Neurotoxicity and Neuroprotective Effects of Polyimides (PI) and Polyphenylenevinylene (PPV) against Hydrogen Peroxide (H_2O_2)

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ABSTRACT

Recently, research and development in the field of drug delivery systems (DDS) facilitating site-specific therapy has reached significant progression. DDS based on polymer micelles, coated micro- and nanoparticles, and various prodrug systems including water-soluble polymer have been prepared and extensively studied as novel drugs designed for cancer chemotherapy and brain delivery. Since polymers are going to be used in human, this study has the interest of testing two types of polymer, polyimides (PI) and polyphenylenevinylene (PPV) on neuronal cells. The objective of this study was to determine the possible neurotoxicity and potential neuroprotective effects of PI and PPV towards SH-SY5Y neuronal cells challenged by hydrogen peroxide (H_2O_2) as an oxidant. Cells were pretreated with either PI or PPV for 1 hour followed by incubation for 24 hour with 100 µM of H_2O_2. MTS assay was used to assess cell viability. Results show that PI and PPV are not harmful within the concentration up to 10 µM and 100 µM, respectively. However, PI and PPV do not protect neuronal cells against toxicity induced by H_2O_2 or further up the cell death.

Keywords: *Neurotoxicity, Neuroprotection, Polyimide (PI), Polyphenylenevinylene (PPV), and Hydrogen Peroxide*

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Introduction

Over the last decade, there have been major advances in understanding the mechanisms involved in the development of central nervous system (CNS) disorders. Simultaneous with these events, huge progress has made been made by researchers to identify the function of peptides and proteins as key molecules in the regulation of most physiological processes, both cellular and the molecular level. Peptides and proteins have turned out to be the main targets in neuropharmaceutical drug design for the treatment of a broad range of CNS disorders for example ischemia, inflammatory CNS diseases, neurodegenerative diseases, and acute and chronic pain syndromes [1 – 6]. Nevertheless, the inability of many existing peptide and protein drugs (P/P drugs) to effectively deliver and sustain within the brain have turned them to be ineffective for the treatment of these clinical problems. The main obstacle to targeting the brain with therapeutics in general (P/P drugs amongst them) is the presence of various barriers, in the first line the blood-brain barrier (BBB), which controls the concentration and entry of solutes into the CNS.

Currently, there is no effective treatment for the majority of brain diseases and it is a great challenge in designing potential P/P drugs with improved potency, selectivity and therapeutic efficacy for treating CNS disorders. The failure of treatment in some cases is due to the inability of P/P drugs to cross the BBB, which represents a limiting factor for the translation of drug research into clinical reality and commercialization. Other undetermined importance factors include the low stability and half-life of the P/P drugs in the blood, their non-selective accumulation, short residence time in the CNS and unspecific distribution of P/P drugs to target sites inside the brain [7].

Nowadays, research and development in the field of drug delivery systems (DDS) facilitating site-specific therapy has achieved significant progression. Safe and nontoxic formulations of a cytotoxic drug based on a polymer, its site-specific delivery and specific activation of the cytotoxic biologically active compound at its target (tumor tissue or tumor cell lines), have become the major goal of such research. DDS based on polymer micelles [8], coated micro- and nanoparticles [9], and various prodrug systems [10] including water-soluble polymer have been prepared and extensively studied as novel drugs designed for cancer chemotherapy and brain delivery [11, 12]. Since polymers are going to be used in human,

this research explored the effects of two types of polymers, polimides (PI) and polyphenylenevinylene (PPV) on neuroblastoma cell line, SH-SY5Y. Generally, the toxicity of these polymers is identified before proceeding to the neuroprotective test because PI and PPV are considered safe to be used if they are not toxic to the cells. This study used hydrogen peroxide (H_2O_2), as an oxidative stress that induced cell death.

