

EXPERIMENTAL STUDY

The effect of melatonin on digoxin-induced cardiac damage in cardiomyocytes

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ABSTRACT

OBJECTIVES: Digoxin is a cardiac glycoside which is widely used in cardiovascular medicine. Oxidative stress, as well as intracellular Ca²⁺ overload, plays an important role in digoxin toxicity. Transient receptor potential vanilloid 1 (TRPV1) channels are found in cardiomyocyte cells and they are activated by reactive oxygen species. We investigated the effects of digoxin toxicity and alterations in Ca²⁺ influx, oxidative stress and apoptosis through TRPV1 channels and modulator role of melatonin in cardiomyocytes.

METHODS: The cells were divided into seven main groups as control, digoxin, digoxin+capsazepine, digoxin+melatonin, digoxin+capsazepine+melatonin, melatonin and melatonin+capsazepine groups. Cells in the groups were stimulated with capsaicin and inhibited with capsazepine in related experiments for activation and inactivation of TRPV1 channels, respectively. We measured cytosolic calcium, intracellular reactive oxygen, mitochondrial depolarization, caspase 9 and caspase 3 levels.

RESULTS: The apoptosis values were significantly lower in the melatonin and digoxin+melatonin groups than in the digoxin group of cardiomyocytes ($p < 0.001$). The cell viability values were higher in the digoxin+capsazepine ($p < 0.001$), digoxin+melatonin ($p < 0.001$) and digoxin+melatonin+capsazepine ($p < 0.001$) groups than in the digoxin group.

CONCLUSION: TRPV1 channels are overactivated during digoxin toxicity and melatonin could show a cardioprotective effect through TRPV1 channel modulation (Fig. 5, Ref. 56). Text in PDF www.elis.sk.

KEY WORDS: digoxin, oxidative stress, cardiomyocyte.

Introduction

Digoxin is a cardiac glycoside which is widely used in heart failure and atrial fibrillation patients as it increases inotropy and decreases chronotropy. It has multiple direct and indirect cardiovascular effects (1, 2). The inhibition of sarcolemma-bound Na⁺/K⁺ ATPase by digoxin leads to an increase in sodium (Na⁺) ion concentration. This cellular Na⁺ overload causes an increase in free calcium (Ca²⁺) concentration mediated by the Na⁺/Ca²⁺ exchanger. When cytoplasmic Ca²⁺ increases above the storage capacity of the sarcoplasmic reticulum, digoxin toxicity occurs (3, 4). Toxic concentrations of digoxin are associated with oxidation of membrane phospholipids and increase in lytic enzymes due to increase in intracellular Ca²⁺ (5–7).

Melatonin is an amphiphilic molecule which protects mitochondria against oxidative stress. It has been demonstrated that melatonin is a potent antioxidant, anti-apoptotic and anti-inflammatory

molecule which has protective effects in various organs including heart (8, 9). Vital role of transient receptor potential vanilloid 1 (TRPV1) channels in modulating heart failure, atherosclerotic heart disease and vascular remodeling has been shown in previous studies. Reactive oxygen species (ROS) activate and potentiate TRPV1 channels. Activating TRPV1 channels repeatedly is associated with increased cytosolic calcium levels, oxidative stress parameters and apoptosis levels (10, 11). Cardiomyocytes are one of main targets of ROS. Melatonin may modulate Ca²⁺ entry via TRPV1 and can affect oxidative stress parameters and cell injury in cardiomyocytes during digoxin toxicity.

To the best of our knowledge, there have been no previous reports on the roles of TRPV1 channels and melatonin during digoxin toxicity in cardiomyocytes. In the present study, we wanted to evaluate the effects of digoxin toxicity and alterations in Ca²⁺ influx, oxidative stress and apoptosis through TRPV1 channels and modulator role of melatonin in cardiomyocytes.

Methods*Cell culture and reagents*

AC16 (Human cardiomyocyte cell line) was obtained from ATCC. Dulbecco's modified Eagle's medium (DMEM) (Sigma) was used for cardiomyocytes culture containing 12 % fetal bovine serum (FBS) (Fisher Scientific, and 1 % penicillin/streptomycin

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(Thermo-Fischer). Cardiomyocyte cells were evenly distributed as 1×10^6 cells in each of 8–10 flasks (filter cap+sterile+ 5 ml; 25 cm²). A humidified incubator was used to incubate cardiomyocytes at 37 °C at 5 % CO₂. After cells had reached 75–85 % confluence, they were incubated with the chemical compounds described in groups section. Cells were examined daily for the evidence of contamination. After treatments, the cells were detached with 0.25 % Trypsin–EDTA for analysis and split into the sterile falcon tubes for analyses.

Cell viability (MTT) dye was purchased from Thermo Fischer (Massachusetts, USA). Dihydrorhodamine-123 (DHR123) obtained from Molecular Probes (OR, USA). Caspase 3 and Caspase 9 substrates were purchased from Biovision (San Francisco, USA). APOPercentage dye with releasing buffer were obtained from Biocolor Ltd. (Northern Ireland), JC1 was purchased from Santa Cruz (Texas, USA), Fura 2 AM was purchased from Calbiochem (Darmstadt, Germany).

Groups

The study was planned with 7 main groups as follows,

- Group 1 (Control): None of the study drugs were used and cardiomyocytes were kept in a flask containing the same cell culture condition.
- Group 2 (DGX): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min (12).
- Group 3 (DGX+CAPZ): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).
- Group 4 (DGX+Mel): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min and then incubated with 300 μM melatonin for 2 h.
- Group 5 (DGX+Mel+CAPZ): Cardiomyocytes were incubated with 0.03 μM Digoxin for 30 min and then incubated with 300 μM melatonin for 2 h and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).
- Group 6 (Mel): Cardiomyocytes were incubated with 300 μM melatonin for periods of 2 h (13).
- Group 7 (Mel+CAPZ): Cardiomyocytes were incubated with 300 μM melatonin for periods of 2 h and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).

