Modulation of H2O2- Induced Neurite Outgrowth Impairment and Apoptosis in PC12 Cells by a 1,2,4-Triazine Derivative

Solaleh Khoramian Tusi[1], Shahnaz Babaei Abraki[1], Mohsen Amini[2], Fariba Khodagholi[1*]

1. Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
2. Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical science, Tehran, Iran.

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ABSTRACT

Introduction: Increased oxidative stress is widely accepted to be a factor in the development and progression of Alzheimer's disease. Triazine derivatives possess a wide range of pharmacological activities including anti-oxidative and anti-inflammatory actions. In this study, we aimed to investigate the possible protective effect of 3-thioethyl-5,6-dimethoxyphenyl-1,2,4-triazine (TEDMT) on H2O2-induced neurite outgrowth impairment and apoptosis in neuron-like PC12 cells.

Methods: We pretreated PC12 cells with 5, 7, and 10 μM of TEDMT followed by adding H2O2 as an oxidative stress agent.

Results: We found that TEDMT contributed to up-regulation of Bcl-2, down regulation of Bax protein and reduction of cleaved Caspase-3 and PARP proteins. Moreover, TEDMT could inhibit the phosphorylation of different mitogen activated protein kinases (extracellular signal-regulated kinase, c-Jun N-terminal kinase and P38 mitogen-activated protein kinase). TEDMT induced heat shock protein 70 while decreased heat shock protein 90 level. Besides we measured six different parameters of neurite outgrowth and complexity. We showed that H2O2 increased cell body area, average neurite width and the proportion of bipolar cells, while decreased average neurite length, the numbers of primary neurites and the ratio of the total neurite branching nodes to the total number of primary neurites.

Discussion: Interestingly, we found that TEDMT not only protects PC12 cell against H2O2-induced apoptosis, but also defends against the destructive effect of oxidative stress on the criteria of neural differentiation. Protective effect of this compound could represent a promising approach for treatment of neurodegenerative diseases.

1. Introduction

Neural cells connected to one another form networks that are the central processing units underlying neural function. These networks are primarily formed during development as maturing neurons extend processes to reach synaptic targets. At the leading edge of these processes are growth cones that recognize and translate a combination of chemical and physical cues into a specific trajectory towards a population of target cells. Within their target region, neuronal processes ramify, furcate, and form synapses with other neurons. Many factors can cause disruption in neurite outgrowth including oxidative stress (de la Monte et al., 2000; Khodagholi et al., 2012). Oxidative stress is the major culprit in neuronal death observed in neurodegenerative diseases, such as Alzheimer's disease (AD) (Behl, 1999) which is primarily a disorder of aging with loss of cognitive function. This disease is characterized biologically by the death of neurons in the forebrain, hippocampus, and cerebral cortex accompanied by the presence of amyloid deposition (Wirak et al., 1991; Shimohama, 2000; Eik et al., 2012).

**** Corresponding Author:***
Fariba Khodagholi, PhD
Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Tel: 0098-21-22429768/ Fax: 0098-21-22432047
E-mail: khodagholi@sbmu.ac.ir

The search is now for small molecules that can cross the brain-blood barrier and inhibit or delay the disruption in neurite outgrowth.

Compounds with antioxidant properties may help prevent or alleviate diseases in which oxidative stress is a primary cause (Lim et al., 2001; Tusi et al., 2011; Joodi, Ansari & Khodagholi, 2011; Asadi et al., 2011; Li et al., 2012). Triazine derivatives are kind of synthetic compounds possessing a wide array of biological activities. Many investigations revealed the anti cancer potential of various types of triazine derivatives (Bekircan et al., 2005). Some of the others have been introduced as anti inflammatory (Saxena et al., 1994), radical scavenger (Iwashita et al., 2003) and β-sheet breaker (Kim et al., 2006), agent. We have previously reported that 3-(ethylthio)-5,6-di-(4- methoxyphenyl)-1,2,4-triazine (TEDMT) (shown in Figure 1) can decrease cell death by attenuating NF-κB and activating the NF-E2- related factor2 antioxidant response element signaling pathway. Additionally, we examined its ability to penetrate the blood-brain barrier by using a reliable model, the artificial neural network and interestingly, we found that this compound can penetrate the blood-brain barrier (Tusi et al., 2010). In the present study, cultured rat pheochromocytoma PC12 cells have been used as an in vitro model system for investigation of neuronal differentiation, neuronal survival, and neurotransmitter secretion (Tischler and Greene, 1978; Rukenstein, Rydel & Greene, 1991). Nerve growth factor (NGF) activates extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) (Kim et al., 2004). PC12 cells respond to neurotrophin NGF and differentiate into sympathetic neuron phenotype and extending axon-like processes called neurites (Parmar et al., 2002). This serves as an excellent model to study the effects of molecules both synthetic and natural that will stimulate the outgrowth of neurites. Since MAPKs play a central role in the signaling pathways of cell proliferation, differentiation, survival, and apoptosis (Waskiewicz and Cooper, 1995), we further evaluated the roles of MAP kinases and heat shock proteins (Hsps) in neuroprotection that was mediated by this triazine derivative.

2. Methods

2.1. Materials

Antibodies directed against HSP70, HSP90, phospho-p38, MAP kinase (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), p38 MAP kinase, SAPK/JNK, Bcl-2, Bax, Caspase-3, Poly ADP ribose polymerase (PARP) and β-actin which are rabbit monoclonal or polyclonal antibodies and have cross reactivity with rat, were obtained from Cell Signaling Technology. Phospho-ERK1/2 and ERK1/2 antibodies were obtained from ABCAM. All the other reagents, unless otherwise stated, were from Sigma Aldrich (St. Louis, MO). Rat pheochromocytoma (PC12) cells obtained from Pasteur Institute (Tehran, Iran). TEDMT was synthesized as previously reported (Tusi et al., 2010).

