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ORIGINAL ARTICLE

Melatonin can mitigate H_2O_2-induced atrophy and promote muscle fiber hypertrophy in morphological level in mouse myoblast cell line*

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ABSTRACT

Objective: It is known that oxidative stress is the main factor in the formation of disuse muscle atrophy which is the most common type of muscle atrophy. The utilization of antioxidants as supplements, particularly the category known as mitochondrial targeting antioxidants (MTA), such as melatonin, have demonstrated significant potential as an advanced therapeutic approach. In this study, we aimed to investigate the effect of melatonin application on the cellular morphology of the C2C12 cell line.

Materials and Methods: In our experiment, we induced oxidative stress using hydrogen peroxide (H_2O_2) to create a model of skeletal muscle atrophy. We established four distinct groups of C2C12 cells, all exposed to the same conditions. These groups included Control (C), Melatonin (M), H_2O_2 (H), and Melatonin + H_2O_2 (MH). The aim was to examine morphological features, specifically myotube diameters, to assess atrophy.

Results: The analysis revealed significant differences in diameters among the groups ($p < 0.05$). The melatonin treatment group not only showed a mitigation of diameter change due to atrophy but also exhibited a significant increase in diameter.

Conclusion: The results suggest that H_2O_2 induces muscle atrophy, and melatonin plays a dual role in maintaining muscle health, protecting against atrophy and promoting hypertrophy, particularly at the morphological level. However, additional research is needed to figure out the details of underlying mechanisms.

Keywords: Melatonin, mitochondria targeted antioxidants, morphological level, H2O2-Induced muscle Atrophy.

INTRODUCTION

Muscle mass accounts for approximately 40-50% of the total body weight in healthy adult humans and plays a crucial role in regulating metabolism in mammals. Skeletal muscle exhibits a highly organized structure that facilitates motion and generates mechanical tension (1). Muscle atrophy, defined as the involuntary loss of 5-10% of muscle mass, involves a reduction in organelles, proteins, and cytoplasm within muscle cells. Consequently, fiber diameter, strength production, and resistance to fatigue are all reduced. This type of atrophy can occur in various pathological conditions such as denervation, AIDS, cancer, and also during the aging process (2). Moreover, the decline in muscle mass can compromise the efficacy of diverse treatments. Consequently, it is essential to conduct molecular studies focused on preventing and managing muscle atrophy or alleviating its effects (3). Additionally, muscle disuse without underlying diseases can also lead to skeletal muscle atrophy (4). The excessive breakdown of proteins in skeletal muscles, coupled with the subsequent decrease in muscle mass, results in increased morbidity and mortality due to both functional losses and impaired energy metabolism (2). Skeletal muscles not only contribute to physical performance but also play a vital role in maintaining overall health across the lifespan. They are actively involved in various metabolic pathways that impact overall well-being. For instance, skeletal muscles are crucial for insulin-dependent glucose uptake, making them essential for maintaining glucose homeostasis (5). Moreover, these muscles play a significant role in metabolic functions such as fatty acid metabolism and glycogen synthesis. Consequently, metabolic disorders affecting skeletal muscles can contribute to insulin resistance, metabolic syndrome, and obesity (6). The structure and functional characteristics of skeletal muscles adapt to meet the body's demands. Changes in metabolic requirements, such as exercise or inactivity, can initiate alterations in muscle mass. Due to the limited capacity for muscle cell proliferation, the regulation of muscle size relies on maintaining a delicate balance between protein synthesis and protein breakdown. Intense mechanical load or stimulation by anabolic hormones shifts this balance towards protein synthesis, resulting in hypertrophy characterized by an increase in fiber diameter. In contrast, under catabolic conditions where protein breakdown surpasses protein synthesis, it results in muscle weakness and atrophy (3). Increased production of reactive oxygen species (ROS) resulting from oxidative stress has been identified as a significant factor in the development of various diseases (7). The association between ROS and disuse-related atrophy was first identified in 1991. Subsequent studies over the past two decades have consistently confirmed this link (8, 9). In skeletal muscle, oxidative stress serves as a well-established mechanism that triggers atrophy, involving multiple conditions and proteolytic processes (10,11). Research suggests that increased production of ROS and impaired redox signaling in skeletal muscle fibers activate major proteolytic systems, initiating wasting (12). Elevated ROS production is primarily observed in the mitochondria of underused muscles (13). Disuse muscle atrophy induces substantial changes in the morphology, function, and content of mitochondria through the activation of catabolic pathways, directly affecting ROS production (3). In order to preserve mitochondrial morphology and function, a variety of antioxidant agents specifically targeting mitochondria have been synthesized (14) and extensively examined for their efficacy in this regard (15). However, none of these approaches have proven unsatisfactory results mainly due to the limited permeability of the mitochondrial membrane requiring substances to be transported through transmembrane carrier systems. Successful synthetic agents targeting mitochondria, like coenzyme Q10 (MitoQ) and mitochondria-targeted vitamin E (MitoE), overcome these membrane barriers by attaching a lipophilic cation, allowing for significant accumulation within mitochondria (16). Certain substances present in our body possess a unique structure that allows them to accumulate within the mitochondria and act as mitochondria-targeted antioxidants (MTAs). Melatonin is one of the most significant examples, which was isolated and identified by Lerner in 1958(17). According to the findings, melatonin has a stronger protective effect compared to MitoQ and MitoE. Additionally, there is evidence that melatonin is present in high levels inside mitochondria and is even produced by mitochondria themselves (14).

