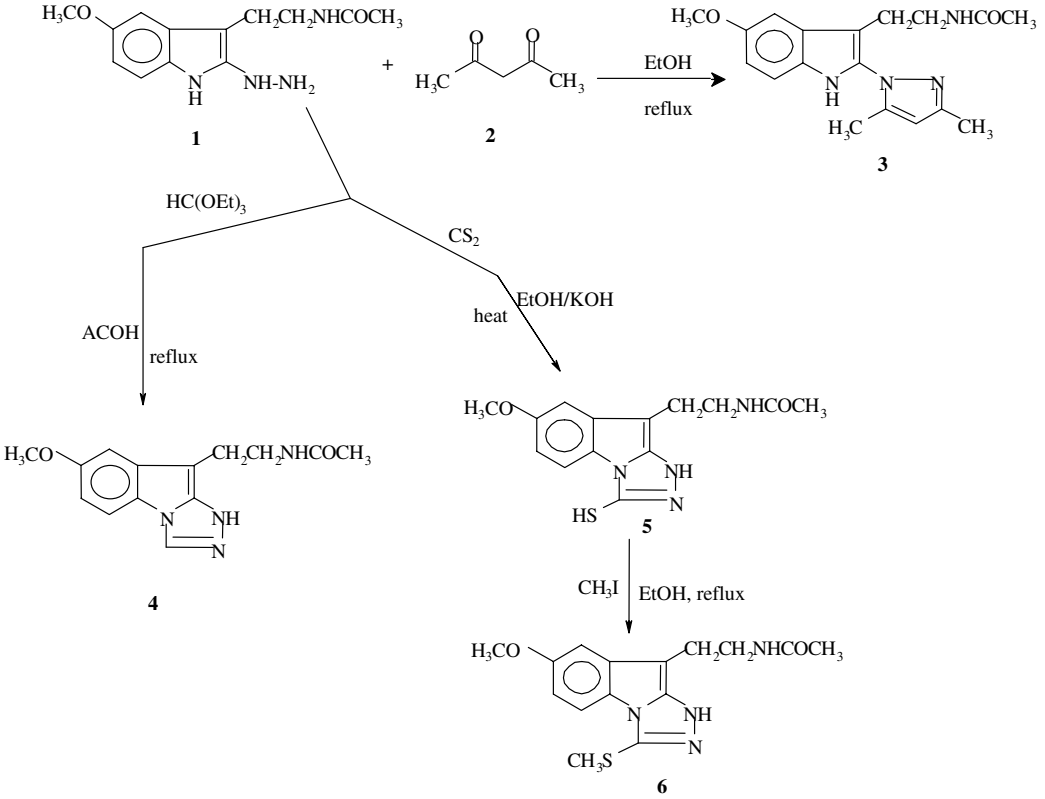
A wide range of biological activities have been attributed to fused triazoles.[17] Hence, the authors were interested to synthesize triazolomelatonin derivatives. Refluxing equimolar amounts of compound (**1**) and triethyl orthoformate in glacial acetic acid resulted in a chromatographically pure product. The triazolo[4,3-*a*]-indole derivative **4** is assigned to this product through elemental analysis and spectroscopic data (Scheme 1). Compound (**1**) also underwent cyclization when heated with carbon disulfide in ethanolic potassium hydroxide solution to yield the corresponding mercaptotriazolo[4,3-*a*]indole derivative **5**. Similar fused triazoles were also formed by the same method.[18] Confirmation for the structure of compound **5** was obtained by studying its reactivity towards the alkylating reagents. Therefore, compound **5** reacted with methyl iodide in ethanolic sodium ethoxide solution under reflux to form the corresponding methylthiotriazolo[4,3-*a*]indole derivative **6** (Scheme 1).

Melatonin (**7**) containing a diazotisable CH group at C-2[16] was coupled with *p*-methylbenzenediazonium chloride **8** in ethanolic sodium acetate solution at 0 °C to give the corresponding 2-methylphenylazomelatonin derivative **9** (Scheme 2). The ^{1}H NMR spectrum of compound **9** showed singlet signal at δ 2.25 ppm for the methyl group and a multiplet at δ 7.32–7.93 (7H) for the aromatic protons. Further confirmation for the structure of compound **9** was obtained by studying its reactivity towards some active methylene reagents to give fused heterocyclic derivatives with potential biological activities. Thus, the reaction of compound **9** with either malononitrile or ethyl cyanoacetate in refluxing ethanolic piperidine solution formed the corresponding aminotriazino[4,3-*a*]indole derivatives **10a**,**b**, respectively (Scheme 2).

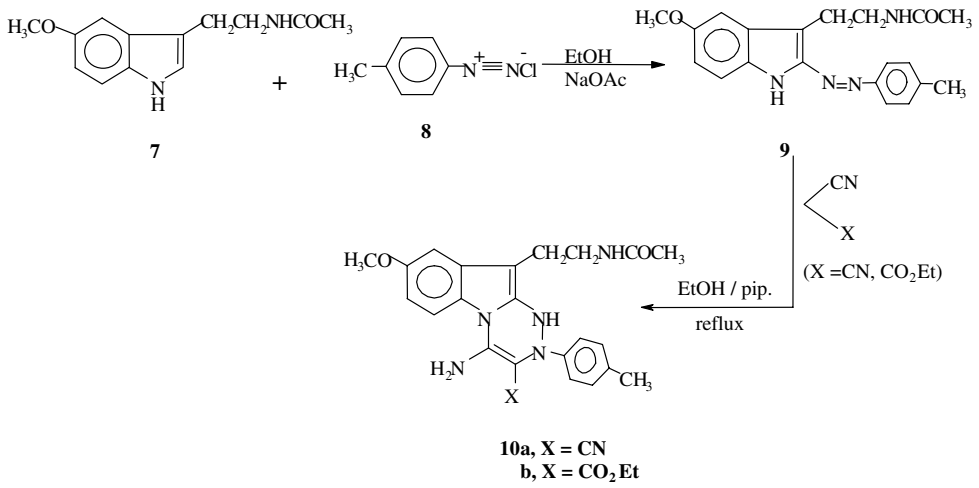
In a similar manner, the reaction of melatonin (**7**) with pyridindiazonium chloride **11** produced the pyridinylazomelatonin derivative **12** under the same experimental conditions. The reaction of compound **12** with either malononitrile or ethyl cyanoacetate in refluxing ethanolic piperidine solution formed the corresponding pyridinylaminotriazino[4,3-*a*]indole derivatives **13a**,**b**, respectively (Scheme 3). The structures of all the novel synthesized melatonin analogs were assigned based on their spectroscopic and elemental analysis data.

2.2. Bioassay

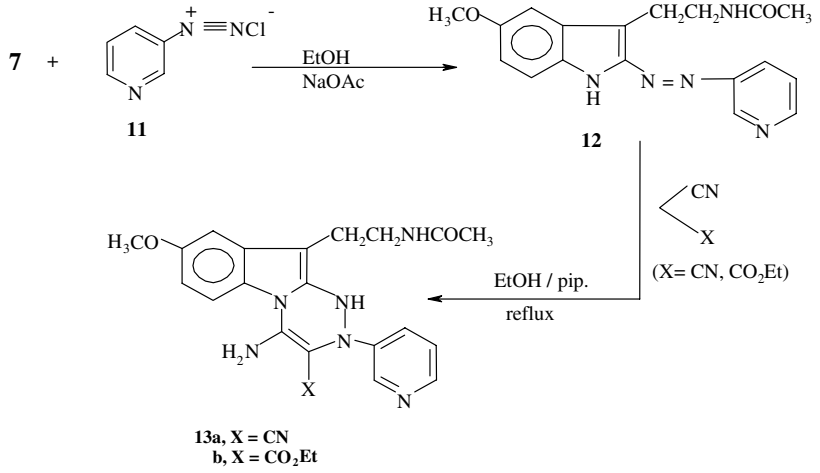
The in vivo potential cardioprotective effect of melatonin and four of its novel synthesized derivatives,



Scheme 1.



