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Phytochemical Ginkgolide B Attenuates Amyloid-β_{1-42} Induced Oxidative Damage and Altered Cellular Responses in Human Neuroblastoma SH-SY5Y Cells

Iqbal Gill[a,1], Sukhchain Kaur[b,1], Navrattan Kaur[a,1], Monisha Dhiman[b] and Anil K. Mantha[a,*]
[a]*Centre for Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Punjab, India*
[b]*Centre for Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Punjab, India*

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Abstract. Oxidative stress is an upsurge in reactive oxygen/nitrogen species (ROS/RNS), which aggravates damage to cellular components viz. lipids, proteins, and nucleic acids resulting in impaired cellular functions and neurological pathologies including Alzheimer's disease (AD). In the present study, we have examined amyloid-β (Aβ)-induced oxidative stress responses, a major cause for AD, in the undifferentiated and differentiated human neuroblastoma SH-SY5Y cells. $A\beta_{1-42}$-induced oxidative damage was evaluated on lipids by lipid peroxidation; proteins by protein carbonyls; antioxidant status by SOD and GSH enzyme activities; and DNA and RNA damage levels by evaluating the number of AP sites and 8-oxo-G base damages produced. In addition, the neuro-protective role of the phytochemical ginkgolide B (GB) in countering $A\beta_{1-42}$-induced oxidative stress was assessed. We report that the differentiated cells are highly vulnerable to $A\beta_{1-42}$-induced oxidative stress events as exerted by the deposition of Aβ in AD. Results of the current study suggest that the pre-treatment of GB, followed by $A\beta_{1-42}$ treatment for 24 h, displayed neuro-protective potential, which countered $A\beta_{1-42}$-induced oxidative stress responses in both undifferentiated and differentiated SH-SY5Y neuronal cells by: 1) hampering production of ROS and RNS; 2) reducing lipid peroxidation; 3) decreasing protein carbonyl content; 4) restoring antioxidant activities of SOD and GSH enzymes; and 5) maintaining genome integrity by reducing the oxidative DNA and RNA base damages. In conclusion, $A\beta_{1-42}$ induces oxidative damage to the cellular biomolecules, which are associated with AD pathology, and are protected by the pre-treatment of GB against Aβ-toxicity. Taken together, this study advocates for phytochemical-based therapeutic interventions against AD.

Keywords: $A\beta_{1-42}$, amyloid-beta, antioxidants, DNA/RNA base damage, ginkgolide B, neuronal differentiation, oxidative stress

INTRODUCTION

The human brain is highly vulnerable to oxidative damage as it utilizes 20% of total oxygen (O_2) consumption of the whole body. The high consumption of oxygen increases chances of over production of reactive oxygen and nitrogen species (ROS/RNS), which ultimately causes damage to the neurons. Being post-mitotic/replication deficient, the antioxidant defense system of neurons can be easily weakened by the overload of ROS/RNS, i.e., oxidative stress [1, 2].

Production and accumulation of amyloid-β (Aβ), the leading cause of oxidative stress in the neurons,

[1]These authors contributed equally to this work.

*Correspondence to: Dr. Anil K. Mantha, PhD, Associate Professor, Centre for Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, 151 001, Punjab, India. Tel.: +91 164 2864 142; E-mails: anilmantha@gmail.com or anil.kumar@cup.ac.in

is one of the important hallmarks of Alzheimer's disease (AD) [3], which leads to the production of increased ROS and death of neurons. All through these years, advancements have been made to understand the cellular and molecular alterations occurring during the pathogenesis of AD, especially in regard to disruption of the antioxidant defense system of the cells. One study in this direction showed that intracellular Aβ interacts with superoxide dismutase 1 (SOD1), resulting in impairment of the enzymatic activity of SOD1 [4]. Further, it has been demonstrated that the loss of copper chaperone for SOD (a metalloprotein required for proper functioning of SOD) causes increased Aβ production accompanied by increased processing of amyloid-β protein precursor (AβPP) at the β-secretase 1 (BACE1) site [5]. This study indirectly suggested the poor activity of SOD resulting in the AD pathogenesis. Another important antioxidant enzyme of the brain viz. glutathione (GSH), known as the redox buffer of the cell, is protective against oxidative stress and serves an important role in neurodegenerative diseases [6]. It has been demonstrated that the loss of GSH from neurons results in increased production of ROS/RNS [7] and also causes apoptotic death of the neurons [8].

Accretion of nucleic acid oxidation results in decreased capacity to repair the nucleic acid damage leading to neurodegeneration and aging [9]. Free radicals like ROS/RNS, produced by intrinsic and extrinsic factors, induce a variety of lesions including DNA stand breaks and oxidized bases including 8-hydroxydeoxyguanosine, 8-hydroxyguanosine (8-OHG), and AP-sites [10, 11]. It is estimated that 10^5 DNA base lesions are produced in a mammalian cell genome each day; out of which 10^4 lesions are oxidized bases and single-strand breaks as reviewed by Hegde et al. [11]. Base excision repair (BER) is the predominant pathway in the nucleus as well as mitochondria that removes these oxidized base lesions. Among the BER enzymes, apurinic/apyrimidinic endonuclease (APE1) is known to be a multifunctional enzyme involved in DNA repair and redox regulation of various transcription factors. Defects in repairing oxidative DNA insults cause accumulation of damaged bases, resulting in a number of neurodegenerative disorders like AD and Parkinson's disease [12]. Oxidative DNA damage is increased both in nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), the latter being more prone to oxidation due to its close proximity to ROS/RNS. The mtDNA also lacks shielding histones and have limited repair mechanisms against oxidative insults [13, 14]. Damage to mtDNA could potentially result in bioenergetic dysfunction and, consequently, to aberrant nerve functions. Without efficient repair ability in the brain, mutations in nDNA and mtDNA may result in neuronal cell death through defects in oxidative phosphorylation [15]. Keeping this in mind, recently our group has shown that over expression of APE1 rescued human neuroblastoma SH-SY5Y cells against Aβ-induced oxidative stress responses and also restored the OXPHOS capacity of SH-SY5Y cells. Further phytochemical ginkgolide B (GB) treatment enhanced the neuroprotective role of APE1 [16].

Being a single-stranded molecule and lacking histones, oxidative insults occur more frequently in RNA as compared to DNA [17]. The oxidized mRNA causes production of abnormal proteins due to errors in translation, leading to neurological disorders such as AD, Parkinson's disease, dementia with Lewy bodies, and xeroderma pigmentosum [18, 19]. Highly reactive hydroxyl radicals, produced in close proximity to the RNA, are responsible for a number of base damages. Most common is the formation of 8-OHG, an ubiquitous marker of oxidative stress [20]. To overcome the damage caused by oxidative stress, many recent studies have demonstrated that various plant secondary metabolites show a vast potential for the treatment of AD-like neurodegenerative diseases [21, 22]. These phytochemicals have antioxidant, anti-amyloidogenic, and neuroprotective properties [23–25]. The antioxidant properties of these plant-based products increase the levels of various antioxidants viz. catalase, SOD, and GSH and play a key role in cell survival [26–28].

The proliferative cell line SH-SY5Y is the most commonly used cell line to study the pathomechanisms of AD; with different protocols being optimized for the differentiation of these cells into neuronal-like cells mimicking the internal environment of the neurons. A study by Forster et al. demonstrated that differentiation initiates the transition from an oxidative stress-resistant cell state to a neuronal cell state with elevated energetic stress and oxidative vulnerability. However, undifferentiated cells have a low metabolic rate and are more resistant to oxidative insults [29]. In the present study, $A\beta_{1-42}$-induced oxidative stress responses in undifferentiated and differentiated SH-SY5Y neurons were studied. This study also focusses on the use of GB, a terpenoid found in Chinese plant *Ginkgo biloba*, to quench the damaging effects of $A\beta_{1-42}$, indicating the neuroprotective potential of GB in regulating oxidative damage to cellular entities, including the DNA and

RNA; and stimulating the neuronal cells to overcome the oxidative stress induced by $A\beta_{1-42}$ as observed in AD toward maintenance of cellular functionality.