2.2 Cell Culture and Experimental Treatments

The characteristic as a sub-population of the total number of cells that met PC12 cells were grown in culture flasks with collagen coating and by using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin, in a humidified atmosphere at 37°C with 5% CO2. Growth medium was changed three times a week. PC12 cells were differentiated by treating with NGF (50 ng/ml) every other day for 6 days. One day after cell differentiation, PC12 cells were plated in 75 cm2 culture flasks and incubated with different concentrations (5, 7 and 10 µM) of TEDMT which previously dissolved in dimethyl sulfoxide (DMSO) at 10-2 M and then diluted in culture medium. After incubation for 3h by this compound, the cells were treated with H2O2 (150 µM) for 24 h.

2.3. Morphological Analysis of Differentiated PC12 Cells

For morphological analysis, PC12 cells were plated in 6-well tissue culture plates and treated by the mentioned way. In follow, random images were acquired from each well, taking two images per well. A minimum of 50 cells per treatment were quantified. Criteria for selection were that the cell body and processes were completely within the field of view, and that the cell body was distinct from neighboring cell bodies. Data analysis was done by Cell^ A program. Cells fitting these criteria were analyzed and their cell body area, average neurite length, average neurite width, number of primary neurites and bipolar morphology were quantified. Cell body area was defined as the area of the cell exclusive of neurite processes. Neurite length was calculated by summing the lengths of the primary process and all associated branches. To establish the average neurite width, the outlines of individual primary neurites were traced, the area was calculated and then divided by the length of the neurite. Primary neurites were defined as clear protrusions from the cell body greater than 10 µM length. Cells were considered "bipolar" if they displayed a cell

body with one process at either end. To evaluate neurite networks, images were analyzed using the cell counter plugin to score all branching nodes in each image. Nodes were defined as sites at which individual neurites branched or separate neurites contacted each other (Frankel et al., 2009).

All measurements expressed as proportions used the number of cells displaying selection criteria described above.

2.4. Acridine Orange/Ethidium Bromide (AO/EB) Double Staining

Cell viability was determined morphologically after staining the cells with acridine orange/ethidium bromide (AO/EB) followed by fluorescence microscopy inspection (Saydam et al., 2003). AO is a cell-permeable nucleic acid selective dye which is taken up by both viable and nonviable cells and emits green fluorescence if intercalates into double stranded nucleic acid. EB is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. Briefly, differentiated PC12 cells (1 × 106 cells/ml) were seeded in a 6-well plate and the next day treated with different concentration (5, 7 and 10 µM) of TEDMT for 3h prior to H2O2-treated. After 24 h incubation, the cells were harvested and washed three times with phosphate buffered saline (PBS) and were adjusted to a density of 1×106 cells/ml of PBS. AO/EB solution (1:1 v/v) was added to the cell suspension in a final concentration of 10 µM. Cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

2.5. Hoechst Staining

PC12 cells were stained with Hoechst 33342 dye to evaluate apoptosis (Lotharius, Dugan & O'Malley, 1999). Briefly, PC12 cells (1×106 cells/ml) were treated with different concentrations (5, 7 and 10 µM) of TEDMT for 3h, followed by adding H2O2 (150µM) for 24h. After incubation, the cells were harvested and washed three times with PBS and were adjusted to a density of 1×106 cells/ml of PBS. Cells were incubated with Hoechst 33342 (1 µg/ml) for 5 min at room temperature. Fluorescence was visualized using an Olympus microscope.

2.6. Western Blot Analysis

Treated PC12 cells were lysed in buffer containing complete protease inhibitor cocktail. The protein concentration was measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. Cell lysate was separated on 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes and probed with specific antibodies, diluted 1:1000. Immunoreactive polypeptides were detected by chemiluminescence using Electrochemiluminescence reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by ImageJ, measuring integrated density of bands after background subtraction.

2.7. Data Analysis

All data are represented as the mean ± S.E.M. (Standard Error Mean) and processed by commercially available software GraphPad Prism® 5.0. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by an appropriate post-hoc test (Newman-Keuls test) to analyze the difference. The statistical significances were achieved when $P< 0.05$.

3. Results

3.1. TEDMT Improved Neurite Growth in Differentiated PC12 Cells

Cell body area, average neurite length and average neurite width were evaluated to monitor cell growth, as described in Figure 2A. As shown in Figures 2B and C, average cell body area increased in H2O2 exposed cells compared to control, whereas pretreatment of cells with TEDMT resulted in a significant decrease in cell body area. Accordingly, as shown in Figure 2D, TEDMT significantly increased neurite length, compared to H2O2-treated cells. Moreover, the average neurite width increased in neurons exposed to 150 µM H2O2 (Figure 2E). Because of variability along the length of

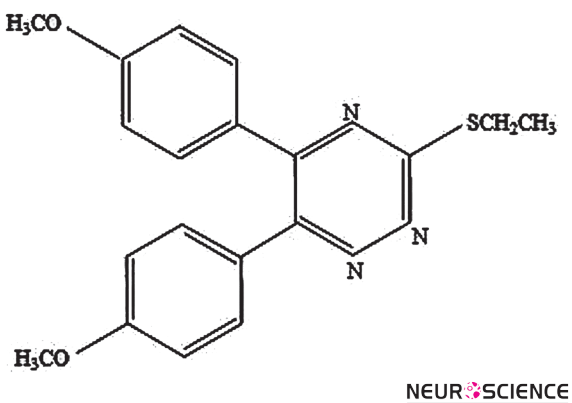
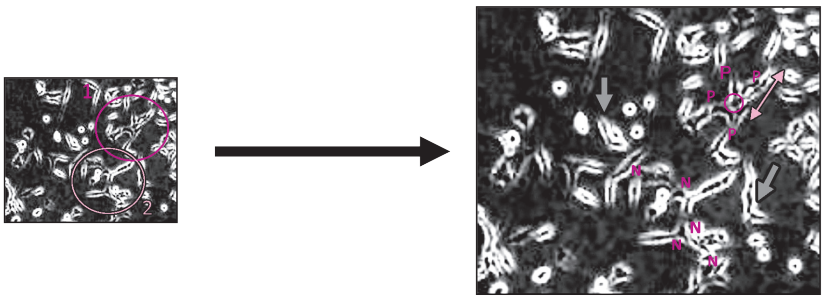
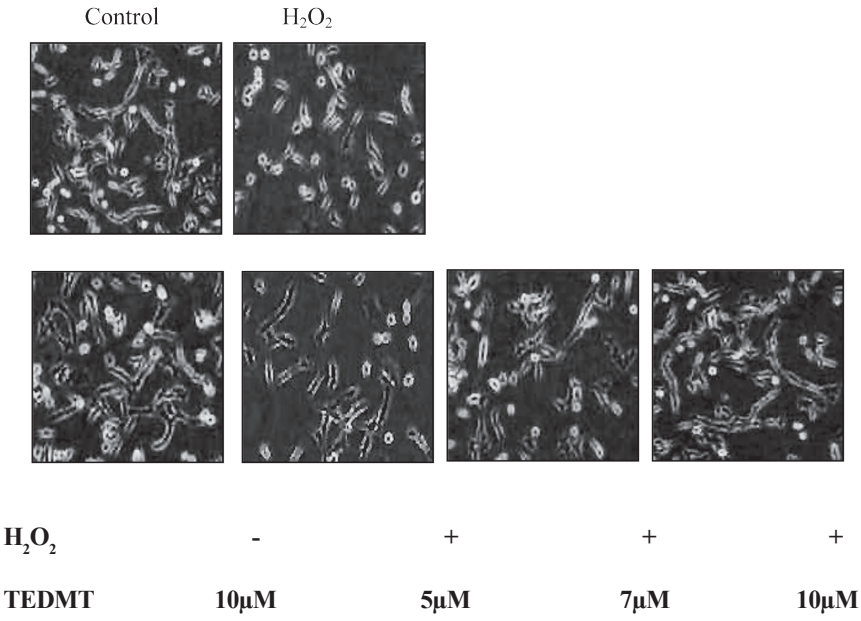


