Melatonin antagonizes oxidative stress-induced apoptosis in retinal ganglion cells through activating the thioredoxin-1 pathway

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Abstract

This study aimed to explore the role of melatonin in oxidative stress-induced injury on retinal ganglion cells and the underlying mechanisms. The immortalized RGC-5 cells were treated with H_2O_2 to induce oxidative injury. Cell viability was measured by Cell Counting Kit-8, and apoptosis was determined by flow cytometry and western blot assays. Reactive oxygen species (ROS), lactate dehydrogenase (LDH), and malondialdehyde (MDA) levels were examined to evaluate oxidative stress levels. In addition, Thioredoxin-1 (Trx1) was silenced in RGC-5 cells using small interfering RNA followed by signaling pathway examination to explore the underlying mechanisms of melatonin in alleviating oxidative injury. Melatonin pre-treatment significantly alleviated H_2O_2-induced apoptosis in RGC-5 cells. Melatonin also markedly reversed the upregulation of cleaved-caspase 3, cleaved-caspase 9, and Bax expression and downregulation of Bcl-2 expression induced by H_2O_2. Further analyses presented that melatonin significantly attenuated the increase of ROS, LDH, and MDA levels in RGC-5 cells after H_2O_2 treatment. Melatonin also abolished the downregulated expression of Superoxide dismutase type 1, Trx1, and Thioredoxin reductase 1, and the reduced activity of thioredoxin reductase in RGC-5 cells after H_2O_2 treatment. Notably, Trx1 knockdown significantly mitigated the protective effect of melatonin in alleviating H_2O_2-induced apoptosis and oxidative stress, while administration of compound C, a common inhibitor of c-Jun *N*-terminal kinase (JNK) signaling, partially reversed the effect of Trx1 silencing, thereby ameliorating the apoptosis and oxidative injury induced by H_2O_2 in RGC-5 cells. Melatonin could significantly alleviate oxidative stress-induced injury of retinal ganglion cells via modulating Trx1-mediated JNK signaling pathway.

Keywords Retinal ganglion cells · Oxidative injury · Melatonin · Trx1 · JNK signaling pathway

Introduction

Reactive oxygen species (ROS) is a subset of nitrogen or oxygen species generated during intracellular aerobic metabolism or immune response [1]. The imbalance between ROS production and clearance results in oxidative stress and induces cell damage, which is associated with the progression of various diseases [2]. Retinal ganglion cells (RGCs) are a kind of special projection neurons located nearby the inner retinal surface of the eyes and play multiple roles in visual signaling delivering, visual information controlling, and photoreceptors and brain communicating [3, 4]. Accumulating studies have suggested that oxidative stress acts a critical role in many ocular neurodegenerative diseases via inducing RGC damage and missing [5]. Hence, more attention has been focused on exploring suitable antioxidant agents that may confer neuroprotection.

Melatonin is a phylogenetically conserved molecule defined as a drug possessing a capacity to increase the amplitude of the circadian rhythms. Increasing evidence suggests that melatonin is generated in every mammalian cell that possesses mitochondria and plays critical roles in many biological activities, including enhancing immune response, maintaining membranes fluidity, anti-inflammation, and regulating mitochondrial homeostasis [6, 7]. Benefiting from its special features, melatonin is considered as

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an ideal antioxidant for preventing oxidative stress-induced injury. For example, as an amphiphilic substance, melatonin can cross through cell membranes to deliver protection to the targets [8]. With very low toxicity, melatonin intake at a high dose of 1 g daily is considered safe [9, 10]. Due to its safety as a drug and its well-documented chronobiotic and cytoprotective properties, melatonin is also introduced for treating neurodegeneration [11, 12], but the exact efficacy and mechanisms remain to be fully elucidated.

In this study, we aimed to explore the effect of melatonin on relieving oxidative stress-induced damage at cell level using an immortalized retinal ganglion cell line RGC-5. Cells were treated with H_2O_2 to mimic the oxidative injury and melatonin pre-treatment was set as the intervening measurement. Then, the underlying efficacy and mechanisms of melatonin administration were determined using small interfering RNA (siRNA)-mediated loss-of-function and kinase inhibitor pathway blockage studies. We identified critical molecules and signaling pathway that contributed to the beneficial roles of melatonin in these investigations, and we hope to provide some new insights in the understanding and therapy of ocular neurodegenerative disease.

Materials and methods

Cell culture and treatment

Murine retinal gallian cell line RGC-5 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone, Beijing, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 0.1% penicillin/streptomycin at 37 °C in 5% CO_2 incubator. After expansion, cells were cultured in the absence or presence of H_2O_2 (100, 200, 300 or 400 µM; Sangon, Shanghai, China) for 24 h to induce an oxidative stress state in cells. For melatonin pretreatment, cells were cultured in medium supplemented with melatonin (10 µM) for 3 h followed by being exposed to H_2O_2 (300 µM) for 24 h.

Lentivirus packaging and infection

The Trx1-specific siRNA (si-Trx1)-expressing plasmid and its corresponding negative control plasmid (si-NC) were generated by Shanghai Genechem. Co., Ltd. (Shanghai, China), and separately transfected into 293 T cells using the Lipofectamine® 3000 kit (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's protocol. Lentivirus was harvested from the culture medium supernatant of plasmids-transfected 293 T cells. For infection, RGC-5 cells were seeded into six-well plate and cultured overnight to reach 70% confluency. Then, si-NC or si-Trx1 lentivirus was

mixed with fresh DMEM at 1:1 volume and added to each well with cells incubated at 37 °C. At 48 h after infections, cells were harvested for confirmation of siRNA silencing efficiency and were subjected to following investigation.

Cell Counting Kit-8 (CCK-8) cell proliferation

RGC-5 cells (transfected or non-transfected) were seeded into 96-well plates with 5×10^3 cells/well. Then, cells were incubated overnight at 37 °C in a humidified incubator with 5% CO_2 in atmosphere. Then, cells were treated with melatonin or H_2O_2 as appropriate with triplicated replicates in each group. After treatment, the viability of cells in different groups was determined using the CCK-8 assay according to the instructions of the manufacturer (Beyotime, Shanghai, China). Finally, the absorption value of each well at 450 nm was measured using a microplate reader (Bio-red, Hercules, CA, USA).

