The effects of Tomatidine in stimulating mitophagy in human fibroblast

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Abstract: Mitochondria are the powerhouse of cells that provide energy in the form of adenosine triphosphate (ATP) via the electron transport chain. Mitochondrial membrane potential (MMP) is generated during the ATP synthesis. MMP is the indicator of cell healthy and mitochondrial function. Loss of MMP is associated with the PINK/Parkin accumulation on the mitochondrial outer membrane leading to the elimination of mitochondria by autophagy. Moreover, reactive oxygen species (ROS) which occur as by-products through ATP production, can cause the damage of mitochondria. Mitochondria have a process to maintain their functions and get rid of the damaged mitochondria called mitochondrial quality control. Mitophagy is a selective elimination of damaged mitochondria via lysosomes. Interestingly, tomatidine has been reported to enhance the lifespan and healthspan of *C. elegans* through mitophagy. However, the effect of tomatidine on mitophagy in human fibroblast cells remains unclear. This study aimed to investigate the effect of tomatidine on stimulating mitophagy and its mechanism in human fibroblasts using the fluorescent staining techniques under the Operetta-CLS high content imaging system. Our result showed that the 15 μM of tomatidine for 48 h significantly stimulated mitophagy compared to control. Moreover, the concentration of tomatidine higher than 10 μM for 24 h significantly decreased MMP levels in a dose-dependent manner.

Keywords: Tomatidine; Mitophagy; Mitochondrial Membrane potential

1. Introduction

Mitochondria are essential components of the cells that provide energy for living in the form of adenosine triphosphate (ATP). The general morphology of mitochondria is composed of two membranes which are inner and outer membranes. Mitochondria generate energy by pumping protons out from the matrix to intermembrane space. This process drives the synthesis of ATP via chemiosmosis oxidative phosphorylation inside the mitochondria. Kreb's cycle produces NADH and FADH2, which carry electrons to the mitochondrial respiratory chain, where the electrons transfer are coupled with the ADP phosphorylation. Molecular oxygen is required in the OXPHOS as a terminal electron acceptor in which reactive oxygen species (ROS) may occur as a by-product from the electron transport chain (ETC) and it is harmful to the mitochondria and cells.

Therefore, mitochondrial quality control is an essential mechanism to protect against mitochondrial damage using to destroy the dysfunctional mitochondria before developing the diseases and preventing cell death [1] . Mitochondrial quality control can be classified into 5 types which are mitophagy [2]. The function of mitophagy is to eliminate damaged or unwanted mitochondria for keeping the balance of mitochondrial homeostasis. The autophagosome is wrapping around damaged mitochondria and fusing with lysosomes for hydrolytic degradation. In addition, mitophagy has a role in developing

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erythrocytes and removing sperm-derived mitochondria [3]. The consequence in the impairment of mitophagy is associated with human diseases [4] such as neurodegenerative diseases: Alzheimer's disease, Parkinson's disease, and Huntington's disease, aging [5], cancer, and cardiovascular diseases [6]. Mitophagy occurs through two major pathways [7]; the first is damage-induced mitophagy (PINK and PARKIN), and the second is developmental-induced mitophagy (BNIP3 and NIX). The central mechanism of mitophagy is triggered by PINK (altered PTEN-induced putative protein kinase 1). In the healthy mitochondria, PINK1 is imported into the inner mitochondrial membrane (IMM) via translocase of the outer (TOM) membrane and inner (TIM) membrane.

Consequently, PINK1 is cleaved by the mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) in the matrix. However, PINK1 cannot enter into the matrix due to the dissipation of MMP when the occurrence of damaged or depolarized mitochondria. As a result, PINK1 is stabilized on the outer mitochondrial membrane and interacts with TOM, and that it is phosphorylated and becomes highly active. Actively, PINK1 further activates the phosphorylation of Parkin and ubiquitin. Later, phosphorylated Parkin further induces microtubule-associated protein 1A/1B-light chain 3 (LC3) following by the mitochondrial degradation within the autophagosome. The impairment of mitophagy is an underlying cause of many diseases, such as neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, cancer, and aging [5, 8, 9].

Curiously, tomatidine, an aglycone of glycoalkaloid tomatine founded in leaves, green or unripe of tomatoes [10], has been reported to enhance lifespan and healthspan of *C. elegans* through mitophagy induction via the SKN-1/Nrf2 pathway [11]. They found that the 25 μM of tomatidine enhanced muscle functions (swimming ability, pharyngeal pumping, and maximum movement velocity) and extended lifespan via mitophagy. In addition, tomatidine induced mitochondrial hormesis by mildly inducing ROS production. Then, it activated the SKN-1/Nrf2 pathway and possibly induced the cellular antioxidant response pathways, followed by increasing mitophagy. Besides, tomatidine has many desirable effects, such as antibacterial [10], anti-inflammatory [12], anticancer [13], and inhibition of skeletal muscle atrophy in cultured skeletal myotubes [14]. However, the effect of tomatidine on mitophagy in human fibroblast cells is still unclear. Therefore, this study aimed to validate the effect and mechanism of tomatidine on stimulating mitophagy in human fibroblast cells using a model to study the protection and modulation of mitochondria by tomatidine.

2. Materials and Methods

Samples.

Primary fibroblast cells were established from skin biopsies of healthy volunteers without a pathogenic mitochondrial DNA mutation in ND4. The cell culture passages used in the experiments were lower than or equal to 7 to prevent cell senescence. The Human Research Protection Unit approved this study with the certificate of approval No. Si 263/2018 (The Faculty of Medicine Siriraj Hospital, Mahidol University).

Cell culture and cell seeding.

Primary fibroblast cells were cultured in 5 mM of glucose-Dulbecco's Modified Eagle's (DMEM) medium supplemented with 10% of fetal bovine serum, 1% of antimicrobial (penicillin and streptomycin), and 0.1% of antifungal (amphotericin B). The cells were incubated at 37°C, 5% of CO_2, and 95% humidity. The cultured medium was changed every other day.

