Role of exogenous melatonin on adriamycin-induced changes in the rat heart

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Abstract. – *Aim:* The protective effect of melatonin on adriamycin (ADM)-induced cardiotoxicity was investigated in the rat heart. Melatonin is a pineal hormone with free radical scavenging activity on oxidants; therefore it may decrease the ADM-induced oxidative stress and cardiotoxicity so that therapeutic efficacy might be enhanced.

Materials and Methods: Wistar rats in 4 groups were treated with saline (control), melatonin (MEL), adriamycin (ADM) and melatonin plus adriamycin (MEL+ADM).

Results: Adriamycin given at a single dose of 15 mg/kg significantly increased lipid peroxidation products as measured by thiobarbituric acid reactive substances (TBARS). Melatonin (5 mg/kg bw) given 2 days before and 7 days after ADM treatment reduced TBARS level. Adriamycin significantly reduced superoxide dismutase activity which was elevated by melatonin treatment. Additionally, ADM significantly increased catalase enzyme activity while melatonin normalized the ADM induced alteration in activity of catalase.

Conclusions: The combined use of ADM and melatonin reduces the threat of cardiomyopathy. Melatonin seems to hold promise as a therapeutic treatment and can be recommended as an adjunct in antitumor therapy as a safe and effective protection against acute ADM-induced cardiotoxicity.

Key Words:

Adriamycin, Melatonin, TBARS, Superoxide dismutase, Catalase.

Introduction

The anthracycline antibiotic adriamycin (ADM; doxorubicin hydrochloride) is one of the most active and broad-spectrum anti-neoplastic agent used to treat various types of cancers including leukaemia, lymphomas and several forms of solid tumors[1]. However, the clinical use of this drug has been seriously limited by its undesirable side effects, especially the dose-dependent cardiotoxicity[2,3]. The induction of adriamycin-induced cardiotoxicity has been ascribed to various mechanisms, including the inhibition of nucleic acid and/or protein synthesis, an imbalance of myocardial electrolytes, alterations in platelet-activating factor and/or prostaglandin activity, alterations of mitochondrial membrane function, mitochondrial DNA damage and dysfunction[4,5], formation of free radicals, induction of apoptosis[6], release of histamine, production of adriamycin metabolites, calcium overload, hyperlipidemia[4,7], and alterations of transcriptional events specific to the myocardium[8]. However, among these, the most plausible hypothesis, seems to be the reduction of the quinine structure of adriamycin and the production of free radicals which induce membrane lipid peroxidation and generate superoxide anions (O_2^-) and hydroxyl radicals (OH) that cause oxidative damage in the myocardium[9,10]. Under aerobic conditions in myocardial cells, redox cycling of the quinone and semiquinone yields $O_n^{.-}$, which are subsequently reduced to hydrogen peroxide (H_2O_2) by the antioxidant enzyme superoxide dismutase. In the presence of Fe^{++}, the H_2O_2 is further reduced to the extremely reactive ·OH which reacts with polyunsaturated fatty acids to yield lipid hydroperoxide. This initiates a lipid radical chain reaction that causes oxidative damage to cell membranes[11]. In fact, lipid peroxidation due to ADM-induced free radical formation has been documented in the pathogenesis of anthracycline-induced cardiotoxicity[2,12-14]. It follows that scavengers that prevent free radicals from reacting with other molecules should decrease the car-

diotoxicity of ADM. Several investigations have demonstrated this protective effect[7,15-19].

It has been shown that melatonin (MEL) acts as a powerful antioxidant and as a free radical scavenger of the hydroxyl radical ($\cdot$OH), peroxyl radicals, and superoxide anions[20-22]. MEL is rapidly converted to 6-hydroxymelatonin in the liver[23]; this major metabolite has 50 times less binding affinity for plasma membranes than melatonin, but it possesses a free radical-scavenging indole ring and therefore, could maintain antioxidant activity. Antioxidant activity of MEL has been also attributed to its ability to chelate with metal ions. Moreover, it was reported that MEL could up-regulate antioxidant enzymes[24]. Kotler et al[25] showed that MEL could increase mRNA levels of antioxidant enzymes glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) up to two fold in rat brain cortex.

The aim of this study was to investigate the protective effects of MEL for acute adriamycin-induced changes in oxidative stress enzyme levels and lipid peroxidation in myocardial cells in a rat model.