Apoptosis detection

Apoptosis was determined using the fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, RGC-5 cells (transfected or non-transfected) were seeded into 6-well plates and incubated overnight. When cells reached 70% confluency, cells were treated with melatonin or H_2O_2 as indicated. After treatment, cells were trypsinized, centrifuged, and resuspended in 200 µl of $1 \times$ binding buffer and then incubated with 5 µl of Annexin V and 10 µl of PI at room temperature for 15 min in a dark environment. Then, cells were washed and analyzed using the FACSCalibur™ flow cytometer (BD, NJ, USA). Apoptosis rate was considered as the percentage of Annexin V-positive cells among all cells.

Reactive oxygen species detection

ROS production in cells was determined using the Reactive Oxygen Species Assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, cells were harvested and incubated with 20 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 0.5 h at 37 °C. Following three washes with PBS, the fluorescence intensity of the cells in each group was measured using the FACSCalibur™ flow cytometer (BD, NJ, USA) with the 485 nm excitation and 530 nm emission filters.

Western blot

After treatment, RGC-5 cells were lysed with Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) containing protease and phosphatase inhibitors

(Beyotime) on ice for 15 min. Then, total protein was isolated from cell lysates by centrifugation and quantified using the Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After being boiled with equal volume of loading buffer, proteins (25 µg/lane) were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently blotted onto polyvinylidene difluoride (PVDF) membrane (Pierce). Following this, membrane was blocked in 5% nonfat milk at room temperature for 1.5 h, and incubated at 4 °C overnight with the following primary antibodies: anti-Trx1 (Proteintech Company, 1: 4000 dilution), anti-C-caspase 3 (Proteintech Company, 1:1000 dilution), anti-C-caspase 9 (Cell Signaling Company, 1:1000 dilution), anti-Bax (Proteintech Company, 1: 8000 dilution), anti-Bcl-2 (Proteintech Company, 1: 6000 dilution), anti-SOD1 (Proteintech Company, 1: 10,000 dilution), anti-TrxR1 (Proteintech Company, 1: 5000 dilution), anti-p-JNK (Proteintech Company, 1: 2000 dilution), anti-JNK (Proteintech Company, 1: 4000 dilution), anti-p-ERK (Proteintech Company, 1: 4000 dilution), anti-ERK (Proteintech Company, 1: 6000 dilution), anti-p-p38 (Proteintech Company, 1: 2000 dilution), anti-p38 (Proteintech Company, 1: 6000 dilution), and anti-β-actin (Proteintech Company, 1: 20,000 dilution). After rinsing the membrane with Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) for three times, membrane was then incubated with the secondary antibody at room temperature for 1 h. Then, membrane was washed with TBST for three times. Finally, protein bands on membrane were visualized using an enhanced chemiluminescent (ECL) detection Kit (Pierce) and quantified using Image J software (NIH, Bethesda, MD, USA). Representative images of bands from one of three independent experiments with similar results are shown.

Quantitative real time PCR (qRT-PCR)

After treatment, total RNA from RGC-5 cells was isolated using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instruction. With 2 µg template, cDNA was synthesized according to the manufacturer's instructions. Then, the amplification of gene was performed using SYBR Green with the following condition: 94 °C for 5 min, 35 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 15 min. With β-actin as the internal control, the relative expression of Trx1 was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were summarized as follows: Trx1, forward primer 5′-CCCTTCTTCCATTCCCTCT-3′, reverse primer 5′-TCCACATCCACTTCAAGGAAC-3′; and β-actin, forward primer 5′-CAACTTGATGTATGAAGGCTTTGGT-3′, reverse primer 5′-ACTTTTATTGGTCTCAAGTCAGTGTACAG-3′.

Lactate dehydrogenase release (LDH) assay

Cell oxidative level was measured by LDH using a commercial detection kit (Cayman Chemical, MI, USA). According to the manufacturer's instructions, RGC-5 cells were pretreated with melatonin and cultured in 96-well plates. Then, the LDH assay solution was added into the cultures and cells were incubated at 25°C for 30 min. The absorbance value was analyzed at 450 nm using a microplate reader (Bio-red, Hercules, CA, USA).

Intracellular malondialdehyde (MDA) assay

RGC-5 cells were cultured in 6-well plates overnight, followed by the treatment with melatonin or H_2O_2 as indicated. Then, the intracellular MDA was detected using the thiobarbituric acid (TBA) method according to the manufacturer's instruction (Nanjing Jiancheng, Nanjing, Jiangsu, China). Each experiment was performed in triplicate and the mean value was calculated.

Thioredoxin reductase activity

The changes of thioredoxin reductase activity were measured using a commercial thioredoxin reductase kit (Cat. no. ab190804, Abcam, USA) following the instruction. Nicotinamide adenine dinucleotide phosphate (NADPH) is used by TrxR to reduce 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). The yellow-colored product TNB was measured spectrophotometrically at 405 nm.

Statistics

Data were calculated using GraphPad Prism 7.0 (GraphPad Prism Software, Boston, MA, USA) and presented as mean ± standard deviation. Comparisons were performed using Student t-test or one-way analysis of variance followed by Turkey's test as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Melatonin pretreatment attenuates H_2O_2-induced apoptosis in RGC-5 cells

To select a suitable concentration of H_2O_2 that could induce optimal cell oxidative injury, RGC-5 cells were treated with gradient concentrations of H_2O_2 (100, 200, 300, or 400 µM) for 24 h followed by cell viability analysis. CCK-8 assay results showed that H_2O_2 significantly decreased RGC-5 cell viability with a dose-dependent manner and H_2O_2 at

300 μM caused a ~ 50% reduction of cell viability in RGC-5 cells (Fig. 1A). According to this finding, H_2O_2 at 300 μM was chosen for the following treatment of cells after melatonin pretreatment for 3 h. Notably, the CCK-8 assay results also revealed that melatonin pretreatment markedly alleviated H_2O_2-induced reduction of cell viability in a dose-dependent manner when the concentration of melatonin was within 10 μM (Fig. 1B). In addition, the apoptosis of RGC-5 cells was also determined by flow cytometry and the results revealed that melatonin pretreatment obviously reversed the elevated apoptosis of RGC-5 cells that was induced by H_2O_2 treatment (Fig. 1C). Taken together, these findings suggested that melatonin reduced oxidative injury in RGC-5 cells.