In CAPZ incubated groups, cardiomyocytes were also blocked by TRPV1 blocker CAPZ (0.1 mM, 30 min) prior to the related analysis in the existence of 1.2 mM calcium in extracellular environment. For all experiments (except for calcium signaling), the cells were further treated with capsaicin (CPSN, 0.1 mM, 10 min) for activation of TRPV1 channel prior to the related analysis. During calcium signaling analysis (Fura-2/AM), the cells were stimulated on 20th cycles with 0.1 mM CPSN in the existence of 1.2 mM calcium in extracellular environment.

Measurements of intracellular calcium concentration

UV light excitable Fura 2 AM (acetoxymethyl ester) dye was used for measuring intracellular calcium level in cardiomyocytes. The relevant experimental procedures were carried out in accordance

with the experimental procedure of Uğuz et al, which included 4 μM Fura 2 AM fluorescent dye for final staining period (14). Fluorescence emission intensity at 510 nm was determined in individual wells using a plate reader equipped with an automated injection system (Synergy™ H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles. During the measurement of intracellular calcium signaling, TRPV1 channels were stimulated by automatic injector with capsaicin (0.1 mM) on 20th cycle. Measurement of Ca²⁺ analysis was performed as modified by Uğuz et al, and Martinez et al, in previous studies (14, 15).

Intracellular ROS production measurement

DHR123 is a non-charged and non-fluorescent dye which easily goes through the cell membrane. The relevant experimental procedures were carried out in accordance with the experimental procedure of Espino et al (16). Inside the cardiomyocyte cell, DHR123 is oxidized to cationic rhodamine 123 (Rh 123) which localizes in the mitochondria and exhibits green fluorescence. Synergy™ H1 automatic microplate reader device was used for determining Rh 123 fluorescent intensities. The analyses were performed at 488 nm (excitation) and 543 nm (emission) wavelengths. We presented the data as fold changes as from their levels before treatment.

Apoptosis assay

The APOPercentage™ cell apoptosis assay was used for the detection and quantification of apoptosis. The APOPercentage dye is actively bound to phosphatidyl-serine lipids and transferred into the cells, which stains apoptotic cells red. The apoptosis analyzes procedure was performed according to manufacturer's instruction and Özdemir et al (11). The cardiomyocytes were analyzed for apoptotic cells detection by spectrophotometry (multiplate reader) at 550 nm (Synergy™ H1, Biotek, USA).

Caspase 9 and caspase 3 activity assays

Caspase 9 and Caspase 3 activity evaluation methods were based on previously reported studies (17, 18). Caspase 9 (AC-LEHD-AMC) and Caspase 3 (ACDEVD-AMC) substrates cleavages were calculated with Synergy™ H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths (excitation/emission). The values were evaluated as fluorescent units/mg protein and shown as fold changes as from their levels before treatment (experimental/control).

Mitochondrial membrane potential (JC1) analyses

JC1 (1 μM), which is a mitochondrial membrane potential fluorescence dye, was evaluated by 485 nm (green) excitation wavelength and emission wavelength of 535 nm, red signal at 540 nm (excitation) and 590 nm (emission) wavelengths (Synergy™ H1, Biotek, USA). Data are presented as emission ratios (590/535). Mitochondrial membrane potential changes were quantified as the integral of the decrease in JC1 fluorescence ratio of experimental/control.

