EXPERIMENTAL STUDY

Cardioprotective effect of melatonin and agomelatine on doxorubicin-induced cardiotoxicity in a rat model: an electrocardiographic, scintigraphic and biochemical study

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ABSTRACT

AIM: The present study aimed to determine the protective effect of melatonin and agomelatine on DOX-induced cardiotoxicity in rats by electrocardiographic, scintigraphic and biochemical methods.

MATERIALS AND METHODS: Forty-nine male Wistar rats were randomly separated into seven groups; control (CON), doxorubicin (DOX), melatonin (MEL), agomelatine (AGO), melatonin+doxorubicin (MEL+DOX), agomelatine+doxorubicin (AGO+DOX) and melatonin+agomelatine+doxorubicin (MEL+AGO+DOX) groups. Cardiotoxicity was induced by intraperitoneal (i.p.) injection of DOX (18 mg/kg daily for three days). Rats receiving MEL and AGO treatment in the DOX-induced cardiotoxicity group received MEL and AGO (40 mg/kg/day, i.p., for seven days). They were injected with doxorubicin (18 mg/kg, i.p.) on days 5, 6, and 7. The rats were given MEL and AGO as substance control (40 mg/kg/day, i.p., for 7 days). On day 8 of the experiment, animals were evaluated by means of electrocardiography (ECG) and 99mtechnetium pyrophosphate (99mTc PYP) scintigraphy and their biochemical parameters [blood urea nitrogen (BUN), creatine kinase (CK), cardiac troponin T (cTnT)] were examined.

RESULTS: DOX-induced acute cardiotoxicity in rats is characterized by conduction abnormalities in the ECG pattern (including decreased P wave and QRS complex duration, increased QT and RR intervals, and ST-segment elevation), increased serum BUN, CK, and cTnT parameters and increased 99mTc PYP uptake (p < 0.001). Pretreatment with MEL, AGO, or MEL+AGO effectively alleviated DOX-induced ECG abnormalities close to normal (p < 0.001). Moreover, serum biochemical evidence and 99mTc PYP uptake values demonstrated that pretreatment with MEL, AGO, or MEL+AGO has the same protective effect against the abnormalities produced in the heart by DOX (p < 0.001).

CONCLUSIONS: MEL and AGO have a potential protective effect on DOX-induced cardiotoxicity. At the same time, this study suggests that 99mTc PYP is a non-invasive method suitable for early determination of DOX-induced cardiotoxicity (Tab. 3, Fig. 5, Ref. 41). Text in PDF www.elis.sk.

KEY WORDS: melatonin, agomelatine, doxorubicin, cardiotoxicity, rat, 99mTc pyrophosphate.

Introduction

Doxorubicin (DOX) is an eminently effective chemotherapeutic drug; however, its clinical use is limited because of its severe cardiotoxicity (1, 2). DOX-induced cardiac toxicity involves an immense clinical spectrum and is explained by early and late effects. Early effects can be detected by electrocardiogram (ECG) changes, namely ST-segment elevation, arrhythmias and sinus tachycardia. Late indications of DOX administration take form of congestive heart failure and cardiomyopathy (1, 3, 4). However, the mechanism underlying DOX-induced cardiotoxicity is not clearly understood. Among various mechanisms, it is generally admitted that DOX induces oxidative stress through enhanced reactive oxygen species (ROS) production and depletion of endogenous antioxidants and this triggers the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes (5, 6, 7).

Many studies have shown that potent antioxidants protect the heart against DOX toxicity by reducing oxidative stress (8, 1). Melatonin (MEL) is a secretory product of the pineal gland of all mammals and an important natural antioxidant. It also influences various biological processes such as circadian rhythms, and cardiovascular, neuroendocrine and immune functions. It may also reduce DOX-induced oxidative stress (9). Recent studies provide direct confirmation that melatonin protects against DOX-induced toxicity by inhibiting ROS production. This implies that MEL or MEL receptor agonists might produce protective effects on DOX-induced cardiotoxicity (1, 10, 11–12).

