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Original Article

Anti-apoptotic and autophagic effect: Using conditioned medium from human bone marrow mesenchymal stem cells to treat human trabecular meshwork cells

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ABSTRACT

Introduction: Glaucoma is a vision-threatening disease associated with accelerated aging of trabecular meshwork (TM) which results in elevated intraocular pressure (IOP). Increased oxidative stress in TM plays an important role in cellular molecular damage which leads to senescence. Autophagy is an intracellular lysosomal degradation process which is activated when cells are under stressful condition, and emerging studies have demonstrated increased expression of modulators of apoptosis and expression of autophagic cascade in ex-vivo TM specimens or cultured TM cells under oxidative stress. Recently, studies have shown neuroprotective and IOP-lowering effects after transplanting mesenchymal stem cells (MSCs) or injecting condition medium (CM) of MSCs into ocular hypertension animal models. However, knowledge of the underlying mechanism accounting for these effects is limited. Using condition medium (CM) from human bone marrow-derived mesenchymal stem cells (BM-MSCs), we investigated the effects of the CM derived from BM-MSCs on TM autophagy and apoptosis.

Methods: H_2O_2 was added to culture medium of human TM cells to mimic oxidative damage in glaucomatous eyes, and the autophagic and anti-apoptotic effects of BM-MSCs-derived CM was explored on the oxidatively damaged cells. Mitochondrial ROS production was examined by MitoSOX™, apoptosis was evaluated using terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) staining, and the expression of proteins involved in autophagy as well as extracellular matrix was investigated via Western blot.

Results: There were no significant differences in TM cell viability when the cells were treated with different concentrations of CM in the absence of oxidative stress. Cell viability was significantly higher in oxidatively damaged TM cells treated with 1X or 5X CM compared to untreated TM cells under oxidative stress. The mitochondrial ROS level significantly increased with oxidative stress, which was mitigated in the CM treatment groups. DNA fragmentation significantly decreased in oxidatively stressed TM cells after treatment with CM. LCB3 II/LCB3 I was significantly elevated in the oxidative stress group compared to the control group and was significantly decreased in the CM treatment groups. Expression of fibronectin was not significantly different among the groups.

Conclusion: The CM derived from human BM-MSCs has the capacity to rescue oxidatively damaged human TM cells associated with decreased autophagy and apoptosis. The BM-MSCs CM has potential for

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slowing down age- and disease-related degeneration of TM in patients with glaucoma, facilitating success in the control of IOP.

1. Introduction

Glaucoma, a leading cause of irreversible blindness worldwide, is a degenerative optic neuropathy that manifests as progressive visual field loss. An estimated 8 million people will suffer from bilateral blindness caused by this disease in the near future [1,2]. Elevated intraocular pressure (IOP) and aging are two of the most important risk factors for glaucoma. Currently, IOP-lowering therapy is the only effective treatment to slow the progression of visual field loss [3,4].

The IOP depends on the balance between the production of aqueous humor from the ciliary process and excretion of it through the trabecular meshwork (TM). As the production of aqueous humor in patients with glaucoma remains comparable to that of individuals without glaucoma [5,6], the balance relies mostly on the function of the TM, a reticular structure in the anterior chamber angle of the eye. Studies on human TM specimens have shown significantly fewer TM cells and increased extracellular matrix (ECM) accumulation in glaucomatous eyes compared to those of age-matched controls [7–9]. In addition, reactive oxygen species (ROS) generated through a light-dependent reaction with melanin in the iris may induce mitochondrial dysfunction and oxidative damage of TM cells, impairing aqueous humor outflow [7–10]. Human studies have shown that glaucomatous eyes have a higher level of oxidative damage to both the nuclear and mitochondrial DNA, which is proportional to the severity of the visual field defect. This phenomenon is present even in eyes with well-controlled IOP treated by glaucoma medication, indicating ongoing oxidative damage in the TM despite treatment [11–14].

Autophagy is a survival reaction when tissue is under stress or environmental change. It has a variety of physiological and pathophysiological roles and acts as a cellular housekeeper to control functional qualities. Autophagy has been reported to be associated with the development of some neurodegenerative diseases and aging [15,16]. Porter et al. demonstrated a decrease in autophagic activity in porcine TM cells using an experimental model mimicking chronic oxidative stress, which is in line with the notion that oxidative stress may decrease autophagic activity [16,17].

