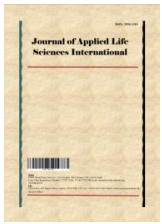

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The Roles of Hydrogen Peroxide Exposure in the Toxic Aggregation of Alpha-synuclein and Translocation of DNA Methyltransferase-1 in Human Neuroblastoma Cell Model of Parkinson's Disease

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Authors' contributions

This work was carried out in collaboration among all authors. Author OAO designed the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Authors SGM and KMJ both assisted in critical editing of the manuscript. Author DPS managed the literature searches and managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Background: Oxidative stress has been implicated in neuronal damage in Parkinson's disease (PD). However, the specific roles of reactive oxygen species such as Hydrogen peroxide (H_2O_2) and Iron in the pathogenesis of PD especially, alpha-synuclein (α-Syn) aggregation and translocation of nuclear DNA Methyltransferase-1(Dnmt1), are yet to be fully understood.
Aims: This study investigated and compared the effects of H_2O_2 and ferrous iron (Fe^{2+}) on α-Syn aggregation and localization of Dnmt1 in human neuroblastoma cells (SH-SY5Y), using a Parkinson's disease model expressing A53T mutation and wild typed (WT) α-Syn respectively.
Materials and Methods: The study was done using CellTox™ assay, Immunocytochemical and Enzyme-linked immunosorbent Assay (ELISA) methods. Statistical analysis of triplicate data were analysed on Microsoft Excel 2010 and Stats Direct© using one-way analysis of variance (ANOVA) and Dunnet comparison tests.
Results: Specifically, 100 µM of H_2O_2 caused significant reduction of cell viability, translocation of Dnmt1 from nucleus into the cytoplasm and expression of relatively higher amount of α-Syn proteins, compared to 500 µM iron after 24 hours treatment. H_2O_2 elicited the highest expression of

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both WT α-Syn (13.7 ± 0.5) ng/ml and (16.0 ± 0.2) ng/ml A53T α-Syn proteins respectively. While Iron caused the expression of (9.1± 1.1) ng/ml and (14.8 ± 1.1) ng/ml of WT and A53T α-Syn proteins respectively. The untreated controls expressed (3.2 ± 0.1) ng/ml and (7.5 ± 0.0) ng/ml of WT and A53T α-Syn proteins respectively. Furthermore, the A53T mutation also promoted the expression and aggregation of α-Syn, as evidenced with the relatively higher amount of A53T α-Syn protein compared to WT α-Syn expressed in control, H_2O_2 and Iron treated cells.

Conclusion: This study demonstrated that H_2O_2 and Fe^{2+} induced α-Syn aggregation and Dnmt-1 translocation, which promotes the pathogenesis of Parkinson's disease. Likewise, the A53T genetic alterations increased the overexpression and aggregation of α-Syn proteins. Hence, novel therapies targeting reactive oxygen species, oxidative stress and mutations may be beneficial for long term treatment of Parkinson's disease.

Keywords: Alpha-synuclein (α-Syn); DNA methyltransferase (Dnmt1); parkinson's disease; hydrogen perioxide (H_2O_2); A53T mutation; wild type (WT); neurotoxicity.

1. INTRODUCTION

Neurological diseases such as Parkinson's diseases (PD) and Dementia with Lewy bodies (DLB) account for about 4% of all deaths worldwide and are often unrecognized, misdiagnosed or ignored as a minor concern in the early stages of diseases [1]. PD is characterized by loses of neurones or degeneration and deposition of cytoplasmic inclusions called Lewy bodies(LB), which mainly contains misfolded, alpha synuclein (α-Syn) aggregates [2]. Although, Alpha-synuclein (α-Syn) aggregation in the brain is been identified as an important pathophysiological characteristic of PD and other neurodegenerative conditions [3,4]. However, the causes of PD are not fully understood, hence the role of environmental neurotoxicants in α-Syn aggregation has recently been of much concerns, given that aggregated α-Syn mediates neurotoxicity in neurons and glial cells [1]. Also, the molecular triggers responsible for initiating and/or propagating α-Syn aggregation and toxicity are poorly understood [5].

Previous post-mortem studies of brain samples from PD patients and α-synuclein transgenic mice showed a reduced level of nuclear DNA methyltransferase-1 [6]. The transfer of nuclear DNA methyltransferase-1(Dnmt-1) enzymes to the cytoplasm results in decreased DNA methylation of α-Syn(*SNCA)*gene at its CpG promoter site with resultant overexpression of α-Syn proteins in presynaptic nerve endings and possible aggregation of α-Syn [6,7]. These events suggest the propensity of α-Syn aggregation increasing with the cytoplasmic sequestration of Dnmt1 [7]. The study of the interaction of metal ions with α-Syn demonstrated that metals ions alter the structure and environment of α-Syn near its tyrosine residue by increasing hydrophobicity or decreasing the net charges and promotes partial folding, as well as the aggregation of α-Syn in *in vitro* and cell culture models of synucleinopathies [8,9]. Previously, a systematic study demonstrated that divalent metal cations such as Fe (II) significantly accelerate α-Syn *in vitro* aggregation under physiological conditions [9]. Similarly, Fe (II) stimulates α-Syn aggregation by inhibiting Nrf2/heme oxygenase-1 (HO-1) [10]. Hence, it might be possible that other chemical agents or environmental conditions that facilitate translocation of nuclear Dnmt1 into the cytoplasm might contribute to α-Syn aggregation and development of PD.

Oxidative stress has been implicated in the etiology of several disease conditions [11], and in the pathogenesis of many neurodegenerative diseases including PD [12,13]. Hydrogen peroxide (H_2O_2), a neutral charged reactive oxygen species that can be produced intracellularly as a metabolite of superoxide (O_2^{2-}) by superoxide dismutase, can also readily pass through cell membrane to produce oxidative stress [14]. At certain concentrations beyond the antioxidant capacity of the cell, reactive oxygen species (ROS) have the potential of altering α-Syn protein structure, leading to cell death due to oxidative stress [15,16,17].

H_2O_2 is commonly used at low concentration (approximately 0.29-1.8M) in several personal care and domestic products such as alcohol-free mouth wash/toothpaste, multi-surface stain cleaner, laundry detergents, general disinfectant, and chlorine-free bleach as well as in peroxide-based hair dyes, hence non-occupational exposure of human to small amounts of H_2O_2 may occur through the use of such products [18]. Also, human exposure to large quantities of hydrogen peroxide is most likely to occur at occupational settings, where high concentrations of H_2O_2 (typically above 10.2M or 35%) are used

in many industrial applications [19]. Although direct neurotoxicity arising from usage or exposure to products with mild to high contents of H_2O_2 is yet to established, however, using ultrasensitive nanoplasmic detection, H_2O_2 from external sources was shown to exert oxidative stress that significantly caused a two-fold increase in α-Syn *in vitro* aggregation, even though these *in-vitro* findings, may not directly reflect *in vivo* or intracellular effects of H_2O_2 on the aggregation of α-Syn [20].