## Melatonin altered the expression of apoptosis-associated proteins in RGC-5 cells

The levels of apoptosis-associated proteins were also determined in H_2O_2 and melatonin treated RGC-5 cells to further consolidate the beneficial roles of melatonin in alleviating oxidative injury. The results presented that H_2O_2 significantly increased the expression of cleaved-caspase 3, cleaved-caspase 9, and Bax, but prominently decreased Bcl-2 expression; while melatonin pretreatment obviously reversed the upregulated expression of cleaved-caspase 3, cleaved-caspase 9, and Bax, as well as the downregulated expression of Bcl-2 induced by H_2O_2
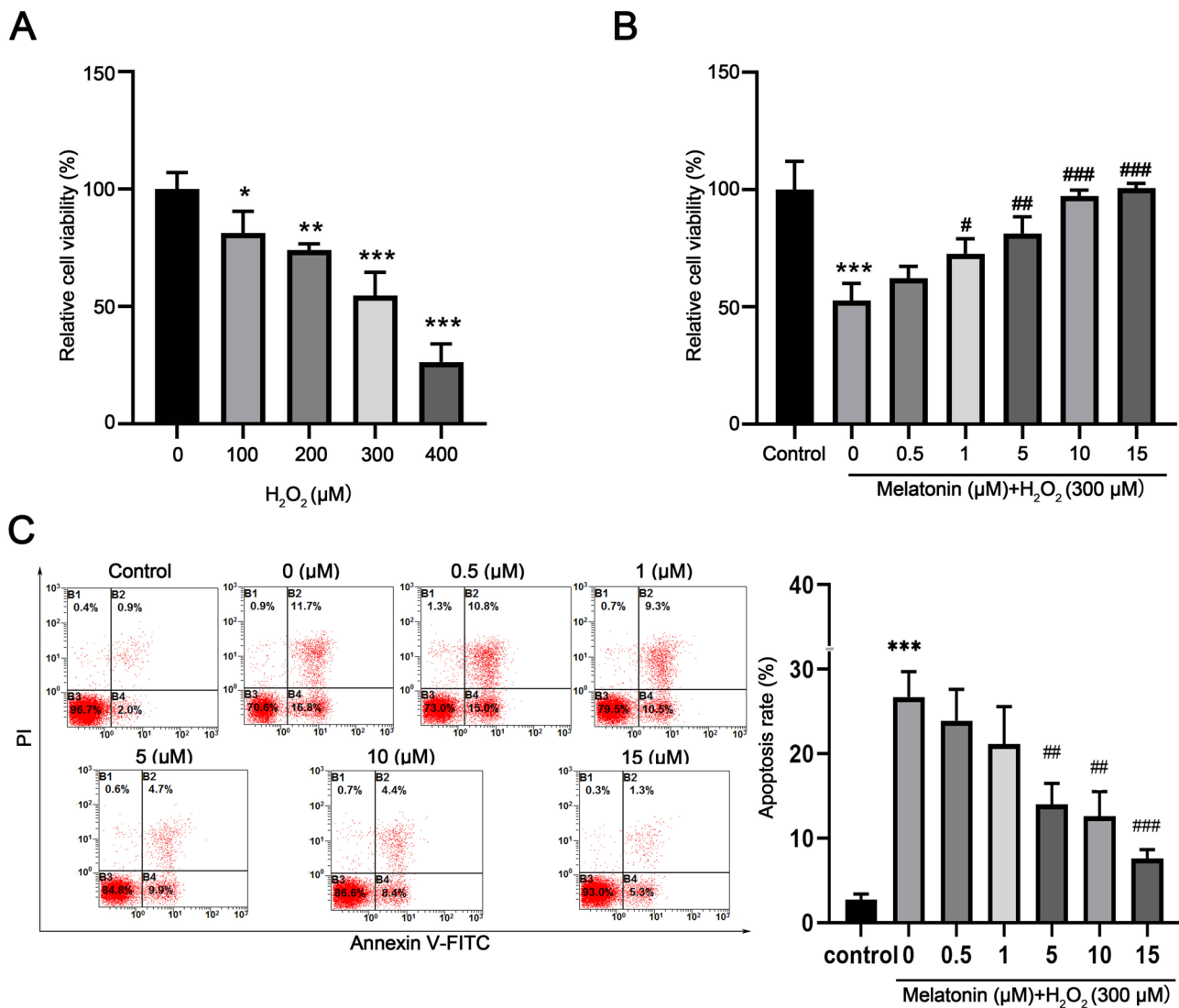
Fig. 1 Melatonin alleviates H_2O_2-induced oxidative injury in RGC-5 cells. A The viability of RGC-5 cells after being treated with different concentrations of H_2O_2 was determined using the CCK-8 method. B The viability of RGC-5 cells after being treated with melatonin and H_2O_2 at the indicated concentrations was determined using the CCK-8 method. C Apoptosis of RGC-5 cells after being treated with melatonin and H_2O_2 at the indicated concentrations was determined using flow cytometry. Compared with the control group, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Compared with the single H_2O_2 treatment group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$