In the field of ophthalmology, stem cell therapy is a promising strategy for the treatment of glaucoma [18–20]. In particular, bone marrow-derived mesenchymal stem cells (BM-MSCs) have been broadly explored as a new e via the secretion of cytokines and growth factors [21–23]. Compared to stem cells themselves, stem cell-derived conditioned medium (CM) has the advantages of being easier to manufacture and simpler to pack and transport [24]. Recent studies have shown the neuroprotective and IOP-lowering effects of transplanting MSCs or injecting the CM of MSCs (MSC CM) into a rat model of ocular hypertension [25]. However, the cellular mechanisms for how MSCs or MSC CM achieve these effects on TM cells have not been discussed in the literature. Here, we investigate the capacity of CM derived from human BM-MSCs to promote the survival and maintain the functions of human TM cells by evaluating cell proliferation, autophagy, and apoptosis.

2. Materials and methods

2.1. Culture of human TM cell

Human TM cells (6590, ScienCell Research Laboratories, USA) were cultured in Trabecular Meshwork Cell Medium (TMCM, 6591, ScienCell Research Laboratories, USA) containing 2% fetal bovine serum (FBS, 0010, ScienCell Research Laboratories, USA), 1% Trabecular Meshwork Cell Growth Supplement (6592, ScienCell Research Laboratories, USA), and 1% penicillin/streptomycin solution (P/S, 0503, ScienCell Research Laboratories, USA). The cultured cells were incubated at 37 °C in a 5% CO_2 atmosphere. The cell culture media was changed every 3 days.

2.2. Preparation of CM from human BM-MSCs

BM-MSCs (7500, ScienCell, USA) were cultured in Mesenchymal Stem Cell Medium (7501, ScienCell, USA) composed of basal growth medium, 5% FBS (0025, ScienCell, USA), 1% Mesenchymal Stem Cell Growth Supplement (7552, ScienCell, USA), and 1% P/S (0503, ScienCell, USA). The cell culture media was changed every 3 days. BM-MSCs at passage 9 were seeded in the culture dish for 16 h and washed three times with PBS. BM-MSCs were cultured in basal medium for an additional 24 h at 37 °C in a 5% CO_2 atmosphere. The medium was collected and concentrated 40x using a 10 kDa centrifugal filter (Amicon Ultra-15, Millipore, USA) [25]. The CM from human BM-MSCs (BM-MSC CM) was stored at 4 °C until use.

2.3. Evaluating the effects of cell proliferation by BM-MSC CM on human TM cells

The cell viability of 1, 5, and 10-fold concentrated BM-MSC CM on TM cells was determined using Cell Counting Kit 8 (CCK-8, Dojindo, USA) after cultivation for 16 h. Human TM cells were cultured at a density of 5000 cells per well in a 96-well plate and maintained for 1 day. The TMCM containing detached cells was removed and replaced with basal growth medium or the BM-MSC CM. The basal growth medium and 1, 5, or 10-fold concentrated BM-MSC CM were then separately added to the wells (200 μl per well) as the culture medium throughout the culture. At 16 h, cells were washed with PBS and then incubated with CCK-8 reagent following the manufacturer's instructions. The optical density (OD) at 450 nm was measured by enzyme-linked immunosorbent assay (ELISA, Sunrise remote, TECAN, USA). The cell viability was calculated using the following formula:

$$\text{Cell viability } (\%) = \frac{OD_{\text{experimental group}} - OD_{\text{blank}}}{OD_{\text{control group}} - OD_{\text{blank}}} \times 100$$

2.4. Constructing an oxidatively damaged CM-treated TM cell model

TM cells were cultured in 24-well plates at a cell density of 10,000/well. After proper adhesion of the cells, 250 μM H_2O_2 were added. After culturing for 30 min, CM was added to a final concentration of 1X or 5X. After another 15.5 h, the mitochondrial ROS production, autophagy-related protein expression, DNA fragmentation, cell viability, and ECM-related protein levels were analyzed. The H_2O_2-damaged TM cells without further treatment was called the H group. The damaged cells treated with 1X and 5X BM-MSC CM were called the H-1X CM and H-5X-CM groups, respectively.