Melatonin's mitochondrial protective effects involve several mechanisms: It acts as a potent radical

scavenger, increases the expression of antioxidant genes, such as superoxide dismutase and glutathione peroxidase.It affects the mitochondrial permeability transition pores (MPTP), preventing the formation of active caspase-3 and apoptosis. and enhances the activity and gene expression of uncoupling proteins (UCPs) leading to reduced ROS production (18). Studies indicate melatonin enhances antioxidant capacity and upregulates antioxidant gene expression in myoblast cells (19).

Experimental models utilizing H_2O_2 have been extensively used in both in vitro and in vivo studies to explore disuse atrophy (20-22). H_2O_2 -induced oxidative stress upregulates atrogenes, encoding muscle proteases, while concurrently inhibiting protein synthesis at transcription and translation stages. This dual effect reduces the production of new proteins in muscle cells, impairing muscle growth and maintenance. Oxidative stress increases the susceptibility of oxidized proteins to proteolysis, as structural alterations make them more prone to proteolysis, exacerbating muscle atrophy (23). H_2O_2 is used to study disuse atrophy models in the C2C12 cell line, derived from myoblasts in the thigh muscles of C3H mice (24). C2C12 cells easily multiply in high-serum conditions. Yet, when they reach over 80% confluence and come into contact, they undergo differentiation during the shift to low-serum media. and have the capacity to form contractile myotubes and characteristic muscle proteins (25). Due to their pure cell line nature, rapid differentiation ability, and the ability to form contractile muscle fibers, make them a preferred choice for in vitro models of muscle diseases.

In this study, we investigated the effectiveness of melatonin in addressing oxidative stress and its impact on muscle atrophy. Specifically, we aimed to examine how melatonin administration influences the morphological changes of the C2C12 cell line exposed to H_2O_2.

MATERIAL AND METHODS

Cultured myogenic cell line

The C2C12 mouse myoblast cell line from the American Type Culture Collection (ATCC) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 20% Fetal Bovine Serum (FBS), 2% L-Glutamine, and 1% streptomycin+penicillin during proliferation at 37°C with 5% CO2 (26). Upon reaching 80% density, cells were transitioned to differentiation medium (DMEM, 2% heat-inactivated horse serum, 1% streptomycin+penicillin, and 1% L-Glutamine) and allowed to differentiate for 7 days growth and myotube formation were observed using a light microscope (27).

Determination of half-maximal inhibitory concentration (IC50) for melatonin and H_2O_2

For cytotoxicity assessment and dosage optimization of H_2O_2 and melatonin treatments on the C2C12 cell line, we utilized the Vybrant MTT Cell Proliferation Assay Kit (28). H_2O_2 was administered at varying concentrations (0.025 mM, 0.05 mM, 0.1 mM, and 0.2 mM) and durations (4, 24, 48, and 72 hours). Melatonin treatments included different concentrations (0.001 mM, 0.05 mM, 0.1 mM, 0.5 mM) for a 24-hour duration (29). Upon determining IC50 values and confirming compatibility with literature, the study progressed with the identified optimal concentrations.