Fig. 2 Melatonin mitigates H_2O_2-induced apoptosis in RGC-5 cells. **A** Expression of cleaved-caspase-3, cleaved-caspase-9, Bax, and Bcl-2 proteins in RGC-5 cells after the indicated treatments were determined by western blotting assay. **B** Quantitative results on the protein levels of target molecules accessed by western blotting assay. Between the indicated groups, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$
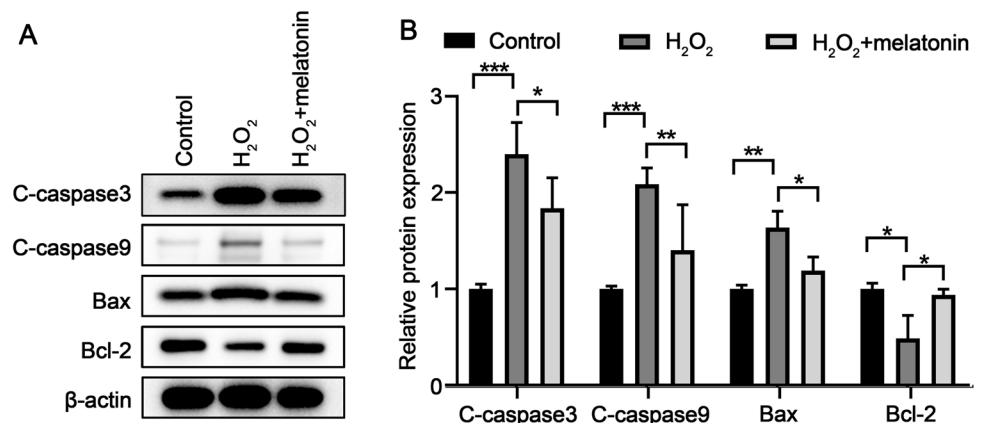
(Fig. 2A, B). Collectively, these findings confirmed that melatonin pretreatment can alleviate oxidative stress-induced injury in RGC-5 cells.

Melatonin pretreatment attenuates oxidative stress in H_2O_2 treated RGC-5 cells, which was associated with the regulation of the Trx1 pathway

To further reveal the molecular mechanisms underlying the roles of melatonin in alleviating oxidative injury, the ROS levels in RGC-5 cells were determined after different treatments. The flow cytometry results revealed that melatonin did not alter the ROS level, but H_2O_2 significantly increased the ROS level in RGC-5 cells. Importantly, melatonin pre-treatment markedly decreased the ROS level in RGC-5 cells stimulated with H_2O_2 (Fig. 3A, B). In addition, another two oxidative stress-associated markers, LDH and MDA, were also determined in melatonin and H_2O_2 treated RGC-5 cells. The results demonstrated that melatonin treatment alone did not change the LDH and MDA levels of RGC-5 cells, but H_2O_2 treatment markedly increased the LDH and MDA levels in RGC-5 cells; while melatonin pretreatment obviously reversed the upregulation of LDH and MDA levels in RGC-5 cells stimulated with H_2O_2 (Fig. 3C, D). Further in-depth analyses showed that H_2O_2 treatment significantly decreased the expression of SOD1, Trx1, and TrxR1 in RGC-5 cells, while melatonin pretreatment obviously attenuated these reductions (Fig. 4A). Moreover, Thioredoxin reductase activity assay also revealed that melatonin pretreatment alleviated the reduction of thioredoxin reductase activity induced by H_2O_2 in RGC-5 cells (Fig. 4B). Therefore, these findings suggested that melatonin ameliorated the oxidative stress-mediated cell injury, which was associated with the modulation of Trx1 expression and thioredoxin reductase activity.

Trx1 knockdown attenuates the protective effects of melatonin against oxidative stress in RGC-5 cells

To further reveal the role of Trx1 in melatonin-mediated protective role, Trx1 was silenced in RGC-5 cells, which was confirmed by qRT-PCR and western blotting assays (Fig. 5A, B). CCK-8 assay results showed that melatonin pretreatment significantly improved the viability of RGC-5 cells after being treated with H_2O_2, while silencing Trx1 markedly reversed the viability-promotive effect of melatonin (Fig. 5C). Flow cytometry analysis showed that melatonin obviously decreased the apoptosis of H_2O_2-stimulated RGC-5 cells, while silencing Trx1 attenuated the protective effects of melatonin (Fig. 5D). Western blotting analysis also revealed that melatonin markedly weakened the upregulated expression of cleaved-caspase 3, cleaved-caspase 9, and Bax, and downregulated Bcl-2 expression, which was induced by H_2O_2 treatment, while inhibiting Trx1 significantly reversed these effects of melatonin (Fig. 5E). In addition, oxidative stress-associated analyses presented that siTrx1 significantly attenuated the protective effects of melatonin in alleviating the upregulations of LDH and MDA levels that were induced by H_2O_2 (Fig. 5F). All these findings suggested that Trx1 knockdown attenuated the protective effects of melatonin in alleviating oxidative stress.

Trx1 contributes to the protective effects of melatonin via regulating the Trx1/JNK/ERK/p38 MAPK signaling pathway

