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The Protective Effect of Melatonin Administration against Adriamycin-induced Cardiotoxicity in Rats

Jin Han*, Chunghee Kim*, Nari Kim, Juhee Park, Youngchurl Yang[1], and Euiyong Kim

Department of Physiology and Biophysics, [1]Department of Anatomy, College of Medicine, Inje University, Busan 614–735, Korea

Adriamycin is a commonly used chemotherapeutic agent for cancer, including acute leukemia, lymphoma, and a number of solid human tumors. However, recent studies have recognized severe cardiotoxicity after an acute dose, which are likely the result of generation of free radicals and lipid peroxidation. Therefore, the clinical uses of adriamycin have been limited. Melatonin, the pineal gland hormone known for its ability to modulate circardian rhythm, has recently been studied in its several functions, including cancer growth inhibition, stimulating the immune system, and acting as an antioxidant and radical scavenging effects. In the present study, we evaluated the effect of melatonin administration on adriamycin-induced cardiotoxicity in rat. Heart slices were prepared using a Stadie-Riggs microtome for the measurement of malondialdehyde (MDA) content used as an index of lipid peroxidation and lactate dehydrogenase (LDH) release as an indicator of lethal cell injury. Serious adriamycin-induced lethality was observed in rat by a single intraperitoneal injection in a dose-dependent manner. A single injection of adriamycin (25 mg/kg, i.p.) induced a lethality rate of 86%, with melatonin (10 mg/kg s.c. for 6 days) treatment reducing the adriamycin-induced lethality rate to 20%. The severe body weight loss caused by adriamycin was also significantly attenuated by melatonin treatment. Treatment of melatonin marked reduced adriamycin-induced the levels of MDA formation and LDH release. A cell damage indicated by the loss of myofibrils, swelling of the mitochondria as well as cytoplasmic vacuolization was seen in adriamycin-treated group. Melatonin attenuated the adriamycin-induced structural alterations. These data provide evidence that melatonin prevents adriamycin-induced cardiotoxicity and might serve as a combination with adriamycin to limit free radical-mediated cardiotoxicity.

Key Words: Adriamycin, Cardiotoxicity, Melatonin, Lipid peroxidation, LDH

INTRODUCTION

Adriamycin (doxorubicin), an anthracyclin antibiotic, is effective against a wide range of human neoplasms such as acute leukemia, lymphoma, and a number of solid human tumors (Singal et al, 1995). However, clinical uses of adriamycin have been restricted due to its toxicity, especially its cardiotoxicity which lead to cardiomyopathy and heart failure (Booser & Hortobagyi, 1994).

Although the pathogenesis of adriamycin-induced cardiotoxicity remains unclear, oxygen free radicals production and lipid peroxidation are considered to be the most important factors (Sarvazyan et al, 1995; Singal et al, 1995). The mechanism involves one-electron reduction of adriamycin for generation of a semiquinone radical. Adriamycin semiquinone radical reduces oxygen to produce superoxide and to regenerate adriamycin. The net result of this process is that adriamycin catalyzes the reduction of oxygen by NADPH, to form a superoxide radical, which is subsequently reduced to hydrogen peroxide (H_2O_2) by the

Corresponding to: Euiyong Kim, Department of Physiology and Biophysics, College of Medicine, Inje University, Gaegeum-dong, Busanjin-gu, Busan 614-735, Korea. (Tel) 82-51-890-6714, (Fax) 82-51-894-4500, (E-mail) phykimey@ijnc.inje.ac.kr
*These authors contributed equally to this work.

antioxidnat enzyme, superoxide dismutase. In the presence of Fe^{2+}, the H_2O_2 is further reduced to the extremely reactive hydroxyl ($\cdot$OH) radical, which can react with polyunsaturated fatty acids to yield lipid hydroperoxide. Biological membranes contain a large amount of polyunsaturated fatty acids, which are susceptible to peroxidative attacks by oxygen free radicals, resulting in lipid peroxidation. Among the many organs where peroxidative tissue damage has been documented, the heart is considered to be one of the most vulnerable to the free radicals produced by adriamycin administration (Singal et al, 1987).