Experimental protocol

After necessary optimizations and determining optimal concentrations, the procedure was implemented with the identified concentrations. The study groups were as follows: control (C) group, melatonin (M) group, H_2O_2, (H) group, melatonin and H_2O_2 (MH) group. Melatonin and H_2O_2 treatments, consistent with MTT assay results and literature, were applied during differentiation. On the 4th day of differentiation, melatonin (0.1 mM, 24 hours) was administered to the M and MH groups. On the 5th day, H_2O_2 (0.05 mM, 24 hours) was applied to the H and MH groups.

Myotube diameter measurement

Myotube thickness was measured using the method described by Van der Meijden et al. (30). Measurements were taken on the 7th day of differentiation using an inverted microscope (Nikon, ECLIPSE TS100). We captured images using a Toupcam Digital Camera (TOUPTEK PHOTONICS, P/N: TP105100A) and used the ToupView 3.7 software program (Toup Tek-Toup View, Version: x64, 3.7.4460). Evaluations of myotube diameters were performed using the ImageJ (NIH) program. In the ImageJ program, the scale length was defined as 200 µm, and measurements were calculated based on this scale. The length of each myotube

segment was considered 100% and divided into 10 equal parts. These parts, representing the cross-sectional (length) equivalent and cross-sectional angles (using the image frame as a reference), were created using the program.

After all calculations were made, the average length of the cross-sections was taken, and the overall average was considered the group average. Figure 1 explains how the Image J program was used to measure the diameter of myotubes.

Statistical analysis

Statistical analysis employed GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Descriptive statistics, mean ± SD deviation was utilized. Group comparisons utilized ANOVA, and Pearson correlation analysis was conducted. A significance level of $p \leq 0.05$ indicated statistical significance.

RESULTS

MTT method results

The melatonin concentrations used in the MTT method and the corresponding cell viability percentages are provided in Table 1.

Based on MTT assay results, it was demonstrated that C2C12 cells exposed to 0.1 mM melatonin for 24 hours maintained 95.72% viability which was suitable for our experimental protocol.

