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Effect of melatonin on the cardiotoxicity of doxorubicin

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Summary. This study was designed to investigate the preventive effect of melatonin on doxorubicin's most important side effect, cardiotoxicity. Forty male albino Wistar rats were utilized and the rats were divided into five groups: group I, 0.9 % NaCl for 4 days; group II, doxorubicin 3 mg/kg/day for 4 days; group III, 2.5 % ethanol for 15 days; group IV, melatonin 6 mg/kg/day for 15 days; and group V, a doxorubicin and melatonin combination were administered intraperitoneally. At the end of the experiment, tissue samples obtained from the cardiac muscle of the left ventricle of the rats were processed for measurement of malondialdehyde and for electron microscopic examination. Malondialdehyde, a product of lipid peroxidation, was found to be significantly higher in the doxorubicin group. However, in the doxorubicin and melatonin combination group the level of malondialdehyde was decreased statistical significant. The histological examination revealed destruction of myofibrils, disorganization of sarcomeres, mitochondrial degeneration and formation of giant mitochondria and lipid accumulation in the doxorubicin group. Also, accumulation of filamentous structures in the sarcoplasma in some of the cells, structural changes in capillaries and an increase in collagen fibers forming bundles were observed. When melatonin was added to the doxorubicin treatment all structural changes were reduced. The cardiotoxic side effect of doxorubicin used as a chemotherapeutic agent and was probably developed as a result of supression of the antioxidant system and lipid peroxidation. Therefore, it could be assumed that the addition of melatonin in the treatment of doxorubicin could prevent the cardiotoxicity of doxorubicin.

Key words: Doxorubicin, Cardiac muscle, Melatonin, Ultrastructure.

Introduction

Doxorubicin is an anthracycline antibiotic with a wide spectrum and is used widely in cancer chemotherapy. Its clinical use has been limited by lethal cardiac toxicity (Myers et al., 1977; Ganey et al., 1991; Agapito et al., 2001; Xu et al., 2001a). Doxorubicin induces important toxic effects in brain, kidney, lung and small intestine and leads to dose-dependent irreversible cardiomyopathy and severe congestive heart failure (Wassermann et al., 1985; Zhou et al., 2001). Oxidative damage to membrane lipids and other cellular components is belived to be a major factor in doxorubicin and other anthracyclines antibiotics. Therefore, many authors reported that the use of natural or synthetic antioxidants would possibly protect against oxidative stress due to doxorubicin and other cytotoxic drugs (Unverferth et al., 1983; DeAtley et al., 1999; Agapito et al., 2001; Dziegiel et al., 2002; Kang et al., 2002; Kocak et al., 2003). Clinical administrations and physiological and biological functions of melatonin have been shown in many studies and its antioxidant property has been frequently confirmed in recent years (Dziegiel et al., 2002).

Although there have been many studies on the protective function of melatonin in doxorubicin-induced cardiotoxicity, these studies have generally dealt with biochemical and hemodynamic changes (Morishima et al., 1998; Wahab et al., 2000; Agapito et al., 2001; Xu et al., 2001a). Nevertheless, there are a few ultrastructural studies related to the protective effect of melatonin on doxorubicin cardiotoxicity. Therefore, this present study was designed to evaulate the ultrastructural effects of melatonin on doxorubicin-induced cardiotoxicity.

Materials and methods

In this study 40 male Wistar rats weighing 170-220 g were utilized. They were kept under a photoperiod of 12 h light : 12 h darkness, in controlled conditions of temperature (22±2 °C), with food and water ad libitum. The rats were distributed in five groups of eight animals each. Group I (control I) was treated intraperitonally with 0.9% NaCl for 4 days. Group II was administered with doxorubicin (Adriblastina-Carlo Erba) dissolved in 0.9% NaCl intraperitonally for 4 days at a dose of 3 mg/kg/day. Group III (control II) was treated intraperitonally with 2.5% ethanol for 15 days. In Group IV, melatonin (Sigma) dissolved in 2.5% concentrated ethanol was administered intraperitonally for 15 days at a dose of 6 mg/kg/day and Group V was treated with a doxorubicin and melatonin combination treatment.

