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The role of toll-like receptors in the protective effect of melatonin against doxorubicin-induced pancreatic beta cell toxicity

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ABSTRACT

Aims: Doxorubicin, an anticancer drug, has a toxic effect on many tissues such as heart, pancreas, liver, kidney, and testis. The aim of current study is to investigate whether melatonin would be protective in doxorubicin-induced beta (β) cell toxicity via HMGB1/TLR2/TLR4/MAPK/NF-κB signaling pathway.

Main methods: Human pancreatic β cell (1.1B4) was used in the present study. Four experimental groups were created as control, melatonin (10 μM), doxorubicin (2 μM) and the combination of melatonin with doxorubicin. Following 24-h treatment, Mitogen-activated protein kinase (MAPKs), Toll like receptors (TLRs) including TLR2 and TLR4, pro-and anti-apoptotic protein expression levels were determined by western blotting. Total anti-oxidant (TAS), oxidant status (TOS) and oxidative stress index (OSI) of the cells as well as superoxide dismutase (SOD) levels were determined. Active caspase-8 activity was measured and TUNEL staining was performed to study apoptotic pathways. Mitochondrial membrane potential (MMP), some protein expressions and F-actin distribution were analyzed.

Key findings: Doxorubicin caused to depolarize MMP, resulting in enhancing apoptosis by activation of caspase-8 via MAPKs/NF-κB pathway via elevation of TOS and decreasing TAS. Also, doxorubicin destroyed F-actin distribution and elevated TLR2 and some apoptotic proteins, including Bax. However, co-treatment of melatonin with doxorubicin could reverse depolarization of MMP and inhibition of apoptosis through MAPK/NF-κB signaling by decreasing TOS and increasing TAS. The co-treatment reversed the alternations of TLR2, TLR4, MAPKs and apoptotic protein expressions induced by doxorubicin.

Significance: Melatonin could be a good candidate against pancreatic β cell toxicity-induced by doxorubicin through TLR2/TLR4/MAPK/NF-κB pathways.

1. Introduction

Doxorubicin is one of the most common chemotherapeutic drugs. It has been used in the treatment of various hematological and solid tumors in children and adults since 1960 [1,2]. However, long-term clinical usage of doxorubicin is limited in the treatment of cancer patients due to its toxicity on several organs such as heart, kidney, liver and pancreas with multipotent underlying mechanisms [3]. Among them, the drug-induced cardiotoxicity and nephrotoxicity are the most studied ones. On the other hand, it should be mentioned that doxorubicin is toxic to pancreatic cells. However, only a small number of researches has been performed on doxorubicin-induced pancreatic β cell toxicity.

Melatonin plays a critical role primarily in the regulation of circadian rhythm. Also, it has several important activities, such as anti-apoptotic, anti-inflammatory, and anti-oxidant. Melatonin can also be

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useful in decreasing adverse or toxic effect of chemotherapeutic drugs on healthy tissues or enhancing the effectiveness of these drugs on cancer cells via several cell-signaling pathways [4]. For example, melatonin may be useful in the protection against chemotherapy induced-nephrotoxicity by reducing apoptosis, inflammation, and oxidative stress [5]. In pancreatic tumor cell line, melatonin injection with chemotherapeutic agents such as doxorubicin resulted in decreased tumor cell viability and increased apoptosis, mitochondrial membrane depolarization in the tumor cells as well as the production of intracellular ROS in the tumor cells. This suggests that melatonin may enhance the beneficial effect of chemotherapeutic agents [6]. However, the effect of melatonin in doxorubicin-induced pancreatic β cells as an untargeted tissue of chemotherapy has not been fully clarified. Besides, the link between cancer and diabetes is known [7]. For example, the incidence of diabetes mellitus in cancer survivor is found to be higher in U.S. population [8]. Doxorubicin could impair the functions of pancreatic β cells, resulting in hyperglycemia, hyperinsulinemia, and systemic insulin resistance [9].

Toll-like receptors (TLRs) are the main activators of innate immunity. TLRs plays a critical role in signal transduction pathways for many pathological and physiological processes [10,11]. For example, TLRs such as TLR2 and TLR4 are of key importance in local pancreatic cancer [12]. In addition, TLR4 plays a crucial role in the synthesis of insulin, secretion and viability of β cells [13]. In addition, TLRs are also critical in pancreatic ductal adenocarcinoma. They are directly linked to several important proteins such as NF-κB, MAPKs, pro-apoptotic and anti-apoptotic proteins [14]. Melatonin has beneficial effects in various diseases via modulating TLRs. A recent study indicates that melatonin attenuates apoptosis via decreasing TLR-4 activation [15]. In addition, it is reported that melatonin can be protective against cardiac injury via suppressing high mobility group box 1 (HMGB1) and TLR2/TLR4 signaling pathways [16]. On the other hand, the underlying mechanism of melatonin's effect on untargeted pancreatic β cells damage in the doxorubicin treatment via HMGB1/TLR2/TLR4/MAPK/NF-κB pathways is unknown.

All in all, it is essential to find an effective strategy to fight the undesired side effects of chemotherapeutic agents by using antioxidant molecules before and/or during chemotherapy. The impact of melatonin on doxorubicin-induced β cell toxicity and the mechanism involved in have never been investigated. In the present study, we aim to examine the potential protective effect of melatonin in doxorubicin-induced pancreatic β cell toxicity and mechanistic pathways underlying its effects.

2. Material and methods

2.1. Cell culture

In cell culture flask, human pancreatic β cell line (1.1B4) was grown in RPMI 1640 medium containing 10% bovine serum albumin, 1% pyruvate, 2 mM glutamine and antibiotic (100 IU/ml penicillin, 0.1 mg/ml streptomycin) with incubation conditions at 37 °C and 5% carbon dioxide. When the cells in the flask grew by 80%, the study was started.

2.2. Experimental groups

Four experimental groups were created to test our hypothesis; control (C), melatonin (M), doxorubicin (D), and the combination of melatonin with doxorubicin (MD). The cells in the C group were not exposed to any drug. Melatonin was administrated to the cells in the M group with a concentration of 10 μM for 24 h [17]. Doxorubicin was administrated to the cells in D group with a concentration of 2 μM for 24 h (Adriblastina vial 10 mg, Pharmacia) because the mean plasma concentration of patient treated with doxorubicin is about 2–6 μM after receiving a standard bolus injection [18]. Melatonin (10 μM) and doxorubicin (2 μM) were administered at the same time to the cells in the MD group.

