Synergism of Docosahexaenoic Acid and Rosuvastatin in the Treatment of Donepezil Intolerant Alzheimer's Disease Population- A Study with SH-SY5Y Cell Line

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KEYWORDS
Alzheimer's disease, neuroinflammation, amyloid beta, docosahexaenoic acid, rosuvastatin, donepezil

ABSTRACT
Background: Alzheimer's disease (AD) has become a global concern. Many times the patients cannot tolerate the first line anti-Alzheimer's drugs. This includes acetylcholinesterase inhibitors such as donepezil. This shifts the focus on repurposing study with drugs which are expected to alleviate AD. The purpose of the study was to evaluate the synergistic therapeutic potential of docosahexaenoic acid (DHA) and rosuvastatin combination against donepezil.
Methods: RT-PCR was performed to find the effects of individual drugs and drug combination on caspase-3 (amyloid beta1-42 induced), IL-1β and TNF-α expression. Nitric oxide, lipid peroxide level and acetylcholinesterase activity were measured by spectrophotometric method.
Results: DHA and rosuvastatin combination was better than donepezil in suppressing expressions of caspase-3 (2^ΔΔCT values for gene expression 0.21 Vs 0.60), IL-1β (2^ΔΔCT values for gene expression 0.13 Vs 0.28) and TNF-α (2^ΔΔCT values for gene expression 0.04 Vs 0.27). Similarly, DHA and rosuvastatin combination was better than donepezil in inhibiting nitric oxide level (73.96% Vs 66.19%). However, donepezil was better than DHA and rosuvastatin combination in suppressing lipid peroxide (72.40% Vs 67.20%) and acetylcholinesterase (91.85% Vs 82.33%).
Conclusions: DHA and rosuvastatin combination was better than donepezil in suppressing most of the study parameters associated with AD. DHA and rosuvastatin combination was synergistic and may emerge as a substitute for donepezil to treat AD. The significance of the finding lies in the fact that the data generated in this research will open newer horizons in AD research. Once proven effective, the combination may be tried to manage mild to severe AD in donepezil intolerant AD population.

1. Introduction

Alzheimer's disease (AD) dating back to 1907 (O'Brien & Wong, 2011), has become a global concern. It has affected more than 35 million people world-wide, and represents 60-80% of all dementia cases (Sarasija et al., 2018). In spite of the huge treatment expenditure of AD, the results are often disappointing, limiting the expectancy of life up to 7-10 years following the manifestation of dementia symptoms (Sathianathan & Kantipudi, 2018; Van der Flier & Scheltens, 2005; Zamberlan et al., 2020). Till date there is no medication which could reverse or completely cure AD. Donepezil becomes the drug of choice for AD due to its ability to treat mild, moderate and advanced AD (Farlow et al., 2010). It is a highly selective inhibitor of acetylcholinesterase (AChE). However, therapeutic response is genotype specific. About 50% of AD population which shows abnormal metabolism of AChE-inhibitors needs higher dose of donepezil, whereas, remaining 50% which shows poor metabolism manifests potential toxicity at low dose (Cacabelos, 2007). Donepezil shows many adverse effects such as insomnia, muscle cramps, fatigue, anorexia, bradycardia and may produce heart block (Kumar et al., 2023). Adverse effects may be observed at a minimum therapeutic dose of 5mg/day (Hashimoto et al., 2000); whereas, for higher therapeutic efficacy a dose of 10-23mg/day may be required (Farlow et al., 2010). This raises concern on the tolerability of donepezil. This underscores the need to develop an alternative therapeutic option for donepezil intolerant AD population. Two drugs which could play a promising role in the treatment of AD are docosahexaenoic acid (DHA) and rosuvastatin. Though DHA was not a promising therapy for mild to moderate AD, yet intravenous administration of DHA after 30 minutes of traumatic brain injury has reduced neurological deficits. DHA could reduce oxidation and deposition of amyloid precursor protein (Thau-Zuchman et al., 2020). DHA is safe at 2g/day dose (Dean et al., 2014). Prophylactic DHA has also been found to reduce amyloid beta (Aβ) production (Grimm et al., 2011). Thus, evaluation of anti-Alzheimer's therapeutic potential of DHA on already deposited Aβ and other mediators was much

warranted. Similarly, prophylactic rosuvastatin has been found to reduce cortical and hippocampal Aβ deposit (Zheng et al., 2020). It is also safe among wide range of patients at 5-40 mg dose range (Shepherd et al., 2004). Prophylactic use of statin had reduced the incidence of AD (Torrandell-Haro et al., 2020) but anti-Alzheimer's therapeutic potential of rosuvastatin on already deposited Aβ and its downstream messengers was not unveiled. Similarly, the therapeutic potential of DHA and rosuvastatin combination was yet to be explored against AD. Combination of DHA and rosuvastatin was expected to produce synergism because combination of omega-3-polyunsaturated fatty acid with rosuvastatin had improved endothelial function, reduced triglyceride and low density lipoprotein (LDL). However, absence of DHA had abolished the vasodilatory effect of statin (Mindrescu et al., 2008). Hence, this research tries to evaluate the efficacy of DHA and rosuvastatin combination against donepezil. The results of the study would help to evaluate our hypothesis, which states that DHA and rosuvastatin combination would be synergistic and might be used in the treatment of donepezil intolerant AD population.

2. Material and Methods

Materials

Amyloid Beta 1-42 (Aβ1-42) (Product No: A9810, purity ≥ 95%, purchased from sigma), lipopolysaccharide (LPS) (Product No: L3024, purchased from Sigma), DHA (Product No: D2226, purity > 97 %, purchased from TCI), rosuvastatin calcium (Product No: R0180, purity > 95%, purchased from TCI). Trypsin-EDTA solution, fetal bovine serum, penicillin/streptomycin, Dulbecco's Modified Eagle Medium (DMEM), Dimethyl sulphoxide (DMSO), F12 medium, phosphate-buffered saline (PBS) were purchased from HiMedia. All other chemicals and reagents of analytical grade were readily available from commercial sources.

Dose selection

Dose of Aβ1-42 (25µM) was finalized based on its ability to induce caspase-3. Concentration of bacterial lipopolysaccharide (LPS) (10ng/ml) was selected based on previous literature (Justin et al., 2020). Dose selection for DHA (70µM) (Géraldine et al., 2010), rosuvastatin (20µM) (Famer & Crisby, 2004) and donepezil (1.5µM) (Zimmermann et al., 2004) was made based on previous MTT assays.

Preparation of drug solution

Aβ1-42, LPS, DHA, rosuvastatin and donepezil stocks were prepared in DMSO and further diluted with DMEM plain media to get the required concentration. Final dilution contains less than 1% DMSO.

Cell culture

SH-SY5Y cell line was procured from National Centre for Cell Science (NCCS), Pune, India. The cell line was authenticated by Dr. Punam Nagvenkar, Scientist D, Cell repository. The adherent type of SH-SY5Y cell line was maintained as per standard protocol. Stock cells were cultured in 1:1 ratio of DMEM and F12 medium supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100µg/ml). The culture was continued in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05 % glucose in PBS). The viability of the cells were checked and seeded in 12 well plates. Undifferentiated cells were differentiated and used for the research purpose (Figure 1).

