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Dimethyl fumarate exerts neuroprotection by modulating calcineurin/NFAT1 and NFκB dependent BACE1 activity in $A\beta_{1-42}$ treated neuroblastoma SH-SY5Y cells

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ABSTRACT

Ample studies indicate that calcineurin, a Ca^{2+}/calmodulin-sensitive phosphatase, plays a key role in the initiation and/or clinical progression of Alzheimer's disease, and alteration of calcineurin has been confirmed in Alzheimer's disease brain, impacting tau proteins and amyloid beta (Aβ) levels and resulting in neuronal cell death. As, it is sensible to deliberate the likelihood of calcineurin inhibition as a pharmacological target in the progress of novel Alzheimer's disease therapies, we investigated the neuroprotective efficacy of dimethyl fumarate (DMF) *via* calcineurin dependent downstream targets in oligomeric $A\beta_{1-42}$ treated neuroblastoma SH-SY5Y cells. DMF pre-treatment reduced LDH release, increased cell survival and decreased calcineurin activity in $A\beta_{1-42}$-incubated cells. DMF was found to block calcineurin dependent apoptosis induced by $A\beta_{1-42}$ through Bcl-2 linked death protein (BAD) and cAMP response element binding (CREB) dephosphorylation; moreover, rescued the opposing effect of $A\beta_{1-42}$ on CREB-driven transcription in cells transfected with the CRE-SEAP reporter gene indicating its efficacy on long term potentiation and synaptic plasticity. DMF reversed $A\beta_{1-42}$-induced enhancement in the active form of nuclear factor of activated T-cells (NFAT1) and further associated beta-site amyloid precursor protein cleaving enzyme-1 (BACE1) expression, BACE1 promoter activity and BACE1 enzymatic activity. DMF exhibited another neuroprotective mechanism against $A\beta_{1-42}$ treatment by reducing nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) dependent BACE1 gene transcription. DMF does not alter either beta amyloid precursor protein (βAPP) or its mRNA levels in SH-SY5Y cells, confirming that it does not possess amyloidogenic effect. Inhibiting molecular events related to $A\beta_{1-42}$-induced calcineurin activity by DMF, may be an approach towards finding suitable treatment for Alzheimer's disease.

1. Introduction

Alzheimer's disease is utmost communal form of progressive dementia, characterized by senile deposits of extracellular amyloid beta (Aβ) peptides; neurofibrillary tangles pertaining to instability of neuronal microtubules and an extensive modification of glial cells,

Abbreviations: Aβ, amyloid beta; AC, adenylate cyclase; ANCOVA, analysis of co-variance; APP, amyloid precursor protein; ATCC, American Type Culture Collection; BACE1, beta-site amyloid precursor protein cleaving enzyme 1; BAD, Bcl-2 linked death protein; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; CCK-8, cell counting kit-8; CREB, cyclic adenosine monophosphate response element binding; DMEM, Dulbecco's minimal essential medium; DMF, dimethyl fumarate; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetra acetic acid; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid; FBS, foetal bovine serum; GAPDH, d-glyceraldehyde-3-phosphate dehydrogenase; GSK-3β, glycogen synthase kinase-3 beta; HFP, 1,1,1,3,3,3-Hexafluoro-2-propanol; LDH, lactate dehydrogenase; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid-2-related factor 2; OD, optical density; mRNA, messenger ribonucleic acid; pBAD, phosphorylated Bcl-2 linked death protein; PBS, phosphate buffered saline; pCREB, phosphorylated cyclic adenosine monophosphate response element binding; PKA, phosphokinase-A; PMSF, phenylmethylsulfonyl fluoride; PP1, protein phosphatase-1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; RIPA, radioimmunoprecipitation assay; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEAP, secreted alkaline phosphatase; TBS, tris buffered saline; TBST, tris buffered saline with tween 20.

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termed as gliosis (Serrano-Pozo et al., 2011). These hallmarks are key appearances of Alzheimer's disease; however, the concluding situation of this ailment still remains indefinable. In amendment to original "amyloid hypothesis", it is established that solvable oligomeric Aβ causes initial cognitive deficits *via* synaptotoxic action conceivably by varying the size, shape and protein composition and expression of the dendritic spines (Lacor et al., 2007).

Calcineurin, likewise recognized in place of protein phosphatase 2B (PP2B), is a calcium (Ca^{2+})-sensitive serine/threonine phosphatase, expressed in neurons and covers near about a percent of entire neuronal protein (Klee et al., 1979). It is directly stimulated by calmodulin, responsive to Ca^{2+} flux and results in impactful cellular processes that influence cell survival and growth (Rusnak and Mertz, 2000). Contained within the list of calcineurin substrates are: cAMP response element binding (CREB) (Bito et al., 1996); Bcl-2 linked death protein (BAD) (Asai et al., 1999); glycogen synthase kinase-3 beta (GSK-3β) (Kim et al., 2009); protein phosphatase-1 (PP1) (Reese and Taglialatela, 2011); microtubule-associated protein tau (Goto et al., 1985) and nuclear factor of activated T cells (NFAT) (Rao et al., 1997).

Alteration of calcineurin has been confirmed in Alzheimer's disease brain, impact tau proteins and Aβ levels and calcineurin proficient of encouraging neuronal cell death *via* direct dephosphorylative activation of the pro-apoptotic BAD (Reese and Taglialatela, 2011). In neuronal cell cultures, oligomeric Aβ treatment increases calcineurin activity and cause a dose-dependent decrease in BAD and phosphorylated BAD (pBAD) levels (Agostinho et al., 2008). The interaction amongst calcineurin and phosphokinase-A (PKA) signaling plays a serious part in the negative-feedback mechanism pertaining to the compensation of excitation or inhibition of neuronal bustle. While calcineurin/NFAT signaling largely controls axon terminal remodeling, whereas PKA/-CREB signaling regulates synaptic vesicle accumulation, numerous evidences propose mutual inhibitory relations amongst the activities of these signaling pathways. Corresponding phosphorylation pathways bring about the stimulation of CREB; phosphorylated CREB (pCREB) translocates to the nucleus and transcribe genes that yield proteins essential for synaptic integrity (Kipanyula et al., 2016). Controlling vital signaling processes including direct or indirect dephosphorylative inactivation of CREB, calcineurin opposes long term potentiation and memory function (Reese and Taglialatela, 2011)

The amyloid precursor protein (APP) undergoes proteolytic rift by the enzymes β- and γ- secretase, thus yield a chief component of plaques *i.e.* Aβ peptide in the brains of Alzheimer's disease sufferers. β-Secretase has been recognized as β-site APP cleaving enzyme 1 (BACE1), predominantly expressed by neurons and is the main enzyme for originating the generation of Aβ, making this enzyme a striking drug target for prevention and treatment of Alzheimer's disease (Sinha et al., 1999). BACE1 mRNA exhibits the highest level of expression in brain neurons (Yan et al., 1999) and it was found that BACE1 gene expression in neurons and astrocytes is encouraged by Aβ treatment through stimulation of calcineurin/NFAT1 and calcineurin/NFAT4 signaling respectively. This BACE1 expression in neurons happens through upregulation of disturbed intracellular calcium homeostasis and a calcium permeable channel formed by membrane-inserted Aβ in astrocytes (Cho et al., 2008; Jin et al., 2012).

Recent clinical description confirms significant decrease in occurrence of dementia in organ transplant patient treated with calcineurin inhibitors (Taglialatela et al., 2015), thereby signifying a key part of calcineurin in Alzheimer's disease beginning and/or clinical progression. Cyclosporine and tacrolimus, selective calcineurin inhibitors were screened for their protective effects on memory dysfunctions in mouse model of streptozotocin- and aging-induced dementia, which may possibly be attributed to antioxidative, anti-inflammatory, anticholinesterase and anti-amyloid effects (Kumar and Singh, 2017). Therefore, it is sensible to deliberate the likelihood of calcineurin inhibition as a pharmacological target in the progress of novel Alzheimer's disease therapies.