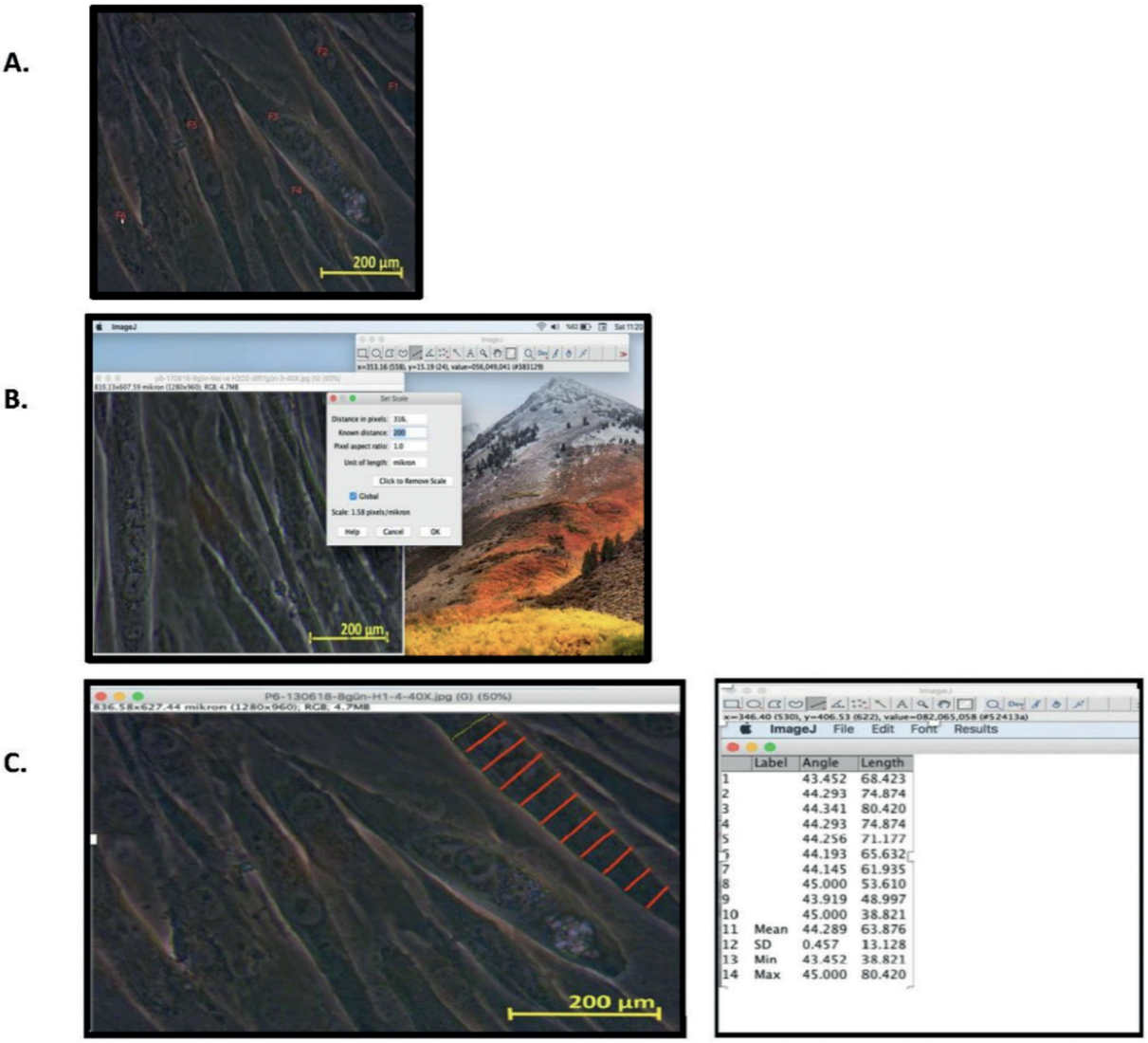

Figure 1. At 7th day of differentiation, myotube segments with clearly defined boundaries were selected and numbered in each microscope field 40X magnification **(A)**. 200µm scale bar displayed in the ImageJ program **(B)**.Measurements displayed on the ImageJ program screen **(C)**.

Table 1. The melatonin concentrations and cell viability percentages at 24h

Melatonin (mM)	10	5	1	0.1	0.01
Viability * %	15.58	50.54	100	95.72	97.74

* viability rates were calculated as a percentage relative to the control.

Table 2. The H_2O_2 concentrations and cell viability percentages at 24h

H_2O_2 (mM)	1	0.5	0.1	0.05	0.001
Viability * %	12.80	74.12	94.21	93.36	98.78

* viability rates were calculated as a percentage relative to the control.

The concentrations of H_2O_2 used in the MTT method and the resulting cell viability percentages are indicated in Table 2.

Based on MTT assay results, the cells exposed to 0.05 mM H_2O_2 for 24 hours showed 93.36% viability, which was deemed an appropriate dose.

Comparing C2C12 cell morphology at different growth and differentiation stages in

Experimental groups

All cell culture imaging was conducted using a Nikon inverted objective microscope under normal light conditions and processed with the Toupview software. Representative examples of the typical proliferation and differentiation patterns of the C2C12 cells used are shown in Figure 2.

Most cells were observed to differentiate into myotubes as expected. The morphology of C2C12 cells in groups (C), (M), (H) and (MH) on the 7th day of differentiation are shown at 10x and 40x magnifications in Figures 3, 4, 5, and 6, respectively.

The measurements taken from 9 different regions of each group in the Image J program, were transferred to Microsoft Excel. The average and standard deviation of the cross-sectional (length) equivalent and angular measurements for each myotube were calculated.

Table 3. Mean myotube diameter and standard deviation (± SD) by experimental group

Group	Mean ± SD	p-values
C	78.12 ± 7.61	C-H: 0.0262, C-M: 0.0338
H	60.83 ± 6.89	H-C: 0.0262, H-M: <0.0001, H-MH: 0.0005
M	94.78 ± 17.68	M-C: 0.0338, M-H: <0.0001
MH	86.69 ± 13.60	MH-H: 0.0005

C: Control; H: H2O2; M: Melatonin; MH: Melatonin +H2O2.

Comparison of morphological measurement values among experimental groups

At the 7th day of cell differentiation in the experimental groups, the average myotube diameter measurements are illustrated in Figure 7.

The experimental group mean myotube diameter values, along with their respective standard deviations (SD), are detailed in Table 3. These values reveal the differences in mean myotube diameters across the experimental groups ($p \leq 0{,}000013$).

The table displays the mean myotube diameters (± SD) for four experimental groups: C, M, H, and MH. These measurements were obtained through statistical analysis using One-way ANOVA and post-hoc Tukey's HSD test to assess differences between the groups. The p-values indicate the statistical significance of the inter-group differences in mean myotube diameters ($p<0.05$).