MATERIALS AND METHODS

Cell culture and differentiation

Human neuroblastoma SH-SY5Y cells gifted by Prof. Pankaj Seth, National Brain Research Centre, Manesar were cultured in complete medium containing Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS)/10% horse serum and 1% penicillin-streptomycin. The SH-SY5Y cells were maintained in an incubator under 5% CO_2 at 37°C [30]. To induce differentiation, SH-SY5Y cells were plated at a density of 5×10^3 cells/cm^2 in a 60-mm dish. From the day one after plating, cells were given sequential treatment with 10 μM *all-trans*-retinoic acid (RA; Sigma) in dark for five days in DMEM/Ham's F-12 (1:1) media supplemented with 1% FBS [31]. Differentiation of SH-SY5Y cells was documented by morphological examination by phase contrast microscope.

Cell treatments

The SH-SY5Y cells were seeded in culture dishes of 60 mm diameter at a density of 5×10^3 cells/cm^2 in a 60 mm dish as described elsewhere [30] for treatment with oxidant $A\beta_{1-42}$ and the phytochemical GB; Sigma).

Preparation and treatment of $A\beta_{1-42}$ peptide

$A\beta_{1-42}$ peptide (GenScript) stock solution was freshly prepared before each treatment at 1 mM in double-distilled deionized H_2O, considered as the soluble form [30]. Cells were treated with 10 μM $A\beta_{1-42}$ for 24 h in different assays.

Preparation and treatment of GB

The phytochemical GB treatment was given to the SH-SY5Y cells at two concentrations, i.e., 10 μM and 20 μM in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. SH-SY5Y cells were also pre-treated with GB for 3 h before the treatment with 10 μM $A\beta_{1-42}$ in further experiments [16].

Measurement of intracellular reactive oxygen species

Cells were seeded overnight in a 96-well plate at a density of 1×10^5 cells/well and given appropriate treatments. Then, the cells were equilibrated in phosphate-buffered saline (PBS) and incubated in the dark for 30 min with 100 μM of H_2DCFDA (Invitrogen). After washing twice with PBS, fluorescence intensity was then read at the excitation wavelength of 478 nm and emission wavelength 518 nm using BioTek (Winooski, VT) microplate reader as per the protocol described [16, 32].

Measurement of intracellular reactive nitrogen species

To measure the intracellular RNS levels, cells were seeded in a 96-well plate at a seeding density of 1×10^5 cells/well, grown overnight, and given appropriate treatments. Then, the cells were equilibrated in PBS and incubated in the dark for 30 min with 20 μM of DAF-FM (Invitrogen). After washing twice with PBS, fluorescence intensity was then read at an excitation wavelength of 478 nm and at an emission wavelength of 515 nm using the BioTek microplate reader as per the protocol described [16, 33].

Nitric oxide release assay

Griess assay is used to determine the amount of nitric oxide (NO) produced by the cells as nitrite accumulation in the cell supernatants. The first step is conversion of nitrate to nitrite utilizing nitrate reductase, followed by the second step involving addition of Griess Reagent (1% sulphanilic acid and 0.1% N-(1-Naphthyl) ethylenediamine) which converts nitrite into deep purple azo compound. Briefly, Griess reagent and the cell culture supernatants were added in 1:1 ratio to each well of a 96-well plate and mixed thoroughly. After 5 min, the absorbance of the colorimetric product formed was read at 540 nm using BioTek microplate reader. The nitrite content of each sample was evaluated from a standard curve obtained using sodium nitrite and was expressed in μM [34].

Preparation of total cell lysates

Briefly, SH-SY5Y cells were harvested and centrifuged at 2000 rpm at 4°C for 10 min. Following centrifugation, the pellet was re-suspended in the lysis buffer containing 20 mM Tris-Cl (pH 7.5),

150 mM NaCl, 1 mM EDTA, 0.1% NP-40, and protease inhibitor cocktail with vortexing every 5 min for 20 min at 4°C. Then, the mixture was centrifuged at 20,000 g for 15 min at 4°C. The supernatant was collected and stored at –20°C for further experiments, followed by protein estimation by the Bradford Method [30].

Measurement of SOD activity

SOD activity was measured using a simple method based on the ability of SOD to inhibit the auto-oxidation of pyrogallol. The final assay mixture contained 0.1 mM sodium phosphate buffer (pH 8), 3 mM EDTA, and 8.1 mM pyrogallol with the protein sample. Initially, protein samples with the assay mixture were added except pyrogallol. The reaction was started by adding pyrogallol and the change in absorbance was measured for 3 min at 420 nm using Shimadzu double-beam spectrophotometer. SOD activity was expressed as U/mg of protein. One unit of SOD activity being defined as the amount of enzyme that causes half- maximal inhibition of auto-oxidation of pyrogallol [35].

Measurement of total glutathione content

The assay is used for determining total GSH in the samples using Ellman's reagent (DTNB). It is based on the reaction between DTNB and GSH producing a yellow colored product. Briefly, proteins were precipitated with 25% trichloroacetic acid (TCA), followed by centrifugation at 6,000 rpm for 10 min. The supernatant obtained was then mixed with DTNB and incubated in dark for 15 min. Change in absorbance was measured for 3 min at 412 nm using BioTek microplate reader. The GSH content was determined from a standard curve made using GSH [36].

Protein oxidation assay

This assay is used for detecting protein carbonyls involving derivatization of the carbonyl group with DNPH, which leads to the formation of a stable hydrazone product. Firstly, proteins were precipitated with 20% TCA, followed by centrifugation at 6,000 rpm for 10 min. The pellets obtained were then mixed with a solution of 10 mM DNPH in 2 N HCl and allowed to stand in the dark for 90 min with vortexing every 15 min. Again, the samples were precipitated with 10% TCA and washed 3 times with ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Finally, the pellets were re-suspended in 6 M guanidium hydrochloride and centrifuged at 6,000 rpm for 3 min; carbonyl content was determined from the change in optical density measured at 385 nm using molar absorption coefficient of 22,000 M^{-1} cm^{-1} using BioTek microplate reader [37].

Lipid peroxidation assay

The measurement of thiobarbituric acid reactive substances (TBARS) is a method for screening lipid peroxidation; with malondialdehyde (MDA) being one of the products of lipid peroxidation. Starting with, the protein samples were mixed with 15% TCA, 0.375% thiobarbituric acid and 0.25 mol/l HCl and heated to 95°C for 45 min. After boiling, the samples were allowed to cool at 4°C for 30 min and centrifuged at 1000 rpm for 10 min. The optical density of supernatants was measured at 532 nm and the MDA content was calculated using 1.56×10^5 M^{-1} cm^{-1} as molar absorption coefficient using BioTek microplate reader. Results were expressed as nmol/mg of protein [38].

Quantification of number of AP sites in the genome

Genomic DNA was isolated from the undifferentiated and differentiated SH-SY5Y cells, which were treated with $A\beta_{1-42}$, GB, and their combinations, and the amount and purity of isolated DNA was determined using Nanodrop reader (Thermo Scientific). DNA oxidation (AP-sites) was determined in the samples with OxiSelect™ Oxidative DNA Damage Quantitation Kit (Cell Biolabs, San Diego, USA) following the manufacturer's protocol using BioTek microplate reader. Results are expressed as AP sites/10^5bp.