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Melatonin treatment was begun 1 day before doxorubicin treatment. When the two drugs were given in combination, melatonin was administered 3 hr before each doxorubicin injection (melatonin was injected at 09:00 am and doxorubicin at 12:00 am). The combination treatment was given for 4 days and then melatonin was continued to be given to the group for 11 days.

At the end of all treatments, cardiac perfusion was performed under anaesthesia for the histological examination of the rats. After perfusion the heart was removed and cardiac tissue samples of the left ventricle were obtained. Tissue samples were immediately placed in 5% glutaraldehyde buffered at pH 7.4 with Millonig phosphate buffer for 4 h and subsequently fixed in 1% osmium tetroxide for 2 hr. The samples were dehydrated in graded ethanol and embedded in araldite. Thin sections were stained with lead citrate and uranyl acetate and examined with a Zeiss EM 10B electron microscope. In addition, tissue samples were taken for biochemical analyses, placed in 0.9% NaCl. Malondialdehyde (MDA) level was determined by the thiobarbituric acid (TBA) method (Ohkawa et al., 1979).

All the results were expressed as mean values ± SEM. Data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls multiple test to compare the groups. A value of $p<0.05$ was accepted as significant.

Results

Biochemical analyses

The malondialdehyde levels measured in the tissues in the experimental group were significantly higher than those of the control group ($p<0.05$), and the malondialdehyde levels measured in the tissues of the doxorubicin+melatonin-treated group were significantly lower than those measured in the group treated merely with doxorubicin ($p<0.05$). The values of MDA are shown in Table 1.

Electron microscopic findings

In group I, which received 0.9% NaCl, group III, which received 2.5% ethanol, and group IV, which received 6 mg/kg/day of melatonin , the nuclei and cytoplasm of myocardial fibers were normal in structure. The nuclei of the cells were regularly outlined and exhibited a normal chromatin content. Myofibrils exhibited a regular sarcomere organization. Cytoplasmic organelles and capillary structures between the myofibrils showed a normal structure (Figs. 1-3).

The samples obtained from group II, which received 3 mg/kg/day of doxorubicin, displayed normal nuclei and sarcoplasmic reticulum. Degenerative changes in mitochondria were more prominent. The number and size of mitochondria increased (Fig. 4). Furthermore, an increase in matrix density, pleomorphism and the presence of numerous giant mitochondria were striking. There was also mitochondrial damage as a lysis of matrix and fragmentation of cristae, resulting in the formation of vacuoles containing membranous structures. Myocardial fibers exhibited disorganization of the sarcomere, disruption of myofibrils and an increase in Z- line density. Numerous and variously-sized lipid droplets were seen where the structural alterations were prominent (Fig. 5). In addition, sarcoplasmic lysis, membranous structures and degeneration of discus intercalaries were prominent features (Fig. 6). In some of the cells, there were filamentous structures forming bundles or small aggregates and sarcoplasma showed degenerative changes (Figs. 7, 8). Also, increment of collagen fibers in the connective tissue was striking (Fig. 9).

In group V, administered with the doxorubicin and melatonin combination therapy, myocardial fibers and discus intercalaries between them generally preserved their normal structure. The nuclei of myocardial fibers were regularly outlined and displayed normal chromatin organization (Fig. 10). A few lipid droplets were dispersed in the sarcoplasma, but they were less prominent in comparison with group II.

In some of the cells mitochondria were increased in number, but mitochondrial degeneration and pleomorphic and giant mitochondria were decreased in number in comparison to the control group (Fig. 11). In this group, myofibrillar degeneration was not seen and sarcomere organization was protected. Blood capillaries and collagen fibers between the myocardial cells had a normal structure (Fig. 12).