When reaching 80-95% of cell confluency, the old-cultured medium was removed and washed twice with PBS. The 1 mL of 0.25% trypsin-EDTA (for T25 flask) was added into the flask and incubated for 3 min. To stop the reaction, 3 mL of fresh media was added. The suspension was transferred into a 15 mL tube, then centrifuged at 3,000 rpm for 5 min. The supernatant was removed, and 3 mL of fresh media was added and transferred into a new flask for cell expansion.

Fibroblast cells were trypsinized, counted by using a hemocytometer. Approximately 4,000 – 5,000 cells were seeded in each well of 96-well plates (PerkinElmer 96-well CellCarrier™, 6005550). After resting the plate on the table for cells falling to the bottom for 10 min. The plate was placed into an incubator and incubated at least 24 h before experiments.

Preparation of tomatidine.

Tomatidine (Sigma, CAS Number 6192-62-7, Lot# WXBC5122V) was dissolved in absolute ethanol (MERCK) to prepare a stock at 25 mM and stored at -20°C with the protection from light. Stocks of tomatidine were freshly prepared every 3 months to protect their destabilization.

Cytotoxicity in tomatidine treatment.

Fibroblasts were cultured on the 96-well plate **(PerkinElmer 96-well CellCarrier™, 6005550)**. Subsequently, cells were exposed to tomatidine in various concentrations (5, 10, 15, 25, and 50 μM) for 24 h. After the treatment, the cytotoxicity of tomatidine was observed by a fluorescent co-staining technique using 1:250 dilution of propidium iodide (PI) (excitation 546 nm/emission 647 nm) (Enzo Life Sciences) to stain nuclei of dead cells and 1:1,000 dilution of Hoechst 33342 (excitation 350 nm/emission 461 nm) (Enzo Life Sciences) for nuclear staining. The Operetta-CLS high-content analysis system (PerkinElmer) was visualized and captured the cytotoxicity events under a 10X magnification air lens. The fluorescent images were analyzed with the Cell-Profiler program for calculating the number of cell death measuring by the overlapping region between Hoechst and propidium iodide (PI) fluorescent signals.

Mitophagy in tomatidine treatment.

Fibroblasts were cultured on the 96-well plate. Subsequently, cells were treated with 15, 25, and 50 μM of tomatidine for 24 and 48 h. Mitophagy activity was measured using a fluorescent co-staining of mitochondria by 1:10,000 dilution of Mito-ID® Red detection kit (excitation 558 nm/emission 690 nm, Enzo Life Sciences), lysosome by 1:625 dilution of Cytopainter Lysosomal Staining Kit (excitation 490 nm/emission 525 nm, Abcam), and 1:1000 dilution of Hoechst 33342 for nuclear staining (excitation 350 nm/emission 461 nm, Enzo Life Sciences). Mitophagy events were visualized and captured under a 40X magnification (water lens) of the Operetta-CLS high-content analysis system (PerkinElmer) after Cell-Profiler analyzed images. Finally, the mitophagy events per cell were calculated by the number of localized mitochondria to lysosome divided by the sum of localized mitochondria and non-localized mitochondria.

Polarization of mitochondrial membrane potential (MMP) in tomatidine treatment.

The fibroblast cells in 96 wells plate were exposed to tomatidine at 5, 10, 15, 25, and 50 μM for 24 h. MMP levels were measured using the MITO-ID Membrane Potential Detection Kit (JC-10) (Enzo Life Sciences). JC-10 is a cationic lipophilic fluorescent dye that developed from JC-1 to improve its water solubility. JC-10 have two form that are J-

aggregates (red fluorescent) (excitation 540 nm/emission 590 nm) and J-monomer (green fluorescent) (excitation 490 nm/emission 525 nm). In healthy mitochondria, JC-10 is presented in J-aggregated form higher than J-monomeric form. In depolarized mitochondrial membrane potential, JC-10 dye returns to J-monomeric form in the cytosol. Therefore, the ratios between red to green fluorescence can reflect MMP status.

The fluorescent co-staining technique between 1:100 dilution of MITO-ID Membrane Potential Detection Kit (JC-10) and 1:1000 dilution of Hoechst 33342 was used to observe the MMP status. After staining, the cells were captured under a 40x magnification water lens with the Operetta CLS high-content analysis system (PerkinElmer). The images were analyzed by using the Cell-Profiler program. The MMP levels were received from mean J-aggregates intensity per cell divided by mean J-monomer intensity per cell. The results were converted to represent relative value compared with vehicle control.

Fluorescent staining

After cell treatments in 96 well plate, the media were removed from the cell culture flask. First, the cells were washed twice with PBS and stained with the mixture of a fluorescent dye (the final volume per well is 50 μL). Next, the cell was stained for 30 min with the protection from light and subsequently washed once with PBS. Finally, the 100 μL of 2% FBS in DMEM without phenol red was added to each well before the fluorescent imaging.

Statistical testing

Group comparison was analyzed by analysis of variance (ANOVA) and post-hoc analysis (Bonferroni) by the Jamovi program (version 1.0.7.0) (https://www.jamovi.org/). The statistic comparison between the two groups was analyzed by using Student's t-test. The results were considered as a statical significant difference when the *P*-value is less than 0.05. The graph was created by GraphPad prism (version 8).

3. Results

Cytotoxicity tasting in tomatidine treatment.

Fibroblast cells were exposed to tomatidine at the concentrations of 5, 10, 15, 25, and 50 μM for 24 h compared with vehicle control. The fluorescent images by Hoechst 33342 and PI staining of tomatidine treatments were shown in Figure 1.