Scheme 2.



Scheme 3.

compounds **3**, **4**, **9** and **10b** against Dox-induced cardiac toxicity in rats was investigated. Cardiac damage was detected by measuring the end product of cardiac lipid peroxidation, the activity of cardiac antioxidative enzymes, serum troponin I and nitric oxide levels. Moreover, cardiotoxicity was further confirmed by measurement of some indices related to heart injury such as serum T_3, T_4, IL-1α, leptin and lipid profile.

2.2.1. Effect of drug administration on serum troponin I and thyroid hormones. Table 1 shows a significant variation of troponin I level among the six control groups (control group, melatonin, compound **4**, compound **9**, compound **3** and compound **10b** treated groups) ($F = 3.64$, $P = 0.01$) using ANOVA test. Troponin I has been shown to be a highly sensitive specific marker of myocardial cell injury.[19] Also Table 1 shows that compound **4** caused significant elevation in serum troponin I level as compared to the other five groups indicating the adverse effect of compound **4** on the heart, whereas T_3 and T_4 levels exhibited insignificant changes among the six groups of normal rats ($F = 0.77$, $P = 0.58$ and $F = 0.62$, $P = 0.68$, respectively). Troponin I and T_4 levels were significantly varied among the six groups of Dox-injected animals ($F = 2.55$, $P = 0.05$ and $F = 3.08$, $P = 0.03$, respectively), whereas T_3 exhibited insignificant change among those groups ($F = 0.38$, $P = 0.85$).

Independent Student's *t*-test revealed significant increase ($P < 0.05$) in troponin I and significant decrease in both T_3 and T_4 levels in Dox-injected group compared with the control group. In general, data in Table 1 show that compounds **3** and **10b** exhibited more protective action against Dox toxicity than compounds **4** and **9**. Hence, the data reported here demonstrated that Dox administration leads to cardiac injury as manifested by the high level of troponin I and low levels of thyroid hormones. These results are in agreement with those of Bertinchant et al.[19] and Van Vleet et al.[20] Dox may affect thyroid gland function via an oxidative stress mechanism, since Dox has been shown to be a potential source of reactive oxygen species (ROS) and free radicals.[21] It has been reported that ROS could inhibit iodine organification (a step of thyroid hormone formation) and its catalytic

Table 1. Effect of melatonin and its derivatives on serum troponin I and thyroid hormones levels in normal and Dox-treated rats

Groups	Troponin I (ng/mL)	T_3 (ng/mL)	T_4 (μg/dL)
Control	9.89 ± 0.24^{a}	0.440 ± 0.12	2.95 ± 0.35
Melatonin	10.48 ± 0.21^{a}	0.307 ± 0.004	2.75 ± 0.17
Compound **4**	11.59 ± 0.20^{b}	0.310 ± 0.02	2.68 ± 0.36
Compound **9**	10.38 ± 0.37^{a}	0.262 ± 0.03	2.07 ± 0.52
Compound **3**	10.10 ± 0.39^{a}	0.352 ± 0.05	2.60 ± 0.63
Compound **10b**	9.78 ± 0.52^{a}	0.242 ± 0.02	2.10 ± 0.14
F-ratio	3.64	0.77 NS	0.62 NS
P-value	0.01	0.58	0.68
Dox	$14.08 \pm 1.79^{*,b}$	$0.188 \pm 0.02^{*,a}$	$1.86 \pm 0.25^{*,a}$
Dox + melatonin	10.09 ± 0.32^{a}	0.237 ± 0.08^{ab}	2.57 ± 0.34^{ab}
Dox + **4**	11.41 ± 0.38^{ab}	0.237 ± 0.03^{ab}	2.28 ± 0.26^{ab}
Dox + **9**	13.10 ± 1.26^{ab}	0.203 ± 0.03^{ab}	2.31 ± 0.19^{ab}
Dox + **3**	10.94 ± 0.42^{a}	0.235 ± 0.06^{ab}	3.11 ± 0.30^{b}
Dox + **10b**	10.97 ± 0.30^{a}	0.260 ± 0.03^{b}	3.12 ± 0.33^{b}
F-ratio	2.55	0.38 NS	3.08
P-value	0.05	0.85	0.03

The different letters indicate statistically significant means (Duncan's multiple range test) at $P < 0.05$.
NS, non-significant.
* Significant difference between Dox group and the control group at $P < 0.05$ using Student *t*-test.

enzyme, thyroid peroxidase.[22] Therefore, decreased serum T_3 level was commonly observed in patients suffering from severe heart failure.[23] T_3 was considered to improve the myocyte contractile process through a cyclic adenosine monophosphate (c-AMP)-induced mechanism. Furthermore, T_3 potentiates the effects of β-adrenergic-receptor stimulation transduction by increasing c-AMP production, Ca^{2+} channel current and Ca^{2+} availability to the myocyte contractile apparatus.[24]

Melatonin is known to be a highly protective agent against chemotherapeutic drugs-induced cardiotoxicity.[25] This finding could be confirmed, in the current study, by the significant reduction in serum troponin I in melatonin-treated group as compared with Dox-injected group (Table 1). In this study, we postulate that compounds **3** and **10b** may protect the thyroid gland against extensive oxidative damage caused by Dox on thyroid hormones synthesis via their highly antioxidant activity.

2.2.2. Effect of drug administration on lipid profile and leptin. Triglycerides (TG), cholesterol, low density lipoprotein cholesterol (LDL-ch) and leptin showed no significant variation among the six groups of normal rats (Table 2). However, compound **10b**-treated group showed a significant decrease in LDL-ch in relation to control, melatonin and compound **4** groups. Table 2 shows also significant variations of leptin and TG among the six groups of Dox-injected rats while choles-

Table 2. Effect of melatonin and its derivatives on the levels of triglycerides, cholesterol and LDL-ch and leptin in normal and Dox-treated rats

Groups	Triglycerides (mg/dL)	Cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	Leptin (ng/mL)
Control	101.87 ± 8.2^{ab}	60.94 ± 5.17	24.99 ± 3.80^{a}	13.06 ± 0.11
Melatonin	106.25 ± 14.20^{a}	65.26 ± 7.83	24.89 ± 3.85^{a}	13.83 ± 0.67
Compound **4**	105.42 ± 5.59^{ab}	66.28 ± 6.41	25.43 ± 5.53^{a}	13.47 ± 0.27
Compound **9**	92.15 ± 12.87^{ab}	61.42 ± 7.31	20.05 ± 2.72^{ab}	12.58 ± 0.21
Compound **3**	88.15 ± 6.40^{ab}	67.44 ± 3.08	18.83 ± 1.64^{ab}	12.76 ± 0.41
Compound **10b**	89.40 ± 6.07^{ab}	58.68 ± 3.94	13.83 ± 0.70^{b}	13.06 ± 0.41
F-ratio	1.83 NS	0.348 NS	1.83 NS	1.35 NS
P-value	0.15	0.878	0.15	0.28
Dox	$119.86 \pm 2.77^{*,c}$	$106.87 \pm 21.45^{*,b}$	$51.79 \pm 3.32^{*,a}$	$14.84 \pm 0.81^{*,b}$
Dox + melatonin	83.26 ± 4.23^{a}	83.92 ± 7.13^{ab}	44.24 ± 6.23^{ab}	12.58 ± 0.27^{a}
Dox + **4**	99.46 ± 6.72^{ab}	88.60 ± 6.99^{ab}	47.26 ± 4.32^{ab}	13.59 ± 0.15^{ab}
Dox + **9**	102.92 ± 8.60^{b}	75.37 ± 9.36^{ab}	46.25 ± 7.10^{ab}	13.83 ± 0.15^{ab}
Dox + **3**	95.60 ± 5.66^{ab}	76.12 ± 10.38^{ab}	41.41 ± 4.37^{ab}	13.59 ± 0.30^{ab}
Dox + **10b**	84.40 ± 3.92^{a}	68.30 ± 1.43^{a}	32.17 ± 4.24^{b}	12.94 ± 0.11^{a}
F-ratio	5.59	1.46 NS	1.69 NS	4.13
P-value	0.001	0.25	0.18	0.01