Dimethyl fumarate (DMF) is a synthetic nuclear factor erythroid-2-related factor 2 (Nrf2) activator that alkylates cysteine residues on Keap-1, prevent Nrf2 ubiquitination and promote subsequent activation of Nrf2 target genes, which work on both antioxidant and inflammatory pathways. DMF is proved to be an oral anti-inflammatory, anti-oxidative and neuroprotective drug in numerous studies (Majkutewicz et al., 2018); additionally, cause to decrease synthesis of the proinflammatory mediators: nitric oxide, interleukin-1β, tumour necrosis factor-α and interleukin-6 in activated microglia and astrocytes *in vitro* (Wilms et al., 2010). DMF has confirmed quick and constant efficiency in clinical trials on patients having relapsing-remitting multiple sclerosis (Kappos et al., 2015). DMF was also found to attenuate intracerebroventricular streptozotocin-induced spatial memory impairment and hippocampal neurodegeneration in rats; moreover, age-dependent effects on cognitive and neuropathological features in the streptozotocin-induced rat model of Alzheimer's disease (Majkutewicz et al., 2018). Campolo et al. (2018) confirms the neuroprotective effect of DMF against Aβ-induced cytotoxicity in human neuronal cells demonstrating the involvement of Nrf2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) system. DMF could represent as a possible therapeutic candidate for restraining the inflammatory and oxidative influence on brain neurons due to amyloid peptides acquaintance.

So far there are very less evidence regarding the potential of calcineurin inhibitors in dementia of Alzheimer's disease type and there is a need to explore the potential of calcineurin inhibitors in dementia as a novel kind of treatment approach. Therefore, the present study has been undertaken to investigate the potential of DMF on calcineurin activity and downstream targets in neuroblastoma SH-SY5Y cells treated with oligomeric $A\beta_{1\text{-}42}$.

2. Materials and methods

2.1. SH-SY5Y human neuroblastoma cell culture

Procurement of SH-SY5Y human neuroblastoma cells was done from American Type Culture Collection (ATCC) (Manassas, VA, USA) (RRID: CVCL_0019). The cells were full-fledged to monolayer in a culture medium comprising 45 % Dulbecco's Minimal Essential Medium (DMEM); 45 % Ham's F12 modified with 2 mM L-glutamine and 1.0 mM sodium pyruvate; 10 % foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. The cells were kept at 37 °C in a 5 % CO_2/95 % humidified air incubator for the specified time. Cells from passage 15 to 25 were used in the experiments. Cells were coated in 96-well plates and grown to 70–80 % confluence for the experiments (Campolo et al., 2018).

2.2. Preparation of oligomeric $A\beta_{1\text{-}42}$

To prepare oligomeric Aβ, lyophilized $A\beta_{1\text{-}42}$ was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol at a concentration of 1 mM, loosely capped and stirred on a magnetic stirrer under a fume hood for 48 h and then used within 36 h. Immediately before treating the cells, stock solution was diluted to 1 μM final concentration in culture medium (Stine et al., 2011).

2.3. Cell treatments

Different groups of cultured SH-SY5Y cells were pre-incubated with DMF (10 and 30 μM), for 2 h, then the cells were stimulated with oligomeric $A\beta_{1\text{-}42}$ (1 μM) for 24 h, for Western blot analysis and biochemical assay. Dose of DMF were based on previous neuroprotective studies in Aβ-induced neuronal cells (Campolo et al., 2018) and pilot studies done in our lab. The SH-SY5Y cell cultures were divided into four different experimental groups *viz.* control cells with normal culture medium, cells stimulated with $A\beta_{1\text{-}42}$ (1 μM), $A\beta_{1\text{-}42}$ (1 μM) + DMF (10 μM) treated cells and $A\beta_{1\text{-}42}$ (1 μM) + DMF (30 μM) treated cells.

2.4. Lactate dehydrogenase cytotoxicity assay and cell viability

The release of lactate dehydrogenase (LDH) from cultured cells was assayed in the culture medium using a commercially available kit (Sigma-Aldrich Co., St. Louis, USA) according to the manufacturer's protocol. Briefly, treated cells (1×10^6) were rapidly homogenized on ice in 500 µl of cold LDH assay buffer and centrifuged at 10,000×*g* for 15 min at 4 °C to remove insoluble material. The soluble fraction (20 µl) was added to a 96 well plate and final volume of 50 µl was made with LDH assay buffer. Microplate reader was utilized to quantify the optical density at 490 nm. The viability of SH-SY5Y cells was evaluated by Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich Co., St. Louis, USA). Briefly, cell suspension (100 µl, 5000 cells/well) in a 96-well plate was dispensed. CCK-8 solution (10 µl) was added to each well and incubated for 4 h. Microplate reader was used to quantify the absorbance at 450 nm.

2.5. Calcineurin activity assay

Calcineurin (PP2B) activity and combined activity of protein phosphatase-1/protein phosphatase 2A (PP1/PP2A) were assayed in cell culture extracts using a commercially available colorimetric kit (Enzo Lifesciences, Lause, Switzerland) employing R-II phosphopeptide as substrate according to the manufacturer's instructions. The detection of free-phosphate released is grounded on the typical Malachite green assay bearing outstanding sensitivity (Harder et al., 1994). Microplate reader was utilized to quantify the optical density at 620 nm.

2.6. Western blot analysis

SH-SY5Y cells were washed for two times with ice-cold phosphate buffered saline (PBS), harvested and were lysed by resuspending in lysis buffer containing Tris−HCl (20 mM, pH 7.5), sodium fluoride (10 mM), sodium chloride (150 µl), 1 % Nonidet P-40 and protease inhibitor cocktail. After 40 min, cell lysates were centrifuged at 16,000×*g* for 15 min at 4 °C. The concentration of the protein extracts was measured by the Bradford assay method using the Bio-Rad Protein Assay kits (Bio-Rad Labs., Gurgaon, Haryana) and bovine serum albumin as standards with a microplate reader. The supernatants were collected, heated at 95 °C for 5 min and run for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12 % SDS-PAGE). The protein bands were electrophoretically transferred to nitrocellulose membranes; then, the nitrocellulose membranes were washed with tris buffered saline (TBS) for three times for 5 min each. The membrane was incubated with blocking solution containing 3 % non-fat dry milk in 0.1 % TBST tris buffered saline with tween 20 (TBST) for 60 min and then washed three times for 5 min each with TBST. The membranes were then incubated in primary antibodies at 4 °C overnight with: anti-BAD (1:1000, Bio-Rad Labs., Gurgaon, Haryana); anti-phospho-BAD (Ser112) (1:1000, Bio-Rad Labs., Gurgaon, Haryana); anti-CREB (1:1000, Sigma-Aldrich Co., St. Louis, USA); anti-phospho-CREB (Ser133) (1:1,000, Sigma-Aldrich Co., St. Louis, USA); anti-GAPDH antibody (1:1000, Sigma-Aldrich, USA) and anti-β-actin antibody (1:5000, Sigma-Aldrich, USA). Then, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse antibody (1:10000, Sigma-Aldrich Co., St. Louis, USA) for 90 min. After the incubation, the membranes were washed three times for 5 min each with TBST. Detection was achieved using enhanced chemiluminescence detection system reagent according to the manufacturer's instructions (ThermoFisher Scientific, Rockford, IL, USA) (Campolo et al., 2017).

2.7. Cell transfection and SEAP assay

Cell transfections were achieved by means of a liposomal mediated plasmid introduction. DMRIE-C (ThermoFisher Scientific, Rockford, IL, USA) liposome was used. Cells at 40–50% confluence received a total of 1.2 pM/ml of DNA attached to DMRIE-C at a ratio of 1:3 and diluted in reduced serum medium (Opti-MEM, ThermoFisher Scientific, Rockford, IL, USA). After 3 h of transfection, the liposome/DNA mix was substituted with fresh culture medium and the cells were allowed at least 48 h of recovery prior to use.

The secreted alkaline phosphatase assay (SEAP) (Sigma-Aldrich Co., St. Louis, USA) was used as a measure of transcription factor activity (Reese et al., 2008). Plasmids containing the SEAP gene downstream of enhancer sequences specific to binding cAMP response element (CRE--SEAP) were transiently transfected as described above. Furthermore, all cells were transfected with additional vector encrypting renilla luciferase to regulate homogeneous transfection proficiency amongst different samples. Twenty-four h after transfection, cells were treated with DMF (10 and 30 µM) for 2 h followed by incubation with 1 µM of oligomeric $A\beta_{1\text{-}42}$, in the presence of the adenylate cyclase (AC) activator forskolin (10 µM) and the release of SEAP in the culture medium (reflecting CREB-driven transcription) assayed 12 h thereafter (Reese et al., 2008). SEAP activity was assayed directly from the culture medium using the chemiluminescent detection kit according to manufacturer's instruction (Sigma-Aldrich Co., St. Louis, USA). SEAP quantity are a direct extent of the outcome of test compounds on the enhancer activity.