2.5. Mitochondrial ROS production

Mitochondrial ROS production was evaluated by MitoSOX™ (M36008, Invitrogen, USA). At the end of culture, cells were collected and 1 mL of 5 μM reagent working solution added. Cells were protected from light and incubated at 37 °C for 30 min. Cells were trypsinized and then washed twice with PBS. Ten thousand cells were analyzed using a BD Biosciences FACSCalibur flow cytometer with excitation and emission wavelengths of 510 and 580 nm, respectively.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic cells were identified by the TUNEL assay (11684795910, Roche, USA) according to the manufacturer's instructions. The cells were washed with PBS and then fixed in a 4% paraformaldehyde solution for 1 h at room temperature. The cells were washed with PBS and then permeabilized using 0.1% Triton X-100 (T8532, Sigma, USA) and 0.1% sodium citrate (71497, Sigma, USA) for 2 min on ice. After washing twice with PBS, 50 μl of the TUNEL reaction mixture was added and then incubated at 37 °C. After 1 h, the samples were washed with PBS and 10,000 cells analyzed using a BD Biosciences FACSCalibur flow cytometer.

2.7. Cell viability

Cell viability was evaluated using CCK-8 as described in Section 2.3. The OD was measured at a wavelength of 450 nm by ELISA. The cell viability was calculated and compared to the control group (100%).

2.8. Western blot assay of proteins involved in autophagy and extracellular matrix

The levels of microtubule-associated protein 1A/1B-light chain 3 (LC3) I and II and fibronectin were detected by Western blot analysis. Cells were collected at the end of culture. The protein concentration in each sample was determined using the bicinchoninic acid (BCA) protein assay kit (500-0001, Bio-Rad, USA) according to the manufacturer's instructions. Total cellular protein was separated on sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dry milk and incubated at 4 °C overnight with specific primary antibodies for fibronectin (F3648, Sigma, USA) or LC3I/LC3II (2775S, Cell Signaling, USA) and GAPDH (8245, Abcam, USA). The membranes were then incubated with secondary anti-rabbit or anti-mouse antibodies conjugated to

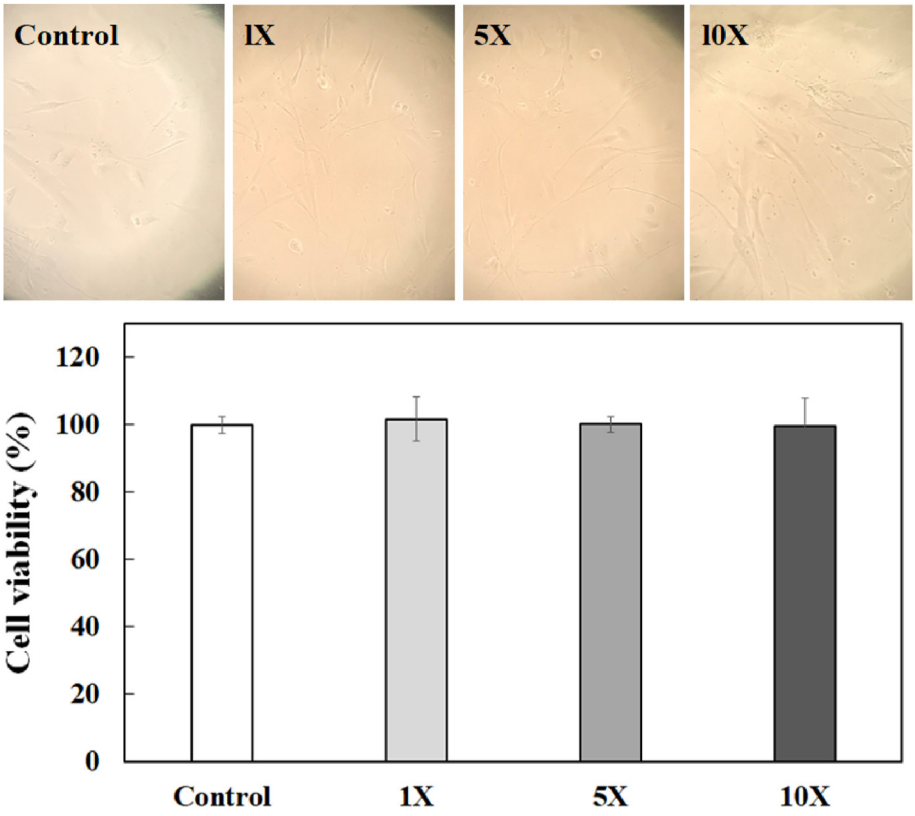

Fig. 1. Cell viability of normal trabecular meshwork (TM) cells and TM cells treated with conditioned medium (CM). Cell viability was evaluated using CCK-8 for untreated TM cells (Control group), and cultured TM cells treated with 1X, 5X, and 10X CM from bone marrow-derived mesenchymal stem cells (BM-MSCs). n = 3, $p > 0.05$.

