Dimethyl fumarate attenuates scopolamine induced amnesia and oxidative stress in mice

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ABSTRACT

Background: Memory and cognition, processes essential for adaptation and survival of organisms can be impaired by oxidative stress resulting from high amounts of reactive oxygen species in the brain. Scopolamine induced amnesia is associated with increased oxidative stress biomarkers both in the whole brain and in discrete areas of the brain involved in memory and cognition. Dimethyl fumarate (DMF) is an immunomodulatory, antioxidant and anti-inflammatory agent currently used for management of remitting relapsing multiple sclerosis.

Objective: In this study, we seek to evaluate the effect of DMF in scopolamine induced amnesia and oxidative stress in a murine model of cognition and memory.

Methods: The effect of DMF on scopolamine induced amnesia in mice following acute and chronic treatment was evaluated using the Y-maze spontaneous alternation performance test as neurobehavioural test index. Levels of catalase, glutathione and malondialdehyde as determinants of brain oxidative stress were determined in isolated brain tissues following chronic treatment with DMF. Dimethyl sulphoxide solution was used as the control drug (vehicle) while donepezil was used as the reference memory enhancing agent.

Results: DMF significantly ($p<0.05$) improved spontaneous alternation performance in the Y-maze test, post acute and chronic drug administration. Levels of catalase and glutathione in isolated brain tissues were significantly increased ($p<0.05$) following treatment with DMF while lipid peroxidation products indicated by malondialdehyde levels were significantly decreased ($p<0.05$).

Conclusion: The results obtained suggest that DMF improves scopolamine induced memory and cognitive deficits and ameliorates oxidative stress in the brain.

Keywords: Y-maze test, catalase, antioxidant, amnesia, glutathione

Le fumarate de diméthyle atténue l'amnésie induite à la scopolamine et le stress oxydatif chez la souris

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RÉSUMÉ

Contexte: La mémoire et la cognition, processus essentiels à l'adaptation et à la survie des organismes, peuvent être altérées par le stress oxydatif résultant de la quantité élevée d'oxygène réactif dans le cerveau. L'amnésie induite par la scopolamine est associée à une augmentation des biomarqueurs du stress oxydatif à la fois dans le cerveau entier et dans des zones distinctes du cerveau impliquées dans la mémoire et la cognition. Le fumarate de diméthyle ou diméthylfumarate (DMF) est un agent immunomodulateur, antioxydant et anti-inflammatoire actuellement utilisé pour le traitement de la sclérose en plaques récurrente-rémittente.

Objectif: Dans cette étude, nous cherchons à évaluer l'effet du DMF sur l'amnésie induite par la scopolamine et le stress oxydatif dans un modèle murin de cognition et de mémoire.

Méthodes: Les effets du DMF sur l'amnésie induite par la scopolamine chez la souris à la suite d'un traitement aigu et chronique ont été évalués en utilisant le test de performance d'alternance spontanée du labyrinthe en Y comme indice de test neurocomportemental. Les taux de catalase, de glutathion et de malondialdéhyde, déterminants du stress oxydatif dans le cerveau, ont été déterminés dans des tissus cérébraux isolés après un traitement chronique au DMF. La solution de diméthylsulphoxyde a été utilisée en tant que médicament témoin (véhicule), tandis que le donépézil a été utilisé comme agent améliorant la mémoire de référence.

Résultats: Le DMF améliore significativement ($p<0,05$) la performance d'alternance spontanée dans le test du labyrinthe en Y, après l'administration aiguë et chronique du médicament. Les taux de catalase et de glutathion dans les tissus cérébraux isolés étaient significativement plus élevés ($p<0,05$) après traitement par le DMF, alors que les produits de peroxydation lipidique indiqués par les taux de malondialdéhyde étaient significativement réduits ($p<0,05$).

Conclusion: Les résultats obtenus suggèrent que le DMF améliore la mémoire induite par la scopolamine et les déficits cognitifs et améliore le stress oxydatif dans le cerveau.

Mots-clés: test du labyrinthe en Y, catalase, antioxydant, amnésie, glutathion

INTRODUCTION

Memory and cognition - processes vital for adaptation and survival- are some of the most important functions of the human brain.[1] Decline in cognitive function is a characteristic feature of neurodegenerative diseases such as Alzheimer's and Parkinson's disease and is often a component of neuropsychiatric disorders such as depression, stress and anxiety.[2-6]

Cognitive dysfunction is associated with diminished cholinergic neurotransmission hence scopolamine - an anti-cholinergic agent - is often employed in studies evaluating anti-amnesic effect of test compounds.[7, 8] Scopolamine-induced amnesia has been demonstrated to aggravate oxidative stress in the brain, and is associated with inactivation of superoxide dismutase, low levels of catalase, glutathione and 2,2-diphenyl-1-picrylhydrazyl. Additionally, higher levels of lipid peroxidation products such as thiobarbituric acid reactive substances and inhibition of adenosine triphosphatase enzymes were observed following treatment with scopolamine.[9-12]