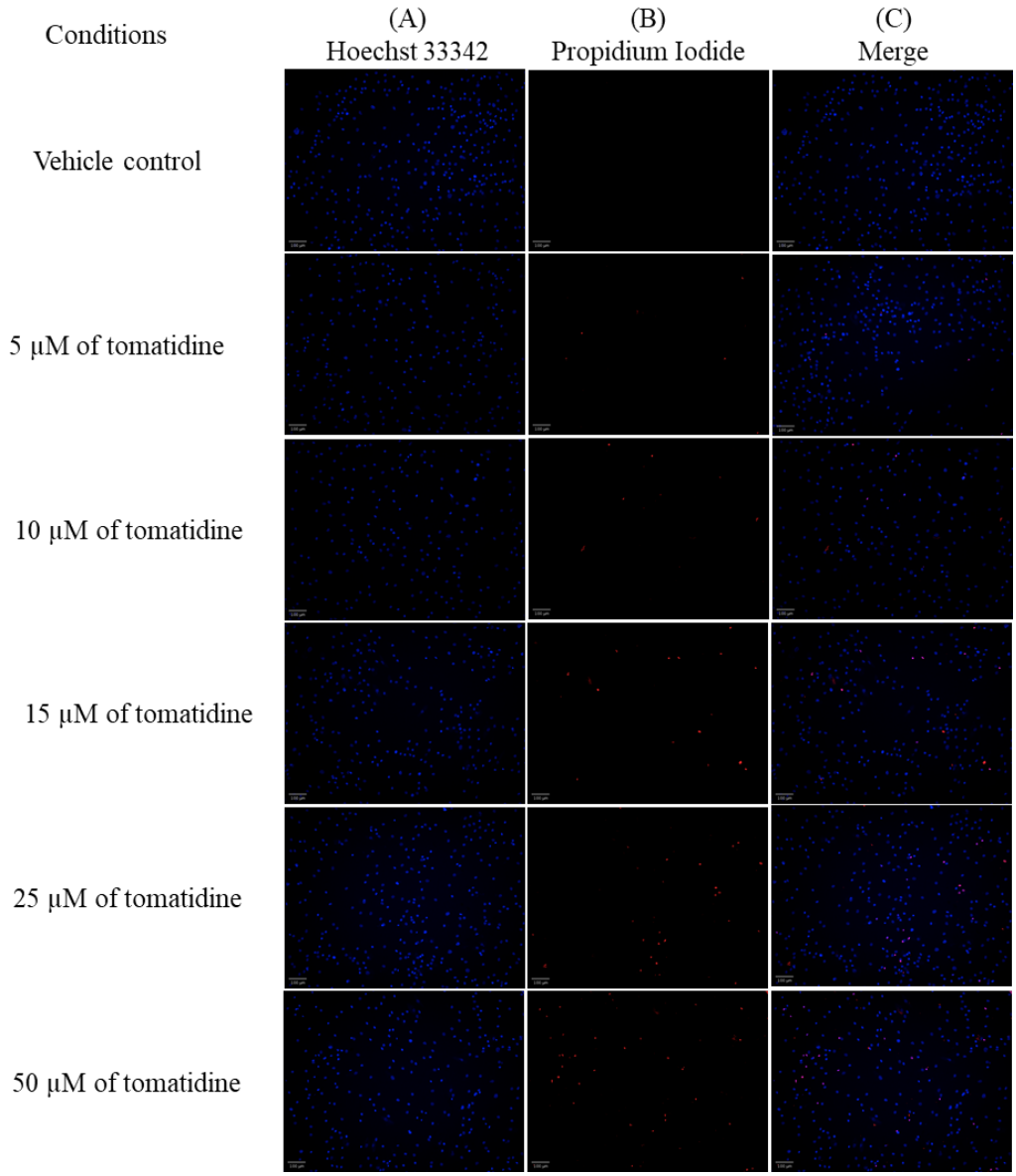

Figure 1. The fluorescent images of cytotoxicity tasting in tomatidine treatments.(A) Hoechst 33342, (B) Propidium Iodide, and (C) The merge channels of Hoechst 33342 and Propidium Iodide. The images were captured under a 10X magnification (air) lens of the Operetta-CLS high content image system. The image scale bar was 100 μm.

From the ANOVA and post-hoc analysis using Bonferroni's test, the cytotoxicity results showed that the percentages of cell survival at 5 μM, 10 μM, and 15 μM of tomatidine treatments were not significantly different at *P*-value 0.05 when compared with vehicle control. The mean ± S.E were 100.7 ± 2.77, 96.6 ± 2.77, 96.7 ± 2.77, and 100 ± 2.77, respectively. Whereas the percentages of cell survival at the 25 μM and 50 μM of tomatidine treatment were significantly decreased at *P*-value = 0.001 and < 0.001, respectively, compared with vehicle control. The mean ± S.E were 82.8 ± 2.77, 74.6 ± 2.77 vs. 100 ± 2.177. We also noticed that the concentrations of tomatidine higher than 25 μM exhibited toxicity to fibroblast cells. The bar graphs of cell survival were represented in Figure 2; the mean ± S.E and *P*-value were represented in table 1.

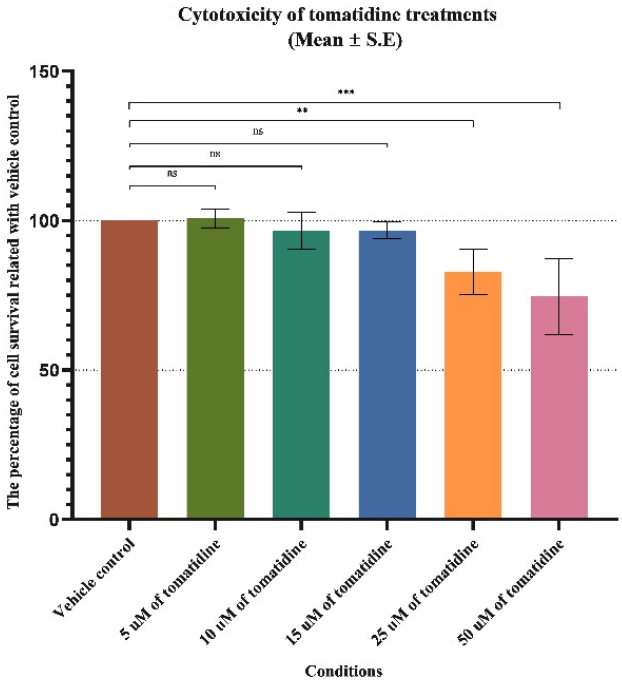

Figure 2. The cytotoxicity of tomatidine treatment in various concentrations for 24 h. The Y-axis represents the percentage of cell survival related to vehicle control, and the X-axis represents the conditions. Three independent experiments have been done. Data were shown as mean ± S.E. Group comparison were analyzed by ANOVA and Post-hoc analysis by Bonferroni, ns. = not significant at *P*-value **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Table 1. The statistical testing in cytotoxicity of tomatidine treatments compared with vehicle control at 24 h represented in mean ± S.E and *P*-value.