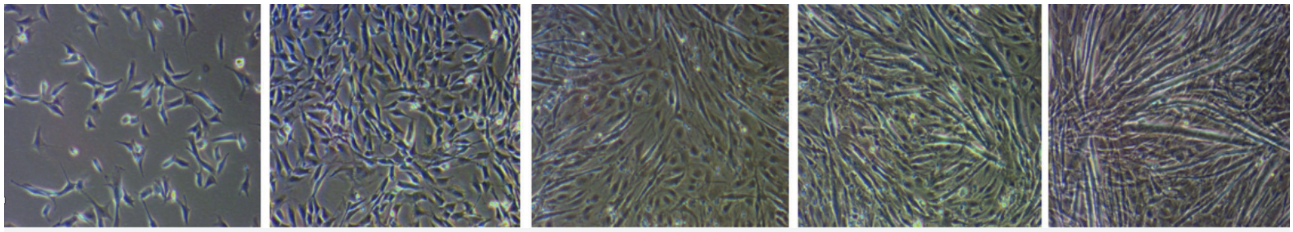

Figure 2. Proliferation of C2C12 Cells and Initiation of Their Differentiation Process. 30% density of cells **(A)**, 80% density of cells **(B)**, first day of differentiation **(C)**, second day of differentiation **(D)**, third day of differentiation **(E)**.

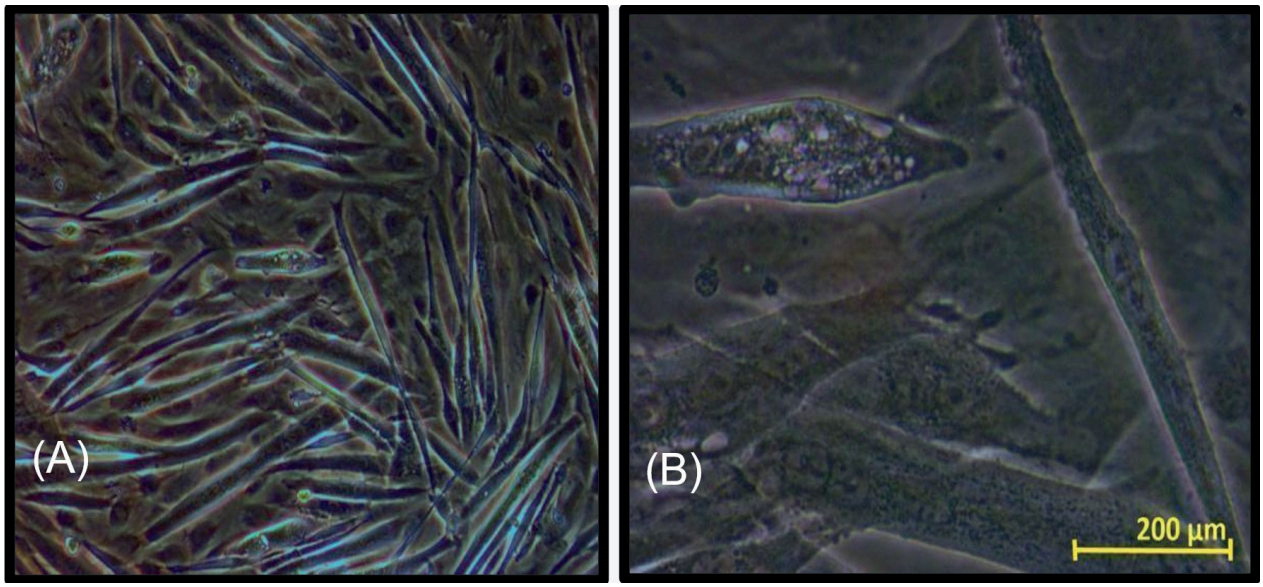

Figure 3. Group C at 10x magnification **(A)**, Group C is displayed at 40x magnification with a 200 µm scale bar **(B)**.