If oxygen radical-induced lipid peroxidation of membrane lipid is indeed a cause of adriamycin-induced cardiotoxicity, then antioxidants should prevent it. Indeed, several antioxidants have been reported to reduce or delay adriamycin-induced cardiotoxicity in experimental studies (Wang et al, 1980; Fujita et al, 1982; Tesoriere et al, 1994).

In recent years, a hormone produced and secreted by the pineal gland in almost all animals and best known for its ability to modulate circardian rhythm (Brzezinski, 1997). There are numerous reports, dealing with the various effects of melatonin, which include inhibition of cancer growth (Blask, 1993), stimulation of the immune system (Maestroni, 1993), oxygen free radical scavenging activity (Marshall et al, 1996). The antioxidant activity of melatonin is ascribed to two different mechanisms: (1) melatonin reduces oxidative stress by stimulating antioxidant enzymes (Antolin et al, 1996; Reiter et al, 1997); (2) melatonin directly scavenges $\cdot$OH radicals (Poeggeler et al, 1994). It has been demonstrated that melatonin protects cells, tissues and organs from oxidative damage induced by a variety of free radical generating agents and processes (Reiter et al, 1997; Reiter, 1998). Melatonin as an antioxidant is effective in protecting membrane lipids, nuclear DNA and protein from oxidative damage both in vivo and in vitro (Reiter et al, 1998a, b).

In the present study we therefore examined whether melatonin could attenuate adriamycin-induced cardiotoxicity in rats as an antioxidant agent. We have examined adriamycin-induced oxidative damage using as criteria the levels of malondialdehyde (MDA) as an index of lipid peroxidation, LDH release as an irreversible cell damage. We then have investigated whether the protective effects of melatonin are mediated through alterations of above criteria.

METHODS

Animals

Male Sprague-Dawley rats weighing between 175 and 240 g each were used in all experiments. They were fed a standard rat chow diet and housed in plastic cages. Food and water were provided *ad libitum*.

Chemicals

Adriamycin (Pharmacia & Upjohn S.P.A., Milan, Italy) was dissolved in a normal saline solution immediately before use in each experiment. Melatonin (Sigma, St. Louis, MO, USA) was dissolved in DMSO (Sigma, St. Louis, MO, USA) and dispensed to normal saline at the final concentrations required by the each protocol. DMSO never exceeded 1%. The chemicals were freshly prepared immediately just before use. All other chemicals used in this study, unless specified otherwise, were from Sigma (St. Louis, MO, USA).

Preparation of heart slices

All animals were administered heparin (300 IU/ml i.p.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.). As soon as optimal anesthesia was achieved, evidenced by lack of eye-blink reflex and foot withdrawal reflex, hearts were rapidly isolated via thoracotomy and the aorta was cannulated. A dissected heart was mounted on a Langendorff apparatus and perfused retrogradely with oxygenated normal Tyrode solution containing 143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4 with NaOH) for 5~6 min until all signs of blood were removed with gentle squeezing of the heart. Thin (0.4~0.5 mm thick) slices of heart were prepared using a Stadie-Riggs microtome and were stored in an ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, and 20 mM Tris-HCl (pH 7.4 with NaOH).

Dose-response relation of adriamycin lethality

Four groups of rat were used to evaluate the dose-response relationship of lethality from adriamycin.

The percentage of animals surviving in each group was plotted against time (days) after adriamycin injection. Each animal received a single i.p. injection of adriamycin. Group 1 is control (1% DMSO) (n=10), group 2 received adriamycin, 5 mg/kg (n=10), group 3 received 15 mg/kg (n=20) and group 4 received 25 mg/kg (n=21).

Alteration in adriamycin lethality

Alterations in the lethality of adriamycin were studied after the administration of melatonin. One group of rats (n=10) is control (1% DMSO). A second group of rats (n=10) received adriamycin alone (25 mg/kg i.p.). A third group (n=10) was injected with melatonin (10 mg/kg s.c.) 1 hr before adriamycin (25 mg/kg i.p.) and every 12 hr thereafter 6 days. A fourth group (n=10) received only melatonin (10 mg/kg s.c.) every 12 hr for 6 days.