Discussion

The results of this study revealed striking structural changes, especially in the mitochondria of the myocardial cells of the rats in the doxorubicin-treated group. It is assumed that the changes have resulted from

Table 1. The values of biochemical studies of control and experimental group as statistical values.

	GROUP I (control I)	GROUP II (Doxorubicin)	GROUP III (control II)	GROUP IV (Melatonin)	GROUP V (Doxorubicin+melatonin)
Malondialdehyde (nmol/ml)	5.51±0.43	14.22±4.25[a]	3.57±0.81	4.67±0.43	5.85±1.10[b]

Values are expressed as mean (X) ± Standard error (SE) from eight animals in each group. [a]: significantly different from Group I ($p<0.05$); [b]: significantly different from Group II ($p<0.05$).

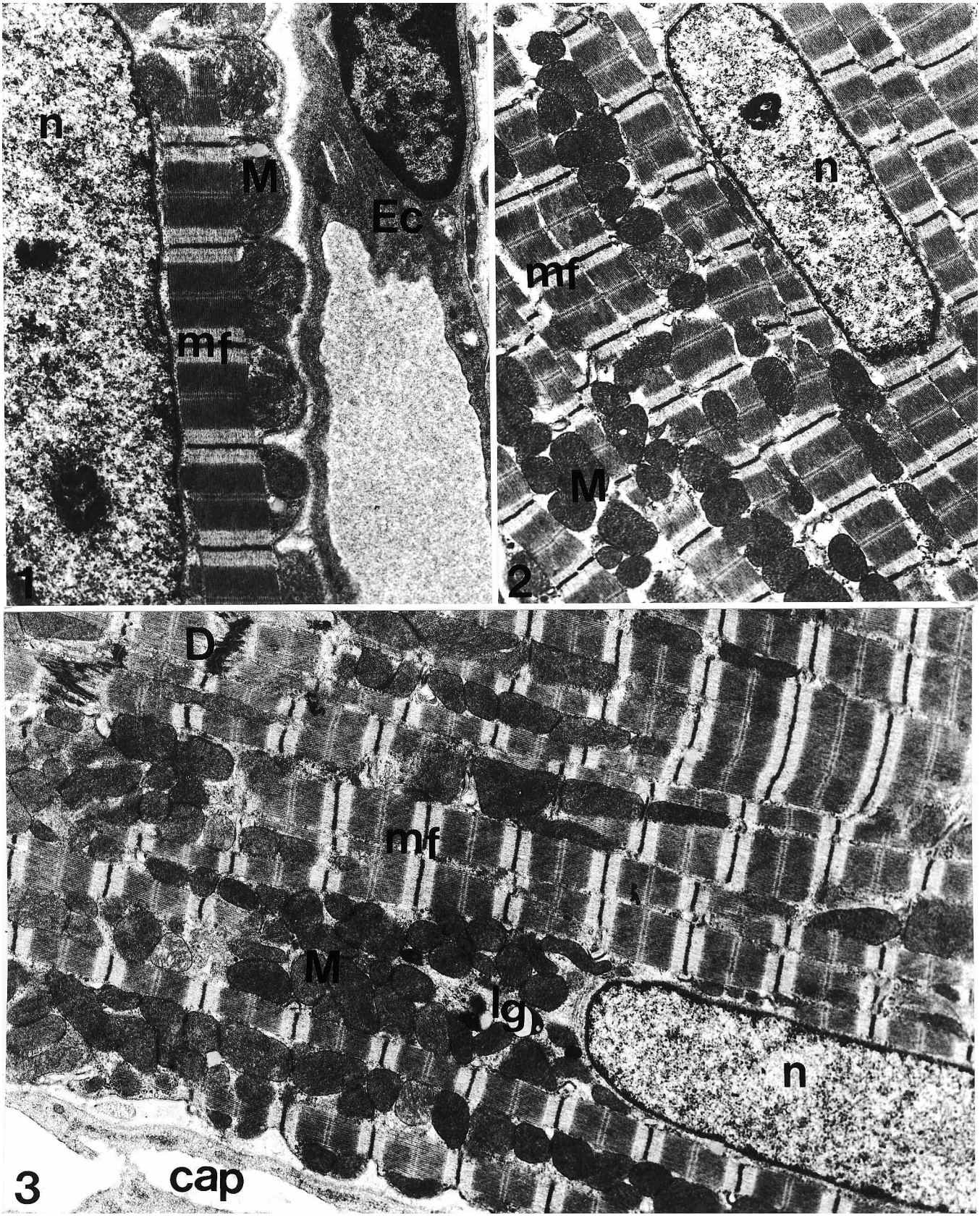

Fig. 1. Group given 0.9% NaCl. Myofibrils (mf), mitochondria (M), nucleus (n), endothelial cell of capillary (Ec). x 12,075

Fig. 2. Group given 2.5% ethanol. Myofibrils (mf), nucleus (n), mitochondria (M). x 9,300

Fig. 3. Group given melatonin. Myofibrils (mf), mitochondria (M), lipofuscin granule (lg), capillary (cap), nucleus (n), discus intercalaries (D). x 9,112.5