Conditions	Mean ± S.E.	*P*-value
Vehicle control	100 ± 2.77	-
5 μM of tomatidine	100.7 ± 2.77	1
10 μM of tomatidine	96.6 ± 2.77	1
15 μM of tomatidine	96.7 ± 2.77	1
25 μM of tomatidine	82.8 ± 2.77	0.001
50 μM of tomatidine	74.6 ± 2.77	< 0.001

The data from cell survival were used to calculate the median lethal concentration (LC_{50}), which the graph represents in Figure 3. The graph was plotted by Microsoft Excel and calculated by using an equation of linear regression (Y = mx + C). Therefore, the LC_{50} from the calculation is 91.380 μM of tomatidine. However, due to the limited solubility of tomatidine, the number of data points was too small for reliability to calculate LC_{50} with confidence.

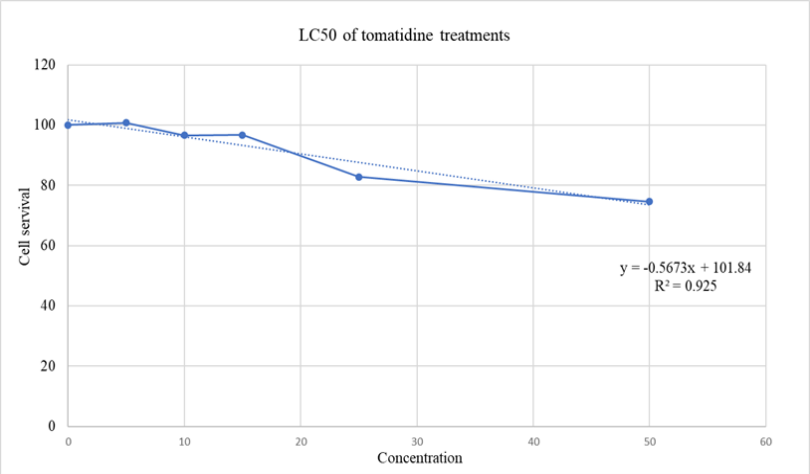

Figure 3. The LC_{50} of tomatidine treatments for 24 h.

In conclusion, tomatidine at 5 μM to 15 μM were not related to the cytotoxic effect to human fibroblast cells, observing the none of statistically significant difference between the number of cell survival of tomatidine treatment compared with the vehicle control (*P*-value = 1.000). However, the results from LC_{50} were 91.38 μM of tomatidine. Therefore, the concentrations of tomatidine for the subsequent experiments should not be over 91.38 μM. However, the 25 μM and 50 μM of tomatidine should be cautious for reducing cell survival.

Mitophagy measurement of tomatidine treatment.

The cell images of the colocalization of mitochondria with lysosomes (indicating mitophagy) by fluorescent co-staining techniques are shown in Figure 4. Fibroblast cell images of fluorescent staining by tomatidine treatment for 24 h and 48 h in various concentrations are shown in Figure 5.

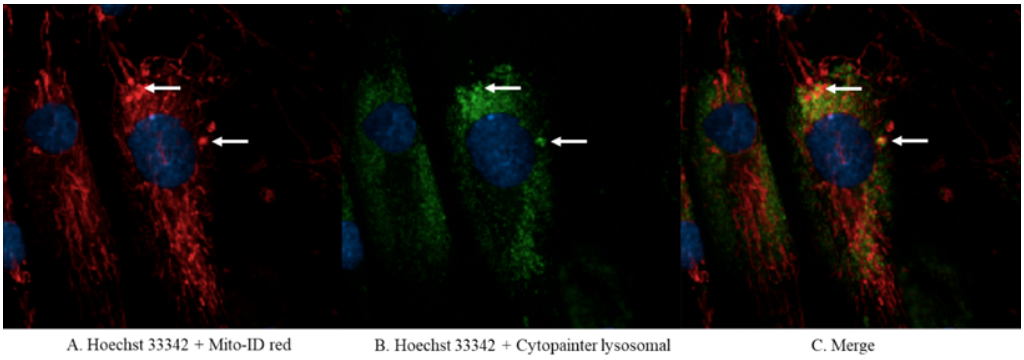

Figure 4. The fluorescent staining of mitophagy event. (A) Hoechst 33342 (nuclear staining) with Mito-ID Red (mitochondrial staining). (B) Hoechst 33342 (nucleus staining) with Cytopainter lysosome (lysosomal staining). (C) The merged channels of Hoechst 33342, Mito-ID Red, and Cytopainter lysosome. Mitophagy was identified as mitochondrial-related to the lysosome.