Although, H_2O_2 is a major cause of oxidative stress and neural induced cell death [21]. However, the dose-dependent neurotoxicity of H_2O_2 is yet to be clearly established in human neuronal cell lines. For instance, [22] and [23] demonstrated low concentrations of H_2O_2 may be beneficial in the cells by initiating cell proliferation/differentiation, activating certain enzymes and transcription factors. Furthermore, H_2O_2 could modulate the expression of antioxidant genes and is generated as a toxin against invading microbes, when phagocytes activate NADPH oxidase to engulf the microbes [24]. Furthermore, the intracellular concentration of H_2O_2 seems to be tightly controlled by the cellular antioxidant defence system [25]. This may explain why endogenously secreted H_2O_2 in human embryonic kidney cells, had a negligible neurotoxic effect in term of α-Syn aggregation [20]. However, [21] reported that cellular exposure to H_2O_2 as low as 30-50 μM below the physiological concentration of 100 μM caused significant oxidative stress and death in human neuroblastoma cells lines. While [26] reported H_2O_2 above physiological concentration at 200 μM resulted in cell death in cultured human SH-SY5Y cells by the activation of the Ras signalling cascade. Other studies suggest prolonged cellular exposure to H_2O_2 might exert deleterious neurotoxicity of oxidative damage, lipid peroxidation, disruption of membrane integrity, cell death arising from dysfunctional cellular functions and increased expression of pro-oxidants associated with apoptosis and necrosis due to its conversion to hydroxyl radicals which may damage several cell components in neuronal functions [27,28]. It is, however, unclear if the effects of H_2O_2 are solely dependent on dose or duration of exposure or whether other environmental or genetic factors have a role in these observed differences in the effect of H_2O_2.

Although, abnormality in subcellular localization of proteins important for the signaling, metabolic or structural properties of the cell have been linked to human diseases involving cell metabolism and protein aggregation including neurodegenerative diseases [29]. However, the role of H_2O_2 in the translocation of nuclear Dnmt1 to the cytoplasm as well in the aggregation of α-Syn in a well-characterized *in vitro* cell model of PD such as SH-SY5Y neuronal cell line [30], is yet to be clearly established. Hence, it is important to investigate the role of oxidative stress arising from H_2O_2 exposure.

Having previously established the induction of α-Syn aggregation by metal ions especially Fe(II) in SH-SY5Y neuroblastoma cells line [31], this study therefore used Fe (II) as a positive control in investigating the role of H_2O_2 in aggregation of α-Syn and translocation of Dnmt1 proteins in the PD *in vitro* model of human SH-SY5Y neuroblastoma cells line. The pathological feature of α-synuclein aggregation in PD, was mimicked using the SH-SY5Y neuroblastoma cells line overexpressing Wild Type (WT) α-syn, as well as those with stable expression of A53T mutations in the α-syn were used [32]. It was hypothesized that neither the overexpression of WT α-syn, nor stable expression of A53T mutant α-syn leads to increased formation of inclusions. While exposure to H_2O_2 over a period of time may promote the death of neuronal cells, *in vitro* aggregation of α-Syn and out-of nucleus translocation of Dnmt1 proteins as observed in PD.

2. MATERIALS AND METHODS

2.1 WT and A53T SH-SY5Y Neuronal Cell Line

Human neuroblastoma SH-SY5Y cells expressing WT and A53T α-Syn were obtained from the Department of Biosciences, Sheffield Hallam University, United Kingdom. The WT α-Syn expressing human neuroblastoma SH-SY5Y cells were then cultured and routinely maintained at split ratio of 1:4 in regular minimum essential media (MEM) (Life Technologies) supplemented with 2 mM L-Glutamine, 10% foetal Calf Serum (FCS) (Life Technologies), 1% Non-Essential Amino Acids (NEAA), and 1% Penicillin/ Streptomycin (Life Technologies).

A53T α-Syn overexpressing SH-SY5Y cell lines (Sigma, UK), were maintained at a split ratio of 1:3 in media as described for cells expressing the WT α-Syn. However, the 1% Penicillin/Streptomycin was replaced with 500 μg/mL of G418 sulphate (Sigma, UK), to select for cells containing the vector [32].

2.2 Preparation of Iron for Cell Treatment

Both WT and A53T cell lines were treated with Iron (II) Chloride ($FeCl_2$). Initial stock solutions of $FeCl_2$ (Sigma, UK) were prepared at a concentration of 500 mM in deionised water. Nitrilotriacetic acid (NTA) was added to the $FeCl_2$ stock solution at a ratio of 2:1 to prevent precipitation of the ferrous iron. The stock was filter sterilised with 5 ml Millex® sterile syringe filter (Sigma, UK) and then diluted 1000 fold with appropriate complete media to obtain a working solution used for treating cells, at a concentration of 500 µM as a standard positive control.

2.3 Plating out of Cells and Treatment with H_2O_2

Aqueous solutions of 30% (weight/volume) of stock Hydrogen peroxides (Sigma, UK) was serially diluted with complete Minimum Essential Media (MEM) to obtain working H_2O_2 concentrations of 200 µM, 100 µM, 32.6 µM and 0 µM.

The SH-SY5Y cells expressing WT and A53T α-Syn were plated out at 300 µL of 10,000 cells per well in 96 well plates and chamber slides. While 100,000 cells per well was plated in 6 well plates. The plated cells were incubated under sterile conditions at 37°C for 24 hours in Thermo Scientific ^{TM} CO_2 incubators (ThermoFisher, UK), to allow for adherence.

After 24 hours incubation, undifferentiated SH-SY5Y cells were treated with 300 µL working concentrations of diluted H_2O_2 and $FeCl_2$ respectively to induce oxidative stress. The untreated control cells expressing WT and A53T α-Syn had 300 µL of complete media .Both pre-treated and untreated control cells were incubated at 37°C for 24 hours, in Thermo Scientific^{TM} CO_2 incubators (ThermoFisher, UK) .Cytotoxic assays and immunoflourence staining were then carried out.

2.4 Determination of Optimal Cytotoxic Concentration of H_2O_2 using Celltox™ Green Assay

To determine the optimal cytotoxicity of H_2O_2 in SH-SY5Y cells, CellTox™ green cytotoxic assay (Promega, USA) was used. The procedure was performed according to the method described by [33] with slight modifcation. Using 25 µL of x1 CellTox^{TM} reagent, added to cells in each wells of the 96 well plates. Which was shielded from ambient light to prevent degradation of CellTox^{TM} reagent. Cells were incubated at room temperature for 15 minutes after mixing using an orbit shaker at 750 rpm for a minute to ensure homogeneity. The cytotoxicity was determined by measuring fluorescence intensity at 490nm excitation and 525 nm emission in a fluorescence plate reader (ThermoScientific Multiskan Ex).

2.5 Immunocytochemistry

In order to investigate the localization of Dnmt1 and aggregation of α-Syn, the H_2O_2 and $FeCl_2$ pre-treated A53T and WT cells were washed with Tris buffered saline (TBS) and fixed with 70% ice-cold methanol (ThermoFisher, UK). Cells were then washed in TBST containing 0.1% Tween-20 in 1x TBS. Unspecific binding sites was blocked using 1% bovine serum albumin (BSA) in TBS. After blocking for 1 hour, cells were washed in PBS-T, cells were further incubated at room temperature (RT) for 1 hour on a shaker with primary antibody 1 uL mouse anti-α-synuclein syn211 monoclonal antibody [(Fisher; AFMA112874) or 2 uL rabbit anti-Dnmt1 polyclonal antibody (Abcam; AB19905)] in 1:2000 diluted with 1% BSA in 2 ml TBS. Background fluorescence was assessed by omitting primary antibodies in certain chambers.

