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Original article

Melatonin does not have any effect on proteasome activity in human dermal fibroblast.

A. N. M. Mamun-Or-Rashid, Wakako Takabe, Masayuki Yagi, Yoshikazu Yonei

Anti-Aging Medical Research Center and Glycation Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, JAPAN

Abstract

***Objectives*:** Protein structure and function is continuously demolished by nonenzymatic glycation and thereby produce advanced glycation end products (AGE). Therefore, the degradation of advanced glycation end products (AGE) cross-links are of great importance. The present study was conducted to evaluate the effects of melatonin-induced human dermal fibroblast (HDF) cells on AGE crosslink degradation and on the cellular proteasome activity.

***Method*:** Glycated human serum albumin (HSA) was taken as AGEs with the advantage of its fluorescence intensity to evaluate degradation in a relatively easy way. A quinine sulfate solution was used as a reference for the calibration of fluorescent materials and the fluorescence intensity was measured at Ex/Em = 370/440 nm. Proteasome activity was measured using a commercial kit.

***Results*:** Our experimental AGEs showed high fluorescence intensity and HDF showed significant reduction of fluorescence. However, melatonin did not change the reduction level, showing that it has no effect on AGE degradation. Proteasome activity also remained unchanged by melatonin treatment.

***Conclusion*:** HDF shows effective AGE degradation and proteasome activity, and melatonin has no effect on it.

***KEY WORDS*:** advanced glycation end product (AGE) degradation, melatonin, proteasome activity, fluorescent AGE.

Introduction

Glycation causes unnecessary changes in protein structure and function by making cross-links between amino acids and sugar, or its derivatives, and then accumulates into body parts and thus play an important role in aging. Some of the glycated proteins are toxic and diminish cell viability. Some act as a ligand to the cell surface receptors and change the intracellular functions. Glycative stress increases with age, and sleep quality falls. Melatonin (N-acetyl-5-methoxytryptamine) is a natural mammalian hormone secreted from the pineal gland, and plays a key role to control sleep cycles and maintain sleep quality. It showed a potential anti-inflammatory and antioxidant role. It has also been reported to have anti-aging properties [1]. Melatonin showed osteoblast-inducing effects on human adipose-derived mesenchymal stem cell [2] and osteoclast-stimulating effects on murine monocyte/macrophage RAW264.7 cells [3]. Melatonin also plays a fundamental role in the female reproduction system [4, 5]. In humans, melatonin secretion declines progressively with age and after menopause [6, 7].

Glycation produces highly fluorescent or non-fluorescent advanced glycation end products (AGEs). Cleaving the non-enzymatic cross-links between proteins and glycating agents is of great importance. Melatonin was reported to have about 15% AGE cross-link degradation activity [8], presenting the potential of reusing the glycated proteins or preventing the negative role of AGEs; although, the melatonin did not show any carbonyl scavenging actions [9].

Cells use the ubiquitin-proteasome system to maintain cellular function, proliferation, and differentiation by degrading regulatory and abnormal proteins. Proteasome activity shows chymotrypsin-like, trypsin-like, and caspase-like activities that are strongly regulated and are basic cellular requirements [10, 11]. Intracellular protein quantity and quality are also maintained by proteasomes [5, 12]. Proteasomal degradation of oxidized proteins does not require ubiquitin

Corresponding author: A. N. M. Mamun-Or-Rashid, PhD
Anti-Aging Medical Research Center and Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3 Tatara Miyakodani, Kyotanabe, Kyoto 610-0394, Japan
TEL& FAX: +81-774-65-6382 E-mail: amamun@mail.doshisha.ac.jp
Co-authors: Takabe W, wtakabe@mail.doshisha.ac.jp;
Yagi M, myagi@mail.doshisha.ac.jp; Yonei Y, yyonei@mail.doshisha.ac.jp

conjugation [13-17]. Therefore, any unexpected or forceful alteration of proteasome activity may play a significant role in cellular systems. Melatonin showed various important cellular activities that require proteasome activities. Here, we observed the effect of melatonin on proteasome activity using HDF to check if melatonin changes proteasome activity or not.