Alterations of adriamycin-induced body weight change and cardiotoxicity

Four groups of rat were used to evaluate the body weight change and cardiotoxicity from adriamycin: control (n=8), adriamycin alone (n=9), melatonin alone (n=10), adriamycin with melatonin (n=10). In the adriamycin group, 15 mg/kg adriamycin was intraperitoneally injected once, while in the adriamycin with melatonin group, rats were treated with melatonin (10 mg/kg s.c.) for 6 consecutive days, starting two days prior to adriamycin injection. To observe any effect of melatonin on normal rats, melatonin (10 mg/kg s.c.) was administered daily for five consecutive days in the melatonin alone group. Body weights were measured every other day. Deaths were recorded daily. To assess the severity of myocardial tissue damage, MDA and LDH levels were assayed using the protocols described below. Six days after adriamycin injection, heart slices were prepared under pentobarbital anesthesia for the measurement of the levels of MDA and LDH described above.

Measurement of LDH release

For the measurement of LDH release, heart slices were centrifuged at 1,000 rpm for 5 min. The pellet was discarded and the supernatant was saved. LDH activity was determined in the supernatant and incubation medium using a LDH assay kit (Asan Pharm. Co., LTD., kyunggee-do, Korea). Final values were expressed as the relative percent of the control group.

Lipid peroxidation assay

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) according to the method of Okawa et al (1979). Heart slices were homogenized in an ice-cold 1.15% KCl (5% wt/vol). A 0.2 ml aliquot of homogenate was mixed with 50 μl of 8.1% sodium dodecyl sulfate, and incubated for 10 min at room temperature. Acetic acid (375 μl, 20%, pH 3.5) and 375 μl of thiobarbituric acid (0.6%) were added. The mixture was heated for 60 min in a boiling water bath. The samples were allowed to cool at room temperature. After addition of *n*-butanol and pyridine (15 : 1, 1.25 ml), the contents were vigorously vortexed and centrifuged at 1,000 rpm for 5 min. The absorbance of the upper, colored layer was measured at 535 nm and 520 nm with a spectrophotometer (Hitach, U-2000, Japan) and compared with freshly prepared 1,1,3,3-tetraethoxypropane standards. Final values were expressed as the relative percent of the control group.

Morphologic study

Three groups of rat were used to evaluate the morphologic alterations in the heart from adriamycin: control (n=2), adriamycin alone (n=3), adriamycin with melatonin (n=3). In the adriamycin group, 15 mg/kg adriamycin was intraperitoneally injected once, while in the adriamycin with melatonin group, rats were treated with melatonin (10 mg/kg s.c.) for 6 consecutive days, starting two days prior to adriamycin injection. Six days after adriamycin injection, the hearts were prepared under pentobarbital anesthesia as described in *Preparation of heart slices* section and fixed by perfusing through the aorta for 10 min with 0.1 M phosphate buffer containing 1.7% glutaraldehyde and 1.6% paraformaldehyde at a pressure of 120 mmHg. The hearts were then excised and stored in fixative. Random samples of heart tissues were postfixed in Epon 812. Ultra-thin sections (50~70 nm) were obtained, stained with uranyl acetate and lead nitrate, and viewed in an electron microscope (Jeol 1200 EX II, Japan).

Statistical analysis

All values are expressed as mean±SE. The differences between mean values were analyzed by Students' *t* test, Dunnett's procedure after analysis of variance or chi square analysis, ANOVA and Kaplan Meier (Mantel-Cox) method. $P<0.05$ was considered statistically significant.

RESULTS

Survival study

Fig. 1 shows dose-response relationship of adriamycin-induced lethality. Lethality after single-dose adriamycin treatment in rats was dose-dependent manner. The lethality rate of each group was as follows: group 1 (control) and group 2 received adriamycin, 5 mg/kg, were 0% (0 out of 10); group 3 received 15 mg/kg was 15% (3 out of 20); group 4 received 25 mg/kg was 86% (18 out of 21).

Fig. 2 shows alteration in the lethality of adriamycin (25 mg/kg i.p.) after the administration of melatonin (10 mg/kg s.c. for 7 days). Treatment of melatonin significantly attenuated adriamycin-induced lethality from 86 to 20% (2 out of 10) ($P<0.05$). Melatonin alone, when given according to this regimen, did not produce lethality in any rats.