Figure 1. Chemical structure of TEDMT.

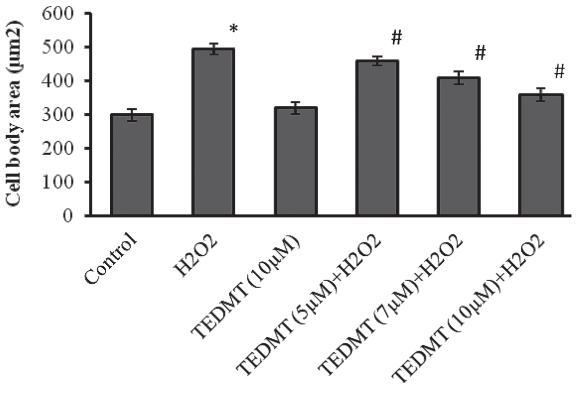
A



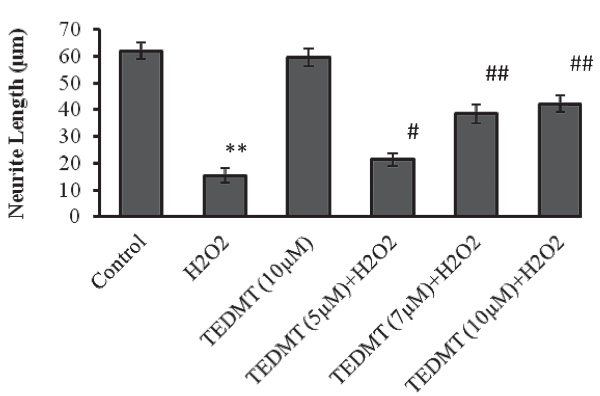
B


Control
H2O2
H2O2 - + + +
TEDMT 10μM 5μM 7μM 10μM

C


Cell body area (um2)
600
500
400
300
200
100
0
*
#
#
#
Control
H2O2
TEDMT (10μM)
TEDMT (5μM)+H2O2
TEDMT (7μM)+H2O2
TEDMT (10μM)+H2O2

D


Neurite Length (um)
70
60
50
40
30
20
10
0
**
#
##
##
Control
H2O2
TEDMT (10μM)
TEDMT (5μM)+H2O2
TEDMT (7μM)+H2O2
TEDMT (10μM)+H2O2