The cells were then washed with TBS-T, and incubated with agitation at room temperature for 1 hour in secondary antibody [1 ul Texas Red® goat anti-mouse polyclonal IgG H+L (Invitrogen; T6390) or 1 ul Alexafluor ® donkey anti-rabbit polyclonal IgG (life technologies; A10043)] at 1:1000 diluted with1% BSA in 2 ml. Secondary antibody incubation with of cells was shielded from ambient light to prevent photo bleaching.

The cells were stained with 0.01 µg/mL DAPI nuclear stain and washed with 1x TBST before mounting with immersion oil (Sigma; 56822), then covers slips added and then stored for about 24 hour in the dark at 4°C to prevent degradation of fluorophore signal and photo bleaching.

Immunostained cells were visualised using Olympus BX60 fluorescent microscope of x 400 magnification (Olympus, USA).Image overlays, contrast and brightness were altered on Ms Photoshop (Microsoft. USA).

2.6 Protein Extraction

After 24 hours of H_2O_2 and $FeCl_2$ treatment of SH-SY5Y cells in 6 well plates, spent media was removed and washed with 1x PBS. Cells were

incubated with 1 mL Cell Lytic™ M reagent (Sigma,UK) with 1 µL of 5M PMSF in isopropanol at 4°C on an MaxQ^{TM}2000 bench top orbit shaker(ThermoFisher, UK). Cells were then collected by cell scraper (ThermoFisher, UK) and centrifuged at 4°C for 15 minutes at 13,000 rpm in a sigma refrigerated centrifuge (Sigma, UK). The supernatant were collected and stored at -20°C.

2.7 Bicinchoninic Acid (BCA) Assay

To determine the concentrations of total protein extract, a range of protein standards was prepared by diluting Bovine Serum Albumin (BSA) (ThermoFisher, UK) with CellLytic™ M reagent (Sigma, UK).Protein standards were at 5, 2.5, 1.25, 1, 0.75, 0.5, 0.25, 0.1 mg/mL. BCA reagent was prepared using a 50 fold diluted 4% copper sulphate solution (w/v) (ThermoFisher, UK) in Bicinchoninic acid (Sigma, UK). 10 µL of standards and the samples to be analysed were added to separate wells of a 96 well plate and 200 µL of BCA reagent also added to each well. The plate was swirled to mix then incubated at room temperature for 30 minutes. Absorbance was recorded on a plate reader (ThermoScientific Multiskan Ex) at 570 nm. Total protein concentrations of samples were derived from protein standard curve.

2.8 ELISA Determination of α-Syn Protein

The Human α-Syn standard (Fisher Scientific, UK), was serially diluted with 250 µL standard diluents buffer to 15, 7.5, 3.75, 1.88, 0.94, 0.47, 0.23 and 0 ng/mL. The protein samples were also diluted in 1:5 with standard diluent buffer. While x25 wash buffer was diluted with deionized water to x1.While all protocols were according to manufacturer's instructions(Fisher Scientific, UK).The 10uL of x100 anti rabbit immunoglobin (IgG) was added to each well strips containing 1mL HRP diluents and thoroughly mixed to a x1 final concentration .

To ensure the binding of antigen to pre-set primary antibodies, 50 µL of standard buffer and protein standard were added to wells of Elisa kit (Sigma.UK), except controls. Further 50 µL protein samples and control were added to wells. 50 µL of α-Syn detection antibodies was further added to all wells except the blank controls. Plated samples were mixed and incubated at room temperature (RT) for three hours to enable detection of α-Syn antibodies.

Solutions in the well was removed and washed with x1 wash buffer. 100 µL of x1 anti rabbit IgG HRP-conjugate antibody was added to each wells except the blank controls to enable secondary antibody conjugation to primary antibody. The ELISA plate was then incubated at RT for 30 minutes. The solution was removed and washed with x1 wash buffer.100 ul stabilized chromogen was added to each well for coloured antibody detection and incubated for 30 minutes in the dark. An 100 µL stop solution was then added to each samples to terminate the reaction and absorbance read in a plate reader (ThermoScientific Multiskan Ex) at 450 nm. The unknown protein concentrations of samples were derived from protein standard curve.

2.9 Statistical Analysis

The mean ± standard deviations (SD) of triplicate data were analysed on Microsoft Excel 2010 and StatsDirect© using one-way analysis of variance (ANOVA) and Dunnet comparison test to compare Hydrogen peroxide (H_2O_2) treated group and control. While mean ± SD of duplicates experiments (n=2), were analysed with Microsoft Excel 2010 and student T-test. Statistically significance was established at $P<0.05$.

3. RESULTS

3.1 Determination of the Effect of Various Concentrations of H_2O_2 on the Viability of SH-SY5Y Cells

To determine the optimum concentration of H_2O_2 effective in reducing cell viability of WT and A53T expressing SH-SY5Y cell lines, the cell viability was assessed using CellTox^{TM} cytotoxicity assay (Promega, USA). Fig. 1 shows the mean absorbance of WT and A53T cells, treated for 24 hours in triplicates, with 300 µL of H_2O_2 at 32.68, 100, 200 µM and 0 µM (lytic control).The cytotoxicity concentration was presented as percentage of the lytic control's absorbance [26]. Fig. 2 indicates H_2O_2 at 32.68, 100 and 200 µM significantly decreased cell viability by 24%, 50%, and 56% in WT cells. While in A53T cells, cell viability decreased by 9%, 49% and 55% of the untreated (0 µM) lytic control values, respectively .Fig. 2 also indicates 100 µM of H_2O_2 reduced cells' survival by 50% and 49% in WT and A53T SH-SY5Y cells respectively. Hence 100 µM H_2O_2 was used for subsequent treatment of the WT and A53T expressing cells SH-SY5Y cells.

To determine the statistical significance and comparison of the effects of H_2O_2 on viability of

WT and A53T SH-SY5Ycells,one-way ANOVA and Dunnett post Adhoc analysis of the absorbance from H_2O_2 treated WT and A53T SH-SY5Y cells was performed. The results in Fig. 2, further indicates 32.68, 100 and 200 µM of H_2O_2 significantly increased the cell cytotoxicity in both WT and A53T SH-SY5Y cells in a dosage dependent manner, compared to the lytic controls. However, no significant differences was observed in the H_2O_2 induced reduction in cell viability of WT cells compared to A53T cells, using ANOVA one way analysis at 95% confidence level. The results were expressed as mean percentage cytotoxicity ± standard error (SEM) of triplicate experiments (n=3).