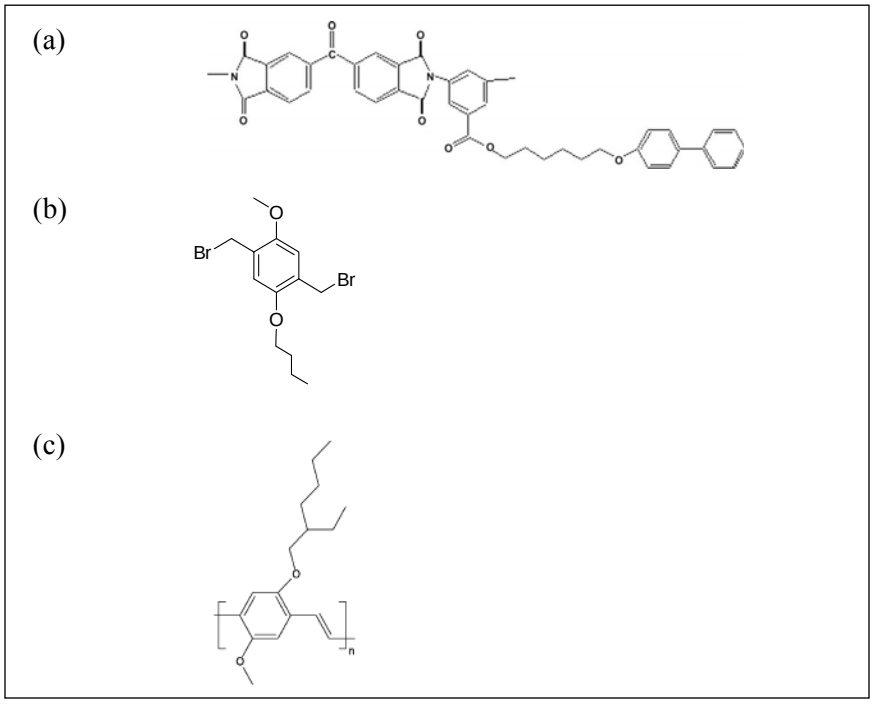

Figure 1: Chemical Structure of (a) Polyimides (PI). (b) Poly(1,4-bis(bromomethyl)-2-butoxy-5-methoxybenzene) (MBu-PPV). (c) Poly(2-Methoxy-5-(2'-Ethyl-Hexyloxy)-p-Phenylenevinylene) (MEH-PPV)

Materials and methods

Materials

Polyimides (PI) and polyphenylenevinylene (PPV) and its derivatives; poly(1,4-bis(bromomethyl)-2-butoxy-5-methoxybenzene) (MBu-PPV) and poly(2-Methoxy-5-(2'-Ethyl-Hexyloxy)-*p*-Phenylenevinylene) (MEH-PPV) were synthesized by Organic Synthesis and Biology laboratory, Institute of Science (IOS), Universiti Teknologi MARA (UiTM) Shah Alam, Selangor.

Cell Culture and Treatments

Human neuroblastoma SH-SY5Y cell lines were purchased from American Type Culture Collection (ATCC). The cells were adapted to grow in Dulbecco's modified Eagle's medium (DMEM) pH 7.4. 2×10^4 cells per ml were seeded in 96 well plates. The cells were differentiated to neural phenotype by adding retinoic acid (RA). After 24 hour, RA was added at a final concentration of 10 µM in MEM-F12 supplemented with 1 % glutamine, 1 % non-essential amino acids, 1 % gentamicin and 10 % heat activated fetal bovine serum in a humidified atmosphere containing CO_2 at 37 ºC. The media was changed after 3 days and cells were used at day 6.

Analysis of H_2O_2 Toxicity (IC_{50} Determination)

Dilutions of H_2O_2 in DMEM were made fresh just prior to each experiment. Small volumes of H_2O_2 were added to each well to make final dilutions of 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM, 10 mM, and 100 mM. The wells were then agitated lightly. Cultures were incubated in a humidified incubator at 37 ºC, 5 % CO_2 with H_2O_2 for 24 hour. Cell viability was assessed by MTS assay. The concentration that caused 50 % reduction of cell viability was determined.

Analysis of Neurotoxicity of PI and PPV

Prior to each experiment, PI and PPV dilutions were made fresh in MEM-F12. Small volumes of PI were added to each well to make final dilutions of 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml, 1 mg/ml and 10 mg/ml, while PPV was diluted to make the concentrations 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml. The wells

were agitated lightly. Cultures were incubated in a humidified incubator at 37 ^{0}C, 5 % CO_2 for 24 hour, and with PI or PPV for 1 hour and cell viability was assessed by MTS reduction assay.

Analysis of Neuroprotective Effects of PI and PPV Against H_2O_2-induced Cell Death

Differentiated SH-SY5Y cells were incubated with PI or PPV for 1 hour prior incubation with 100 μM H_2O_2 that cause 50 % cell viability (IC_{50} determination earlier) for 24 h. Cell viability was assessed by MTS assay.

