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Cytotoxic and cytoprotective effects of tryptamine-4,5-dione on neuronal cells: a double-edged sword

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Cytotoxic and cytoprotective effects of tryptamine-4,5-dione on neuronal cells: a double-edged sword

Serotonin (5-hydroxytryptamine) is a putative substrate for myeloperoxidase, which may convert it into the reactive quinone tryptamine-4,5-dione (TD). In this study, we found that the viability of human SH-SY5Y neuroblastoma cells treated with 25 μM TD was increased to approximately 117%. On the other hand, the cell viability was significantly decreased by exposure to TD (150–200 μM), with an increase in intracellular reactive oxygen species (ROS). Interestingly, pretreatment of SH-SY5Y cells with 100 μM TD prevented cell death and suppressed intracellular ROS generation evoked by the addition of hydrogen peroxide (H_2O_2). Expression of the phase-II antioxidant enzyme NAD(P)H: quinone oxidoreductase 1 and heme oxygenase 1 were upregulated by TD at a concentration of 50–100 μM. Nuclear factor erythroid 2-related factor 2 (Nrf2), the regulator of these enzyme, was translocated from the cytosol to the nucleus by 100 μM TD. In summary, moderate concentrations of TD may increase the self-defense capacity of neuronal cells against oxidative stress.

Keywords: word; quinone; cytotoxicity; neuronal cells; NAD(P)H: quinone oxidoreductase 1 (NQO1); heme oxygenase 1 (HO-1)

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Introduction

The monoamine neurotransmitter serotonin [5-hydroxytryptamine (5HT)] is released from neurons in the midline of the brainstem and then distributed throughout the brain [1]. 5HT is thought to be involved in the regulation of various biological processes, such as mood, affection, cognition, aggression, satiety, sleep, and other autonomic functions [1, 2]. 5HT is easily oxidized by superoxide anion radical [3] and peroxidases [4, 5], a reaction that also produces the reactive quinone tryptamine-4,5-dione (TD), which is a possible neurotoxin [3, 5–8]. The generation of TD was confirmed in the brains of zebrafish treated with methyl mercury [9]. Some quinone compounds, such as tetrachlorobenzoquinone and dopamine quinone, have a variety of toxic effects because of their potential to alkylate proteins and/or DNA and form

reactive oxygen species (ROS) [10, 11]. However, some quinone compounds confer protective effects against oxidative stress at relatively low concentrations [12].

Myeloperoxidase (MPO) is a heme-containing, inflammatory enzyme present in myeloid cells such as neutrophils, microglia, and macrophages [13]. It is secreted during inflammation by activated and pro-inflammatory subsets of these immune cells [14]. Inflammatory cells in the brain produce MPO at high levels [15, 16], and MPO expression is increased in brain tissue showing Alzheimer's neuropathology [17]. 5HT may be a preferred substrate of MPO [4, 5]. Therefore, the oxidization of 5HT by MPO may contribute to certain neurodegenerative diseases.

Dryhurst et al. performed pioneering studies on characteristics of TD such as its stability and reactivity [18–20]. Recently, we reported the formation of TD by MPO [21] and the site-specific adduction of TD on to a model thiol protein [22]. We also showed that some cytoskeletal proteins, such as α- and β-tubulins, vimentin, and neurofilament-L, are preferentially modified by TD in SH-SY5Y neuroblastoma cells [23]. Among them, tubulins form microtubules that provide structural support to a cell and have important functions in processes such as mitosis, cell motility, and intracellular transport [24]. When we exposed purified tubulins to synthetic TD, their self-polymerization was initially enhanced, but the progression of this polymerization was subsequently suppressed [23]. These findings suggest that the modification of cellular proteins by TD modulates their functions. However, the effect of TD on cell viability and gene expression in neuronal cells still remains unclear.

In this study, we investigated the cytotoxicity of TD in SH-SY5Y cells and the effect of TD on the expression of NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase 1(HO-1) in the cells. We found that moderate concentrations of TD

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enhanced the adaptive response of SH-SY5Y cells to hydrogen peroxide (H_2O_2)-induced oxidative stress.