3.2 Immunocytochemical (ICC) Detection of α-syn Aggregation and in H_2O_2 and $FeCl_2$ (Fe^{2+}) Treated SH-SY5Y Cells

3.2.1 Localization and aggregation of α-Syn within the cytoplasm of Iron and H_2O_2 treated SH-SY5Y cells

Previous study suggests that under normal physiological conditions, *in vitro* α-Syn aggregation is more favourable and may not be true representation of *in vivo* [34]. Hence, further study using H_2O_2 treatment is necessary. The induction of α-Syn aggregation were established in iron (Fe) treated SK-N -SH neuroblastoma cells [10,31]. There are however reported disparity in the translocation of α-Syn, from plasma membrane to cytoplasm [35], from cytoplasm to the nucleus [36] and from cytoplasm onto mitochondria surface [37]. Given that it is still unclear how cellular localization of α-syn affects its pathology and where the aggregation of α-syn is initiated [38], this study therefore, used immunofluorescence staining and fluorescence microscopy to investigate the localization and aggregation of α-Syn in Fe^{2+} and H_2O_2 treated WT and A53T overexpressing SH-SY5Y cells, using anti- α-Syn monoclonal primary antibody and Texas red secondary antibody.

The result shown in the fluorescence microscopy images at x400 magnification (Figs. 2a and 2b) indicates both SH-SY5Y cells expressing WT and A53T α-Syn treated with H_2O_2 and Fe^{2+} had aggregation of α-Syn accumulated within the cytosol as indicated with arrows diagrams. While the untreated control SH-SY5Y cells overexpressing WT and A53T mutations showed the absence of α-Syn in the nucleus but rather dispersed α-Syn around the cytosol. No nuclear staining of α-Syn was observed in both $Fe^{2+}$and H_2O_2 treated SH-SY5Y cells as well as in the untreated control.

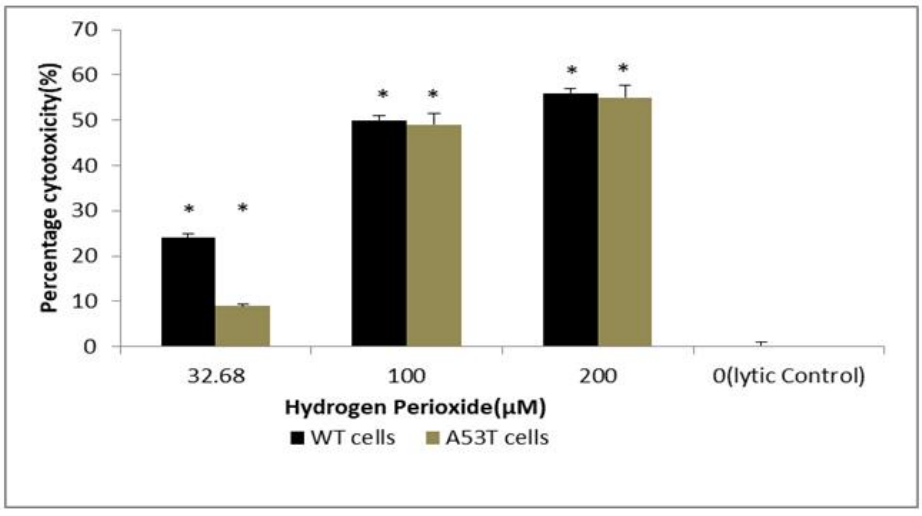

Fig. 1. Comparison of the effect of H_2O_2 induced increase in cytotoxicity in SH-SY5Y cells expressing WT and A53T mutations. The cultured SH-SY5Y cells were treated with H_2O_2 at 32.68,100,200 µM for 24 hours. Cytotoxicity was assessed using CellTox™ assay and presented as percentage of untreated (0 µM) control. Cytotoxicity significantly increased in both WT and A53T as H_2O_2 concentration increases from 32.68, 100 to 200 µM compared with lytic control (*p<0.05). However no significant difference in cytotoxicity of H_2O_2 treated WT cells compared to the treated A53T SH-SY5Y cells. The results were expressed as means ± SEM of triplicate experiments analysed with one way ANOVA

3.2.2 Cytoplasmic translocation of Dnmt1 in iron and H_2O_2 treated SH-SY5Y cells

The role of H_2O_2 in translocation of nuclear Dnmt1 to the cytoplasm was investigated with ICC to determine the localization of Dnmt1 in Fe^{2+} and H_2O_2 treated SH-SY5Y cells. Using anti-dnmt1 polyclonal primary antibody and Alexa fluor secondary antibody under similar conditions to those described above for the immunostaining of α-Syn. This study demonstrated that H_2O_2 caused the movement of Dnmt1 from the nucleus into the cytoplasm, similar to the observation in Fe^{2+} treated cells. As shown in Figs. 3a and 3b there was no nuclear staining of Dnmt1 in H_2O_2 and Fe^{2+} treated A53T and WT cells, unlike the untreated cells with nuclear stained Dnmt1. Furthermore, accumulation and localization of Dnmt1 within the cytosol of both $FeCl_2$ and H_2O_2 treated SH-SY5Y cells was observed as indicated by arrowed diagrams (Figs. 3a and 3b). While Dnmt1 was dispersed and localized within the nucleus in untreated SH-SY5Y cells.

The Fig. 3b indicates cytosolic translocation of Dnmt1 in WT SH-SY5Y cells.

3.3 Quantification of Total Protein and α-Syn Expressed in H_2O_2 and $FeCl_2$ Treated SH-SY5Y Cells

3.3.1 Increased expression of total protein by H_2O_2 treated SH-SY5Y cells

Considering that the interactions of α-Syn with some other proteins such as synphilin-1 have being found to play an essential role in regulating the cytosolic aggregation of α-Syn and formation of Lewy body inclusions [39,40]. Hence, in order to investigate and normalize the level of α-Syn expressed by WT and A53T cells, the total proteins expressed by Fe^{2+} and H_2O_2 treated SH-SY5Y cells, was determined with Bicinchronic acid (BCA) assay using Bovine serum albumin (BSA) protein standard. Fig. 4 shows protein concentrations of treated and untreated cells as derived from the BCA standard curve and expressed as mean ± SD. Fig. 4 also indicates H_2O_2 treated WT cells expressed the highest amount of total proteins at 3.4 ± 0.4 mg/ml in the cell lysates, compared to the H_2O_2 treated A53T cells with total protein of 2.2 ± 0.1 mg/ml. However, Fe^{2+} treated WT SH-SY5Y cells expressed the same amount of total protein at 2.8 ± 0.6 mg/ml as the A53T Fe^{2+} treated cells at 2.8 ± 0.4 mg/ml. However, more proteins were expressed in untreated A53T cells with higher total protein of 1.5 ± 0.3 mg/ml compared to the untreated WT SH-SY5Y cells expressing a lower total protein of 1.3 ± 0.4 mg/ml. Although treatment with 500 µM $FeCl_2$ and 100 µM H_2O_2 increased total protein expression compared to the untreated controls in WT and A53T cells. However no significant difference between the total proteins expressed by WT and A53T cells when compared by unpaired T-test ($P<0.05$, where n=4).