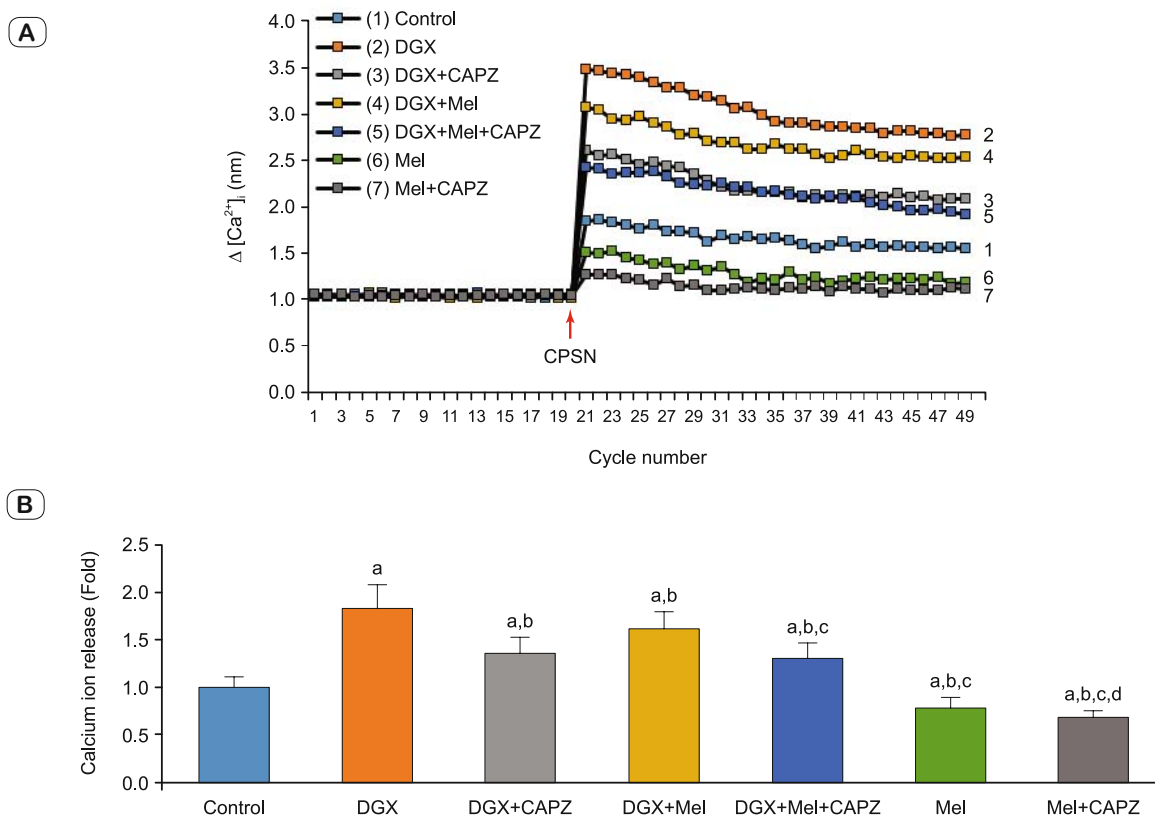


Fig. 1A, B. The effect of Digoxin (0.03 μ M for 30min) and Mel (300 μ M for 2 hrs) on cytosolic calcium levels in cardiomyocyte cells. Cells are stimulated by capsaicin (CPSN 0.1 mM and on 20th cycle) but blocked with capsazepine (CAPZ 0.1 mM for 30 min; mean \pm SD and n = 10) *p < 0.001 vs control, ^bp < 0.001 vs DGX, ^cp < 0.001 vs DGX+Mel and ^dp < 0.001 vs Mel. DGX: digoxin group, DGX+CAPZ: digoxin+capsazepine group, DGX+Mel: digoxin+melatonin group, DGX+Mel+CAPZ: digoxin+melatonin+capsazepine group, Mel: melatonin group, Mel+CAPZ: melatonin+capsazepine group.

Cell viability (MTT) assay

Cell viability was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After treatments with chemical compounds as described in group section, the cardiomyocytes were washed and then incubated with fresh DMEM containing MTT (0.5 mg/ml) at 37 °C for 90 min (19). Then, the supernatants were removed, and Dimethyl-sulfoxide was added to dissolve the formazan crystals. Optical density was estimated by Synergy™ H1 automatic microplate reader device (Biotek, USA) at a test wavelength of 490 nm and reference wavelength of 650 nm to nullify the effect of cell debris. The obtained data are shown as fold changes as from their levels before treatment (experimental/control).

Statistical analysis

The values are presented as means \pm standard deviations (SD). To compare the different treatments, statistical significance was calculated by one-way analysis of variance (ANOVA) and Mann-Whitney U test. All data were analyzed by SPSS statistical program (version 9.05 software, SPSS Inc. Chicago, Illinois, USA) and p < 0.05 was considered significant.

Results

Effects of digoxin toxicity and melatonin on cytosolic calcium levels in cardiomyocytes

The effect of digoxin toxicity and melatonin administrations on cytosolic calcium levels in cardiomyocyte cells are shown in Figure 1a, b. The TRP Vanilloid 1 channel blocker capsazepine was used to evaluate intracellular Ca^{2+} increase through TRPV1 channels in digoxin toxicity model of cardiomyocytes. As shown in Figure 1b, the Ca^{2+} concentration in cardiomyocytes was greater in the digoxin group when compared with the control (p < 0.001). The Ca^{2+} level was lower in melatonin and melatonin+capsazepine groups than in the control (p < 0.001). Also, cytosolic Ca^{2+} concentration was lower in the digoxin+capsazepine, digoxin+melatonin and digoxin+melatonin+capsazepine groups than in the digoxin group (p < 0.001).

In addition, cytosolic Ca^{2+} concentration in the cardiomyocytes was markedly lower in the digoxin+melatonin+capsazepine group, compared to the digoxin+melatonin group (p < 0.001).

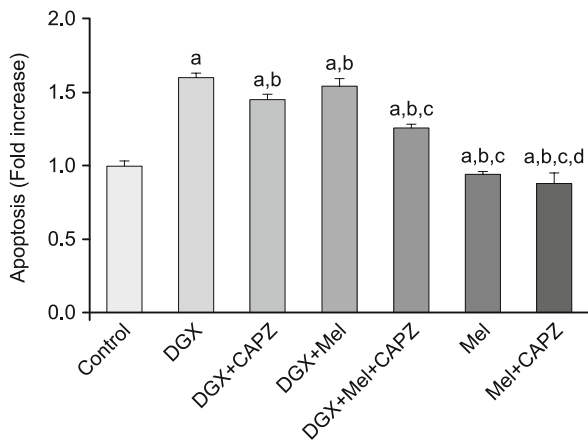


Fig. 2. The effect of Digoxin (0.03 μ M, 30min) and Mel (300 μ M, 2 hrs) on apoptosis levels in the cardiomyocyte cells. Cells are stimulated by capsaicin (CPSN 0.1 mM for 10 min) but blocked with capsazepine (CAPZ 0.1 mM for 30 min; mean \pm SD and n = 10) ^ap < 0.001 vs control, ^bp < 0.001 vs DGX, ^cp < 0.001 vs DGX+Mel and ^dp < 0.001 vs Mel. DGX: digoxin group, DGX+CAPZ: digoxin+capsazepine group, DGX+Mel: digoxin+melatonin group, DGX+Mel+CAPZ: digoxin+melatonin+capsazepine group, Mel: melatonin group, Mel+CAPZ: melatonin+capsazepine group.