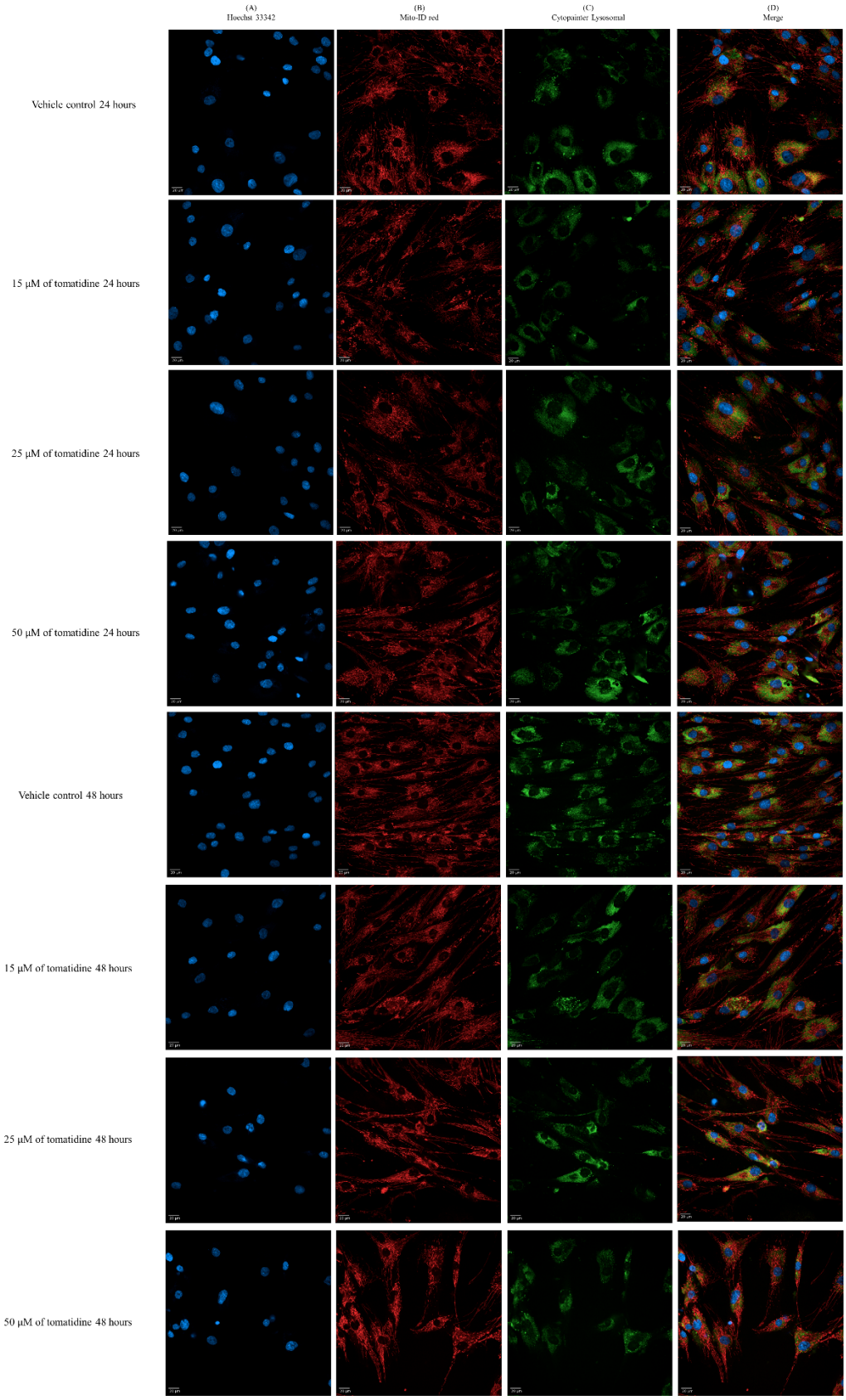

Figure 5. The fibroblast cells with fluorescent staining of mitophagy events. Cells were treated with tomatidine at 15, 25, and 50 µM and vehicle control for 24 h and 48 h. Mitophagy events were visualized under a 40X magnification (water lens) of the Operetta-CLS high content imaging system. The image scale was 20 µm.

Our results showed that tomatidine treatments at the concentrations of 15 μM and 25 μM for 24 h the levels of mitophagy event were even lower than vehicle control and did not show an effect on stimulating mitophagy when compared with the vehicle control. Whereas the 50 μM of tomatidine treatment for 24 h significantly stimulated mitophagy compared with the vehicle control. Furthermore, tomatidine treatments at 15, 25, and 50 μM for 48 h significantly stimulated the mitophagy events compared with vehicle control (Figure 6). The mean ± S.E and *P*-value of the levels of mitophagy events of tomatidine treatments compared with vehicle control for 24 h and 48 h were represented in Table 2.

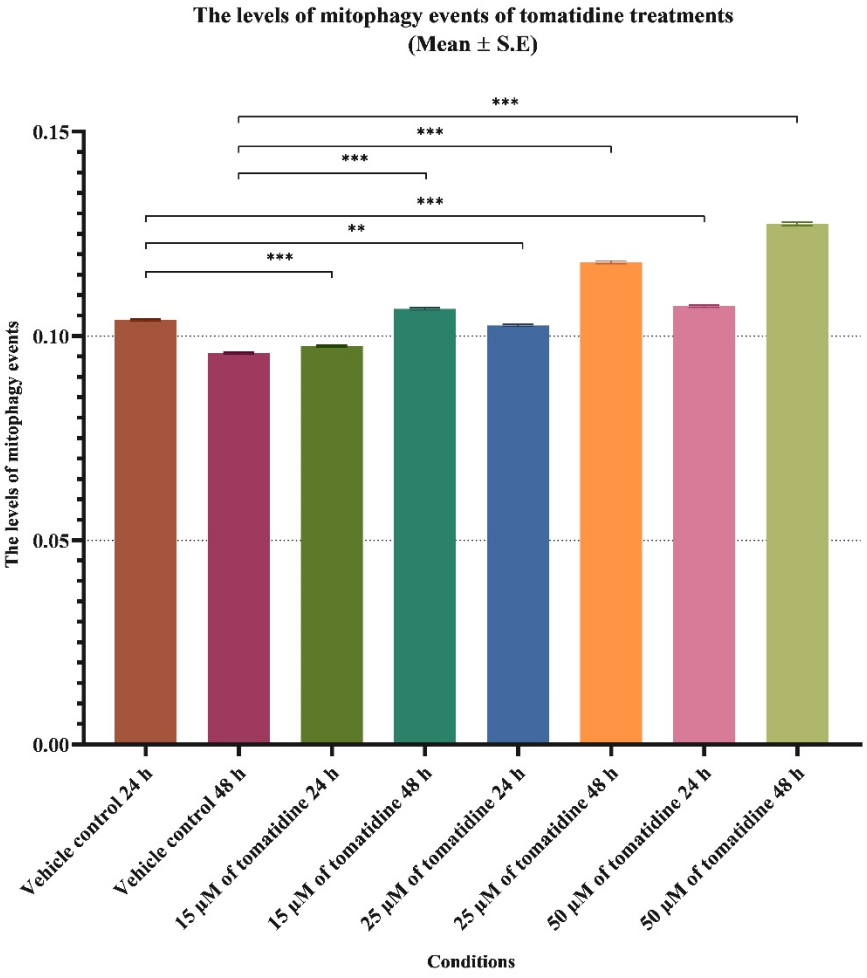

Figure 6. The effect of tomatidine treatment on mitophagy in fibroblast cells. The Y-axis represents mitophagy events calculated by the number of localized mitochondria to lysosome divided by the sum of localized mitochondria and non-localized mitochondria. The X-axis represents the tomatidine treatments for 24 h and 48 h in various conditions. The graph represents in mean per cell, and the experiments were performed in quintuplicate individuals. Group comparisons were analyzed by ANOVA and Post-hoc analysis by Bonferroni, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale error bars represent S.E.