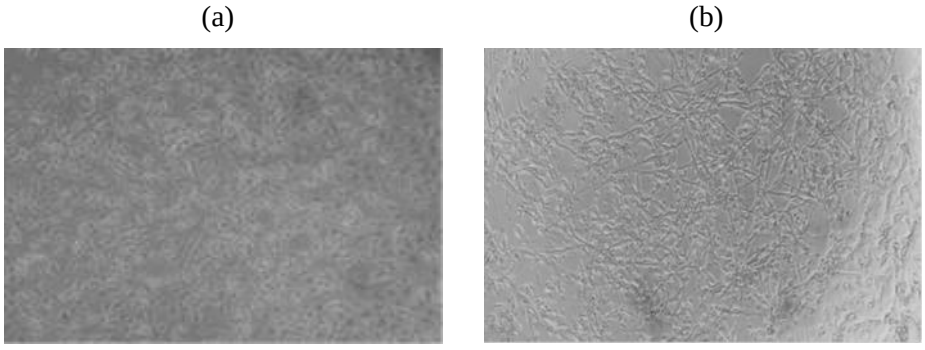

Figure 1: (a) Undifferentiated cells (b) Differentiated cells.

Treatment groups

Differentiated SH-SY5Y cells were divided into 6 groups. Group 1: Control, Group 2: Aβ/LPS/Ethanol treatment, Group 3: Aβ/LPS/Ethanol + DHA treatment, Group 4: Aβ/LPS/Ethanol + Rosuvastatin calcium treatment, Group 5: Aβ/LPS/Ethanol + DHA and Rosuvastatin calcium combination treatment, Group 6: Aβ/LPS/Ethanol + Donepezil hydrochloride treatment.

Caspase-3 assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 10 x 10^6 cells/ml using media containing 10% FBS. To each well of the 6 well microtiter plate, 100µl of the diluted cell suspension (10,00,000 cells/well) was added. After 24hrs, Aβ (25µM) was added and incubated for 24hrs at 37°C, 5% CO_2. After incubation, the cells were treated with test drugs as per the group distribution and were incubated at 37°C for 48hrs in 5% CO_2 atmosphere. Later, the cells were taken for RNA isolation (Schmittgen & Livak, 2008).

Sample preparation and RNA isolation

Sample preparation and RNA isolation were done as per standard protocol (Schmittgen & Livak, 2008). SH-SY5Y cells were washed twice with PBS and 2 ml of TRIzol (per T25 flask) was added to the adherent cells and transferred to the tube and vortexed. Samples were allowed to stand for 5 minutes at room temperature. Following this, 0.2ml of chloroform was added per 1 ml of TRIzol used. The tube was closed and shaken vigorously for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes. The resulting mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C. The colourless upper aqueous phase was transferred to a new clean tube. Added 0.5 ml of isopropanol per 1ml of TRIzol used, mixed gently by inverting the sample 5 times and incubated at room temperature for 5 minutes. This is followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. Supernatant was discarded and the RNA pellet was washed by adding 1ml of 70% ethanol and mixed gently by inverting the sample a few times. Again centrifuged for 5 minutes at 14,000 rpm at 4°C. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5 minutes at 55°C. The pellet was then resuspended in 25 µl of diethylpyrocarbonate (DEPC) treated water.

RT-PCR study

A semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Applied Biosystems to determine the levels of caspase-3 and Beta-Actin (Schmittgen & Livak, 2008). The cDNA was synthesized from 2 µg of RNA using the Prime script RT reagent kit (TAKARA) with oligo dT and random hexamer primer according to the manufacturer's instructions. The reaction volume was set to 20µl and cDNA synthesis was performed at 50°C for 30 minutes. This was followed by RT inactivation at 85°C for 5 minutes and finally the c-DNA was diluted with twice the volume (1:2). Primer sequence used for the study is reported below (Table 1). The PCR primers used for β-Actin and caspase-3 are same as the primers used by Isaq *et al.* (Isaq et al., 2023).

Table 1. List of different PCR primers used in the study.

Gene	Primer pair	Sequence	Tm	Product size (bp)
β-Actin[$]	FP	TCCTCCTGAGCGCAAGTACTCT	62.1	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	62.4	
Caspase-3[$]	FP	ACATGGCGTGTCATAA AATACC	51.88	162
	RP	CACAAAGCGACTGGATGAAC	51.83	
TNF-α	FP	GAGAAGGGTGACCGACTCAG	59.83	176
	RP	GGTTGAGGGTGTCTGAAGGA	60.09	
IL-1β	FP	TGTACCTGTCCTGCGTGTTG	60.25	198
	RP	AGACGGGCATGTTTTCTGCT	60.25	

[$]Same primers for β-Actin and caspase-3 have been used for research purpose by Isaq *et al*.

Determination of relative fold expression of target gene by the comparative CT Method (ΔΔ CT Method)

Relative expression of target gene in relation to housekeeping gene Actin was determined by the comparative CT method. Delta CT for each treatment was calculated using the formula (Schmittgen & Livak, 2008) given below.

Delta Ct = Ct (target gene) – Ct (reference gene)

To compare the individual samples from treated group with untreated control, delta delta CT was calculated using the formula given below.

Delta delta Ct = delta Ct (treatment group) – average delta Ct (control group)

Fold change in target gene expression for each treatment was calculated using the following formula.

Fold change = 2^ (-delta delta Ct)

Cytokine (IL-1β and TNF-α) assay

Treatment condition, sample preparation, RNA isolation, RT-PCR study techniques and calculation for expression levels of IL-1β and TNF-α remain the same, as it was for caspase-3 study, except for the inducing agent, primers used (Table 1) and treatment exposure. SH-SY5Y cells were treated with LPS (10ng/ml) for 4hrs at 37ºC, 5% CO_2 to induce the expression of IL-1β and TNF-α. After 4 hours of treatment with LPS, cells were treated with the therapeutic agents for 24 hours and subjected to the estimation of the expression level of cytokines.

Nitric oxide assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 5 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000 cells/well) was added. After 24hrs, LPS (10ng/ml) was added and allowed to interact for 4hrs and different test drugs were added to the microtiter plates as per the group distribution. The plates were then incubated at 37ºC for 48hrs in 5% CO_2 atmosphere. Following this, 200µl of cell supernatant was taken and the level of nitric oxide was estimated from the absorbance of nitrite at 540 nm using standard procedure (Justin et al., 2020; Green et al., 1982). The standard calibration curve was plotted with different concentrations of sodium nitroprusside ranging from 20µM - 0.625µM. Percentage inhibition of nitric oxide was estimated by using the formula given below.

% Inhibition = {(OD of LPS treated group - OD of drug treated group)/ OD of LPS treated group} X 100.

