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RESEARCH ARTICLE

Putative effect of melatonin on cardiomyocyte senescence in mice with type 1 diabetes mellitus

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Abstract

Background To date, many investigators have tried to clarify the molecular mechanism of cardiovascular injuries after T1D. In present study, we evaluated the possible effects of melatonin on the levels of aging-related factors in the heart tissue of streptozotocin-induced diabetic mice.

Methods 40 male mice were enrolled in this study and randomly allocated into 4 groups (n = 10) as follows: Control group (C), Control group + melatonin (CM), Diabetic group (D), Diabetic + melatonin (DM) group. Single Streptozotocin (50 mg/kbW) was applied for the induction of T1D. 3 mg/kg melatonin was injected intraperitoneally twice a week for consequent four weeks. After the completion of this period, the animals were sacrificed and their heart tissue was obtained for histological examination (IHC analysis of vWF and α-SMA cells), aging and inflammation-related gene analysis.

Result Hematoxylin and Eosin staining indicated cardiomyocyte toxicity in T1D mice. IHC analysis of vascular tissue showed the detachment of vWF and α-SMA cells and disintegration into the vascular lumen. Additionally, real-time PCR assay showed the up-regulation of β-galactosidase and suppression of SOX2, Klotho, and Telomerase genes in T1D mice compared to the control group ($p < 0.05$). We noted that melatonin administration can revert these condition and closed near-to-control levels. Along with these conditions, the levels of IL-1β were also decreased after melatonin treatment.

Conclusions In general, one can hypothesize that modulation of different effectors associated with aging is beneficial to alleviate cardiac injuries under hypergylcemic condition. Melatonin can exert its therapeutic effects, in part, through anti-aging capacity.

Keywords Type 1 diabetes mellitus · Aging · Inflammation · Melatonin · Cardiac tissue · Pathological changes

Introduction

Type 1 diabetes (T1D), a familiar metabolic disorders, is indicated by massive destruction of pancreatic beta cells, insulin insufficiency and hyperglycemic condition [1, 2]. It has been shown that the recruitment of immune cells and inflammatory responses exacerbate the loss of insulin-producing cells [3, 4]. The continuity of hyperglycemic conditions in the diabetic patients can affect the function of several tissues such as cardiovascular tissue by the

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induction of inflammatory signaling pathways and *oxidative* and *nitrosative* stress [5–7]. Inflammatory mediators such as *Interleukin*-1β (*IL-1β*), C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), etc. are increased upon the initiation of hyperglycemic condition which participate in the progression of cardiovascular diseases [8, 9]. It was suggested that the alteration of oxidative/nitrosative status can dysregulate the activity of host cells, triggering the pathological injury [10, 11]. Of note, the accumulation of reactive oxygen species (ROS) leads to acceleration of aging changes and consequent cell dysfunction [12]. Due to complex nature of diabetic conditions, different strategies should be considered for the alleviation of metabolic disorders.

Melatonin is touted as natural hormone, derived from tryptophan, secreted mainly by pineal gland with pleiotropic effects [13]. A plethora of functions related to melatonin such as anti-inflammatory, −aging and -oxidant effects have been documented in various tissues [13–16]. Noteworthy, this hormone can stimulate the activity of antioxidant enzymes and can stabilize microsomal membranes, causing resistance to oxidative injuries [15, 17]. One of most post-complications related to the diabetic condition is the aging phenomenon. The promotion of aging changes can alter the levels of certain molecules, effectors inside the cells. These features are indicated by the malfunction of several organelles, DNA mutation, accumulation of exhausted by products [18]. Whether and how melatonin can modulate the expression of aging-related factors inside the cardiac tissues upon the onset of diabetic condition is subject of debate and needs more investigations. Here, we tried to address the possible therapeutic outcomes associated with melatonin on diabetic heart in the mouse model.

Material and methods

Animal issues

The procedures of this study was adopted to the guidelines published by the National Institutes of Health for Laboratory Animal Care (revised 1996). Local Ethics Committee of Tabriz University of Medical Sciences was approved this study (No: IR.TBZMED.VCR.REC.1399.178).

T1D induction and animal groups

Forty male BALB/c mice (8–10 weeks old), weighting 25–30 g, were enrolled to the study. The mice were maintained in standard condition with 12:12 light–dark cycle with free access to chewing food and water. Seven days after accommodation, mice were randomly divided into 4 groups (each in 10) as follows: Control (C), Melatonin (CM), Diabetic (D), Diabetic + Melatonin (DM) groups.