Materials and methods

Melatonin was obtained from Sigma-Aldrich (St. Louis, MO). Test products were dissolved into dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) and a very low amount (2 µL/mL) was used in all cell culture experiments to avoid DMSO's cytotoxicity.

Cell culture and reagents

Human dermal fibroblast (HDF) was purchased from Cell Application Inc., San Diego, CA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), penicillin 100 units/mL, streptomycin 100 µg/mL and amphotericin B 25 µg/mL (Gibco, El Paso, TX) at 37 °C under the condition of 5% CO_2.

Evaluation of cell viability

Cell viability was evaluated using CCK-8 (Dojindo, Kumamoto, Japan). HDF cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated for 24 hours. Melatonin stock was prepared in DMSO as 50 mM and then serially diluted to prepare the desired stock. Melatonin stock solutions were used along with regular media (DMEM) to prepare 100, 1, 0.01, 0.0001 µM melatonin in media and used to replace in cell culture. An equal volume (2 µL/mL) of DMSO was used as vehicle control. After 2 days of incubation at 37 °C under the condition of 5% CO_2, a 10% volume of CCK-8 solution was added to the culture medium and the cells were incubated for 1 h. Absorbance at 450 nm was then measured using a Varioscan® Flash microplate reader.

Glycated-HSA preparation

To prepare glycated-HSA, we used 8 mg/mL HSA along with 33 mM glycolaldehyde (Glycol) or glyceraldehyde (Glycer) in a 0.05 M phosphate buffer (pH 7.4) and incubated at 60 °C for 40 hours. After that, we removed the remaining unreacted glycating agents and phosphate buffer using Amicon ultra-4 10K (Millipore, Darmstadt, Germany) centrifugal devices according to the manufacturer's instruction. Briefly, 4 mL of protein mixture was placed into the centrifugal devices and centrifuged at 7500 ×g for 15 minutes. The mixture was then washed using sterile milliQ and centrifuged again to collect glycated proteins. The amount of protein was measured using the BCA protein assay (Thermo Scientific, Rockford, IL) [18,19].

Measurement of fluorescent AGEs

Purified protein samples (AGEs) 150 µg/mL in PBS were used to measure fluorescence intensity. A quinine sulfate solution was used as a reference for the calibration of fluorescent materials and the fluorescence intensity was measured at Ex/Em = 370/440 nm using a Varioscan® Flash (Thermo Scientific, Waltham, MA) microplate reader [18,19].

Proteasome activity

Proteasome activity was measured using a proteasome activity assay kit (Abcam) as per the manufacturer's protocol. Briefly, protein lysates were extracted from the experimental HDF cells in 0.5% NP40 after 48 hours of treatment. The kit takes advantage of the chymotrypsin-like activity of the proteasome, utilizing an aminomethylcoumarin (AMC)- tagged peptide substrate which releases free, highly fluorescent AMC in the presence of proteolytic activity. The kit uses a specific proteasome inhibitor, MG-132, to suppress all proteolysis due to proteasomes differentiating proteasome activity from other protease activity present in the samples. Fluorescence was measured with the previously mentioned microplate reader after 20-50 min at fluorometric Ex/Em = 350/440 nm at 37 °C. Proteasome activity was subsequently calculated according to the manufacturer's instructions.

$$Proteasome\ Activity = \left(\frac{B}{(T_2 - T_1) \times V} \right) \times D.$$

Where B = Amount of AMC in the sample well (pmol)
V = Sample volume added into the reaction well (µL)
T_1 = Time (min) of the first reading (RFU_1 and $iRFU_1$)
T_2 = Time (min) of the second reading (RFU_2 and $iRFU_2$)
D = Sample dilution factor
RFU = Total proteolytic activity
iRFU = Proteolytic activity due to non-proteasome activity (proteasome inhibitor)
$\Delta RFU = (RFU_2 - iRFU_2) - (RFU_1 - iRFU_1)$

One unit of proteasome activity is defined as the amount of proteasome, which generates 1.0 nmol of AMC per minute at 37 °C.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). All statistical analyses were performed using the Tukey-Kramer test for intergroup comparison in all the experiments. Differences were considered significant at a significance level of 5%.