Table 2. Mean, standard error of the mean (SE), and *P*-value of the levels of mitophagy events by tomatidine treatment in fibroblast cells compared with the vehicle control.

Conditions	Mean ± S.E.	*P*-value
Vehicle control 24 h	$0.1039 \pm 2.74 \times 10^{-4}$	-
Vehicle control 48 h	$0.0957 \pm 2.40 \times 10^{-4}$	-
15 μM of tomatidine 24 h	$0.0975 \pm 2.47 \times 10^{-4}$	< .001
15 μM of tomatidine 48 h	$0.1066 \pm 2.55 \times 10^{-4}$	0.016
25 μM of tomatidine 24 h	$0.1025 \pm 2.65 \times 10^{-4}$	< .001
25 μM of tomatidine 48 h	$0.1180 \pm 2.82 \times 10^{-4}$	< .001
50 μM of tomatidine 24 h	$0.1072 \pm 2.68 \times 10^{-4}$	< .001
50 μM of tomatidine 48 h	$0.1274 \pm 3.20 \times 10^{-4}$	< .001

Tomatidine treatments in the same concentrations with different time durations (24 and 48 h) were compared (Figure 7). The bar graphs of tomatidine treatment at 15 μM, 25 μM, and 50 μM showed a similar pattern. Tomatidine treatment for 48 h showed a statistically significant difference at *P*-value < 0.001 of the levels of mitophagy events higher than 24 h.

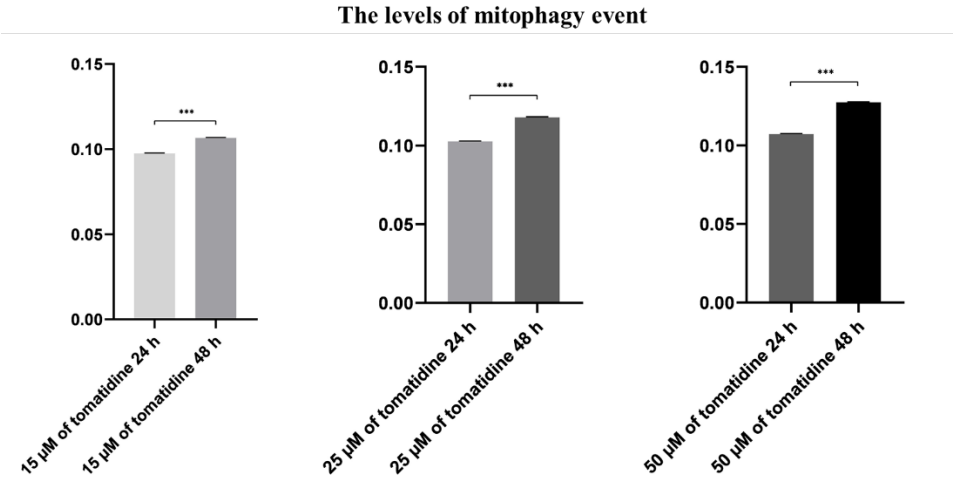

Figure 7. The effect of tomatidine treatment in the same concentrations on mitophagy levels in fibroblast cells compared between 24 and 48 h. The graph was presented as mean values with the standard error of the mean (S.E.). Statistical significances were considered when ***$P < 0.001$ (Student's t-test analysis).

The effect of tomatidine treatments on mitophagy was compared at a simultaneous time in various concentrations. The bar graph showed the same pattern of tomatidine treatment for 24 h and 48 h. The increase of mitophagy levels was associated with the dose-dependent manner of tomatidine.

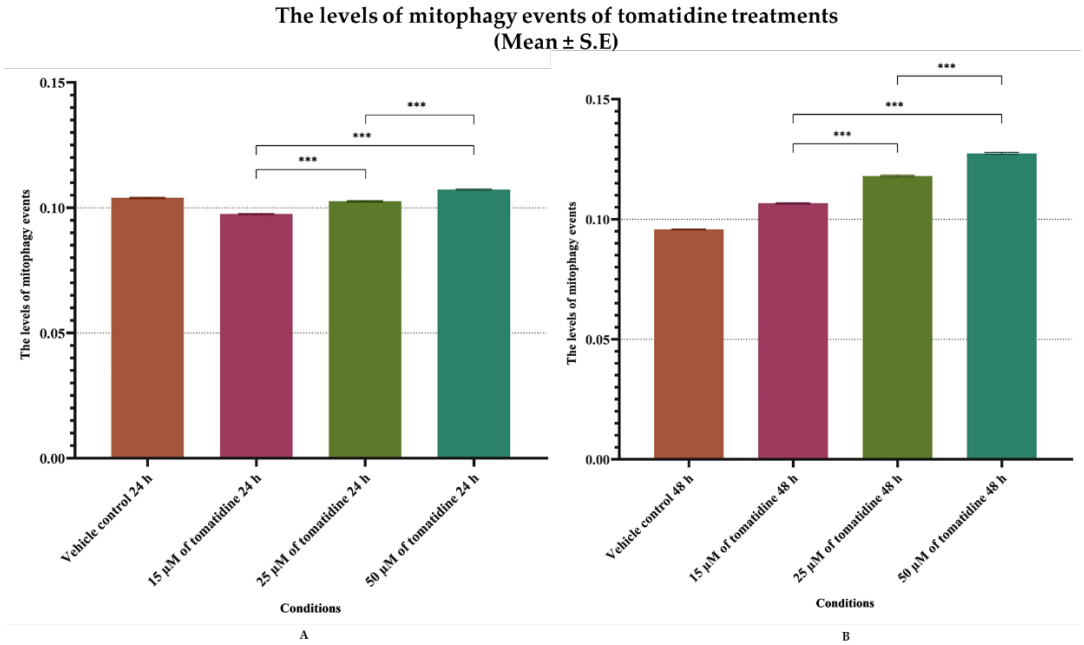

Figure 8. The bar graph represents a comparison of tomatidine treatments in various concentrations of 24 h (A) and 48 h (B) on mitophagy events. Data showed mean ANOVA and Post-hoc analysis by Bonferroni analyzed ± S.E. Group comparison within tomatidine treatments± S.E. Group comparison within tomatidine treatments, *** *P*-value = < 0.001.