Quantification of number of 8-OHG sites in the RNA

Firstly, RNA was isolated from the samples using TRIzol (Invitrogen) as per manufacturer's instructions, followed by an additional phenol chloroform extraction and ethanol precipitation. The concentration and integrity of RNA was measured by using Nanodrop reader (Thermo Scientific) and stored at –20°C for further use. 8-OHG was quantified in RNA samples using the OxiSelect Oxidative RNA Damage ELISA kit (Cell Biolabs, San Diego, USA) with minor modifications. Beforehand, the extracted RNA

was digested using Nuclease S1, followed by treatment with alkaline phosphatase at 37°C for 1 h. The reaction mixture was then centrifuged at 6,000 g for 5 min at 4°C, and the supernatant obtained was used for 8-OHG quantification using BioTek microplate reader.

Statistical analysis

All the data were analyzed using Student's *t*-test and represented as mean ± standard deviation ($n = 3$ or more). The data was considered statistically significant when $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

RESULTS

Retinoic acid induced differentiation of human neuroblastoma SH-SY5Y cells

Exposure of *all-trans*-RA resulted in differentiation of SH-SY5Y cells into more characteristically neuronal morphology. Cells seeded on day 0 exhibited compact morphology, which changed over 3–5 days on exposure to 10 μM RA with the formation of axon and dendrite-like projections and shrinkage of the cytoplasm. RA treatment was extended up to day 8 to observe the morphological difference between differentiated and undifferentiated SH-SY5Y cells. Confirmation of morphologically differentiation of SH-SY5Y cells was documented using phase contrast microscopy (Fig. 1).

Assessment of Aβ1-42-induced oxidative stress and phytochemical modulation by the pretreatment of ginkgolide B in undifferentiated and differentiated SH-SY5Y cells

Measurement of intracellular ROS levels

Intracellular ROS levels were measured using H_2DCFDA fluorescent dye as a result of oxidative stress induced by 100 μM H_2O_2 (positive control) and 10 μM of $A\beta_{1-42}$, and further modulation by phytochemical GB at two concentrations, i.e., 10 μM

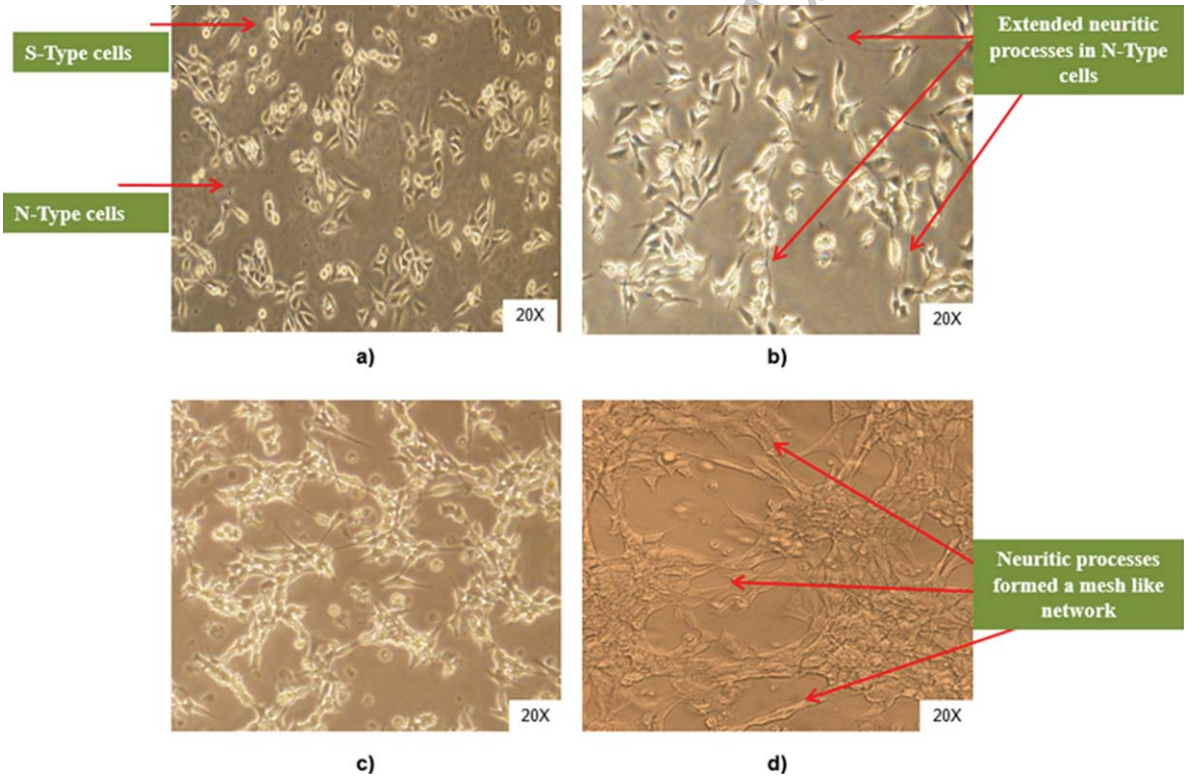

Fig. 1. Effect of *all-trans*-retinoic acid-induced differentiation on morphological appearance of SH-SY5Y cells. a) Phase contrast images of SH-SY5Y cells after retinoic acid (10 μM) treatment for 0, 2, 3, and 8 days. b) Neurite extensions were evident in 2nd and 3rd day cells (arrows). The above pictures (a-c) were taken with FSX 100 fluorescence microscope (Olympus); and (d) day 8, the neuritic processes forming a mesh-like network in differentiated SH-SY5Y cells.

and 20 μM. ROS levels were found to be increased by 28% ($p \leq 0.01$) and 33% ($p \leq 0.01$) on treatment with 10 μM GB in undifferentiated and differentiated SH-SY5Y cells, respectively, as compared to the respective untreated control SH-SY5Y cells. Treatment with $A\beta_{1-42}$ increased the ROS levels significantly ($p \leq 0.01$) by two times in undifferentiated SH-SY5Y cells and by a fold change of 1.6 ($p \leq 0.001$) in $A\beta_{1-42}$-treated differentiated SH-SY5Y cells as compared to their respective control cells. Interestingly, treatment with H_2O_2 at a physiological concentration (100 μM) did not increase the ROS levels to an extent as in the differentiated neurons, suggesting a balance between the free radical generating, H_2O_2- generating and H_2O_2- metabolizing enzymes in these two type of cells. In comparison with $A\beta_{1-42}$-treated cells, pre-treatment with GB (10 μM) followed by treatment with $A\beta_{1-42}$ led to a decrease in ROS levels by 25% ($p \leq 0.05$) and 16% in undifferentiated and differentiated SH-SY5Y cells, respectively (Fig. 2). This points toward the attenuation of ROS levels in both undifferentiated and differentiated SH-SY5Y cells on pre-treatment of GB (10 μM) prior to $A\beta_{1-42}$ treatment.