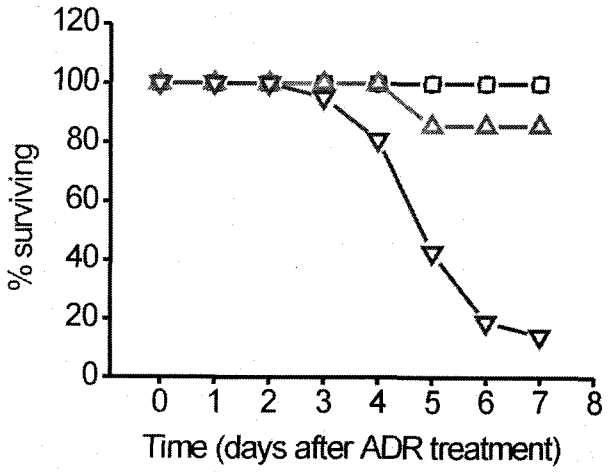

Fig. 1. Effect of three different doses of adriamycin on lethality. ○, represents a control (n=10). □, represents a single dose of adriamycin, 5 mg/kg i.p. (n=10). △, represents a single dose of adriamycin, 15 mg/kg i.p. (n=20). ▽, represents a single dose of adriamycin, 25 mg/kg i.p. (n=21).

Body weight change

Fig. 3 shows the effect of melatonin on the alteration of adriamycin-induced body weight changes. Adriamycin injection significantly decreased mean body weight on day 7 by 16% of the initial weight compared to controls ($P<0.05$). In contrast, the body weight of adriamycin with melatonin treatment group decreased by 5%. Treatment with melatonin ameliorated the adriamycin-induced body weight loss ($P<0.05$). However, melatonin alone did not have any significance on body weight in control group.

Changes in the levels of MDA

MDA is a final product of lipid peroxidation and can be used as an index for determining the extent of lipid peroxidation. Fig. 4 shows the effect of melatonin on the alteration of adriamycin-induced lipid peroxidation. Significantly elevated levels of lipid

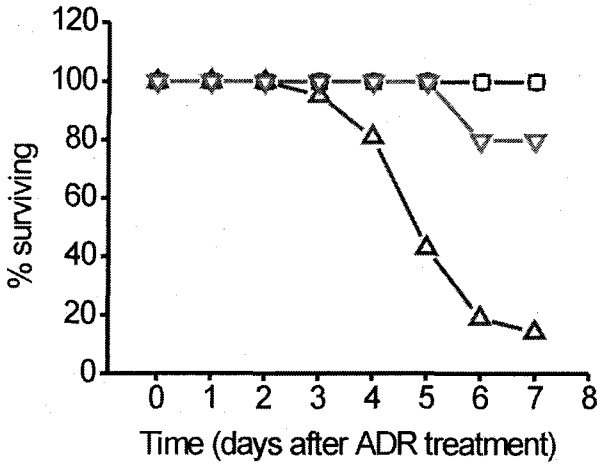

Fig. 2. Effect of melatonin on adriamycin-induced lethality. ○, represents the percentage of animals surviving at various times in control (n=10). △, represents the percentage of animals surviving at various times after a single dose of adriamycin, 25 mg/kg i.p. (n=21). ▽, represents the percentage of animals surviving at various times after a single dose of adriamycin, 25 mg/kg i.p. in the group treated with melatonin, 10 mg/kg s.c.1 hr before adriamycin and every 12 hr thereafter for 6 days (n=10). □, represents a single dose of melatonin, 10 mg/kg s.c. (n=10). This graph demonstrates that treatment with melatonin reduces the death rate from adriamycin toxicity.