Lipid peroxide assay

Lipid peroxide assay was performed using thiobarbituric acid method, where malondialdehyde content (MLD) indicated the extent of lipid peroxidation (Justin et al., 2020; Ohkawa et al., 1979). The monolayer cell culture was trypsinized and treated with LPS in the same manner as it was for nitric oxide assay. Following treatment with therapeutic agents cells were washed once with 1X PBS and cell lysates were prepared. Cell lysate (0.2ml) was taken and 0.8ml of saline, 0.5ml of butylhydroxytoluene (BHT) and 3.5ml thiobarbituric acid (TBA) reagent (0.8%) were added and incubated at 60°C. This was followed by cooling and further centrifuged at 2000 rpm for 10 min. Absorbance of the sample was measured at 532 nm using a microplate reader. The standard calibration curve was plotted with different concentrations of MLD ranging from 50µM - 1.56µM.

Percentage inhibition of lipid peroxide was estimated by using the formula given below.

% Inhibition = {(OD of LPS treated group - OD of drug treated group)/ OD of LPS treated group} X 100.

AChE activity

AChE activity was measured as per standard protocol (Ellman et al., 1961; Sun et al., 2017); acetythiocholine iodide which was used as a substitute for acetythiocholine chloride. The monolayer cell culture was trypsinized and the cell count was adjusted to 5 x 10^5 cells/ml using media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000 cells/well) was added. After 24hrs, the supernatant was removed, the monolayer was washed once with medium and 100µl of 500mM ethanol (EtOH) was added for 24hrs to induce AChE. After incubation, test drugs were added to the microtiter plates as per the group distribution. The plates were then incubated at 37ºC for 48hrs in 5% CO_2 atmosphere. Treatment samples were removed from the plates and washed once with 1X PBS. Then 100µM acetythiocholine iodide was added to each microtiter well. The contents were further incubated for 5 minutes. After incubation, 180µl of 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB) reagent from the stock of 10mg/ml was added. The absorbance was measured at 412 nm. Percentage inhibition of AChE was estimated using the formula given below.

% Inhibition = {(OD of EtOH treated group - OD of drug treated group)/ OD of EtOH treated group} X 100

Statistical analysis

The significance of the difference was determined using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparisons test, using GraphPad Prism 4 and Microsoft Excel 2010. $p < 0.05$ is considered significant at 95% confidence interval.

3. Results and Discussion

Reduction in caspase-3 expression

Treatment of SH-SY5Y with Aβ has increased the expression of caspase-3 to about 11.63 folds compared to control group. DHA alone could not significantly reduce the expression of caspase-3. Treatment with rosuvastatin and the combination of DHA with rosuvastatin following Aβ1-42 treatment had significantly reduced caspase-3 expression to 0.45 and 0.21 folds respectively. All these values are significant ($p < 0.05$) compared to Aβ treated group. Donepezil also had significantly reduced the expression of caspase-3 to 0.60 fold compared to Aβ treated group. Combination of DHA and rosuvastatin was synergistic and more effective than donepezil in reducing caspase-3 expression (Table 2 and Figure 2). Though combination of DHA and rosuvastatin did not show any statistical significance at 95% confidence interval over the donepezil treated group ($p > 0.05$), yet the combination may be used as an alternative to donepezil due to its higher therapeutic significance in reducing caspase-3 expression.

Table 2. Relative gene expression of caspase-3 in differentiated SH-SY5Y cells following drug treatment.

Treatment	β-Actin†	Caspase-3†	Delta CT (ΔCT)	Delta Delta CT (ΔΔ CT)	Fold change (2^ΔΔCT)
Control	18.25	23.53	5.28	0.00	1.00 ± 0.000
Aβ1-42	19.50	21.24	1.74	-3.54	11.63 ± 6.845
DHA	19.87	24.49	4.62	-0.66	1.58 ± 0.998
Rosuvastatin	18.35	24.77	6.42	1.14	0.45 ± 0.225*
DHA + Rosuvastatin	19.38	26.88	7.50	2.22	0.21 ± 0.085*
Donepezil	19.89	25.89	6.00	0.72	0.60 ± 0.000*

Values are expressed as mean ± SD (n = 2). *Values are significant as compared to Aβ treated group. *p < 0.05. †Represents the mean of two readings (data not shown).

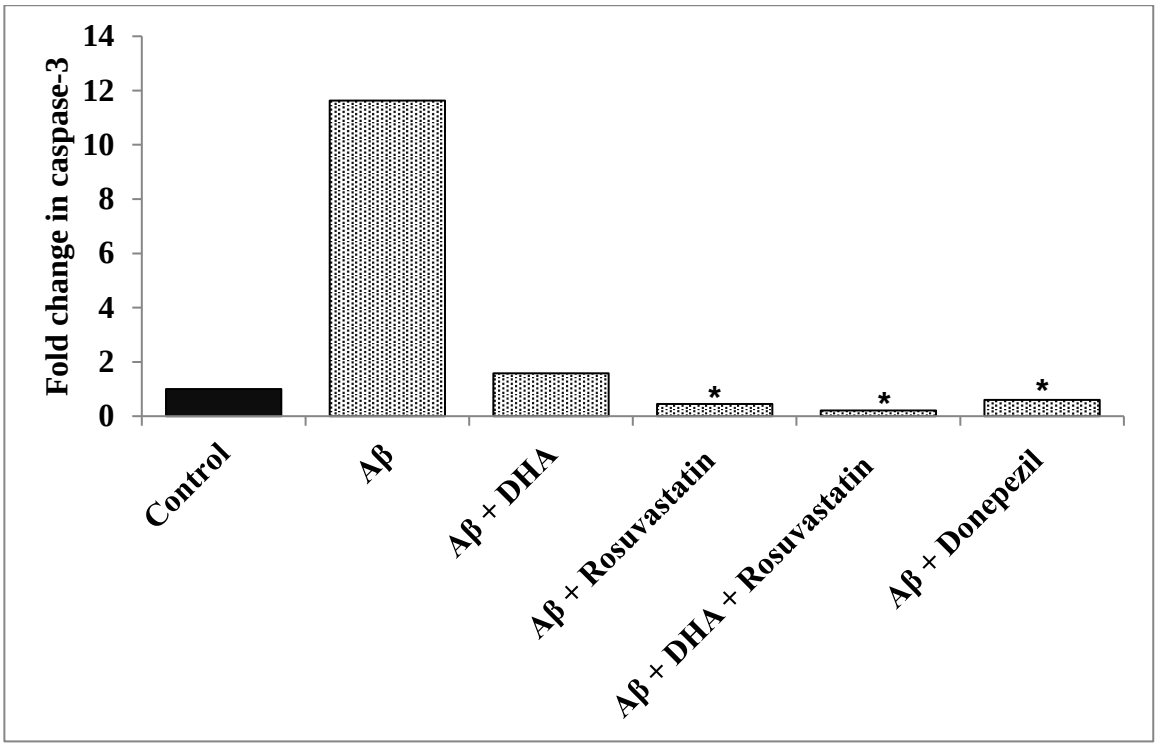

Figure 2: Effects of DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil on Aβ induced caspase-

3 expression. *$p < 0.05$.