2.8. NFAT1 expression in nuclear extracts

Harvested cells were resuspended in a hypotonic buffer containing 1 mM EGTA, 1 mM EDTA and pH 7.4, 10 mM Tris and a protease inhibitor cocktail containing aprotinin (2 mg/ml), phenylmethylsulfonyl fluoride (100 mg/ml) and leupeptin (2 mg/ml). The mixture was incubated for 30 min on ice, homogenized and centrifuged for 15 min at 500 *g*. Pellets were collected and incubated with RIPA buffer for 15 min on ice and centrifuged to get nuclear extracts. Supernatants were centrifuged for 30 min at 17,000 *g* and upper layers as cytosolic extracts were isolated. RIPA buffer was used to excerpt membrane extracts from pellets. All solutions included protease inhibitor cocktail, PMSF and dithiothreitol (Cho et al., 2008).

The expression of NFAT1 was assessed by Western blot analysis (as described in section 2.6) using anti-NFAT1 antibody (1:1,000, Sigma-Aldrich Co., St. Louis, USA) in the nuclear fraction of treated SH-SY5Y cells.

2.9. BACE1 expression and BACE1 promoter transactivation

As NFAT1 activation is known to cause BACE1 expression (Cho et al., 2008), we thought to screen the potential effect of DMF pre-treatment on $A\beta_{1\text{–}42}$-induced BACE1 protein expression and BACE1 promoter activity in SH-SY5Y cells. The expression of BACE1 in treated SH-SY5Y cells was assessed by Western blot analysis (as described in section 2.6) using anti-BACE1 polyclonal antibody (1:1,000, Sigma-Aldrich Co., St. Louis, USA). The full-length 5′ promoter region of the human BACE1 gene was subcloned into the luciferase reporter vector pGL3basic and used to measure BACE1 promoter transactivation as described previously (Christensen et al., 2004). Transfection of SH-SY5Y cells grown in 12-well plates was done with promoter construct (1 µg) using Lipofectamine 2000 (ThermoFisher Scientific, India). After 3 h of transfection, the liposome/DNA mix was substituted with fresh culture medium and 24 h after transfection cells were treated with DMF (10 and 30 µM) for 2 h followed by incubation with 1 µM of oligomeric $A\beta_{1\text{-}42}$. The cells were lysed after 24 h and the luciferase assay was conducted as per the Dual Luciferase Reporter Assay System (Promega, Madison, USA).

2.10. β-Secretase enzyme activity

To investigate whether BACE1 enzymatic activity was altered by calcineurin-NFAT1 signaling and treatment with DMF has any consequence on it, the assessment of β-secretase activity was done on cell

membrane fractions isolated from $A\beta_{1-42}$ treated neuroblastoma cells. *In vitro* peptide cleavage fluorometric assay for measurement of BACE1 enzymatic activity was performed as per instructions (Abcam Fluorometric Assay Kit, ab65357). Samples (30 µl of membrane fractions) were added to 96-well plates and the volume was adjusted to 50 µl/well with extraction buffer in presence or absence of the β-secretase-specific inhibitor (Sigma-Aldrich Co., St. Louis, USA). To each well, 50 µl of reaction buffer was added and incubated for 10 min at 37 °C. Then, the β-secretase-specific substrate (10 µM) (Sigma-Aldrich Co., St. Louis, USA) was added to all samples, incubated for 1 h at 37 °C in dark and the β-secretase-specific activity corresponding to specific inhibitor sensitive fluorescence was recorded at each time point at 335 nm and 495 nm excitation and emission wavelengths.

2.11. NFκB-regulated BACE1 gene transcription

As $A\beta_{1-42}$ oligomers are known to induce NF-κB signaling (Tom et al., 2019), further enabling BACE1 expression and APP processing (Chen et al., 2012), we thought to elucidate another possible mechanism of action of DMF in BACE1 gene transcription mediated through NFκB signaling. Transfection of SH-SY5Y cells grown in 12-well plates was done with plasmid pBACE1-NFκB and pNFκB (1 µg) using Lipofectamine 2000 (ThermoFisher Scientific, India). After 3 h of transfection, the liposome/DNA mix was substituted with fresh culture medium and 24 h after transfection cells were treated with DMF (10 and 30 µM) for 2 h followed by incubation with 1 µM of oligomeric $A\beta_{1-42}$. The cells were lysed after 24 h and the luciferase assay was conducted as per the Dual Luciferase Reporter Assay System (Promega, Madison, USA).

2.12. β-Amyloid precursor protein processing

APP modulates cell growth, motility, neurite outgrowth and cell survival (O'Brien and Wong, 2011). Hence, it was thought worth to investigate the effect of DMF on βAPP processing. After treatment with various concentrations of DMF (10 and 30 µM), the cells were harvested and Western blot analysis (as discussed in Section 2.6) using anti-APP antibody (1:1,000, Sigma-Aldrich Co., St. Louis, USA) and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) were performed to quantity βAPP and mRNA expression levels, respectively.

2.13. Semiquantitative reverse transcription polymerase chain reaction

RT-PCR was carried out as described formerly (Permpoonputtana and Govitrapong, 2013). DMF treated cells were harvested and total RNA was isolated using TRI® reagent (Sigma-Aldrich Co., St. Louis, USA) conferring to the manufacturer's protocol. Reverse transcription was carried out by means of a reverse transcription kit and 2 µg of total RNA on a thermal cycler (The Eppendorf Mastercycler). PCR amplification was performed using primer sets (Table 1) specific for APP or D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich Co., St. Louis, USA). The PCR conditions were: 95 °C for 4 min; followed by 30 cycles of 95 °C for 45 s; 58 °C for 45 s; 72 °C for 45 s; a final extension step for 5 min at 72 °C and then holding at 4 °C. The amplification products were separated by electrophoresis in a 1 % agarose gel and visualized using BlueView™ Nucleic Acid Staining Solution (Sigma-Aldrich Co., St. Louis, USA). The PCR bands on the gel were visualized and imaged using an image analysis system (Bio-Rad, California, USA). The strengths of definite PCR bands were computed relative to GAPDH bands augmented from the identical cDNA.

Table 1
Primer arrangements for reverse transcription polymerase chain reaction.

Gene	Primer sequence (5′–3′)	Amplicon (bp)	Amplicon T^m (°C)
APP	Forward: GCTGGCCTGCTGGCTGAACC Reverse: GGCGACGGTGTGCCAGTGAA	387	79
GAPDH	Forward: TCTTCTTTTGCGTCGCCAG Reverse: GGGGGCAGAGATGATGACC	414	80

APP: amyloid precursor protein; GAPDH: D-glyceraldehyde-3-phosphate dehydrogenase.

2.14. Statistical analysis

Statistical analysis was accomplished by means of GraphPad Prism Version 7.0 for Windows. (GraphPad Software Inc., San Diego, CA). Communication of results was done as Mean ± S.E.M. Significance was assessed through a one-way analysis of co-variance (ANCOVA) trailed by Tukey's multiple evaluation trials. Probability (p) values of less than 0.05 were considered as significantly different.

3. Results

3.1. DMF ameliorates $A\beta_{1-42}$-induced calcineurin activity and cell apoptosis

Fig. 1(A) indicates that the release of LDH (an index of cell death) from SH-SY5Y cells was significantly increased ($p < 0.001$) by treatment with $A\beta_{1-42}$ (1 µM) for 24 h, in comparison with control cells, which was significantly blocked by DMF (10 and 30 µM) pre-treatment ($p < 0.001$ for both doses). There was no significant difference in the LDH release between DMF (10 µM) and DMF (30 µM) treated cells incubated with $A\beta_{1-42}$ (1 µM), indicating that both the doses of DMF exerted similar effect on LDH release [$F(3, 20) = 39.09$, $p < 0.0001$, $R^2 = 0.8543$]. CCK-8 assay was used to govern viability of cells. There was a significant decrease ($p < 0.001$) in survival of cells treated with $A\beta_{1-42}$ in comparison with control cells. Pre-treatment with DMF (10 and 30 µM) significantly diminished ($p < 0.001$ for both doses) the effect of $A\beta_{1-42}$ on cell death. Interestingly, DMF at the dose of 30 µM exhibited significantly enhanced cell survival ($p < 0.001$) when compared to DMF 10 µM dose in $A\beta_{1-42}$ (1 µM) treated cells, indicating a dose dependent effect [$F(3, 20) = 237.50$, $p < 0.0001$, $R^2 = 0.9727$] [Fig. 1(B)].