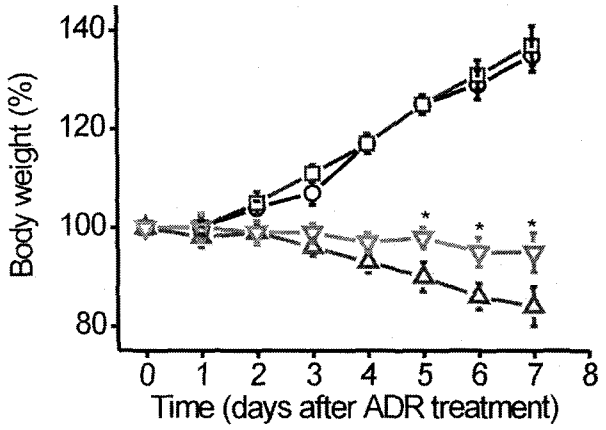

Fig. 3. Time course of body-weight changes in rats. Data are mean±SE of twelve experiments. ○: the percentage of animals body-weight at various times in the control group; △: the percentage of animals body-weight at various times after a single dose of adriamycin, 25 mg/kg i.p.; ▽: the percentage of animals body-weight at various times after a single dose of adriamycin, 25 mg/kg i.p. in the group treated with melatonin, 10 mg/kg s.c.1 hr before adriamycin and every 12 hr thereafter for 6 days; □: a single dose of melatonin, 10 mg/kg s.c. Melatonin treatment attenuates adriamycin-induced body-weight loss, although melatonin alone did not have any affect on body weight change in normal rats.

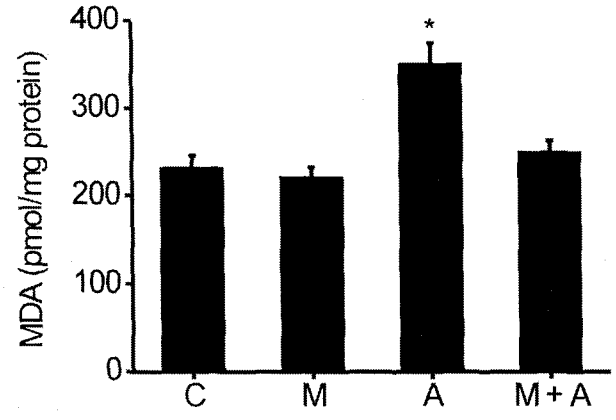

Fig. 4. Effect of melatonin treatment on adriamycin- induced changes of lipid peroxidation as indicated by MDA. C, control (n=8); M, melatonin (10 mg/kg s.c. for 5 days) (n=10); A, adriamycin (15 mg/kg i.p.) (n=9); M+A, melatonin (10 mg/kg s.c. for 5 days)+adriamycin (15 mg/kg i.p.) (n=10). Data are mean±SE. $*P<0.05$ compared with C, M and M+A groups.

peroxidation were observed in the group received only adriamycin (15 mg/kg i.p.) when compared to those of the control group ($p<0.05$). When the adriamycin-injected animals were pretreated with melatonin (10 mg/kg s.c. for 5 days), the levels of lipid peroxidation were lowered with respected to the group received only adriamycin ($P<0.05$). Melatonin alone did not affect the levels of lipid peroxidation.

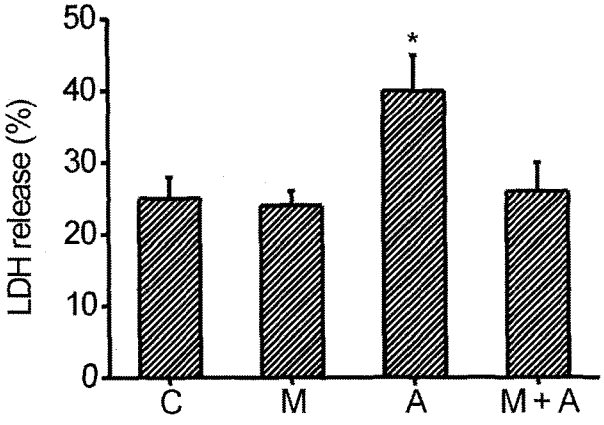

Fig. 5. Effect of melatonin treatment on adriamycin-induced changes of LDH release. C, control (n=8); M, melatonin (10 mg/kg s.c. for 5 days) (n=10); A, adriamycin (15 mg/kg i.p.) (n=9); M+A, melatonin (10 mg/kg s.c. for 5 days)+adriamycin (15 mg/kg i.p.) (n=10). Data are mean±SE. $*P<0.05$ compared with C, M and M+A groups.