Reduction in IL-1β expression

Treatment of SH-SY5Y with LPS had increased the expression of IL-1β to about 3.23 folds. Treatment with DHA, rosuvastatin and the combination of DHA with rosuvastatin following LPS treatment had significantly reduced IL-1β expression to 0.56, 0.20 and 0.13 folds respectively. All these values are highly significant ($p < 0.01$) compared to LPS treated group. This is supported by previous finding where DHA had reduced IL-1β production by differentiated macrophages (Solanki et al., 2013). Donepezil had also reduced the expression of IL-1β to 0.28 fold, which is highly significant. Combination of DHA and rosuvastatin was synergistic and more effective than donepezil in reducing IL-1β expression (Table 3 and Figure 3). Though DHA and rosuvastatin combination did not show any statistical significance ($p > 0.05$) at 95% confidence interval over the donepezil treated group, yet the combination may be used as an alternative to donepezil due to its higher therapeutic significance in reducing IL-1β expression.

Table 3. Relative gene expression of IL-1β in differentiated SH-SY5Y cells following drug treatment.

Treatment	β-Actin†	IL-1β†	Delta CT (ΔCT)	Delta Delta CT (ΔΔ CT)	Fold change (2^ΔΔCT)
Control	14.68	16.79	2.11	0.00	1.00 ± 0.000
LPS	13.48	13.90	0.42	-1.69	3.23 ± 1.014
DHA	16.00	18.95	2.95	0.84	0.56 ± 0.024**
Rosuvastatin	14.17	18.57	4.40	2.30	0.20 ± 0.012**
DHA + Rosuvastatin	13.82	18.92	5.1	2.99	0.13 ± 0.056**
Donepezil	14.20	18.13	3.93	1.82	0.28 ± 0.024**

Values are expressed as mean ± SD (n = 2). **Values are highly significant as compared to LPS treated group. **$p < 0.01$. †Represents the mean of two readings (data not shown).

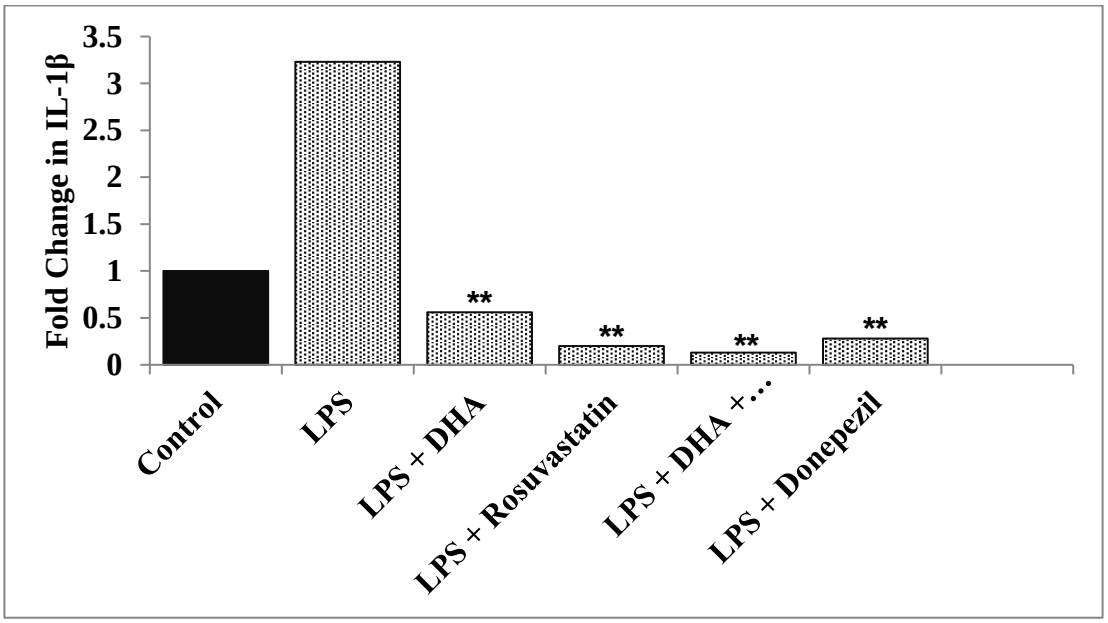

Figure 3: Effects of DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil on LPS induced IL-1β expression. **$p < 0.01$.

Reduction in TNF-α expression

Treatment of SH-SY5Y with LPS had increased the expression of TNF-α to about 4.50 folds. Treatment with DHA, rosuvastatin and the combination of DHA with rosuvastatin following LPS treatment had significantly reduced TNF-α expression to 0.40, 0.06 and 0.04 folds respectively. All these values are extremely significant ($p < 0.001$) as compared to LPS treated group. This is supported by previous finding where DHA had reduced TNF-α production by differentiated macrophages (Solanki et al., 2013). Donepezil had reduced the expression of TNF-α to 0.27 fold, which is also extremely significant. Combination of DHA and rosuvastatin was synergistic and more effective than donepezil in reducing TNF-α expression (Table 4 and Figure 4). Though

DHA and rosuvastatin combination did not show any statistical significance ($p > 0.05$) at 95% confidence interval over the donepezil treated group, yet the combination may be an alternative to donepezil due to its higher therapeutic significance in reducing TNF-α expression.

Table 4. Relative gene expression of TNF-α in differentiated SH-SY5Y cells following drug treatment.

Treatment	β-Actin†	TNF-α†	Delta CT (ΔCT)	Delta Delta CT (ΔΔ CT)	Fold change (2^ΔΔCT)
Control	14.68	22.46	7.78	0.00	1.00 ± 0.000
LPS	13.48	19.09	5.61	-2.17	4.50 ± 0.022
DHA	16.00	25.10	9.10	1.32	0.40 ± 0.175***
Rosuvastatin	14.17	25.91	11.74	3.96	0.06 ± 0.012***
DHA + Rosuvastatin	13.82	26.06	12.24	4.46	0.04 ± 0.022***
Donepezil	14.20	23.87	9.67	1.89	0.27 ± 0.109***

Values are expressed as mean ± SD (n = 2). ***Values are extremely significant compared to LPS treated group. ***$p < 0.001$. †Represents the mean of two readings (data not shown).

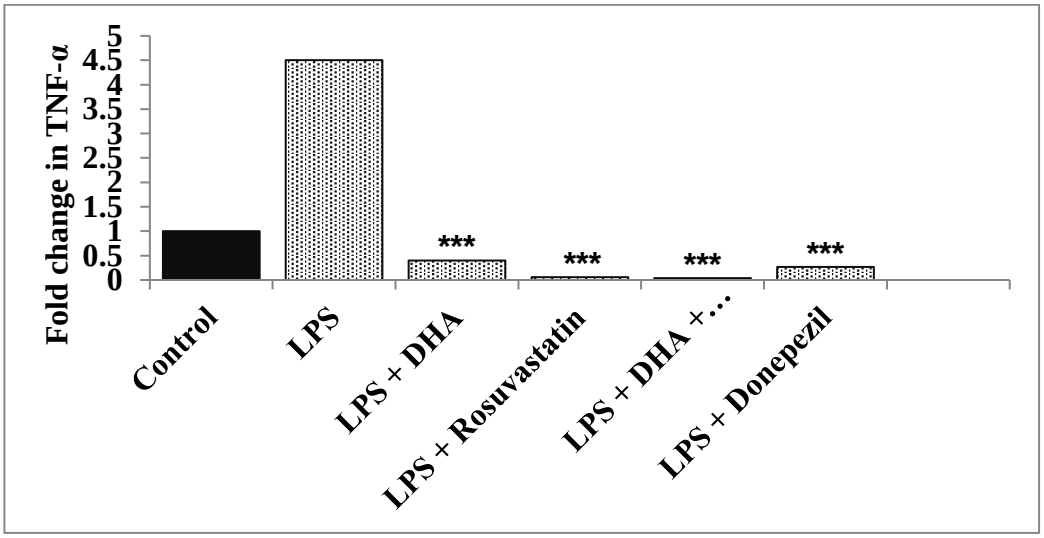

Figure 4: Effects of DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil on LPS induced TNF-α expression. ***$p < 0.001$.