High adenosine triphosphatase demand resulting in disproportionately high levels of oxygen consumption relative to the small size of the brain, coupled with a high percentage of readily oxidizable poly unsaturated fatty acids and low levels of antioxidant defense mechanisms make the brain vulnerable to oxidative processes. Constitutive oxidative processes in the brain generate reactive oxygen species which when produced in high amounts can overwhelm the intrinsic protective defense systems.[13-15] Compounds which can restore the oxidative system in favor of antioxidant biomarkers are expected to be neuroprotective in disorders resulting from increased oxidative stress.[16-18]

Dimethyl fumarate (DMF), a fumaric acid ester with antioxidant properties was found to be neuroprotective via anti-inflammatory mechanisms and preservation of blood brain barrier integrity in murine models of haemorrhagic and ischaemic stroke.[19-21] Both in *in vitro* and *in vivo* studies of oxidative stress, DMF prevented oxidative damage by up-regulating haem oxygenase-1 enzyme [22], enhanced neuronal survival and protected glial cells from oxidative damage [23] and activated the nuclear factor erythroid - derived factor-2/antioxidant response element (Nrf2/ARE) pathway leading to increased production of nicotinamide adenine dinucleotide phosphate quinone oxidoreductase and phosphorylation of Nrf2.[19, 24]

In this study, we sought to evaluate the effect of DMF in scopolamine induced amnesia and oxidative stress in a murine model of cognition and memory.

METHODS

Reagents/Chemicals

Scopolamine hydrobromide, dimethysulphoxide (DMSO), DMF, acetyl thiocholine, bovine serum albumin, thiobarbituric acid and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Aldrich, St. Louis, MO, USA. Donepezil was purchased from Pfizer, while sodium hydroxide, hydrochloric acid, tris HCl, sulfosalicylic acid, trichloroacetic acid, 99% chloroform, dipotassium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride were purchased from JHD Chemicals, Guangdong, China.

Animals

Swiss albino male mice weighing 18-26 g procured from the University of Benin Animal House, Benin City, Edo State were used for the study. They were kept in plastic cages at the Animal House of the Department of Pharmacology and Toxicology, University of Benin, Benin City. They were maintained under standard controlled environment and were allowed free access to feed (Top feeds® Growers Mash, Super-Deluxe Animal Feed Mills Co. Ltd, Nigeria) and clean water *ad libitum*. Handling of the animals was done according to standard protocols for the use of laboratory animals of the National Institute of Health [25] and ethical approval (EC/FP/018/10) from the Institutional Ethics Committee was obtained for the study.

Evaluation of the effects of DMF on memory and cognition

The effects of DMF on scopolamine induced amnesia was carried out in two phases following acute and chronic drug administration.

Evaluations after acute administration

In this phase of the study, thirty six (36) animals in groups of six (6) animals were used, they were randomly divided into the following treatment groups:

Group I: Naive animals (no treatment)
Group II: Scopolamine only
Group III: Scopolamine + Vehicle
Group IV: Scopolamine + DMF 50 mg/kg
Group V: Scopolamine + DMF 100 mg/kg
Group VI: Scopolamine + Donepezil (0.5 mg/kg)

Animals in group I received no drug treatment and

served as naive animals while those in group II received only scopolamine intraperitoneally. Mice in groups III-VI were treated with 0.7 mg/kg scopolamine [26] intraperitoneally and thirty minutes later with the vehicle (DMSO solution), 50 mg/kg DMF, 100 mg/kg DMF [19] and 0.5 mg/kg donepezil [26] respectively via the oral route. One hour post drug administration, each animal was placed in the centre of the Y-maze and entry into each arm was scored by observers unaware of drug treatment. An arm entry was scored when all four paws were placed in the arm. Spontaneous alternation (SA), defined as consecutive entries into all three arms, was measured as an index of working memory, by calculating the ratio of the actual number of alternations to the possible/maximum number of alterations using the following formula:

SA= (Actual Alternations/Maximum Alternations)*100

After each animal was removed, the apparatus was cleaned and wiped with 70% alcohol. Each animal was used only once.[27,28]

Evaluations after chronic administration

Drug administration and neurobehavioural evaluation

The methods of Umukoro and Kaur [15, 26] with some modifications were used for this phase of the study. Before the start of the study, animals were placed on the rotarod (Ugo Basile, Gemonio Italy; 47600 V04) set at a constant speed of 20 rpm to check for neurological deficits. Animals which fell off the horizontal bar in less than 30 seconds were excluded from the study. On the 1st day of the study, each animal was placed in the Y-maze and spontaneous alternation calculated as described above to obtain baseline values.