Results

Melatonin did not induce HDF cell death in our experimental condition. At a higher concentration (100 µM), melatonin may reduce mitochondrial activity (***Fig. 1***). Glycated-HSA that shows high fluorescence intensity (excitation at 370 nm, emission at 440 nm), was prepared.

Glycated-HSA were used to check their effect on cell survival. Here, we found that none of our experimental HSA-AGEs killed the cell; instead, they showed higher activity (*Fig.2*), which could be due to the experimental AGEs being used by HDF cells.

Following this, we checked the effect of melatonin on the HSA-AGEs fluorescence reduction and found that HDF cell can reduce fluorescence intensity in both HSA-Glycol and HSA-Glycer. However, melatonin has no effect on the reduction (*Fig. 3*).

Next, we checked whether proteasome is responsible for this reduction or not by using melatonin or vehicle-treated cell lysate incubating with AGEs. Here, we found that there is a reduction but not due to the proteasome; instead, NP-40 may be responsible for this reduction (*Fig. 4*).

Finally, we checked melatonin-treated HDF cells proteasome activity by using a commercial kit and found it to have no effect (*Fig. 5*).

Discussion

The protein degradation pathway by the cellular proteasome is a key process to maintain cell cycle, gene expression, responses to oxidative stress, and cell survival. Proteasome degrade abnormal, misfolded, oxidized, denatured proteins and then this degradation products are either reused by the cell or removed from the cell [11-13,15-17,20]. Human fibroblast cells fall into a limited number of *in vitro* cultures and reach a state of irreversible growth arrest called replicative senescence. The proteasome function is interrupted during the replicative senescence in human fibroblast [21] and with age in different rat organs [22]. Earlier studies reported melatonin as a proteasome inhibitor [23]. Melatonin was mentioned to inhibit proteasomes of renal cancer cells [24]. HDF cell can reduce the HSA-AGE's fluorescence intensity in our experimental conditions. Melatonin was found to degrade AGEs in experimental condition [8], but could not induce HDF cell to degrade more fluorescent AGEs (*Fig. 3*). Although melatonin was previously regarded as an inhibitor of the ubiquitin-proteasome system [25], our different results may be due to different cells with different experimental conditions.

Glycative stress, oxidative stress increase, and sleep quality fall with aging. The decline in sleep quality induces blood glucose spikes, and subsequently, an aldehyde spark, thus resulting in a formation of carbonylated protein or advanced glycation end products (AGEs) [1]. The intensity of skin autofluorescence mainly derived from AGEs is shifted higher in the individual with short sleep duration of fewer than 6 hours [26]. The frequency of glucose spikes increases when the sleep duration is markedly short [1]. Less or almost no actions of melatonin, however, are reported for the preventive effect of AGE formation or it's adverse effects [27]. Additionally, in this study, melatonin did not show any actions on proteasome function (*Fig. 5*). By reviewing these findings, the main mechanism of melatonin can be considered by enhancing decomposition of AGEs [8], especially the cleaving effect of the α-diketone structure of AGEs.

Melatonin also has actions to ameliorate glucose spikes [28], however, its mechanism still remains unclear. Our hypothesis currently states that a lower amount of AGEs, reduced by decomposition effect of melatonin, influences insulin secretion from pancreatic β-cell by load reduction of AGEs. If the amount of AGEs is reduced, ER stress also may be reduced, insulin secretion recovered, and as a result, glucose spikes could be ameliorated. Further study is needed for the elucidation.