2.3. Western blotting

To prepare cell lysate, human pancreatic β cells were centrifuged at 3000 rpm for 5 min, and then the pellet was washed with 2 ml of 1 × PBS in RIPA buffer (Cat. No. sc-24948; Santa Cruz Biotechnology) and was centrifuged again at 3000 rpm for 5 min, then incubated on ice for 10 min. The lysates were stored at − 20 °C until used. The protein levels were determined according to Broadford assay. 100 μg protein sample was mixed with 4 × SDS loading buffer and then separated on a 4–12% Bis-Tris gel. After running, proteins were transferred to a PVDF membrane. We used primary antibodies against the following proteins: HMGB1, TLR2, TLR4, NF-κB (sc-8008, 1/500), PNF-κB (sc-136548, 1/300), Bcl-2 (sc-7382; 1/100), SAPK/JNK (CS 9258; 1/1000), p38 (CS 9212;1/1000), ERK1/2 (CS 4695; 1/1000), pERK1/2 (CS 9101; 1/250), pJNK (CS 4671; 1/250), p-p38 (CS 4511; 1/250), TLR4 (ab22048; 1/500), AMPK (ab32047; 1/1000), APAF-1 (1/500), Caspase 3 (ab1899; 1/100), BAX (MA5-16322; 1/100), Cytochrome-*c* (ab90529; 1/1000), and p-AMPK (ab133448; 1/1000). Alkaline phosphatase-conjugated secondary antibodies (anti-mouse and anti-rabbit 1/3000) was used. Signals were detected using the NCIP/BNP kit (Cat. No. ES0006; Millipore, Boston, MA, USA).

2.4. Biochemical parameters

2.4.1. Total oxidant level

Total oxidant status (TOS) was measured using a commercial kit (Rel Assay Diagnostic, Cat No: RL0024; Gaziantep, Turkey) with a BioTek μQuant microplate spectrophotometer according to the manufacturer's instructions. The principle of assay depends on the reduction of dark blue green colored 2, 29-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical to colorless reduced ABTS form by antioxidant in the sample. Total antioxidant level of the sample is related to the change of absorbance at 660 nm. A stable antioxidant solution (Trolox Equivalent) is used to calibrate the assay. The results are expressed as millimoles Trolox equivalent per liter (mmol Trolox equivalent/L).

2.4.2. Total antioxidant level

Total antioxidant status (TAS) was measured using a commercial kit (Rel Assay Diagnostic, Cat No: RL0017; Gaziantep, Turkey) with a BioTek μQuant microplate spectrophotometer according to the manufacturer's instructions The assay is based on the oxidation of the ferrous ion to ferric ions by oxidant in the sample. The oxidation reaction is enhanced by the glycerol molecules in the reaction medium. In the acidic medium, the ferric ions makes a colored complex with xylenol orange. The intensity of color depends on the total amount of oxidant molecules. Hydrogen peroxidase is used to calibrate the assay. The results are expressed in terms of micro molar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L).

2.4.3. Oxidative stress index

Oxidative stress index (OSI) was determined by the ratio between TOS and TAS levels measured in six samples per group. To calculate OSI, the unit of TAS were converted to μmol/L. Then OSI value was calculated as: OSI = (TOS, μmol H_2O_2 equivalent/L) / (TAS, μmol Trolox equivalent/L) * 100.

2.4.4. Superoxide dismutase measurement

Superoxide dismutase (SOD) levels were also measured using a commercial kit according to the manufacturer's instructions (Sigma, #19160-1KT-F). Briefly, 20 μl of sample solution in 2 well were added and then 200 μl of WST working solution was added to each well. Added 20 μl of enzyme working solution to each well was mixed and incubated at 37 °C for 20 min. After incubation, the plate was read at

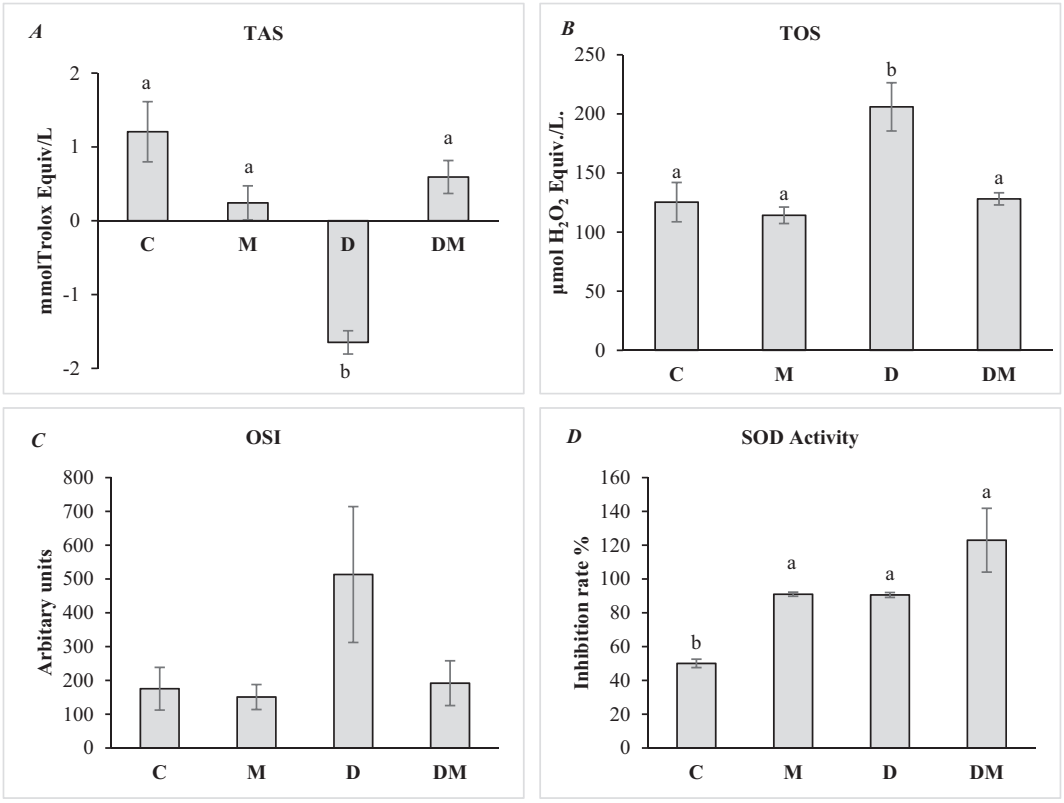

Fig. 1. The effect of melatonin on oxidative stress in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin; TAS: Total Antioxidant Status; TOS: Total Oxidant Status; OSI: Oxidative Stress Index; SOD: Superoxide Dismutase. Different letters indicate significant differences between groups ($p < 0.05$).

450 nm and SOD activity (inhibition rate %) was calculated using the following formula: SOD activity (inhibition rate %) = {[(Ablank 1 − Ablank 3) − (Asample − Ablank 2)] / (Ablank 1 − Ablank 3)} × 100

2.4.5. Total and phosphorylated NF-κB measurement

Total and phosphorylated NF-κB levels in cell lysates were measured using a commercial kit according to manufacturer's instructions (Invitrogen, 85-86083-11). Briefly, after cells cultivation (n = 8 per groups), proteins were isolated from the samples using lysis solution in the kit. NF-κB levels was measured by using the 96-well plate in the kit. The results were given as absorbance values at 450 nm.