Materials and Methods

This study was carried out at Inonu University, Medical Biology and Genetics laboratories in accordance with the Guide for the Care Use of Laboratory Animals published by the United States National Institutes of Health [US NIH publication no: 83-23 revised 1996]. Thirty two Wistar rats with body weight ranging from 175 to 225 g were divided into 4 groups, each consisting of 8 animals. The animals were maintained under controlled temperature (20±2°C) and light conditions (a 12 h light-12 h dark cycle). Food and water were provided *ad libitum*. All drugs and placebos were administered intraperitoneally at a fixed time between 09:00 to 11:00 hours. Melatonin (Sigma Chemical Co, Saint Louis, MO, USA) was dissolved in a minimum volume of ethanol (0.001%) and then diluted with physiological saline. ADM was dissolved in physiologic saline. Experimental groups were treated as follows: Rats in group C (control) received a daily injection of saline containing a minimum volume of ethanol for 7 consecutive days. Rats in group MEL (melatonin only) were given 5 mg/kg melatonin daily for 7 days. Rats in group ADM (adriamycin only) were injected with a single dose of 15 mg/kg adriamycin on day 1. Rats in group MEL+ADM (melatonin plus adriamycin) received 5 mg/kg melatonin for two consecutive days prior to a single dose of 15 mg/kg ADM plus a 5 mg/kg dose of melatonin on day 1 and then received a daily injection of MEL for six consecutive days. All rats were sacrificed at the end of the seventh day with an intraperitoneal injection of sodium pentobarbital. For all groups at the time of sacrifice, the heart of each rat was excised and rinsed in saline to remove residual blood. The atria and large vessels were discarded and the ventricles stored at –70ºC for the biochemical analyses.

Sample Preparation

Tissue samples were divided into four equal parts for homogenisation. An Ultra-Turrax homogeniser (model T 25, Janke and Kunkel, Staufen**,** Germany) 9500 rpm (4 × 10 s at 4°C) was used. The homogenates were centrifuged at 1000 × g for 10 min at 4°C (Microcentrifuge 157.MP, Ole Dich, Denmark) and supernatants were collected. One portion of the heart was homogenised in a ratio of one part wet tissue to 9 ml of cold 1.15% KCl. An aliquot of 0.5 ml of the 1:9 homogenate was used for the thiobarbituric acid reactive substances (TBARS) assay. The remaining three parts were homogenised as above (i.e., 1:9) but with the addition of 0.02 M potassium phosphate (pH 7.0) as a buffer into the solution. The buffered homogenate was equally divided for catalase (CAT) and SOD activity determination. The TBARS levels, SOD and CAT activity were measured with a Shimadzu model UV/Visible1601 spectrophotometer (Shimadzu Corporation, Nakagyou-ku, Kyoto, Japan). All chemicals were of analytical grade and were purchased from Sigma (St Louis, MO, USA) unless otherwise stated.

Thiobarbituric Acid Reactive Substances (TBARS) level

Lipid peroxidation in hearts was determined using the thiobarbituric acid (TBA) reactive substances method for the estimation of malondialdehyde (MDA) content[26]. Hearts were homogenized in 10% trichloroacetic acid (TCA) at 4°C. 0.2 ml homogenate was mixed with the following: 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 30% acetic acid (pH 3.5) and 1.5 ml 0.8% TBA. The reaction mixture was heated for 60 min at 95°C and then cooled on ice. 1.0 ml water and 5.0 ml *n*-butanol: pyridine (15:1 v/v) was added

and centrifuged at $4000 \times g$ for 10 min. The absorbance of the developed colour in the organic layer was measured at 532 nm. Commercially available 1,1,3,3,-tetraethoxypropane was used as a standard for MDA.

Superoxide Dismutase (SOD) Activity

SOD enzyme activity was measured by the indirect spectrophotometric assay described by McCord and Fridovich[27]. SOD catalyses the dismutation of superoxide radicals. SOD activity was determined in the supernatant using its inhibitory action on the superoxide-depended reduction of cytochrome c by a xanthine/xanthine oxidase mixture. In a total volume of 1.1 ml the reaction mixture contained phosphate buffer (20 mM, pH 7.0), 0.1 M EDTA, 50 μM xanthine and 10 μM cytochrome c. The reaction was initiated by adding xanthine oxidase, and the rate of cytochrome c reduction was monitored spectrophotometrically at 550 nm.