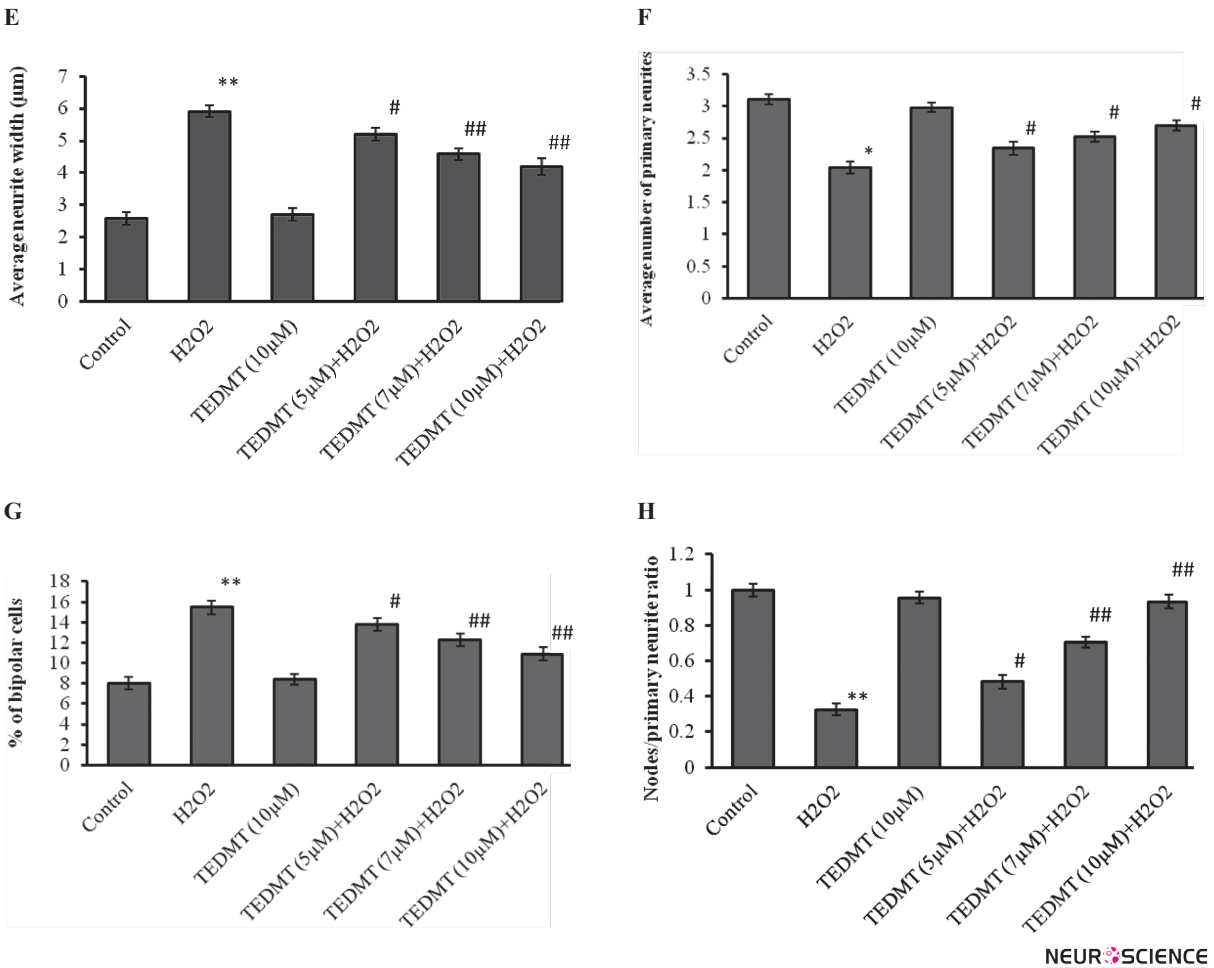
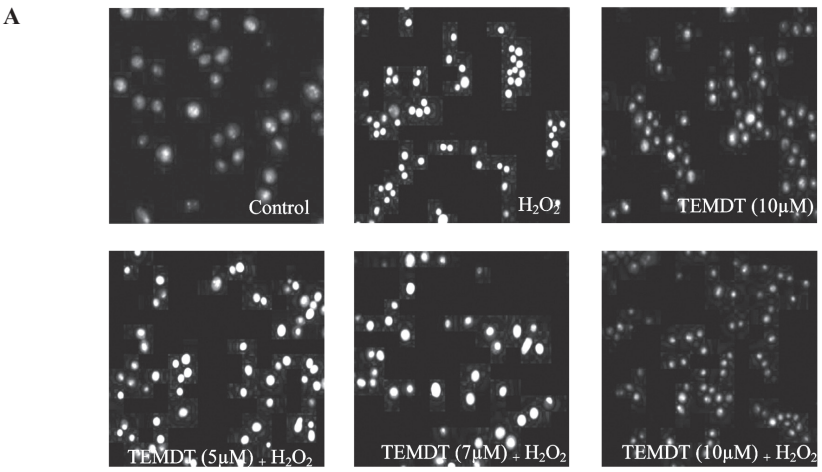
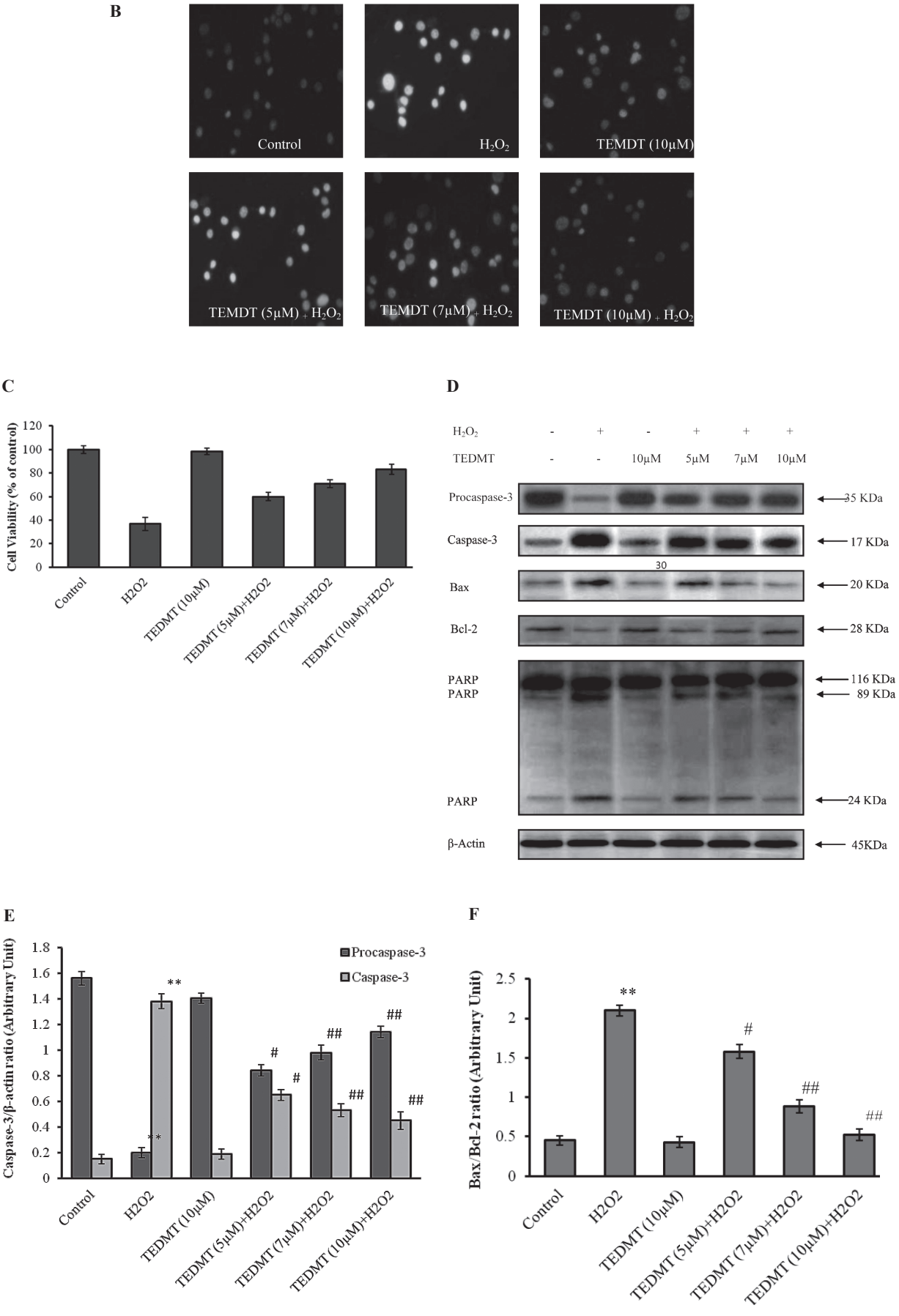