It was found that calcineurin activity (PP2B activity) was significantly increased ($p < 0.001$) by $A\beta_{1-42}$ treatment in SH-SY5Y cells in comparison with untreated cells and significantly blocked by DMF pre-treatment at the dose of 10 µM and 30 µM ($p < 0.01$ and $p < 0.001$ respectively). DMF at the dose of 30 µM exhibited significantly decreased PP2B activity ($p < 0.05$) when compared to 10 µM dose of DMF in $A\beta_{1-42}$ (1 µM) treated cells, indicating a dose dependent effect [$F(3, 20) = 39.09$, $p < 0.0001$, $R^2 = 0.8543$] [Fig. 1(C)]. Conversely, no consequence was detected on the joint activity of PP1/PP2A, two further protein phosphatases that in conflict with calcineurin are Ca^{2+}/calmodulin unresponsive (Mansuy and Shenolikar, 2006) in SH-SY5Y cells treated with $A\beta_{1-42}$ either in the presence or absence of pre-incubation with DMF ($p > 0.05$). This indicated that DMF does not have any direct significant effect ($p > 0.05$) on the combined activity of PP1/PP2A in SH-SY5Y cells [$F(3, 20) = 6.83$, $p = 0.0024$, $R^2 = 0.5063$] [Fig. 1(D)].

3.2. DMF blocks calcineurin activated apoptosis induced by $A\beta_{1-42}$ through BAD and CREB dephosphorylation and regulate CREB-driven transcription

Calcineurin activation induces apoptosis through dephosphorylation of BAD, hence dephosphorylation of BAD is pro-apoptotic (Virdee et al., 2000). SH-SY5Y cells treated with $A\beta_{1-42}$ resulted in BAD dephosphorylation. Western blot studies indicated that there is a related decrease of levels of phosphorylated-BAD (pBAD) and total BAD in cells treated with oligomeric $A\beta_{1-42}$ ($p < 0.001$). Pre-treatment with DMF (10 and 30 µM) reduced $A\beta_{1-42}$-induced rise of calcineurin activity in a dose dependent manner as evident from pBAD and BAD expression ($p < 0.01$). CREB is a

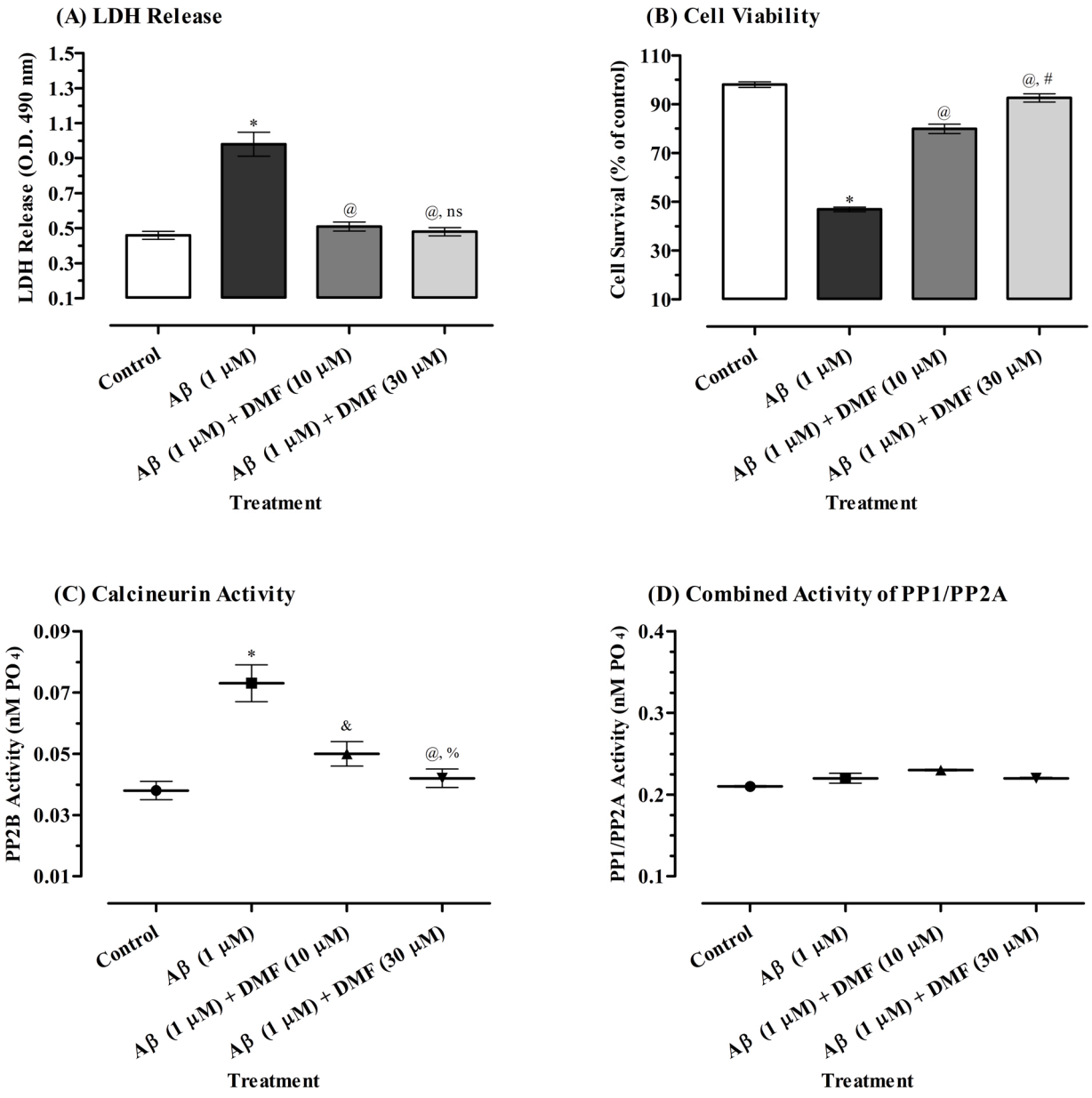

Fig. 1. Effect of pre-treatment with dimethyl fumarate on (A) lactate dehydrogenase release; (B) cell survival; (C) calcineurin (PP2B) activity and (D) combined activity of PP1/PP2A in neuroblastoma SH-SY5Y cells treated with oligomeric $A\beta_{1\text{-}42}$. Results are expressed as Mean ± S.E.M.; n = 6 in individual group. Data was analyzed by one way repeat measure ANCOVA followed by Tukey's multiple comparison test. Significance: *$p < 0.001$ when compared with control group; $^{\&}p < 0.01$, $^{@}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ treated group and $^{ns}p > 0.05$, $^{\%}p < 0.05$, $^{\#}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ + DMF (10 μM) treated group. Aβ: amyloid beta; DMF: dimethyl fumarate; LDH: lactate dehydrogenase; O.D.: optical density; PP1: protein phosphatase-1; PP2A: protein phosphatase-2A; PP2B: protein phosphatase-2B (calcineurin).

transcription factor crucial for long term potentiation and synaptic plasticity and the phosphorylative stimulation of CREB is negatively controlled by calcineurin (Hotte et al., 2007). Phosphorylation of CREB was decreased in SH-SY5Y cells treated with $A\beta_{1\text{-}42}$ ($p < 0.001$). Pre-treatment with DMF (10 and 30 μM) dose dependently blocked the effect of $A\beta_{1\text{-}42}$ on CREB phosphorylation in cells as evident from Western blotting ($p < 0.05$) [Fig. 2(A, B)].

Treatment of cells with the AC activator forskolin induces PKA-promoted CREB transcriptional activity; treatment of these cells with $A\beta_{1\text{-}42}$ significantly ($p < 0.01$) opposed the AC-stimulated release of SEAP. To determine whether induction of calcineurin may play a role in $A\beta_{1\text{-}42}$-promoted decrease of active CREB, SH-SY5Y cells were exposed to $A\beta_{1\text{-}42}$ in the presence of DMF (10 and 30 μM). The results illustrated in Fig. 2(C) show that opposing effect of $A\beta_{1\text{-}42}$ on forskolin-induced CREB-driven transcription in cells transfected with the CRE-SEAP reporter gene was significantly rescued ($p < 0.01$ and $p < 0.001$ for mentioned doses respectively) by treatment of cells with DMF in a dose dependent manner [$F(4, 25) = 76.07$, $p < 0.0001$, $R^2 = 0.9241$] [Fig. 2 (C)].