Analysis of Cell Viability

Cell viability was assessed with Cell Titer 96* Aqueous Non-Radioactive Cell Proliferation Assay (MTS, Promega, USA). The MTS assay is a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)2-(4 sulfophenyl)-2H-tetrazolium (MTS) by dehydrogenase enzymes found in metabolically active cells (Chemicon, USA). The amount of colored formazan product is proportional to the number of viable cells. Briefly, 20 μl MTS solution was added to each well and incubated in humidified incubator at 37 ^{o}C in 5 % CO_2 for 2 - 4 hours in the dark. The quantity of formazan product present was determined by measuring the absorbance at 490 nm with a micro titer plate reader (GloMax Integrated Systems by Promega USA). Values were expressed as the percentage of optical density of control cells in the absence of H_2O_2 and/or PI or PPV.

Statistical Analysis

Each experiment was carried out in triplicates with at least 3 independent cultures with comparable results. Data was reported as mean ±SD of at least three experiments. Comparison between control and treatment of PI and PPV were made by Student's t-test. $P < 0.05$ was considered statistically significant.

Results and Discussion

H_2O_2 Toxicity

The human SH-SY5Y cell line, derived from child neuroblastoma, is commonly used as an *in vitro* model to study the related neurodenegerative diseases such as Parkinson's and Alzheimer's disease [13, 14]. This is due to the fact that the cells express several markers for those diseases, such as dopaminergic markers, including tyrosine hydroxylase (TH) and the dopamine transporter (DAT) [15, 16]. However, in undifferentiated state, the cells are immature neuroblasts that maintain the characteristics of stem cell and aggressively proliferate for a long period of time [15, 16]. Because of this reason, the cells are differentiated with retinoic acid (RA), either alone, or in combination with other agents, such as growth factors and phorbolester, to stop mitosis and enhance the neuronal and dopaminergic characteristics of the cells [15, 17]. In this study, the SH-SY5Y cell line was differentiated to neuron-like cells with RA alone before proceeding with the treatment at day 6.

Hydrogen peroxide (H_2O_2), a byproduct of oxidative stress derives from the enzymatic or spontaneous dismutation of superoxide anions, which are the byproducts of a wide and ubiquitous variety of oxidases [18]. H_2O_2 has been known for a long time to be toxic for numerous cell types [19, 20], [21]. Because of its high membrane permeability [22, 23] intracellularly formed H_2O_2 could induce its toxic effects not only within its cell of origin, but also in neighboring cells. Several findings indicate that neurons are particularly vulnerable to this mild oxidizing agent [24 – 27]. H_2O_2 directly stimulate endonuclease activity in renal tubular epithelial cells, which lead to DNA fragmentation and cell death [28]. In this study, we explored the effect of H_2O_2 on SH-SY5Y, the possible neurotoxicity and potential neuroprotective effects of PI and PPV by determining cell viability as measured by the MTS reduction assay.

MTS reduction assay has been used to reflect the number of viable cell [29]. Figure 2 shows the percentage of viable neuron-like cells after exposure to H_2O_2 as measured by the MTS assay. The result showed that at concentrations higher than 1 µM, H_2O_2 was toxic as it decreased the number of cell viability. At 100 µM, 50 % cell viability was observed. It was the IC_{50} for the SH-SY5Y cell line and the concentration was used in determining the neuroprotective effects of PI and PPV. Previous study by

Musalmah et al. [30] showed that H_2O_2 was cytotoxic at concentrations more than 100 µM as it decreased the cell viability as well as it increased the number of cell loss.