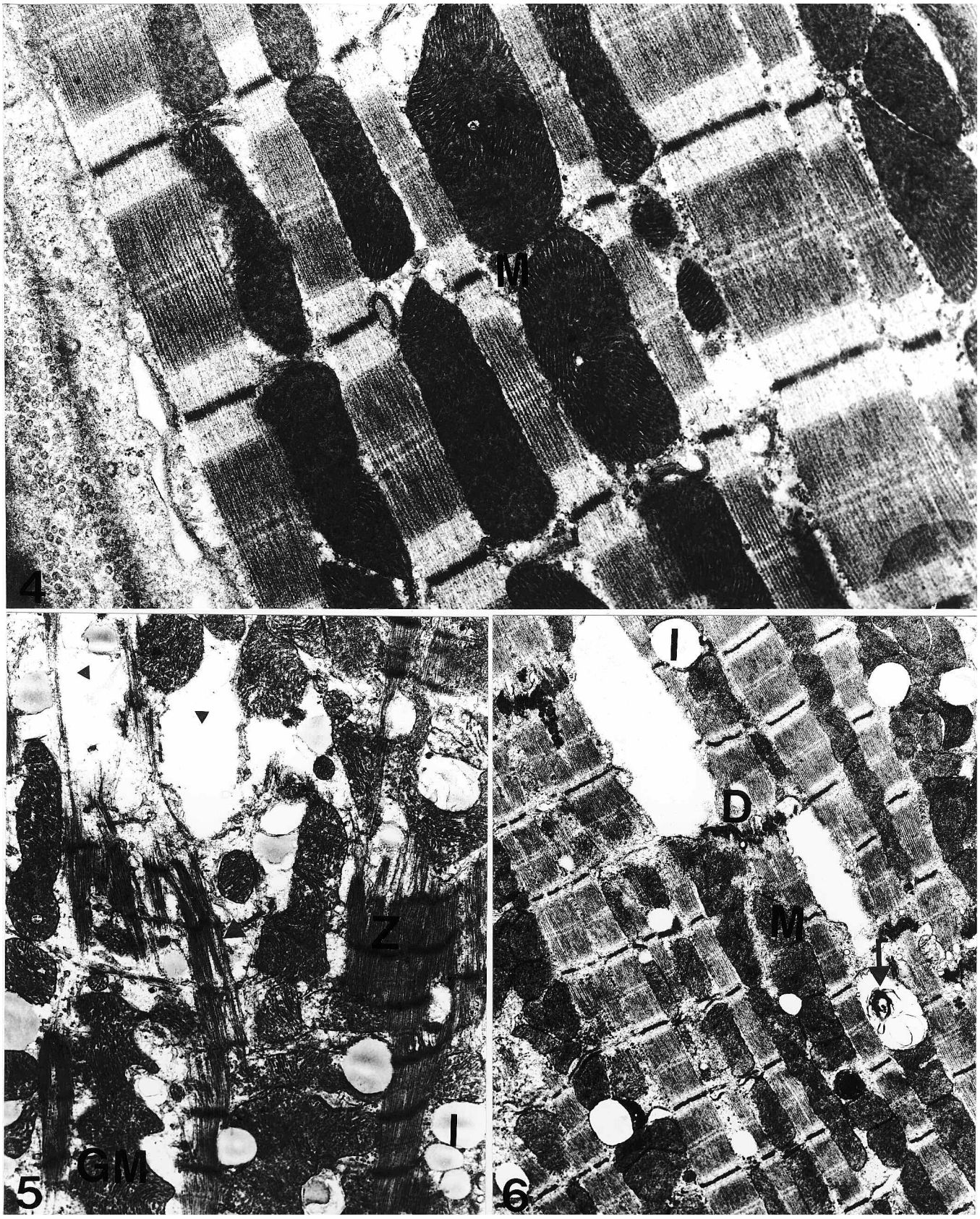

Fig. 4. Group given doxorubicin. Different shaped and sized electrondense mitochondria (M) appear between the myocardial fibers. x 22,500

Fig. 5. Group given doxorubicin. Myofibrillar loss and disorganization (arrow), lytic areas (arrow head) and an increase in Z-line density (Z) are seen. Lipid droplets (l), giant mitochondria (GM). x 9,300

Fig. 6. Group given doxorubicin. Lipid droplets (l), mebranous structures (arrow) and degeneration of discus intercalaries (D) are seen. Mitochondria (M). x 9,300