2.5. TUNEL staining

The pancreatic β cells were grown up on a coverslip and created groups. After the incubation period, the cells fixed in 4% paraformaldehyde solution for 1 h at room temperature. Then, the cells incubated in permeabilization solution (0.1% Triton-X-100 in 0.1% sodium citrate), and the apoptotic cells labeled with a terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) kit from Roche (Cat. No. 1684795; Mannheim, Germany) according to the manufacturer's instructions. All images were taken by an Olympus BX51 microscope equipped with a DP72 camera and controlled with Olympus DP2-TWAIN software. The apoptotic (TUNEL-positive) cells were quantified using the ImageJ.

2.6. Determination of active caspase-8 by immunofluorescence method

In this analysis, IETD-FMK (FITC-IETD-FMK), a FITC-linked caspase-8 inhibitor, was used as a marker. FITC-IETD-FMK is non-toxic and binds irreversibly to activated Caspase 8 found in apoptotic cells. Active caspase-8 was determined by immunofluorescence method according to manufacturer protocol (Roche). Briefly, for each coverslip, one µL FITC-IETD-FKM was added to 500 µL of DMEM. Cells were incubated at a 37 °C for 75 min in FITC medium. Following the completion of the incubation period, washing was carried out twice using a washing solution for 5 min each. Later, the samples were left to dry in the dark at room temperature for 10 min. Then, they were scanned and imaged at 20×, 40×, and 100× by using fluorescence microscopy.

2.7. F-actin filaments

The F-actin filament is so crucial for pancreatic β cell function due to insulin secretion. That is why the F-actin structure were investigated. Pancreatic β cell were grown on coverslips and created groups. After 24 h treatment, the cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 1 h. Then, they were permeabilized in 0.1% (vol/vol) Triton X-100 in PBS at room temperature for 15 min. After blocked in 5% BSA in PBS at room temperature for 60 min, Alexa Fluor 595 phalloidin, and nuclei with 4′,6-diamidino-2-phenylindole dichloride (DAPI) (Invitro Molecular Probes, OR, USA) were used for actin filaments visualization by using an Olympus BX51 microscope equipped with a DP72 camera controlled via Olympus DP2-TWAIN software.

2.8. Mitochondrial membrane potential

Pancreatic β cells were grown on a coverslip. Mitochondrial membrane potential (MMP) was measured by a using kit (Sigma-Aldrich; #CS0390) according to the manufacturer's instructions. Briefly, they were incubated with JC-1 dye at 37 °C for 40 min. The dye can easily cross the membrane to form red aggregated for healthy mitochondria having a high MMP. The cell with low MMP, described as unhealthy,

can accumulate as fluorescence monomers represented as green.

2.9. Statistical analysis

Data were analyzed using SPSS (SPSS Statistical Software; SPSS Inc., Chicago, IL, and v. 22.0). Data are expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA), followed by a post hoc protected Tukey test was used for comparisons between the groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Oxidatives stress

To investigate the effect of doxorubicin and melatonin cotreatment on the oxidative status of the cells, we measured TAS, TOS, OSI, and SOD activity of the cells treated with doxorubicin and melatonin. The results revealed that doxorubicin significantly decreased the TAS of the cells compared to other groups. Treatment of the cells with melatonin in DM groups increased the TAS of the cells (Fig. 1A). Also, doxorubicin significantly increased the TOS of the cells in comparison to other groups. On the other hand, melatonin improved the TOS in DM groups when compared to D group (Fig. 1B). There were no significant differences in OSI and SOD activity among groups (Fig. 1C–D).

3.2. Nuclear factor-kappaB (NF-κB) levels

To determine the role of NF-κB in the effect of melatonin, we measured the total and phosphorylated NF-κB in the doxorubicin-induced pancreatic β cell. NF-κB plays a crucial role in the regulation of inflammation, apoptosis, immunity, and progression of cancer cells. Doxorubicin significantly decreased the level of total and phospho-NF-κB in D group when compared to the C group. Melatonin alone or combined with doxorubicin did not affect the level of total and phospho- NF-κB (Fig. 2).

3.3. Mitochondrial membrane potential

We also measure MMP to determine whether melatonin is protective against mitochondrial damage induced by doxorubicin. The intensity of red fluorescence presents the health status of mitochondria. In other words, the cells with healthy mitochondria reflect more intense yellow light and/or red fluorescence under fluorescence microscopy. Green fluorescence and/or reduction in the intensity of fluorescence dye means that MMP is dissipated or mitochondria are unhealthy. In C and M groups, the cells had normal healthy mitochondria with high MMP (Fig. 3A,a and B,b). Treatment of cells with doxorubicin disrupted the MMP, indicated by an increase green fluorescence and a decrease in the intensity of red fluorescence when compared to C and M groups (Fig. 3C,c). However, melatonin preserved the MMP in the cells treated with doxorubicin, increasing the intensity of fluorescence dye and the number of the cells when compared to D group (Fig. 3D,d).

3.4. F-actin distribution

Actin filaments are one of the crucial proteins in the determination of cell morphology and dynamics in healthy and cancer cells as well. Chemotherapeutic agents disrupt mainly filamentous actin of cells. Therefore, the effect of melatonin on F-actin distribution in doxorubicin-treated cells was investigated. The cells in C and M groups had normal F-action distribution (Fig. 4A–B). The treatment of the cell with doxorubicin destroyed F-actin, indicated by an explosive filopodia formation (Fig. 4C). However, melatonin treatment improved the actin filament in doxorubicin-treated cells, indicated by the fact that filopodia formation in DM group was almost disappeared (Fig. 4D).

3.5. Active caspase-8

The activation of caspase enzymes (proteolytic activities of these enzymes) refers to the early stage of apoptosis, responsible for the death of cells in many physiological and pathological conditions. That is why; we determined the caspase-8 activity in the cell treated with doxorubicin and melatonin. In control and melatonin groups, caspase-8 activity was observed as expected in the physiological process (Fig. 5A,a–B,b). Doxorubicin resulted in increased activity of caspase-8 activity, as indicated by the increasing number of the cell reflecting red fluoresce light compared to C and M groups (Fig. 5C,c). Melatonin treatment led to a reduction of caspase-8 activity in the cell treated with doxorubicin when compared to D groups (Fig. 5D,d). Also, as shown in Fig. 6, the number of active caspase 8 increased in D group.

3.6. Apoptotic cell counts

There was no significant difference between C and M group (Figs. 7Aa, Bb, and 8). Doxorubicin significantly increased the apoptotic cell death when compared to C group. However, the combination of melatonin with doxorubicin attenuated the apoptotic cell death in contrast to D group (Figs. 7Cc, Dd, and 8).