Changes in the level of LDH release

The release of LDH is considered to be an indicator of myocardial damage by adriamycin. The levels of LDH release were significantly ($P<0.05$) elevated after a single injection of adriamycin (15 mg/kg i.p.) reaching 40% as compared with the control group (25%). Treatment with melatonin (10 mg/kg s.c. for 5 days) decreased significantly the levels of LDH release by 45% ($P<0.05$). However, melatonin alone did not have any significant effect on the levels of LDH release in normal rats.

Morphologic study

Morphological changes in the adriamycin group were typical for adriamycin-induced cardiomyopathy and included swelling of mitochondria, vacuolization of the cytoplasm, and loss of myofibrils (Fig. 6A). Ultrastructure of hearts from the melatonin+adriamycin group was indistinguishable from that of the control group (data not shown) and had regular

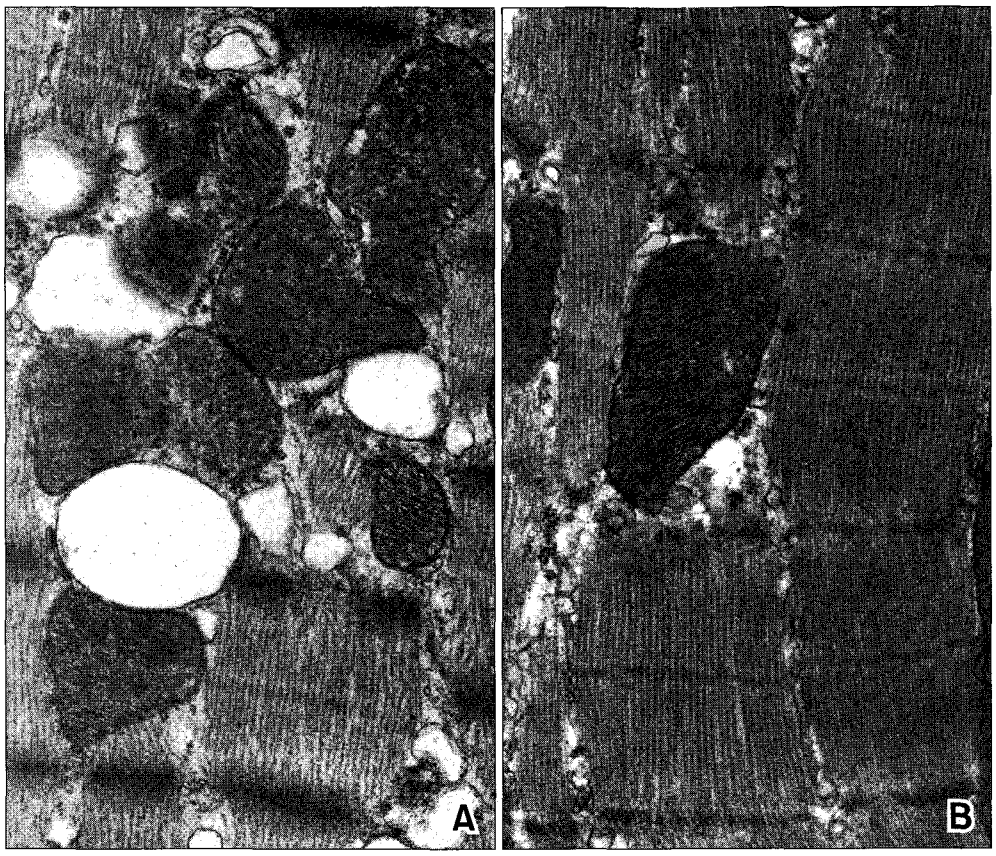

Fig. 6. A. Electron micrograph of adriamycin-induced changes included loss of myofibrils, swelling of mitochondria. Vacuolization is also apparent. B. Hearts from the melatonin and adriamycin group did not show any of these changes. Mitochondria, myofibrils, and other cellular details are normal. Magnification, 15000.

myofibrillar arrangement, and preserved mitochondria (Fig. 6B).