Agomelatine (AGO) is a melatonergic M1 and M2 receptor agonist and also a serotoninergic (5-HT2C) receptor antagonist (13).
It displays a high affinity for M1 and M2 melatonin receptors and mimics the role of MEL in antioxidant properties (14). Many studies have shown that AGO has a protective effect on brain, kidneys, testes, and ovaries. It protects from ischemia-reperfusion injury by enhancing antioxidant properties. A new study by Jia et al (15), demonstrated that AGO protects the heart against myocardial ischemia-reperfusion injury.

However, the protective effect of AGO on DOX-induced cardiotoxicity has not been examined. Therefore, we have investigated the possible cardioprotective effects of AGO alone and in combination with MEL against DOX-induced cardiotoxicity by electrocardiographic, scintigraphic and biochemical methods.

Materials and methods

Chemicals

DOX hydrochloride was purchased from Sandoz Pharmaceutical Industry, Turkey. MEL and AGO were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). MEL and AGO were dissolved in 1 % ethyl alcohol. The required doses were determined in accordance with previous studies and were administered i.p. in a volume of 1 mL for 7 days (12, 15).

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Fig. 1. Limb lead at position II during ECG recording. The negative electrodes are attached to the dermal layers of the front leg paws and the positive electrode is attached to that of the left hind leg paw.

Fig. 2. ECG patterns in the control group (CON, A), doxorubicin administration group (DOX, B), melatonin+doxorubicin group (MEL+DOX, C), agomelatin+doxorubicin group (AGO+DOX, D), and melatonin+agomelatin+doxorubicin group (MEL+AGO+DOX, E). The DOX group had a significant elevation of ST segment amplitude and prolongation of QT interval compared to the control group (** p < 0.001) but MEL+DOX, AGO+DOX, and MEL+AGO+DOX did not demonstrate significant changes compared to the control group. Similarly to the CON group, the pre-treatment groups of MEL+DOX, AGO+DOX and MEL+AGO+DOX groups had a significantly decreased ST-segment amplitude and QT interval duration compared to DOX group (** p < 0.001).
Tab. 1. The statistical analysis of electrocardiographic parameters for all groups.

| Groups          | P wave (duration, sec) | QRS complex (duration, sec) | QT interval (duration, sec) | RR interval (duration, sec) | ST segment amplitude (mV) |
|-----------------|------------------------|-----------------------------|-----------------------------|-----------------------------|--------------------------|
| CON             | 0.03±0.01              | 0.06±0.02                   | 0.13±0.01                   | 0.05±0.03                   |
| DOX             | 0.02±0.08              | 0.03±0.01                   | 0.25±0.02                   | 0.18±0.03                   |
| MEL             | 0.03±0.01              | 0.06±0.02                   | 0.13±0.02                   | 0.05±0.04                   |
| AGO             | 0.03±0.01              | 0.06±0.02                   | 0.14±0.02                   | 0.05±0.03                   |
| MEL+DOX         | 0.03±0.01              | 0.06±0.02                   | 0.15±0.02                   | 0.06±0.01                   |
| AGO+DOX         | 0.03±0.01              | 0.06±0.02                   | 0.14±0.01                   | 0.06±0.03                   |
| MEL+AGO+DOX     | 0.03±0.01              | 0.06±0.01                   | 0.12±0.01                   | 0.05±0.01                   |

CON = control, DOX = doxorubicin, MEL = melatonin, AGO = agomelatine, and MEL+DOX = melatonin+doxorubicin, AGO+DOX = agomelatine+doxorubicin, and MEL+AGO+DOX = melatonin+agomelatine+doxorubicin. *p<0.001 in all groups as compared to CON groups, *p<0.001 in MEL+DOX, AGO+DOX, MEL+AGO+DOX groups as compared to DOX groups.

Animals

Forty-nine adult male Wistar rats, weighing 225–280 g, were used. They were obtained from University of Gaziosmanpa Experimental Research Centre. The local ethics committee of Gaziosmanpa University (2017/15) approved all experimental procedures. All animals were housed in a temperature-controlled (23 ± 12°C) environment under a 12-h light/dark cycle and 50% humidity, and with free access to tap water and standard laboratory pellet diet.