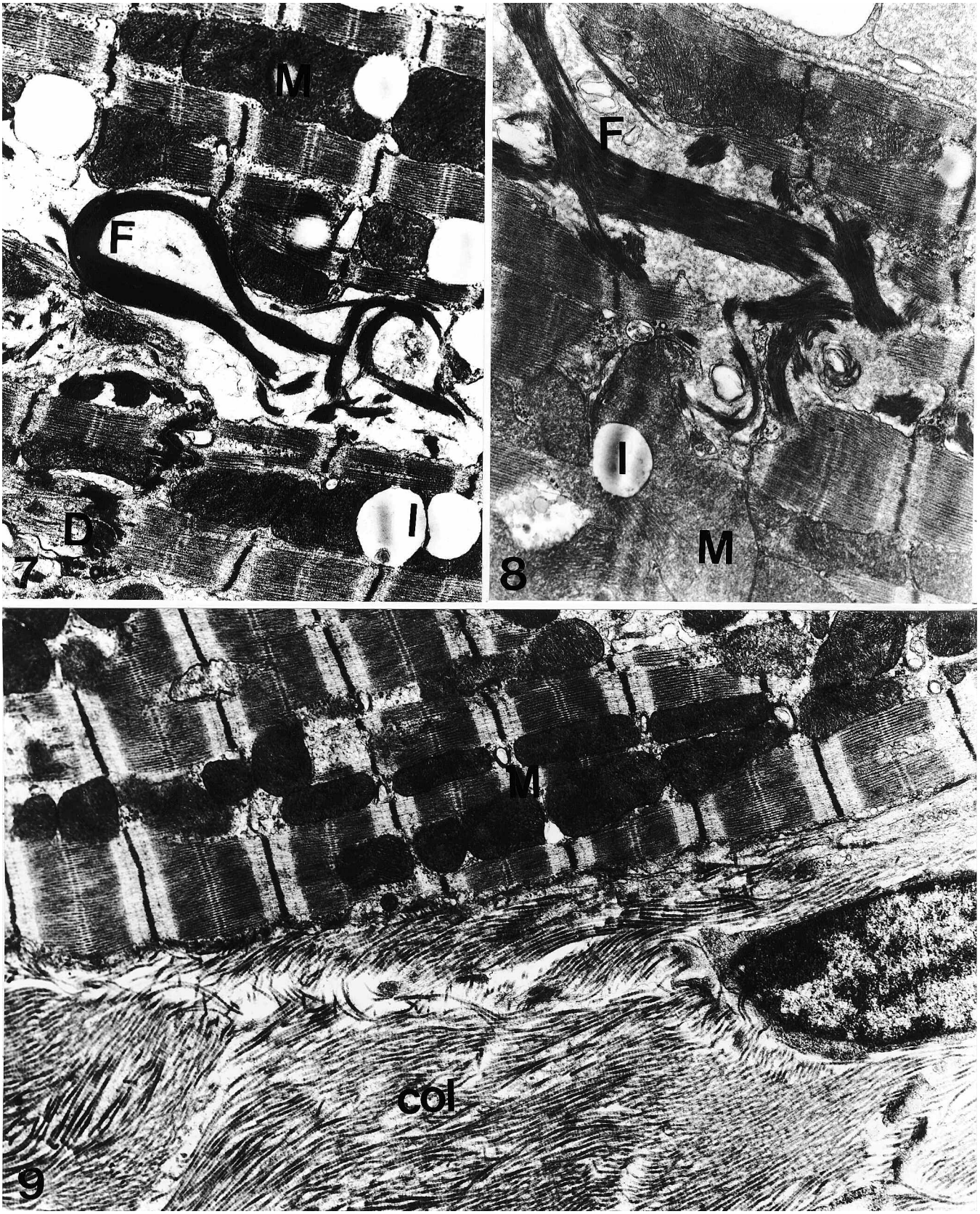

Fig. 7. Group given doxorubicin. Lipid droplets (I) between myofibrils, increased matrix density of mitochondria (M) and filamentous bundles (F) are observed. Discus intercalaries (D). x 15,000

Fig. 8. Group given doxorubicin. Filamentous structures forming bundles (F), lipid droplets (I), mitochondria (M). x 18,600

Fig. 9. Group given doxorubicin. Collagen fibers increased in connective tissue (col) and increased matrix density of mitochondria (M). x 13,950