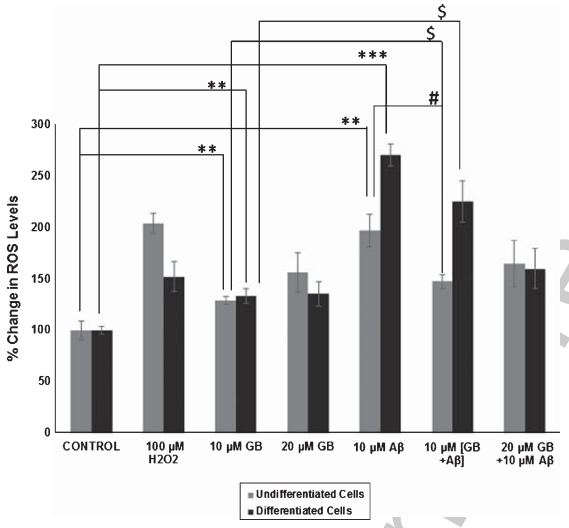

Fig. 2. Measurement of intracellular ROS production after $A\beta_{1-42}$-induced oxidative stress in undifferentiated and differentiated SH-SY5Y cells by H_2DCFDA and phytochemical modulation by the pre-treatment of ginkgolide B (GB). Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, GB/Aβ treated cells compared with control; $^{\#}p \leq 0.05$, GB + Aβ treated cells compared with Aβ-treated cells; $^{\$}p \leq 0.05$, GB + Aβ treated cells compared with GB-treated cells. Results are presented as mean ± SD ($n = 3$).

Measurement of intracellular RNS levels

The fluorescence-based DAF-FM assay was used to measure intracellular RNS levels in undifferentiated and differentiated SH-SY5Y cells after 24 h of treatment with GB and $A\beta_{1-42}$ and their combinations. A 14% rise in RNS levels was observed when undifferentiated SH-SY5Y cells were treated with GB (10 μM) as compared with the untreated control. When the differentiated SH-SY5Y cells were treated with 10 μM GB, there was a significant ($p \leq 0.01$) decrease in RNS levels by 39% as compared to the respective RA-induced differentiated SH-SY5Y control cells. Treatment with $A\beta_{1-42}$ increased the RNS levels significantly, by more than 2 folds in both undifferentiated ($p \leq 0.001$) and differentiated ($p \leq 0.01$) SH-SY5Y cells. A similar trend in the RNS levels after H_2O_2 treatment was seen as in the ROS levels. But, treatment with $A\beta_{1-42}$ did not increase the levels of RNS in the differentiated neurons as compared to normal SH-SY5Y cells. This could be due the balance between the free radical generating and free radical metabolizing enzymes in countering this nitrosative stress in the neuronal cells. It might be the scenario that the role of peroxisomal antioxidant defense in the neuronal cells could also be a factor leading to this differential effect; which needs further investigation. The pretreatment of GB (10 μM) followed by $A\beta_{1-42}$ treatment of SH-SY5Y cells showed a decrease in RNS levels by two times in both undifferentiated ($p \leq 0.01$) and differentiated ($p \leq 0.01$) SH-SY5Y cells as compared to respective $A\beta_{1-42}$-treated cells (Fig. 3), pointing toward the attenuating potential of GB by reducing the levels of RNS in the presence of $A\beta_{1-42}$-induced oxidative stress.

Measurement of extracellular NO levels

This assay was performed to measure the extracellular NO levels in SH-SY5Y cells in the presence of oxidative stress induced by $A\beta_{1-42}$. On treatment with 10 μM GB, the extracellular NO levels were found to rise by 1.8 folds in undifferentiated SH-SY5Y cells; whereas an increase by 18% was seen in differentiated cells as compared to their respective untreated control cells. Treatment with $A\beta_{1-42}$ increased the NO levels significantly by 24% ($p \leq 0.01$) in undifferentiated SH-SY5Y cells and by a fold change ($p \leq 0.01$) in differentiated SH-SY5Y cells, respectively, as compared to their respective control SH-SY5Y cells. In comparison with $A\beta_{1-42}$-treated cells, the 10 μM [GB + β_{1-42}]-treated undifferentiated

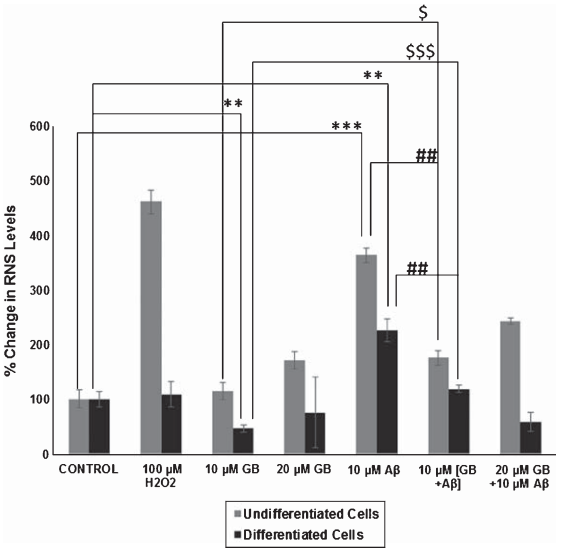

Fig. 3. Measurement of intracellular RNS produced after $A\beta_{1-42}$-induced oxidative stress in undifferentiated SH-SY5Y cells by DAF-FM and phytochemical modulation by GB. Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, GB/Aβ treated cells compared with control; $^{\#\#}p \leq 0.01$, GB+Aβ treated cells compared with Aβ-treated cells; $^{\$}p \leq 0.05$, $^{\$\$\$}p \leq 0.001$ GB+Aβ treated cells compared with GB-treated cells. Results are presented as mean ± SD ($n = 3$).

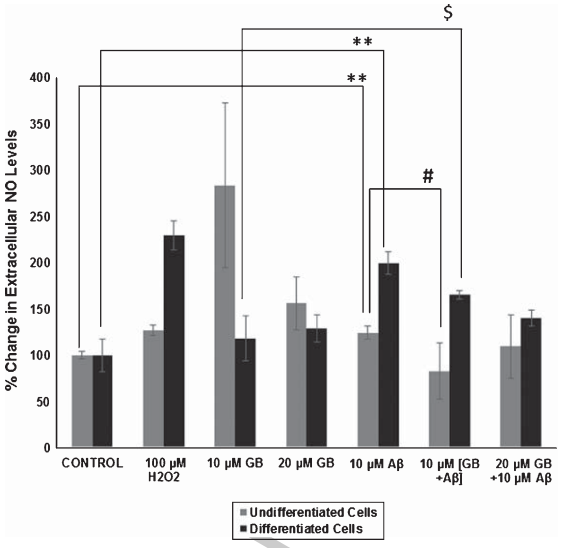

Fig. 4. Measurement of extracellular NO produced after $A\beta_{1-42}$-induced oxidative stress in undifferentiated and differentiated SH-SY5Y cells by Griess reagent and phytochemical modulation by the pre-treatment with GB. Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, GB/Aβ treated cells compared with control; $^{\#}p \leq 0.05$, GB + Aβ treated cells compared with Aβ-treated cells; $^{\$}p \leq 0.05$, GB + Aβ treated cells compared with GB-treated cells. Results are presented as mean ± SD ($n = 3$).

SH-SY5Y cells shown decreased NO levels by 33% ($p \leq 0.05$); whereas a decrease of 16% was observed in 10 μM [GB + $A\beta_{1-42}$]-treated differentiated SH-SY5Y cells (Fig. 4). This shows the levels of NO are being regulated by GB in the presence of $A\beta_{1-42}$-induced oxidative stress in AD.

Estimation of SOD activity

The activity of the free-radical scavenging enzyme SOD in the presence of oxidative stress by $A\beta_{1-42}$ and pre-treatment with GB was measured. Treatment with phytochemical GB (10 μM) led to an increase in SOD activity by 19% in undifferentiated SH-SY5Y cells whereas a 26% increase ($p \leq 0.01$) in SOD activity was observed in differentiated SH-SY5Y cells as compared to the respective untreated control. Upon $A\beta_{1-42}$ treatment, an increase in SOD activity by 32% ($p \leq 0.01$) in undifferentiated SH-SY5Y cells was observed on comparison with the untreated control cells. In contrast, $A\beta_{1-42}$ treatment led to a significant decrease in SOD activity by 44% ($p \leq 0.01$) in differentiated SH-SY5Y cells as compared to the untreated control cells. When compared with $A\beta_{1-42}$-treated differentiated cells, an increase by 1.2 folds ($p \leq 0.01$) was seen in 10 μM [GB + $A\beta_{1-42}$]-treated differentiated SH-SY5Y cells (Fig. 5). This indicates the role of GB in modifying the activity of SOD in presence of Aβ stress.