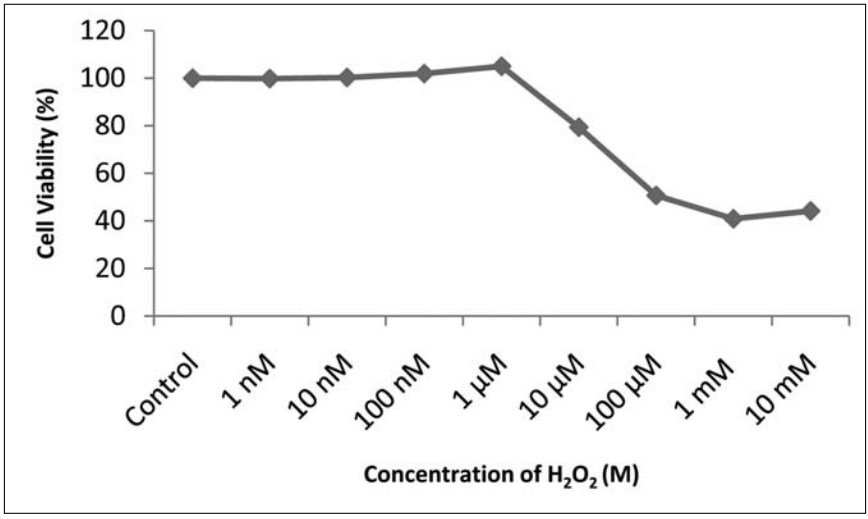

Figure 2: Effects of H_2O_2 Towards Viability of Neuron-like Cells as Assessed by the MTS. The Cells Line were First Differentiated and Cultured for Approximately 6 Days. The Cells were then Exposed to Increasing Concentrations of H_2O_2 for 24 Hour at 37 °C. No Significant Difference in Viable Cell Number between Control and H_2O_2 was Observed when the Concentration of H_2O_2 was Less than 1 µM. Cell Viability Decreased by 50 % when Exposed to 100 µM of H_2O_2. The IC_{50} of H_2O_2 for SH-SY5Y Cell Line was Found to be 100 µM. Data is Presented as Means ±SD (n = 3).

PI and PPV Toxicity

Figure 3 shows the neurotoxicity of PI on SH-SY5Y, as assessed by the MTS assays. Treatment with PI for 24 hour did not result in significant cell loss for a concentration range from 1 ng/ml to 10 mg/ml. The result shows that no significant difference in cell viability observed compare to control. A study by Kontush [31] showed that, Amyloid-β peptide, an antioxidant, has been reported to become a pro-oxidant at high concentration. The present data showed that high concentration (100 µg/ml) of PPV (MBu and MEH), but not PI, is toxic to the cells. The result (Figure 4) shows no significant difference in cell viability observed compared to control for

the concentration range from 1 ng/ml to 1 µg/ml for either MBu-PPV or MEH-PPV. There were more than 50 % reductions in viable cell number at concentration 100 µg/ml of both MBu-PPV and MEH-PPV. On the other hand, incubation with PI did not result in significant reduction in the number of viable cells up to 10 mg/ml concentration. However, at 10 µg/ml of MBu-PPV and MEH-PPV, the cell viability was more than 100 %. Further studies need to be done in order to understand why there is a huge reduction from concentration of 10 µg/ml to 100 µg/ml of MBu-PPV and MEH-PPV.

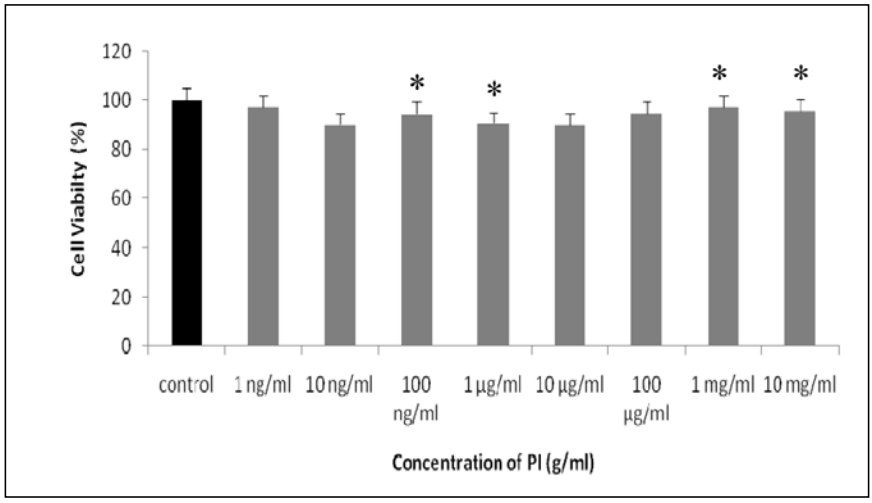

Figure 3: Neurotoxicity of PI on SH-SY5Y. SH-SY5Y Cell Line was Incubated with Increasing Concentrations of PI for 24 Hour at 37 ºC. Prior PI Addition, Slight Difference in Cell Viability was Observed for a Concentration Range from 1 ng/ml to 10 mg/ml. The Results Show that no Significant Difference in Cell Viability Observed Compared to Control. *Denotes $P < 0.05$ Compared to Controls. Data are Presented as Mean ± SD, from 3 Independent Experiments of Triplicate Wells (n = 3)