Reduction in nitric oxide level

Treatment of SH-SY5Y with LPS has increased nitrite production to 11.33 µM. DHA, rosuvastatin and the combination of DHA with rosuvastatin had reduced nitrite levels to 5.71, 4.25, 2.95 µM respectively. These values are extremely significant ($p < 0.001$) compared to LPS treated group. These values represent an inhibition of 49.60 %, 62.46 % and 73.96 % respectively. This is similar to previous finding where DHA had reduced nitric oxide production as well as expression of nitric oxide synthase in murine macrophage (Komatsu et al., 2003). Donepezil had reduced nitric oxide level to 66.19 %. DHA and rosuvastatin combination was synergistic and more effective compared to donepezil in reducing nitric oxide level (Table 5 and Figure 5). Though DHA and rosuvastatin combination did not show any statistical significance ($p > 0.05$) at 95% confidence interval over the donepezil treated group, yet the combination may be an alternative to donepezil due to its higher therapeutic significance in reducing nitric oxide level. The absorbance of standard nitrite (Table 6) and its calibration curve (Figure 6) are shown below.

Table 5. Nitric oxide levels in different treatment groups.

Treatment	Absorbance at 540 nm						Mean ± SD	% Inhibition
	n=1	Nitrite (µM)	n=2	Nitrite (µM)	n=3	Nitrite (µM)		
Control	0.188	3.06	0.162	2.52	0.202	3.35	2.98 ± 0.43	-
LPS	0.588	11.45	0.512	9.85	0.647	12.69	11.33 ± 1.42	0.00
DHA	0.262	4.61	0.258	4.53	0.423	7.99	5.71 ± 1.97***	49.60
Rosuvastatin	0.235	4.05	0.225	3.84	0.275	4.88	4.25 ± 0.55***	62.46

DHA + Rosuvastatin	0.186	3.02	0.174	2.77	0.188	3.06	2.95 ± 0.15***	73.96
Donepezil	0.242	4.19	0.228	3.90	0.204	3.40	3.83 ± 0.40***	66.19

Nitric oxide values are expressed as mean ± SD (n = 3). ***Values are extremely significant compared to LPS treated group. ***$p < 0.001$.

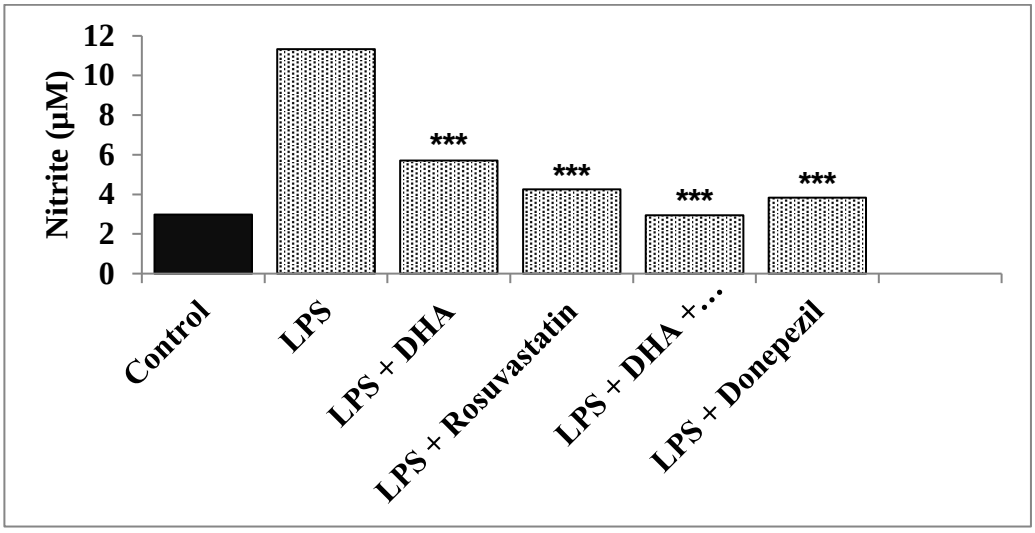

Figure 5: Effects of DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil on LPS induced nitric oxide production. ***$p < 0.001$.

Table 6. Absorbance of nitrite standard

Standard	Concentration (µM)	Absorbance at 540 nm
Standard nitrite	0	0.026
	0.625	0.066
	1.25	0.109
	2.5	0.178
	5	0.285
	10	0.512
	20	0.996

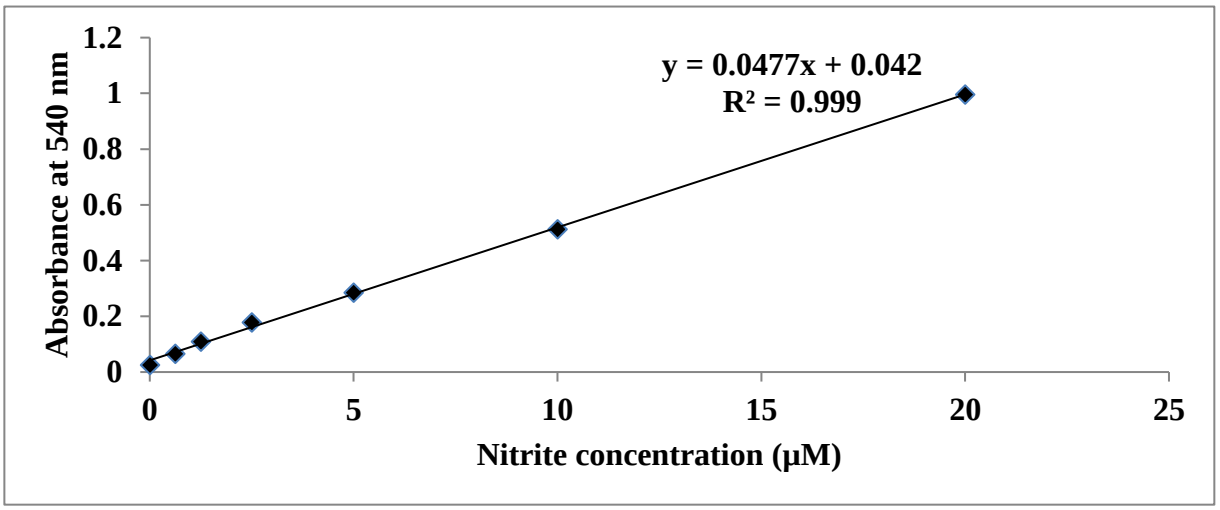

Figure 6: Standard nitrite calibration curve.