To induce T1D, mice were intraperitoneally received streptozotocin (STZ; 50 mg/kg; single dose) after 8 h-fasting according to our previous studies [2, 19]. Before the injection, STZ was dissolved in 0.1 mmol/L sodium citrate buffer (pH = 4.5). To confirm the efficiency our protocol, blood samples were taken from tail vein after 72 h and the systemic levels of glucose measured using glucometer strip. In this study, glucose levels more than 250 mg/dL was considered as diabetic condition. Also, glucose concentrations were carefully measured upon induction of T1D and melatonin intake. The injection of melatonin was started two weeks after the confirmation of type 1 diabetes. In CM, and DM groups, 3 mg/kg melatonin was injected intraperitoneally twice a week for consequent four weeks [20, 21]. Two days after the last injection, at the end of the experiment, mice were anaesthetized with a ketamine-xylazine cocktail and sacrificed. The cardiac samples were taken for histopathological studies and gene expression analysis (Fig. 1).

Histological examination

We monitored the potency of melatonin in the alleviation of cardiac tissue injury after T1D. To this end, left ventricles were isolated, fixed in 10% buffered-formalin solution and introduced to histological examinations. The common features of tissue injury were monitored using Hematoxylin-Eosin (H & E) staining. 5-μm thick sections were stained with H& E and the existence of pathological changes were indicated pre- and post-melatonin treatment.

We also investigated the possibility of vascular injury (either endothelial and smooth muscle cells) by monitoring the protein levels of von Willbrand factor (vWF) and alpha-smooth muscle actin (α-SMA) which are indicated to endothelial cells (ECs) and pericytes, respectively. For immunohistochemistry (IHC) staining, the slides were exposed to 3% H_2O_2 to inhibit the endogenous activity. For antigen retrieval, samples were treated with citrate (pH = 6.5) for 20 min. Thereafter, slides were incubated with anti-human vWF (Dako) and α-SMA (Dako) antibodies for 1 h and washed three times with phosphate-buffered saline (PBS). Then, HRP-tagged secondary antibodies were used for 1 h at RT. Following PBS washes, the slides were stained with DAB as chromogen agent. Finally, vWF^+ and $\alpha\text{-}SMA^+$ vessels were monitored under the light microscopy (Olympus; Model BX41; Japan) and compared between the experimental groups.

Quantitative real-time PCR (qRT-PCR) analysis

Here, we aimed to measure the expression of SOX2, Klotho, β-galactosidase, Telomerase and IL-1β using real-time

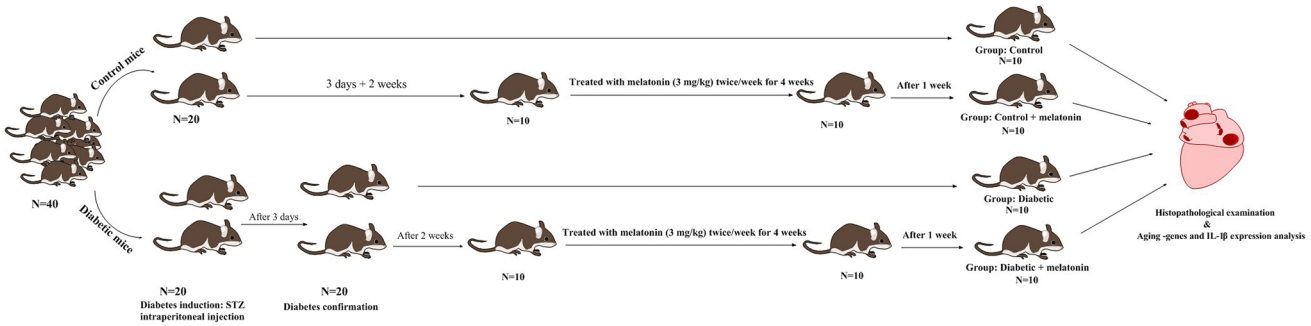

Fig. 1 Flow chart of experimental design

PCR analysis. Briefly, one week after injection, left ventricles were quickly removed and RNAs isolated using RNA extraction kit (Roche, Germany) and standard protocols. Quality and quantity of extracted RNA were monitored using a PicoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Appropriate primers were designed using Perl Primer Software and were blasted in terms of product specificity (Table 1). Reverse transcription reaction was done by cDNA Synthesis Kit (TaKaRa). Each real-time PCR reaction was performed by the mixture of 1 µl cDNA sample, 10 µl SYBR Green master mix (TaKaRa), 7 µl DEPC water and 1 µl primers on Corbett Rotor-Gene 6000 instrument (Australia). CT values were normalized to β-actin as a housekeeping gene and expression rate calculated using $2^{-\Delta\Delta Ct}$ method [22, 23].