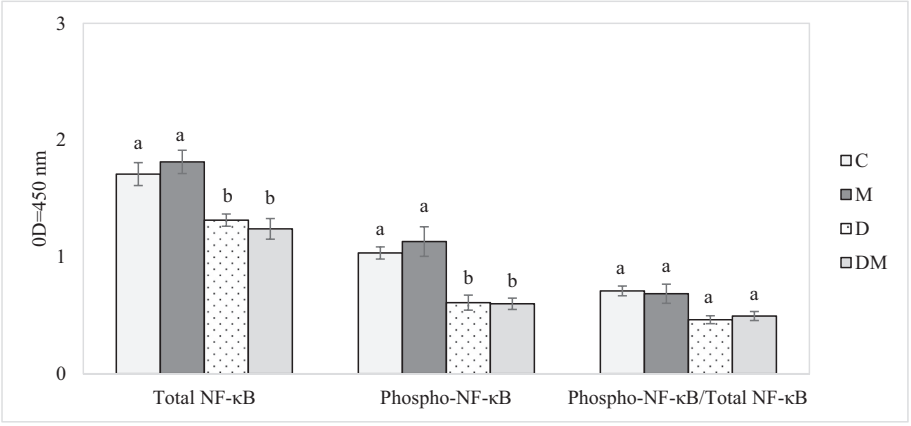

Fig. 2. The effect of melatonin on NF-κB levels in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin. Different letters indicate significant differences between groups ($p < 0.05$).

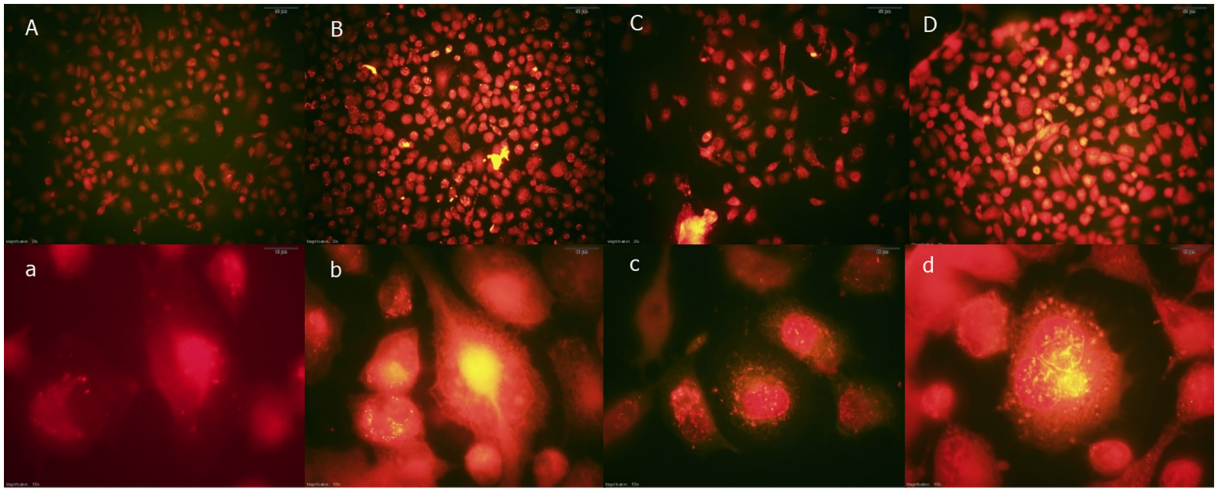

Fig. 3. The effect of melatonin on mitochondrial membrane potential in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: Capital letters: 20×; Small letters: 100×; A,a: Control; B,b: Melatonin; C,c: Doxorubicin; D,d: Doxorubicin + Melatonin.

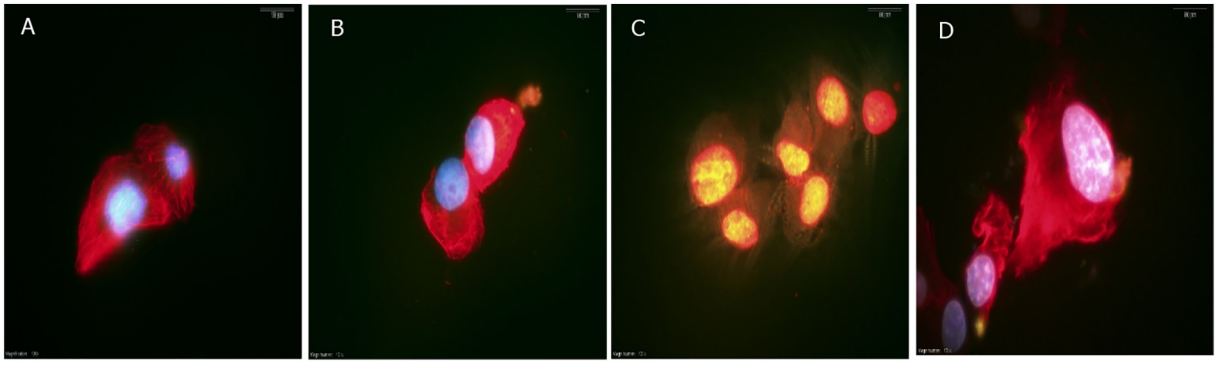

Fig. 4. The effect of melatonin on F-actin distribution in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: A: Control; B: Melatonin; C: Doxorubicin; D: Doxorubicin + Melatonin.

3.7. Protein expressions

3.7.1. HMGB1

The result of the present study indicated that there was no significant alternation on HMGB1 protein level between groups (Fig. 9a).

3.7.2. TLR2 and TLR4

In comparison to C group, melatonin affected neither TLR2 nor TLR4 protein expressions in M group. However, doxorubicin significantly downregulated TLR4 but upregulated TLR2 protein expressions in D group. On the other hand, the combination of melatonin with