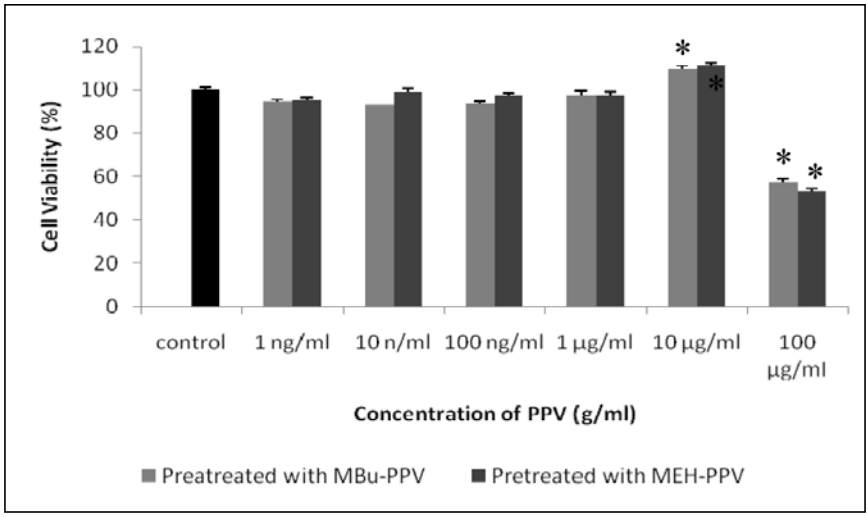

Figure 4: Neurotoxicity of PPV on SH-SY5Y. SH-SY5Y Cell Line were Incubated with Increasing Concentrations of PPV for 24 h at 37 ºC. The Result Shows that no Significant Difference in Cell Viability Observed Compared to Control for the Concentration Range from 1 ng/ml to 1 µg/ml for either MBu-PPV or MEH-PPV. A Slight Increase in Cell Viability was Shown at the Concentration of 10 µM for both MBu-PPV and MEH-PPV. However, there was a Huge Reduction in Cell Viability at the Highest Concentration, 100 µg/ml. *Denotes $P < 0.05$ Compared to Controls. Data are Presented as mean ± SD, from 3 Independent Experiments of Triplicate Wells (n = 3)

Neuroprotective Effects of PI and PPV on H_2O_2-Induced Cytotoxicity

Figure 5 and 6 show the neuroprotective effects of PI and PPV against H_2O_2-induced cell loss in SH-SY5Y cell line, respectively. Cell viability was determined using the MTS reduction assay. The results show that both compounds, PI and PPV, were incapable to protect against cell loss and did not further up the cell death at concentration range from 1 ng/ml to 10 mg/ml and 100 µg/ml, respectively. Previous study reported that a compound can be considered in having a potential neuroprotective effects if the results lay in between positive and negative results [32]. As seen on the data, all concentrations did not have a potential neuroprotective effect towards neuron-like cells. However, both PI and PPV also do not cause further cell death after H_2O_2 treatment. This indicated that PI and PPV had

no ability to protect neuronal cells against H_2O_2-induced cell loss or either further up the cell death.

Earlier study by Kang *et al.,* [33] used SH-SY5Y human neuroblastoma cells to test the neuroprotective effects of four structurally related flavones: wogonin, chrysin, apigenin, and luteolin against oxidative stress-induced cell death. Luteolin and apigenin significantly inhibited cell death induced by hydrogen peroxide, resulting in an increase in the cell. Chrysin at a concentration of 50 μM was able to inhibit cell death but by far lesser extent than that of luteolin and apigenin. Wogonin, however, had no effect on hydrogen peroxide-induced cell death.

As reported earlier, the high biodegradability by gastrointestinal, plasma and tissue peptidases are among the factors of low bioavailability of peptides. Various chemical strategies have been developed to try and overcome the limitations of peptides to increase their in vivo plasma residence time [34]. With further studies, PI and PPV could be the candidates to carry peptides to overcome the limitation of peptides to increase their in vivo plasma residence time or as prodrugs for improved CNS delivery.