In conclusion, tomatidine treatment stimulated mitophagy events in human fibroblasts and the levels of mitophagy events associated with dose-dependent in tomatidine treated for 48 h.and time-dependent. Thus, the suitable and safe concentration for human fibroblast cells that could stimulate mitophagy events was 15 µM of tomatidine for 48 h.

Mitochondrial membrane potential (MMP) testing.

We examined how mitophagy events occurred by measuring the mitochondrial membrane potential, which was a trigger that leads to mitophagy [2, 3]. The mitochondrial membrane potential was detected using MITO-ID Membrane Potential Detection Kit and observing under 20X magnification of Operetta-CLS high content image system. Tomatidine in various concentrations (5 µM, 10 µM, 15 µM, 25 µM, and 50 µM) were treated for 24 h. The fluorescent images were shown in Figure 9.

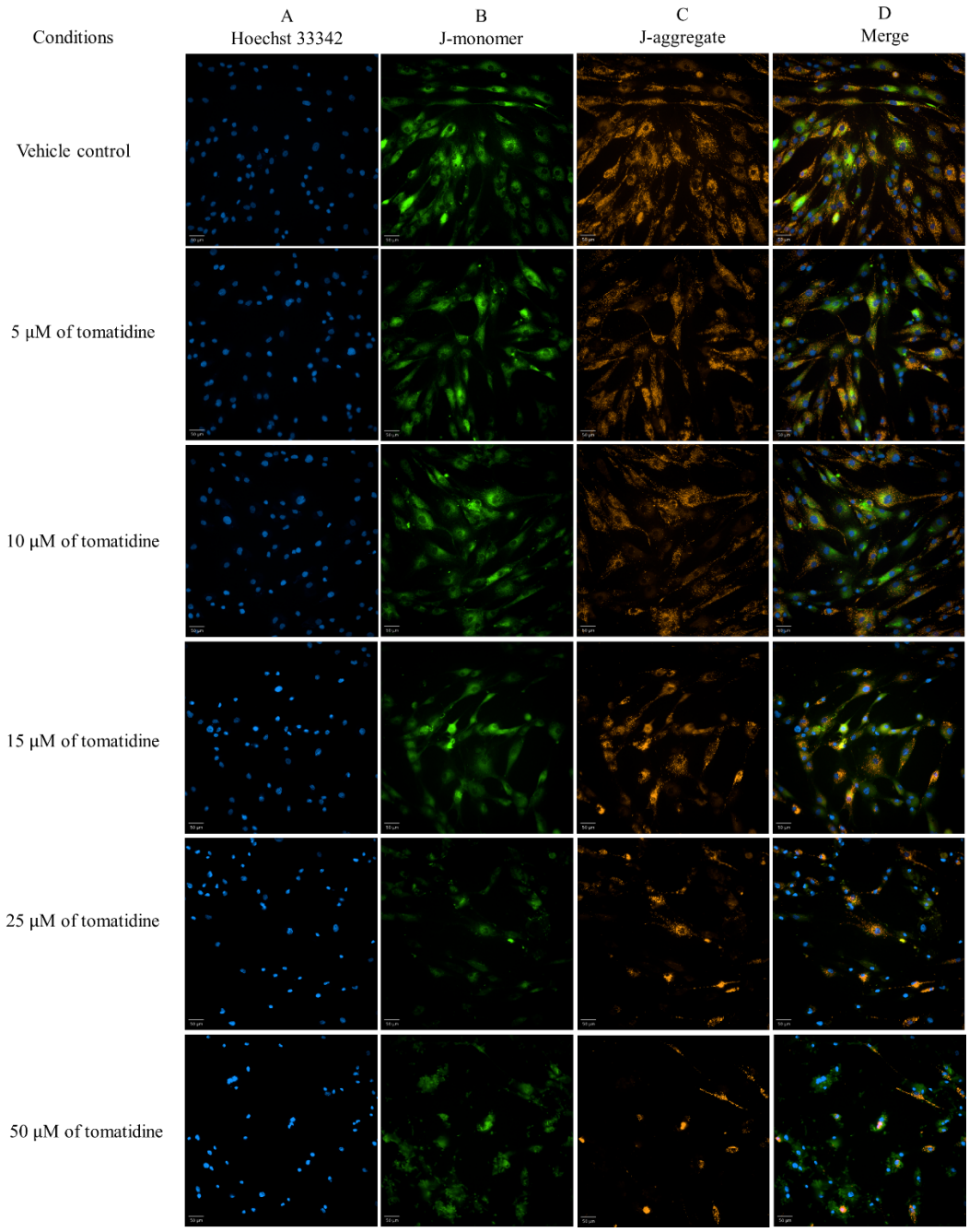

Figure 9. The fluorescent staining of MMP in various conditions. (A) Hoechst 33342, (B) J-monomer form of JC-10, (C) J-aggregates form of JC-10, and (D) The merge channels of Hoechst 33342, J-monomer, and J-aggregates. MMP testing were visualized under a 20X magnification of the Operetta-CLS high content imaging system. The image scale was 50 µm.

The bar graph represented mean ± S.E relative to vehicle control in the levels of MMP after treatment with various concentrations of tomatidine (Figure 10). At 5 μM of tomatidine treatment, the levels of MMP decreased, but this was not significantly different from that of vehicle control at *P*-value = 1.00. The concentrations higher than 5 μM of tomatidine treatment significantly decreased MMP compared to vehicle control at a *P*-value less than 0.001. In the group of tomatidine treatment, the trend of MMP levels decreased in a dose-dependent manner. The statistical testing and mean ± S.E of tomatidine treatments were presented in Table 3.