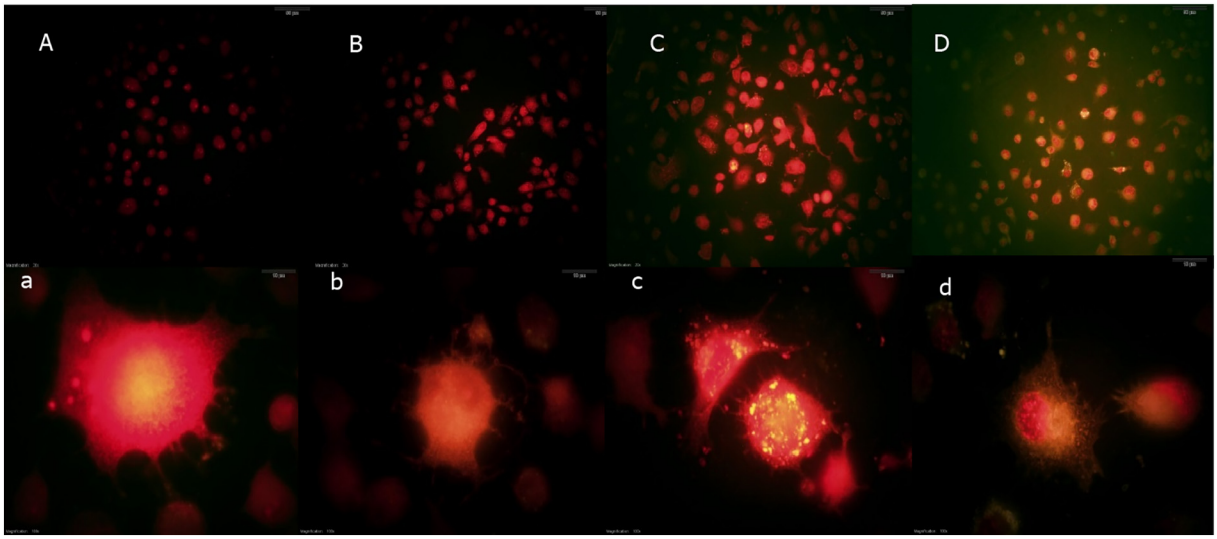

Fig. 5. The effect of melatonin on active caspase-8 in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: Capital letters: 20×; Small letters: 100×; A,a: Control; B,b: Melatonin; C,c: Doxorubicin; D,d: Doxorubicin + Melatonin.

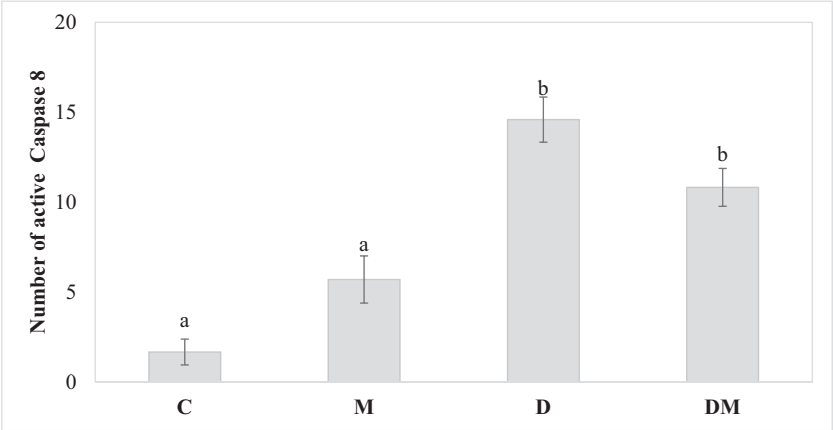

Fig. 6. The effect of melatonin on active caspase 8 in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin. Different letters indicate significant differences between groups ($p < 0.05$).

doxorubicin improved TLR2 and TLR4 expressions in DM group (Fig. 9b and c).

3.7.3. Total and phosphorylated 5′ adenosine monophosphate-activated protein kinase (AMPK)

Doxorubicin resulted in the downregulation of AMPK expression in D group, although melatonin improved the expression of AMPK in DM group in compared to the D group (Fig. 9d). Also, doxorubicin decreased the expression of pAMPK in D group while melatonin restored the expression pAMPK in DM group (Fig. 9e).

3.8. Mitogen-activated protein kinases (MAPKs)

3.8.1. Total and phosphorylated Jun N-terminal kinases (JNK)

Doxorubicin increased the expressions of JNK and pJNK in D group. Moreover, melatonin did not alter JNK and pJNK expressions in DM group (Fig. 10a, b).

3.8.2. Total and phosphorylated p38

p38 is one of the four well-known subfamilies of MAPKs (ERK1/2, ERK5, JNKs, and p38s) The protein expression of p38 was upregulated in D group, whereas it was improved in DM group (Fig. 10c). Likewise, doxorubicin led to an increase in the expression of pP38 in D group. On the other hand, melatonin restored it in DM group (Fig. 10d).

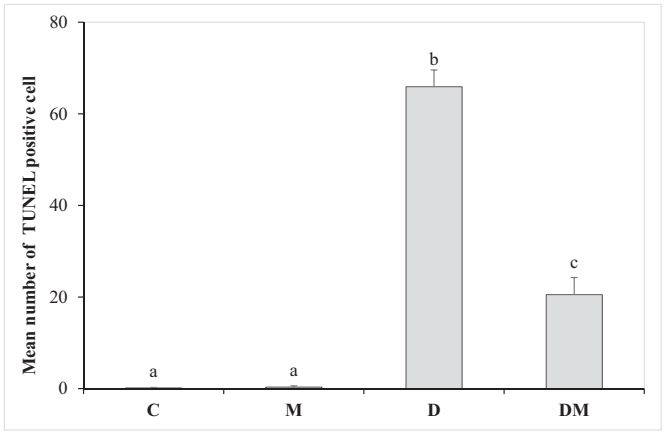

Fig. 8. The effect of melatonin on apoptosis in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin. Different letters indicate significant differences between groups ($p < 0.05$).

3.8.3. Total and phosphorylated extracellular signal-regulated kinases 1/2 (ERK1/2)

Doxorubicin resulted in upregulation of ERK1/2 expression in D

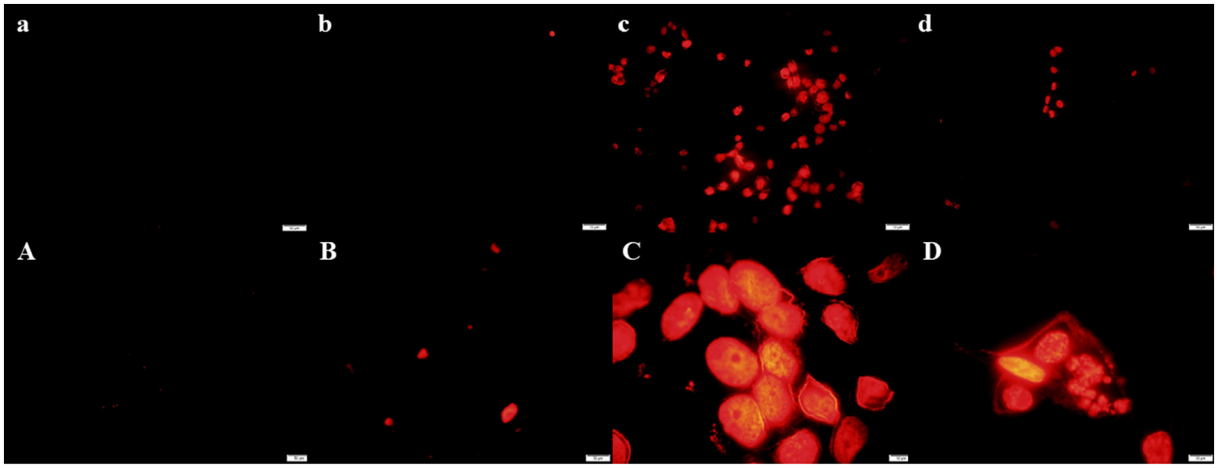

Fig. 7. The effect of melatonin on apoptosis in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: Small letters: 20×; Capital letters: 100×; A,a: Control; B,b: Melatonin; C,c: Doxorubicin; D,d: Doxorubicin + Melatonin.