Materials and Methods

Materials

All polymerase chain reaction (PCR) primers used and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 nutrient mixture, Cell Count Reagent SF, Chemi-Lumi One L, and a protein bicinchoninate (BCA) assay kit were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Mammalian protein extraction reagent (M-PER), HALT protease inhibitor cocktail and ProLong® Gold were purchased from Thermo Fisher Scientific (MA, USA). Block Ace was obtained from Sumitomo Dainippon Pharma. (Osaka, Japan). Antibodies were obtained from the following sources: rabbit anti-NQO1 antibody was purchased from Abcam (ab34173; Cambridge, UK); rabbit anti-HO-1 antibody was obtained from Enzo Life Sciences, (BML-HC3001; New York, USA); rabbit anti-β-actin antibody was procured from Medical and Biological Laboratories. (PM053; Nagoya, Japan); and goat anti-rabbit antibody was obtained from DAKO Japan (P0448; Tokyo, Japan); rabbit Nrf2 antibody was procured from Santa Cruz Biotechnology (sc-722; CA, USA); goat anti-FITC-labeled anti-rabbit immunoglobulin was obtained from Sigma-Aldrich Japan K.K. (F-9887; Tokyo, Japan). Propidium iodide (PI) solution was obtained from Dojindo (Kumamoto, Japan). *Tert*-butylhydroquinone (*t*BHQ) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Potassium nitrosodisulfonate, 0.4% (*w*/*v*) trypan blue solution, serotonin, H_2O_2, and ISOGEN I were purchased from Wako Pure Chemical Industries (Osaka, Japan).

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Cell culture

The SH-SY5Y neuroblastoma cell line was obtained from European Collection of Authenticated Cell Cultures (Salisbury, UK). The cells were maintained in DMEM/Ham's F-12 nutrient mixture medium containing 10% fetal bovine serum (FBS).

Cell viability

SH-SY5Y cells (1.0×10^4 cells) were pre-incubated in a 96-well plate for 24 h, and then treated with various concentrations of TD or 5HT in FBS-free DMEM/Ham's F12 nutrient mixture medium for 30 min. The FBS-free medium was then replaced with flesh medium containing 10% FBS, and the cells were incubated for an additional 24 h. After treatment, cell viability was assessed by trypan blue exclusion assay. To investigate the protective effect of TD against H_2O_2-induced cytotoxicity, after a 24 h incubation, the medium was exchanged for FBS-free medium containing 100 μM H_2O_2, and the cells were incubated for 12 h prior to assess the cell viability by Cell Count Reagent SF. The method was according to the manufacturer's instruction.

Synthesis of quinones

TD was freshly synthesized from 5HT using a method previously described with minor modifications [18, 20]. Briefly, 1 mg of 5HT was dissolved in 500 μl of water and then added to a tube containing 6.3 mg of potassium nitrosodisulfonate, known as Frémy's salt. After 5 min, the mixture was applied to a C18 solid-phase extraction (SPE) column (Discovery® DSC-18, 500 mg; Sigma-Aldrich Japan K.K.). The SPE column was washed with 3 ml of water and eluted with 1% formic acid in water/acetonitrile (25/75). Eluate containing a purple substrate (TD) was collected in a tube. TD concentration was measured by absorbance at 350 nm with the molar

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extinction coefficient [25]. Five hundred microliters of water without 5HT were processed as described above, and used as a negative control sample (vehicle).

RNA extraction

Cells (2.5×10^5/well) were seeded into six-well culture plates, and treated with samples or vehicle alone for 30 min in FBS-free medium. The medium was replaced with flesh medium containing 10% FBS and then incubated for a range of times. Total RNA was extracted using ISOGEN I according to the manufacturer's instruction. Reverse transcribed cDNA was prepared using a PrimeScript™ II 1st-Strand cDNA Synthesis Kit (Takara Bio Inc., 6210A; Shiga, Japan), and this cDNA was subjected to quantitative reverse-transcription (qRT)-PCR amplification.

Quantitative real-time reverse-transcription polymerase chain reaction

qRT-PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: 5′-TTCCATCACCACTGGTGGCAG-3′ (forward) and 5′-TCCTGCCTGGAAGTTTAGGTC-3′ (reverse) for NQO1; 5′-AGCTTGGTCTAGAGTGAAAA-3′ (forward) and 5′-GAGGCAGAATCATGAGATAT-3′ (reverse) for HO-1; and 5′-CTGCACCACCAACTGCTTAGC-3′ (forward) and 5′-CTTCTGGGTGGCAGTGATGGC-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR products were amplified using an Applied Biosystems 7500 Real-Time PCR System. The temperatures and times of the cycling program were as follows: 10 min initial preheating at 95°C, followed by 40 cycles including 15 s at 95°C (denaturing), then 60°C (annealing) for 1 min. Relative levels of gene expression in each sample were calculated using the comparative ΔΔCt method.