To further reveal the underlying mechanisms, the alteration of the candidate signaling pathways (including the JNK, ERK, and p38 MAPK pathways) was evaluated in H_2O_2 and melatonin treated RGC-5 cells. The results suggested that melatonin significantly decreased the phosphorylation levels of JNK, ERK, and P38, but did not alter the total protein levels of JNK, ERK, and p38
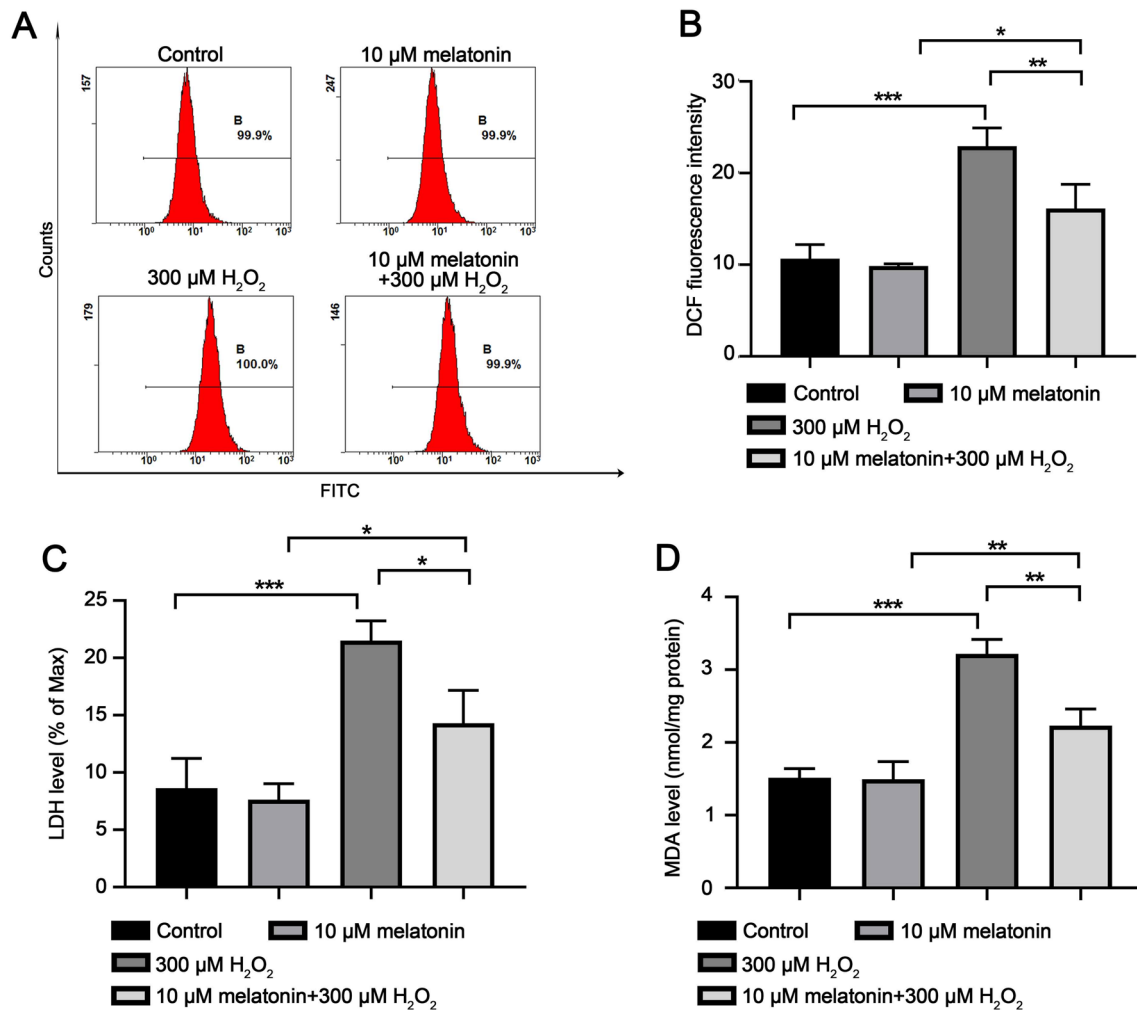
Fig. 3 Melatonin attenuates the oxidative stress in H_2O_2-stimulated RGC-5 cells. **A** Intracellular ROS was determined by flow cytometry assay, and representative flow profile is shown. **B** Quantitative result on the fl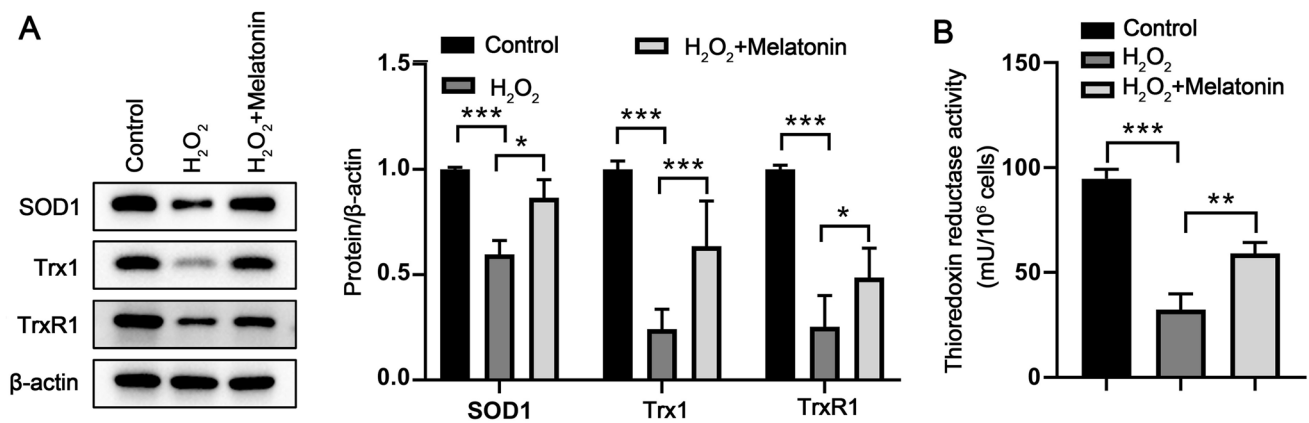uorescence intensity of DCF in flow cytometry assay. **C** LDH level in RGC-5 cells after the indicated treatments with H_2O_2 and melatonin; **D** MDA level in RGC-5 cells after the indicated treatments with H_2O_2 and melatonin. Between the indicated groups, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

in RGC-5 cells stimulated with H_2O_2. However, silencing Trx1 remarkably abrogated the effects of melatonin in decreasing the protein levels of phosphorylated JNK, but not that of phosphorylated ERK or phosphorylated p38 (Fig. 6A). Compound C, a common inhibitor of JNK, was used to further confirm whether the JNK signaling was involved in modulating H_2O_2-induced oxidative stress. We found that siTrx1 markedly aborted the protective effects of melatonin in increasing proliferation and decreasing apoptosis of RGC-5 cells after H_2O_2 treatment, while compound C treatment partially reversed the effects of Trx1 knockdown in aborting the protective effects of melatonin (Fig. 6B–D). These results suggested that melatonin can alleviate oxidative stress-induced cell injury via regulating the Trx1/JNK/ERK/p38 MAPK signaling pathway in RGC-5 cells.