3.3.2 Increased expression of α-Syn protein in H_2O_2 treated SH-SY5Y cells

Cytosolic α-Syn aggregates were detected by immunofluorescence staining in this study. Similarly, previous study by [41], identified the presence of α-Syn containing exosomes in cell media. Likewise,α-Syn released by SH-SY5Y cells was also previously recovered by centrifugation of cell culture [42]. Hence, the amount of α-Syn expressed by H_2O_2 and Fe^{2+} treated SH-SY5Y cells, was quantified in their cell lysate and spent media, using Enzymes Linked Immunosorbent Assay (ELISA). The assay was optimized using 50 µL Human α-Syn standard (ThermoFisher, UK) at different concentrations and the absorbance of 50 µL of various protein samples diluted in 1:5 with standard diluent were read. The generated standard curve was used to determine the concentrations of α-Syn in the treated and untreated SH-SY5Y cells lysate .The experiment was conducted in duplicates. The results in Fig. 5 are mean α-Syn concentrations ±SD. Fig. 5 shows that compared to the untreated control and Fe^{2+} treated (positive control) cells, H_2O_2 treatment induced the expression of higher amount of α-Syn protein in both WT and A53T SH-SY5Y cell lines at 13.7 ± 0.5 ng/ml and 16.0 ± 0.2 ng/ml respectively. Likewise, H_2O_2, $FeCl_2$ treated and untreated A53T SH-SY5Y cell lines, respectively expressed relatively higher amount of α-Syn at 16.0 ± 0.2, 14.8 ± 1.1 and 7.5 ± 0.0 ng/ml compared to the WT SH-SY5Y cells expressing smaller amount of α-Syn at 13.7 ± 0.5, 9.1 ± 1.1 and 3.2 ± 0.1 ng/ml respectively.

Comparing the percentage of α-Syn in total proteins of the WT and A53T expressing cells treated with H_2O_2 and $FeCl_2$ as well as untreated control. Fig. 6 expresses the percentage of α-Syn, normalized in total protein of WT and A53T treated and untreated control. The result in Fig. 6 also showed that H_2O_2 increased the amount of α-Syn in total protein expressed by both WT and A53T cells with $42.5x10^{-5}$ % and $73.2x10^{-5}$ % respectively, compared to the $FeCl_2$ and untreated, WT and A53T controls. Although H_2O_2

treatment induced expression of a higher percentage of α-Syn in SH-SY5Y cell lines expressing A53T compared to the WT SH-SY5Y cells.

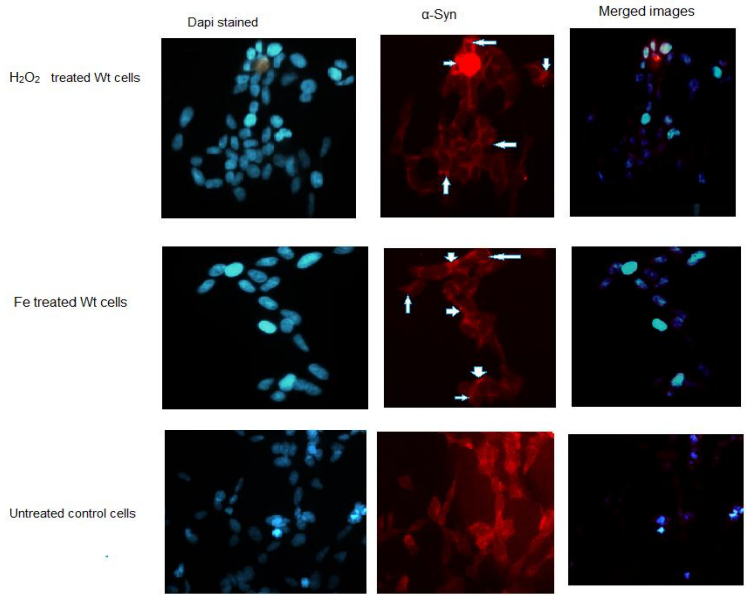

Fig. 2a. Immunoflourescence staining of SH-SY5Y cells expressing Wt mutations indicating Dapi nuclear stain(blue) and α-Syn antibody (Texas Red), as well as merged images under x400 magnifications to provide details of alpha synuclein aggregation after treatment with 100 μM H_2O_2 and 500 μM Fe^{2+}. White arrow indicates regions of cytoplasmic localization and aggregation of α-Syn

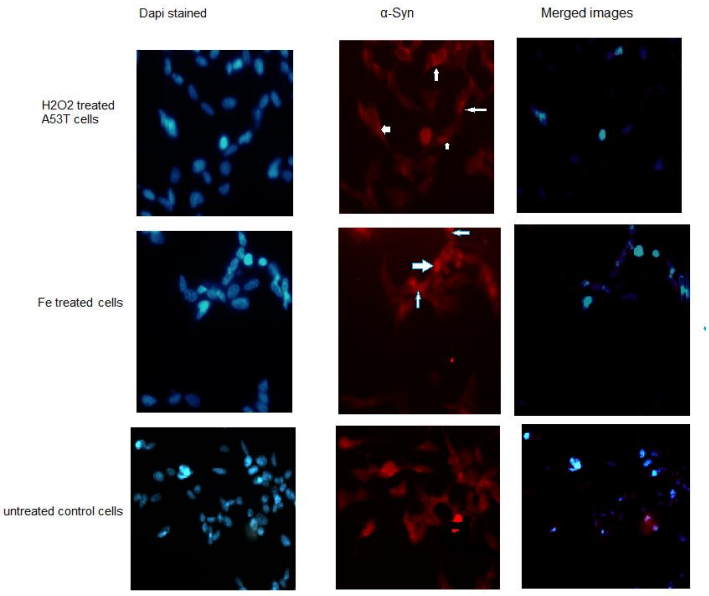

Fig. 2b. Immunostaining of SH-SY5Y cells expressing A53T mutations indicating Dapi nuclear stain (blue) and α-Syn antibody (Texas Red), as well as merged images from immunofluorescence microscope of x400 magnifications showing increased α-Syn presence, aggregation and dispersed α-Syn within the cytosol of A53T cells treated with 100μM H_2O_2, 500 μM $Fe2^+$ and untreated control respectively. The white arrow indicates regions of cytoplasmic localization of α-Syn aggregates

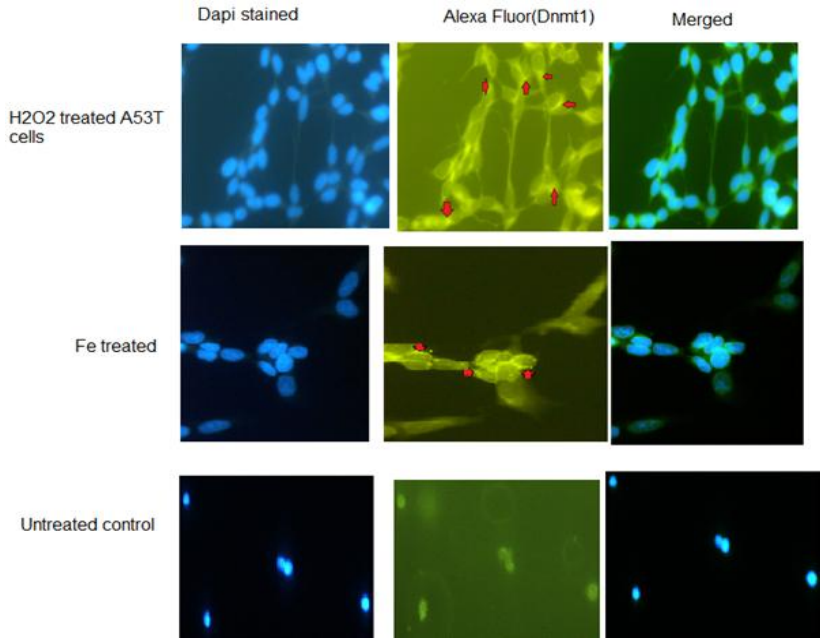

Fig. 3a. Flourescent microscope imaging of H_2O_2 and Fe^{2+} treated SH-SY5Y cells expressing A52T mutation, showing H_2O_2 and Fe^{2+} induced the accumulation and translocation of Dnmt1from nuclei to the cytosol. The Dapi nuclear stain(blue) and Alexa fluorgreen (anti-Dnmt1 antibody), merged images and red arrows indicates regions of cytoplasmic translocation and aggregation of Dnmt1