Statistical analysis

The analysis was conducted using Graph Pad Prism software (Ver. 8). All quantitative data were presented as mean ± SEM and analyzed using One-way ANOVA. In present study, the p value <0.05 was considered as a significant level.

Results

Melatonin blunted heart injury after diabetic changes

Histological evaluation of obtained samples revealed the promotion of pathological modifications in heart tissue after induction of the T1D. According to bright-field imaging data, cardiomyocyte cytotoxicity was found that indicated necrotic changes after exposure to T1D (Fig. 2). These features were blunted in diabetic mice received Melatonin (DM group). We also monitored protein levels of vWF (an endothelial marker) and α-SMA (typical smooth muscle cell marker) in diabetic hearts pre- and post-melatonin treatment. Data showed the injury of vWF^+ cells indicated with remarked cell detachment from underlying layer and slaughtering into the vascular lumen (Fig. 2B). Bright-field imaging displayed the lack of endothelial cell layer injury in diabetic heart treated with melatonin. Along with these data, occurrence of T1D loosened the integrity of vascular pericytes (α-SMA^+ cells), showing the injury of vascular wall. The exposure of cardiac vessels to T1D led to the loss of pericyte-to-pericyte interaction (Fig. 2C). Again, we found that melatonin can reduce the injury of α-SMA^+ cells in the vascular wall under diabetic condition.

Table 1 Primers list

Gene	Primer sequence (5′-3′)	
	Forward	Reverse
IL-1β	*TGGACCTTCCAGGATGAGGACA*	GTTCATCTCGGAGCCTGTAGTG
Sox2	GCGGAGTGGAAACTTTTGTCC	GGGAAGCGTGTACTTATCCTTCT
β-galactosidase	CGGATACCCCGCTTCTACTG	AGTTCCAGGGCACGTACATC
Telomerase reverse transcriptase	TGGATGCTACTGTTGAGCCG	GAGACCCACACACCTGGTAG
klotho	CACGCCGAGCAAGACTCACTG	TTGATGTCGTCCAACACGTAGGC
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