3.3. DMF opposes $A\beta_{1\text{-}42}$-induced activation of BACE1 expression and promoter activity through up-regulation of calcineurin/NFAT1 signaling pathway

To examine the effect of DMF on Aβ-induced NFAT1 expression in SH-SY5Y cells, Western blotting studies were conducted. After exposure

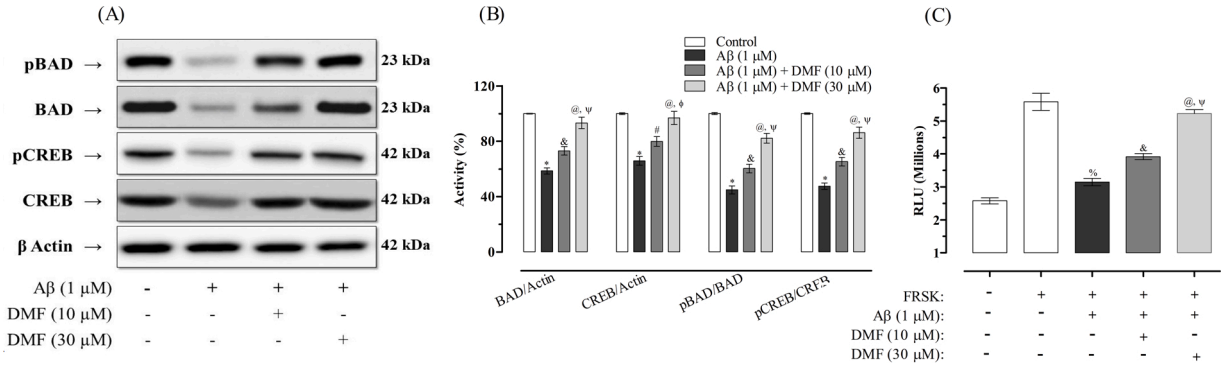

Fig. 2. Calcineurin dependent effect of pre-treatment with dimethyl fumarate on BAD and CREB dephosphorylation and CREB transcriptional activity in neuroblastoma SH-SY5Y cells treated with oligomeric $A\beta_{1\text{-}42}$. (A) Illustrative Western blot distinguishing phosphorylated BAD and phosphorylated CREB in total protein isolates from SH-SY5Y cells pre-incubated with DMF (10 and 30 μM), for 2 h, then treated with $A\beta_{1\text{-}42}$ (1 μM) for 24 h as shown. The blots were stripped and re-probed for total BAD, total CREB and β-actin to control for equal protein loading in each lane. (B) Relative expression of various proteins *viz.* BAD/Actin, CREB/Actin, pBAD/BAD and pCREB/CREB. (C) SEAP activity assay in the culture medium of SH-SY5Y cells transiently transfected with pCRE-SEAP reporter gene and treated with DMF (10 and 30 μM) for 2 h followed by incubation with 1 μM of oligomeric $A\beta_{1\text{-}42}$, in the presence of the adenylate cyclase (AC) activator forskolin (10 μM), and the release of SEAP in the culture medium (reflecting CREB-driven transcription) assayed 12 h thereafter. Results are expressed as Mean ± S.E.M.; n = 6 in individual group. Data was analyzed by one way repeat measure ANCOVA followed by Tukey's multiple comparison test. Significance: $^{\%}p < 0.01$, $^{*}p < 0.001$ when compared with control group; $^{\#}p < 0.05$, $^{\&}p < 0.01$, $^{@}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ treated group or FRSK + $A\beta_{1\text{-}42}$ treated group in respective figures and $^{\Phi}p < 0.01$, $^{\Psi}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ + DMF (10 μM) treated group or FRSK + $A\beta_{1\text{-}42}$ + DMF (10 μM) treated group in respective figures. Aβ: amyloid beta; BAD: Bcl-2 linked death protein; CREB: cAMP response element binding; DMF: dimethyl fumarate; FRSK: forskolin; SEAP: secreted alkaline phosphatase assay.

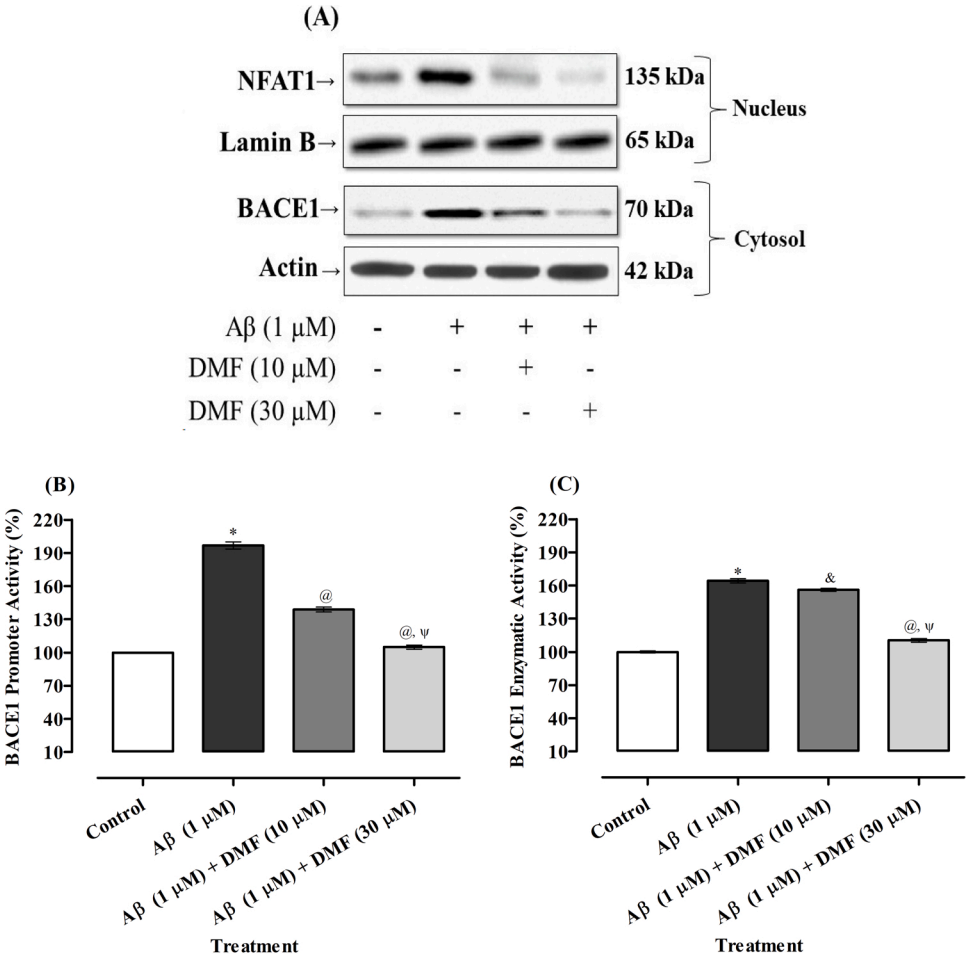

Fig. 3. Calcineurin dependent effect of pre-treatment with dimethyl fumarate on BACE1 and NFAT1 expression in neuroblastoma SH-SY5Y cells treated with oligomeric $A\beta_{1\text{-}42}$. (A) Nuclear and cytosolic extracts were isolated from SH-SY5Y neuroblastoma cells treated with DMF (10 and 30 μM) for 2 h followed by incubation with 1 μM of oligomeric $A\beta_{1\text{-}42}$. Treatment with $A\beta_{1\text{-}42}$ induced an increase of dephosphorylated NFAT1 (active form) and BACE1 in the nucleus and cytosol respectively, which was found to be settled by DMF treatment. (B) Aβ-induced BACE1 promoter activity was blocked by DMF pretreatment as evident from luciferase assay. (C) Aβ-induced BACE 1 enzymatic activity used for *in vitro* cleavage assay for β-secretase activity, but blocked by DMF. Results are expressed as Mean ± S.E.M.; n = 6 in individual group. Data was analyzed by one way repeat measure ANCOVA followed by Tukey's multiple comparison test. Significance: $^{*}p < 0.001$ when compared with control group; $^{\&}p < 0.01$, $^{@}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ (1 μM) treated group and $^{\Psi}p < 0.001$ when compared with $A\beta_{1\text{-}42}$ + DMF (10 μM) treated group. Aβ: amyloid beta; BACE1: β-site amyloid precursor protein cleaving enzyme-1; DMF: dimethyl fumarate; NFAT1: nuclear factor of activated T cells-1.