Catalase Activity (CAT)

CAT activity was measured spectrophotometrically by the method of Luck[28]. Samples were diluted 1:10 in cold potassium dihydrogen phosphate buffer (pH 7.0) incubated with occasional shaking for 10 min at 4°C and centrifuged followed by a repeated extraction. Supernatants were combined, and 0.04 ml was added into 3 ml 0.01 M of H_2O_2-phosphate buffer, mixed well and read on a spectrophotometer equipped with a constant temperature unit attachment. Blank readings were performed in a control cuvette that included enzyme solution as in the experimental sample but with H_2O_2-free phosphate buffer. Decrease in absorbance measurements at 240 nm are followed until the optical density reaches 0.450. The time (Δt) required for the optical density to reach 0.400 was recorded and used to obtain the value k from the formula $0.1175/\Delta t$. The value (k) is then converted to CAT units.

Total Protein Assay

Protein was measured by the Lowry procedure[29], using bovine serum albumin as a standard.

Statistical Analysis

Data are presented as mean values ± SEM. The Mann-Whitney U-test was used for determining the significance of the results. Treatment differences with *p*-values less than 0.05 are considered as significant.

Results

Tissue TBARS concentration was significantly elevated after a single injection of ADM in group ADM compared to the control group. TBARS level of group MEL+ADM was significantly lower than that of group ADM. On the other hand, there were no significant differences among TBARS levels of groups C, MEL, MEL + ADM (Figure 1).

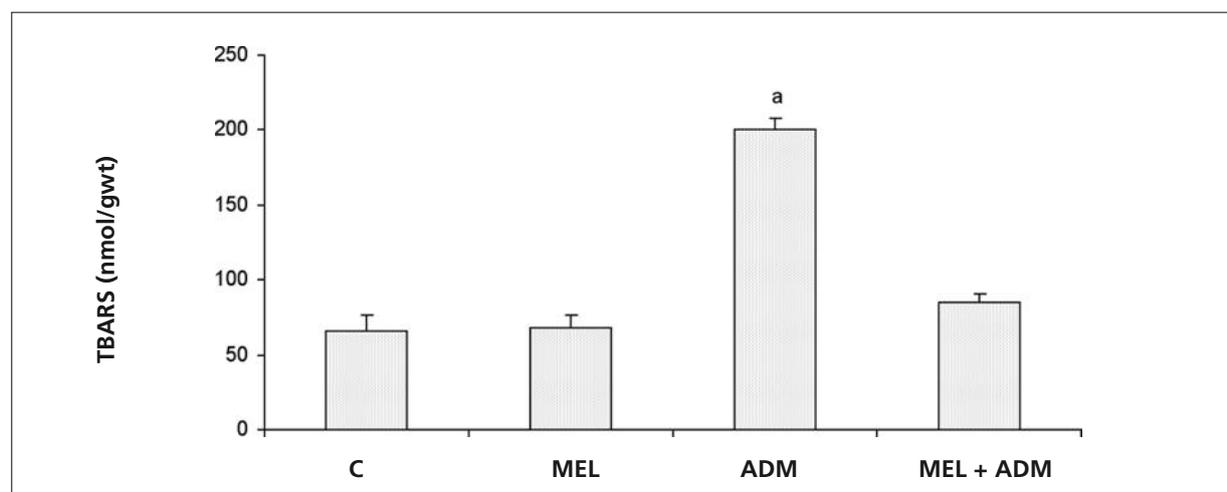While SOD activity in group ADM were significantly lower than in control, there was a sig-

Figure 1. Cardiac tissue thiobarbituric acid reactive substances (TBARS) levels in the treatment groups. C (Control), MEL (Melatonin), ADM (Adriamycin), MEL+ADM (Melatonin plus Adriamycin). p was significant at 0.05 level when compared to control.

nificant increase in SOD activity of group MEL+ADM compared to those of control and group ADM (Figure 2).

Cardiac tissue CAT activity was significantly increased in response to adriamycine alone (group ADM) as compared to control. However, there was no significant difference in CAT activity between control and group MEL+ADM (Figure 3).

Discussion

In the present study, it was evaluated whether melatonin would protect against ADM-induced toxicity when administered 2 days before ADM was given. Results of lipid peroxidation test, as assessed by tissue TBARS level, showed that single administration of ADM at 15 mg/kg caused lipid peroxidation following 7 days post-injection. Overproduced reactive oxygen species (ROS) could result in lipid peroxidation which is indirectly shown by increased TBARS level.