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Detection of intracellular ROS

SH-SY5Y cells (2.7×10^4 cells) were plated on to a chamber slide. and left to attach overnight. After treatment with CM-H_2DCF-DA (5 μM) in FBS-free medium for 30 min, the cells was washed then incubated with samples or vehicle for 30 min in FBS-free medium. Fluorescence originating from intracellular ROS was observed using a fluorescence microscope. To evaluate the effect of TD or 5HT pretreatment, samples or vehicle alone were added to the FBS-free medium for 30 min, the medium was then replaced with flesh medium containing 10% FBS. After incubation for 24 h, the cells were exposed to 200 μM H_2O_2 in FBS-free medium and cultured for 6 h. After a 30 min incubation with CM-H_2DCF-DA (5 μM), ROS generation in the cells was measured using a fluorescence microscope. Quantitative data analysis was performed using ImageJ software (ver. 1.41 o). To quantify the ROS level, 50 cells from each treatment were chosen, and the fluorescence intensities were measured and then compared with those of untreated control cells. Each data point represents the mean ± standard deviation of 50 cells and data are representatives of experiments performed in triplicate.

Western blotting analysis

Cells (2.5×10^5/well) were seeded into six-well culture plates, and treated with samples or vehicle alone for 30 min in FBS-free medium, and then medium was replaced with flesh medium containing 10% FBS. After incubation for a range of times, the cells were washed with phosphate buffered saline and lysed using M-PER with HALT protease inhibitor cocktail, and then centrifuged. Protein concentrations in the lysates were determined using a BCA protein assay kit. The protein samples were mixed with loading buffer containing 2-mercaptoethanol, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel, and electrotransferred on to an Immobilon-P Transfer Membrane (EMD Millipore

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Corp., Billerica, MA, USA). The membrane was blocked with 4% Block Ace in aqueous solution for 1 h at room temperature. After washing the membrane three times for 10 min each with Tris-buffered saline containing 0.05% Tween-20 (TTBS), the membrane was incubated with a rabbit polyclonal antibody against NQO1 (1: 2,000 dilution), HO-1 (1: 1,000 dilution), and ß-actin (1: 2,000 dilution) overnight at 4ºC. After washing, the membrane was incubated with anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (1: 10,000 dilution) in TTBS for 1 h at room temperature. After washing, antigens on the membrane were visualized using Chemi-Lumi One L. A gel image was captured using LAS-1000 Plus Lumino Image Analyzer, and were analyzed using Image Gauge software (ver. 3.4) (Fujifilm, Tokyo, Japan).

Immunocytochemistry

SH-SY5Y cells (2.5×10^4 cells) were seeded into a chamber slide, and exposed to 100 µM samples, 50 µM *t*BHQ (positive control), or vehicle alone for 30 min in FBS-free medium; the medium was then replaced with flesh medium containing 10% FBS. After incubation for 3 h, the wells were washed with PBS three times and fixed in methanol at −20°C for 10 min. Fixed cells were permeabilized by incubation in 0.5% Triton X-100 before blocking with 3% BSA in PBS. The slide was washed with PBS three times again and treated with anti-Nrf2 antibody (1:50 dilution) in PBS containing 0.5% BSA for 2 h at room temperature. After washing three times with PBS, anti-FITC-labeled anti-rabbit immunoglobulin (1:100 dilution) and 60 ng/ml PI in PBS were added to cells, which were incubated at room temperature for 1 h in the dark. After washing three times again with PBS, the cells were mounted with an antifade reagent (ProLong Gold) and subjected to analysis by confocal microscopy.

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Statistical analysis

Each data was statistically analyzed using Tukey HSD or Games-Howel post hoc test after ANOVA with SPSS (ver. 22.0) software. A P-value less than 0.05 was considered significant.

Results and Discussion

Effect of TD on cell viability and ROS generation

TD is a toxic compound that may contribute to neurodegeneration [6, 8]. First, we examined the effect of TD on the viability of SH-SY5Y cells. As shown in Fig. 1A, TD promoted cell proliferation at a concentration of 25 μM. However, higher concentrations of TD (150–200 μM) significantly decreased cell viability. Conversely, 5HT, the parent molecule of TD was used as a control and had no notable effects on the cell viability.