The different letters indicate statistically significant means (Duncan's multiple range test) at $P < 0.05$.
NS, non-significant.
* Significant difference between Dox group and the control group at $P < 0.05$ using Student *t*-test.

terol and LDL-ch did not differ significantly among these groups. Dox treatment also resulted in significant increase in both leptin and triglycerides levels compared with the control group. Both Dox + melatonin and Dox + **10b** groups exhibited significant reduction in leptin level compared to the group injected with Dox alone. Regarding the triglycerides level, melatonin and its derivatives produced significant reduction in serum triglycerides level compared to Dox-treated group. Furthermore, compound **10b** could significantly decrease the elevation of cholesterol and LDL-ch caused by Dox injection. The increased levels of triglycerides, cholesterol and LDL-ch induced by Dox in our study are consistent with previous results.[21,26] In Dox-treated animals lipoprotein lipase (LPL), an insulin-activated enzyme mainly responsible for plasma triglycerides clearance, is decreased[21] resulting in increased plasma levels of very low density lipoprotein triglycerides,[27] which is a risk factor in atherorsclerosis. Extrahepatic LPL, such as in the heart, is involved in the uptake of triglycerides rich lipoproteins from the circulation.[28]

In our study, both melatonin and compound **10b** administration decreased the serum triglycerides level. These results suggest that lipoprotein lipase might be restored by melatonin and **10b**. Our results concerned with melatonin coincide with the finding of Nishida et al.[29] Melatonin and compound **10b** also suppressed the high level of serum cholesterol as well as serum LDL-ch in Dox-treated animals. Melatonin results are in agreement with the previous reports.[30] The lowering action of melatonin on cholesterol resulted in a significant decrease in LDL-ch in Dox-treated animals. These data suggest that melatonin and compound **10b** may enhance hepatic clearance of serum LDL-ch. Dox has been reported to inhibit lecithin cholesterol acyl transferase (LCAT).[21] Melatonin and compound **10b** may also increase the activity of LCAT, which is inhibited by Dox-treatment. LCAT has been reported to be synthesized in the liver and secreted into the blood where it is responsible for the synthesis of the major portion of cholesterol ester.[31]

Concerning leptin, the data in the present study showed a significant elevation in serum leptin level associated with high levels of serum triglycerides, cholesterol and LDL-ch levels in Dox-treated group. A study of Meisel et al.[32] suggested that leptin may be involved in the acute response to oxidative stress. The results of Tamer et al.[33] revealed a significant positive correlation between leptin level and the concentration of both cholesterol and LDL-ch in patients with myocardial infarction. A study of Wallace et al.[34] showed that the plasma leptin level can predict the risk of coronary heart disease (CHD) and they interpreted this finding on the basis of the known relationship between leptin and C-reactive protein, a marker of inflammation and a predictor of CHD risk. Another interpretation visualizes high levels of leptin and plasma triglycerides as an established risk factor of coronary events.[35]

Administration of melatonin or compound **10b** could modulate the level of serum leptin that was increased by Dox. These results are in analogy with previous studies, which demonstrated the suppression of plasma leptin by melatonin administration in middle aged male rats.[36]

2.2.3. Effect of drug administration on oxidant–antioxidant status. Table 3a shows that serum IL-1α exhibited insignificant change among the six control groups ($F = 1.81$, $P = 0.16$) while in Dox-treated groups, IL-1α exhibited significant change ($F = 4.69$, $P = 0.005$). Also, Dox treatment decreased the level of IL-1α (Table 3a) indicating immunosuppressive effect of this drug. Several evidences revealed the lymphoid toxicity by Dox. Dox was found to induce neutropenia by its suppressive effects on myeloid progenitor cells (granulocyte/monocyte colony forming units)[37] and to produce

Table 3a. Effect of melatonin and its derivatives on the oxidant status in control and Dox-treated rats

Groups	IL-1α (ng/mL)	MDA (nmol/g tissue)	NO (μmol/L)
Control	2.34 ± 0.43	14.83 ± 0.81^{b}	48.95 ± 2.49
Melatonin	3.92 ± 0.73	12.58 ± 0.27^{a}	48.70 ± 4.64
Compound **4**	2.99 ± 0.10	13.59 ± 0.15^{ab}	48.60 ± 4.03
Compound **9**	2.22 ± 0.99	13.83 ± 0.15^{ab}	57.20 ± 7.82
Compound **3**	2.14 ± 0.68	13.59 ± 0.30^{ab}	50.90 ± 3.41
Compound **10b**	2.58 ± 0.02	12.94 ± 0.11^{a}	47.25 ± 3.80
F-ratio	1.81 NS	4.13	0.58 NS
P-value	0.16	0.01	0.71
Dox	1.65 ± 0.03^{a}	25.21 ± 3.82^{*,b}	79.90 ± 11.64*
Dox + melatonin	1.97 ± 0.27^{ab}	20.05 ± 2.73^{ab}	57.40 ± 4.91
Dox + **4**	1.49 ± 0.10^{a}	25.42 ± 5.54^{b}	68.90 ± 14.64
Dox + **9**	1.73 ± 0.04^{a}	24.90 ± 3.84^{b}	65.77 ± 8.66
Dox + **3**	1.68 ± 0.05^{a}	18.82 ± 1.62^{ab}	67.36 ± 13.72
Dox + **10b**	2.96 ± 0.50^{b}	13.83 ± 0.72^{a}	66.40 ± 11.99
F-ratio	4.96	1.84 NS	0.35 NS
P-value	0.005	0.15	0.87

The different letters indicate statistically significant means (Duncan's multiple range test) at $P < 0.05$.
NS, non-significant.
* Significant difference between Dox group and the control group at $P < 0.05$ using Student *t*-test.

atrophy of thymus and secondary lymphoid organs in mice.[38] IL-1Ra, a cytokine produced during inflammatory responses, but has no IL-1 activity, prevents binding of IL-1α and IL-1β to their receptors in order to modulate IL-1 activity.[39] Recently, IL-1Ra is used as a sensitive marker of clinical instability in patients with coronary artery diseases.[40]

In the present study, administration of either melatonin or compound **10b** to Dox-injected rats revealed a protective action against lymphoid organ toxicity as indicated by the improvement of IL-1α secretion. The melatonin results are in analogy with those of Pioli et al.,[41] which revealed that melatonin could restore the impaired T-helper cell activity in immunodepressed mice through enhancing antigen presentation by splenic macrophages to T-cells and increase the expression of MHC class II molecules and production of IL-1α.

Table 3a also shows significant variations of cardiac malondialdehyde (MDA) ($F = 4.13$, $P = 0.01$) among the six examined control groups, while serum nitric oxide (NO) did not significantly differ among these groups. The data show also significant decrease in the level of MDA in both melatonin and compound **10b** groups compared with the control group. A significant reduction in MDA was observed in Dox + **10b** group compared with Dox, Dox + **4** and Dox + **9** groups. Compared to the control group, both cardiac MDA and serum NO exhibited significant increase ($P < 0.05$) in rats injected with Dox alone.