Reduction in lipid peroxide level

Treatment of SH-SY5Y with LPS has increased MLD production to 41.92 µM. DHA, rosuvastatin and the combination of DHA with rosuvastatin had reduced MLD levels to 8.81, 5.93, 4.28 µM respectively. These values are extremely significant ($p < 0.001$) compared to LPS treated group. These values represent an inhibition

of 32.48 %, 54.55 % and 67.20 % respectively. DHA and rosuvastatin combination was synergistic. However, donepezil had reduced lipid peroxide production by 72.40 %, which is more than the synergistic effect of DHA and rosuvastatin (Table 7 and Figure 7). Lipid peroxide inhibition value of donepezil treated group did not show statistical significance ($p > 0.05$) over DHA and rosuvastatin combination treated group at 95% confidence interval. This allows DHA and rosuvastatin combination to be the alternative for donepezil. The absorbance of MLD (Table 8) and its calibration curve (Figure 8) are shown below.

Table 7. Lipid peroxide levels in different treatment groups.

Treatment	Absorbance at 532 nm						Mean ± SD	% Inhibition
	n=1	MLD (µM)	n=2	MLD (µM)	n=3	MLD (µM)		
Control	0.229	11.56	0.256	14.18	0.248	13.41	13.05 ± 1.35	-
LPS	0.529	40.69	0.547	42.44	0.549	42.63	41.92 ± 1.07	0.00
DHA	0.195	8.26	0.211	9.82	0.196	8.36	8.81 ± 0.87***	32.48
Rosuvastatin	0.163	5.16	0.175	6.32	0.175	6.32	5.93 ± 0.67***	54.55
DHA + Rosuvastatin	0.154	4.28	0.157	4.57	0.151	3.99	4.28 ± 0.29***	67.20
Donepezil	0.148	3.70	0.146	3.50	0.147	3.60	3.60 ± 0.10***	72.40

Lipid peroxide (MLD) values are expressed as mean ± SD (n = 3). *** Values are extremely significant compared to LPS treated group. ***$p < 0.001$.

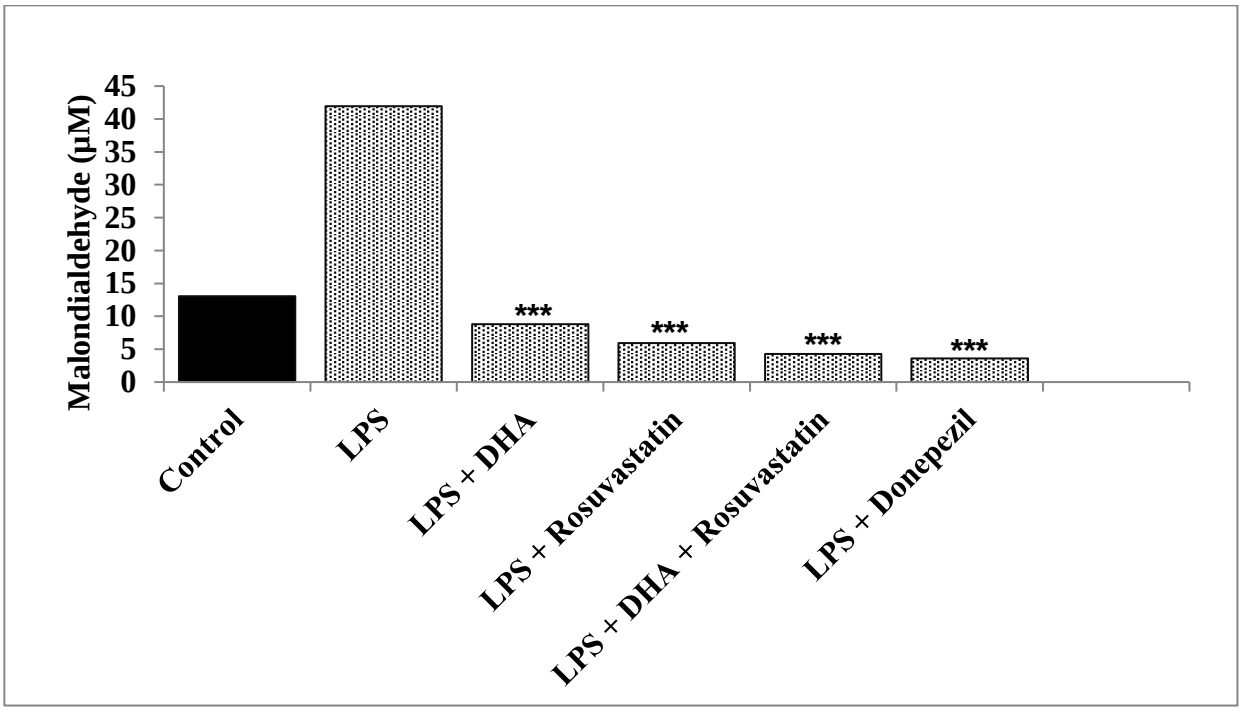

Figure 7: Effects of DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil on LPS induced malondialdehyde production. ***$p < 0.001$.

Table 8. Absorbance of malondialdehyde standard

Standard	Concentration (µM)	Absorbance at 532 nm
Malondialdehyde	0	0.099
	1.56	0.129
	3.13	0.148
	12.5	0.239
	25	0.374
	50	0.624