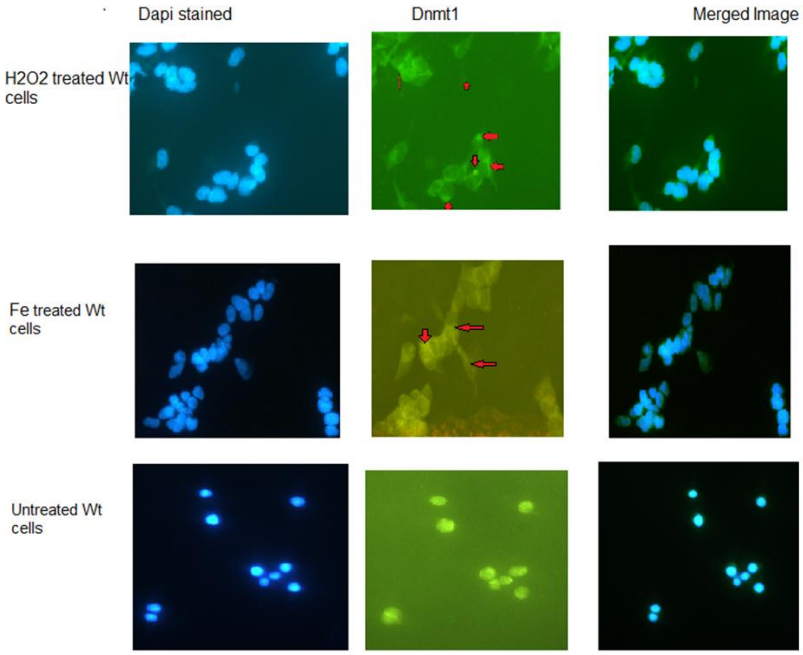

Fig. 3b. Immunoflourescent images of H_2O_2 and Fe^{2+} treated SH-SY5Y cells expressing WT mutation. The images under x400 magnifications indicates that the treatment induced accumulation and translocation of Dnmt1from nuclei to the cytosol. The Dapi nuclear stain(blue) and Alexa fluor green(anti-Dnmt1 antibody) , merged images and red arrows indicates regions of cytoplasmic translocation and accumulation of Dnmt1

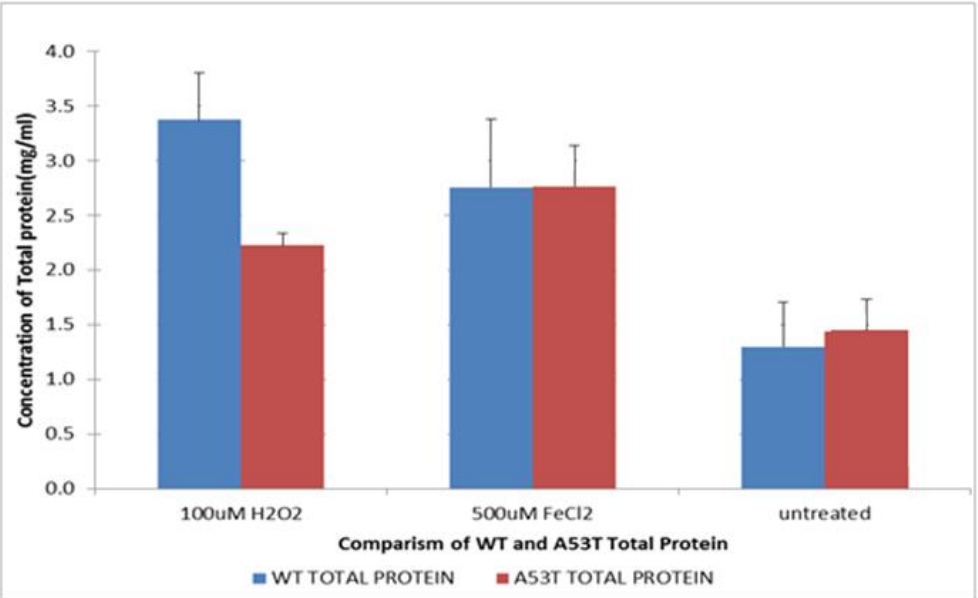

Fig. 4. Quantification of total protein by BCA in H_2O_2 and $FeCl_2$ treated as well as untreated WT and A53T cells. Indicates treatment significantly increased total protein expressed by SH-SY5Y cells. However no significant difference (at P<0.05) exist between total protein expressed by WT and A53T cells. The results was expressed as a mean ± SD, where n=4

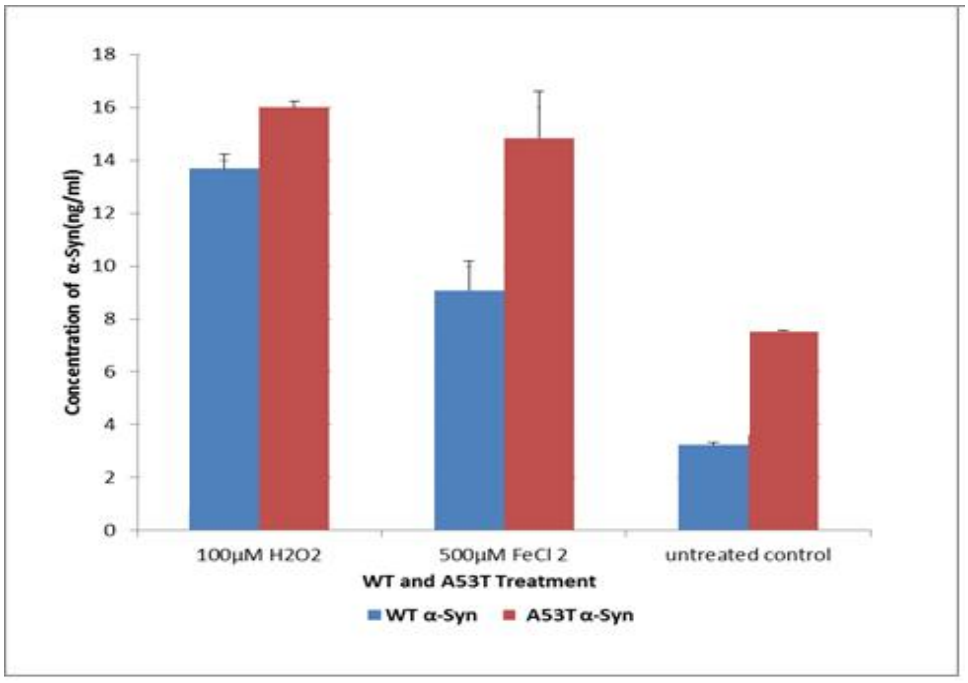

Fig. 5. Determination of α-Syn by ELISA in H_2O_2, $FeCl_2$ treated and untreated SH-SY5Y cells lines. Results expressed as mean ± SD treated and untreated WT and A53T cells indicates H_2O_2 and $FeCl_2$ increased α-Syn in both WT and A53T cells compared to the control. Using unpaired t-test, no statistical difference (P<0.05) exist between α-Syn protein expressed by WT and A53T cells. Where n=2