Lipid peroxidation due to ADM treatment was shown by several studies[14,18,30]. Distinct mechanisms were reported to be involved in ADM-induced cardiotoxicity. These include the increased oxidative stress via elevated reactive oxygen and nitrogen species, decrease in antioxidant defence mechanism, alterations in myocardial energy metabolism, ion gradients, cellular Ca^{++} regulation, apoptosis and up-regulation of inducible Nitric Oxide Synthase (iNOS) gene expression[1,14,31,32]. However, oxidative stress, through the excessive free radical generation, has been the widely accepted mechanism or at least the initial step leading to cardiotoxicity[33]. ADM accumulates in the mitochondrion where quinone ring of ADM could easily undergo redox cycling by mitochondrial enzymes. Electrons produced during these reactions are captured by oxidizing agents leading to generation of ROS[3]. MEL given 2 days before the single 15 mg/kg ADM administration and then continuing MEL injections for 7 days were able to mitigate the cardiotoxic effect of ADM. MEL is able to effectively reduce the oxidative stress and lipid peroxidation in various toxic conditions including ADM-induced cardiotoxicity[34,35]. Othman et al.[30] reported that MEL is capable of preventing iron-mediated free radical generation via controlling iron and iron binding proteins in ADM toxicity. It has been also known that melatonin has a powerful free radical scavenger and antioxidant activity, and it is a direct scavenger of ROS and RNS including hydroxyl radical, H_2O_2, singlet oxygen, nitric oxide, peroxynitrite anion and peroxy nitrous acid[36,37]. Although protection from ADM-induced lipid peroxidation by melatonin could be attributed to its radical scavenging activity, MEL is also known to induce antioxidant enzymes especially under oxidative stress[24].
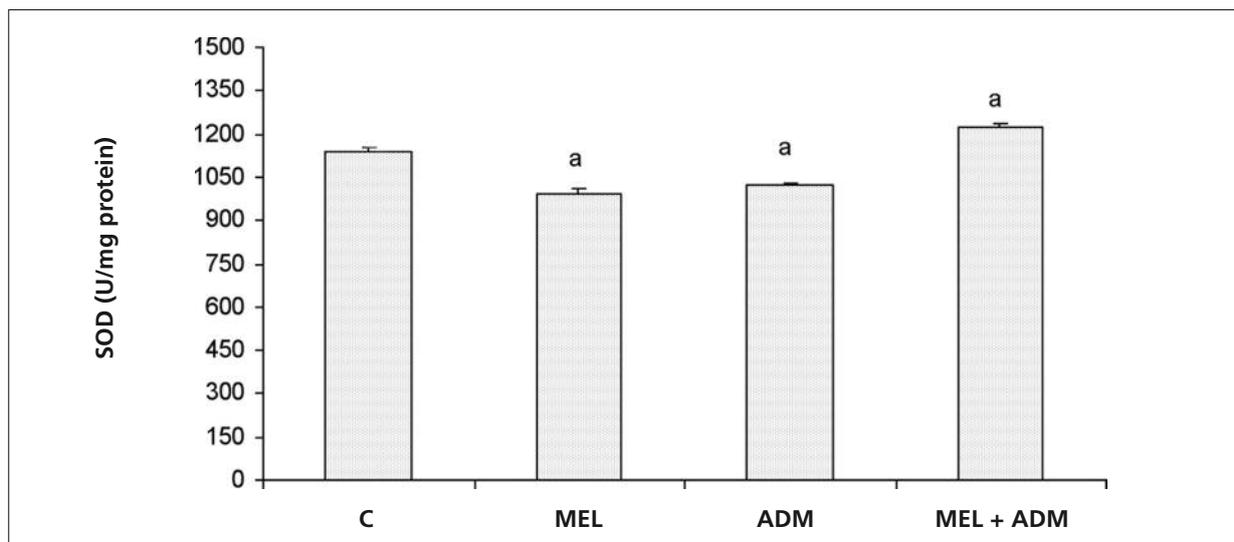