Figure 2. Effect of TEDMT on H2O2-induced disruption of neurite outgrowth in differentiated PC12 cells. (A) The criteria of PC12 differentiation is shown on two neurons (left image) of a sample image. The "P" on right image indicates the primary neuritis of a neuron 1. The yellow arrow shows the length of a neurite, extent of elongation, and membrane-enclosed protrusions of cytoplasm. The green circle in the rightimage shows the cell body. Neurite width is not equal in all parts of the neurons; thus, the average neurite width must be calculated by dividing cellbody area to average neurite length. At the right image, the blue arrows shows two bipolar cells. The letter "N" indicates the nodes, the sites at which individual neuritesbranched or separate neurites contacted each other. (B) NGF-differentiated PC12 cells were pretreated with 5, 7, and 10 µM TEDMT for 3 h and then exposed to H2O2 (150 µM). Criteria were quantified at 24 h; (C) average neurite length; (D) cell body area; (E) average neurite width; (F)number of primary neurites per cell; (G) percent of bipolar cells; and (H) the ratio of nodes to primary neurites. *P<0.05; **P<0.01 significantly different from control cells. #P<0.05; ##P<0.01 significantly different from H2O2- treated cells.




B
Control
H2O2
TEMDT (10μM)
TEMDT (5μM) + H2O2
TEMDT (7μM) + H2O2
TEMDT (10μM) + H2O2
C
Cell Viability (% of control)
120
100
80
60
40
20
0
Control
H2O2
TEDMT (10μM)
TEDMT (5μM)+H2O2
TEDMT (7μM)+H2O2
TEDMT (10μM)+H2O2
D
H2O2 - + - + + +
TEDMT - - 10μM 5μM 7μM 10μM
Procaspase-3
Caspase-3
Bax
Bcl-2
PARP
PARP
PARP
β-Actin
35 KDa
17 KDa
30
20 KDa
28 KDa
116 KDa
89 KDa
24 KDa
45KDa
E
Caspase-3/β-actin ratio (Arbitrary Unit)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Procaspase-3
Caspase-3
**
**
#
#
#
##
##
##
##
Control
H2O2
TEDMT (10μM)
TEDMT (5μM)+H2O2
TEDMT (7μM)+H2O2
TEDMT (10μM)+H2O2
F
Bax/Bcl-2 ratio (Arbitrary Unit)
2.5
2
1.5
1
0.5
0
**
#
##
##
Control
H2O2
TEDMT (10μM)
TEDMT (5μM)+H2O2
TEDMT (7μM)+H2O2
TEDMT (10μM)+H2O2

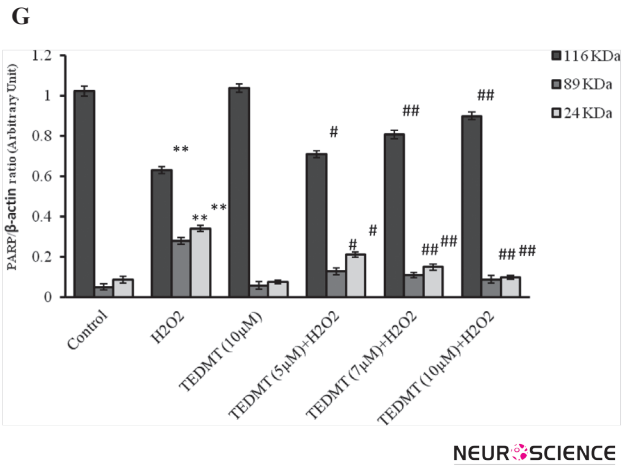


Figure 3. Determination of cell death using morphological evaluation and western blot analysis. The cells were exposed to different concentrations of TEDMT for 3 h followed by exposure to 150 μM of H2O2 for 24 h. (A) AO/EB double staining and (B) Hoechst 33342. (C) The number of stained cells was counted in 10 randomly selected fields. Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures. (D) Caspase-3, Bax, Bcl-2 and PARP response to different concentrations of TEDMT in PC12 cells pretreated for 3 h and then exposed to H2O2 (150 μM) for 24 h. Twenty μg proteins were separated on SDS-PAGE, Western blotted, probed with anti-caspase-3, -Bax, Bcl-2 and PARP antibodies and reprobed with anti-β-actin antibody. (E) The densities of Procaspase-3, caspase-3, (F) Bax and Bcl-2 were measured, the ratios to β-actin and Bax:Bcl-2 ratio were calculated. (G) The densities of PARP bands were measured and the ratios to β-actin were calculated. (One representative Western blot was shown; n=3). The mean of three independent experiments is shown. **P<0.01 significantly different from control cells. #P<0.05; ##P<0.01 significantly different from H2O2- treated cells.