Some quinone compounds are considered to have the potential to induce an increment of intracellular ROS [10, 11]. Consequently, we examined intracellular ROS generation in SH-SY5Y cells treated with TD using the redox-sensitive fluorescent reagent, CM-H_2DCF-DA. Treatment with TD (100 or 200 μM) for 30 min significantly increased the generation of ROS (Fig. 1B). Although some reports showed that 5HT elevates intracellular ROS levels in bovine pulmonary artery smooth muscle cells and Chinese hamster ovary cells [26, 27], the increase in ROS was negligible in this study. Thus, while TD at lower concentrations (to 25 μM) promotes cell proliferation, it induces cytotoxicity and ROS generation in higher concentrations (100–200 μM).

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Pretreatment with TD reduced cytotoxicity and ROS generation induced by H_2O_2

Mattson et al. suggest that mild stress, such as low levels of neurotoxins in fruits and vegetables, cause beneficial effects, activating adaptive cellular stress response pathways [28]. Therefore, we next examined whether a moderate stress by TD enhanced the adaptive biologic response. The cells were pretreated with TD or 5HT prior to exposure to H_2O_2, which served as a precursor for highly reactive free radicals and is known to induce toxicity *via* apoptosis in SH-SY5Y cells [29]. As shown in Fig. 2A, H_2O_2 treatment caused decrease in the cell viability. However, pretreatment with 100 μM TD restored the cell viability by approximately 10 %. Although treatment with 200 μM H_2O_2 enhanced ROS generation as evaluated by fluorescence levels (Fig. 2B), pretreatment with 100 μM TD exhibited a significant suppressive effect on ROS generation compared with that of the vehicle with H_2O_2-treated cells. In contrast, pretreatment with 5HT did not induce a protective effect against either cytotoxicity or ROS generation. These results suggest that the protection against cytotoxicity induced by TD may, at least in part, be due to the suppression of intracellular ROS generation. However, it is reported that the 5HT concentration in peripheral cerebrospinal fluid from patients with neurodegenerative diseases and healthy controls is approximately 1–5 nM [30], which is lower than those used in the model experiments. Therefore, it is necessary to measure physiological TD levels and investigate their effects on toxicity or functionality toward neuronal cells *in vitro* and *in vivo.*

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TD increased NQO1 and HO-1 expression

One of the key molecules of the adaptive biologic response is nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression of phase-II antioxidant genes [31]. Because previous reports have shown that some electrophilic

chemicals activate phase-II drug-metabolizing enzymes [12, 32], we examined whether TD and 5HT affected the mRNA and protein levels of NQO1 and HO-1 in SH-SY5Y cells. As shown in Fig. 3, TD significantly increased the mRNA and protein expressions of both NQO1 and HO-1 compared with 5HT treatment. Because phase-II antioxidant enzymes are thought to contribute to protection against H_2O_2-induced oxidative stress [33], the protective effect of TD may be at least partly due to the induction of these genes.

TD stimulates the translocation of Nrf2 to nucleus in SH-SY5Y cells

To assess the role of Nrf2 in TD-induced expression of NQO1 and HO-1, we examined the change of its localization in cells after treatment with TD (100 μM). Although Nrf2 immunostaining was mostly distributed in the cytoplasm in vehicle control and 5HT, Nrf2 molecules were translocated to the nucleus by treatment with TD (Fig. 4). Similar translocation was obtained in positive control cells treated with *t*BHQ, a known inducer of Nrf2 signaling [34]. A recent review showed that Kelch-like ECH-associated protein 1 (Keap1), which regulates the active degradation of Nrf2, is a sensor and chemical target for electrophiles and oxidants [31]. For example, some compounds, such as xenobiotics (*e.g.*, 1,2-naphthoquinone and 2-tert-butyl-1,4-benzoquinone) and phytochemicals (*e.g.*, isothiocyanate and zerumbone), activate Nrf2 through the chemical modification of Keap1 [32, 35–37]. Thereafter, downstream enzymes and proteins, such as NQO1 and HO-1, are upregulated [36, 38, 39]. TD exhibits a high reactivity for the thiol moiety [22], and this study shows that 100 μM TD produces a detectable level of intracellular ROS (Fig. 1B). Therefore, TD may directly or indirectly modulate Keap1. Besides the Keap1/Nrf2 pathway, these genes are elevated by the activation of various factors that possess cysteine thiols, such as phosphatidylinositol 3-kinase (PI3K)–Akt [40, 41]. Indeed, it has been reported that *tert*-butylhydroquinone

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stimulates PI3K–Akt signaling and upregulates Nrf2-dependent antioxidant response-element activity [42, 43]. These issues are currently under investigation in our laboratory.