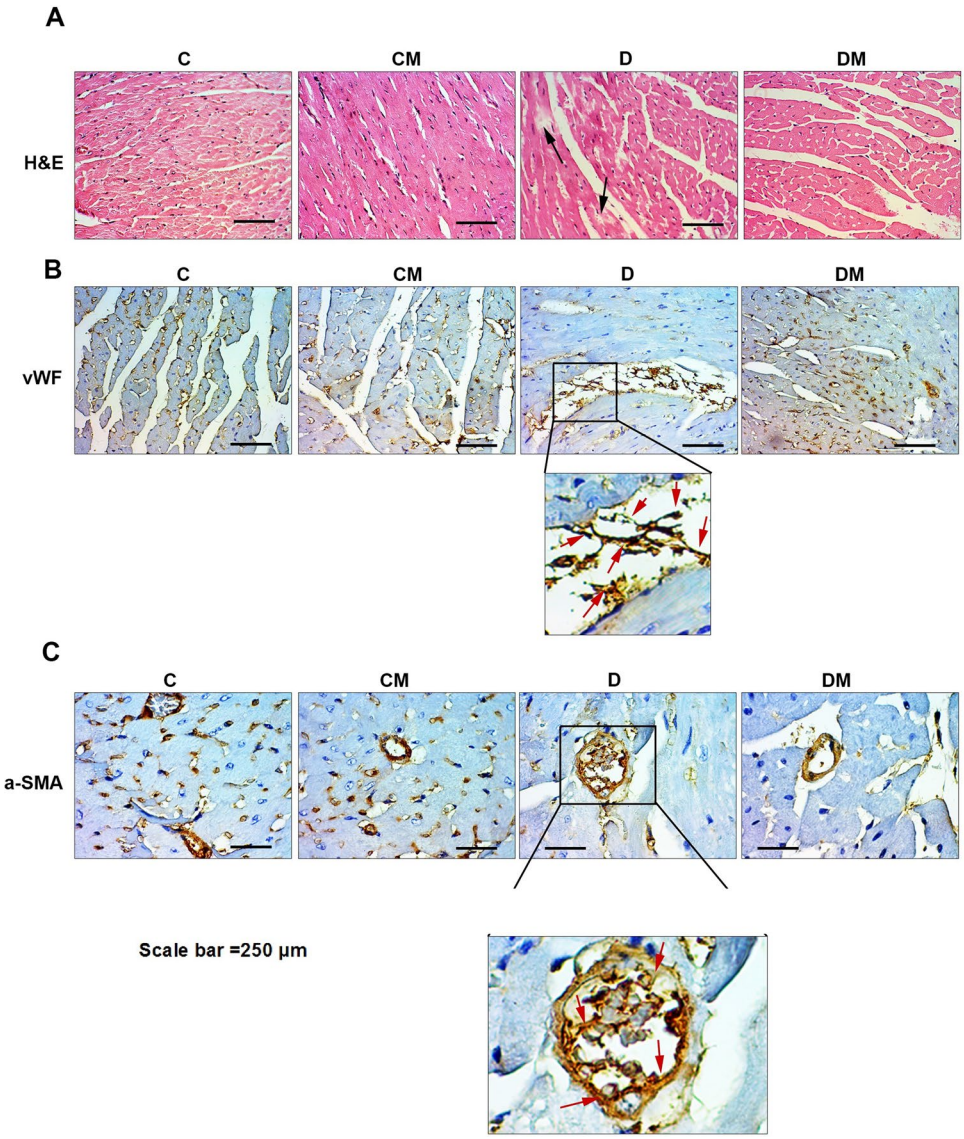

Fig. 2 H & E staining of diabetic cardiac tissue after treatment with melatonin (**A**). Data revealed necrotic changes and vacuolar degeneration in murine cardiomyocytes under T1D (black arrows). Imaging showed that melatonin is eligible to revert these conditions. IHC analysis of vascular α-SMA and vWF in cardiac vessels in diabetic condition and melatonin-treated groups (**B** and **C**). Data showed that T1D can detach vascular vWF cells (endothelial cell layer) and slaughtering into the lumen space. Like these changes, the promotion of T1D loosened the integrity of α-SMA$^+$ cells (vascular pericytes). Again, we found that melatonin can reduce the injury of α-SMA$^+$ cells in the vascular wall under diabetic condition

Melatonin altered aging-related gene expression in diabetic mice

Based on qRT-PCR data, T1D increased the transcription of aging related genes such as β-galactosidase. These changes coincided with the suppression of SOX2, Klotho, and Telomerase genes in the diabetic mice ($p < 0.001$; Fig. 3a-d). The injection of melatonin to the diabetic mice reversed these changes in which the expression of β-galactosidase increased significantly compared to the diabetic mice. By contrast, melatonin was able to increase the expression of SOX2, Klotho, and Telomerase genes, leading to activation of genes related to postponement of aging changes in comparison with the diabetic mice ($p < 0.05$ to $p < 0.01$; Fig. 3a-d). These data demonstrated that melatonin can postpone aging in murine cardiomyocytes exposed to the diabetic condition.

Melatonin reduced pro-inflammatory status in diabetic hearts

Along with the evaluation of aging-related genes, we evaluated inflammatory status by monitoring IL-1β levels pre- post-melatonin administration. We noted that IL-1β was induced in cardiac tissue by the promotion of T1D, indicating the inflammatory status compared to the control group ($p < 0.001$; Fig. 4). No statistically significant differences were found in the levels of IL-1β between the control and CM groups ($p > 0.05$). As expected, the transcription of IL-1β was significantly decreased after melatonin injection to the diabetic mice in comparison with the DM group ($p < 0.001$; Fig. 4). These data indicated that melatonin could suppress pro-inflammatory response under the diabetic conditions.