DISCUSSION

In the present study, we used a single intraperitoneal (i.p.) injection of adriamycin. We observed a marked increase in lethality following adriamycin administration, body weight loss and increased the levels of lipid peroxidation and LDH release, accompany by the characteristic structural alterations. The effectiveness of melatonin was demonstrated by the reduced body weight loss, amelioration of the increase in lipid peroxidation and LDH release, an increased survival rate and prevention of cardiomyopathic changes.

Adriamycin, a quinone containing anthracycline antibiotic with potent anticancer activity (Fig. 7), also produces a lethal cardiotoxicity after several weeks or

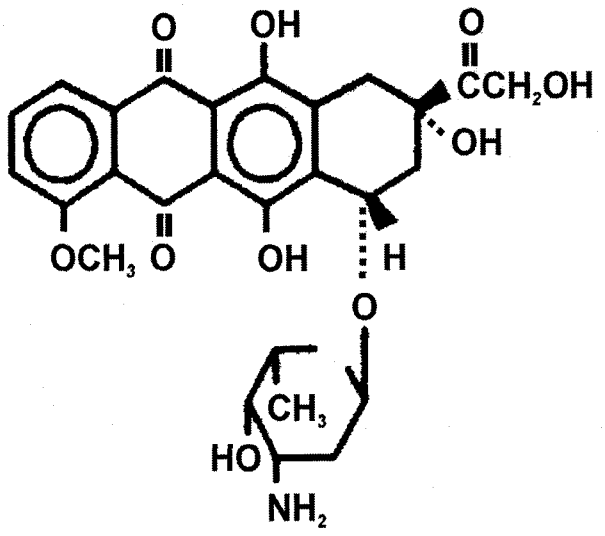

Fig. 7. The chemical structural formula of adriamycin. Aminosugar is linked to tetracyclic aglycone through a glycosidic bond. Presence of quinone ring confers radical production ability.

months of treatment, and sometimes even after the therapy has been completed (Lefrak et al, 1973). The most characteristic morphological alterations in adriamycin-induced cardiotoxicity include the loss of fibrils, dilation of the sarcoplasmic reticulum, cytoplasmic vacuolization, swelling of mitochondria and increased number of lysosomes (Jaenke & Fajardo, 1977; Von Hoff et al, 1979). It has been known that loss of myofibrils and vacuolization of the cardiac myocytes are two important ultrastructural markers for adriamycin-induced cardiomyopathy, which can be reproducibly seen in the myocardium of rats exposed to adriamycin (Fig. 6A). It is likely that rats simulate most of the clinical and hemodynamic changes (Siveski-Iliskovic et al, 1994). The functional refractoriness of adriamycin-induced cardiomyopathy and heart failure observed in human has been observed in rats (Weinberg & Singal, 1987; Tong et al, 1991). Thus, in the present study, we used rat as an animal model of adriamycin-induced cardiomyopathy.

Since the first report of adriamycin-induced cardiomyopathy (Bonadonna et al, 1970), a number of mechanisms have been proposed to explain the development of adriamycin-induced cardiomyopathy, including the inhibition of nucleic acid and protein synthesis (Buja et al, 1973; Arena et al, 1974), release of vasoactive amines (Bristow et al, 1980), changes in adrenergic function (Tong et al, 1991), abnormalities in the mitochondria (Gosalvez et al, 1979), lysosomal alterations (Singal et al, 1985), altered sarcolemmal Ca^{2+} transport (Singal & Pierce, 1986), changes in adenylate cyclase, Na^{+}-K^{+} ATPase and Ca^{2+}-ATPase (Singal & Panagia, 1984), imbalance in myocardial electrolytes (Olson et al, 1980), free radical formation (Kalyanaraman et al, 1980; Doroshow, 1983; Singal et al, 1987), reduction in myocardial antioxidant enzyme activities (Siveski-Iliskovic, 1994), lipid peroxidation (Myers et al, 1977; Singal et al, 1985; Singal et al, 1987), depletion of non-protein tissue sulfhydryl compounds (Olson et al, 1980; Odom et al, 1992) and apoptosis (Kumar et al, 1999), indicating that the cause of adriamycin-induced cardiomyopathy is probably multi-factorial and complex. Nevertheless, it has been generally accepted that oxygen free radical mechanisms may contribute to most of these cardiotoxicity of adriamycin (Myers et al, 1977; Doroshow et al, 1983; Singal et al, 1987; Kaul et al, 1993; Siveski-Iliskovic, 1994). In the present study, increased levels of free radicals due to adriamycin were detected by an increase in tissue MDA formation which is a breakdown product of lipid peroxidation (Fig. 4). These results are consistent with the previous studies reported by other investigators (Myers et al, 1977; Singal, 1987). Moreover, adriamycin-induced free radical formation has been detected directly by electron spin resonance spectroscopy (Alegria et al, 1989; Iliskovic et al, 1999).