In summary, we showed that TD induces both the generation of intracellular ROS and cytotoxicity. However, the pretreatment of neuronal cells with TD upregulates mRNA and protein expression of NQO1 and HO-1 to confer resistance to H_2O_2-induced cytotoxicity. Further studies on the physiologic roles of TD are necessary to elucidate these novel aspects of the function of serotonin.

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Disclosure statement

The authors report no conflicts of interest.

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Figure legends

Fig. 1. The effect of TD or 5HT exposure on cell viability (**A**) and intracellular ROS generation (**B**) in SH-5Y5Y cells. **A**: SH-SY5Y cells were exposed to the indicated concentrations of samples (TD/5HT) or vehicle in FBS-free medium for 30 min, and the medium was then replaced with flesh medium containing 10% FBS to remove any unreacted TD. "Vehicle" indicates a solvent-only control. After a 24 h incubation, the cells were subjected to a cell viability assay. The data are expressed as mean ± standard deviation (n = 5) and represent percentage control. Statistical significance was set at ** $p < 0.01$ and *** $p < 0.001$ versus vehicle using the one-way ANOVA and Tukey post-hoc test. **B**: SH-5Y5Y cells were pre-loaded with CM-H2DCF-DA for 30 min, and then treated with vehicle (a), 200 μM H_2O_2 (b), 100 μM TD (c), 200 μM TD (d), 100 μM 5HT (e), or 200 μM 5HT (f) for 30 min. H_2O_2 served as a positive control. This experiment was repeated three times and similar results were achieved. *Images*: DCF-derived fluorescence in SH-SY5Y cells. Scale bar indicate 70 μm. *Graph*: Quantification of ROS levels by measuring DCF-derived fluorescence in cells. Fluorescent intensity of each image was quantified using ImageJ and expressed as relative to the control (a); values are means ± standard deviation from 50 cells in triplicate experiments. ** $p < 0.01$ and *** $p < 0.01$ indicate significant difference vs. vehicle using the one-way ANOVA and Games-Howel post-hoc test.

Fig. 2. The effect of pretreatment with TD or 5HT on H_2O_2-induced cytotoxicity (**A**) and ROS generation (**B**). **A**: SH-SY5Y cells were treated with either vehicle or samples (TD/5HT) in FBS-free medium for 30 min, and then the medium was replaced with flesh medium containing 10% FBS. After incubation for 24 h, the cells were further incubated for 12 h in the presence or absence of 100 μM H_2O_2, and then subjected to a cell viability assay. Vehicle indicates the same as in Fig. 1. The values presented are

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means ± standard deviation (n = 6) and are expressed as percentages relative to H_2O_2-untreated control cells. **Significantly different (the one-way ANOVA and Tukey post-hoc test) from vehicle, $p < 0.01$. **B**: The cells were treated with either vehicle or 100 μM samples (TD/5HT) in the same way as the above and incubated for 24 h. After the medium was refreshed, 200 μM H_2O_2 was added, and the cells were incubated for a further 6 h. CM-H_2DCF-DA (5 μM) was added to the treated cells for 30 min. *Images*: The fluorescent signal was photographed using a fluorescence microscope. The results shown are representative images of experiments performed in triplicate. Scale bar indicate 70 μm. *Graph*: Quantification of ROS in cells pretreated with or without TD followed by H_2O_2. Intracellular ROS generation was determined by DCF fluorescence and the intensity was then calculated as described in Fig. 1B legend. The data is expressed as relative to vehicle control without H_2O_2. ** $p < 0.01$ and *** $p < 0.01$ indicate significant difference vs. vehicle without H_2O_2; # $p < 0.05$ indicates significant difference vs. vehicle with H_2O_2 using the one-way ANOVA and Games-Howel post-hoc test.

Fig. 3. Time- and concentration-dependent changes in the expression of the NQO1 and HO-1 in SH-5Y5Y cells exposed to TD or 5HT. **A**: The cells were treated with either TD (closed circles), 5HT (closed squares) or vehicle (open circles) in FBS-free medium for 30 min, and the medium was then replaced with flesh medium containing 10% FBS. After incubation for the indicated time intervals, NQO1 and HO-1 expression in the cells was analyzed using real-time PCR. **B**: The cells were treated in the same way as the above, and incubated for 6 h in medium containing 10% FBS. The expression of NQO1 and HO-1 was calibrated by measuring GAPDH expression, as described in the Materials and Methods. **C**: The cells were treated with 100 μM TD/5HT or vehicle for

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30 min in FBS-free medium, and the medium was replaced with flesh medium containing 10% FBS. After incubation for the indicated time intervals, the cell extracts were analyzed by Western blotting. The results shown are representative of at least two independent experiments. The values are presented as mean ± standard deviation (n = 3) and represent percentage control. Statistical significance was set at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus vehicle using the one-way ANOVA and Tukey post-hoc test.