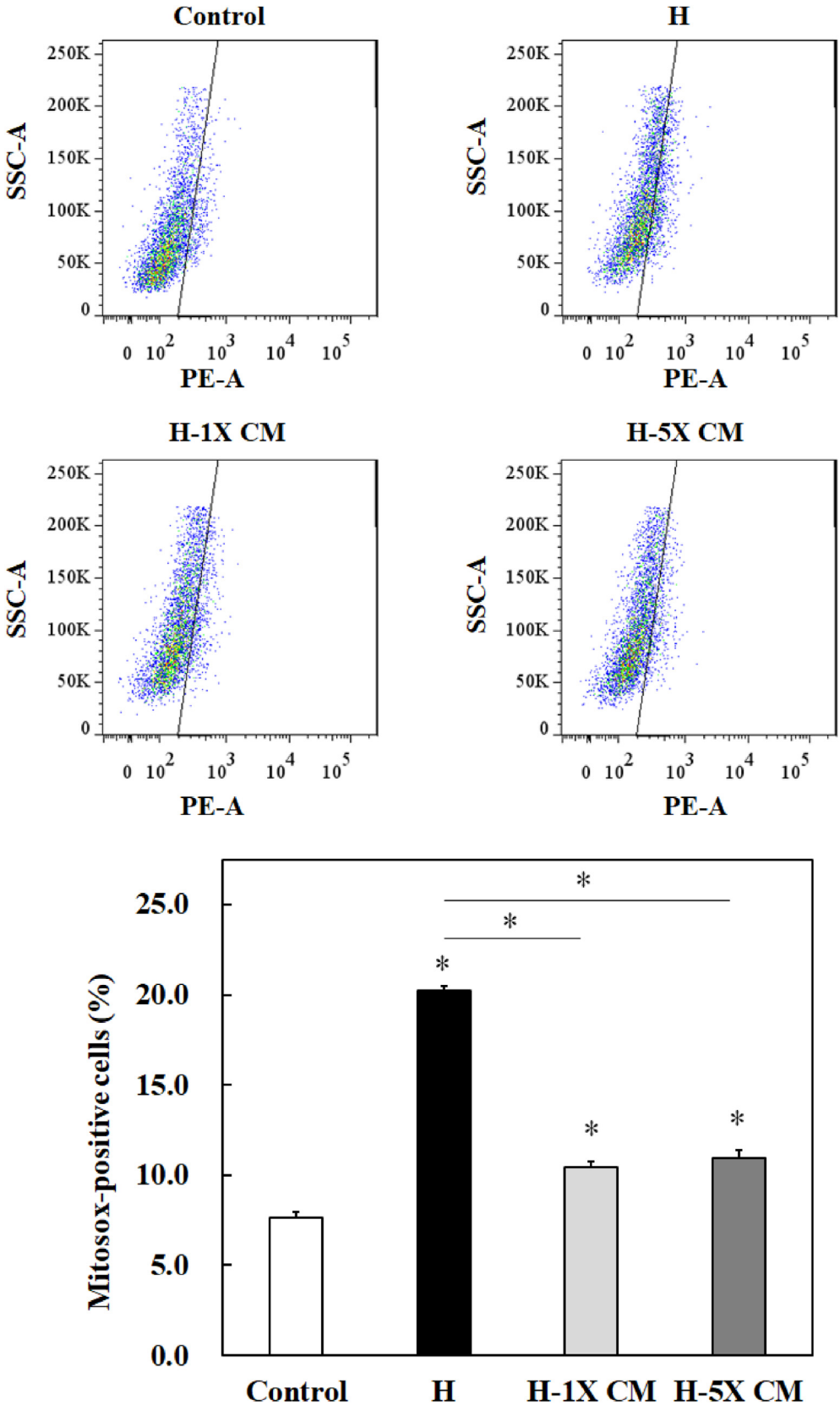

Fig. 2. Flow cytometric analysis of normal, oxidative-stressed, and conditioned medium (CM)-treated trabecular meshwork (TM) cells. The mitochondrial reactive oxygen species content was evaluated by flow cytometry of normal TM cells (without treatment, Control), TM cells exposed to 250 μM H_2O_2 (H group), and TM cells under H_2O_2 -induced oxidative stress treated with 1X CM (H-1X CM group) or 5X CM (H-5X CM group). n = 3, *$p < 0.05$.