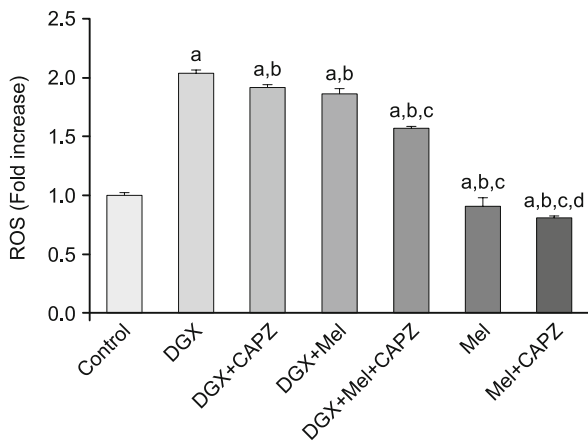


Fig. 3. The effect of Digoxin (0.03 μ M, 30min) and Mel (300 μ M, 2 hrs) on reactive oxygen species (ROS) levels in the cardiomyocyte cells. Cells are stimulated by capsaicin (CPSN 0.1 mM for 10 min) but blocked with capsazepine (CAPZ 0.1 mM for 30 min; mean \pm SD and n = 10) ^ap < 0.001 vs control, ^bp < 0.001 vs DGX, ^cp < 0.001 vs DGX+Mel and ^dp < 0.001 vs Mel. DGX: digoxin group, DGX+CAPZ: digoxin+capsazepine group, DGX+Mel: digoxin+melatonin group, DGX+Mel+CAPZ: digoxin+melatonin+capsazepine group, Mel: melatonin group, Mel+CAPZ: melatonin+capsazepine group.

Effects of digoxin toxicity and melatonin on apoptosis levels in cardiomyocytes

Effects of digoxin toxicity and melatonin administrations on apoptosis levels are shown in Figure 2. The apoptosis values were higher in the digoxin group than in the control. The apoptosis values were lower in melatonin and digoxin+melatonin group than in the digoxin group of cardiomyocytes (p < 0.001). Also, the values were lower in the digoxin+melatonin+capsazepine group

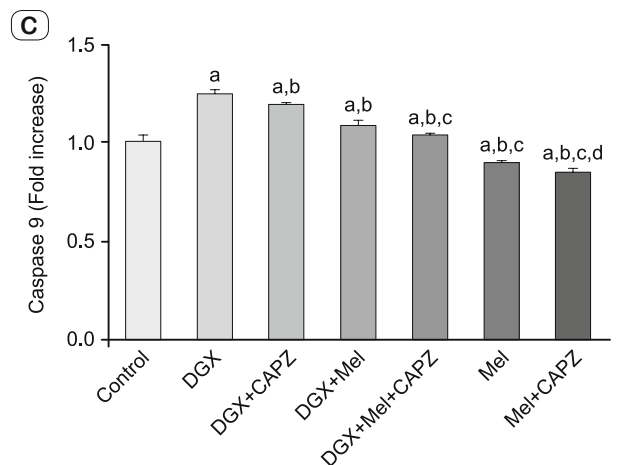
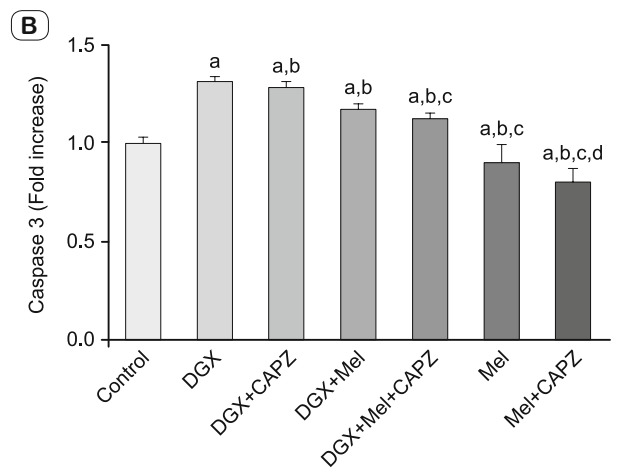
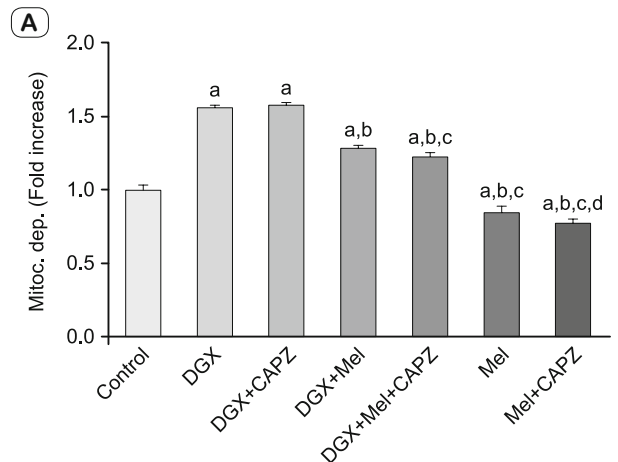


Fig. 4 A-C. The effect of Digoxin (0.03 μ M, 30min) and Mel (300 μ M, 2 hrs) on mitochondrial membrane depolarization (A), caspase 3 (B), caspase 9 (C) levels in the cardiomyocyte cells. Cells are stimulated by capsaicin (CPSN 0.1 mM for 10 min) but blocked with capsazepine (CAPZ 0.1 mM for 30 min; mean \pm SD and n = 10) ^ap < 0.001 vs control, ^bp < 0.001 vs DGX, ^cp < 0.001 vs DGX+Mel and ^dp < 0.001 vs Mel. DGX: digoxin group, DGX+CAPZ: digoxin+capsazepine group, DGX+Mel: digoxin+melatonin group, DGX+Mel+CAPZ: digoxin+melatonin+capsazepine group, Mel: melatonin group, Mel+CAPZ: melatonin+capsazepine group.