with $A\beta_{1-42}$ oligomer, the active form of NFAT1 *i.e.* the levels of dephosphorylated NFAT1 were enhanced in the nuclear fraction of the neuroblastoma cells, the effects were significantly found out to be reversed with DMF pre-treatment in a dose dependent manner [Fig. 3 (A)]. As NFAT1 activation is known to cause BACE1 expression (Cho et al., 2008), the potential effect of DMF pre-treatment on $A\beta_{1-42}$ induced BACE1 expression and promoter activity was screened in SH-SY5Y cells. Treatment with DMF at the dose of 10 and 30 μM significantly and dose dependently reduced BACE1 protein expression [Fig. 3(A)] and BACE1 promoter activity ($p < 0.001$ for both doses) brought by $A\beta_{1-42}$ introduction in neuroblastoma SH-SY5Y cells [$F(3, 20) = 413.60$, $p < 0.0001$, $R^2 = 0.9841$] [Fig. 3(B)]. To investigate whether BACE1 enzymatic activity was altered by calcineurin-NFAT1 signaling and treatment with DMF has any consequence on it, the assessment of β-secretase activity was done on cell membrane fractions isolated from $A\beta_{1-42}$ treated neuroblastoma cell lines. It was found that Aβ treatment significantly ($p < 0.001$) enhanced BACE1 enzymatic activity and DMF (10 and 30 μM) pre-treatment significantly reduced ($p < 0.01$ and $p < 0.001$ respectively) the activity in a dose dependent manner [$F(3, 20) = 466.30$, $p < 0.0001$, $R^2 = 0.9859$] [Fig. 3(C)].

3.4. DMF exhibits neuroprotection against $A\beta_{1-42}$ treatment by reducing NFκB mediated BACE1 gene transcription

To evaluate the possible role of DMF on NFκB-regulated BACE1 gene transcription, neuroblastoma SH-SY5Y cells were transfected with pBACE1-NFκB or pNFκB followed by oligomeric $A\beta_{1-42}$ for the activation of NFκB signaling. Dual luciferase revealed that oligomeric $A\beta_{1-42}$ significantly enhanced ($p < 0.001$) pBACE1-NFκB and pNFκB transcription activity relative to respective control. Contrarily, DMF treatment (10 and 30 μM) dose dependently elicits a significant reduction ($p < 0.001$) in NFκB signaling dependent BACE1 gene transcription [$F(3, 20) = 362.50$, $p < 0.0001$, $R^2 = 0.9819$ for pBACE1-NFκB transcription activity] [$F(3, 20) = 1130.00$, $p < 0.0001$, $R^2 = 0.9941$ for pNFκB transcription activity] (Fig. 4).

3.5. DMF is non-amyloidogenic: exhibit no effects on βAPP processing or mRNA levels

APP modulates cell growth, motility, neurite outgrowth and cell survival (O'Brien and Wong, 2011); hence, the effect of DMF on βAPP processing was investigated. As revealed from Western blot analysis [Fig. 5(A)] and semiquantitative RT-PCR [Fig. 5(B)], DMF at dose of 10 and 30 μM did not exhibit any significant effect on either βAPP protein expression [$F(2, 15) = 0.8839$, $p = 0.4336$, $R^2 = 0.1054$] nor mRNA levels [$F(2, 15) = 1.0310$, $p = 0.3806$, $R^2 = 0.1209$] in human neuroblastoma SH-SY5Y cells.

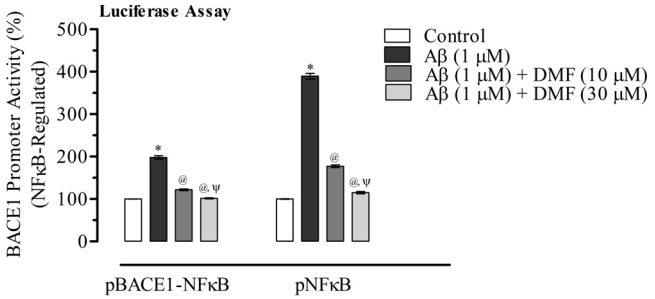

Fig. 4. Effect of DMF on oligomeric $A\beta_{1-42}$ stimulated NFκB dependent BACE1 gene transcription in neuroblastoma SH-SY5Y cells. Treatment with $A\beta_{1-42}$ induced an increase in NFκB dependent BACE1 promoter activity in neuroblastoma cells transfected with pBACE1-NFκB or pNFκB, which was found to be settled by DMF treatment. Results are expressed as Mean ± S.E.M.; n = 6 in individual group. Data was analyzed by one way repeat measure ANCOVA followed by Tukey's multiple comparison test. Significance: *$p < 0.001$ when compared with control group; $^{@}p < 0.001$ when compared with $A\beta_{1-42}$ (1 μM) treated group and $^{\Psi}p < 0.001$ when compared with $A\beta_{1-42}$ + DMF (10 μM) treated group. Aβ: amyloid beta; BACE1: β-site amyloid precursor protein cleaving enzyme-1; DMF: dimethyl fumarate; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells.

4. Discussion

Outcomes of the present research demonstrated that oligomeric $A\beta_{1-42}$ induced cell death, enhanced calcineurin activity by increasing BAD dephosphorylation and reducing CREB phosphorylation in SH-SY5Y cells. Moreover, $A\beta_{1-42}$ treatment enhanced the active form of NFAT1 that cause increased BACE1 levels, BACE1 promoter activity, BACE1 enzymatic activity and NFκB signaling dependent BACE1 gene transcription in SH-SY5Y cells. All these $A\beta_{1-42}$-induced effects were found to be reversed by DMF ((Fig. 6).

Reese et al. (2008) discovered that only oligomeric Aβ and not monomeric or fibrillar Aβ, produces calcineurin dependent impact in producing/worsening Alzheimer's disease pathology in neuronal cells by Ca^{2+}/calmodulin-dependent calcineurin activation; henceforth, only oligomeric isoform of $A\beta_{1-42}$ has been used in our study and the oligomers were prepared as per the method described by the researchers. LDH is a constant cytoplasmic enzyme existing in all cells and upon damage of plasma membrane it is quickly released into the cell culture supernatant. Therefore, the level of LDH is utilized as an unfailing biochemical indication for the impairment of neuronal plasma membrane integrity (Kumar et al., 2018). Acquaintance of SH-SY5Y cells to $A\beta_{1-42}$, cytotoxicity and LDH leakage was induced. Application of CCK-8 is an index of mitochondrial viability since the leakage levels of LDH are decreased by metabolically live mitochondria (Liu et al., 2016). The results indicated that DMF exerted a noteworthy involvement in SH-SY5Y cell's mitochondrial viability and was effective in averting LDH release from SH-SY5Y cells. Our findings are in agreement with a study by Campolo et al. (2018) showing that pre-treatment of SH-SY5Y cells with DMF prior to $A\beta_{1-42}$ peptide exposure significantly decreased cell death, assessed using MTT assay. They also discovered that DMF itself does not have any effect on cell viability (Campolo et al., 2018).

We first examined the expression of calcineurin (PP2B), PP1 and PP2A in the SH-SY5Y neuroblastoma cells treated with $A\beta_{1-42}$ and the protective effect of DMF on their expression. It was found that 30 μM DMF treatment increased percent cell survival and further reduced calcineurin expression in $A\beta_{1-42}$-induced cells when compared to the 10 μM treatment, addressing a dose dependent effect. This outcome on calcineurin activity was in accordance with the differential effect produced by the macrolactam natural product, tacrolimus in blocking the oligomeric Aβ-induced rise of calcineurin activity in neuronal cells (Reese et al., 2008). Tacrolimus turns as an influential and clinically beneficial immunosuppressant *via* interruption of signaling actions facilitated by calcineurin in T lymphocytes. It forms a molecular complex with the intracellular FK506-binding protein-12, thereby getting the capability to interact with calcineurin and subsequently dephosphorylation of various substrates (Dumont, 2000). DMF was also found out to be immunosuppressant in numerous studies by acting on specific memory and effector T cell subsets, limiting their survival, proliferation, activation, cytokine production and altering phosphorylation of some targets (Wu et al., 2017). The immunosuppressive similarity of DMF and FK506, including the ability of effecting the phosphorylation of various biomolecules, may be considered to compare the similar effect of tacrolimus on calcineurin activity with that of DMF.