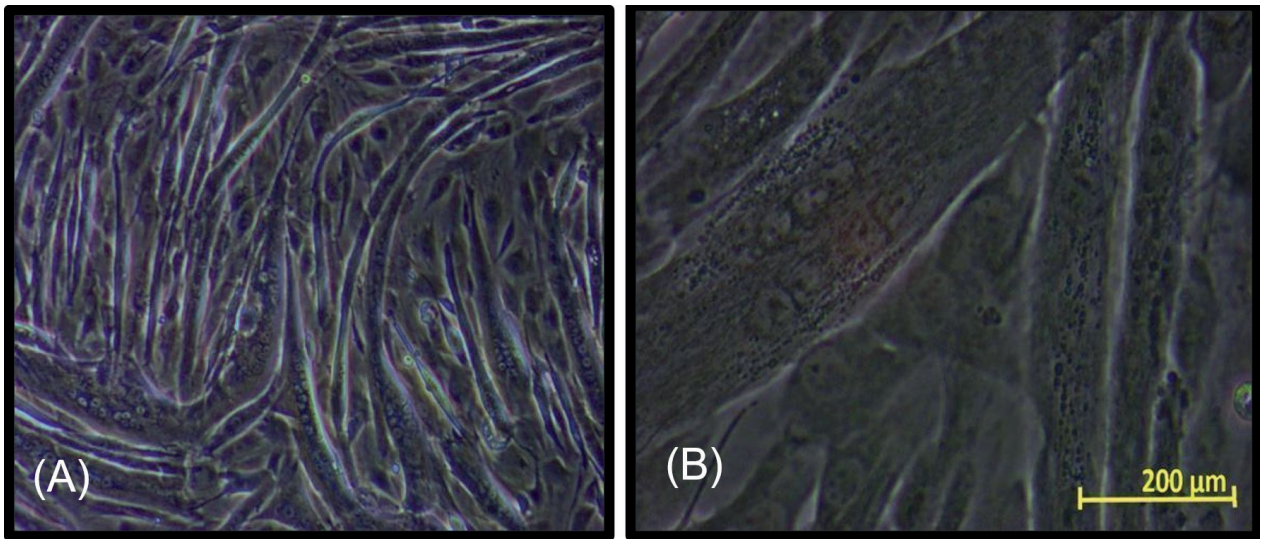

Figure 4. Group M at 10x magnification **(A)**, Group M is displayed at 40x magnification with a 200 µm scale bar **(B)**.

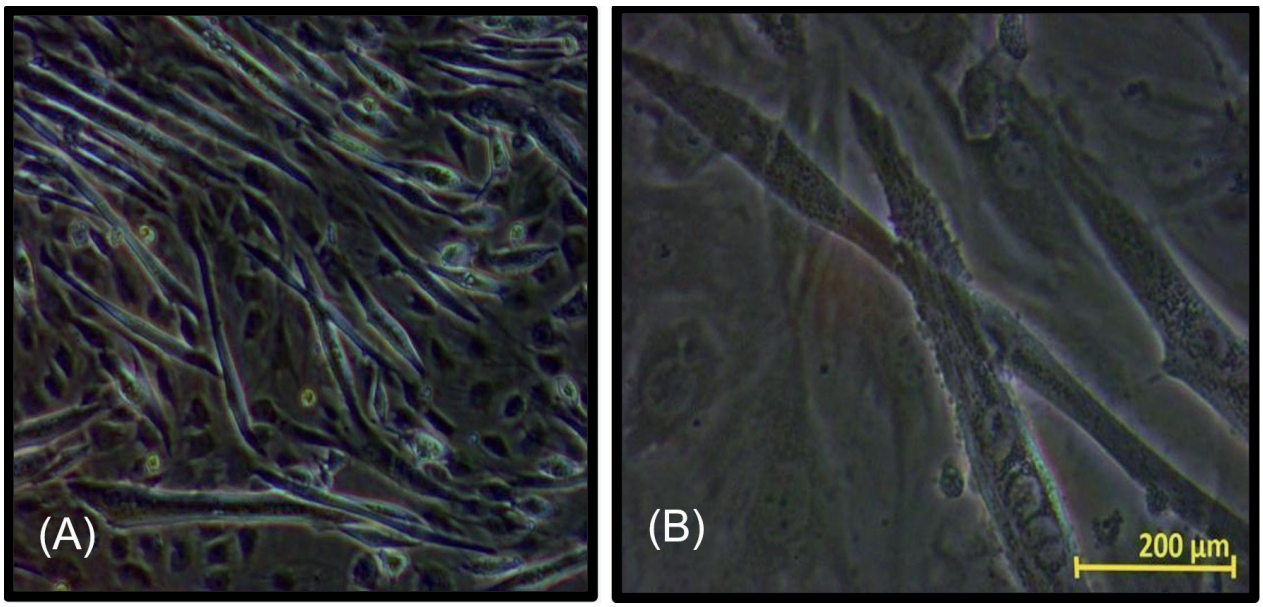

Figure 5. Group H at 10x magnification **(A)**, Group H is displayed at 40x magnification with a 200 µm scale bar **(B)**.