Experimental design

The animals were randomly divided into 7 groups of 7 rats per group (n = 7×7): Group I (Control) served as control group and animals received saline 1 ml/kg of body weight, i.p. for 7 days. Group II (DOX) served as DOX group, in which the animals received a total cumulative dose of 18 mg/kg, body weight, i.p. daily at 9:00 a.m. for three days in the study. Group III (MEL) animals received melatonin treatment (40 mg/kg body weight, i.p.) for 7 days.

Group IV (AGO) animals received agomelatine treatment (40 mg/kg body weight, i.p.) for 7 days.

Group V (MEL + DOX) animals received melatonin treatment (40 mg/kg body weight, i.p.) for 7 days and were injected with DOX (cumulative dose of 18 mg/kg, i.p.) daily, at 9:00 a.m. on days 5, 6 and 7.

Group VI (AGO + DOX) animals received agomelatine treatment (40 mg/kg body weight, i.p.) for 7 days and were injected with DOX (cumulative dose of 18 mg/kg, i.p.) daily, at 9:00 a.m. on days 5, 6 and 7.

Group VII (MEL+AGO+DOX) animals received melatonin and agomelatine treatment (40 mg/kg body weight, i.p.) for 7 days and were injected with DOX (cumulative dose of 18 mg/kg, i.p.) daily, at 9:00 a.m. on days 5, 6 and 7.

Electrocardiography

At the end of the treatment protocol, animals were anesthetized and sedated with a combination of ketamine (100 mg/kg; i.p.) + xylazine (10 mg/kg; i.p.). Anesthesia was assessed clinically by pedal reflex. During electrocardiographic (ECG) recordings, rectal temperatures were maintained at 37.5°C by a thermostatically controlled heating blanket. In all animals, 10 min after anesthesia, three needle electrodes were inserted under the skin of the animals for the limb lead at position II. As depicted in Figure 1, the lead II may be achieved in rats by attaching the negative electrodes to the dermal layer of both front leg paws and positive electrode to the left hind leg paw. ECG parameters were recorded for 1 minute by using the MP-150 multi-channel physiological analysis system (MP 150, BIOPAC Systems Inc.; USA). The changes in duration of P wave (sec), QRS complex (sec), QT interval (sec), RR interval (sec) and amplitude of ST-segment (mV) were determined.

Scintigraphic images

1 mCi 99mTc Technetium pyrophosphate (99mTc PYP) radiopharmaceutical was administered i.v. to anesthetized rats. One hour after 99mTc PYP radiopharmaceutical administration, static imaging with a dual head gamma camera (E-CAM, Siemens, Germany) was performed in anterior and posterior positions with 2.55 zoom factor. The radioisotope uptake heart area was measured by drawing equal rectangular regions of interest (ROI) and 99mTc PYP uptake was calculated by the semi-quantitative method to all study groups.

Biochemical assays and measurements of cardiac enzymes

At the end of the experiment, all rats were deeply anesthetized under light anesthesia and their blood samples were collected into tubes. After 30 min, these tubes were centrifuged at 3,000 rpm for 10 min; blood samples were separated and transferred to Eppendorf tubes for biochemical analysis of creatine kinase (CK), blood urea nitrogen (BUN), and cardiac troponin T (cTnT) levels. Then the rats were killed under anesthesia.

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0; SPSS, USA). The groups of parametric variables were compared by using one-way ANOVA followed by Tukey post hoc test. The groups of nonparametric variables were compared by Kruskal Wallis test. All the values are presented as the mean ± standard error of the mean (SEM). Graphs were sketched using GraphPad Prism (software, USA) version 7 software. The values of p < 0.05 were considered to indicate a statistically significant difference.

Results

One animal from the DOX-only treated group died prior to the termination of experiment. Pretreatment with 40 mg/kg i.p. of MEL and AGO totally prevented the mortality.
Electrocardiography

Electrocardiographic patterns (P wave duration, QRS complex duration, QT interval, RR interval duration, ST-segment amplitude) of the control and experimental groups are displayed in Figure 2 and Table 1. Rats treated with MEL-only, AGO-only and saline controls group demonstrated a normal pattern on ECG while the DOX-only group, when compared with the control group, displayed a significant decrease in duration of both QRS complex and P wave (p < 0.001), increase in QT interval (p < 0.001), and RR interval (p < 0.001) and elevation in ST-segment amplitude (p < 0.001). MEL+DOX, AGO+DOX and MEL+AGO+DOX groups did not demonstrate significant changes in ECG parameters as compared to control group (p > 0.05).