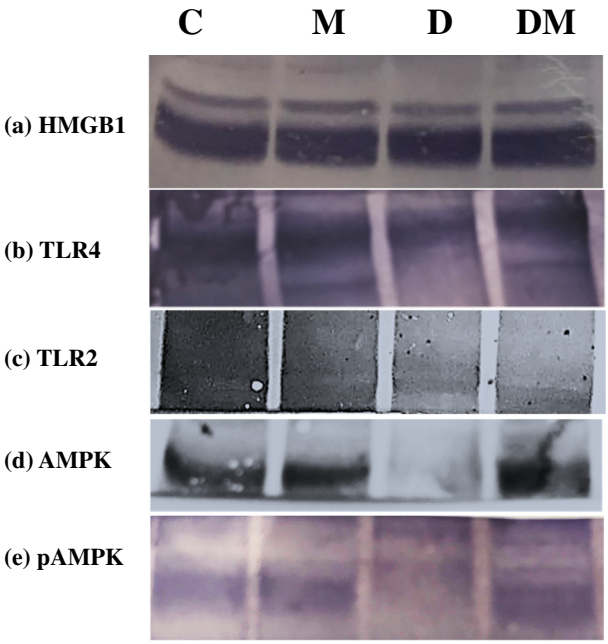

Fig. 9. The effect of melatonin on some protein expressions in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin.

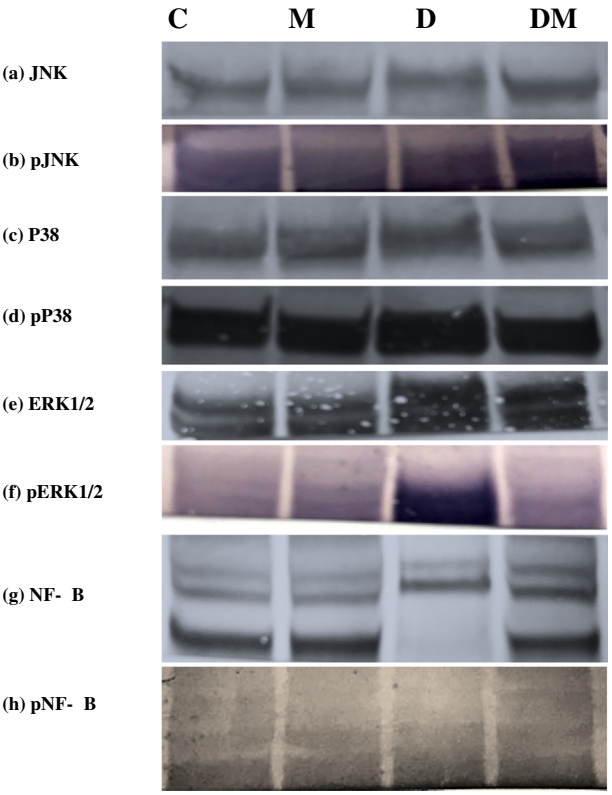

Fig. 10. The effect of melatonin on MAPK protein expression in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin.

group while melatonin attenuated its expression in DM group (Fig. 10e). Similarly, a marked increase in the expression of pERK1/2 levels in D group was observed. On the other hand, melatonin improved it in DM group (Fig. 10f).

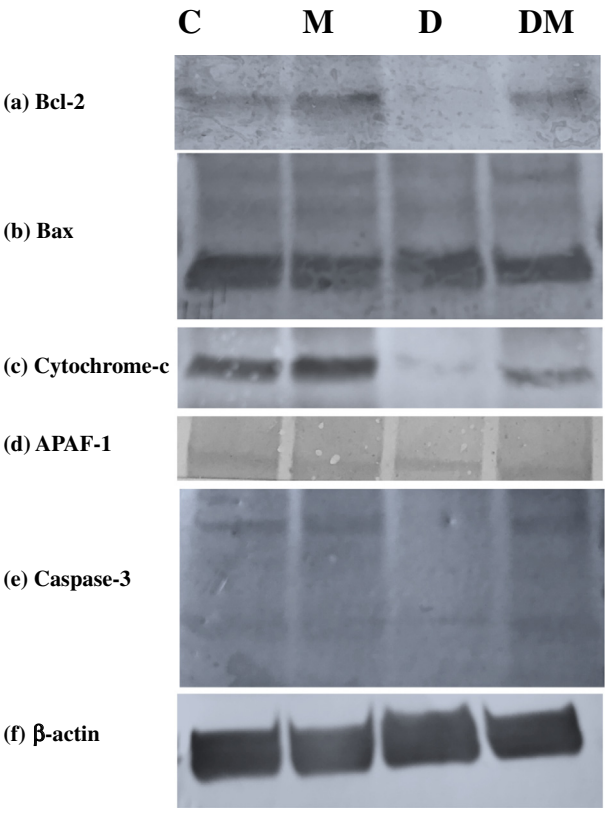

Fig. 11. The effect of melatonin on apoptotic proteins in 1.1B4 pancreatic beta cells treated with doxorubicin.
Abbreviations: C: Control; M: Melatonin; D: Doxorubicin; DM: Doxorubicin + Melatonin.

3.9. Total and phosphorylated nuclear factor kappa B (NF-κB)

Doxorubicin attenuated total NF-κB protein expression compared to the C group. Melatonin improved it in the MD group in contrast to the D group (Fig. 10g). However, there was no significant difference in pNF-κB protein expression levels between groups (Fig. 10h).

3.10. Apoptotic proteins

3.10.1. Bcl-2

In comparison to C group, melatonin increased the expression of Bcl-2 protein in M groups. However, doxorubicin reduced it D group. Cotreatment of melatonin and doxorubicin led to the increase in the expression of Bcl-2 protein in DM group (Fig. 11a).

3.10.2. Bax (BCL2-associated X protein)

Doxorubicin upregulated the expression of BAX in D group. However, melatonin restored it in DM group compared to D group (Fig. 11b).

3.10.3. Cytochrome-c

Cytochrome-*c* is a critical molecule that initiates the mitochondrial-dependent apoptosis. Doxorubicin downregulated the expression of cytochrome-*c* in D group while melatonin improved it in DM group (Fig. 11c).

3.10.4. Apoptotic protease activating factor 1 (APAF1)

The expression of APAF1 increased in D group. On the other hand, melatonin improved the expression of APAF1 in DM group (Fig. 11d).

3.10.5. Caspase-3

Caspase-3 is another essential protein that regulates apoptotic pathways. In D group, the expression of full length caspase-3 (35 kDa) was downregulated, but up regulated the cleavage (17 kDa) of caspase-3 while melatonin improved its both expressions in DM (Fig. 11e).