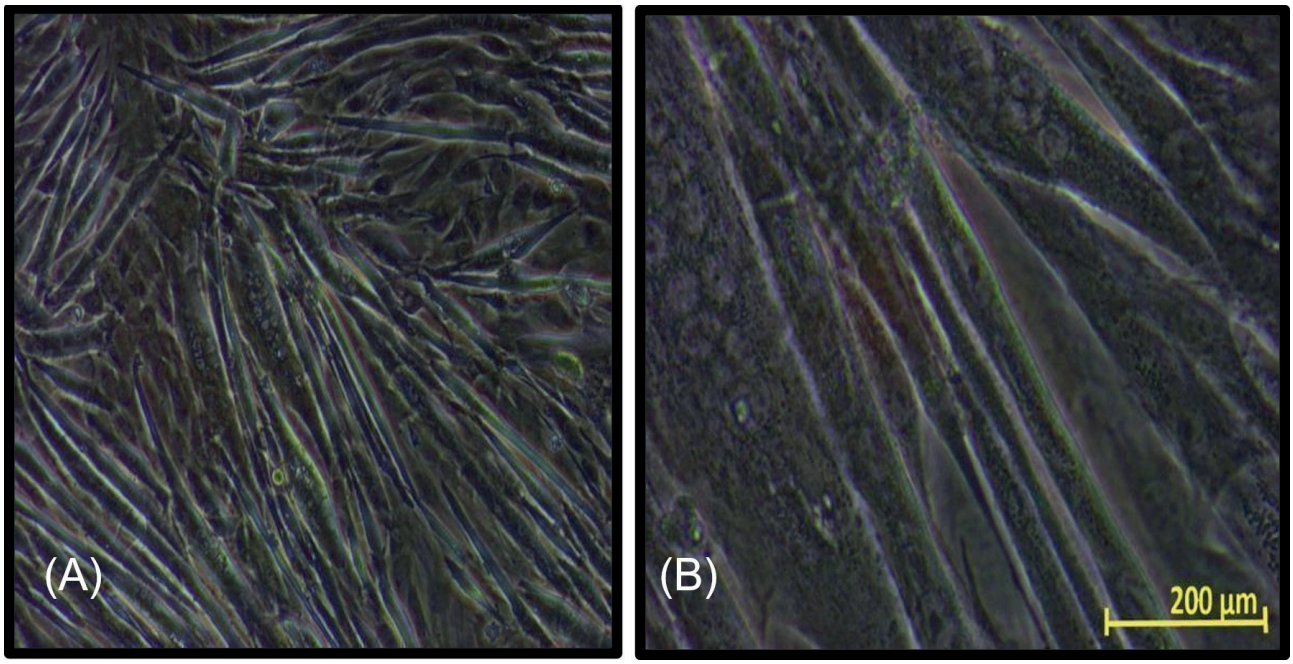

Figure 6. Group MH at 10x magnification **(A)**, Group MH at 40x magnification with a 200 µm scale bar **(B)**.

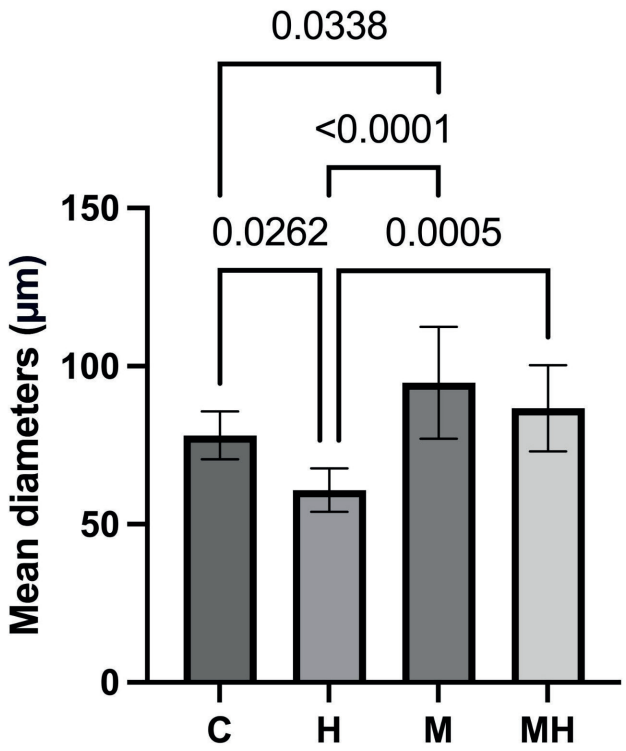

Figure 7. The average myotube diameters at the 7th day of differentiation for the experimental groups with standard deviation bars and p values