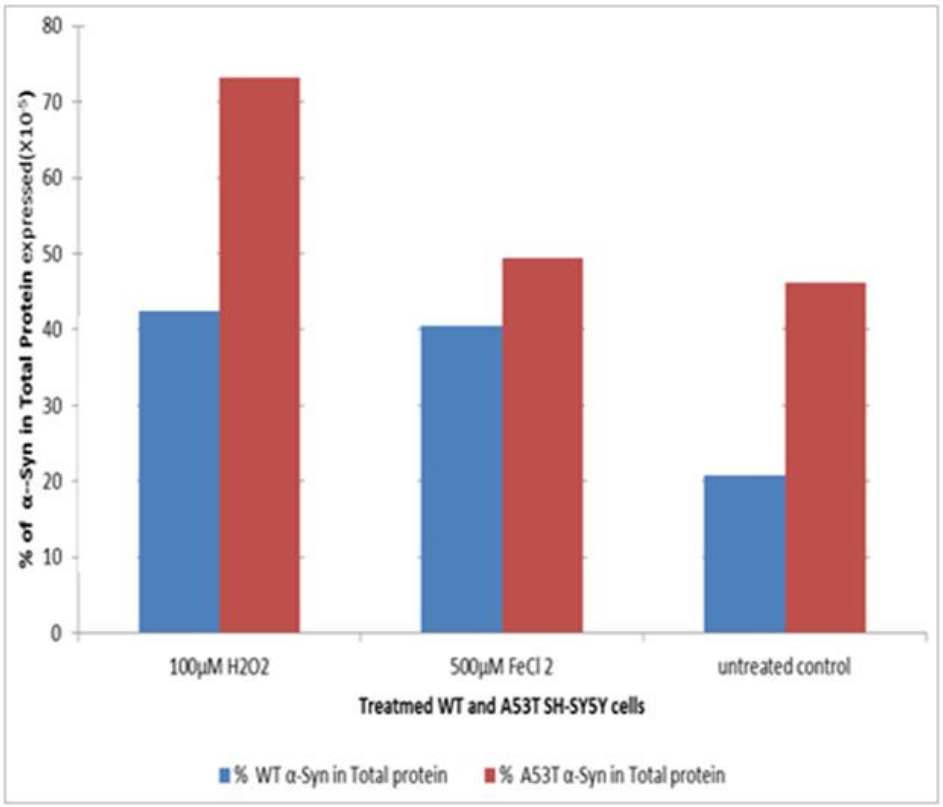

Fig. 6. Percentage composition of WT and A53T α-Syn in total protein expressed by cell lysate of SH-SY5Y cells treated with 100 μM H_2O_2, $FeCl_2$ treated and media untreated control. With an observed higher percentage of α-Syn protein expressed in H_2O_2 treated A53T cells compared to the Fe^{2+} and untreated controls of the A53T cells. However, similar percentage of α-Syn protein was expressed in H_2O_2 and $Fe2^+$ treated WT SH-SY5Y cells

4. DISCUSSION

4.1 Optimum H_2O_2 Concentration Induced Significant Cytotoxicity

This study demonstrated the dose dependent reduction of the viability of SH-SY5Y cells by H_2O_2 treatment 100 μM of H_2O_2 significantly induced cytotoxicity on the WT and A53T SH-SY5Y cells. This result is consistent with the findings of [43] and [44], where 100 μM H_2O_2 was found to reduce survival rate to 50% in yeast cells expressing A53T α-Syn and on SH-SY5Y cells expressing WT α-Syn respectively. Although [45] reported a 50% decrease on SH-SY5Y cells viability with a higher concentration of 200 μM H_2O_2. This discrepancy could be due to the duration dependent effect of H_2O_2 on cell viability [46]. According to [47], differences in cell plating density may contribute to the variation in H_2O_2 cytotoxic concentration reported in different studies. Furthermore, the discrepancies of H_2O_2 dose toxicity may be related to different experimental conditions, different cells or different detection methods.

Similar to what was previously reported by [21] and [26], the observed cytotoxicity of 100μM H_2O_2, characterized by significant death of the SH-SY5Y neuronal cells in this study, arise from prolonged exposure to H_2O_2 resulting in oxidative stress induced apoptosis and necrosis [23].

This observed concentration and time dependent H_2O_2 induced neuronal cell death can result from apoptosis and necrosis through the activation of Mitogen activated protein (MAP) kinases such as protein kinase B (PKB), extracellular signal regulated kinase (Erk), Jun N-terminal kinase (JNK) via the intrinsic (mitochondrial dependent) and extrinsic (death receptor mediated) pathways [48].

These findings may suggest that environmental factors that raise the intracellular concentration of

H_2O_2 above 1 μM is considered to induce oxidative stress and might contribute to the development of several diseases including PD [47]. The use of H_2O_2 in household products and for industrial purpose should therefore be limited, while safer alternatives may be desirable.

4.2 H_2O_2 Treatment Induced Aggregation of α-Syn and Translocation of Nuclear Dnmt1 to the Cytoplasm

This study further demonstrated that 100 μM H_2O_2 increased cytosolic presence of α-Syn and translocation of Dnmt1 from the nucleus to the cytoplasm in SH-SY5Y cells overexpressing WT and A53T α-Syn.

Using Dapi nuclear stain, the immunocytochemical images of the H_2O_2 treated SH-SY5Y cells overexpressing WT and A53T α-Syn identified cytosolic α-Syn accumulations and a shift in nuclear Dnmt1 to the cytoplasm. This observation is consistent with the findings of [20] and [6], where H_2O_2 caused oxidative stress resulting to *in vitro* aggregations of α-Syn, while nuclear Dnmt1 was reported to be localized along with α-Syn in the cytosol.

The increased aggregation of α-Syn in this study, could be associated with the translocation of Dnmt1 into cytoplasm in SH-SY5Y cells, similar to what was reported in PD and DLB human brain cells, where DNA hypomethylation of the CpG islands at intron 1 promoter sequence of *SNCA* gene further promoted the expression and aggregation α-Syn [6].

While the observed translocation of Dnmt1 in this studies, could be attributed to increased intracellular concentration of the H_2O_2, that binds and modified target biomolecules including DNA to induced oxidative stress to nucleic acid, transport and transcription factors, phospholipids and proteins such as α-Syn and Dnmt1 within the cytoplasm and nucleus [49,50]. Similarly, H_2O_2 induced oxidative signalling could initiate the interactions and redistributions of a NAD dependent deacetylase called Sirtuin 1, as well as Dnmt1, 3B and transcription factors such as NF-$_k$B and Nrf$_2$ within or outside the nucleus to induce aberrant methylation of DNA [51]. Such aberrant DNA methylation may also account for the observed up-regulated expression of α-Syn proteins in this study [52]. This is consistent with the findings that H_2O_2 induced decreased levels of DNA methylation in a dose dependent manner and significant changes in the in DNA methylation with at least 10 days of exposure to H_2O_2.