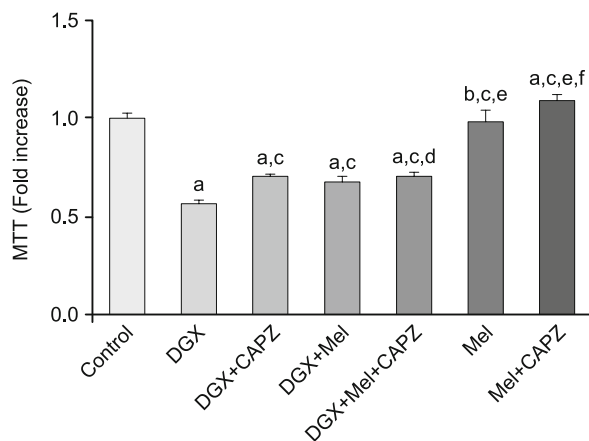


Fig. 5. The effect of Digoxin (0.03 μ M, 30min) and Mel (300 μ M, 2 hrs) on MTT levels in the cardiomyocyte cells. Cells are stimulated by capsaicin (CPSN 0.1 mM for 10 min) but blocked with capsazepine (CAPZ 0.1 mM for 30 min; mean \pm SD and n = 10) ^ap < 0.001 and ^bp < 0.05 vs control, ^cp < 0.001 vs DGX, ^dp < 0.05 and ^ep < 0.001 vs DGX+Mel and ^fp < 0.001 vs Mel. DGX: digoxin group, DGX+CAPZ: digoxin+capsazepine group, DGX+Mel: digoxin+melatonin group, DGX+Mel+CAPZ: digoxin+melatonin+capsazepine group, Mel: melatonin group, Mel+CAPZ: melatonin+capsazepine group.

when compared with the digoxin+melatonin group of cardiomyocytes (p < 0.001)

Effects of digoxin toxicity and melatonin on intracellular ROS production in cardiomyocytes

Intracellular ROS production of groups are shown in Figure 3. The ROS production values of the digoxin group were greater than those in the control group. The values of digoxin+capsazepine (p < 0.001), digoxin+melatonin (p < 0.001) and digoxin+melatonin+capsazepine (p < 0.001) groups were lower than those in the digoxin group. Also, the ROS production was markedly lower in the digoxin+melatonin+capsazepine group when compared to the digoxin+melatonin group (p < 0.001).

Effects of digoxin toxicity and melatonin on caspase 3 and 9 activities, mitochondrial depolarization levels and cell viability (MTT) values in cardiomyocytes

Levels of mitochondrial membrane depolarization, caspase 3–9 activities of groups are shown in Figures 4 a, b, and c, respectively. It has been shown that caspase 3 and 9 activities have an important role in the mitochondrial apoptotic pathways. Also, they are associated with mitochondrial cytochrome c release during the apoptotic cascade (20, 21).

We showed the MTT values in Figure 5. Cell viability values in the digoxin group were lower than those in the control (p < 0.001). The values were higher in the digoxin+capsazepine (p < 0.001), digoxin+melatonin (p < 0.001) and digoxin+melatonin+capsazepine (p < 0.001) groups than in the digoxin group.

Discussion

Digital glycosides are used for patients with heart failure and arrhythmia worldwide, although excessive doses could cause cardiac adverse effects (22). Intracellular Ca^{2+} overload and oxidative stress play an important role in digoxin toxicity. It has been shown that digitalis-induced Na^+ accumulation causes an increase in Ca^{2+} through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. As a result of this internal Ca^{2+} overload, the net increase in intracellular Ca^{2+} activates further calcium release from the sarcoplasmic reticulum. The toxic effects have been seen when the sarcoplasmic reticulum storage capacity is exceeded. Intracellular Ca^{2+} overload is related to persistent inhibition of the Na^+/K^+ ATPase and is associated with a rise in cell automaticity, impaired homeostasis and cell death. Adverse toxic effects are characterized by arrhythmia as well as apoptosis. Digitalis-induced arrhythmogenic effects are evaluated in previous studies but digitalis-induced apoptosis is not yet well understood (23, 24). In a study by Qubaassine et al, it has been shown that digoxin induced cell death in adult rat cardiomyocytes (25). Also, it has been described that toxic concentrations of digitalis cause a sustained elevation of internal Ca^{2+} levels that provoke apoptotic cell death (7).

Cardiac glycosides bind to Na^+/K^+ ATPase and it opens the mitochondrial ATP-sensitive K^+ channel which leads to an increase in ROS. The reactive oxygen species are associated with inhibition of mitochondrial permeability transition which decreases myocyte viability. Cytochrome c release from mitochondria is a critical apoptotic event and this important event was induced by digoxin. Consistent with cytochrome c release induced by digoxin, is the activation of caspase 9 and caspase 3. It is followed by proteolytic process with suggested oxidative stress-induced apoptosis (26–28).

Thus, we hypothesized that TRPV1 channels could be involved in glycoside-induced apoptosis. We think that the mechanism of digoxin to induce apoptotic cell death either in cultured cells or in live organism could follow a common pathway, in which Ca^{2+} plays a principal role. This intracellular Ca^{2+} overload has been linked to mitochondrial dysfunction. We also think that digitalis-induced cell death is mainly apoptotic although a mixed mechanism of cell death cannot be ruled out.

In the current study, we showed that TRPV1 channels in cardiomyocytes are activated by digoxin administration. TRP channels are a family of unique ion channels which affect important cellular functions and signaling pathways. Also, they are primary targets for several potential drugs. They are known to play critical roles in cells' survival as well as their development. TRP channels can be divided into seven subfamilies as TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPN (NOMPC) family (29, 30). They are activated by intracellular and extracellular messengers, chemical and mechanical stimuli and osmotic stress. Their main difference from voltage-dependent channels is that they are weakly sensitive to membrane potential. Some channels are activated by intracellular Ca^{2+} load whereas others are constitutively open (31, 32). Calcium has an important role as a second messenger in cardiac function including cardiac energy homeostasis, as well as