Statistical analysis revealed significant differences in the mean myotube diameters between the groups. Group H exhibited a 23% reduction in myotube diameter compared to group C (p = 0.0262). Group H had significantly smaller myotube diameters compared to all other groups. Specifically, group H showed significantly smaller diameters than group M ($p < 0.0001$) and group MH ($p = 0.0005$). Conversely, group M had significantly larger diameters compared to group C ($p = 0.0338$). However, no significant difference in myotube diameters was observed between group M and group MH.

DISCUSSION

Our study reveals that H2O2 causes muscle atrophy in C2C12 cell line, and melatonin effectively prevents this atrophy. This aligns with the potential therapeutic benefits of melatonin in mitigating oxidative stress-related conditions (31, 32). The ability of melatonin to counteract the harmful effects of H2O2 on muscle cells suggests its potential as a protective agent against disuse-related muscle atrophy. The cells included in our study, having the same passage number, underwent uniform conditions throughout their growth and differentiation phases. Therefore, it can be stated that the observed differences between the groups are attributed to the experimental protocol. In our study, we used 0.05 mM H_2O_2 for 24 hours based on MTT assay results and a prior study (27). This led to a 23% reduction in myotube diameter in our atrophy model compared to the control —a recognized indicator of atrophy in the literature (33). We applied 0.1 mM melatonin for 24 hours, consistent with recommended literature dosage and our MTT results (29). Despite previous

evidence of melatonin's antioxidant and anti-apoptotic effects in the C2C12 cell line (29, 34) and its positive impact on muscle wasting, the morphological analysis of muscle atrophy remains unexplored in in vitro research. The 2023 study conducted by Ming Su et al. provides evidence that melatonin enhances muscle regeneration in the stages during the process of skeletal muscle differentiation (35). In our study, we demonstrated the impact of melatonin on muscle atrophy in the C2C12 cell model at the morphological level. Within the group MH, the morphological measurements showed an obvious reversal of muscle atrophy when compared to the group H.This outcome is consistent with previous studies investigating the effects of melatonin on muscle atrophy (31,32,36). Moreover, the group M exhibited a statistically significant increase in fiber diameter compared to the group C. This finding, for the first time implies that melatonin not only prevents atrophy but also induces hypertrophy in C2C12 cell line, which is consistent with a vary recent in vivo study indicating that melatonin has the potential to stimulate skeletal muscle growth and induce muscle fiber hypertrophy primarily through the upregulation of genes associated with skeletal muscle hypertrophy (37). These data allude to a dual role for melatonin in maintaining muscle health, encompassing protection against atrophy and promotion of hypertrophy. Furthermore, a study demonstrated that assessing biochemical markers of atrophy, such as creatine kinase and lactate dehydrogenase, in conjunction with atrogenes, can provide more detailed insights into atrophy (38). Examining these markers alongside atrogene expression is a suitable approach for a more comprehensive evaluation of atrophy. Moreover, to enhance our comprehension of melatonin's role in preventing atrophy and promoting hypertrophy, particularly beyond atrogenic pathways, it is crucial to assess the expression of genes associated with skeletal muscle hypertrophy.

In summary, our study shows that H_2O_2 induces muscle atrophy in C2C12 cells, but melatonin not only prevents this atrophy morphologically but also promotes hypertrophy. This underscores melatonin's potential as a versatile therapeutic agent in muscle health and disease prevention.

Author contribution

Study conception and design: NKA, YK, MTB; data collection: NKA, YK, MTB; analysis and interpretation of results: NKA, YK, MTB; draft manuscript preparation: NKA. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Hacettepe University Animal Experiments Ethics Committee (Approval No. 52338575-98 on 22 August 2017), confirming that the study on the commercially available C2C12 mouse myoblast cell line, obtained from American Tissue Cell Culture, does not require ethics committee approval.

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Conflict of interest

The authors declare that there is no conflict of interest.

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