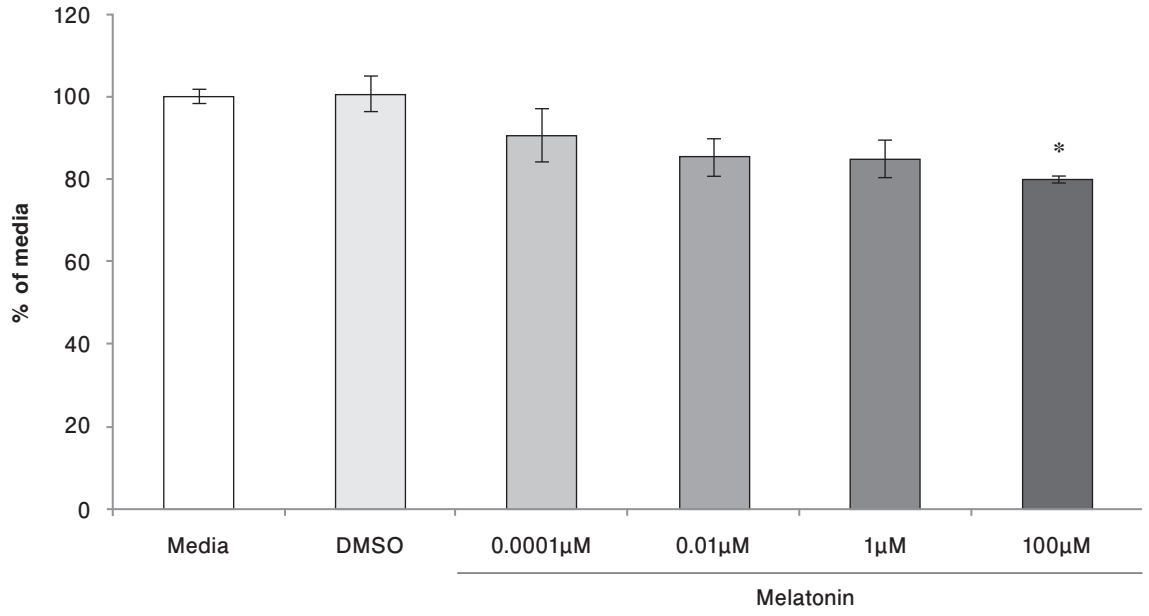

Fig. 1. **Effect of melatonin and vehicle on cell viability.**

HDF cells were treated with DMEM containing 10% FBS and antibiotics with or without differing doses of melatonin, and were incubated at 37 °C and 5% CO_2 for 2 days. WST-8 assay. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. HDF, human dermal fibroblast; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; WST, water-soluble tetrazolium-salt; SEM, standard error mean.

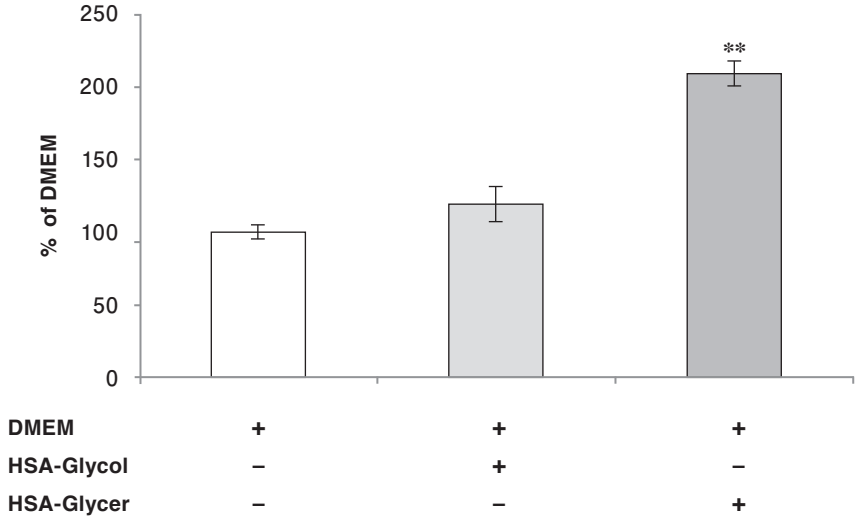

Fig. 2. **Effect of HSA-AGEs on cell viability.**

HDF cells were treated with DMEM containing antibiotics, with or without 150 μg/mL HSA-Glycol or HSA-Glycer, and were incubated at 37 °C and 5% CO2 for 2 days. WST-8 assay. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. HDF, human dermal fibroblast; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; AGEs, advanced glycation end products; Glycol, glycolaldehyde; Glycer, glyceraldehyde; WST, water-soluble tetrazolium-salt; SEM, standard error mean.