Table 3b shows that cardiac superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase activities did not significantly differ among the six control groups. Among the six Dox-injected groups SOD only exhibited significant variation. Table 3b shows also significant reduction in SOD, GPX and catalase activities in Dox-injected group compared with the control group. The increased formation of cardiac MDA and serum NO, and the decreased activity of cardiac antioxidant enzymes (SOD, GPX and catalase) on Dox injection asserted that Dox-induced oxidative damage in the heart tissue. The significant increase in cardiac MDA and serum NO levels in Dox-injected rats coincides with the previous study of Naidu et al.[42] and Pacher et al.[43], respectively. Also, the significant inhibition of GPX, SOD and catalase activities by Dox was supported by the finding of Dziegiel et al.[44] The generation of peroxynitrite is also reported to be involved in the Dox-induced oxidative process.[45] Peroxynitrite is a reactive oxidant produced from nitric oxide.[43] NO is a free radical formed during the oxidation of L-arginine to L-citrulline in a reaction catalyzed by NO synthase.[46] NO and its oxidation product, peroxynitrite, contribute to the injury process by directly damaging the tissue or by initiating additional immunologic reactions that result in damage.[47] In the present study the increased level of NO in Dox-injected rats may be attributed to the stimulation of the inducible nitric oxide synthase expression activity, which is responsible for NO production. The increased oxidative stress on cardiac tissue results in exhaustion of the antioxidant enzymes that are responsible to scavenge the liberated toxic free radicals. Therefore, the decrease of the cardiac antioxidant enzyme activities (SOD, GPX and catalase) was observed in Dox-injected rats (Table 3b).

Melatonin could reduce cardiac MDA (Table 3a) and this is consistent with Mayo et al. results.[48] The mechanism by which melatonin inhibits lipid peroxidation probably involves the direct scavenging of the reactive free radicals, especially OH and ONOO independent or associated with the inhibition of lipid peroxidation.[49] Melatonin could distribute readily in all subcellular compartments due to its solubility in both water and lipids. As such, it can pass through the cell membrane easily and enter cardiac cells to remove free radicals and

Table 3b. Effect of melatonin and its derivatives on antioxidant status in control and Dox-treated rats

Groups	SOD (u/mg protein)	GPX (u/mg protein)	Catalase (u/mg protein)
Control	0.89 ± 0.04	55.17 ± 10.28^{ab}	2.36 ± 0.08^{a}
Melatonin	0.80 ± 0.07	54.00 ± 5.35^{ab}	2.53 ± 0.64^{ab}
Compound **4**	0.82 ± 0.05	45.72 ± 7.09^{a}	1.66 ± 0.65^{a}
Compound **9**	0.84 ± 0.10	45.10 ± 4.57^{a}	1.78 ± 0.32^{a}
Compound **3**	0.88 ± 0.03	74.97 ± 10.22^{b}	2.44 ± 0.96^{a}
Compound **10b**	0.87 ± 0.14	74.60 ± 12.03^{b}	3.66 ± 0.15^{b}
F-ratio	0.22 NS	2.38 NS	1.02 NS
P-value	0.94	0.08	0.43
Dox	$0.382 \pm 0.06^{*,b}$	$32.17 \pm 4.24^{*,a}$	$0.59 \pm 0.06^{*,a}$
Dox + melatonin	0.657 ± 0.09^{a}	47.25 ± 4.31^{ab}	1.66 ± 0.70^{ab}
Dox + **4**	0.375 ± 0.03^{b}	44.25 ± 6.22^{ab}	1.18 ± 0.50^{ab}
Dox + **9**	0.507 ± 0.07^{ab}	41.42 ± 4.38^{ab}	1.21 ± 0.42^{ab}
Dox + **3**	0.515 ± 0.04^{ab}	46.25 ± 7.11^{ab}	2.75 ± 0.29^{b}
Dox + **10b**	0.830 ± 0.02^{c}	51.70 ± 3.31^{b}	2.04 ± 0.39^{b}
F-ratio	9.09	1.7 NS	1.02 NS
P-value	0.001	0.18	0.43

The different letters indicate statistically significant means (Duncan's multiple range test) at $P < 0.05$.
NS, non-significant.
* Significant difference between Dox group and the control group at $P < 0.05$ using Student *t*-test.

limits breakdown of membrane lipids,[50] proteins in the cytosol, and DNA in the nucleus.[51] In addition to its antioxidant activity, melatonin may also exert its effects via melatonin 1 receptor in the heart.[50]

Melatonin could decrease NO level (Table 3a) and this may be attributed to the potential inhibitory effect of melatonin on nitric oxide synthase activity[52] by suppressing the activity of nuclear factor (NF) kappa β in macrophage, which regulates the expression of nitric oxide synthase (iNOS) gene.[53] Both melatonin and compound **10b** could stimulate the activity of the cardiac antioxidant enzymes SOD, GPX and catalase (Table 3b). These results asserted the antioxidant properties of melatonin.[54]

2.2.4. Summary. From the results obtained in the present study, it can be stated that, melatonin and its novel derivatives **3**, **4**, **9** and **10b** showed antioxidant activities with various intensities depending on the structure of each compound. Compound **10b** has the strongest antioxidant activity, which exceeds that of the parent reference, melatonin, followed by compound **3** then **9**. Since the damaging impact of Dox on cardiac cells is dependant mainly on the peroxidative decomposition of structural membrane lipids,[42] the tested compounds **3**, **9** and **10b** probably act as scavengers of free radicals.

The indole moiety of the melatonin molecule is the reactive centre of interaction with oxidants due to its high resonance stability and very low activation energy barrier towards the free radical reactions. However, the methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity.[55]

In addition to the indole moiety, the methoxy and amide side chains of the parent melatonin molecule, the pyrazole side chain at C-2 in compound **3**, the 4-methyl phenyl side chain at C-2 in compound **9** and the fused triazine moiety of compound **10b**, which contain amino and methyl phenyl side chains, may be responsible for the antioxidant effect of these derivatives. This suggestion is supported by previous reports, which demonstrated the powerful antioxidant and free radical scavenging properties of some, pyrazoles,[56,57] compounds containing the substituted phenyl and amino side chains,[58,59] and triazines.[57]

In conclusion, the present study has demonstrated that melatonin and its derivatives **3** and **10b** could exhibit pronounced antioxidative and protective activities. These encouraging results warrant further studies in this direction with the possibility to formulate a potent cardioprotective prescription against cardiotoxicity caused by chemotherapeutic drugs.

3. Experimental

3.1. Synthesis

The appropriate precautions in handling moisture-sensitive compounds were undertaken. All melting points were uncorrected, the IR spectra expressed in cm^{-1} and recorded in KBr pellets on a Pa-9721 IR spectrometer. ^1H and ^{13}C NMR spectra were obtained on a Varian EM-390 90 MHz spectrometer in DMSO-d_6 as solvent and TMS as internal reference. Chemical shifts (δ) are expressed in ppm. Mass spectra were recorded on Kratos (75 eV) MS equipment. Elemental analyses were carried out by Microanalytical Data Unit at The National Research Centre, Giza, Egypt. All the elemental analyses results were in a range of ±0.35%.