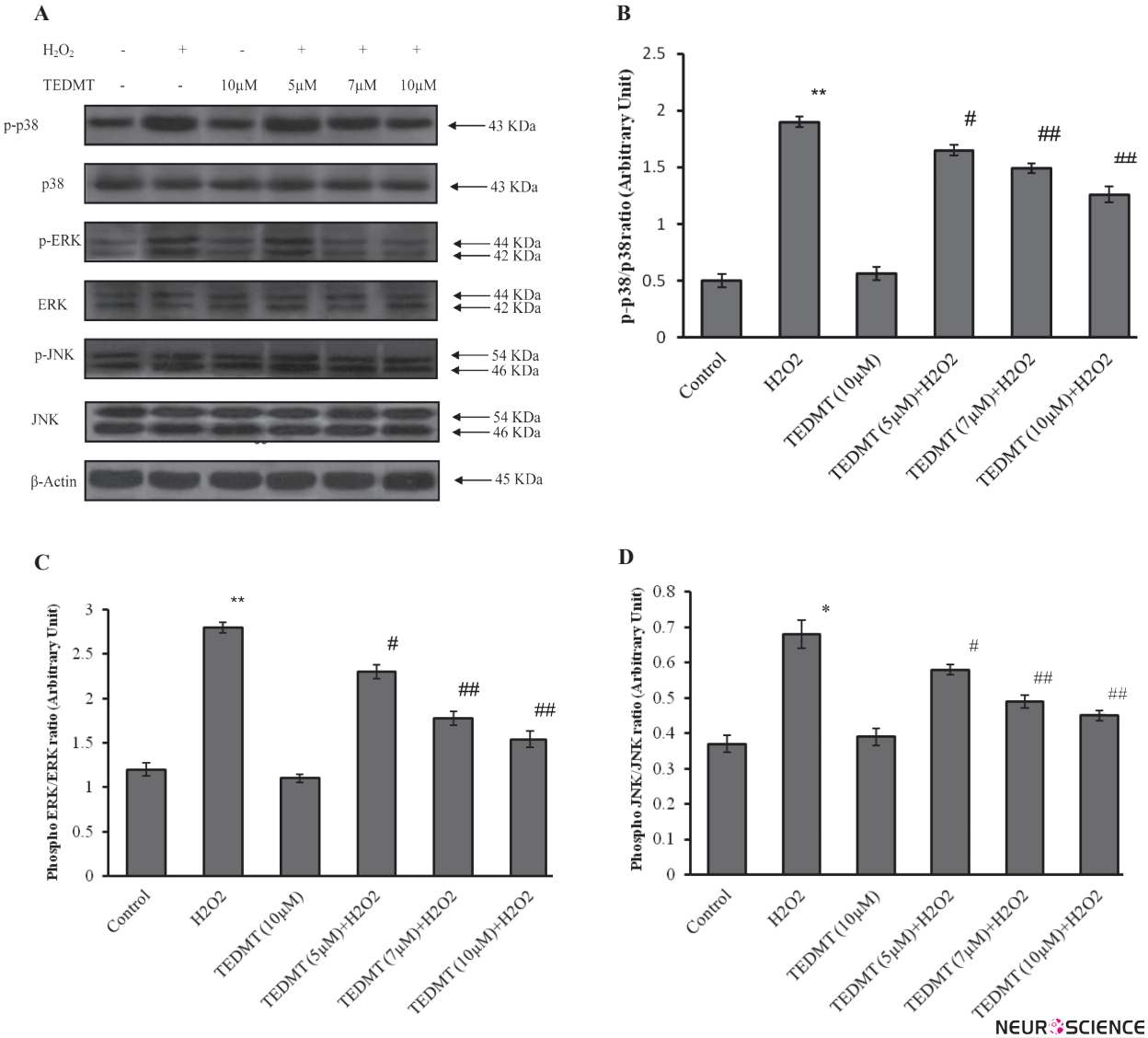
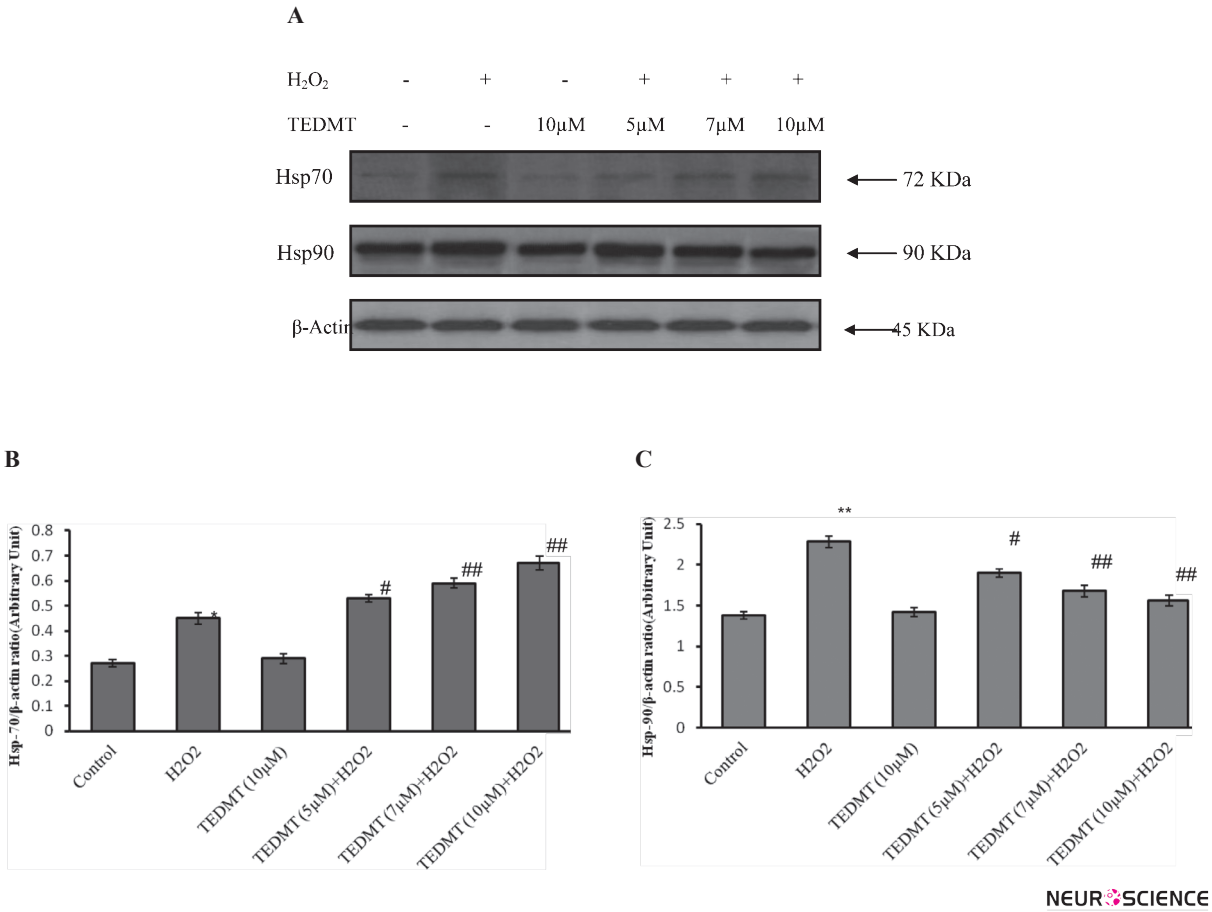