cell death. Transient receptor potential (TRP) proteins are responsible for Na⁺ and Ca²⁺ conducting channels which cause changes in the Ca²⁺ homeostasis and mediate longer lasting modulation of Ca²⁺ levels (33, 34). TRPV1, a member of vanilloid TRP family, is expressed in many organs including heart, kidney, brain, dorsal root ganglions and sensory neurons. It is a ligand-gated, homotetrameric, non-selective cation channel which is activated by heat (over 42 °C), capsaicin (pungent substance from chili peppers) and low extracellular PH (35–37). Capsazepine is a competitive antagonist of capsaicin and it specifically blocks the TRPV1 channel (38). It has been demonstrated that TRPV1 channels are found in the ventricles of heart in the cardiovascular system and have an important role in modulating cardiovascular diseases including atherosclerosis, congestive heart failure and systemic hypertension (39–42). Authors have reported that activation of TRPV1 channels could aggravate heart failure (43, 44). Also, it has been shown that the toxicity of digoxin was amplified in congestive heart failure as a consequence of Na⁺/Ca²⁺ exchanger upregulation (45). Furthermore, previous studies have demonstrated that activation of TRPV1 channels have a dual role in the progression of heart failure (42).

Although oxidative stress has an important role in digoxin toxicity, its role in TRPV1 channel activation has not been investigated yet. Dai et al, showed that extracellular ROS activated TRPV1 channels in apoptosis which is induced by hypoxia in hippocampal neurons (46). Also, authors of previous studies evaluated the role of TRPV1 channel activation in oxidative stress, apoptotic cell injury, and increased cytosolic free Ca²⁺. Chuang et al demonstrated that TRPV1 channel activation by capsaicin was increased in oxidative stress (47). Similarly, Hu et al, showed that increased intracellular Ca²⁺ entry and activation of NADPH oxidase was associated with TRPV1-induced oxidative stress. On the other hand, oxidative stress causes activation of ion channels by phosphorylation and this process is involved in Ca²⁺ influx through TRPV1 channels (48). We observed that digoxin toxicity increased oxidative stress, intracellular Ca²⁺ entry and apoptosis in cardiomyocytes. In the current study we showed that intracellular ROS production and Ca²⁺ levels were increased through TRPV1 channel activation by digoxin toxicity and capsaicin stimulation in cardiomyocytes.

Also, we demonstrated that melatonin reduced ROS, apoptosis, and Ca²⁺ entry through modulation of TRPV1. Melatonin is a pineal secretory product, strong antioxidant and an anti-apoptotic molecule which can pass through all cellular compartments easily (49, 50). It participates in the antioxidant and anti-apoptotic processes directly or indirectly. Melatonin has protective effects in many organs including heart, brain and liver (51–53). Furthermore, protective effects of melatonin during oxidative stress and apoptosis have been shown before in previous studies (8). Moreover, it has been demonstrated that it decreased the effectiveness of voltage-operated Ca²⁺ channels and induced the Ca²⁺-dependent ATPase in cardiac sarcolemma (54). In addition, the protective effect of melatonin in reperfusion arrhythmias has been reported previously and melatonin was suggested to be used as an adjunct therapy in myocardial infarction (55). In a study by Kahya et al, the effect of melatonin on oxidative stress parameters, apoptosis levels and intracellular calcium entry via TRPV1 channels in dia-

betic rats has been evaluated. They found that the modulation of this channel by melatonin involves neuroprotective activities in diabetic rats (56). In the present study, we observed that melatonin could influence intracellular homeostasis during digoxin toxicity. We found that melatonin administration has beneficial effects on apoptosis levels and intracellular ROS production during digoxin toxicity in cardiomyocytes.

Study limitations

Mainly, we did not evaluate the concentration response to distinguish effects of therapeutic versus toxic levels of digoxin on the molecular mechanism studied in the present study. Also, we could not perform an electrophysiological study and evaluate whether digoxin toxicity changes also the expression of TRPV1 channels in cardiomyocytes.

Conclusion

In conclusion, we showed that TRPV1 channels are overactivated by ROS and mediate the lethal cytosolic Ca²⁺ increase in digoxin toxicity. Melatonin could show a cardioprotective effect through TRPV1 channel modulation in cardiomyocytes.

References

1. Ambrosy AP, Butler J, Ahmed A, Vaduganathan M, van Veldhuisen DJ, Colucci WS et al. The use of digoxin in patients with worsening chronic heart failure: reconsidering an old drug to reduce hospital admissions. *J Am Coll Cardiol* 2014; 63: 1823–1832.
2. Fuster V, Ryden LE, Cannon DS, Crijns HJ, Curtis AB, Ellenbogen KA et al. ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Revise the 2001 guidelines for the management of patients with atrial fibrillation): developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society. *Circulation* 2006; 114: e257–354.
3. Gonano LA, Sepúlveda M, Rico Y, Kaetzel M, Valverde CA, Dedman J et al. Calcium–calmodulin kinase II mediates digitalis-induced arrhythmias. *Circ Arrhythm Electrophysiol* 2011; 4: 947–57.
4. Skou JC. The energy coupled exchange of Na⁺ for K⁺ across the cell membrane. The Na⁺, K⁺-pump. *FEBS Lett* 1990; 268: 314–324.
5. McConkey DJ, Lin Y, Nutt LK, Ozel HZ, Newman RA. Cardiac glycosides stimulate Ca²⁺ increases and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells. *Cancer Res* 2000; 60: 3807–3812.
6. Ramirez Ortega M, Maldonado Lagunas V, Melendez Zajgla J, Carrillo Hernandez JF, Pastelín Hernandez G, Picazo Picazo O et al. Proliferation and apoptosis of HeLa cells induced by in vitro stimulation with digitalis. *Eur J Pharmacol* 2006; 534: 71–76.
7. Ramirez Ortega M, Zarco G, Maldonado V, Carrillo JF, Ramos P, Ceballos G et al. Is digitalis compound-induced cardiotoxicity, mediated through guinea pig cardiomyocytes apoptosis? *Eur J Pharmacol* 2007; 566: 34–42.