Hence, it is plausible that uncontrolled exposure to exogenous sources of H_2O_2 may be linked to oxidative stress in several disease conditions and is specifically involved in the pathogenesis of neurodegenerative diseases such as PD [16,17].

The oxidative stress arising from increased exposure to reactive species such as H_2O_2 can also alter the post translational modifications of nuclear/cytoplasmic proteins and transport factors such as exportin 1 and importin β/α ,as well as lead to translocation or redistribution of modified proteins and biomolecules across the nucleus [53]. Hence, oxidative damage arising from exposure to H_2O_2 may contribute the propensity of the modified proteins forming cytotoxic aggregate and resulting in eventual death of neurones associated with PD [54].

Furthermore, this study confirmed through the immunoflourence images that just like H_2O_2, $FeCl_2$ treatment of WT and A53T SH-SY5Y cells (Figs. 3 and 4) promotes some accumulation of α-Syn and Dnmt1 within the cytoplasm of the SH-SY5Y cells. This is consistent with the suggestion that N-terminal region of WT and A53T α-Syn tend to strongly binds metal, leading to increased aggregation of α-Syn [55]. This study therefore demonstrated that exposure of neuronal cell lines to 500 μM Fe^{2+} and 100 μM H_2O caused oxidative stress with observed the aggregation of α-Syn and translocation of nuclear Dnmt1 into the cytoplasm, in undifferentiated SH-SY5Y neuroblastoma cells, expressing WT and A53T α-Syn. This observed α-Syn aggregation is consistent with the findings of [16] that reported intracellular α-Syn aggregation upon exposure of a different type of cell; BE-M17 neuroblastoma cells to the combination of iron and H_2O_2.

Iron is demonstrated to promote α-Syn aggregations, by binding non-toxic α-Syn at its negatively charged C-terminal, to alter secondary structure of the α-Syn, leading to partial misfolding and increased formation of oligomeric α-Syn that become toxic aggregates [15]. Likewise, exposure to toxic amount of reactive species such as H_2O_2 and Iron forms hydroxyl ion free radicals in neuronal cells, which promotes oxidative stress mediated main chain fragmentation and side chain oxidation of α-Syn protein leading to reactive carbonyl (aldehyde and ketone) formations [56]. The carbonyl formed are important markers of oxidative

damage to α-Syn in post mortem PD brain samples, and are known to increase α-Syn susceptible to N-terminal lysine alteration, inter or intra-molecular cross linkage and oligomerization into toxic aggregates [57].

Furthermore, the observed increased α-Syn formation and aggregation arising from H_2O_2 and Iron toxicity may suggest inhibited or dysfunctional proteasome degradation of the aggregated α-Syn, resulting in reduced degradation and increased accumulation of α-Syn oligomer due to the down regulation of catalase expression [58,59]. These effects are shown to account for oxidative stress induced degenerative of neurones in PD, as well as disruption of lipid and mitochondria membranes and ions homeostasis in neuronal cells [60].

4.3 Enhanced Expression of A53T α-Syn Compared to WT α-Syn

The amount of α-Syn present in the cell lysate of H_2O_2 treated and untreated cells were quantified by Elisa method. The result in Fig. 5 indicates that relative higher amount of A53T α-Syn compared to WT α-Syn was expressed in H_2O_2 and $FeCl_2$ treated and untreated SH-SY5Y cells. This finding is consistent with previous reports suggesting more A53T α-Syn were expressed and aggregated in iron treated neuroblastoma cells and in *in vitro* conditions respectively, compared to WT α-Syn [61,16]. Which suggests A53T mutation may promote the effect of H_2O_2 and Iron in the toxic aggregation of α-Syn in neuronal cells. This might also explain why A53T mutations increased neurotoxicity of α-Syn in PD [62].

Although, [63] reported the expression of α-Syn protein was reduced by A53T mutations in familiar PD. However, the observed higher expression and aggregation of A53T α-Syn compared to WT α-Syn in this study may suggest increased formation of α-Syn beyond the ability of the neuronal cells to appropriately proteolyze α-Syn aggregates by degrading or chaperoning them [64]. Similarly, epigenetic alterations resulting in reduced methylation of the Cytosine-Guanine (CpG) region on the intron 1 promoter of *SNCA* gene can account for overexpression of *SNCA* gene and the observed aggregation of α-Syn [52].

Apart from the reported translocation of Dnmt1, possibly accounting for the overexpression of α-Syn(*SNCA)* proteins in the neuronal cell lines of this study, which resulted in the observed aggregation of α-Syn [51]. H_2O_2 induced oxidative stress could also alter nucleobases by converting 5-methylcytosine into 5-hydroxymethylcytosines, which in turn inhibits Dnmt1 and cause improper methylation pattern within the CpG promoter sequence of α-Syn gene [65]. This may cause hypo-methylation of α-Syn gene, resulting in up-regulated expression of α-Syn gene and the higher amount of α-Syn proteins expressed by H_2O_2 treated SH-SY5Y cells in this study [66]. Consequently, these findings suggest that novel therapy and antioxidants targeting toxic levels of H_2O_2 and Iron as well as A53T alteration of α-Syn gene might improve the management of PD.

Studies by [67,68], suggests that factors such as altered metal homeostasis and transglutaminase-2 (an highly expressed protein cross linked with enzymes in PD) could also enhance the aggregation and toxicity of α-Syn. Likewise, though α-Syn in exosome was not quantified in this study, it was possible that α-Syn expression might also have been upregulated by exosome mediated cell-to-cell transfer of extracellular α-Syn, responsible for spread of α-Syn to other cells as PD progresses [69].

5. RECOMMENDATIONS

Future study with differentiated SH-SY5Y cells will further elucidate the molecular involvement of H_2O_2 in the hyper expression of *SNCA* gene and Dnmt 1 methylation. While quantification of cytoplasmic Dnmt1 in H_2O_2 treated SH-SY5Y cells may also provide further insight into how H_2O_2 mediated hyper expression of *SNCA* mRNA affect the localization of Dnmt-1 for developing possible therapeutic target for H_2O_2 mediated epigenetic interactions with α-Syn in Parkinson's disease management.

6. CONCLUSION

This study has expanded the scope of understanding in the aggregation and translocation of α-Syn and Dnmt1 in H_2O_2 treatment of SH-SY5Y cells overexpressing WT and A53T α-Syn. The finding of this study reveals through immunocytochemical and ELISA determinations that exogenous H_2O_2 of 100 μM, promotes the cytoplasmic aggregation and increased concentration of α-Syn, as well as cytosolic translocation of Dnmt-1, in a manner similar to what was observed of Fe (II) in this study.

These findings suggest the important roles of H_2O_2 induced oxidative stress in α-Syn aggregation and Dnmt-1 localization in promoting the pathogenesis of Parkinson's disease. Therapeutic targets of α-Syn aggregation and cytosolic translocation of Dnmt-1 caused by H_2O_2 or other oxidative stress agents may improve the management of PD.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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