When compared to the DOX group, the MEL+DOX, AGO+DOX and MEL+AGO+DOX groups demonstrated a significant increase in duration of both QRS complex and P wave (Fig. 2 and Tab. 1; p < 0.001 and p < 0.001; respectively), and a significant decrease in QT interval, RR interval duration and ST-segment-amplitude (Fig. 2 and Tab. 1; p < 0.001, p < 0.001, and p < 0.001, respectively). As opposed to the DOX group, the pretreatment with MEL and AGO importantly attenuated the DOX-induced abnormalities in ECG parameters.

Scintigraphic images

99mTc PYP scintigraphy images of all seven groups are shown in Figure 3. The DOX-treated group showed an increase in 99mTc PYP uptake as compared to the CON group. The DOX groups significantly increased the 99mTc PYP radiopharmaceutical uptake as compared with the CON group (***p < 0.001, respectively). Pretreatment of DOX-induced toxic rats with MEL, AGO, or combination of MEL and AGO resulted in a significantly decreased 99mTc PYP radiopharmaceutical uptake, when compared to the DOX group (Fig. 4, Tab. 2; p < 0.001, p < 0.001, and p < 0.001, respectively).

Biochemical assays

DOX group demonstrated an increase in BUN (p < 0.001), CK (p < 0.001, cTnT (p < 0.001) as opposed to the CON group. The MEL+DOX, AGO+DOX and MEL+AGO+DOX groups showed a significant decrease in BUN, CK, cTnT (Fig. 5 and Tab. 3; p < 0.001, p < 0.001, and p < 0.001, respectively) when compared to the DOX group.

Discussion

The electrocardiographic results revealed that DOX-induced cardiotoxicity is associated with ECG abnormalities. In the present study, DOX intoxication significantly decreased the duration of both QRS complex and P wave, increased the RR interval, QT interval and brought about ST segment elevation. In line with the present study, previous studies demonstrated that the duration of P wave (16) and QRS complex (1, 16) decreased, while the RR interval duration (1, 17, 18, 19–20) increased. P wave, QRS complex duration, and RR interval are frequently transient and no specificity to DOX-induced toxicity can be assigned to them. Many studies are in agreement with our study by reporting that QT interval prolongation and ST segment elevation brought about by DOX administration have been relatively characteristic ECG findings as a result of DOX-induced cardiotoxicity (1, 16, 17, 18, 19–20–21). The prolongation of QT interval and ST segment elevation may be linked to the cellular membrane damage owing to oxidative stress (22, 23). Treatment with MEL, AGO, and MEL+AGO increased the QRS complex and P wave, and decreased the RR interval, QT interval and ST-segment elevation almost to normal. The ECG changes induced by MEL and AGO might take place owing to their protective effect on myocardium or cell membrane stabilizing action.

Myocardial cells injured by a transient episode of ischemia show an acute marked increase in calcium. Most of this increased calcium is found in mitochondria in form of calcium phosphate...
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(24). \(^{99m}\text{Tc}\) PYP was originally used in order to image acute myocardial infarcts in both animals and humans. Today, \(^{99m}\text{Tc}\) PYP continues to be the most commonly used radiopharmaceutical for myocardial injury because this agent is stable, has good blood clearance properties, and high tagging efficiency, generally gives good bone images and should work equally well (25, 26). The uptake of \(^{99m}\text{Tc}\) PYP into calcium deposits may have an important role in the concentration of radionuclides in the necrotic myocardium. Myocardial scintigraphy images are generally obtained 60–90 min after injection. Post-injection imaging time needs to be fitted in the “time window” between marked bone uptake and high blood concentration (27, 28). Cardiac accumulation patterns of pyrophosphate labeled with \(^{99m}\text{Tc}\) in rats one hour to seven days after coronary artery ligation were studied by myocardial scintigraphy, light microscopy, imaging the isolated heart and direct measurement of tissue activity. Results demonstrated that myocardial cells that are taking up \(^{99m}\text{Tc}\) PYP are irreversibly damaged, and the disappearance of \(^{99m}\text{Tc}\) PYP uptake coincides with the removal of necrotic cells by phagocytes (29). In our study, we showed that \(^{99m}\text{Tc}\) PYP radiopharmaceuticals uptake was increased in the DOX-induced necrotic myocardium. Treatment with MEL and AGO led to an important decrease in the levels of \(^{99m}\text{Tc}\) PYP radiopharmaceutical uptake in the heart area in rats suffering from cardiotoxicity induced with DOX.