4. Discussion

The present study focuses on the possible cellular underlying mechanisms of melatonin's impact on doxorubicin-induced pancreatic β cell toxicity. Main findings of the study are (i) doxorubicin increased TOS levels and decreased TAS levels in pancreatic β cells, however, co-administration of melatonin with doxorubicin improved the TOS and TAS levels, (ii) doxorubicin disrupted MMP and F-action organization in pancreatic β cells whereas administration of melatonin with doxorubicin restored MMP and F-action organization in doxorubicin-induced pancreatic β cells, (iii) doxorubicin increased the number of active caspase 8 and apoptotic cells in pancreatic β cells, on the other hands, melatonin attenuated apoptosis in doxorubicin-induced pancreatic β cells, (iv) doxorubicin increased the protein expression levels of MAPKs (JNK, pJNK, p38, pp38, ERK1/2, pERK1/2), however, melatonin improved the protein expression levels of these proteins in doxorubicin treated pancreatic β cells, (vi) doxorubicin attenuated the protein expression levels of cytochrome-*c*, Bcl-2, and APAF-1 although melatonin improved the expression levels of the proteins in doxorubicin-induced pancreatic β cells, (vii) doxorubicin increased the expression levels of caspase-3 and BAX in pancreatic β cells, however, melatonin improved the protein expression in doxorubicin-treated pancreatic β cells, (viii) doxorubicin decreased the protein expressions of TLR2 and TLR4, AMPK, and pAMPK in pancreatic β cell, on the other hand, melatonin restored the expression levels of this protein in doxorubicin-induced pancreatic β cells.

It is known that doxorubicin has a highly toxic effect on both targeted and non-targeted cells even at a dose of 1 μM. Pancreatic β cells have been indicated to have regeneration capacity, but it needs to be noted that generation capacity is very low [7]. That is why the loss of β cells results in irreversible dysfunction of tissue. Herein, it is essential to clarify the underlying mechanisms of doxorubicin's toxicity on pancreatic β cells as well as the effective strategies to reduce or treat the undesired toxic effect of doxorubicin on off-target cells.

In the present study, we hypothesized that HMGB1 initiates the toxic effect of doxorubicin via TLRs based on that HMGB1 underlies the mechanism of doxorubicin-induced cardiotoxicity in a recent study [19]. The study reported that doxorubicin increased the release and upregulation of HMGB1 via down-regulating YAP expression, resulting in increased expression of c-PARP and c-caspase-3 protein. So, doxorubicin induces apoptosis and autophagy in cardiomyocyte [19]. However, our result suggests that HMGB1 might not play a critical role in doxorubicin-induced pancreatic β cells damage because doxorubicin administration did not change the expression of HMGB1. Therefore, we believe that other TLR agonists such as heat shock protein [20] or oxidative stress [21] probably initiate activation of TLRs involved in the underlying mechanism of doxorubicin-induced β cells toxicity. It was shown that melatonin attenuated serum HMGB1 releasing in hepatic ischemia/reperfusion [22]. In the current study, however, melatonin did not alter HMGB1 protein expression.

TLRs play a critical role in signal transduction pathways for many pathologic processes [12,23]. In addition, they are important for regulation of physiological functions. For example, it was indicated that TLR4 could regulate insulin secretion and viability of pancreatic β cell [13]. Therefore, current study focuses on the role of TLR2 and TLR4 in pancreatic β cell dysfunction-induced by doxorubicin. A previous study showed that doxorubicin caused dilated cardiomyopathy (DCM) via TLR2 and TLR4. The authors provided that attenuation of TLR2 decreased developing of DCM in doxorubicin-treated mice; however, blocking of TLR4 promoted DCM. The result illustrates that TLR2 and TLR4 have distinct roles in the progression of DCM-induced by doxorubucin [24]. Furthermore, another study also supported the participation of TLR2 in doxorubicin-induced toxicity in cardiomyocytes, but not TLR4 [25]. Our results are consistent with these results since doxorubicin gave rise to elevation of TLR2 expression while decreasing TLR4 expression in the present study. Melatonin treatment elevated the TLR4 and decreased TLR2 protein expressions in pancreatic β cell treated with doxorubicin in the present study. On the other hand, there are opposite results in other studies in which it was indicated that melatonin treatment has been indicated to suppressed TLR4/NF-κB pathways in colitis [26], the blood-brain barrier damaged in rats [27]. In addition, a previous study showed that TLR4 triggered caspase-3 in hypoxic microglia [15]. The downstream molecule of TLR4 pathways is NF-κB. Consistently, our study supported the result of a previous study in which melatonin treatment attenuated TLR4 [28]. It was suggested that melatonin exerts its effect via regulation of TLR2 and TLR4 pathways during Helicobacter pylori (*H. pylori*) infection. The infection has been identified into two phases for reciprocal activation of TLR2 and TLR4. The first phase is the initiation stage in which TLR2 has to recognize *H. pylori* in circulation. In the second phase, TLR4 has been dominantly expressed [29]. The other option is that AMPK regulates TLR4 expression, relying on finding of a recent study which showed that AMPK suppresses TLR4 in rat heart treated with lipopolysaccharide [30]. Our results suggest that doxorubucin enhanced TLR2; however, attenuated TLR4 and AMPK. However, melatonin has opposite effects on TLRs and AMPK in the combination of melation and doxorubicin. So, doxorubicin-induced pancreatic β cell toxicity could probably cause the reciprocal activation of TLRs. A recent study reported that doxorubucin triggers apoptosis by caspase activation in pancreatic β cells. This study also pointed out that caspase-3 activation is TLR4-dependent and TLR4-expression is suppressed by melatonin [15]. However, a controversial result is present because it was suggested that TLR4 protects heart via survivor activating factor enhancement (SAFE) pathway which involves tumor necrosis factor alpha and the signal transducer and activator of transcription 3 [31].