To prevent or mitigating the cardiotoxic side effects of adriamycin, the approach has been to develop a combination therapy with a known antioxidant or an iron chelator which will reduce oxidative stress (Myers et al, 1977; Shimpo et al, 1991). While most antioxidants and iron chelators failed to show complete protection against adriamycin-induced cardiomyopathy, in the present study, we provide considerable evidence that administration of melatonin with adriamycin may be a truly efficacious combination therapy. Melatonin has been known to be a direct free radical scavenger of the highly toxic hydroxyl radical (Stasica et al, 1998). Melatonin can also neutralize several other reactive oxygen free radicals as an endogenous antioxidant (Pieri et al, 1994; Cuzzocrea et al, 1997; King & Scaiano, 1997; Noda et al, 1999). It has been suggested that the protective effect melatonin may be related to the actions of the indoleamine. In addition to its direct free radical scavenging effect, melatonin plays a role as an indirect antioxidant by stimulating the mRNA levels and the activities of superoxide dismutase (Kotler et al, 1998), glutathione peroxidase and reductase (Pablos et al, 1998). Since melatonin is highly lipophlic (Costa et al, 1994) as well as somewhat hydrophilic (Shida et al, 1994), it easily enters cells and subcellular compartments where it prevents oxidative damage to a variety of molecules (Reiter, 1998). If adriamycin induces membrane peroxidation in cardiac tissue as a mechanism of cardiotoxicity, then the abilities of melatonin described above may be of considerable importance in detoxifying adriamycin-generated peroxides and preventing the formation of cardiac lesions.

The result of present study demonstrates that treatment with melatonin offers complete protection against adriamycin-induced: lethality (Fig. 2); alterations in body weight (Fig. 3); increase in lipid peroxidation levels (Fig. 4); and elevation of LDH release levels as a biochemical sign of this cardiotoxicity (Fig. 5). Lipid peroxidation measured by MDA has been gen-

erally accepted as an indicator of oxidative stress resulting from free radical overproduction and reduction in antioxidant reserve. Adriamycin treatment caused a significant increase in myocardial lipid peroxidation, which was completely prevented by melatonin, indicating that the antioxidant property of melatonin may be directed against lipid-radical chain reaction (Fig. 4). The protective effects against adriamycin-induced cardiotoxicity as observed with melatonin administration in this study was comparable to that obtained in rats with other chemoprotectants (Mohamed et al, 2000; Mostafa et al, 2000; Nagi & Mansour, 2000). Our findings are also consistent with previous studies where melatonin protects against oxidative damage of other reactive oxygen species-generating agent (Qi et al, 1999).

Our results suggest that melatonin may be a useful adjuvant to prevent adriamycin toxicity in cancer chemotherapy. The anticancer activity of adriamycin and lipid peroxidation are reportedly independent, and antioxidants such as vitamin E and vitamin C have been reported to reduce lipid peroxidation and cardiotoxicity of adriamycin (Myers et al, 1977; Fujita et al, 1982). In this context, we believe that the ameliorating effect of melatonin on adriamycin toxicity may not act by affecting the antitumor effect of adriamycin. However, further studies are needed to examine whether or not the antioxidant effect of melatonin interferes with the antineoplastic effect of adriamycin.

In conclusion, the results of the present study demonstrate that melatonin has a protective effect against adriamycin-induced lethality, body weight loss, lipid peroxidation and LDH release levels, and morphological changes in the rat heart implying that the cardiotoxicity of adriamycin relates to its ability to generate free radical scavenging and antioxidant activities of the indole. The findings provide substantial evidence to support clinical trials of using melatonin to prevent adriamycin-induced cardiotoxicity.

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