Fig. 4. Effect of TD on the subcellular distribution of Nrf2 in SH-SY5Y cells. The cells were treated with TD (100 µM) or vehicle in FBS-free medium for 30 min, and the medium was then replaced with flesh medium containing 10% FBS. Cells treated with 50 µM *t*BHQ were used as positive control. After incubation for 3 h, cells were fixed with methanol and permeabilized with 0.5% Triton X-100 and then blocked with PBS containing 3% BSA. The cells were then incubated with antibody (anti-Nrf2), followed by secondary antibody (FITC labeled). The nuclei were stained with propidium iodide (PI). Arrows indicate nucleus. Scale bar shows 20 µm. This experiment was repeated three times and similar results were achieved.

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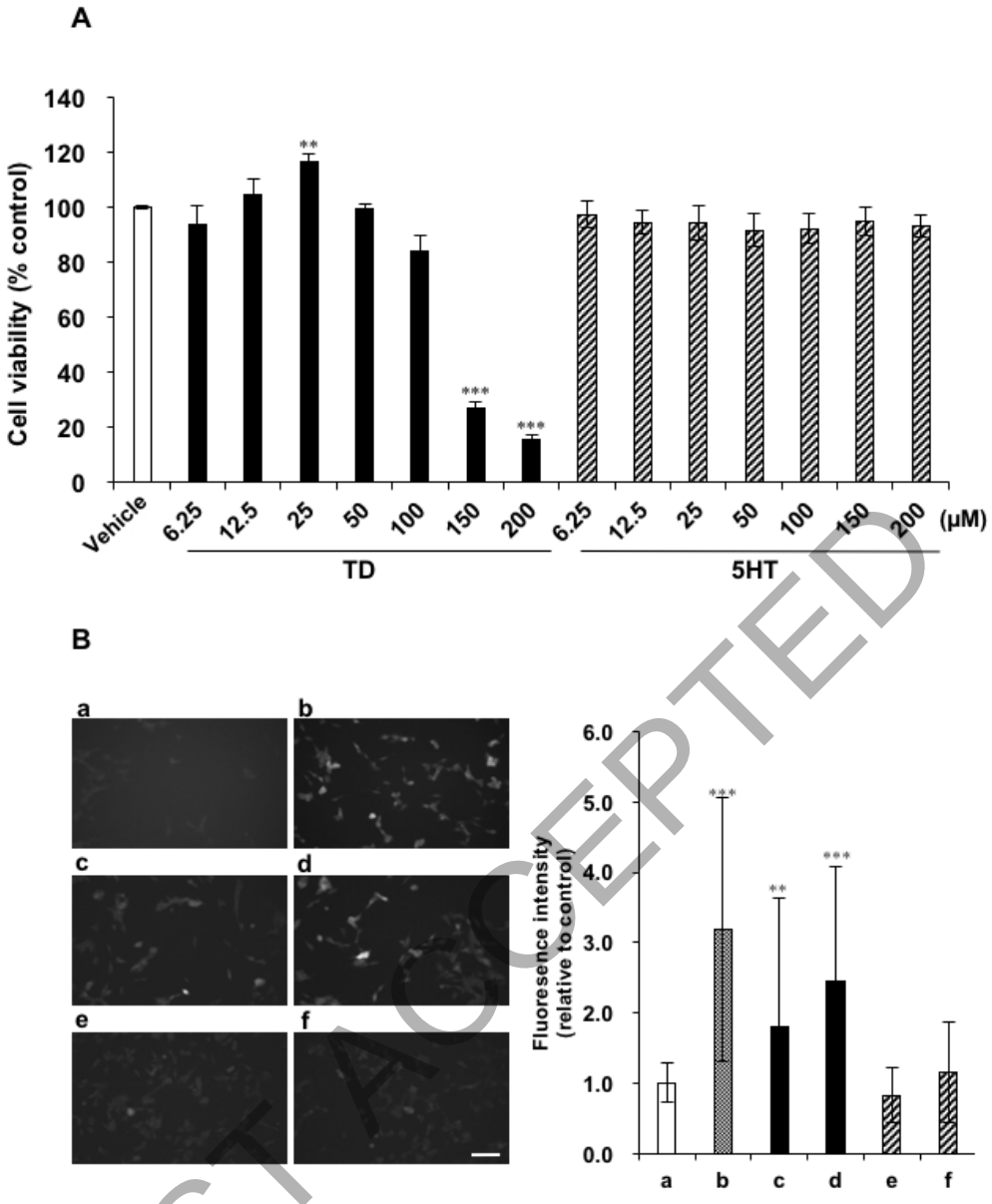