Figure 4. Effects of TEDMT on H2O2-induced phosphorylation of MAPKs in PC12 cells. (A) PC12 cells were pretreated with 5, 7 and 10 TEDMT for 3 h and then exposed to H2O2 (150 µM) for 24 h. Twenty micrograms of proteins were separated on SDS−PAGE, Western blotted, probed with phosphorylated MAPK antibodies, and reprobed with anti-β-actin antibody. (One representative Western blot was shown; n =3.) The densities of phospho-ERK, ERK (B), phospho-JNK, JNK (C), phospho-p38, and p38 (D) bands were measured, and the ratio to total levels was calculated. The mean of three independent experiments is shown. *P<0.05; **P<0.01 significantly different from control cells. #P<0.05; ##P<0.01 significantly different from H2O2-treated cells.



NEUROSCIENCE

Figure 5. Western blot analysis to measure the effects of TEDMT on Hsp70 and Hsp90 in PC12 cells. A) Hsp70 and Hsp90 response to TEDMT(5, 7 and 10 µM) onPC12 cells pretreated for 24 h and then exposed to H2O2 (150 µM) for 24 h. Twenty µg proteins were separated on SDS-PAGE, western blotted, probed with anti-Hsp70 and Hsp90antibodies and reprobed with anti-β-actin antibody. (One representative western blot was shown; n=3). B) The densities of Hsp70 and (C) Hsp90 bands were measured and the ratio was calculated. The mean of three independent experiments is shown. *P<0.05; **P<0.01 significantly different from control cells. #P<0.05; ##P<0.01 significantly different from H2O2- treated cells.

the neurite, the total area of the neurite was divided by the neurite length to calculate average neurite width. This ratio was significantly lower in TEDMT-treated cells, after 24 h (Figure 2E).

3.2. TEDMT Increased Neurite Complexity in Differentiated PC12 Cells

Specific parameters of morphological complexity were also measured. First, the numbers of primary neurites (>10 µm) emanating from individual cell bodies were measured. As shown in Figure 2F, the numbers of primary neurites per cell body decreased in H2O2-treated cells. In contrast, the proportion of cells with the very simple bipolar morphology of a cell body and only two neurites increased in H2O2-treated cells, compared to control cells (Figure 2G). Pretreatment of cells with TEDMT significantly and concentration-dependently increased the numbers of neurites per cell, and thus decreased the proportion of bipolar cells (Figure 2F, G). We also calculated the ratio of the total neurite branching nodes to the total number of primary neurites. This ratio decreased in H2O2-treated cells, while it increased significantly in the presence of TEDMT (Figure 2H).

3.3. Morphological Evaluation of Cell Viability and Apoptosis

AO/EB staining discriminates live cells from dead ones on the basis of membrane integrity. Although it does not stain healthy cells, it can be used to identify non-viable cells that have much more permeable membranes. In this method, viable cells show uniform bright green nuclei with organized structure, while non-viable cells have orange to red nuclei with condensed or fragmented chromatin. In addition, Hoechst 33342 which applies to detect apoptotic nuclei is a DNA stain that binds preferentially to A-T base-pairs and shows DNA fragmentation and condensation of chromatin, which causes bright fluorescence. The results, obtained from AO/EB double staining and Hoechst staining, are presented in Figure 3A, 3B and 3C. Analysis of stained cells indicated that pretreatment of cells with different concentrations of TEDMT (5, 7, and 10 μM) significantly and dose-dependently increased the extent of cell viability and decreased the apoptotic cell death compared to the cells exposed solely to H2O2. TEDMT (10 μM) had no toxic effect when used alone.

3.5. TEDMT Modulated Bax/Bcl-2 Ratio, Caspase-3 Activation and PARP-1 Cleavage in PC12 Cells

In order to confirm the protection observed by TEDMT against apoptotic cell death, we investigated the protein levels of Bax, Bcl-2, cleaved caspase-3 and PARP-1 using western blot analysis. Caspases are major players of apoptotic cell death. Activated caspase-3 is responsible for the execution of apoptosis. The Bax/Bcl-2 ratio in H2O2-exposed cells that pretreated with TEDMT, as well as the cleavage of caspase-3 (Figures 3D, 3E and 3F), reduced compared to the H2O2-treated cells, demonstrating the ability of TEDMT to suppress caspase-dependent apoptotic cascade. In addition, the level of cleaved PARP decreased in the presence of TEDMT (Figures 3D and 3G).

3.6. TEDMT Decreased MAPKs Phosphorylation in PC12 Cells

In order to further investigate the molecular mechanisms, we examined TEDMT's effect on H2O2-induced MAPK phosphorylation using western blot analysis. MAPK cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation (Akiyama et al., 2004; Fábián et al., 2006; Waetzig et al., 2008). As shown in Figure 4, phosphorylation of p-38, ERK1/2 and JNK increased in cells treated with H2O2 for 24 h by about 3.80, 2.33 and 1.83 fold, respectively. However, TEDMT pretreatment for 3 h reduced phosphorylation of p38, ERK1/2 and JNK levels in H2O2-exposed PC12 cells in a dose-dependent manner (Fig. 4). No significant changes were detected in the level of total ERK, JNK and p38 kinase in the cells treated with H2O2, in the presence and/or absence of TEDMT.

3.7. TEDMT Upregulated Hsp70 Level and Inhibited Hsp90 Induction in PC12 cells

Hsps are highly conserved, ubiquitously expressed family of stress response proteins that pharmacological agents targeting molecular chaperones have mainly focused on them, especially Hsp70 and Hsp90. Coordinated upregulation of Hsp expression, called the HSR, helps cells to adapt to a wide range of environmental challenges and maintains their viability. It is widely accepted that increased Hsp70 in response to oxidative stress is essential to maintain cell viability (Sato, Saito & Matsuki, 1996; Ayala and Tapia, 2008; Calabrese et al., 2004). Western Blotting analysis of PC12 cells indicated that TEDMT pretreatment could elevate Hsp70 level in H2O2-treated cells (Figures 5A and 5B).