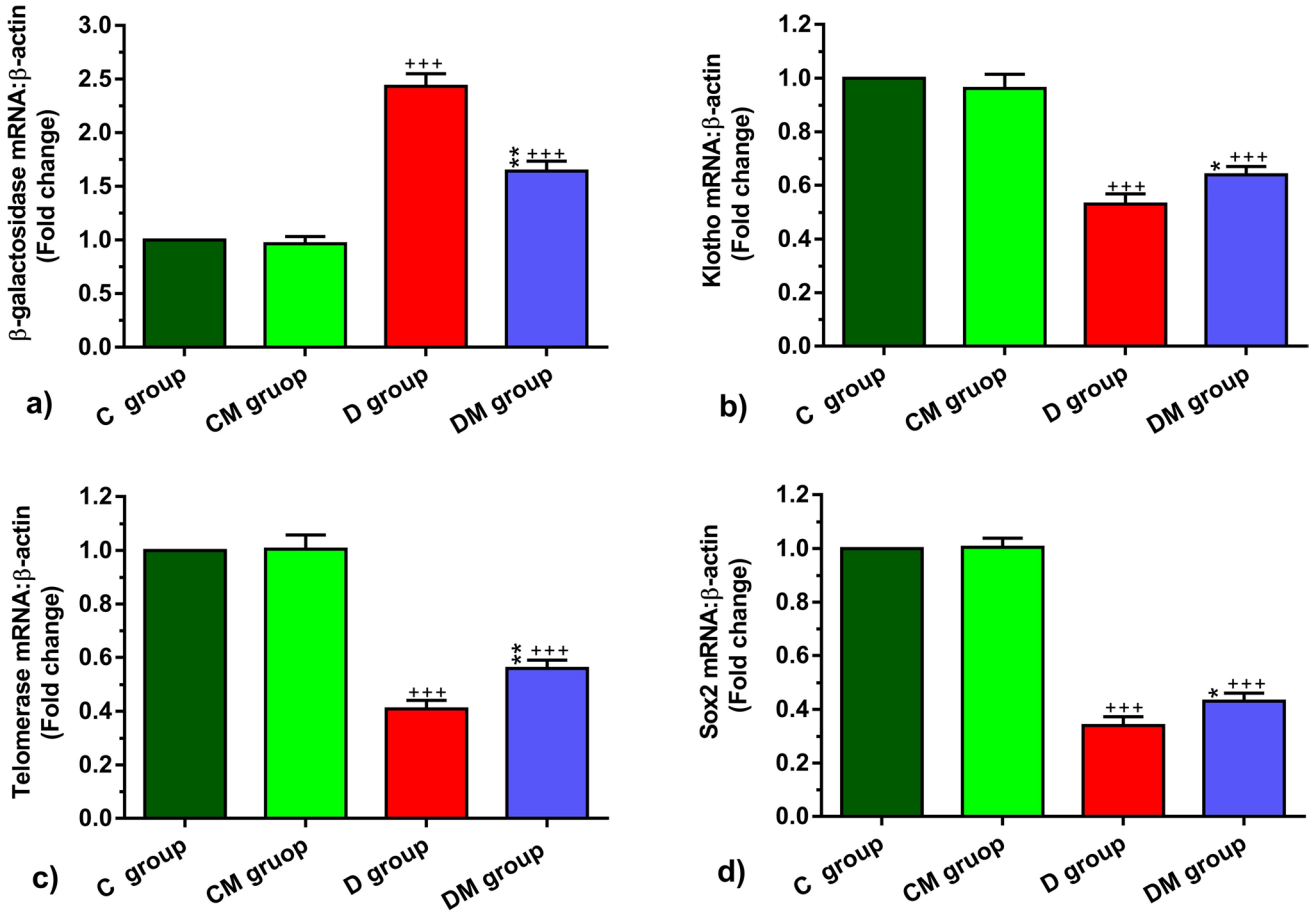

Fig. 3 Measuring the transcription of *β-galactosidase* (**a**), Klotho (**b**), Telomerase (**c**) and *Sox2* (**d**) mRNA in the cardiac tissues of control animals (C group), control animals received melatonin (CM group), diabetic animals (D group), diabetic animals received melatonin (DM group) (for each group, n = 10). Bars represent the mean ± SEM. Statistical differences between control and diabetic groups: +++; $p < 0.001$. Statistical differences between D group and DM group: *; $p < 0.05$ and **; $p < 0.001$

Discussion

The occurrence of different pathologies and metabolic disorders can alter the normal physiology of several organs that may lead to excessive tissue exhaustion and injury. Of these metabolic conditions, diabetes mellitus (DM), either T1D or T2D, is integral to cardiovascular disease and myocardiopathy, leading to high rate of mortalities [24]. Whether and how diabetic condition can accelerate the cardiomyocyte injury is at the center of attention. Besides, different underlying mechanisms involved in the cardiomyocyte injury following T1D and T2D have not been fully addressed [25]. According to our recently published data and other experiments, the promotion of cardiomyocyte injury following T2D and T1D have been proved [20, 26]. To reduce pathological outcomes, numerous attempts have been done in experiments and clinical setting by using therapeutic agent and several biomolecules. Of these components, melatonin is blindly prescribed in metabolic conditions while different underlying therapeutic effects remain unanswered [20]. Of note, we reported aging of cardiomyocytes occurred upon the onset of T2D [26]. It was suggested that melatonin can slow down cellular aging in several tissues by the modulation of redox system and organelles' injury [27]. Commensurate with these descriptions, one could hypothesize that this hormone can be effective in the modulation of aging-related changes in cardiac tissue with the T1D. This experiment was done to determine the effect of melatonin on aging status in heart tissue of the experimental T1D mouse model.