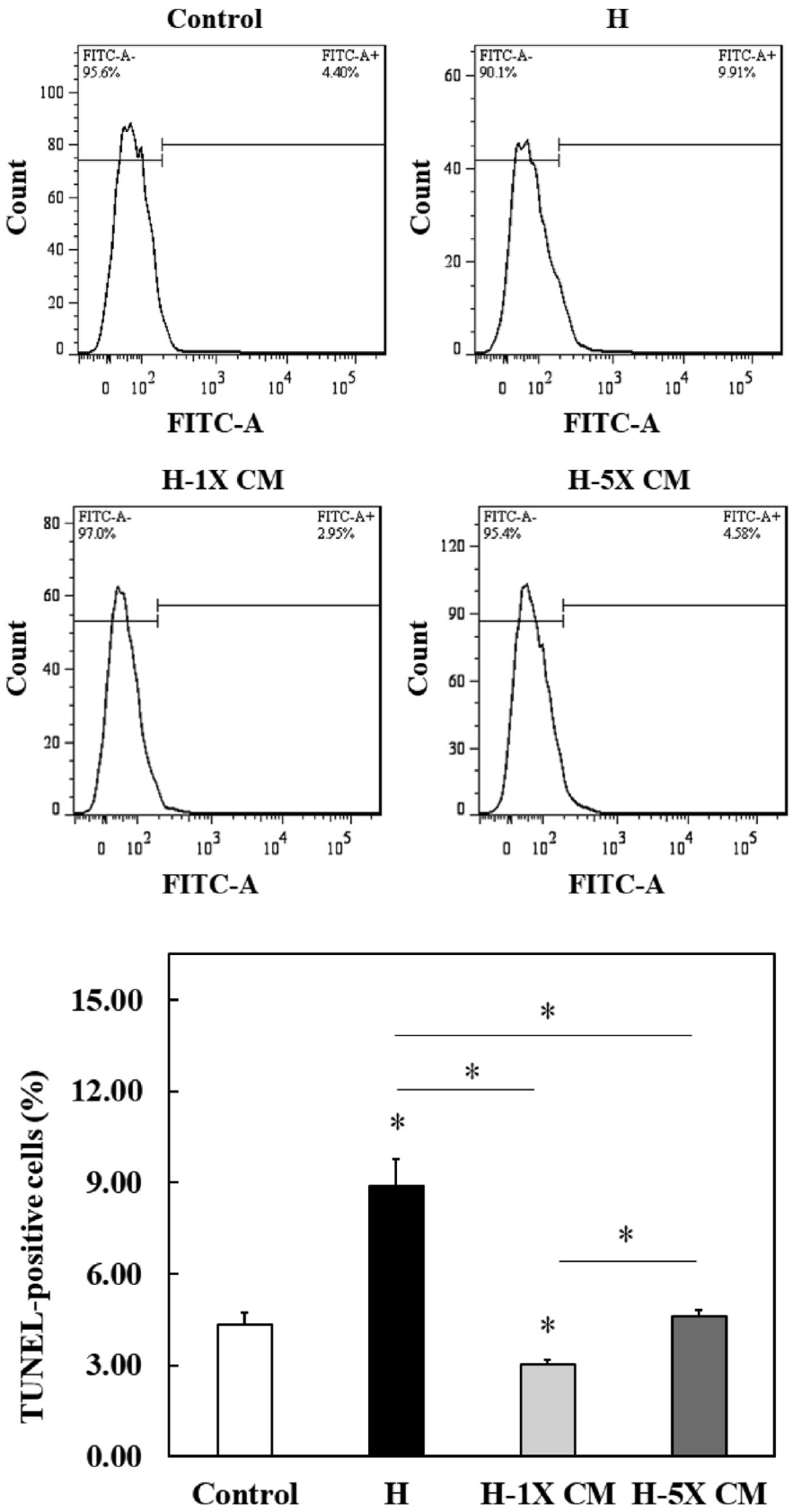

Fig. 3. TUNEL staining of normal, oxidative-stressed, and conditioned medium (CM)-treated trabecular meshwork (TM) cells. TUNEL staining was performed on normal TM cells (without treatment, Control), TM cells exposed to 250 μM H_2O_2 (H group), and TM cells under H_2O_2 -induced oxidative stress treated with 1X CM (H-1X CM group) or 5X CM (H-5X CM group). n = 3, *$p < 0.05$.