Hsp90 inhibitors increase the activity of the transcription factor HSF-1 and thus lead to increased expression of stress-induced proteins such as Hsp70. The interplay between Hsp90 and HSF-1 can be regarded as a molecular switch that can activate a cytoprotective stress-response (Luo, Rodina & Chiosis, 2008). Therefore, we further analyzed the effect of TEDMT on Hsp90 level. We observed that while H2O2 increased Hsp90 level by 1.65 fold, compared to control, TEDMT showed a dose dependent inhibitory effect on Hsp90 induction (Figures 5A and 5C).

4. Discussion

Oxidative stress represents the imbalance of redox equilibrium between the production of free radicals and the ability of cells to defend against them (Jones, 2006). There is considerable evidence supporting that oxidative stress is implicated as a major cause of cellular injuries in a variety of human diseases including neurodegenerative disorders. Intake of chemical compounds, that are able to scavenge free radicals, might serve as a feasible method to augment the antioxidant capacity and protect cells from oxidative damage (Bub et al., 2003). Our previous study demonstrated that TEDMT was effective in defending PC12 cells against H2O2-induced cell death. In current study we investigated the effect of TEDMT

on H2O2-induced impairment of neurite outgrowth. We further evaluated the involvement of Hsps and MAPK molecules.

Axons elongate and apply their normative explorations activity of growing in order to be directed to their correct target that is required for formation of central nervous system network (Díez-Revuelta et al., 2010). It has been reported that neural network is disrupted by oxidative stress in many neurodegenerative disease, such as AD (Gillespie 2003). Neuronal cells are able to sense the surrounding and form branches in response to molecular information from extracellular milieu that instruct the maturation process and induce neurite regeneration in these pathological situations (Díez-Revuelta et al., 2010). Thus, promoting neurite outgrowth is assumed to be rudimentary for reconstructing the defective neural network (Gillespie 2003; Tohda et al., 2004). Neurite outgrowth in cultured neurons is considered as an indication of neuroregenerative potential (Mitchell et al., 2007). Therefore, finding new compounds that promote neurite outgrowth against the toxicity of H2O2 is a crucial approach in treatment of neurodegenerative diseases. The search is now for small molecules that can cross the brain-blood and induce neurite outgrowth. Here, we found that TEDMT, a triazine derivative that its ability to cross the blood brain barrier has been proved in our laboratory through artificial neural network (Tusi et al., 2010), improves neurite outgrowth and complexity. In the present study we found that H2O2 decreases neurite length, while in the presence of TEDMT, the cells could conserve the criteria of differentiation. The rescue of damaged neurons and the stimulation of neurogenesis are attractive strategies for the treatment of neurodegenerative diseases, because the lack of normal neurite outgrowth might reflect the dysfunction of molecules or proteins that are important for maintaining a normal neuronal process. Hence TEDMT possess neurite outgrowth promoting factors and helps PC12 cells challenged with oxidative stress, which suggests that it may be beneficial in AD.

From the other side, it has been shown that addition of NGF to PC12 cells causes the rapid and sustained activation of extracellular signal regulated kinases 1 and 2 (ERK1⁄2), which are members of the MAPK family. Sustained activation leads to neurite outgrowth and the development of many of the phenotypic characteristics of PC12 cells (Greene and Tischler, 1976; Kao et al., 2001; Rakhit, Pyne & Pyne, 2001). The NGF-mediated differentiation of PC12 cells also requires the participation of p38 MAPKs. A recent study showed that the inhibition of NGF-induced p38 MAPK activation blocked neurite outgrowth in PC12 cells, although the activation of ERK1⁄2 induced by NGF was essential for NGF-mediated p38 MAPK phosphorylation (Yung et al., 2008). These studies show that MAPKs pathway is essential to induce neurite outgrowth, however, in the present study we found that TEDMT treatment prior to H2O2 exposure, decreased phosphorylation of ERK, JNK and p38 MAPK which are induced by H2O2. It shows that depending on the time frame and the context of the experiment, MAPKs may play a protective or threatening role.

Oxidative stress induces a variety of cellular defense responses which are both genetically and non-genetically regulated. As another important cellular component in cell fate, we measured the level of Hsps. A wealth of information about Hsp90 inhibitors has been generated in cancer studies (Neckers, 2002; Maloney and Workman, 2002). Parallels with cancer studies can be drawn to neurodegenerative disorders where the detrimental consequences of an inefficient degradation of proteins are equally dramatic. In addition, recently beneficial effects of Hsp70 induction as a therapeutic approach in AD have been investigated as well (Ayala and Tapia, 2008; Kalmar and Greensmith, 2009). Since in our experiment, increase of HSP70 and decrease in the HSP90 amount are related to TEDMT treatment, this compound can be a promising agent in treating AD. Recent reports have revealed that HSP90 regulates key steps in the HSF1 activation-deactivation process. When Hsp90 levels are depleted by Hsp90 inhibition, HSF1 is released and is able to enter the nucleus and begin transcribing genes containing heat shock elements and reinforcing Hsp synthesis. Interestingly, we found that this compound could decrease HSP90 level in a dose-dependent manner. Our results supported the view that PC12 cells are particularly sensitive to compromised chaperone levels and that the pharmacological manipulation of chaperones might be a therapeutic strategy for delaying or preventing the progressive degeneration of PC12 cells (Behl and Schubert, 1993; Quigney, Gorman & Samali, 2003; Ahn and Jeon, 2006).

Although caution should be taken in extrapolating our results in PC12 cells to other cell types, it is noteworthy that TEDMT treatment leads to improve in neurite outgrowth and complexity. To further explore the neuroprotection potential of TEDMT, additional studies in models of primary neurodegenerative diseases are warranted that is now in progress in our laboratory.

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Abbreviations

AD, Alzheimer's disease; AO/EB, Acridine orange/ethidium bromide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; Hsps, heat shock proteins; MAPK, mitogen-activated protein kinase; NGF, Nerve growth factor; PBS, phosphate buffered saline; TEDMT, 3-thioethyl-5,6-dimethoxyphenyl-1,2,4-triazine.

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