Discussion

Eye is one of the organs required rich oxygen and nutrient supply, which makes it sensitive to oxidative stress [2, 13]. RGCs, an important cell type in the eye, play critical role in visual delivery and their apoptosis causes neurodegenerative vision loss [14]. Abnormal accumulation of oxidative stress leads to retinal neurodegeneration, which is responsible for several ocular diseases, including glaucoma, diabetic retinopathy, age-related macular degermation [15, 16]. Hence, attenuating oxidative stress-induced RGCs injury and loss is a key direction for the treatment of neurogenerative diseases.

Melatonin is an indoleamine produced by the pineal gland during night and is widely known to show excellent antioxidant ability in multiple bioactivities [17]. Initially, melatonin was identified as a chronobiotic to modify the amplitude

Fig. 4 Melatonin increases the expression of Trx1 and enhances the activity of thioredoxin reductase in H_2O_2-stimulated RGC-5 cells. **A** Expression of SOD1, Trx1, and TrxR1 in RGC5 cells after the indicated treatments wi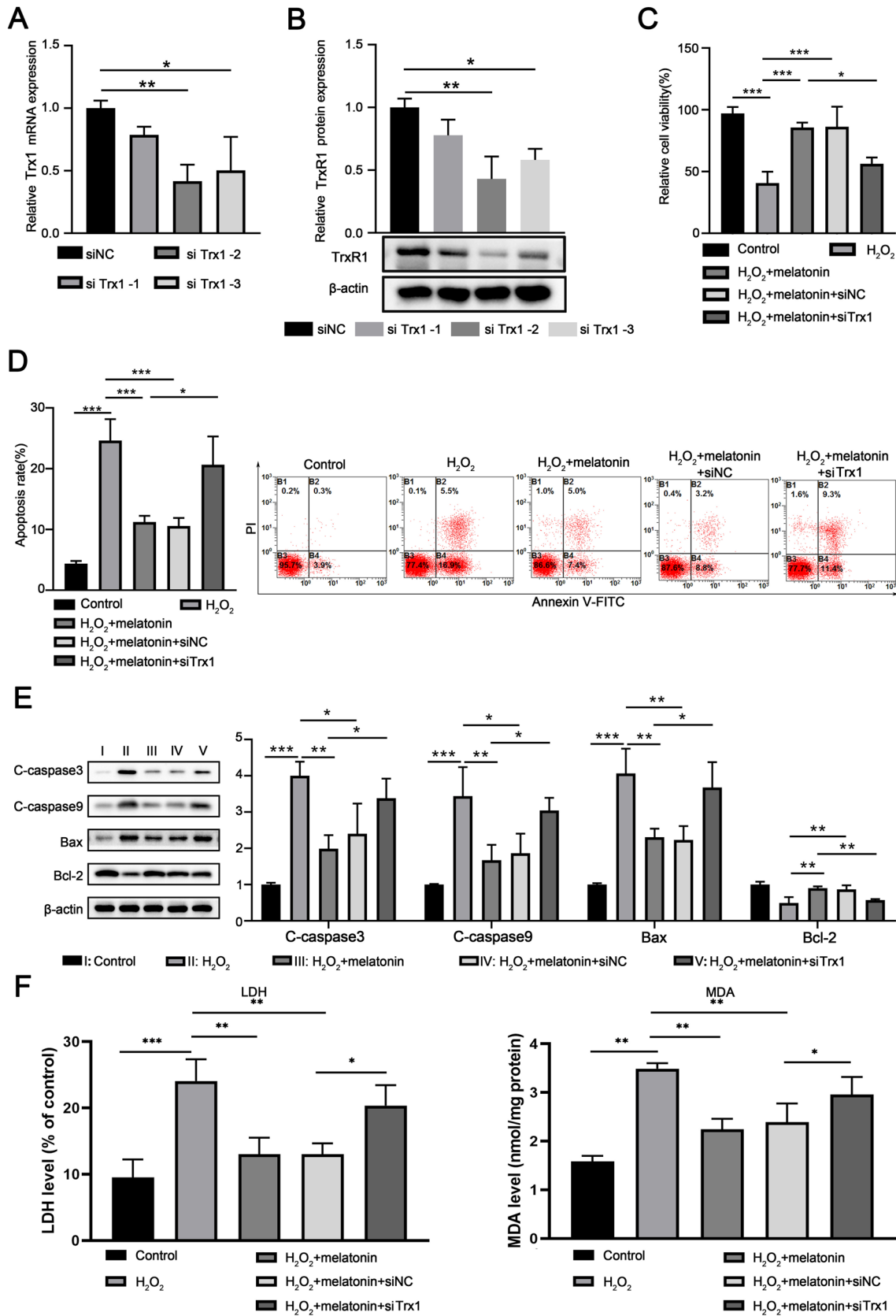th H_2O_2 and melatonin was quantitated by western blotting assay. **B** Thioredoxin reductase activity of RGC5 cells after the indicated treatments with H_2O_2 and melatonin was measured. Between the indicated groups, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

and phase of biological rhythms [18]. Mechanically, melatonin also functions as a free radical scavenger to eliminate the oxidative stress in both animals and plants, and reverses the damage caused by low degree inflammatory [19, 20]. Previous studies showed that melatonin acts as a modulator of degenerative and regenerative signaling pathways in oxidative stress-associated diseases such as RGC damage [21]. Mitochondrion is the key site for ROS production, and oxidative injury of DNA and RNA in retina is significantly associated with the ganglion cell mitochondria [22]. Multiple pieces of evidence from the in vitro and in vivo studies also suggested that melatonin exerts neuroprotective and antiapoptotic effects via reducing oxidative stress-induced injury [23]. For example, in hypoxia–ischemia injured retinal neurons, melatonin treatment significantly improved amplitudes and implicated a-wave and b-wave, and decreased apoptosis levels via increasing BDNF expression and its downstream phospho-TrkB/Akt/ERK/CERB signaling pathway [24]. In addition, melatonin mitigates acute intraocular hypertension-induced RGC pyroptosis via NF-κB/NLRP3 axis [25]. In the current study, we also identified that melatonin pre-treatment significantly decreased H_2O_2-induced apoptosis in RGC-5 cells. In addition, melatonin pre-treatment significantly attenuated H_2O_2-induced upregulation of ROS, LDH, and MDA. These findings suggested that melatonin acts as a promising neuroprotective agent for ocular neurodegenerative diseases via alleviating oxidative stress-induced injury.