Controlled by cAMP, both PP1 and PP2A are fragment of the serine/threonine phosphoprotein phosphatase domain and exhibit critical functions in modulating phosphorylation status and appropriate signal transduction in numerous vital molecular pathways all over the body (Leslie and Nairn, 2019). In Alzheimer's pathology, Aβ disturbs PP1 processing (Knobloch et al., 2017) and PP2A is affected primarily

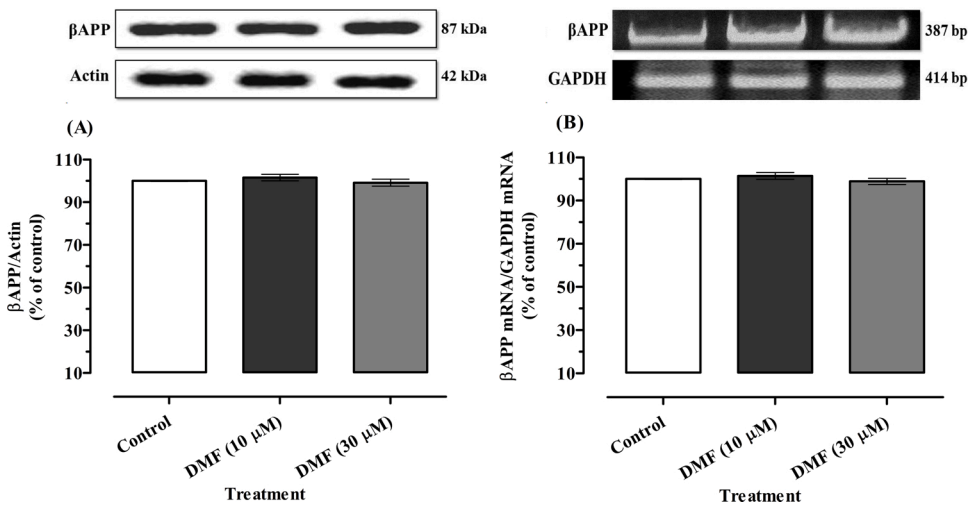

Fig. 5. Effect of pre-treatment with dimethyl fumarate on βAPP expression and mRNA levels in neuroblastoma SH-SY5Y cells. Western blot analysis was used to determine the effect of DMF pre-treatment on βAPP expression and semiquantitative RT-PCR for detection of alteration in mRNA expression of βAPP. Band densities from the Western blots and semi-quantitative PCR were standardized to actin and GAPDH respectively and their ratios were calculated as a fraction of the corresponding value of the control group. Results are expressed as Mean ± S.E.M.; n = 6 in individual group. Data was analyzed by one way repeat measure ANCOVA followed by Tukey's multiple comparison test. βAPP: beta amyloid precursor protein; Aβ: amyloid beta; DMF: dimethyl fumarate; GAPDH: D-glyceraldehyde-3-phosphate dehydrogenase.

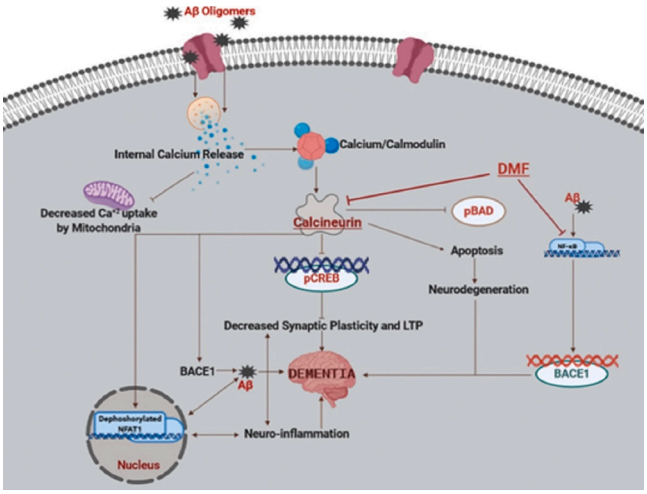

Fig. 6. Effect of DMF on calcineurin intermediated consequences on $A\beta_{1-42}$-induced neurotoxicity. Aβ infiltration cause facilitated internal calcium release that binds to calmodulin to activate calcineurin. Calcineurin further channelize through many pathways like BAD mediated apoptosis and CREB dependent loss in synaptic plasticity and LTD, causing dementia of Alzheimer's disease. BACE1 gene expression in neurons and astrocytes is encouraged by Aβ treatment through stimulation of calcineurin/NFAT1 and calcineurin/NFAT4 signaling respectively. This BACE1 expression in astrocytes happens through calcium permeable channel formed by membrane-inserted Aβ and through upregulation of disturbed intracellular calcium homeostasis in neurons. DMF causes a protective effect on calcineurin mediated neurotoxicity. Aβ: amyloid beta; BACE1: β-site amyloid precursor protein cleaving enzyme-1; pBAD: phosphorylated Bcl-2 linked death protein; pCREB: phosphorylated cAMP response element binding; DMF: dimethyl fumarate; LTP: long term potentiation; NFAT1: nuclear factor of activated T cells-1.

through tau phosphorylation (Sontag and Sontag, 2014), causing disturbance of long-term potentiation and differentiation. Moreover, an increment in PP2A activity decreases Aβ toxicity instead of Aβ production itself, which suggests that the lethal properties of Aβ could be intensely amended without lessening Aβ production (Wu et al., 2017). As evident from the study by Ramser et al. (2013), oligomeric Aβ did not cause any effect on levels of PP1/PP2A in neuronal cells, therefore we checked for the ability of DMF itself to cause any alteration in PP1/PP2A activity. We found that the combined activity of PP1/PP2A was unaltered by $A\beta_{1-42}$-induced cells with or without pre-treatment with DMF in neuroblastoma SH-SY5Y cell lines. Yao et al. (2013) illustrated that Aβ-induced neurotoxicity is through both pre-synaptic and post synaptic dysfunction. Moreover, PP1/PP2A only participates in Aβ-induced acute postsynaptic disruption, whereas PPP2B is involved in both presynaptic and post synaptic dysfunction. Further it was established that PP1/PP2A mainly functions in Aβ-induced post synaptic but not presynaptic dysfunction (Yao et al., 2013). Hence, this fact can be a suitable explanation for the results of our study related to effect on Aβ and DMF on PP1/PP2A activity at cell culture level.

BAD is an adherent of pro-apoptotic Bcl-2 family, dephosphorylative stimulation of BAD through calcineurin causes its disassociation from cytosolic framework proteins and movement towards the mitochondrion, resulting in disruption of the anti-apoptotic function of Bcl-2, thus introducing a sequencer of cell death (Yang et al., 2004). Moreover, calcineurin dependent phosphorylation pathways cause activation of CREB; phosphorylated CREB (p-CREB) translocate to the nucleus and result in transcription of genes that yield proteins apparently crucial for synaptic plasticity, long term potentiation and memory function (Snyder et al., 2005). In our experiments it was shown that oligomeric $A\beta_{1-42}$ decreased BAD phosphorylation and induced cell death and likewise reduced CREB phosphorylation and CREB-dependent transcriptional action in SH-SY5Y cells. Pre-treatment with DMF abolished these outcomes related to $A\beta_{1-42}$-induced neurotoxicity. The results of the present study specify that $A\beta_{1-42}$-induced neural mitochondrial dysfunction and calcineurin activation, and calcineurin acts as an upstream moderator of NFκB stimulation, further elevating the protein expression levels of IL-1β (Li et al., 2017), which is a traditional indicator of neuroinflammation and may consequence in cognitive impairments. As DMF is a well-known potent inhibitor of NFκB pathway (Kastrati et al., 2016), it is proposed that any action exerted by DMF is likely to be mediated by inhibition of both calcineurin and NFκB in $A\beta_{1-42}$-induced neurotoxicity.

The effect of $A\beta_{1-42}$ on CREB-dependent transcriptional action was measured by the release of SEAP in the culture medium by transiently transfecting with a SEAP reporter gene vector construct driven by pCRE-SEAP. It was previously known that CRE-SEAP activity was strikingly, though not significantly, decreased by $A\beta_{1-42}$ treatment of SY5Y cells and very low as close to background standards (Reese et al., 2008). Hence, the cells were treated with AC-activator forskolin. Forskolin significantly brought up with PKA-endorsed CREB transcriptional activity, as reflected by enhanced SEAP release in the culture medium of CRE-SEAP-transfected SH-SY5Y cells. Negative regulation of phosphorylated CREB by $A\beta_{1-42}$ was shown by a lessening of pCREB-driven transcriptional activity, that is the release of SEAP in culture media (Reese et al., 2008), which was reversed with DMF pre-treatment.