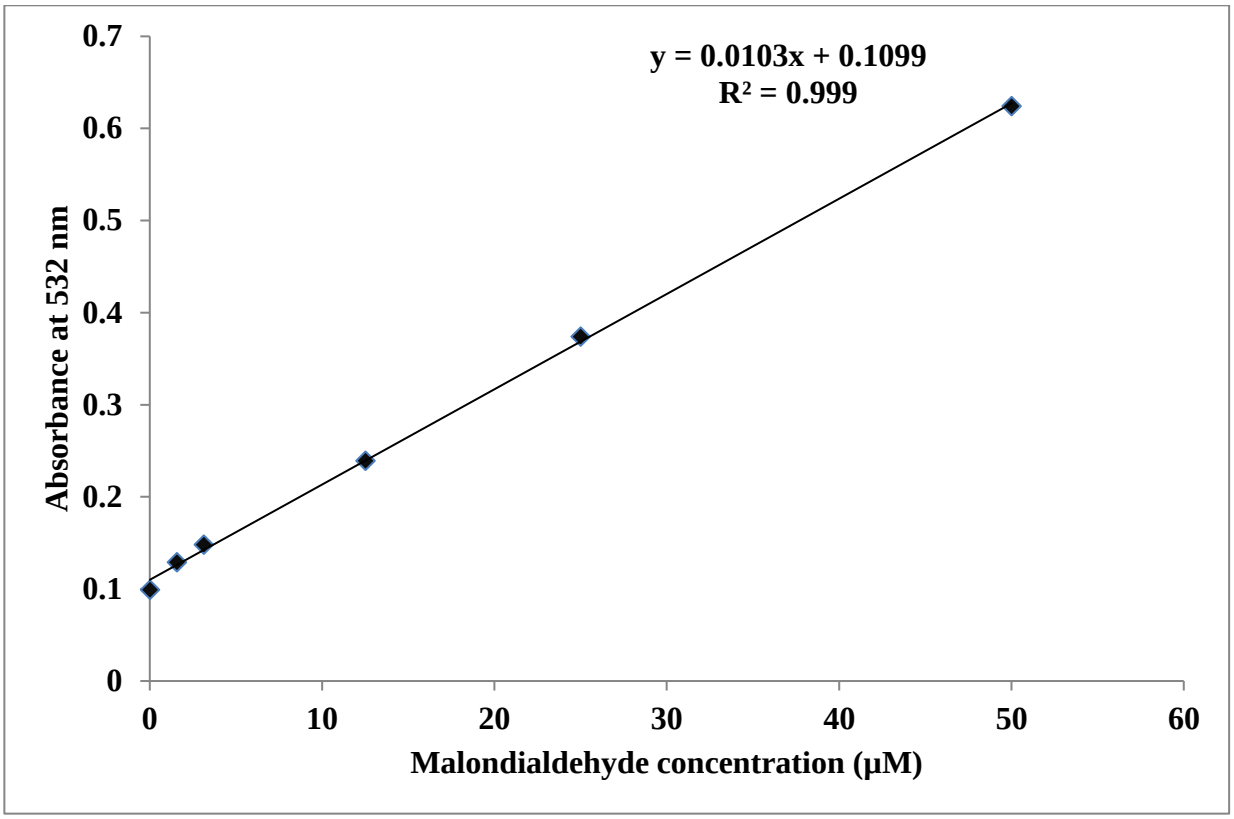

Figure 8: Standard malondialdehyde calibration curve.

Reduction in AChE

Treatment of SH-SY5Y with DHA, rosuvastatin and the combination of DHA with rosuvastatin had shown significant inhibition of AChE as compared to EtOH treated group. Percentage inhibitions of AChE by DHA, rosuvastatin and the combination of DHA with rosuvastatin were found to be 73.37%, 79.89% and 82.33% respectively. These values are extremely significant ($p < 0.001$). However, donepezil had shown maximum inhibition of AChE, which was 91.85% and better than any other therapeutic group. However, combination of DHA and rosuvastatin was synergistic (Table 9 and Figure 9). AChE inhibition value of donepezil treated group was extremely significant ($p < 0.001$) at 95% confidence interval over DHA and rosuvastatin combination treated group. Hence, in spite of therapeutic synergism, DHA and rosuvastatin combination cannot be a substitute for donepezil to inhibit AChE.

Table 9. AChE inhibition in different treatment groups.

Treatment	Absorbance at 412 nm						Mean ± SD
	n=1	% inhibition	n=2	% inhibition	n=3	% inhibition	
Control	0.292	-	0.298	-	0.267	-	-
EtOH	0.964	0.00	0.984	0.00	0.994	0.00	0.00
DHA	0.234	75.73	0.289	70.63	0.261	73.74	73.37 ± 2.57***
Rosuvastatin	0.174	81.95	0.219	77.74	0.199	79.98	79.89 ± 2.10***
DHA + Rosuvastatin	0.162	83.20	0.189	80.79	0.169	83.00	82.33 ± 1.33***
Donepezil	0.065	93.26	0.091	90.75	0.084	91.55	91.85 ± 1.28***

AChE inhibition values are expressed as mean ± SD (n = 3). ***Values are extremely significant compared to EtOH treated group. ***$p < 0.001$.

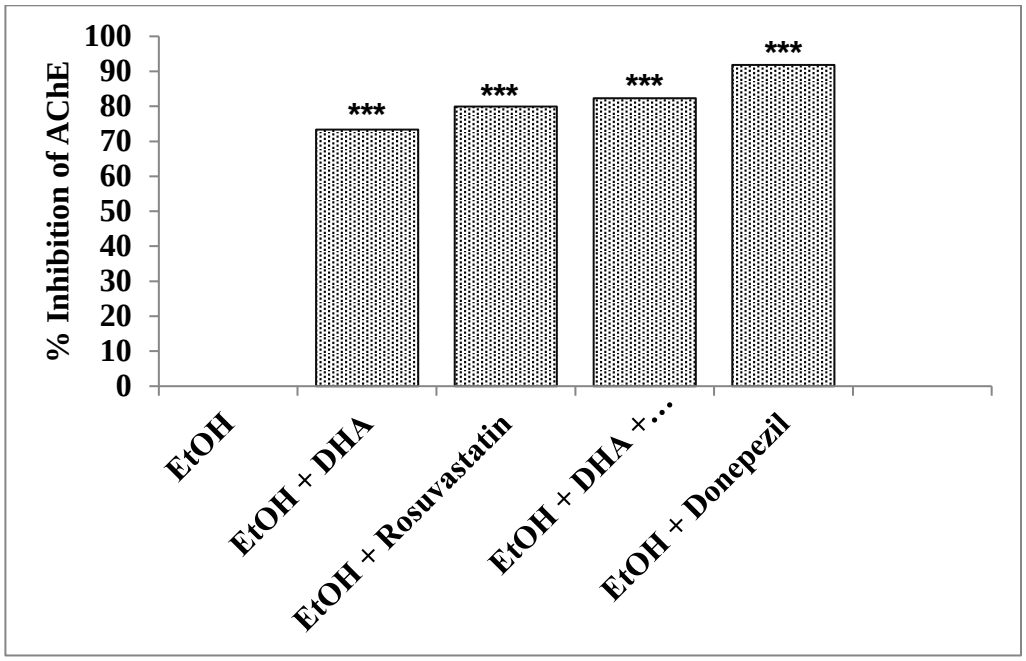

Figure 9: Percentage inhibition of AChE in DHA, rosuvastatin, DHA and rosuvastatin combination, donepezil treated groups. ***$p < 0.001$.

The results of caspase-3 expression study show that DHA and rosuvastatin combination was synergistic and better than any therapeutic group in suppressing caspase-3 expression. Rosuvastatin therapy stands next to the combination therapy in suppressing caspase-3 and shows higher therapeutic efficacy than donepezil. The significance of the result of combination therapy can be understood from the finding that Caspase-3 plays a role in neuronal death and plaque formation in AD (Su et al., 2001). Expression of synaptic procaspase-3 was significantly higher in human brain with AD compared to control. Human brain with AD has shown an increased active caspase-3 concentration in postsynaptic density fractions compared to the control; proving the role of caspase-3 in the progression of AD through synapse degeneration (Louneva et al., 2008). Blockade of caspase-3 had attenuated cleavage of amyloid precursor protein (APP) and Aβ mediated dendritic spine loss (Park et al., 2020). These findings prove that caspase-3 suppression becomes one of the most desired functional characteristics of anti-Alzheimer's drugs.