Trx1, one type of thioredoxin (Trx), is a ubiquitously expressed redox protein acting as a major cellular protein disulfide reductase in regulating disulfide bond reduction, denitrosylation, transnitrosylation, and other redox post-translational modifications [26]. Trx1 is richly expressed in nervous system of mammalians [27] and plays critical roles in the regulation of protein thiols homeostasis and ROS signaling [28]. It was reported that reducing Trx1 expression attenuated the downstream protein expression and altered cellular redox state, thereby promoting RGC death [29, 30]. In addition, Li et al. revealed that salidroside significantly increased the expression of Nrf2 and Trx1, thereby alleviating the infarction rate, apoptosis, and MDA formation in rats with middle cerebral artery occlusion and a cell model of oxygen–glucose deprivation/reoxygenation [31]. Liu et al. also found that Bakuchiol treatment obviously alleviated early brain injury induced by subarachnoid hemorrhage via reducing ROS level and mitochondria injury, and increasing SOD and GSH activities and Trx1 expression [32]. Moreover, a recent study showed that melatonin significantly increased Trx1 expression to promote the osteogenic differentiation of bone marrow derived stem cells in osteoporotic mice, thereby reducing bone loss in osteoporosis [33]. Taken together, these studies suggested that Trx1 was a downstream target of melatonin. In this study, we also found that melatonin significantly aborted the downregulation of SOD1 and Trx1 expression, as well as the decreased thioredoxin reductase activity in H_2O_2-induced RGC-5 cells, suggesting that melatonin could alleviate the oxidative injury of RGCs via increasing Trx1 expression.

A previous study demonstrated that melatonin significantly suppresses endoplasmic reticulum stress and mitochondrial dysfunctions via activating Trx1, thereby inhibiting TXNIP/NLRP3 signaling pathway [34]. Tang et al. have documented that melatonin enhances inner blood-retinal barrier via suppressing p38/TXNIP/NF-κB signaling pathway, thereby attenuating the inflammation in diabetic retinopathy [35]. Since Trx1 may directly inhibit ASK1 and the corresponding ASK1-JNK/P38 pathway leading to anti-apoptosis and anti-inflammation, while TXNIP may inhibit

◄Fig. 5 Silencing Trx1 attenuates the effects of melatonin in alleviating H$_2$O$_2$-induced oxidative stress in RGC-5 cells. **A** Confirmation of Trx1 knockdown in RGC-5 cells using RT-qPCR assay. **B** Confirmation of Trx1 knockdown in RGC-5 cells using western blotting assay. **C** The viability of RGC-5 cells after transduction of siNC or siTrx1 lentivirus and the indicated treatments with H$_2$O$_2$ and melatonin was determined using the CCK-8 method. **D** Intracellular ROS level in RGC-5 cells after the indicated transduction and treatments was determined using flow cytometry assay. **E** Expression of cleaved-caspase-3, cleaved-caspase-9, Bax, and Bcl-2 proteins in the indicated RGC-5 cells was determined by western blotting assay. **F** LDH and MDA levels were determined in the indicated RGC-5 cells. Between the indicated groups, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

Trx1 activity from inducing brain ischemic stroke under oxidative stress conditions, this indicates that Trx1 may protect against ischemic stroke [36]. It was reported that transduced Tat-Trx1 played a protective role against oxidative stress-induced neuronal cell death via ASK1-MAPK signal pathway [37]. In addition, Trx1 was found to mediate neuroprotection of Schisanhenol against MPP + -induced apoptosis via suppression of ASK1-P38-NF-κB pathway in SH-SY5Y cells [38]. Melatonin also protects I/R-induced injury in lung via inhibiting pro-inflammatory response and apoptosis, as well as regulating the JNK, p38 MAPK, and Nrf2 signaling pathways [39]. Thus, these melatonin-associated signaling pathways were also evaluated in this study. The results suggested that melatonin significantly decreased the oxidative stress level and apoptosis in H$_2$O$_2$-induced RGC-5 cells via decreasing the phosphorylation of JNK, p38, and ERK proteins, while silencing Trx1 or compound C treatment partially reversed the effects of melatonin. These results together suggest that melatonin alleviates oxidative stress-induced RGC injury via regulating the Trx1/JNK/ERK/p38 MAPK signaling pathway.

A couple of limitations of this study must be noticed. First, more in vitro cell models rather than RGC-5 are needed to validate our conclusions. RGC-5 is a widely used cell line which has been characterized as expressing RGC markers and exhibiting ganglion cell-like behavior in culture [40]. RGC-5 cells were widely used as a tool for studying the RGC-relevant pathogenesis by many researchers [41–43],

as RGC-5 represents the only available immortalized cell line that has been introduced as being of RGC origin [44]. Recent studies brought some concerns regarding the origin and nature of the cells, as RGC-5 cells were identified to be of mouse origin and their expression of RGC characteristics was questioned by some investigators [44]. In addition, as a transformed cell line, dedifferentiation of RGC-5 cells might be inevitable during the long-time culture with high passage numbers or progressive subculturing. Therefore, primary RGCs are an ideal choice of model to study the physiological processes and molecular mechanisms underlying the beneficial roles of melatonin in decreasing oxidative injury. However, primary RGC cells are difficult to purify and they can only survive a few days [44]. Second, more in vivo experiments are needed to confirm the protective roles of melatonin against oxidative injury of RGC cells. Third, the current model of oxidative stress was induced by H$_2$O$_2$, and the in vitro and in vivo models with other approaches of generating oxidative injury can help to supplement the beneficial roles of melatonin in ameliorating oxidative injury of RGCs under both physiological and pathological conditions. For example, studies in several ocular diseases associated with oxidative stress and RGC degenerations, such as glaucoma, hereditary optic atrophy, inflammatory optic neuritis, ischemic optic neuropathy, traumatic optic neuropathy, and drug toxicity [45], can be conducted. However, due to the time-consuming nature of these in vivo animal studies, our findings can only be further tested in a more biologically relevant background in the future.