TLRs have a pivotal role in MAPK/NF-κB pathways [29]. TLRs have two intracellular adapter proteins named as MyD88 and TRIF dependent. MyD88 has been suggested to involve the MAPK/NF-κB pathway [22]. TLR-4 dependent MAPK activation has two distinct MAPK pathways [32]. First is the activation of JNK and P38 via TAK1 [33], and the second is the activation of ERK1/2 by Tpl2. In addition, TAK1 leads to release of NF-κB. NF-κB is one of transcription factor family member. ERK1/2 activation by TLRs is transient based on the fast degradation of Tpl2 [32]. ERK1/2, known as anti-apoptotic MAPK member [33], has been reported to participate in cell proliferation induced by the growth factor, but p38 and JNK associate with apoptosis-induced by stress [34]. Also, JNK and p38, known as pro-apoptotic members of MAPK, play a role in proliferation and differentiation, which are well known as effectors of ERK1/2 [33]. ERK1/2 exerts a pro-apoptotic influence in various tissue by triggering both intrinsic and extrinsic apoptotic pathways [34]. Our results suggested that doxorubucin triggered all MAPK pathways; however, melatonin diminished the activation of p38 and ERK1/2. In the human pancreatic carcinoma cell line, activation of MAPKs such as JNK and ERK1/2 resulted in increased protein expressions of Bax and cleavage of Cas-3 and decreased protein expression of Bcl-2 as well as the inhibition of NF-κB, which underlie the cellular mechanism of melatonin's protective effect [35]. Also, a previous study indicated that doxorubicin increased the expression of pro-apoptotic proteins such as Bax and Cas-3 and decreased the expression of anti-apoptotic proteins such as Bcl-2. Also, melatonin prevented doxorubicin-induced apoptosis in both H9c2 cells and mice [36]. Consistent with the mentioned study, our results demonstrated that co-treatment of melatonin with doxorubicin was effective against doxorubicin-induced apoptosis in β cells, as shown by improvement in Bcl-2, Bax, and Cas-3 expressions. Apoptotic markers such as Bax, Bcl-2 and cleavage Cas-3 were in agreement with a previous study [35]. Based on this

knowledge; the family member has a sophisticated effect on intracellular pathways.

There is a link between AMPK and MAPK, such that the activation of AMPK triggers ERK1/2 initiated cardiac fibroblast proliferation [34]. In contrast, it has been reported that ERK1/2 is inhibited under glucose deprivation [34]. Indeed, the interaction of these two proteins is so sophisticated because ERK1/2 inhibited AMPK as well. The energy levels of the cell are so crucial for the interaction [34]. However, another study indicated that AMPK activation resulted in p38 activation in tissues, e.g., heart [37]. AMPK is related with both pro- and anti-apoptotic pathways via regulating ERK1/2. ERK1/2 interacts with many proteins, especially cytoskeleton components, apoptosis-related protein or transcription factors [34]. Our F-actin results showed that doxorubucin had a detrimental effect on the cytoskeleton, probably related with ERK1/2 in the present study. On the other hand, melatonin reversed the effect of doxorubicin on F-actin because melatonin can easily diffuse into intracellular space and organelle. Melatonin is well established to have antioxidant features [38] by elevating SOD and glutathione peroxidase. Moreover, melatonin has been reported to promote the AMPK phosphorylation via adenosine 3′,5′-cyclic monophosphate (cAMP) activity. Furthermore, AMPK is suggested to boost SOD and GPx activities as well [39]. Even so, more elaborate studies are required for understanding the mechanistic molecular interaction between AMPK and MAPK.

Mitochondrial dysfunction has a pivotal role in the development of doxorubucin's toxicity [29]. That is why the present study determined MMP by using a JC1 dye. Doxorubicin depolarized MMP and suppressed AMPK, but melatonin ameliorated MMP in the present study. We believe that the effect of melatonin on MMP may associate with AMPK. Because a recent study provided that melatonin has promoted mitochondrial functions and structure via AMPK/PGC1α pathway, resulting in attenuation of apoptotic cell lost [36]. Melatonin also results in enhancing mitochondrial function by ATP production [38]. Based on these effects of melatonin on oxidative stress parameters and AMPK, melatonin could attenuate adverse effect of doxorubicin on pancreatic β cell. A recent study indicates that AMPK plays an essential role in doxorubicin-induced cardiotoxicity. Doxorubicin-induced apoptosis and oxidative stress disrupted mitochondrial functions by impressing AMPK and PGC1α activity. On the other hand, melatonin attenuated apoptosis and oxidative stress induced by doxorubicin through activating AMPK and PGC1α, indicated by that the inhibition of AMPK and/or PGC1α ameliorated the protective effect of melatonin on doxorubicin-induced toxicity [36]. Consistently, in our study, melatonin improved the expression of both total and phosphorated AMPK in doxorubicin-induced β cells. Also, doxorubicin disrupts mitochondrial membrane potential, resulting initiation of apoptotic [40]. In the present study, we explored the effect of doxorubicin treatment and its co-treatment with melatonin on pancreatic β cells. The results indicated that doxorubicin disrupted mitochondrial membrane potential and melatonin treatment revealed mitochondrial membrane potential. In consistent with our results, a previous study showed that co-treatment of melatonin with doxorubicin preserved the mitochondrial membrane potential in pancreatic tumor line, indicating that melatonin can exert a synergetic effect in the treatment of cancer [6].

Apoptosis induced by doxorubicin occurs via different mechanisms depending on cells types such as normal or cancer. In endothelial cells, doxorubicininduced apoptosis via reactive oxygen species such as H_2O_2 although it induced apoptosis via a mechanism depending on the activation of p53 in tumor cells [41]. In contrast, it was suggested that the underlying mechanism of doxorubicin-induced pancreatic β cells might be related to DNA damage rather than excess production of H_2O_2 [7]. So, in the present study, it was shown that apoptosis plays an essential role in doxorubicin-induced toxicity in pancreatic β cells and melatonin may be protective against doxorubicin-induced toxicity due to its antioxidant and anti-apoptotic features. The adverse effect of doxorubicin is mainly related to alleviated oxidative stress in a cell [42]. Based on our findings, doxorubicin enhanced TOS levels and melatonin has a strong antioxidant activity. Moreover, doxorubicin increased the oxidative stress in β cell, and melatonin exerted antioxidant activity in doxorubicin-induced pancreatic damage, indicating by that melatonin improved the TAS and TOS levels in β cells treated with doxorubicin. Our results are consistent with previous studies, which suggest that melatonin treatment revealed doxorubicin-induced oxidative stress *in vivo* and *in vitro* [42,43]. A previous study indicates that doxorubicin decreased serum total antioxidant activity in rats and administration of resveratrol, antioxidant molecules, improved it [44]. Also, in the pancreatic tumor line, co-treatment of melatonin with doxorubicin resulted in decreased production of reactive oxygen species, indicating that melatonin can exert a synergetic effect in the treatment of cancer [6]. Similarly, our results showed that doxorubicin decreased total antioxidant capacity and increased total oxidant status of pancreatic β cells. Co-administration of melatonin with doxorubicin revealed the oxidant and antioxidant status of the cells, suggesting that melatonin may be effective in the improvement of oxidative stress-induced by doxorubicin in pancreatic β cells.

All in all, our results indicated that melatonin exerts a protective effects against the doxorubicin-induced toxicity in the pancreatic β cells due to its antioxidative, antiapoptotic and anti-inflammatory effects. Therefore, melatonin would be a good candidate for the unexpected or adverse effects of doxorubicin in untargeted organs, especially in pancreas. In addition, targeting TLR2 and TLR4 might be a hopeful therapeutic potential for attenuating undesired effects of doxorubicin in untargeted cells.

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Declaration of competing interest

All authors have no conflicts of interest.

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