3.1.1. *N*-[2-(2-(3,5-Dimethylpyrazol-1-yl)-5-methoxyindol-3-yl)ethyl]acetamide (3). A mixture of compound (**1**) (1.31 g, 0.005 mol) and acetyl acetone **2** (0.5 g, 0.005 mol) in absolute ethanol (30 mL) was boiled under reflux for 4 h until all starting materials had disappeared as indicated by TLC. Then the reaction mixture was concentrated under vacuum, whereby the resulted oily product was triturated with ethyl acetate. The solid product so formed was filtered off, dried and crystallized from dioxane. Pale brown crystals, yield 1.32 g (76%), mp 198–199 °C; $C_{18}H_{22}N_4O_2$ (326.402), IR (υ/cm^{-1}): 3448–3380 (2NH), 3050 (CH– aromatic), 2975 (CH_3), 2860 (CH_2), 1705 (C=O). ^1H NMR (δ ppm): 1.77 (s, 3H, $COCH_3$), 2.15 (s, 6H, $2CH_3$), 3.05 (t, 2H, CH_2), 3.37 (t, 2H, CH_2), 3.82 (s, 3H, OCH_3), 6.75 (s, 1H, pyrazole H-4), 7.32–7.80 (m, 3H, aromatic-H), 10.12 (s, 1H, 1NH, D_2O-exchangeable). ^{13}C NMR (δ): 22.6 ($CO\mathit{C}H_3$), 26.3, 26.8 ($2CH_3$), 24.5, 39.2 ($2CH_2$), 55.9 (OCH_3), 131.6 (C-2), 111.8 (C-3), 100.3 (C-4), 152.9 (C-5), 110.7 (C-6), 109.6 (C-7), 131.5, 128.7 (fused-C), 133.2, 106.3, 133.7 (pyrazole–C), 169.5 (C=O). MS (*m*/*z*, %): 326 ($M^{+\bullet}$, 55%), 231 [$M^{+\bullet}-C_5H_7N_2$ (dimethylpyrazole fragment), 40%], 95 ($C_5H_7N_2$, 34%).

3.1.2. *N*-[2-(6-Methoxy-1,2,4-triazolo[4,3-*a*]indol-4-yl)ethyl]acetamide (4). Equimolar amounts of compound (**1**) (1.31 g, 0.005 mol) and triethyl orthoformate (0.74 g, 0.005 mol) in glacial acetic acid (35 mL) were boiled under reflux for 3 h. The reaction mixture was concentrated under vacuum, cooled at room temperature, poured onto ice. The solid product so formed was filtered off, dried and crystallized from ethanol. Brown powder, yield 0.97 g (72%), mp 175–176 °C; $C_{14}H_{16}N_4O_2$ (272.311). IR (υ/cm^{-1}): 3440–3387 (2NH), 3035 (CH– aromatic), 2970 (CH_3), 2860 (CH_2), 1710 (C=O). ^1H NMR (δ ppm): 1.82 (s, 3H, $COCH_3$), 2.95 (t, 2H, CH_2), 3.36 (t, 2H, CH_2), 3.85 (s, 3H, OCH_3), 6.65 (s, 1H, pyrazole H-1), 7.54–7.92 (m, 3H, aromatic-H), 9.92, 10.22 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 272 ($M^{+\bullet}$, 35%), 241 ($M^{+\bullet}-OCH_3$, 42%), 214 ($M^{+\bullet}-NHCOCH_3$, 57%).

3.1.3. *N*-[2-(1-Mercapto-6-methoxy-1,2,4-triazolo[4,3-*a*]-indol-4-yl)ethyl]acetamide (5). To a mixture of compound **1** (1 g) and carbon disulfide (5 mL) in ethanol (30 mL), potassium hydroxide (0.5 g) was added. The reaction mixture was heated under reflux for 6 h, cooled at room temperature, diluted with water and acidified with dilute hydrochloric acid. The formed solid product was filtered off, dried and crystallized from methanol. Yellow crystals, yield 0.85 g (70%), mp 205–207 °C; $C_{14}H_{16}N_4SO_2$ (304.372). IR (υ/cm^{-1}): 3420–3360

(2NH), 3045 (CH– aromatic), 2977 (CH_3), 2865 (CH_2), 2590 (SH), 1710 (C=O). ^{1}H NMR (δ ppm): 1.84 (s, 3H, $COCH_3$), 3.04 (t, 2H, CH_2), 3.34 (t, 2H, CH_2), 3.83 (s, 3H, OCH_3), 3.89 (s, 1H, SH, D_2O-exchangeable), 7.63–7.95 (m, 3H, aromatic-H), 9.64, 10.32 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 304 ($M^{+\bullet}$, 27%), 287 ($M^{+\bullet}-SH$, 40%), 246 ($M^{+\bullet}-NHCOCH_3$, 68%).

3.1.4. *N*-[2-(1-Methylthio-6-methoxy-1,2,4-triazolo[4,3-*a*]-indol-4-yl)ethyl]acetamide (6). To ethanolic sodium ethoxide solution [prepared by dissolving sodium metal (0.5 g) in 30 mL absolute ethanol] compound **5** (1.52 g, 0.005 mol) was added and the solution was heated under reflux for 15 min. Then, methyl iodide (0.70 g, 0.005 mol) was added and the refluxing was continued for additional 3 h. The reaction mixture was then cooled at room temperature, poured onto ice and neutralized with dilute hydrochloric acid. The formed solid product was filtered off, dried and crystallized from DMF. Pale brown crystals, yield 1.22 g (77%), mp 225–226 °C; $C_{15}H_{18}N_4SO_2$ (318.402). IR (υ/cm^{-1}): 3430–3365 (2NH), 3035 (CH– aromatic), 2975 (CH_3), 2870 (CH_2), 1715 (C=O). ^{1}H NMR (δ ppm): 1.85(s, 3H, $COCH_3$), 2.43 (s, 3H, SCH_3), 2.98 (t, 2H, CH_2), 3.35 (t, 2H, CH_2), 3.80 (s, 3H, OCH_3), 7.71–7.98 (m, 3H, aromatic-H), 9.60, 10.17 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 318 ($M^{+\bullet}$, 62%), 271 ($M^{+\bullet}-SCH_3$, 37%).

3.1.5. *N*-[2-(5-Methoxy-2-*p*-methylphenylazoindol-3-yl)-ethyl]acetamide (9), *N*-[2-(5-methoxy-2-pyridin-3-ylazo-indol-3-yl)ethyl]acetamide (12). *General procedure:* a solution of melatonin (1.16 g, 0.005 mol) in ethanol (30 mL) containing sodium acetate (0.5 g) was cooled to 0–5 °C, then treated gradually with a cold solution of either *p*-methylbenzenediazonium chloride **8** or pyridindiazonium chloride **11** [either compound **8** or **11** was prepared by the addition of sodium nitrite solution to appropriate quantities of hydrochloric acid and *p*-toludine or 3-aminopyridine, respectively]. After the addition of the diazonium salt, the reaction mixture was stirred at room temperature for 30 min. The precipitated product was separated upon dilution with cold water, filtered off, washed several times with water, dried and crystallized.

Compound **9**: red crystals from EtOH/H_2O mixture (7:3 v/v), yield 1.38 g (79%), mp 188 °C; $C_{20}H_{22}N_4O_2$ (350.422). IR (υ/cm^{-1}): 3455–3389 (NH), 3050 (CH– aromatic), 2970 (CH_3), 2865 (CH_2), 1698 (C=O). ^{1}H NMR (δ ppm): 1.79 (s, 3H, $COCH_3$), 2.25 (s, 3H, CH_3), 2.89 (t, 2H, CH_2), 3.40 (t, 2H, CH_2), 3.82 (s, 3H, OCH_3), 7.32–7.93 (m, 7H, aromatic-H), 9.82 (s, 1H, NH, D_2O-exchangeable). MS (*m*/*z*, %): 350 ($M^{+\bullet}$, 48%), 231 ($M^{+\bullet}-C_7H_7N_2$, 46%).

Compound **12**: buff crystals from EtOH, yield 1.17 g (70%), mp 233–235 °C; $C_{18}H_{19}N_5O_2$ (337.382). IR (υ/cm^{-1}): 3445–3380 (NH), 3045 (CH– aromatic), 2985 (CH_3), 2875 (CH_2), 1710 (C=O). ^{1}H NMR (δ ppm): 1.92 (s, 3H, $COCH_3$), 2.80 (t, 2H, CH_2), 3.55 (t, 2H, CH_2), 3.80 (s, 3H, OCH_3), 7.52–8.50 (m, 7H, aromatic and pyridine–H), 9.98 (s, 1H, NH, D_2O-exchangeable). MS (*m*/*z*, %): 337 ($M^{+\bullet}$, 54%), 231 ($M^{+\bullet}-C_5H_4N_3$, 23%).