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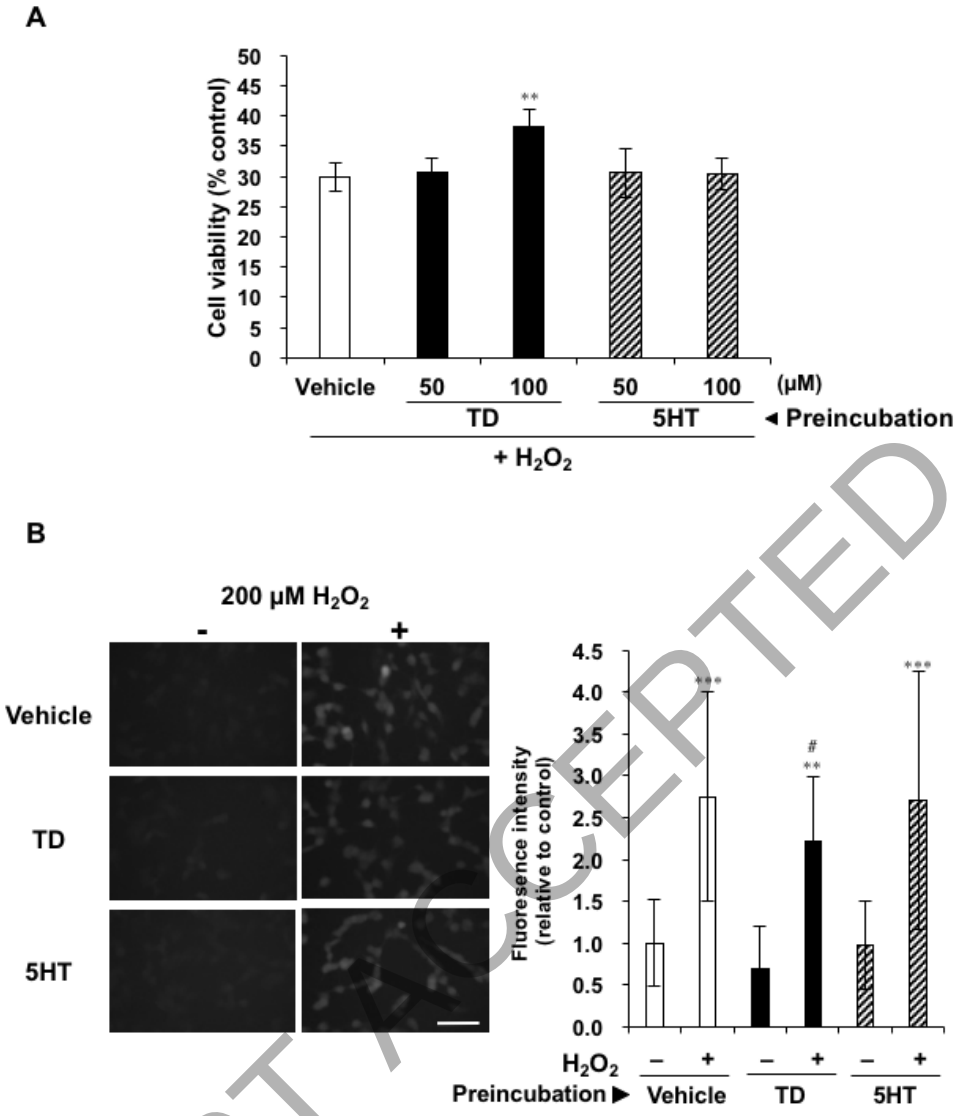