Determination of total glutathione content

Content of GSH, which functions both as a free radical scavenger as well as a substrate for glutathione peroxidase, was measured. A significant increase in the GSH content by 1.2 folds ($p \leq 0.01$) was observed in GB (10 μM)-treated differentiated SH-SY5Y cells in comparison to the untreated control cells. In the presence of oxidative stress induced by $A\beta_{1-42}$, a decrease in GSH content by 39% ($p \leq 0.01$) and 43% was observed in undifferentiated and differentiated cells, respectively, as compared to their respective untreated control cells. Both undifferentiated and differentiated SH-SY5Y cells, which were treated with 10 μM [GB + $A\beta_{1-42}$], were observed to possess increased GSH content by 1.4 folds ($p \leq 0.001$) and 2.9 folds ($p \leq 0.001$), respectively, as compared to their respective $A\beta_{1-42}$-treated SH-SY5Y control cells (Fig. 6). This shows that oxidative stress-like conditions, together with phytochemical

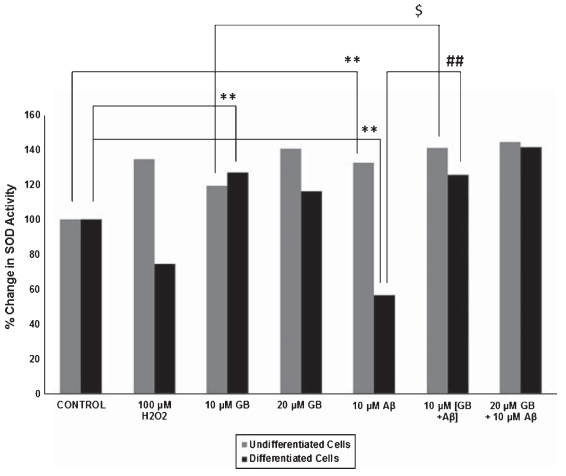

Fig. 5. Measurement of SOD activity in undifferentiated and differentiated SH-SY5Y cells after $A\beta_{1-42}$-induced oxidative stress and phytochemical modulation by the pre-treatment of GB (expressed as U/µg of the protein). Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, GB/Aβ treated cells compared with control; $^{\#\#}p \leq 0.01$, GB + Aβ treated cells compared with Aβ-treated cells; $^{\$}p \leq 0.05$, GB + Aβ treated cells compared with GB-treated cells. Results are presented as mean ± SD ($n = 3$).

pre-treatment, stimulate and prepare the neuronal cell for protection from free radical damage during adverse cellular stress conditions as observed in AD.

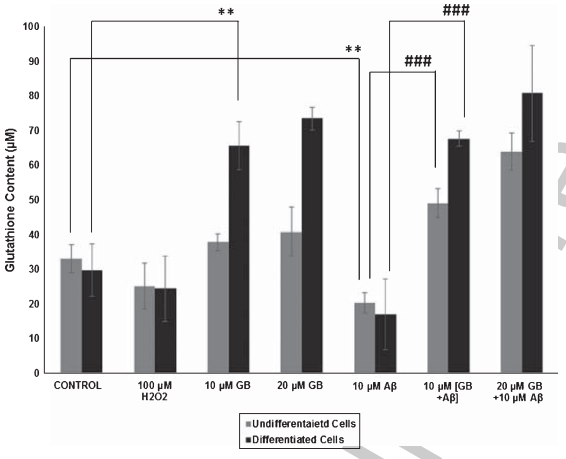

Fig. 6. Measurement of total glutathione (GSH) levels in undifferentiated and differentiated SH-SY5Y after $A\beta_{1-42}$-induced oxidative stress and phytochemical modulation by the pre-treatment of GB (expressed as concentration of glutathione, µM). Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, GB/Aβ treated cells compared with control; $^{\#\#\#}p \leq 0.001$, GB + Aβ treated cells compared with Aβ-treated cells. Results are presented as mean ± SD ($n = 3$).

Lipid peroxidation: TBARS assay

TBARS assay was carried out to measure the lipid peroxidation in the undifferentiated and differentiated SH-SY5Y cells. This assay is based on the reaction between MDA, the end product of lipid peroxidation and thiobarbituric acid. On treatment with the phytochemical GB (10 µM), the MDA content was found to be increased by a mere 36% and 21% (non-significant) in undifferentiated and differentiated SH-SY5Y cells, respectively, as compared to their respective control SH-SY5Y cells. In presence of $A\beta_{1-42}$-induced oxidative stress, a 2-fold ($p \leq 0.05$) and 6-fold increase ($p \leq 0.001$) in lipid peroxidation was observed in undifferentiated cells and differentiated SH-SY5Y cells, respectively, as compared to the respective untreated SH-SY5Y control cells. Pre-treatment with GB followed by $A\beta_{1-42}$ stress led to a decrease in lipid peroxidation (MDA content) significantly by 66% ($p \leq 0.05$) and 87% ($p \leq 0.001$) in undifferentiated and differentiated cells, respectively, when compared to the respective $A\beta_{1-42}$-treated SH-SY5Y cells (Fig. 7), showing the damaging effects of Aβ-induced oxidative stress on lipids and the protective effect displayed by the phytochemical GB.

Protein oxidation: Carbonyl content

As a marker of oxidative stress, the level of protein oxidation as a result of Aβ-induced oxidative injury was measured. When the SH-SY5Y cells were treated with GB at a concentration of 10 µM, a decrease in protein carbonyl content was seen in both undifferentiated and differentiated SH-SY5Y cells after 24 h of time (not significant) as compared to the control cells. In the presence of oxidative stress induced by $A\beta_{1-42}$, a rise in protein oxidation by 70% was seen in undifferentiated SH-SY5Y cells, whereas, a significant increase in protein oxidation by 5-folds ($p \leq 0.01$) was observed in differentiated SH-SY5Y cells as compared to the respective untreated control differentiated SH-SY5Y cells. Further, pre-treatment with GB (10 µM) prior to $A\beta_{1-42}$ treatment led to a decrease in protein oxidation (carbonyl content) by 0.4-fold in undifferentiated SH-SY5Y cells and by 1-fold ($p \leq 0.001$) in differentiated SH-SY5Y cells when compared with their respective $A\beta_{1-42}$-treated control SH-SY5Y cells (Fig. 8), showing the protective role of GB in attenuating the Aβ-induced protein oxidative damage in the neuronal cells by which the normal cellular functionality can be maintained.

Measurement of AP-sites produced in the DNA

The levels of DNA damage were quantified by measuring the number of AP sites produced per 10^5 base pairs. When the undifferentiated and differentiated SH-SY5Y cells were treated with 10 μM GB, a rise by 0.4-fold ($p \leq 0.01$), was seen in undifferentiated cells, whereas, a mere increase in the AP-sites by 20% was seen in the differentiated SH-SY5Y cells when compared to the respective untreated control SH-SY5Y cells. Following, a treatment with $A\beta_{1-42}$ increased the DNA damage by two times ($p \leq 0.01$) in undifferentiated SH-SY5Y cells, whereas Aβ stress led to an increase in AP-sites by ≈0.5-fold in differentiated cells as compared to the respective untreated control SH-SY5Y cells. Pre-treatment with GB prior to $A\beta_{1-42}$ treatment led to lesser number of AP-sites produced in both undifferentiated and differentiated SH-SY5Y cells by 36% ($p \leq 0.05$) and 18%, respectively, when compared to the AP-sites produced in the cells which are treated with 10 μM $A\beta_{1-42}$ (Fig. 9), advocating for the extent of DNA damage modulation by the phytochemical pre-treatment in the presence of Aβ-induced oxidative stress in AD.