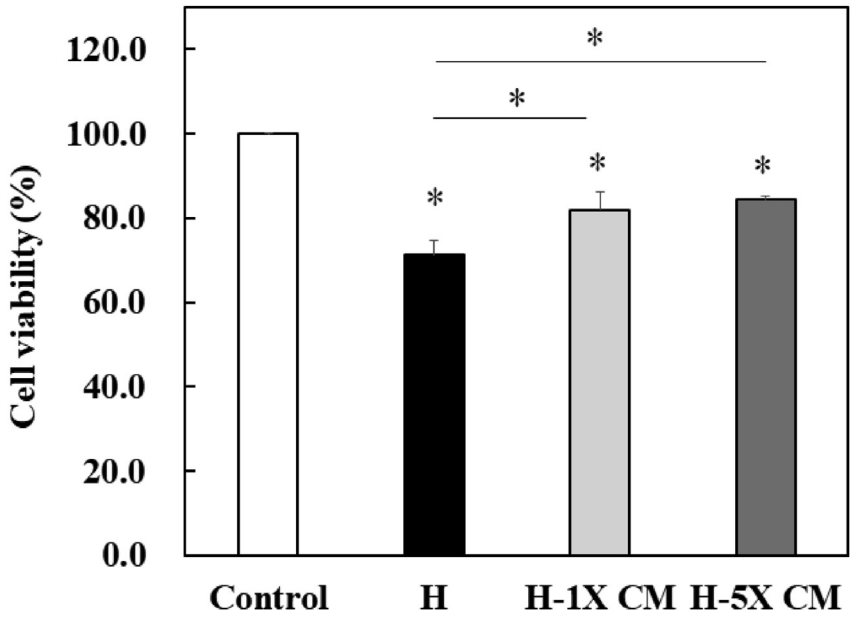

Fig. 4. Cell viability of normal, oxidative-stressed, and conditioned medium-treated trabecular meshwork (TM) cells. Cell viability was evaluated using CCK-8 for untreated TM cells (control), TM cells exposed to 250 μM H_2O_2 (H group), and TM cells under H_2O_2 -induced oxidative stress treated with 1X CM (H-1X CM group) or 5X CM (H-5X CM group). n = 3, *p < 0.05.

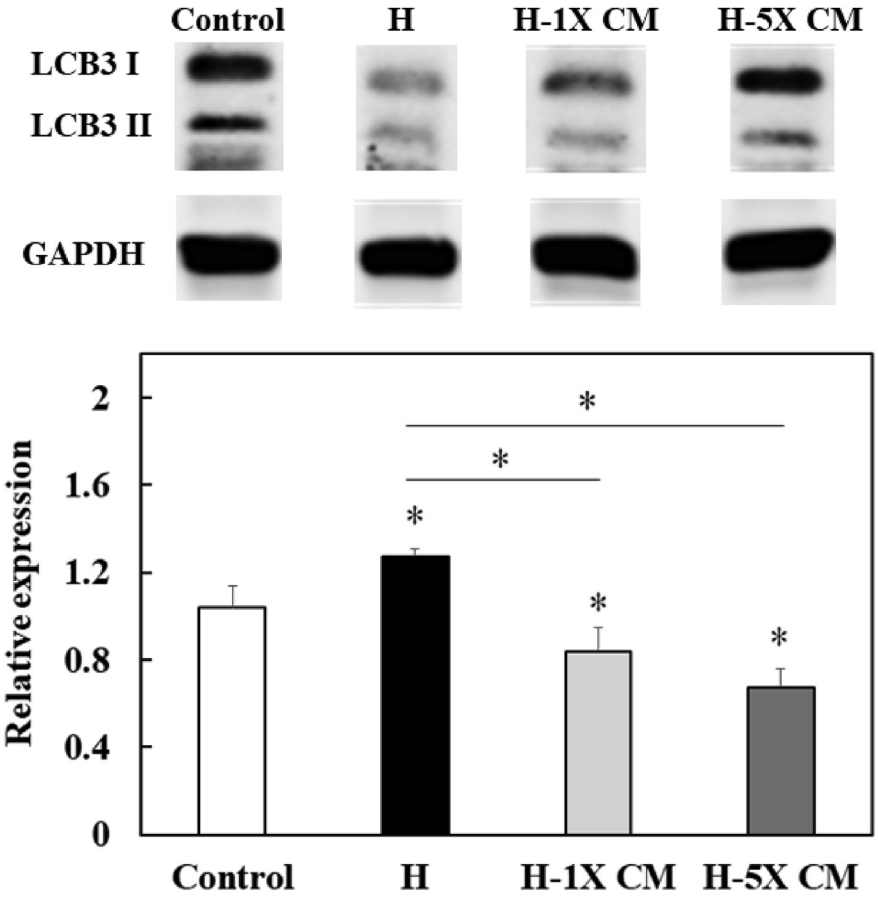

Fig. 5. LCB3 II/LCB3 I protein expression of normal, oxidative-stressed, and conditioned medium (CM)-treated trabecular meshwork (TM) cells. LCB3 II/LCB3 I protein expression was examined by Western blot analysis and the relative ratio determined in normal TM cells (without treatment, Control), TM cells exposed to 250 μM H_2O_2 (H group), and TM cells under H_2O_2 -induced oxidative stress treated with 1X CM (H-1X CM group) or 5X CM (H-5X CM group). n = 3, *p < 0.05.