3.1.6. General method for preparation of compounds 10a,b and 13a,b. To a solution of compound **9** (0.7 g, 0.002 mol) or compound **12** (0.67 g, 0.002 mol) in ethanol (35 mL) containing an amount of catalytic piperidine (2 mL) either malononitrile (0.13 g, 0.002 mol) or ethyl cyanoacetate (0.23 g, 0.002 mol) was added. The reaction mixture, in each case, was heated under reflux for 4 h until all starting materials had disappeared as indicated by TLC, and then left to cool at room temperature, poured over ice/water mixture and neutralized with dilute hydrochloric acid. The formed solid product, in each case, was filtered off, dried and crystallized from the appropriate solvent.

3.1.6.1. *N*-[2-(1-Amino-2-cyano-7-methoxy-3-*p*-methylphenyl-1,2,4-triazino[4,3-*a*]indol-5-yl)ethyl]acetamide (10a). Brown crystals, from EtOH, yield 0.56 g (68%), mp 191–192 °C; $C_{23}H_{24}N_6O_2$ (416.482). IR (υ/cm^{-1}): 3385–3290 (NH_2, 2NH), 3030 (CH– aromatic), 2975 (CH_3), 2853 (CH_2), 2225 (CN), 1695 (C=O). ^{1}H NMR (δ ppm): 1.73 (s, 3H, $COCH_3$), 2.32 (s, 3H, CH_3), 3.04 (t, 2H, CH_2), 3.45 (t, 2H, CH_2), 3.79 (s, 3H, OCH_3), 6.12 (s, 2H, NH_2, D_2O-exchangeable), 7.45–8.05 (m, 7H, aromatic-H), 8.89, 9.52 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 417 ($M^{+\bullet}$+1, 55%), 325 [$M^{+\bullet}-(C_6H_4-CH_3)$, 71%].

3.1.6.2. *N*-[2-(1-Amino-2-ethoxycarbonyl-7-methoxy-3-*p*-methylphenyl-1,2,4-triazino[4,3-*a*]indol-5-yl)ethyl]-acetamide (10b). Reddish brown crystals, from DMF, yield 0.64 g (70%), mp 155–156 °C; $C_{25}H_{29}N_5O_4$ (463.543). IR (υ/cm^{-1}): 3420–3290 (NH_2, 2NH), 3040 (CH– aromatic), 2975 (CH_3), 2869 (CH_2), 1735, 1698 (2C=O, ester C=O, $COCH_3$). ^{1}H NMR (δ ppm): 1.13 (t, 3H, ester CH_3), 1.79 (s, 3H, $COCH_3$), 2.30 (s, 3H, CH_3), 2.95 (t, 2H, CH_2), 3.40 (t, 2H, CH_2), 3.85 (s, 3H, OCH_3), 4.25 (q, 2H, ester CH_2), 6.42 (s, 2H, NH_2, D_2O-exchangeable), 7.32–7.90 (m, 7H, aromatic-H), 9.71, 10.12 (2s, 2H, 2NH, D_2O-exchangeable). ^{13}C NMR (δ): 14.9 (CH_3, ester group), 20.2 (CH_2, ester group), 20.8 ($C_6H_4-\mathit{C}H_3$), 22.8 ($CO\mathit{C}H_3$), 24.8, 40.6 ($2CH_2$), 55.4 (OCH_3), 132.3 (C-2), 110.5 (C-3), 111.3 (C-4), 152.3 (C-5), 110.9 (C-6), 110.2 (C-7), 133.0, 129.1 (fused aromatic-C), 127.2 (*C*–CO_2Et), 169.5, 173.2 (2C=O), 150.3, 112.2, 112.8, 129.0, 129.5, 130.2 (phenyl–C). MS (*m*/*z*, %): 463 ($M^{+\bullet}$, 42%), 372 [$M^{+\bullet}-(C_6H_4-CH_3)$, 54%].

3.1.6.3. *N*-[2-(1-Amino-2-cyano-7-methoxy-3-pyridin-3-yl-1,2,4-triazino[4,3-*a*]indol-5-yl)ethyl]acetamide (13a). Brown powder, from EtOH, yield 0.53 g (66%), mp 225–227 °C; $C_{21}H_{21}N_7O_2$ (403.442). IR (υ/cm^{-1}): 3435–3295 (NH_2, 2NH), 3035 (CH– aromatic), 2970 (CH_3), 2855 (CH_2), 2220 (CN), 1695 (C=O). ^{1}H NMR (δ ppm): 1.80 (s, 3H, $COCH_3$), 2.93 (t, 2H, CH_2), 3.38 (t, 2H, CH_2), 3.85 (s, 3H, OCH_3), 6.32 (s, 2H, NH_2, D_2O-exchangeable), 7.45–8.43 (m, 7H, aromatic and pyridine–H), 8.94, 9.25 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 404 ($M^{+\bullet}$, 35%), 326 ($M^{+\bullet}-C_5H_4N$, 76%), 78 (C_5H_4N, 100%).

3.1.6.4. *N*-[2-(1-Amino-2-ethoxycarbonyl-7-methoxy-3-pyridin-3-yl-1,2,4-triazino[4,3-*a*]indol-5-yl)ethyl]acetamide (13b). Yellow crystals, from MeOH, yield 0.64 g (72%), mp 201–202 °C; $C_{23}H_{26}N_6O_4$ (450.502). IR (υ/cm^{-1}): 3430–3290 (NH_2, 2NH), 3040 (CH– aromatic), 2975 (CH_3), 2873 (CH_2), 1730, 1705 (2C=O, ester C=O, $COCH_3$). ^{1}H NMR (δ ppm): 1.15 (t, 3H, ester CH_3), 1.82 (s, 3H, $COCH_3$), 3.02 (t, 2H, CH_2), 3.40 (t, 2H, CH_2), 3.83 (s, 3H, OCH_3), 4.25 (q, 2H, ester CH_2), 5.92 (s, 2H, NH_2, D_2O-exchangeable), 7.42–8.53 (m, 7H, aromatic and pyridine–H), 8.93, 9.62 (2s, 2H, 2NH, D_2O-exchangeable). MS (*m*/*z*, %): 451 ($M^{+\bullet}$, 45%), 373 ($M^{+\bullet}-C_5H_4N$, 64%), 78 (C_5H_4N, 100%).

3.2. Bioassay

Adriamycin, doxorubicin hydrochloride (Dox), was purchased from Pharmacia of Upjohn S.P.A. Research Laboratories. Male Sprague-Dawley rats (72 rats), weighing 200–250 g, were supplied by the Animal House at National Research Centre. Animals were maintained under 12/12 h light/dark cycle at 20 ± 1 and fed with standard laboratory diet and water ad libitum.

3.2.1. Experimental design. The animals were randomly assigned to 12 groups (each of six rats) as follows: control, melatonin and each one of its derivatives **3**, **4**, **9** and **10b**, Dox, Dox + melatonin, Dox + **3**, Dox + **4**, Dox + **9** and Dox + **10b**. A single dose of Dox (15 mg/kg B.W.) dissolved in water was injected intraperitoneally.[60] Melatonin and its derivatives were dissolved in 2% Tween and injected intraperitoneally in a dose of 5 mg/kg B.W./day[61] for 10 consecutive days starting 3 days prior to Dox injection. Normal healthy animals received melatonin or each of its derivatives alone (5 mg/kg B.W./day) for 10 consecutive days so as to confirm the safety of melatonin and its derivatives.