Figure 2. Cardiac tissue superoxide dismutase (SOD) activity in the treatment groups. C (Control), MEL (Melatonin), ADM (Adriamycin), MEL+ADM (Melatonin plus Adriamycin). p was significant at 0.05 level when compared to control.
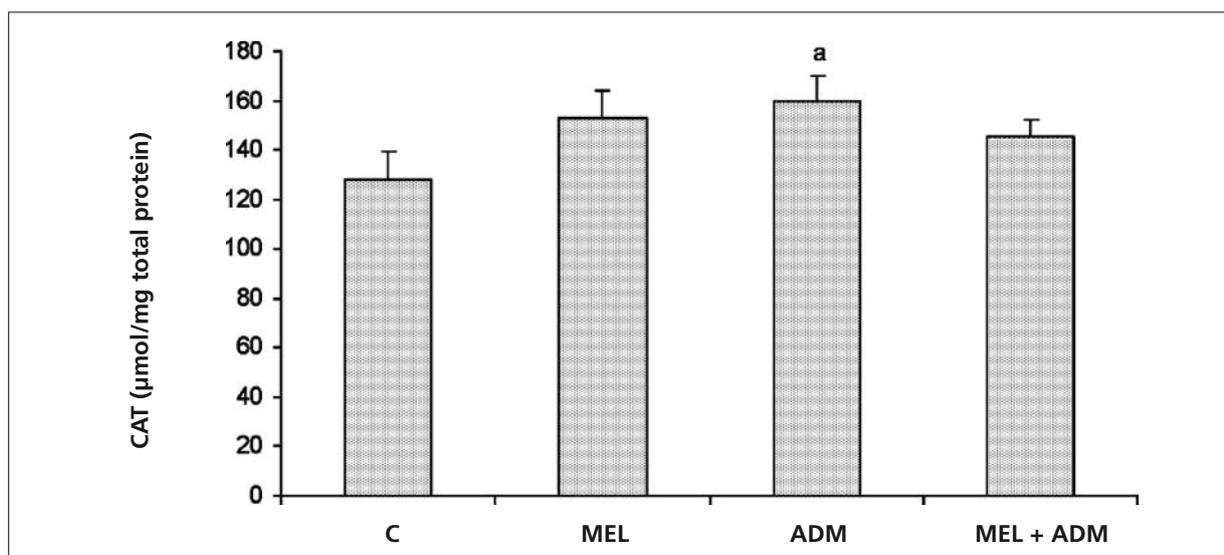
Figure 3. Cardiac tissue catalase (CAT) activity in the treatment groups. C (Control), MEL (Melatonin), ADM (Adriamycin), MEL+ADM (Melatonin plus Adriamycin). p was significant at 0.05 level when compared to control.

ADM has been reported to alter the activities of some important antioxidants including SOD and CAT enzymes which play some critical roles in dealing with the oxidative stress[3]. However, results of previous studies with respect to alterations in antioxidant enzymes appear to be controversial since there is no simple response in antioxidant enzyme activities following noxious stimuli. For example, it was reported that ADM significantly decreased cardiac SOD, CAT and glutathione peroxidase (GSH-Px) activities. MEL significantly increased ADM-induced reductions in these enzyme activities[34]. On the other hand, Dzigel et al.[38] reported increased activities of SOD, CAT and GSH-Px activities following ADM treatment.

In the present study, ADM significantly decreased SOD activity at 15 mg/kg at a single administration compared to control. However, 7 day melatonin treatment significantly increased the SOD activity of group MEL+ADM compared to rats receiving ADM alone. Increase in SOD activity that was observed along with MEL implies that prior melatonin treatment against ADM treatment helps to maintain redox homeostasis of the SOD activity. Cole et al.[39] reported that cardiac mitochondrial injury due to ADM can be ameliorated in animals overexpressing manganese-SOD enzyme. Othman et al.[30] reported that while ADM caused lipid peroxidation, the activity of CAT enzyme was increased by ADM treatment. The increase in CAT activity by ADM was normalized by MEL which is in accordance with the results of current study. Similarly, we note that increased CAT activity parallels that of lipid peroxidation and MEL normalized the CAT activity. There was no significant difference in CAT activity between the control and MEL+ADM treatment groups. The largest part of peroxide molecules produced by SOD is reduced to H_2O by CAT enzyme. Melatonin has been known to induce upregulation of antioxidant enzymes but at the same time it can induce down regulation of pro-oxidant enzymes[36]. Although the activities of antioxidant enzymes are usually elevated under oxidative stress conditions, cells may also react to highly elevated oxidative stress in an opposite manner so that the antioxidant enzyme activities can be reduced due to damage to the molecular machinery which is required to induce the antioxidant enzymes[24]. Therefore, the regulation of the antioxidant enzymes could depend on many factors and conditions during the exposure to the exogenous oxidant or antioxidant agents.