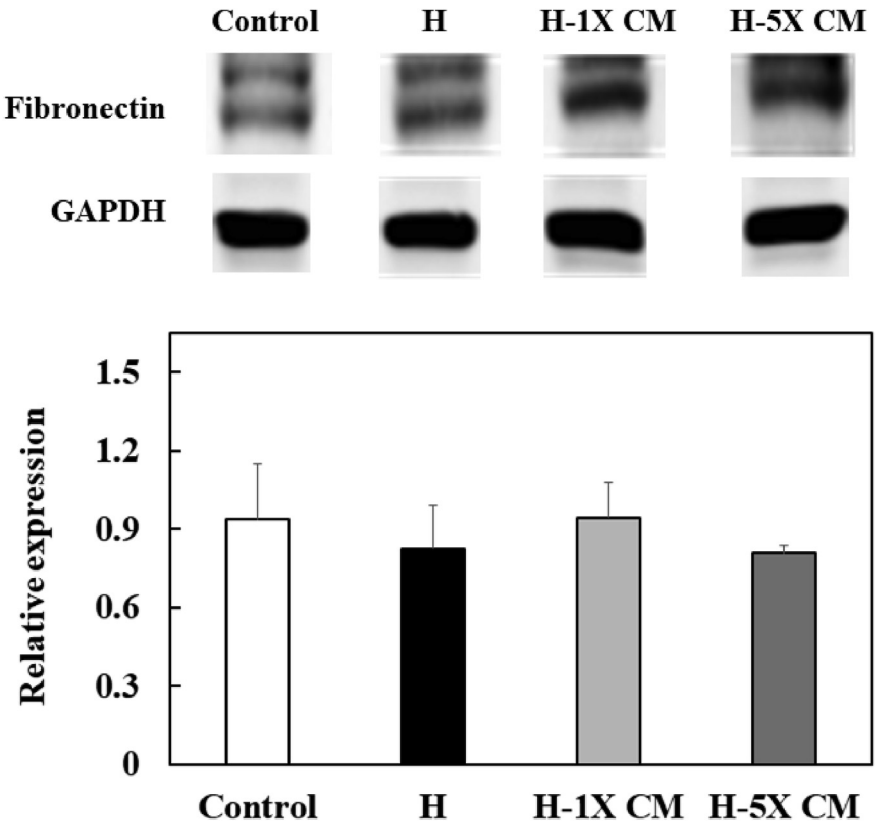

Fig. 6. Fibronectin expression in normal, oxidative-stressed, and conditioned medium (CM)-treated trabecular meshwork (TM) cells. Western blot of fibronectin in normal TM cells (without treatment, Control), TM cells exposed to 250 μM H_2O_2 (H group), and TM cells under H_2O_2 -induced oxidative stress treated with 1X CM (H-1X CM group) or 5X CM (H-5X CM group). n = 3, *$p < 0.05$.

horseradish peroxidase. For data quantification, the samples were analyzed using the UVP BioSpectrum Imaging System.

2.9. Statistical analysis

Statistical analysis was performed using the Student's t–test, one-way or two-way analysis of variance (ANOVA) test, and Tukey's test as appropriate. Data are reported as the means ± standard deviation (SD) of at least three experiments. Significance was set to $p < 0.05$.

3. Results

3.1. Cellular viability of TM cells

After culturing for 16 h under different concentrations of BM-MSC CM, TM cell viability was not significantly different between the control and 1X, 5X, and 10X CM treatments (Fig. 1). Therefore, we used 1X and 5X CM concentrations for TM culture under oxidative stress and analyzed the mitochondrial function, cell apoptosis, cell autophagy, and cellular ECM protein expression. In order to check the rescue effect of the basal growth medium without BM-MSC CM, concentrated basal growth medium was then added to a final concentration of 1X or 5X. The cell viability of the control, the H_2O_2-damaged TM cells (H group), and the damaged cells treated with 1X and 5X concentrated basal growth medium (H-1X BM and H-5X BM groups) was determined (Supplement 1).