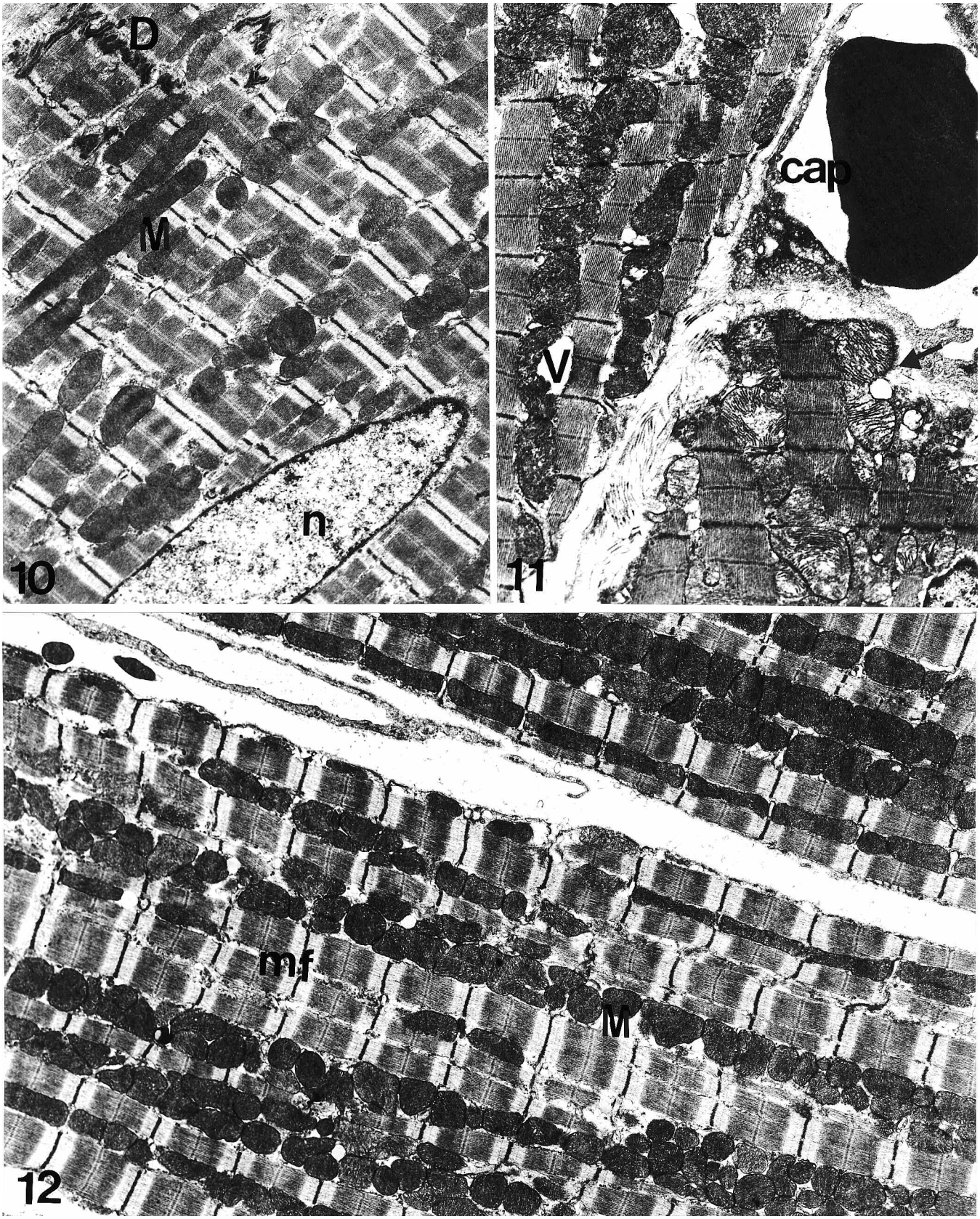

Fig. 10. Group given doxorubicin plus melatonin. Different shaped and sized mitochondria (M), nucleus (n) and discus intercalaries (D) are seen. x 7,500

Fig. 11. Group given doxorubicin plus melatonin. Normal sarcomere organization, mitochondrial degeneration (arrow) and vacuolization (V) are observed. Capillary (cap). x 9,300

Fig. 12. Group given doxorubicin plus melatonin. Myofibrils (mf) are seen in normal structure. Mitochondria (M). x 7,087.5

the free radicals arising from the effects of doxorubicin, and that the mitochondrial structures were impaired due to free radicals causing lipid peroxidation in mitochondrial membranes.

It is known that changes in the mitochondrial membrane stability and permeability are caused by lipid peroxidation; the process results in the decrease in enzymatic activities which are essential for oxidative phosphorylation and ATP production. Thus, all metabolic reactions diminish and this condition gradually leads to the death of the cell. The present study revealed that the increase in the number and the volume of the mitochondria could be a compensatory response of the cell in order to supply the energy needed for all the reactions involving the metabolic activity of the cell. In addition, the related studies indicated that the production of free-radical metabolites stimulated by doxorubicin caused lipid peroxidation in the mitochondrial membranes (Pollakis et al., 1983) and increment of intracellular respiratory activity (Mailer and Petering, 1976; Demant and Jensen, 1983) and ATP level (Seraydarian and Artaza, 1979). Furthermore, alteration of mitochondrial calcium regulation (Zhou et al., 2001), mitochondrial swelling (Nicolay et al., 1986; Zhou et al., 2001) and high density of mitochondria caused by increased matrix volume (Wassermann et al., 1985) had been previously reported. Additionally, the presence of variably-sized lipid droplets in the cytoplasm of myocardial cells might be related to mitochondrial damage induced by doxorubicin, decreased ATP production and the ß-oxidation mechanism in the damaged mitochondria due to the enzymatic changes. In fact, Singal et al. (1987) have reported that there was lipid accumulation in the cell after doxorubicin treatment.