8. **Asghari MH, Abdollahi M, de Oliveira MR, Nabavi SM.** A review of the protective role of melatonin during phosphine-induced cardiotoxicity: focus on mitochondrial dysfunction, oxidative stress and apoptosis. *J Pharm Pharmacol* 2017; 69: 236–243.
9. **Vazan R, Ravingerova T.** Protective effect of melatonin against myocardial injury induced by epinephrine. *J Physiol Biochem* 2015; 71: 43–49.
10. **Crouzin N, Ferreira MC, Cohen-Solal C, Barbanel G, Guiramand J, Vignes M.** Neuroprotection induced by vitamin E against oxidative stress in hippocampal neurons: involvement of TRPV1 channels. *Mol Nutr Food Res* 2010; 54: 496–505.
11. **Özdemir ÜS, Nazıroğlu M, Şenol N, Ghazizadeh V.** Hypericum perforatum attenuates spinal cord injury induced oxidative stress and apoptosis in the dorsal root ganglion of rats: involvement of TRPM2 and TRPV1 channels. *Mol Neurobiol* 2016; 53: 3540–3551.
12. **Watanabe H, Honda Y, Deguchi J, Yamada T, Bando K.** Usefulness of cardiotoxicity assessment using calcium transient in human induced pluripotent stem cell-derived cardiomyocytes. *J Toxicol Sci* 2017; 42: 519–527.
13. **Celik O, Nazıroğlu M.** Melatonin modulates apoptosis and TRPM2 channels in transfected cells activated by oxidative stress. *Physiol Behav* 2012; 107: 458–465.
14. **Uguz AC, Nazıroğlu M, Espino J et al.** Selenium modulates oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through regulation of calcium release and caspase-3 and -9 activities. *J Membr Biol* 2009; 232: 15–23.
15. **Martinez NA, Ayala AM, Martinez M, Martinez-Rivera FJ, Miranda JD, Silva WL.** Caveolin-1 regulates the P2Y2 receptor signaling in human 1321N1 astrocytoma cells. *J Biol Chem* 2016; 291: 12208–.
16. **Espino J, Pariente JA, Rodríguez AB.** Role of melatonin on diabetes-related metabolic disorders. *World J Diabetes* 2011; 2: 82–91.
17. **Bejarano I, Redondo PC, Espino J, Rosado JA, Paredes SD, Barriga C et al.** Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. *J Pineal Res* 2009; 46: 392–400.
18. **Espino J, Bejarano I, Redondo PC, Rosado JA, Barriga C, Reiter RJ et al.** Melatonin reduces apoptosis induced by calcium signaling in human leukocytes: Evidence for the involvement of mitochondria and Bax activation. *J Membr Biol* 2010; 233: 105–118.
19. **Övey IS, Nazıroğlu M.** Homocysteine and cytosolic GSH depletion induce apoptosis and oxidative toxicity through cytosolic calcium overload in the hippocampus of aged mice: involvement of TRPM2 and TRPV1 channels. *Neuroscience* 2015; 284: 225–233.
20. **Shi Y.** Apoptosome: the cellular engine for the activation of caspase-9. *Structure* 2002; 10: 285–288.
21. **Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES et al.** Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479–489.
22. **Bauman JL, Didomenico RJ, Galanter WL.** Mechanisms, manifestations, and management of digoxin toxicity in the modern era. *Am J Cardiovasc Drugs* 2006; 6: 77–86.
23. **Olej B, dos Santo NF, Leal L, Rumjanek VM.** Ouabain induces apoptosis on PHA-activated lymphocytes. *Biosci Rep* 1998; 18: 1–7.
24. **Yeh JY, Huang WJ, Kan SF, Wang PS.** Inhibitory effects of digitalis on the proliferation of androgen dependent and independent prostate cancer cells. *J Urol* 2001; 166: 1937–1942.
25. **Oubaassine R, Weckering M, Kessler L, Breidert M, Roegel JC, Eftekhari P.** Insulin interacts directly with Na⁺/K⁺ATPase and protects from digoxin toxicity. *Toxicology* 2012; 299: 1–9.
26. **Wieckowski M, Vysokikh M, Dymkowska D, Antonsson B, Brdieszka D, Wojtczak L.** Oligomeric c-terminal truncated Bax preferentially releases cytochrome c but not adenylate kinase from mitochondria, outer membrane vesicles and proteoliposomes. *FEBS Lett* 2001; 505: 453–459.
27. **Ekert PG, Read SH, Silke J, Marsden VS, Kaufmann H, Hawkins CJ et al.** Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol* 2004; 165: 835–842.
28. **Ishida, H, Hirota Y, Genka C, Nakazawa H, Nakaya H, Sato T.** Opening of mitochondrial K (ATP) channels attenuates the ouabain-induced calcium overload in mitochondria. *Circ Res* 2001; 89: 856–858.
29. **Nilius B, Owsianik G.** The transient receptor potential family of ion channels. *Genome Biol* 2011; 12: 218.
30. **Kaneko Y, Szallasi A.** Transient receptor potential (TRP) channels: a clinical perspective. *Br J Pharmacol* 2014; 171: 2474–2507.
31. **Venkatachalam K, Montell C.** TRP channels. *Annu Rev Biochem* 2007; 76: 387–417.
32. **Jie Zheng.** Molecular Mechanism of TRP Channels. *Compr Physiol* 2013; 3: 221–42.
33. **Flockerzi V, Nilius B.** TRPs: truly remarkable proteins. *Handb Exp Pharmacol* 2014; 222: 1–12.
34. **Bers DM.** Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* 2008; 70: 23–49.
35. **Gao F, Liang Y, Wang X, Lu Z, Li L, Zhu S et al.** TRPV1 activation attenuates high-salt diet-induced cardiac hypertrophy and fibrosis through PPAR- δ upregulation. *PPAR Res* 2014; 2014: 491963.
36. **Kassmann M, Harteneck C, Zhu Z, Nürnberg B, Tepel M, Gollasch M.** Transient receptor potential vanilloid 1 (TRPV1), TRPV4, and the kidney. *Acta Physiol (Oxford)* 2013; 207: 546–564.
37. **Khatibi NH, Jadhav V, Charles S, Chiu J, Buchholz J, Tang J et al.** Capsaicin pre-treatment provides neurovascular protection against neonatal hypoxic-ischemic brain injury in rats. *Acta Neurochir Suppl* 2011; 111: 225–230.
38. **van den Worm E, de Vries A, Nijkamp FP, Engels F.** Capsazepine, a vanilloid receptor antagonist, inhibits allergen-induced tracheal contraction. *Eur J Pharmacol* 2005; 518: 77–78.
39. **Li L, Wang F, Wei X, Liang Y, Cui Y, Gao F et al.** Transient receptor potential vanilloid 1 activation by dietary capsaicin promotes urinary sodium excretion by inhibiting epithelial sodium channel α subunit-mediated sodium reabsorption. *Hypertension* 2014; 64: 397–404.
40. **Lang H, Li Q, Yu H, Li P, Lu Z, Xiong S, et al.** Activation of TRPV1 attenuates high salt-induced cardiac hypertrophy through improvement of mitochondrial function. *Br J Pharmacol* 2015; 172: 5548–5558.
41. **Li BH, Yin YW, Liu Y, Pi Y, Guo L, Cao XJ et al.** TRPV1 activation impedes foam cell formation by inducing autophagy in oxLDL-treated vascular smooth muscle cells. *Cell Death Dis* 2014; 5: e1182.
42. **Randhawa PK, Jaggi AS.** TRPV1 channels in cardiovascular system: A double edged sword? *Int J Cardiol* 2017; 228: 103–113.
43. **Lu S, Xu D.** Cold stress accentuates pressure overload-induced cardiac hypertrophy and contractile dysfunction: role of TRPV1/AMPK-mediated autophagy. *Biochem Biophys Res Commun* 2013; 442: 8–15.