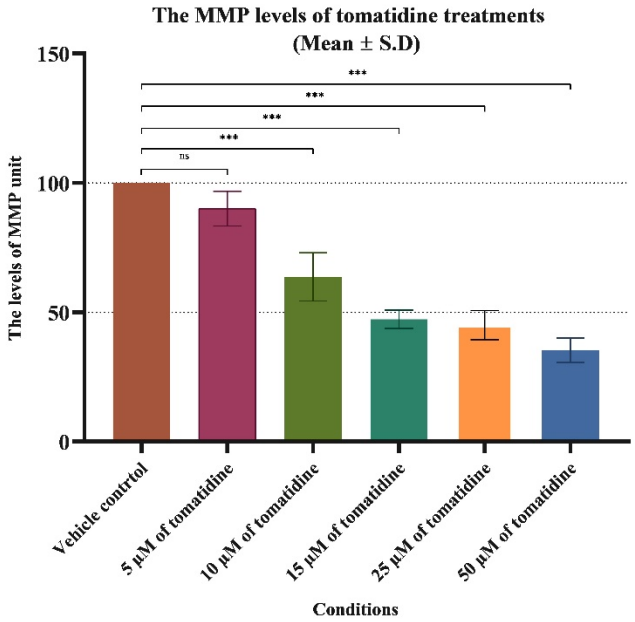

Figure 10. The levels of mitochondrial membrane potential according to tomatidine treatment in various concentrations. The experiments were performed in triplicate. Group comparisons were analyzed by ANOVA and post-hoc analysis by Bonferroni's test, ns. = not significant at *P*-value 0.05 and *** *P*-value = < 0.001.

Table 3. The mean ± S.E and statistical testing of tomatidine treatments.

Conditions	Mean ± S.E	*P*-value
Vehicle control	100 ± 5.48	-
5 μM of tomatidine	90 ± 5.48	1
10 μM of tomatidine	63.7 ± 5.48	< 0.001
15 μM of tomatidine	47.3 ± 5.48	< 0.001
25 μM of tomatidine	45.0 ± 5.48	< 0.001
50 μM of tomatidine	35.5 ± 5.48	< 0.001

In conclusion, tomatidine treatment at all concentrations could decrease the levels of MMP. The decrement of MMP manifested in a dose-dependent manner.

4. Discussion

This study used a human fibroblast cell because it contains the same amount of mitochondria as an axonal neuron. Thus, the study in human fibroblasts is a pre-clinical investigation applicable to developing that can be applied to develop new drug treatments for neurodegenerative diseases.

Cytotoxicity was detected by fluorescent technique instead of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [15] due to MTT assay base on the enzyme activity, which is succinate tetrazolium dehydrogenase in mitochondrial to convert the tetrazolium compound (yellow color) to water-insoluble formazan (purple color), for the reason that we do not know the tomatidine were interferences with the enzyme activity. MTT assay had to incubate for 4 h for enzyme activity. Nevertheless, the fluorescent technique had to incubate only 30 min., which is more comfortable and practical. The concentration of tomatidine higher than 15 μM was affected cell survival, decreasing compared with vehicle control in human fibroblast cells. In a previous study, the cytotoxicity of tomatidine in primary chondrocytes at 20 μM has had a difference in cell viability at P-value < 0.05 compared with a control group [16]. The cytotoxicity of tomatidine in different cell studies gave similar results. The limitation for calculated LC50 by linear regression was the solubility of tomatidine. Therefore, we cannot use the concentration of tomatidine higher than 50 μM.

Mitophagy events were detected by using high-throughput fluorescent microscopy (Operetta-CLS) rather than transmission electron microscopy (TEM, one of the best advice to provide direct evidence for mitophagy because the TEM had the limitation in quantitative studies, for example, the limited of cells number, conditions of tomatidine treatments and biased in selection for scoping during capture. Another disadvantage of fluorescent electron microscopy is the technique measurement of the mitochondrial related to lysosomes. Fluorescent electron microscopy cannot measure the lysosome engulf the mitochondria. The effect of tomatidine treatment on mitophagy in our study in human fibroblast and previous study in *C. elegans* is concordant in which tomatidine could stimulate mitophagy. However, the differences are the concentrations and time durations used in the experiments.

MMP results were different; in the *C. elegans* study, the results showed that tomatidine treatment in primary rat cortical neurons at 4 μM of tomatidine for 24 h significantly increased in MMP at P-value < 0.001 when compared with control. On the other hand, in this study, the results showed that tomatidine decreased the level of MMP at 10 μM of tomatidine for 24 h and significantly decreased compared to vehicle control at a P-value less than 0.001. MMP measurement in *C. elegans* was assessed by the TMRM dye at 40 nM for 15 min and did not inform the machine and calculation method used to observe. This discrepancy could be more sensitive to changes in MMP of the dye that we used together with a higher concentration of tomatidine in our experiment.

In the future study, we will investigate the molecular mechanism to explain how tomatidine stimulates mitophagy; for example, tomatidine stimulates mitophagy by inducing mitochondrial fragmentation, increasing ROS, etc. To verify the effect of tomatidine in stimulating mitophagy, we will use the transmission electron microscopy (TEM), mitochondrial mass, and Western blotting analysis for detection of mitochondrial proteins and study the incubation time that can maintain a balance between mitophagy (elimination of mitochondria) and mitochondrial biogenesis (formation of mitochondria).

5. Conclusions

The suitable concentrations of tomatidine for human fibroblasts are 5-15 μM. The lowest concentration of tomatidine that can stimulate mitophagy is 15 μM treating for 48 h. Tomatidine treatment for 48 h. at all concentrations had shown the levels of mitophagy as dose-dependent. With an increase in the time for tomatidine treatment, the level of mitophagy had risen. Thus, tomatidine can decrease the levels of MMP in a dose-dependent manner in human fibroblast cells.

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