The results of IL-1β expression study show that DHA and rosuvastatin combination was synergistic and better than any therapeutic group in suppressing IL-1β expression. Rosuvastatin therapy stands next to the combination therapy in suppressing IL-1β and shows higher therapeutic efficacy than donepezil. The significance of the result of combination therapy can be understood from the finding that IL-1β has a direct association with AD and plays core role in the pro-inflammatory pathway of AD (Xie et al., 2015). Study has shown that pathological tau activates IL-1β and plays a crucial role in neuroinflammation and AD (Jiang et al., 2021). Certain study has found that sustained IL-1β overexpression is the reason behind aggravated tauopathy, which is a hallmark event in AD. Interestingly, the aggravating effect of IL-1β on tauopathy is not affected by reduced Aβ burden (Ghosh et al., 2013). This shows that pathological tau and IL-1β can trigger each other. The significance of IL-1β can be understood from the finding that oligomeric Aβ can activate microglia and cause neurotoxicity by promoting maturity of microglial IL-1β (Parajuli et al., 2013). Owing to these findings IL-1β becomes another crucial therapeutic target for anti-Alzheimer's drugs.

The results of TNF-α expression study show that DHA and rosuvastatin combination was synergistic and better than any therapeutic group in suppressing TNF-α expression. Rosuvastatin therapy stands next to the combination therapy in suppressing TNF-α and shows higher therapeutic efficacy than donepezil. The significance of the result of combination therapy can be understood from the finding that TNF-α plays a central role in the progression of AD (Chang et al., 2017). It is responsible for the production of extracellular Aβ and α-synuclein deposits by AD neurons (Whiten et al., 2020). Chronic increase in TNF-α at preplaque stages can produce deleterious symptoms at later stages of AD. Early treatment of elevated level of TNF-α can alleviate synaptic pathology/deficit in AD (Cavanagh et al., 2016). This makes TNF-α a prominent therapeutic target for AD.

The results of nitric oxide study show that DHA and rosuvastatin combination was synergistic and better than any therapeutic group in suppressing nitric oxide level. Donepezil therapy stands next to the combination therapy in suppressing nitric oxide. The significance of the result of combination therapy can be understood from the finding that nitric oxide induced neurotoxicity is mediated by superoxide or hydroxyl radicals. Reaction between nitric oxide and superoxide generates peroxynitrite, leading to neurotoxicity (Mohanakumar & Steinbusch, 1998; Guzik et al., 2002; Hayashi et al., 2004). Hence, excess production of nitric oxide should be suppressed irrespective of its dual role in AD (Azargoonjahromi, 2023). This makes nitric oxide another desirable therapeutic target for AD.

The results of lipid peroxide study show that DHA and rosuvastatin combination was synergistic and better than DHA or rosuvastatin treatment and could significantly suppress lipid peroxide level. However, donepezil therapy was slightly better than the combination therapy. Nevertheless, the significance of the result of combination therapy can be understood from the finding that lipid peroxidation can affect protein and other biomolecules and play a significant role in AD (Shichiri, 2014). AD brain shows increased lipid peroxidation compered to age matched control (Montine et al., 2002). These findings make lipid peroxide a therapeutic target for AD.

The results of AChE study show that DHA and rosuvastatin combination was synergistic and better than DHA or rosuvastatin treatment and could significantly suppress AChE activity. However, donepezil therapy was slightly better than the combination therapy. Nevertheless, the significance of the result of combination therapy can be understood from the finding that AChE can promote Aβ and tau dysregulation. This will eventually aggravate AD (García-Ayllón et al., 2011). Thus, irrespective of the finding about the limited usefulness of AChE inhibition (Marucci et al., 2021), AChE becomes one of the therapeutic targets for AD. In this regard it is noteworthy that caspase-3 suppression by AChE-inhibitors will alleviate AD by suppressing APP cleavage, senile plaque and tau tangle formation (Płóciennik et al., 2015). DHA and rosuvastatin combination is better than donepezil in suppressing caspase-3 expression. Hence, DHA and rosuvastatin combination may be an alternative to donepezil irrespective of its lower effect on AChE. This study also shows that effect of DHA on AChE inhibition is contradictory to previous assumption where AChE modulation was not thought as the function of DHA (Willis et al., 2009).

Irrespective of the positive outcome of the combination therapy, there are some limitations of the study. It is associated with Aβ1-42. It has been found that synthetic Aβ peptides are less toxic compared to the cell derived Aβ peptides. Aβ peptides derived from cell show maximum resemblance with CSF or brain derived Aβ peptides. However, they are not also exactly similar (Varshavskaya et al., 2022). Hence, mimicking the pathological condition of AD with synthetic Aβ may be a challenging task. Next limitation is associated with LPS. It has been found that lipid A which is a stable component of LPS structure can show slight chemical differences. Such differences may occur even in the most conservative lipid A. This will lead to diverse immunogenicity of LPS. Further complication may arise from the LPS purification method. LPS purification methods such as "gel-filtration chromatography, ion-exchange chromatography and phenol extracted" may affect its immunogenic and pro-inflammatory properties (Skrzypczak-Wiercioch & Sałat, 2022). This may have impact on the results of the research.

The direction of future research will be towards the evaluation of the effect of combination therapy on anterograde and retrograde axonal transport of mitochondria, mitochondrial respiration and synaptic release of neurotransmitters. The significance of these parameters is evident from the finding that Aβ plaques disrupt anterograde and retrograde mitochondrial movement in the axon, leading to synapse degeneration in brain. This leads to AD (Bhatia et al., 2022; Calkins & Reddy, 2011). AD represents a decreased mitochondrial performance which is marked by a decreased mitochondrial respiration (Fišar et al., 2019) and decreased concentration neurotransmitter such as acetylcholine (Francis, 2005).

The novelty of the present study lies in the fact that nutraceuticals provide many benefits including therapeutic and nutritional. They are also known to reduce the toxic effects of pharmaceuticals including anticancer drugs (Puri et al., 2022). DHA has been found to delay muscle wasting and improve muscle homeostasis (Lee et al., 2020). This makes DHA a perfect combination for rosuvastatin because long term use of rosuvastatin induces myopathy (El-Ganainy et al., 2017). The combination seems to be a new hope in the management of AD because many newer clinical trials have failed. This makes the choice of drugs for the management of AD very narrow. Only a handful of drugs such as "tacrine, donepezil, carbalatine, galantamine, memantine, and lecanemab" are currently managing AD (Peng et al., 2023). Hence, DHA and rosuvastatin combination seems to be a novel

combination for the treatment of AD. However, further research is required to assess complete safety and therapeutic efficacy of the combination.

4. Conclusions

DHA and rosuvastatin combination was synergistic and better than donepezil in reducing caspase-3, IL-1β, TNF-α expression and nitric oxide level, except for lipid peroxide and AChE activity. AChE-inhibitors target caspase-3 and consequently suppress APP cleavage, senile plaque and tau tangle formation. All these effects contribute to the alleviation of AD. Hence, combination of DHA and rosuvastatin can be the substitute for donepezil. This is due to the finding that DHA and rosuvastatin combination is better than donepezil in suppressing caspase-3. Based on the preliminary results we accept the alternative hypothesis and reject the null hypothesis. Hence, it is hypothesized that the combination may be used to treat AD in donepezil intolerant patients. However, further research is required to explore the safety profile and therapeutic potential of the combination for its long term use.

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