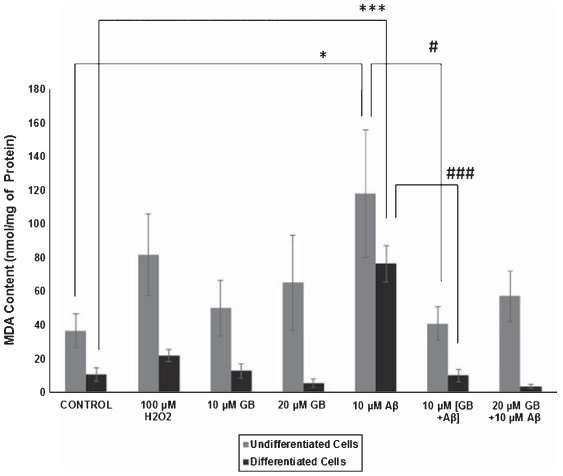

Fig. 7. Measurement of MDA levels in undifferentiated and differentiated SH-SY5Y cells after $A\beta_{1-42}$-induced oxidative stress and phytochemical modulation by the pre-treatment of GB. The MDA levels were expressed as concentration of MDA content (nmol/mg protein). Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{*}p \leq 0.05$, $^{***}p \leq 0.001$, GB/Aβ treated cells compared with control; $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$, GB + Aβ treated cells compared with Aβ-treated cells. Results are presented as mean ± SD ($n = 3$).

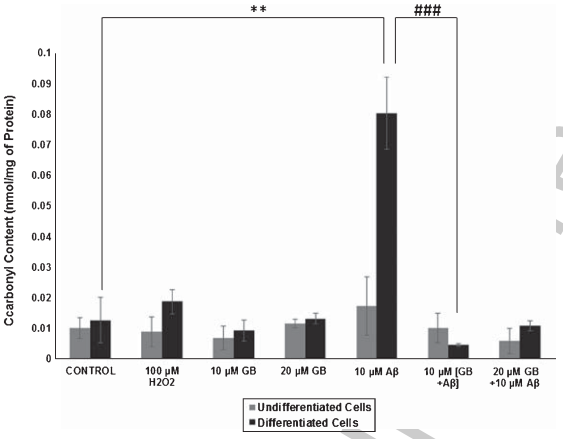

Fig. 8. Measurement of protein carbonyl content in undifferentiated and differentiated SH-SY5Y cells after $A\beta_{1-42}$-induced oxidative stress and phytochemical modulation by the pre-treatment of GB (expressed as concentration of protein carbonyl/mg protein). Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, GB/Aβ treated cells compared with control; $^{\#\#\#}p \leq 0.001$, GB + Aβ treated cells compared with Aβ-treated cells. Results are presented as mean ± SD ($n = 3$).

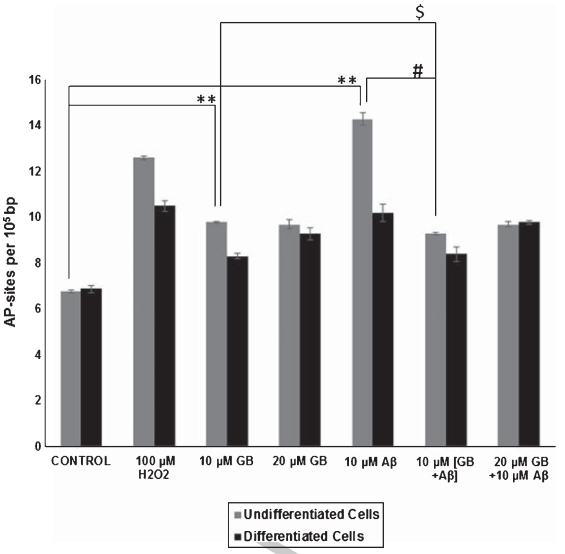

Fig. 9. Measurement of number of AP-sites produced in DNA after $A\beta_{1-42}$-induced oxidative stress responses in undifferentiated and differentiated SH-SY5Y cells; and phytochemical modulation by the pre-treatment of GB. Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{**}p \leq 0.01$, GB/Aβ treated cells compared with control; $^{\#}p \leq 0.05$, GB + Aβ treated cells compared with Aβ-treated cells; $^{\$}p \leq 0.05$, GB + Aβ treated cells compared with GB-treated cells. Results are presented as mean ± SD ($n = 3$).

Measurement of RNA damage via 8-OHG content

Finally, the levels of RNA damage were quantified by the number of 8-OHG sites produced in

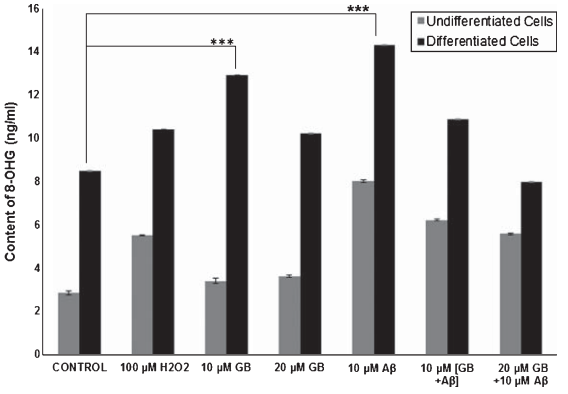

Fig. 10. Measurement of number of 8-OHG sites produced after $A\beta_{1\text{-}42}$-induced oxidative stress in undifferentiated and differentiated SH-SY5Y cells and phytochemical modulation by the pre-treatment of GB. Student's *t*-test was performed to evaluate the significance of the results. Data was statistically significant at $^{***}p \leq 0.001$, GB/Aβ treated cells compared with control. Results are presented as mean ± SD ($n = 3$).

RNA extracted from undifferentiated and differentiated SH-SY5Y cells. Treatment of undifferentiated SH-SY5Y cells with GB (10 µM) led to a mere increase of 8-OHG sites by 19% whereas in differentiated SH-SY5Y cells led to rise in 8-OHG sites by 0.5-fold ($p \leq 0.001$) when compared to the respective untreated SH-SY5Y control cells. There was a rise in RNA damage in terms of 8-OHG sites produced by 1.8-fold in undifferentiated cells on treatment with 10 µM $A\beta_{1\text{-}42}$ as compared to the untreated control SH-SY5Y cells. An increase of 68% ($p \leq 0.001$) was seen in 8-OHG sites produced in response to $A\beta_{1\text{-}42}$ stress in differentiated SH-SY5Y cells as compared to the untreated control cells. Pre-treatment with 10 µM GB in the presence of 10 µM $A\beta_{1\text{-}42}$ stress led to a decrease in 8-OHG sites produced by ≈0.25-fold in both undifferentiated and differentiated SH-SY5Y cells when compared to the respective SH-SY5Y cells treated with 10 µM $A\beta_{1\text{-}42}$ alone (Fig. 10). These observations point toward the damaging effects caused by the deposition of Aβ in AD; which may have a direct role on protein expression as well as functionality.