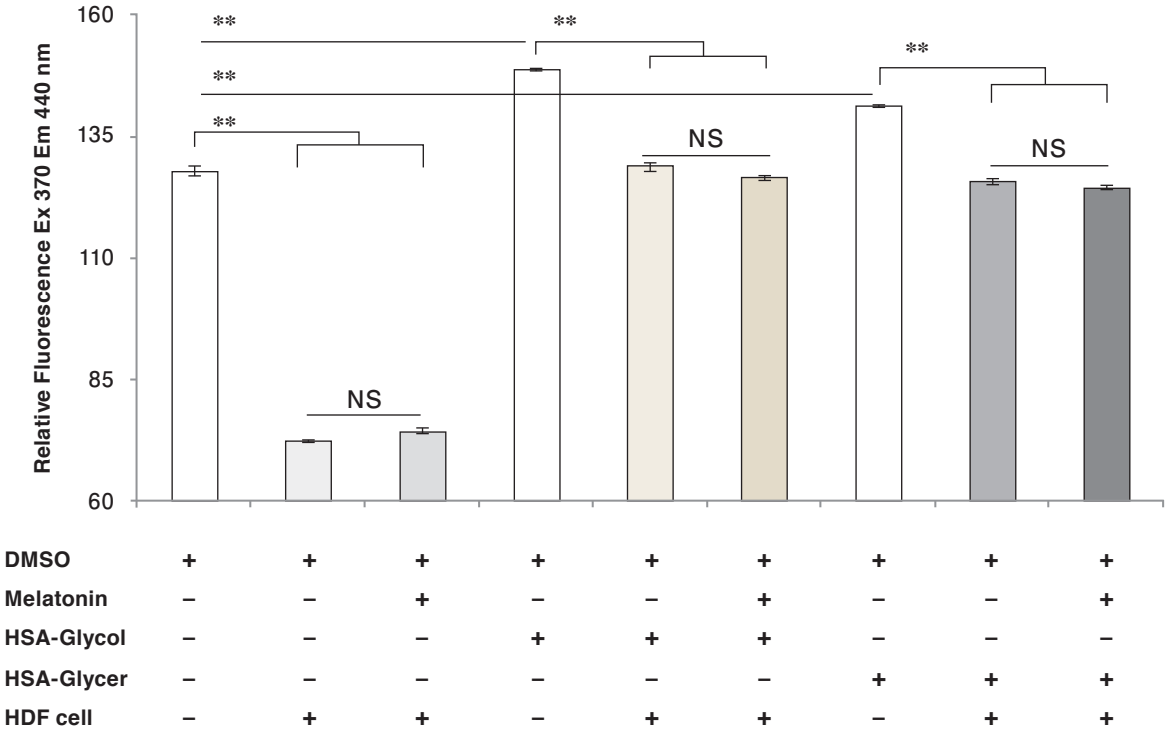

Fig. 3. **Effect of melatonin on HSA-Glycol and HSA-Glycer breakdown by HDF.**

HDF cells were treated with DMEM containing antibiotics, with or without 150 μg/mL HSA-Glycol or HSA-Glycer and/or melatonin 100 μM and incubated at 37 °C and 5% CO_2 for 2 days. Same conditioned media were also incubated without cells to normalize the incubation effect. Then, the culture media was collected and centrifuged at 5,000 rpm for 2 min to remove any cells. An equal volume of supernatant media (200 μL) was used to measure fluorescence intensity at Ex 370, Em 440 nm. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. HDF, human dermal fibroblast; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; Glycol, glycolaldehyde; Glycer, glyceraldehyde; SEM, standard error mean.