3.2. Anti-apoptotic effect of TM cultured with BM-MSC CM under oxidative stress

TM cells were exposed to 250 μM H_2O_2 for 30 min, and then cultured with or without CM. After 16 h, mitochondrial ROS were significantly increased in the H group but decreased in the H-1X CM and H-5X CM groups (Fig. 2). In addition, TUNEL assay (Fig. 3) showed increased DNA fragmentation in TM cells exposed to 250 μM H_2O_2, which significantly decreased after treatment with 1X or 5X CM. TM cell viability decreased under H_2O_2-induced oxidative stress, but treatment with 1X or 5X CM significantly increased the cell viability over the H group (Fig. 4).

3.3. Effects on autophagy and ECM protein expression

Fig. 5 shows the protein expression of LCB3 II and LCB3 I. The LCB3 II/LCB3 I ratio represents the effect of autophagy. The LCB3 II/LCB3 I ratio was significantly elevated in the H group compared to control and significantly decreased in the H-1X CM and H-5X CM groups compared to the H group. Elevated IOP may be caused by the excessive expression of fibronectin. In Fig. 6, the expression of TM fibronectin was not significantly different among the H group, H-1X CM group, and H-5X CM group.

4. Discussion

We analyzed the treatment effect of BM-MSC CM on oxidatively damaged TM cells by evaluating apoptotic and autophagic effects in

the model. The results showed that 1X and 5X CM from MSCs decreases the mitochondrial ROS production after oxidative damage and decreases apoptosis-related DNA fragmentation. As many studies have demonstrated a major role of oxidative stress in the pathogenesis of glaucoma [26–28], we used an oxidatively damaged TM cell model to simulate glaucomatous TM cells, which are characterized by increased expression of oxidative markers and diminished antioxidant potential [29–31].

In recent years, some research has shown that TM-derived MSCs are progenitors of the mature TM and play a key role in regenerating diseased TM tissue. Furthermore, studies have shown that TM-derived MSCs possess similarities with MSCs derived from other tissues, including surface markers, cytoskeletal constituents, and transcription factor expression [25,32]. In the field of ophthalmic research, CM has been reported to stimulate the proliferation of corneal endothelial cells and maintain their phenotypes [33,34]. In the present study, cellular viability after CM treatment of TM cells did not vary by CM concentration.

The therapeutic potential of BM-MSCs has been broadly studied, including in the treatment of ocular diseases [21–25]. Li et al. [21] described a beneficial effect of BM-MSCs on TM cells under oxidative stress, with the potential to predict candidate genes associated with this process. In another study [25], injection of CM from BM-MSCs significantly decreased the IOP in a laser-induced rat model of open angle glaucoma. We used BM-MSCs because they share a neuroprotective presentation and possess growth factors or cytokines with MSCs. After treating oxidatively damaged human TM cells with BM-MSC CM, we found a high level of correlation between autophagy and cellular functions. Thus, MSC CM could be a promising strategy for the treatment of glaucoma.

Under stressful conditions, autophagy occurs as an intracellular lysosomal degradation process. It is a highly evolutionarily conserved method of cellular degradation and recycling, eliminating damaged cellular constituents and providing raw materials for energy and substrates for reconstruction in the body [35]. Defects in autophagy have been associated with the progressive deterioration that occurs during aging [15,16]. Some studies have shown an autophagic effect in TM senescence [36–38]. The autophagolysosome, as the final product of autophagy, degrades under the actions of lysosomal enzymes and transforms into a phagophore [39]. Activation of the microtubule-associated LC3 binding system is involved in elongation of the phagophore. LC3-II is specifically associated with autophagosome formation and used as a marker of autophagosome accumulation [40]. Therefore, the function of autophagy can be presented by evaluating LCB3 II and LCB3 I protein expression.

5. Conclusion

The cell viability analysis showed no cytotoxic effects when TM cells were cultured with 1, 5, and 10-fold concentrated CM from BM-MSCs. Treatment of H_2O_2-damaged TM cells with CM improved cell viability by decreasing the mitochondrial ROS and apoptosis. H_2O_2-damaged TM cells treated with MSC CM had low levels of autophagy based on the LCB3II/LCB3I ratio and normal levels of fibronectin. These results suggest that CM from human BM-MSCs has potential for promoting the survival and maintenance of human TM functions.

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.12.002.

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