44. Thilo F, Liu Y, Schulz N, Gergs U, Neumann J, Loddenkemper C et al. Increased transient receptor potential vanilloid type 1 (TRPV1) channel expression in hypertrophic heart. *Biochem Biophys Res Commun* 2010; 401: 98–103.
45. Gaughan JP, Furukawa S, Jeevanandam V, Hefner CA, Kubo H, Margulies KB et al. Sodium/ calcium exchange contributes to contraction and relaxation in failed human ventricular myocytes. *Am J Physiol* 1999; 277: H714–724.
46. Dai Z, Xiao J, Liu SY, Cui L, Hu GY, Jiang DJ. Rutaecarpine inhibits hypoxia/reoxygenation-induced apoptosis in rat hippocampal neurons. *Neuropharmacology* 2008; 55: 1307–1312.
47. Chuang HH, Lin S. Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. *Proc Natl Acad Sci USA* 2009; 106: 20097–20102.
48. Hu F, Sun WW, Zhao XT, Cui ZJ, Yang WX. TRPV1 mediates cell death in rat synovial fibroblasts through calcium entry-dependent ROS production and mitochondrial depolarization. *Biochem Biophys Res Commun* 2008; 369: 989–993.
49. Reiter RJ, Garcia JJ, Pie J. Oxidative toxicity in models of neurodegeneration: responses to melatonin. *Restor Neurol Neurosci* 1998; 12: 135–142.
50. Baydas G, Reiter RJ, Akbulut M, Tuzcu M, Tamer S. Melatonin inhibits neural apoptosis induced by homocysteine in hippocampus of rats via inhibition of cytochrome c translocation and caspase-3 activation and by regulating pro-and anti-apoptotic protein levels. *Neuroscience* 2015; 135: 879–886.
51. Vazan R, Ravingerova T. Protective effect of melatonin against myocardial injury induced by epinephrine. *J Physiol Biochem* .2015; 71: 43–49.
52. Kireev R, Bitoun S, Cuesta S, Tejerina A, Ibarrola C, Moreno E et al. Melatonin treatment protects liver of Zucker rats after ischemia/reperfusion by diminishing oxidative stress and apoptosis. *Eur J Pharmacol* 2013; 701: 185–193.
53. Kilic U, Yilmaz B, Ugur M, Yüksel A, Reiter RJ, Hermann DM et al. Evidence that membrane-bound G protein-coupled melatonin receptors MT1 and MT2 are not involved in the neuroprotective effects of melatonin in focal cerebral ischemia. *J Pineal Res* 2012; 52: 228–235.
54. Diez ER, Prados LV, Carrión A, Ponce ZA, Miatello RM. A novel electrophysiologic effect of melatonin on ischemia/reperfusion-induced arrhythmias in isolated rat hearts. *J Pineal Res* 2009; 46: 155–160.
55. Dominguez-Rodriguez A, Abreu-Gonzalez P, Garcia-Gonzalez MJ, Kaski JC, Reiter RJ, Jimenez-Sosa A. A unicenter, randomized, double-blind, parallel-group, placebo-controlled study of Melatonin as an Adjunct in patients with acute myocardial Infarction under-going primary Angioplasty The Melatonin Adjunct in the acute myocardial Infarction treated with Angioplasty (MARIA) trial: study design and rationale. *Contemp Clin Trials* 2007; 28: 532–539.
56. Kahya MC, Naziroğlu M, Övey IS. Modulation of Diabetes-Induced Oxidative Stress, Apoptosis, and Ca²⁺ Entry Through TRPM2 and TRPV1 Channels in Dorsal Root Ganglion and Hippocampus of Diabetic Rats by Melatonin and Selenium. *Mol Neurobiol* 2017; 54: 2345–2360.

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