It is thought that the giant mitochondria observed in the experimental group in our study could appear as an adaptative response to the energy required for the intracellular reactions. Furthermore, the adjacent mitochondria might fuse and try to decrease intracellular reactive oxygen radical levels by decreasing the consumption of oxygen. These findings seem to be closely related to those appearing in the related literature (Matsuhashi et al., 1997; Wakabayashi et al., 1997; Wakabayashi, 2002). These studies revealed that oxidative stress induced by free radicals plays an important role in the formation of megamitochondria based on morphological and biochemical changes in mitochondrial membranes. In accordance, Wakabayashi (2002) reported that mitochondria try to decrease the level of intracellular oxygen radicals by decreasing the consumption of oxygen via formation of megamitochondria.

In the doxorubicin-treated group, it was also observed that there were various degenerative changes in sarcomere structures, particularly thin filaments, Z discs and discus intercalaries. It might be thought that these ultrastructural changes may be related to doxorubicin interaction with actin which is a major component of the Z discs and thin filaments. Previously, it has been reported that doxorubicin effects on the myocardial cell membrane, nucleic acids of DNA and cardiac myofibril proteins forming cytoskeleton and fine filaments (Lewis and Gonzalez, 1986; Mariano et al., 1986). Most of the related studies have reported that myofibrils damage (Jaenke, 1976; Mortensen et al., 1986; Torti et al., 1986; Singal et al., 1987; Tong et al., 1991; Qintana et al., 1994; Susman et al., 1997; Suzuki et al., 1997; Xu et al., 2001a,b; Zhou et al., 2001; Zeidán et al., 2002) and that there are structural changes in discus intercalaries (Nicolay et al., 1986; Zeidán et al., 2002) could be a result of the usage of doxorubicin. Mariano et al. (1986) reported that doxorubicin caused actin polymerisation, and Lewis and Gonzales (1986) reported the inhibition of protein synthesis and the decrease in the level of cytoplasmic actin.

In the doxorubicin-treated group, the collagen fibers between the myocardial cells were increased and formed thick bundles. This phenomenon might be thought to be due to the fact that lipid peroxidation caused by doxorubicin could stimulate collagen synthesis. The experimental and clinical studies have already shown the relationship between fibrosis and lipid peroxidation (Parola et al., 1993; Poli and Parola, 1997). Parola et al. (1993) reported that oxidative reactions directly stimulate procollagen Type1 gene expression which contributes to the development of fibrosis.

In the present study, melatonin was utilised as an antioxidant agent in order to eliminate the effects of free radicals. The antioxidative effects of melatonin were believed to play an important role in the improvement of myocardial structures. Furthermore, the normalization of malondialdehyde levels by the concurrent treatment with melatonin might have resulted from the inhibitory effect of melatonin on lipid peroxidation. These results are also similar to the observation of other authors (Morishima et al., 1998; Wahab et al., 2000; Agapito et al., 2001; Xu et al., 2001a; Dziegiel et al., 2002).

In conclusion, this study revealed that lipid peroxidation was developed and that the antioxidant system was suppressed due to doxorubicin and that as a result, ultrastructural changes became prominent. Additionally, the doxorubicin+melatonin combination treatment prevented cellular damage, which were caused by the melatonin effect on the free radicals and by the inhibition of lipid peroxidation.

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