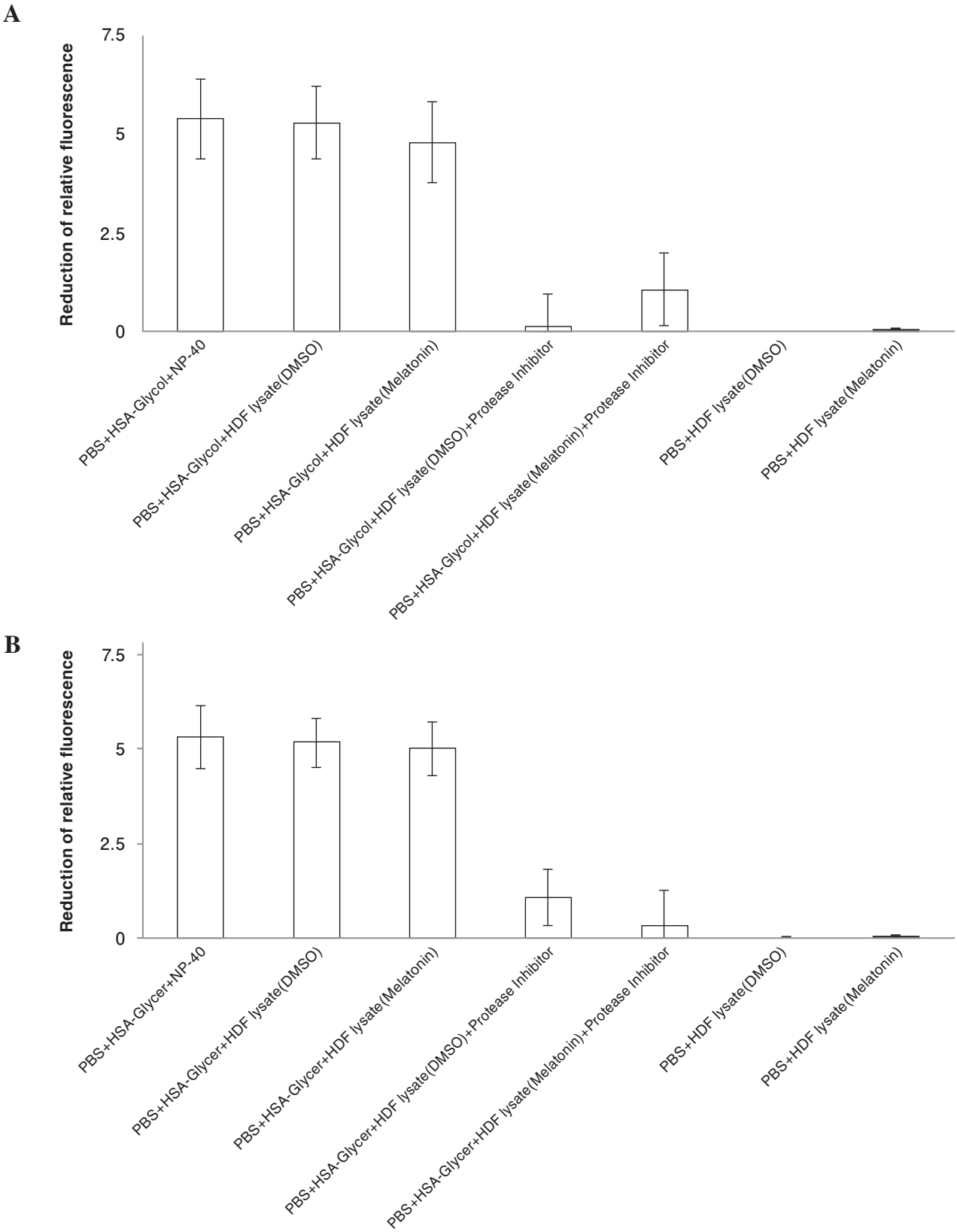

Fig. 4. **Effect of melatonin treated HDF lysate on HSA-Glycol and HSA-Glycer fluorescence reduction.**

HDF cells were treated with DMEM containing antibiotics, with DMSO or melatonin 100 μM and incubated at 37 °C and 5% CO_2 for 3 days. Then, treated cells were collected and cell lysate was prepared using NP-40. An equal amount of cell lysate (15 μg) was taken along with 150 μg/mL HSA-Glycol or HSA-Glycer, and fluorescence (F_1) were measured. After incubation at 37 °C for 20 hours, F_2 was measured. The F_2 value was subtracted from F_1 to measure the fluorescence reduction by cell lysate or NP-40. A) HSA-Glycol, B) HSA-Glycer. All data are shown as means ± SEM, n = 6. *$p < 0.05$, **$p < 0.01$, Tukey-Kramer test. HDF, human dermal fibroblast; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; Glycol, glycolaldehyde; Glycer, glyceraldehyde; SEM, standard error mean.