Cytosolic NFAT proteins are dephosphorylated by calcineurin and translocate to the nucleus to alter gene transcription involved in cytokine production and inflammation (Macian, 2005) and only NFAT1 was activated by $A\beta_{1\text{-}42}$ peptide in SH-SY5Y cells (Cho et al., 2008), hence we studied the effect of DMF on NFAT1 in nuclear fraction. The protective effect of DMF on $A\beta_{1\text{-}42}$-induced NFAT1 expression may be due to one or more of the mechanisms *viz.*: inhibition of enzymatic activity of calcineurin; inhibition of binding of calmodulin to calcineurin or active center of calcineurin; inhibition of NFAT-DNA binding; inhibition of NFAT dephosphorylation (active form) or enhancement of re-phosphorylation (inactive form) and nuclear export of NFAT; disruption of calcineurin-NFAT binding and inhibition of calcineurin-dependent NFAT transactivation. Some compounds like kaempferol, gossypol, dibefurin, dipyridamole *etc.* are known to exert one or more of the aforementioned effects (Sieber and Baumgrass, 2009) and DMF may be considered to be compared for the similar effect on NFAT expression. Moreover, Aβ treatment is known to cause NFAT dependent increase in extracellular glutamate (causing glutathione depletion) and increasing the likelihood of excitotoxic cell death (Abdul et al., 2009), on the other hand DMF has been known to protect from oxidative glutamate toxicity, augment the major cellular antioxidant glutathione and recycling, nuclear translocation of transcription factors and the expression of antioxidant genes (Albrecht et al., 2012). As evident from our study, inhibition of Aβ-induced NFAT activity in SH-SY5Y cells by DMF may be a mechanistic cause for decrease in Aβ-dependent elevations in glutamate and neuronal death, causing neuroprotection. Cho et al. (2008) studied protective effects of a calcineurin inhibitor, cyclosporin A on the BACE1 expression in SH-SY5Y neuroblastoma cells. Cyclosporin A treatment blocked aggregated $A\beta_{1\text{-}42}$-stimulated activation and nuclear translocation of NFAT1 following up-regulation of BACE1 expression. Moreover, in primary cortical cultures, a calcium ionophore stimulated generation of more $A\beta_{1\text{-}42}$ was also reversed by cyclosporin A treatment. These $A\beta_{1\text{-}42}$ and calcium ionophore generated effects were brought due to an increased intracellular calcium concentration and that this process is mediated through the calcineurin-NFAT1 signaling pathway (Cho et al., 2008). Considering that ameliorating effect of cyclosporin A (1 μM) on $A\beta_{1\text{-}42}$-induced NFAT1-mediated BACE1 expression was similarly observed with that of DMF (30 μM) in our study, it is likely that both the compounds exert similar effects on calcium homeostasis and calcineurin dependent pathways and play a crucial role in protecting aging and Alzheimer's disease pathogenesis.

In our study DMF blocked $A\beta_{1\text{-}42}$-induced BACE1 enzymatic and promoter activity caused due to overexpression of NFAT1. We propose that DMF may interfere with a putative NFAT-binding site within the BACE1 gene promoter region confirming hinderance in direct binding of NFAT1 to the BACE1 gene promoter or may cause a reconciliation of intracellular calcium homeostasis. BACE1 mRNA levels (promoter activity) was enhanced by $A\beta_{1\text{-}42}$ and restored by DMF treatment indicating that DMF regulated at the transcriptional level. Secondly, DMF might have altered Aβ-induced BACE1 immunoreactivity or BACE1 transport in axons and dendrites, leading to reduced transport of BACE1 to lysosomal compartments. In fact, this hypothesis is corroborated by a finding by Mamada et al. (2015) that chloroquine treatment induces a similar alteration in BACE1 immunoreactivities in neurites (Mamada et al., 2015).

There is a positive correlation amongst the activity of NFκB and BACE1. Two operational NFκB-binding elements have been discovered in the human BACE1 promoter area. Chen et al. (2012) found that NFκB p65 expression cause enhanced BACE1 promoter activity and BACE1 transcription, whereas interference of NFκB p65 reduced BACE1 gene expression in p65 knockout cells. Furthermore, NFκB p65 expression conducts up-regulated β-secretase cleavage and Aβ generation. NFκB is known to be adhered with BACE1 activation, the BACE1 gene promoter has an intricate structure with various transcription factor binding sites, including NFκB (Buggia-Prevot et al., 2008). According to Chen et al. (2012), human BACE1 promoter region consists of four putative NFκB binding locations. Our results confirmed that they are effective, which is also consistent with former results (Wang et al., 2015). We demonstrated that NFκB activation, induced by $A\beta_{1\text{-}42}$, could enhance BACE1 promoter activity and expression. On the other hand, we demonstrated that DMF interferes with NFκB activity in promoter level. Our results are in concordance with the similar study by Huang et al. (2020) indicating that NFκB mediates the downregulation of curcumin on BACE1 transcriptional levels (Huang et al., 2020). Here, we provide evidence that targeting NFκB linked BACE1 transcriptional activity by DMF confers anti-inflammatory changes and support the notion that inhibiting the pathway might be therapeutically beneficial in Alzheimer's disease.

Taken together, our results clearly demonstrate that NFκB signalling facilitates BACE1 gene expression and APP processing, and increased BACE1 expression mediated by NFκB signalling in the brain could be one of the novel molecular mechanisms underlying the development of Alzheimer's disease in some sporadic cases. Our study suggests that inhibition of NFκB-mediated BACE1 expression may be a valuable drug target for Alzheimer's disease therapy.

Neuronal bustle modifies numerous pathways of APP processing, signifying worthy roles for activity induced APP cleavage results in amending neuronal excitability and synaptic plasticity. The finding that cathepsin B can act as a β-secretase (Hook et al., 2009) drove us to screen effect of DMF on APP processing. It was found that DMF does not alter either βAPP protein or mRNA levels in human neuroblastoma SH-SY5Y cells, establishing the fact that DMF does not possess neurotoxic effect. Besides β- and γ-secretases, APP can also be cleaved by some other proteases, such as caspases, aspargine endopeptidase (δ-secretase), the recently identified η-secretase or other unidentified proteases to generate toxic fragments and/or impact further processing (Haytural et al., 2019). Since APP is extensively proteolytically processed, it is desirable to screen the effect of potential therapeutic candidates like DMF on the full-length APP or an APP processing product *viz.* sAPPα or β-*CTF* (Muresan and Ladescu Muresan, 2015). In the nonamyloidogenic pathway, the production of a neuroprotective and neurotrophic factor sAPPα is dependent on α-secretase (ADAM10) activity and expression and sAPPα is known to be stimulated by PKC activation. In the amyloidogenic pathway, the production levels of β-*CTF* of APP (C99) is dependent upon γ-secretase to generate Aβ peptide. Moreover, C99 expression can be decreased by inhibition of NFκB activation (Panmanee et al., 2015). Therefore, in order to widely investigate the effect of DMF on APP processing, many studies are inside the scope.

5. Conclusion

Present research indicates that DMF averted oligomeric $A\beta_{1\text{-}42}$-induced rise in calcineurin activity that endorse a dual signaling pathway, which can lead to either decreased CREB activity or to BAD promoted cell death as well as NFAT1 and NFκB dependent BACE1 expression in SH-SY5Y human neuroblastoma cells. DMF can shield the effect of $A\beta_{1\text{-}42}$ on synaptic plasticity and memory, independently of neuronal death, *via* a signaling pathway relating increased calcineurin activity. In conclusion, through the data presented here we propose that by means of DMF, inhibiting molecular events related to $A\beta_{1\text{-}42}$-induced enhanced calcineurin activity may be an alternative approach towards finding suitable strategy for cure of neurotoxic events leading to senile dementia, as the disruption of calcium homeostasis plays a crucial role in aging and Alzheimer's disease pathogenesis.

CRediT authorship contribution statement

Mithun Singh Rajput: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Funding acquisition, Resources. **Nilesh Prakash Nirmal:** Methodology, Formal analysis, Investigation, Writing - review & editing, Resources, Supervision. **Devashish Rathore:** Methodology, Resources. **Rashmi Dahima:** Writing - review & editing, Resources, Supervision.

Declaration of Competing Interest

We declare that we do not have any conflict of interest.

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