DISCUSSION

Through this study, our focus resides primarily on the Aβ-induced oxidative stress which is an important determinant in the etiology of neuronal death and AD pathogenesis. Our work seeks to provide insight that $A\beta_{1\text{-}42}$ induces oxidative stress, its pathomechanism, manifestation of proteins, lipids, and nucleic acids and their oxidation as studied in human neuroblastoma SH-SY5Y cells. The present and previous studies from our laboratory and many others show that Aβ has neurotoxic properties inducing production of free radicals that attack brain cell membrane and initiate cellular damage [39–41]. As an experimental model for AD, SH-SY5Y cells were firstly differentiated using *all-trans*-RA for five days. Neurite extensions could be observed in these differentiated SH-SY5Y cells after five days of RA-treatment, which is an important feature of morphological differentiation in comparison to undifferentiated SH-SY5Y cells. Similar features were observed in various studies on differentiation of SH-SY5Y cells with 10 µM *all-trans*-RA along with the expression of neuronal marker GAP-43 [31, 42].

Following differentiation, oxidative damage induced by $A\beta_{1\text{-}42}$ was evaluated; several lines of evidences have indicated that various forms of Aβ interfere with the neuronal membrane, causing oxidation of lipids and proteins, resulting in the generation of ROS and RNS in the AD brain [43]. The current study has demonstrated that upon exposure to $A\beta_{1\text{-}42}$, a significant increase in intracellular ROS and RNS levels was noted in both undifferentiated and differentiated SH-SY5Y cells. These results are in accordance with various studies that show $A\beta_{1\text{-}42}$ plays a role of neurotoxic agent leading to production of ROS in SH-SY5Y cells [44, 45]. $A\beta_{1\text{-}42}$, as a neurotoxic NO stimulator, was also observed when both undifferentiated and differentiated SH-SY5Y cells were treated with $A\beta_{1\text{-}42}$ for 24 hr. In the AD brain, this might be the scenario, which activates microglia and astrocytes to produce toxic inflammatory mediators such as cytokines, NO, and ROS resulting in many neurodegenerative disorders including AD [46–48].

Ginkgo biloba, the living fossil, extracts have been reported to exhibit neuroprotective role against oxidative stress, testified to improve metabolic energy pathways and stabilize mitochondria by inhibiting the action of Aβ, but the underlying mechanism(s) is/are not clearly understood [16, 49–51]. In the present study, the phytochemical GB pretreatment showed significant attenuation of both ROS and RNS production in $A\beta_{1\text{-}42}$-treated undifferentiated and differentiated SH-SY5Y cells. Pretreatment of phytochemical GB also attenuated the NO production to a greater extent against $A\beta_{1\text{-}42}$-induced response. Our results are in accordance with the findings that showed the neuroprotective effect of GB against

$A\beta_{1-42}$-induced cell apoptosis and ROS/RNS accumulation in human neuroblastoma SH-SY5Y cells [16, 52]. In addition, the neuroprotective role of GB has been reported against NO-induced toxicity in rat hippocampal cultured cells and rat brain [53, 54]. Thus, it is clear that GB has neuroprotective ability which can protect the neuronal cells from the effect of Aβ-induced ROS/RNS by scavenging the free radicals.

Increased level of oxidative damage is often accompanied by reduced levels of antioxidant defense mechanisms in the brain. In the present study, $A\beta_{1-42}$ could be seen as an influential oxidant that decreases the SOD activity significantly in differentiated SH-SY5Y cells; while pretreatment of GB restores the SOD activity. These results were found to be consistent with previous existing reports showing that $A\beta_{1-42}$ treatment in differentiated SH-SY5Y cells reduce the SOD activity and the pre-treatment of dicaffeoylqunic acid, a phytochemical, significantly increased the SOD activity [55]. Another study also demonstrated that the phytochemical Ginseng attenuated the methamphetamine-induced oxidative stress and increased the SOD activity, providing protection against cytosolic and mitochondrial oxidative damage in SH-SY5Y cells [56].

Further, the effect of another important antioxidant, i.e., GSH, was studied. Chen et al. [57] showed that there was a reduction in total GSH content in differentiated PC12 and IMR-32 cells upon treatment with Aβ. Additionally, they also showed that treatment of *Centella asiatica* increased the total GSH levels and pointed toward the decreased accumulation of Aβ during oxidative stress in the presence of the phytochemical [57]. Our study also reports that GSH levels were decreased significantly in the differentiated SH-SY5Y cells treated with $A\beta_{1-42}$. Pretreatment with GB attenuated the action of $A\beta_{1-42}$ and restored the GSH levels in both differentiated and undifferentiated SH-SY5Y cells. Taken together, the previous studies and the present study suggest that Aβ-induced oxidative stress causes imbalance in the antioxidant defense system of the neurons. But, the effect of Aβ can be halted by using different plant secondary metabolites.

Another drastic effect of oxidative stress is the peroxidation of fatty acids, which alters the confirmation of the membrane and ultimately affects the signal transduction across neurons. MDA and 4-hydroxy-2-nonenal are the two main end-products of lipid peroxidation. In relation to this, Fallarini et al. studied the effect of clovamide and rosmarinic acid treatments on TBARS levels in Tert-butylhydroperoxide-treated differentiated SH-SY5Y cells, and these treatments were found to decrease the TBARS levels significantly [58]. Another study showed that with the pretreatment of thymoquinone, a bioactive compound, a significant decrease in TBARS content in $A\beta_{25-35}$-treated differentiated PC-12 cells occurs [59]. In the present study, lipid peroxidation (MDA content) was found to be increased significantly with the treatment of $A\beta_{1-42}$ in differentiated SH-SY5Y cells. The pretreatment of GB and $A\beta_{1-42}$ treatment demonstrated an increase in the MDA levels in differentiated SH-SY5Y cells. Increase in ROS/RNS level lead to oxidative stress, and which can be correlated with the results of lipid peroxidation (MDA content), i.e., increase in levels of ROS/RNS, directly related to increase in MDA levels [60].

Formation of protein carbonyls is the main marker of protein oxidation in the neurons which modifies the normal protein structure and alters their normal functioning [61–63]. Here, $A\beta_{1-42}$ treatment in differentiated SH-SY5Y cells recorded higher levels of protein carbonyls. The present study also found that the pretreatment of phytochemical GB, followed by $A\beta_{1-42}$ treatment in differentiated SH-SY5Y cells, resulted in a decrease in the protein carbonyl content. Similarly, other reports showed that the pre-treatment of tocopherol, NAC, and *Lycium barbarum* polysaccharides leads to decreased levels of protein carbonyls in advanced glycation end product-treated differentiated SH-SY5Y and PC-12 cells, respectively [63, 64].

Because of the critical role of DNA in cellular function, oxidative damage to DNA may be one of the most important factors in neuronal degeneration in AD. Earlier investigations have shown that various forms of Aβ, i.e., $A\beta_{25-35}$ and $A\beta_{1-42}$, are capable of inducing oxidative DNA damage in primary cortical culture by escalating the amount of 8-OHG and number of AP sites, which can all be attenuated upon co-incubation with nicotinamide adenine dinucleotide [65]. In the present study, we also report that the treatment with $A\beta_{1-42}$ caused significant augmentation in the ROS/RNS and NO levels in the differentiated SH-SY5Y neuronal cells, and further elevated the DNA damage. The number of AP-sites/10^5 base pairs increased in both undifferentiated and differentiated SH-SY5Y cells. While pretreatment of GB reduced the number of AP-sites produced as compared with that of $A\beta_{1-42}$ treatment in both undifferentiated and differentiated SH-SY5Y cells. These results are consistent with various other