Pathological injury such as loss of cardiomyocyte integrity was achieved during T1D. These conditions coincided with the promotion of cell inflammation indicated by enhanced IL-1β [28]. In support of our data, Dallak and co-workers showed that the injection of insulin in the T1D rats can diminish cardiomyocyte injury via the suppression of both TNF-α and IL-6 cytokines and regulation of oxidative status [29]. Another important finding related to our data was that T1D can accelerate cardiomyocyte aging via

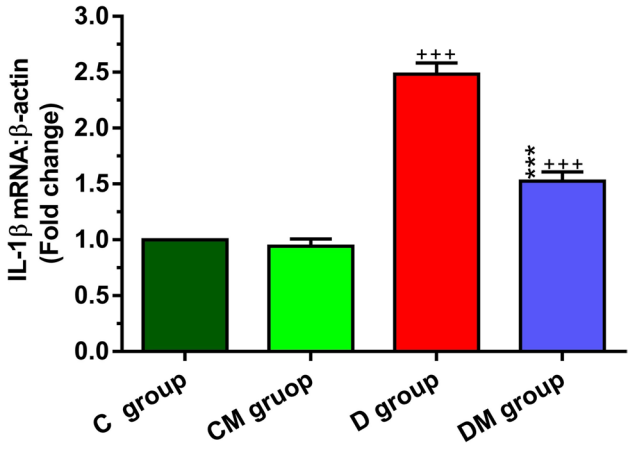

Fig. 4 The levels of IL-1β mRNA expression in the cardiac tissues of control animals (C group), control animals received melatonin (CM group), diabetic animals (D group), diabetic animals received melatonin (DM group) (for each group, n = 10). Bars represent the mean ± SEM. Statistical differences between control and diabetic groups: +++; p < 0. 001.Statistical differences between D group and DM group: ***; p < 0. 001

the down-regulation of anti-aging factors such as SOX2, Klotho, and telomerase levels. By contrast, the expression of β-galactosidase, an aging marker, was induced, showing cardiomyocyte exhaustion [30]. These data support a notion that the promotion of aging is one of key factor that lead to myocardium injury in T1D patients. Of note, we showed that 4-week administration of melatonin in diabetic mice can diminish the pathological changes in hearts, reduce IL-1β level, reverse aging-related genes and close near-to-control levels. Based on the previously published data, continues production of pro-inflammatory cytokines such as ILs are in part associated with the acceleration of cell aging via the up-regulation of NF-κB. Besides, intracellular accumulation of free radicals like reactive oxygen radicals can exacerbate aging procedure [31]. As a correlate, the reduction of pro-inflammatory cytokines after melatonin treatment is thought to reduce aging-related changes. One of therapeutic effects related to melatonin would be that this hormone can decrease the production and accumulation of free radicals and promotion of anti-oxidant defense against different insulting conditions such as T1D [32]. Regarding anti-aging properties, it has been shown that suppression of P53 and NF-κB signaling pathway exerted by melatonin can postpone cellular senescence [32]. In line with our finding and other data, it is logical to propose that aging procedure is one of the T1D complications in cardiac tissue induced by inflammation and melatonin can delay this phenomenon by the regulation of control of inflammatory cytokine and thereby aging procedure. Here, we did not assess the expression of insulin-related signaling effectors and their relationship with inflammation and aging procedure pre- and post-melatonin application. Further studies are required to examine the modulation of insulin-related signaling effectors by melatonin use and alteration of anti-aging and aging mechanisms.

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Authors' contributions M. F., H. R-U., M.H., A.R., J.R., performed several analyses and prepared draft. M.A., and R.R supervised the study.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval This study was approved by Local Ethics Committee of Tabriz University of Medical Sciences.

Consent for publication None declared.

Conflict of interest The authors declare that they have no conflict of interest.

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