Conclusion

In summary, we found that melatonin significantly decreased the oxidative injury of RGCs via regulating Trx1-mediated JNK/ERK/p38 MAPK signaling pathway. Despite that these findings remain validated by in vivo studies under a more biologically relevant context, our findings provide new insights for the understanding of using melatonin to reduce oxidative stress-caused injury in ocular neurodegenerative diseases.
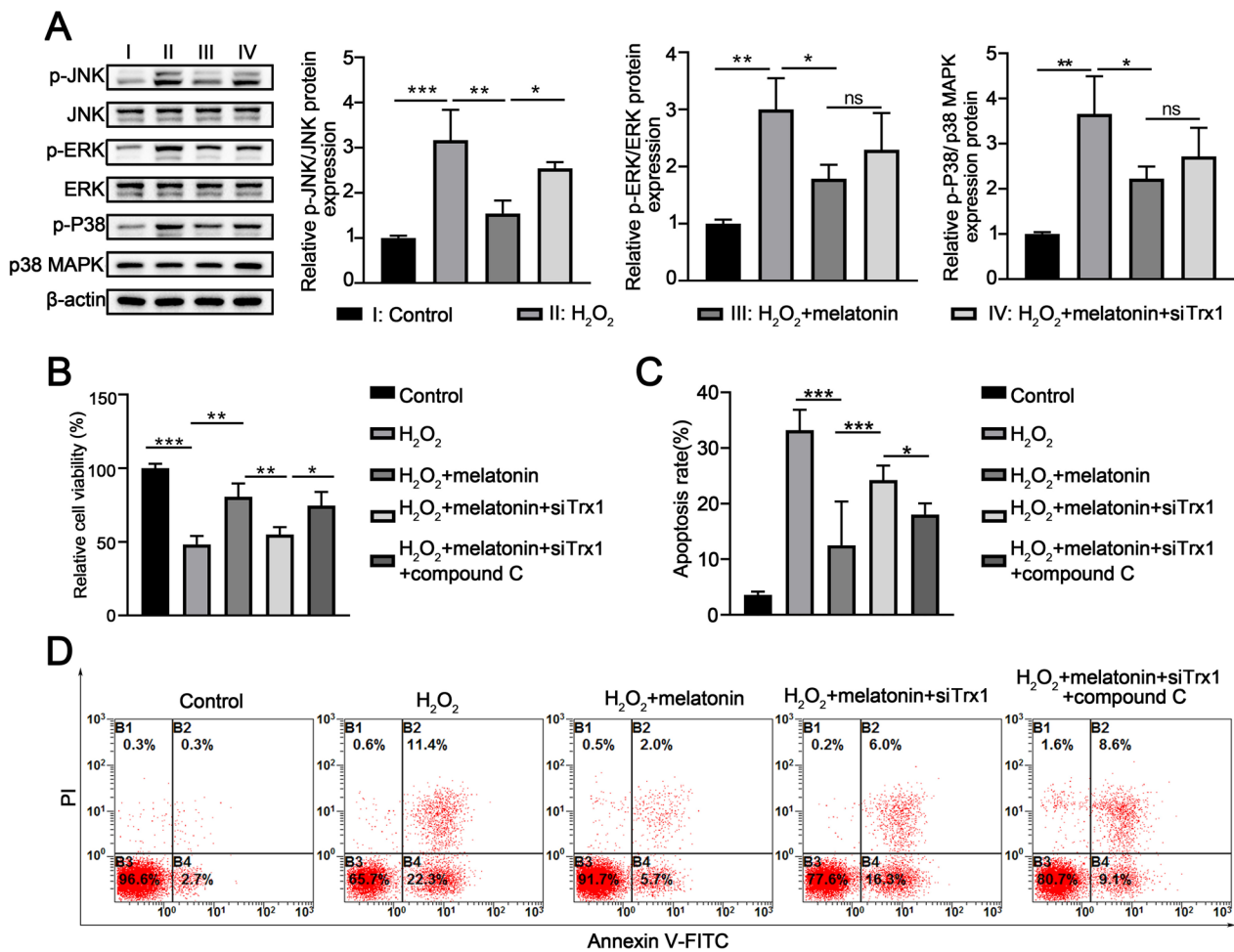
Fig. 6 Melatonin mitigates H_2O_2-induced oxidative injury in RGC-5 cells via regulating the Trx1/JNK/ERK/p38 MAPK signaling pathway. **A** Expression of the total and phosphorylated JNK/p38/ERK proteins in RGC-5 cells after transduction of siTrx1 lentivirus and the indicated treatments with H_2O_2 and melatonin was determined by western blotting assay. **B** The viability of RGC-5 cells after transduction of siTrx1 lentivirus and the indicated treatments with H_2O_2

and melatonin, or/and compound C was determined using the CCK-8 method. **C**, **D**, Apoptosis of RGC-5 cells after the indicated transduction and treatments was quantitated by flow cytometry assay. The apoptosis rate was summarized (**C**), and representative flow profile is shown (**D**). Between the indicated groups, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

Data availability The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Declarations

Conflicts of interest All authors have completed the ICMJE uniform disclosure form. All the authors declare that they have no competing interests.

Ethics approval and consent to participate The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Xi'an Jiaotong University. Animal care and study were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

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