Many studies asserted that cardiac lesions induced by DOX could be due to the reactive oxygen species production (7, 30), increase in lipid peroxidation and decrease in endogenous antioxidants, thus resulting in increased oxidative stress (31, 32). Increased oxidative stress leads to the development of various cellular changes in the myocardium, causing rupture of the cell membrane so that the cellular enzymes leak out (33). Moreover, elevated levels of these biomarker enzymes (BUN, of CK, cTnT) are an indicator of the severity of DOX-induced myocardial damage and can be estimated in blood samples (34). Studies have demonstrated that DOX-induced cardiotoxicity causes an elevation in levels of these biomarker enzymes (18). The treatment with MEL and AGO caused a significant decrease in the levels of \(^{99m}\text{Tc}\) PYP radiopharmaceutical uptake in the heart area in rats suffering from cardiotoxicity induced with DOX.

Fig. 5. CON = control, DOX = doxorubicin, MEL = melatonin, AGO = agomelatine, and MEL+DOX = melatonin+doxorubicin, AGO+DOX = agomelatine+doxorubicin, and MEL+AGO+DOX= melatonin+agomelatine+ doxorubicin. The DOX groups had a significant increase in BUN, CK, and cTnT in comparison to the control group (1076, 975, 294; ***p < 0.001, respectively). Pretreatment of DOX-induced toxic rats with MEL, AGO, or combination of MEL and AGO significantly decreased the levels of BUN, CK and cTnT when compared to the DOX group (** p < 0.01).
by means of reducing oxidative stress and enhancing antioxidant properties, AGO has been demonstrated to protect brain in cerebral ischemia-reperfusion injury and pentylenetetrazole-induced kindling epilepsy in rats, reduce testicular damage in STZ-induced Type I diabetic rats (36, 37, 38–39). In our previous study, we showed that Dox-induced anxiety and depression decreased after agomelatine and melatonin treatments (40). AGO is M1, M2 receptors agonist and 5-HT2C receptors antagonist and its affinity for MEL receptors is higher as opposed to the 5-HT2C receptor (14). The repressive effect of AGO on DOX-induced cardiotoxicity was similar to that of MEL.

In this study, electrocardiographic, biochemical and nuclear imaging data indicate that pretreatment with AGO alleviated DOX-induced cardiotoxicity similar to that of MEL. In the early diagnosis of the DOX-induced cardiotoxicity, ECG can be determined as an increase in ST and QT interval and biochemical BUN, CK, and cTcT increase. DOX-induced cardiotoxicity $^{99m}$Tc PYP uptake was assessed for this and another study (41). The $^{99m}$Tc PYP uptake increase may be helpful in assessing the clinical diagnosis in DOX-induced cardiotoxicity.

**Conclusion**

Experimental studies on rats induced with oxidative stress have shown that AGO acts as an antioxidant in the same way as MEL. Therefore, we extensively evaluated the cardioprotective effects of MEL and AGO in DOX-induced cardiotoxicity through various electrophysiologic, scintigraphic, and biochemical parameters. The present study affirmed that a cumulative dose of DOX (18 mg/kg/i.p) induces cardiotoxicity in rats as proved by the increase in mortality, ECG changes, increased $^{99m}$Tc PYP uptake values and levels of cardiac marker enzymes (BUN, CK, cTcT). Our results demonstrated that MEL and AGO could protect the heart against the undesired effects of DOX.

**Limitations**

The fact that the protective effect of melatonin and agomelatine on DOX-induced cardiotoxicity should have been confirmed by histopathological findings is a limitation to our study.

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