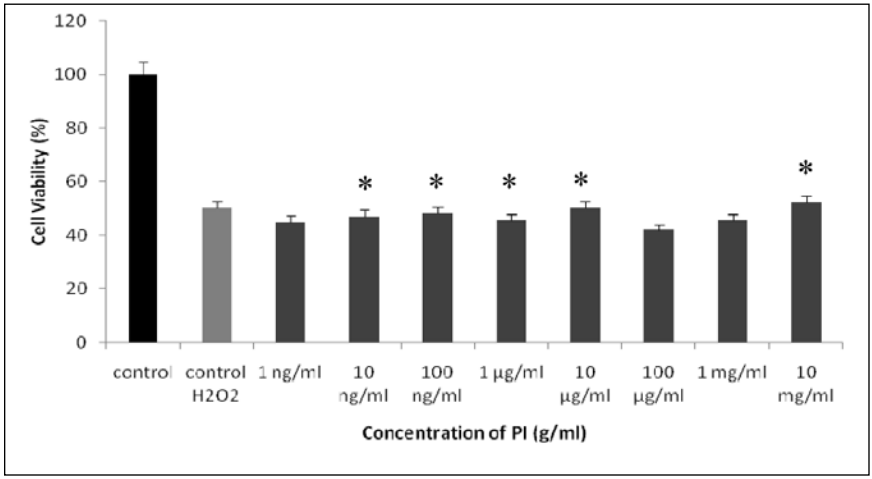

Figure 5: Neuroprotective Effects of PI against H_2O_2-induced Cell Loss in SH-SY5Y Cell Line. Cells were Pretreated with Different Concentrations of PI for 1 Hour before Exposure to 100 μM H_2O_2 for 24 hour at 37 ºC. Cell Viability was Determined using the MTS Reduction Assay. From the Result, it shows that this Compound was Incapable to Protect against Cell Lost or Further Killing the Cells at a Concentration Range from 1 ng/ml to 10 mg/ml. *Denotes $P < 0.05$ Compared to Control. Data is Presented as means ±SD, n = 3

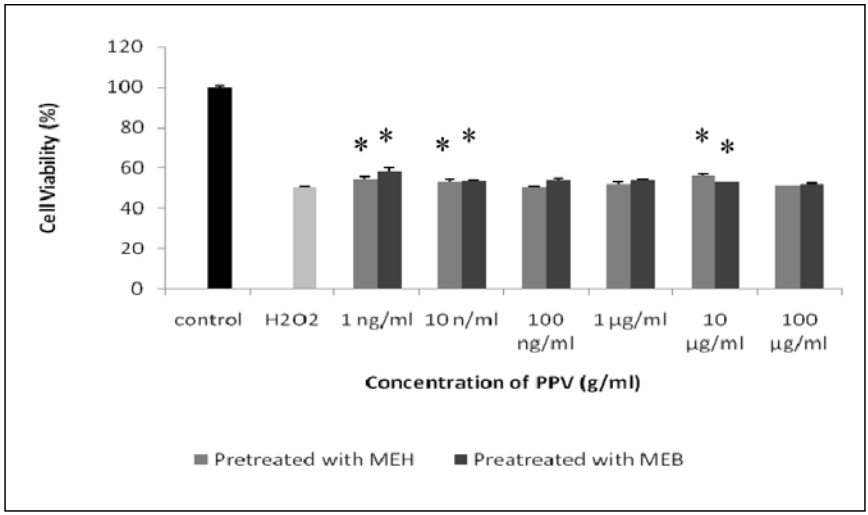

Figure 6: Neuroprotective Effects of PPV against H_2O_2-induced Cell Loss in SH-SY5Y Cell Line. Cells were Pretreated with Different Concentrations of PPV (either MBu or MEH) for 1 Hour before Exposure to 100 µM H_2O_2 for 24 hour at 37 ºC. Cell Viability was Determined using the MTS Reduction Assay. The Result shows that MBu-PPV and MEH-PPV were Incapable to Protect against Cell Lost or Further Up the Cell Death at Concentration Range from 1 ng/ml to 100 µg/ml. *Denotes $P < 0.05$ Compared to Control. Data is Presented as means ±SD, n = 3

Conclusion

The present findings showed that both PI and PPV are not toxic to the SH-SY5Y cells, and could not protect the cells against H_2O_2-induced cell injury as well as did not further up the cell death after H_2O_2 treatment. In addition, PI and PPV are safe to be used, as they are not toxic to the cells up to concentration of 10 mg/ml and 10 µg/ml, respectively. More studies need to be done such as apoptosis and histochemistry in order to understand the possible neurotoxicity of PI and PPV to be the candidates in the drug delivery system.

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