3.2.2. Sample collection. At the end of the experimental period (10 days) blood samples were collected using capillary tubes from retro-orbital plexus of the individuals of all groups after being fasted for 12 h.[62] Blood samples were left to clot then centrifuged at 3000 rpm for 15 min to separate the sera, which were stored at −20 °C until analysis could be completed. After blood collection, all animals were rapidly sacrificed and the hearts were dissected and immediately homogenized in 50 mM ice-cold phosphate buffer (pH 7.4) to give 10% homogenate (w/v). The homogenate was centrifuged at 3200 rpm for 20 min in cooling centrifuge at 0 °C. The supernatant (10%) was used for the determination of lipid peroxidation. The supernatant was further diluted with phosphate buffer (20% dilution) to determine the antioxidant enzyme activities (catalase, superoxide dismutase and glutathione peroxidase) and protein content.

3.2.3. Biochemical analyses. Quantitative estimation of serum troponin I was carried out by enzyme linked immunosorbent assay (ELISA)[63] using kit purchased from DRG international Inc., USA. An immunoenzymometric assay for serum leptin measurement was done according to Friedman and Halaas method[64] using kit produced by BioSource. Europe S.A. Kits from Randox Laboratories Ltd Co. were used for the determination of serum total cholesterol,[65] LDL-cholesterol[66] and triglycerides.[67] Quantitative determination of serum 3,5,3′-triiodothyronine (T_3) and thyroxin (T_4) was carried out using ELISA procedure according to the methods of Tietz[68] and Robbins,[69] respectively, using kits of Adaltis Italia S.P.A. Serum nitrate concentration as a stable end product of nitric oxide was determined photometrically on microtitre plate according to the method of Moshage et al.[70] using kit produced by RfD System GmbH (Germany). Serum interleukin-1alpha (IL-1α) was measured by ELISA procedure of Krakauer and Krakauer[71] using Cytimmune Co. Kit. Lipid peroxidation in cardiac homogenate was estimated by measurement of malondialdehyde by spectrophotometric assay[72] using Oxis Research^{TM} kit. The level of lipid peroxides was expressed as nmol malondialdehyde/gm tissue. Total glutathione peroxidase activity was determined in cardiac homogenate using reduced glutathione and cumene hydroperoxide as substrate using 20 μL diluted supernatant by the modified method of Paglia and Valentine.[73] Superoxide dismutase activity was determined by red formazan dye reduction procedure[74] using 50 μL diluted supernatant. Spectrophotometric assay was used for the determination of catalase activity in cardiac homogenate supernatant according to the method of Aebi[75] using kit produced by Oxis Research^{TM} Inc. The specific activities of glutathione peroxidase, superoxide dismutase and catalase were expressed as units of activity/mg protein. The protein content of heart tissue was measured applying the method of Lowry et al.[76]

3.2.4. Statistical analysis. The Statistical Package for the Social Sciences (SPSS version 7.5) was used in data analysis. Data were expressed as mean ± SE. One way analysis of variance (ANOVA) was used to compare the six control groups together as well as the six groups injected with Dox together. When significant it was followed by Duncan's multiple range test[77] to clarify the significance between the individual groups. Student *t*-test was used to compare the Dox group with the control group.[78] *P* values less than 0.05 were considered significant.

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References and notes

1. Morishima, I.; Matsui, H.; Mukawa, H.; Hayashi, K.; Tobi, Y.; Okumura, K.; Ito, T.; Hayakawa, T. *Life Sci.* **1998**, *63*, 511–521.
2. Kitakaze, M. *Drugs Ther.* **2001**, *15*, 199–200.
3. Goormaghtigh, E.; Brasseur, R.; Ruysschaert, J. M. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 314–320.
4. Singal, P. K.; Pierce, G. *Am. J. Physiol.* **1986**, *250*, H419–H425.
5. Singal, P. K.; Panagia, V. *Res. Commun. Chem. Pathol. Pharmacol.* **1984**, *43*, 67–77.
6. Gewirtz, D. A. *Biochem. Pharmacol.* **1999**, *57*, 727–741.