In conclusion, whatever mechanism of MEL is operating in this study the results provide an evidence for a role of MEL in cardioprotection against adriamycin induced lipid peroxidation. However, alterations in antioxidant enzyme activities against ADM and MEL could depend on redox homeostasis of the particular antioxidant enzymes and degree of oxidative stress. A decrease in adriamycin cardiotoxicity would be of considerable value by improving the therapeutic benefit of the drug.

References

1) OUTOMURO D, GRANA DR, AZZATO F, MILEI J. Adriamycin-induced myocardial toxicity: new solutions for an old problem? Int J Cardiol 2007; 117: 6-15.

2) SIMUNEK T, STERBA M, POPELOVA O, ADAMCOVA M, HRDINA R, GERSL V. Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacol Reports 2009; 61: 154-171.

3) TAKEMURA G, FUJIWARA H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Prog Cardiovasc Dis 2007; 49: 330-352.

4) DAVIES KJ, DOROSHOW JH. Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. J Biol Chem 1986; 261: 3060-3067.

5) OLSON RD, MUSHLIN PS. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. FASEB J 1990; 4: 3076-3086.

6) KUMAR D, KIRSHENBAUM L, LI T, DANELISEN I, SINGAL PK. Apoptosis in isolated adult cardiomyocytes exposed to adriamycin. Ann N Y Acad Sci 1999; 874: 156-168.

7) ILISKOVIC N, SINGAL PK. Lipid lowering: an important factor in preventing adriamycin-induced heart failure. Am J Pathol 1997; 150: 727-734.

8) JEYASEELAN R, POIZAT C, WU HY, KEDES L. Molecular mechanisms of doxorubicin-induced cardiomyopathy. Selective suppression of Reiske iron-sulfur protein, ADP/ATP translocase, and phosphofructokinase genes is associated with ATP depletion in rat cardiomyocytes. J Biol Chem 1997; 272: 5828-5832.

9) FABER M, COUDRAY C, HIDA H, MOUSSEAU M, FAVIER A. Lipid peroxidation products, and vitamin and trace element status in patients with cancer before and after chemotherapy, including adriamycin. A preliminary study. Biol Trace Elem Res 1995; 47: 117-123.

10) SINGAL PK, SIVESKI-ILISKOVIC N, HILL M, THOMAS TP, LI T. Combination therapy with probucol prevents adriamycin-induced cardiomyopathy. J Mol Cell Cardiol 1995; 27: 1055-1063.

11) MIMNAUGH EG, TRUSH MA, BHATNAGAR M, GRAM TE. Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin. Biochem Pharmacol 1985; 34: 847-856.

12) LUO X, EVROVSKY Y, COLE D, TRINES J, BENSON LN, LEHOTAY DC. Doxorubicin-induced acute changes in cytotoxic aldehydes, antioxidant status and cardiac function in the rat. Biochim Biophys Acta 1997; 1360: 45-52.

13) ODOM AL, HATWIG CA, STANLEY JS, BENSON AM. Biochemical determinants of adriamycin toxicity in mouse liver, heart and intestine. Biochem Pharmacol 1992; 43: 831-836.

14) KIM SY, KIM SJ, KIM BJ, RAH SY, CHUNG SM, IM MJ, KIM UH. Doxorubicin-induced reactive oxygen species generation and intracellular Ca2+ increase are reciprocally modulated in rat cardiomyocytes. Exp Mol Med 2006; 38: 535-545.

15) MORISHIMA I, MATSUI H, MUKAWA H, HAYASHI K, TOKI Y, OKUMURA K, ITO T, HAYAKAWA T. Melatonin, a pineal hormone with antioxidant property, protects against adriamycin cardiomyopathy in rats. Life Sci 1998; 63: 511-521.

16) CAO Y, KENNEDY R, KLIMBERG VS. Glutamine protects against doxorubicin-induced cardiotoxicity. J Surg Res 1999; 85: 178-182.

17) VENKATESAN N. Curcumin attenuation of acute adriamycin myocardial toxicity in rats. Br J Pharmacol 1998; 124: 425-427.