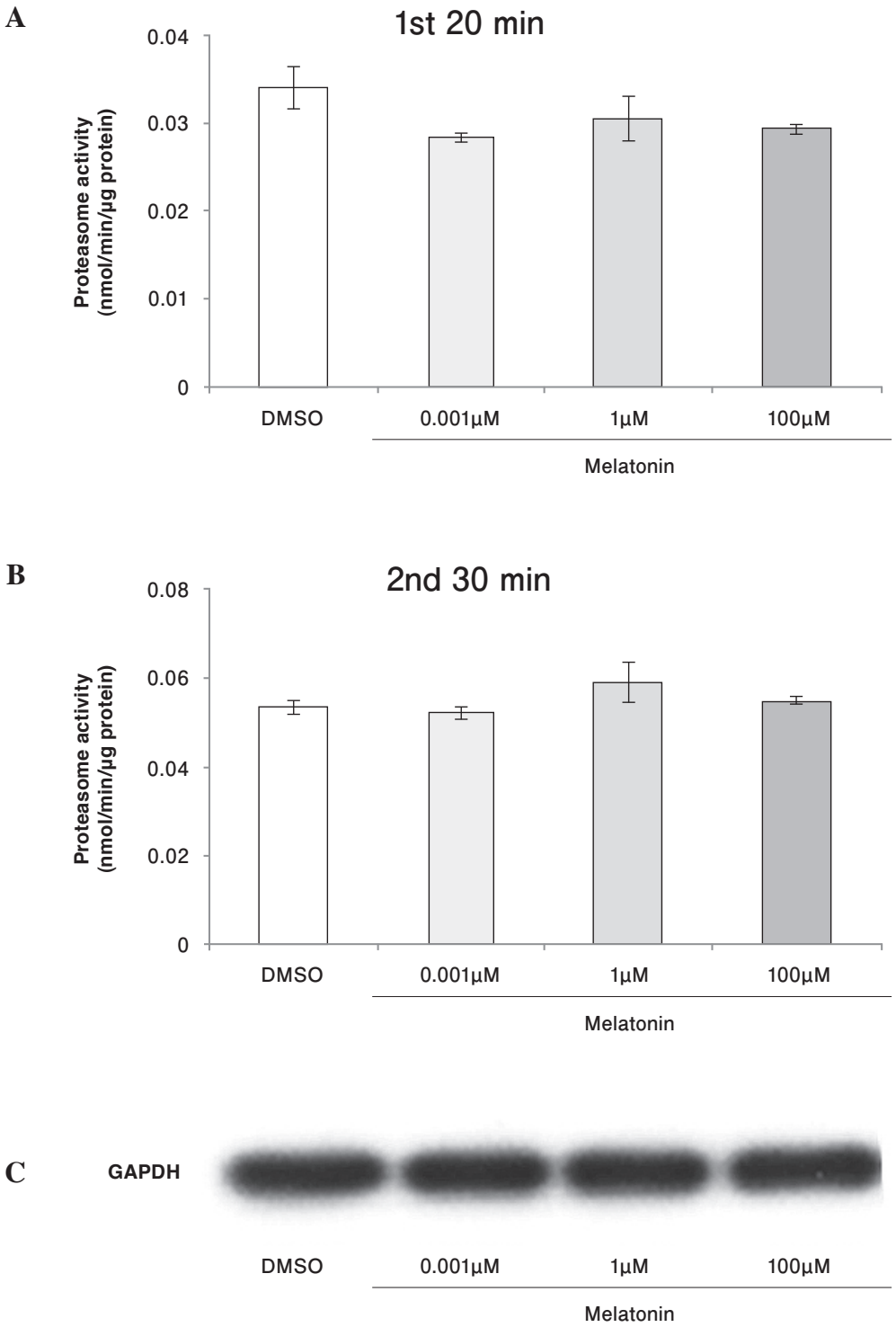

Fig. 5. **Effect of melatonin on proteasome activity.**

The HDF cells were treated and prepared as described in the materials and method section. A) First 20 min of incubation, B) Another 30 min of incubation, C) Western blot of GAPDH. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. HDF, human dermal fibroblast; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error mean.

Conclusions

In this study, melatonin showed almost no effect on proteasome function under this experimental condition. These results indicate the importance of the decomposition effect on AGEs and supporting effect on glucose regulation in the mechanism of melatonin, which reduces the glycative stress.

Acknowledgments

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Conflict of interest

The authors claim no conflict of interest in this study.

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