7. Gille, L.; Nahl, H. *Free Radical Biol. Med.* **1997**, *23*, 775–782.
8. Arola, O. J.; Saraste, A.; Pulkki, K.; Kallajoki, M.; Parvinen, M.; Voipio-Pulkki, L. M. *Cancer Res.* **2000**, *60*, 1789–1792.
9. Davies, K. J.; Doroshow, J. H. *J. Biol. Chem.* **1986**, *261*, 3060–3067.
10. Li, T.; Danelisen, I.; Singal, P. K. *Mol. Cell Biochem.* **2002**, *232*, 19–26.
11. Heiman, M. L.; Porter, J. R. *Horm. Res.* **1980**, *12*, 104–112.
12. Reiter, R. J.; Tan, D. X.; Manchester, L. C.; Qi, W. *Cell Biochem. Biophys.* **2001**, *34*, 237–256.
13. Karbownik, M.; Reiter, R. J. *Cancer Invest.* **2002**, *20*, 276–286.
14. Tsia, P. L.; Hu, M. K. *J. Pharm. Pharmacol.* **2003**, *55*, 1655–1660.
15. Liu, X.; Chen, Z.; Chua, C. C.; Ma, Y.; Yungberg, G. A.; Hamdy, R.; Chua, B. H. L. *Am. J. Physiol., Heart Circ. Physiol.* **2002**, *283*, H254–H263.
16. Doss, S. H.; Mohareb, R. M.; Elmegeed, G. A.; Luca, N. A. *Pharmazie* **2003**, *58*, 607–613.
17. Dobosz, M.; Wujec, M.; Wasko, A. *Acta Pol. Pharm.* **2003**, *60*, 41–44.
18. Sherif, M. S.; Mohareb, R. M.; Shams, H. Z.; Gaber, H. M. M. *J. Chem. Res. (S)* **1995**, 434–435.
19. Bertinchant, J. P.; Polge, A.; Juan, J. M.; Oliva-Lauraire, M. C.; Giuliani, I.; Marty-Double, C.; Burdy, J. Y.; Fabbro-Peray, P.; Laprade, M.; Bali, J. P.; Granier, C.; de la Coussaye, J. E.; Danzat, M. *Clin. Chim. Acta* **2003**, *329*, 39–51.
20. Van Vleet, J. F.; Ferrans, V. J.; Badylak, S. F. *Am. J. Vet. Res.* **1982**, *43*, 2173–2182.
21. Geetha, A.; Catherine, J.; Sankar, R.; Shyamala Devi, C. S. *Indian J. Exp. Biol.* **1990**, *28*, 1071–1074.
22. Sugawara, M.; Sugawara, Y.; Wen, K.; Giulivi, C. *Exp. Biol. Med.* **2002**, *227*, 141–146.
23. Hamilton, M.; Stevenson, L. V.; Luu, M.; Walden, J. A. *J. Am. Coll. Cardiol.* **1990**, *16*, 91–95.
24. Meldrum, D. R.; Cleveland, J. C.; Sheridan, B. C.; Rowland, R. T.; Banerjee, A.; Harken, A. H. *Ann. Thoras. Surg.* **1996**, *61*, 1273–1280.
25. Reiter, R. J.; Tan, D. X.; Sainz, R. M.; Mayo, J. C.; Lopez-Burillo, S. *J. Pharm. Pharmacol.* **2002**, *54*, 1299–1321.
26. Iliskovic, N.; Singal, P. K. *Am. J. Pathol.* **1997**, *150*, 727–734.
27. Ginsberg, H.; Grundry, S. M. *Diabetologia* **1982**, *23*, 421–425.
28. Nestel, P. J.; Havel, R. J.; Benzman, A. J. *J. Clin. Invest.* **1963**, *42*, 1313–1321.
29. Nishida, S.; Segawa, T.; Murai, I.; Nakagawa, S. *J. Pineal Res.* **2002**, *32*, 26–33.
30. Sewerynek, E. *Neuroendocrinol. Lett.* **2002**, *23*, 79–83.
31. Rifai, N.; Bachorik, P. S.; Albers, J. J. In *Tietz Textbook of Clinical Chemistry*; Burtis, C. A., Ashwood, E. R., Eds., 1999; Vol. 3, Chapter 25.
32. Meisel, S. R.; Ellis, M.; Pariente, C.; Pauzner, H.; Liebowitz, M.; David, D.; Shimon, I. *Cardiology* **2001**, *95*, 206–211.
33. Tamer, L.; Ercan, B.; Unlu, A.; Sucu, N.; Pekdemir, H.; Eskandari, G.; Atik, U. *Indian Heart J.* **2002**, *54*, 692–696.
34. Wallace, A. M.; McMahon, A. D.; Parkard, C. J.; Kelly, A.; Shepherd, J.; Gaw, A.; Sattar, N. *Circulation* **2001**, *104*, 3052–3056.
35. Haffner, S. M.; Mykkanen, L.; Rainwater, D. L.; Karhapaa, P.; Laakso, M. *Obes. Res.* **1999**, *7*, 164–169.
36. Wolden-Hanson, T.; Mitton, D. R.; McCants, R. L.; Yellon, S. M.; Wilkinson, C. W.; Matsumoto, A. M.; Rasmussen, D. D. *Endocrinology* **2000**, *141*, 487–497.
37. Lynch, D. H.; Rubin, A. S.; Miller, R. E.; Williams, D. E. *Cancer Res.* **1993**, *53*, 1565–1570.
38. Eppstein, D. A.; Kurahara, C. G.; Bruno, N. A.; Terrell, T. G. *Cancer Res.* **1989**, *49*, 3955–3960.
39. Arend, W. P. *Immunologist* **1997**, *5*, 197–201.
40. Aggarwal, A.; Schneider, D. J.; Terrien, E. F.; Sobel, B. E.; Dauerman, H. L. *Am. J. Cardiol.* **2004**, *93*, 6–9.
41. Pioli, C.; Caroleo, M. C.; Nistico, G.; Doria, G. *Int. J. Immunophamacol.* **1993**, *15*, 463–468.
42. Naidu, M. U.; Kumar, K. V.; Mohan, I. K.; Sundaram, C.; Singh, S. *Indian J. Exp. Biol.* **2002**, *40*, 894–900.
43. Pacher, P.; Liaudet, L.; Bai, P.; Mabley, J. G.; Kaminski, P. M.; Virag, L.; Deb, A.; Szabo, E.; Ungvari, Z.; Wolin, M. S.; Groves, J. T.; Szabo, C. *Circulation* **2003**, *107*, 896–904.
44. Dziegiel, P.; Murawska-Cialowicz, E.; Jethon, Z.; Jonuszewska, L.; Podhorska-Okolow, M.; Surowiak, P.; Zawadzki, M.; Rabczynski, J.; Zabel, M. *J. Pineal Res.* **2003**, *35*, 183–187.
45. Vasquez-Vivar, J.; Marasek, P.; Hogg, N.; Masters, B. S. S., Jr.; Pritchard, K. A., Jr.; Kalyanaraman, B. *Biochemistry* **1997**, *36*, 11293–11297.
46. Knowles, R. G.; Moncada, S. *Biochem. J.* **1994**, *298*, 249–258.
47. Vuillaume, M. *Mutat. Res.* **1987**, *186*, 43–72.
48. Mayo, J. C.; Tan, D. X.; Sainz, R. M.; Lobez-Burillo, S.; Reiter, R. J. *Free Radical Res.* **2003**, *37*, 543–553.
49. Reiter, R. J. *New Physiol. Sci.* **2000**, *15*, 246–250.
50. Dubocovich, M. L. *Trends Pharmacol. Sci.* **1995**, *16*, 50–56.
51. Reiter, R. J.; Tang, L.; Garcia, J. J.; Munoz-Hoyos, A. *Life Sci.* **1997**, *60*, 2255–2271.
52. Leon, J.; Vives, F.; Crespo, E.; Camacho, E.; Espinosa, A.; Gallo, M. A.; Escames, G.; Acuna-Castroviejo, D. *J. Neuroendocrinol.* **1998**, *10*, 297–302.
53. Gilad, E.; Wong, H. R.; Zingarelli, B. *FASEB J.* **1998**, *12*, 685–693.
54. Reiter, R. J.; Acuna-castroviejo, D.; Tan, D. X.; Burkhardt, S. *Ann. N.Y. Acad. Sci.* **2001**, *939*, 200–215.
55. Tan, D. X.; Reiter, R. J.; Manchester, L. C.; Yan, M. T.; El-Sawi, M.; Sainz, R. M.; Mayo, J. C.; Kohen, R.; Allegra, M.; Hardeland, R. *Curr. Top. Med. Chem.* **2002**, *2*, 181–197.
56. Ezure, T.; Kanayama, T.; Nishino, C. *Biochems. Pharmacol.* **2001**, *62*, 335–340.
57. Iwashita, A.; Maemoto, T.; Nakada, H.; Shima, I.; Matsuoka, N.; Hisajima, H. *J. Pharm. Exp. Ther.* **2003**, *307*, 961–968.
58. Tampo, Y.; Tsukamoto, M.; Yonaha, M. *FEBS Lett.* **1998**, *430*, 348–352.
59. Dubuissona, M. L.; Reesa, J. F.; Marchand-Brynaert, J. *Mini. Rev. Med. Chem.* **2004**, *4*, 421–435.
60. Mostafa, M. G.; Mima, T.; Ohnishi, S. T.; Mori, K. *Planta Med.* **2000**, *66*, 148–151.
61. Kocak, G.; Erbil, K. M.; Ozdemir, I.; Aydemir, S.; Sunar, B.; Tuneal, M.; Atalay, S. *Can. J. Cardiol.* **2003**, *19*, 535–541.
62. Schermer, S. *The Blood Morphology of Laboratory Animals*, 3rd ed.; F. A. Davi: Philadelphia, 1967, p 42.
63. Bodor, G. S. *J. Clin. Immunoassay* **1994**, *17*, 40–44.
64. Friedman, J. M.; Halaas, J. L. *Nature* **1998**, *395*, 763–770.
65. Richmond, W. *Clin. Chem.* **1973**, *19*, 1350–1356.
66. Pisani, T.; Gebski, C. P.; Leavy, E. T. *Arch. Pathol. Lab. Med.* **1995**, *119*, 1127–1135.
67. Jacobs, N. J.; Van Denmark, P. *J. Arch. Biochem. Biophys.* **1960**, *88*, 250–255.

68. Tietz, N. W. *Fundamentals of Clinical Chemistry*; Saunders: Philadelphia, 1987, p 588.
69. Robbins, J. Thyroxin-Binding Protein in Serum. In *Laboratory Diagnosis of Endocrine Diseases*; Sunderman, Sunderman, Eds.; Warren H. Green: St Louis, MO, 1971, p 211.
70. Moshage, H.; Rok, B.; Huizenga, J. R.; Jansen, P. I. M. *Clin. Chem.* **1995**, *41*, 892–896.
71. Krakauer, T.; Krakauer, H. *J. Leukocyte Biol.* **1991**, *50*, 123–130.
72. Esterbauer, H.; Schaure, R. J.; Zollner, H. *Free Radical Biol. Med.* **1991**, *11*, 81–128.
73. Paglia, D. E.; Valentine, W. N. *J. Lab. Clin. Med.* **1967**, *70*, 158–169.
74. Suttle, N. F. *Vet. Rec.* **1986**, *119*, 519–522.
75. Aebi, H. *Methods Enzymol.* **1984**, *105*, 121–126.
76. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, P. T. *J. Biol. Chem.* **1951**, *193*, 265–275.
77. Duncan, D. B. Multiple ranges and multiple *F*-test. *Biometrics* **1955**, *11*, 1–42.
78. Snedicor, G. W.; Cochran, W. J. *Statistical Methods*, 6th ed.; The Iowa State University Press: Ames, Iowa, USA, 1967, p 104.