18) KOCAK G, ERBIL KM, OZDEMIR I, AYDEMIR S, SUNAR B, TUNCEL M, ATALAY S. The protective effect of melatonin on adriamycin-induced acute cardiac injury. Can J Cardiol 2003; 19: 535-541.

19) ERTEN SF, KOCAK A, OZDEMIR I, AYDEMIR S, COLAK A, REEDER BS. Protective effect of melatonin on experimental spinal cord ischemia. Spinal Cord 2003; 41: 533-538.

20) REITER RJ, TANG L, GARCIA JJ, MUNOZ-HOYOS A. Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sci 1997; 60: 2255-2271.

21) REITER RJ, TAN DX. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart. Cardiovasc Res 2003; 58: 10-19.

22) GILAD E, CUZZOCREA S, ZINGARELLI B, SALZMAN AL, SZABO C. Melatonin is a scavenger of peroxynitrite. Life Sci 1997; 60: PL169-174.

23) RAYNAUD F, MAUVIARD F, GEOFFRIAU M, CLAUSTRAT B, PEVET P. Plasma 6-hydroxymelatonin, 6-sulfatoxymelatonin and melatonin kinetics after melatonin administration to rats. Biol Signals 1993; 2: 358-366.

24) RODRIGUEZ C, MAYO JC, SAINZ RM, ANTOLIN I, HERRERA F, MARTIN V, REITER RJ. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004; 36: 1-9.

25) KOTLER M, RODRIGUEZ C, SAINZ RM, ANTOLIN I, MENENDEZ-PELAEZ A. Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. J Pineal Res 1998; 24: 83-89.

26) OKHAWA H, OOHISHI N, YAGI N. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Ann Biochem 1979; 95: 351-358.

27) McCORD JM, FRIDOVICH I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244: 6049-6055.

28) LUCK H. Catalase. New York: Academic Press, 1963.

29) LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

30) OTHMAN AI, EL-MISSIRY MA, AMER MA, ARAFA M. Melatonin controls oxidative stress and modulates iron, ferritin, and transferrin levels in adriamycin treated rats. Life Sci 2008; 83: 563-568.

31) LIU B, LI H, QU H, SUN B. Nitric oxide synthase expressions in ADR-induced cardiomyopathy in rats. J Biochem Mol Biol 2006; 39: 759-765.

32) WU S, KO YS, TENG MS, KO YL, HSU LA, HSUEH C, CHOU YY, LIEW CC, LEE YS. Adriamycin-induced cardiomyocyte and endothelial cell apoptosis: in vitro and in vivo studies. J Mol Cell Cardiol 2002; 34: 1595-1607.

33) SCHIMMEL KJ, RICHEL DJ, VAN DEN BRINK RB, GUCHELAAR HJ. Cardiotoxicity of cytotoxic drugs. Cancer Treat Rev 2004; 30: 181-191.

34) AHMED HH, MANNAA F, ELMEGEED GA, DOSS SH. Cardioprotective activity of melatonin and its novel synthesized derivatives on doxorubicin-induced cardiotoxicity. Bioorg Med Chem 2005; 13: 1847-1857.

35) MAHIEU S, CONTINI MDEL C, GONZALEZ M, MILLEN N. Melatonin reduces oxidative damage induced by aluminium in rat kidney. Toxicol Lett 2009; 190: 9-15.

36) TENGATTINI S, REITER RJ, TAN DX, TERRON MP, RODELLA LF, REZZANI R. Cardiovascular diseases: protective effects of melatonin. J Pineal Res 2008; 44: 16-25.

37) LIN HW, LEE EJ. Effects of melatonin in experimental stroke models in acute, sub-acute, and chronic stages. Neuropsychiatr Dis Treat 2009; 5: 157-162.

38) DZIEGIEL P, MURAWSKA-CLALOWICZ E, JETHON Z, JANUSZEWSKA L, PODHORSKA-OKOLOW M, SUROWIAK P, ZAWADZKI M, RABCZYNSKI J, ZABEL M. Melatonin stimulates the activity of protective antioxidative enzymes in myocardial cells of rats in the course of doxorubicin intoxication. J Pineal Res 2003; 35: 183-187.

39) COLE MP, CHAISWING L, OBERLEY TD, EDELMANN SE, PIASCIK MT, LIN SM, KININGHAM KK, ST CLAIR DK. The protective roles of nitric oxide and superoxide dismutase in adriamycin-induced cardiotoxicity. Cardiovasc Res 2006; 69: 186-197.

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