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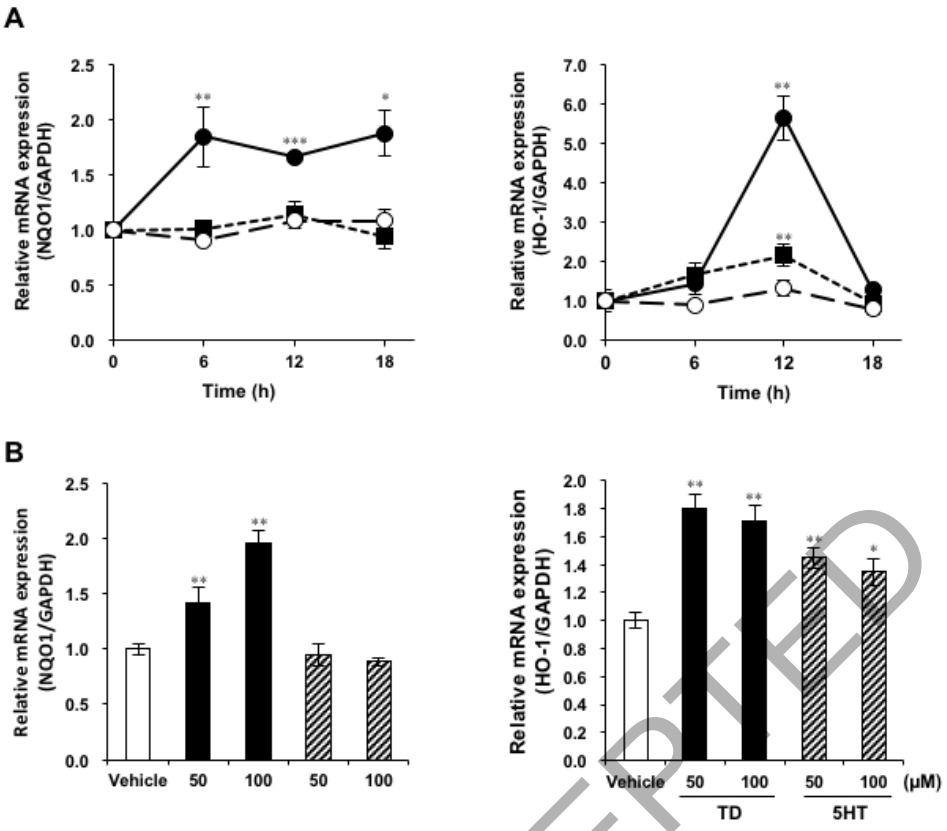

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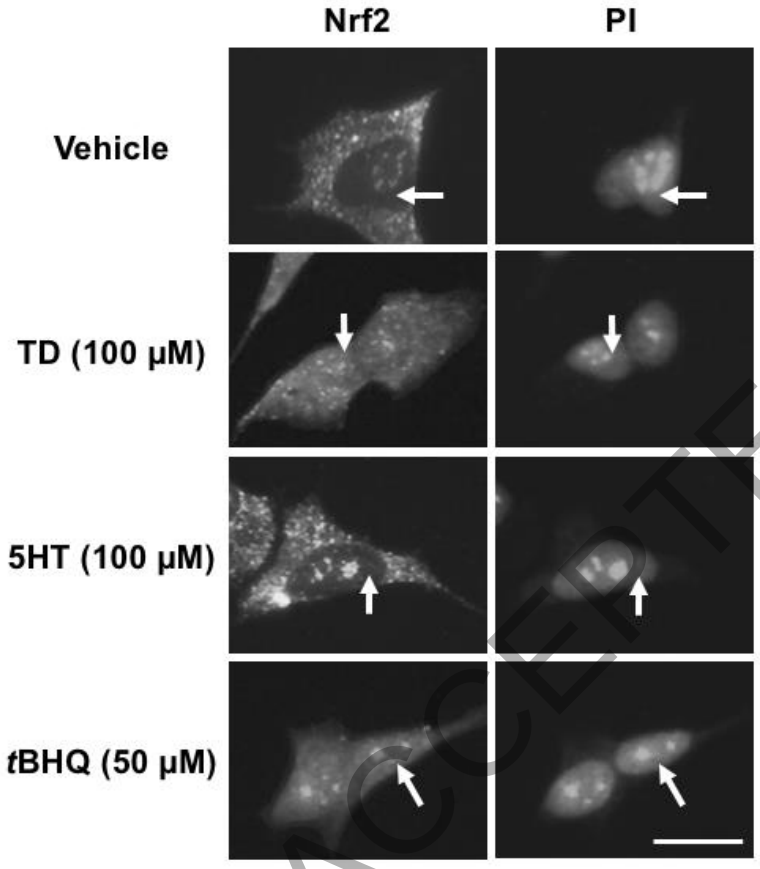

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