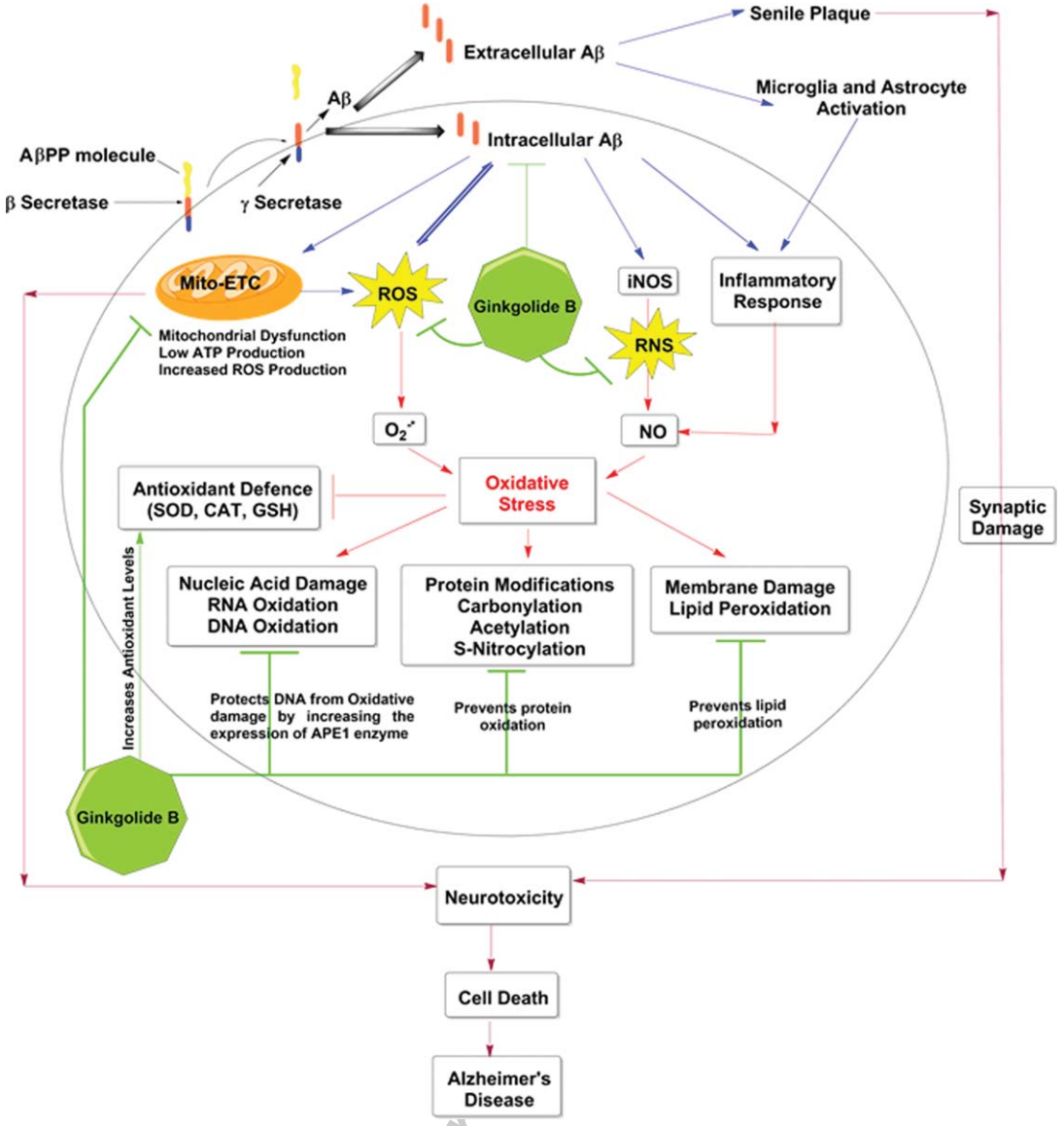

Fig. 11. A model summarizing the phytochemical modulation against Aβ-induced oxidative stress by ginkgolide B on various cellular processes studied and suggestive are: mitochondrial dysfunction, antioxidant defense mechanisms, oxidation of nucleic acids, protein oxidation, lipid peroxidation and others in AD pathology.

reports that showed $A\beta_{1\text{-}42}$ treatment stimulating ROS production, causing oxidation of DNA leading to the production of 8-oxo-G and AP-sites; which are linked to the pathogenesis of several age-related and chronic diseases [66]. A protective role of GB has also been reported against H_2O_2–induced DNA damage in yeast cells [67]. In addition, dietary supplementation of watermelon juice bestowed notable radioprotection against oxidative DNA damage by a mitigating number of AP sites in the brain, lung, and liver tissue of mice [68].

Oxidative stress also results in oxidation of RNA leading to the loss of normal levels of proteins, protein function, and production of defective proteins, leading to protein aggregation, a common feature of neurodegenerative disorders [14]. In the present study, we also found that $A\beta_{1\text{-}42}$ augmented RNA damage (8-OHG) levels in both undifferentiated and differentiated SH-SY5Y cells with the latter being more susceptible. A number of studies suggest the accumulation of $A\beta_{1\text{-}42}$ in the cytosol [69, 70], triggering oxidative stress, which ought to make the RNA

in the cytoplasm more prone to oxidation than the nuclear DNA. This is thought to happen because of the single-stranded nature, absence of the protective histones, and lack of hydrogen bonding in the bases of the RNA molecule. This is in line with a study which showed that RNA oxidation takes place in vulnerable neurons in the earliest stage of cognitive impairment in AD brain [71]. Thus, taking into consideration these studies and based on our data, there occurs a higher rate of oxidation of RNA in the neuronal SH-SY5Y cells. These results are supported by the study of Ding et al., which showed that H_2O_2 treatment elevated the levels of RNA oxidation in primary neurons [72]. Whereas, with the pretreatment of GB, followed by Aβ_{1-42} treatment, the reduction in the 8-OHG levels was observed as compared to respective controls. In this regard, based on previous studies and the current study, GB acts to protect against oxidative DNA and RNA damage by restoring the antioxidant defense and modulating the ROS/RNS levels (current study), increasing endothelial SIRT-1 expression, reducing Nrf2 expression [73] and Akt phosphorylation [74]; inducing astrocytic erythropoeitin expression and upregulating HIF-1α expression [75]; reducing necrotic and apoptotic cell death [76]; and reducing the oxidation of DNA and RNA (current study) via checking the number of oxidized base lesions generated in the DNA (AP-sites) and RNA (8-oxo G sites), which could be attributed to its effect on the activity of BER-pathway enzymes viz. APE1 and its redox regulation toward repairing the damaged DNA and RNA. Further, GB has a profound effect on the mitochondria due to its effect in modulating the activities of the mitochondrial complexes (I, III, and IV) in the presence of Aβ-induced oxidative stress [16]. This is attributable to its effects leading to an increase in the level of APE1 as a cell survival strategy in the mitochondria. Taken together, along with our previous study [16)] and the current study, this points toward the therapeutic potential of GB in regulating the oxidative DNA and RNA damage with the involvement of APE1 toward neuroprotection against Aβ-induced oxidative stress in AD from a new point of view.

In conclusion, the experimental data presented here suggests that Aβ-induced oxidative stress elevates the oxidative damage of lipids, proteins and nucleic acids in differentiated human neuroblastoma SH-SY5Y cells. It is also concluded that the differentiated cells are highly vulnerable to oxidative damage exerted by the deposition of Aβ in AD. Additionally, this study demonstrates that the phytochemical GB can modulate Aβ-induced oxidative damage to cellular biomolecules like proteins, lipids, DNA, and RNA and also strengthen the antioxidant defense system in differentiated neurons (Fig. 11). Further, phytochemical GB based studies can be extended to monitor Aβ-induced oxidative damage possibly via inhibiting Aβ accumulation, modulation of tau phosphorylation, induction of growth factors, acting as an anti-inflammatory agent and as a potential therapeutic agent for slowing down the onset and progression of AD.

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