Forty-eight (48) mice, randomly divided into six (6) groups of eight (8) animals per group, were used for this phase of the study. In the first week of the study, mice in group I received no drug treatment, while animals in groups II-VI were subjected to daily treatment with 0.7 mg/kg scopolamine intraperitoneally. Neurobehavioural evaluation using the Y-maze test as previously described was carried out on the seventh day of drug treatment.

In the second week of the study, mice in group I received no drug treatment, animals in group II received scopolamine (0.7 mg/kg) intraperitoneally, while animals in groups III-VI were subjected to daily treatment with the vehicle, DMF 50 mg/kg, DMF 100 mg/kg and donepezil 0.5 mg/kg, respectively, via the oral route. Spontaneous alternation using the Y-maze test as previously described was carried out on the last day.

In the third week of the experiment, animals in groups III to VI were treated daily with scopolamine followed thirty minutes later by the vehicle, DMF 50 mg/kg, DMF 100 mg/kg and donepezil 0.5 mg/kg. Animals in group I did not receive any drug treatment, while those in group II received only scopolamine for the entire week. Cognitive function was assessed in the Y-maze test.

Biochemical evaluations

Twenty four hours after the Y-maze test, animals were anesthetized with chloroform, euthanized and whole brains were isolated, washed carefully, homogenized in phosphate buffer (pH 7.4) and centrifuged for 30 minutes at 12,000 rpm. Supernatant layers were carefully separated and used for biochemical assays.

Protein determination assay

Protein was measured in brain homogenate using bovine serum albumin as standard, according to the method described by Lowry et al.[29]

Estimation of acetylcholinesterase activity

Following the method of Elman *et al*., [30] brain homogenate (400 µl) was added to a mixture of 100 µl 5,5'-dithiobis-(2-nitrobenzoic acid) solution and 2.6 ml phosphate buffer (pH 8). The sample was mixed thoroughly and absorbance measured at 412 nm to obtain basal values. Twenty micro liters (20 µl) of acetylthiocholine was added to the solution and absorbance measured every 2 minutes for a period of 10 minutes. Acetylcholinesterase enzyme activity was calculated using the formula:

$$R = 5.74 \times 10^{-4} \times A/CO$$

Where, R = Rate in moles of substrate hydrolyzed /minute/gm tissue

A = Change in absorbance/min

CO = Original concentration of the tissue (mg/ml)

Estimation of brain catalase levels

Catalase levels in the brain were assayed following the method of Claiborne.[31] Changes in absorbance levels of the assay mixture made up of 1.95 ml phosphate buffer, 1 ml hydrogen peroxide and 0.05 ml brain homogenate were recorded at 240 nm for each sample. Catalase levels are expressed as micromoles of hydrogen peroxide consumed per minute per milligram of protein.

Estimation of brain glutathione levels

Following the method of Ellman,[32] 1 ml of brain homogenate was added to 1 ml of 4% sulphosalicylic acid and kept at 4°C for 1 hour. Thereafter the mixture was centrifuged, 2.7 ml of phosphate buffer and 0.2 ml 5,5′-dithiobis (2-nitrobenzoic acid) were added to 1 ml of the supernatant and the resultant colour changes were measured at 412 nm.

Estimation of brain lipid peroxidation

Malondialdehyde (MDA) content, a measure of lipid peroxidation was estimated in the form of thiobarbituric acid reactive substance following the method described by Wills.[33] Brain homogenate (0.5 ml) was added to an equal volume of tris hydrochloric acid and the mixture incubated at room temperature for 2 hours. Thereafter 1 ml of 10% trichloroacetic acid was added, the mixture was centrifuged at 1,500 g for 10 minutes and 1 ml of thiobarbituric acid was added to an equal volume of the resultant supernatant. This mixture, in test tubes, was boiled for 10 minutes and allowed to cool. Distilled water (1 ml) was then added and the absorbance changes were measured at a wavelength of 532 nm using an ultra violet spectrophotometer. A calibration curve using malondialdehyde was generated and the results obtained are expressed as nanomoles of MDA per milligram of protein.

Statistical analysis

The results were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test using Sigma® Stat version 11.0 (California, USA). A difference was considered significant at $p < 0.05$.The results are presented as mean ± standard error of mean (SEM).

RESULTS

Evaluations after acute drug administration

Y-Maze Spontaneous Alternation Test

In the acute phase of the study, both doses of DMF improved spontaneous alternation performance in the Y-maze test, this was significantly ($p<0.05$) different from scopolamine and vehicle treated animals but not different from donepezil treated animals and naive mice. Data is presented in Fig 1.

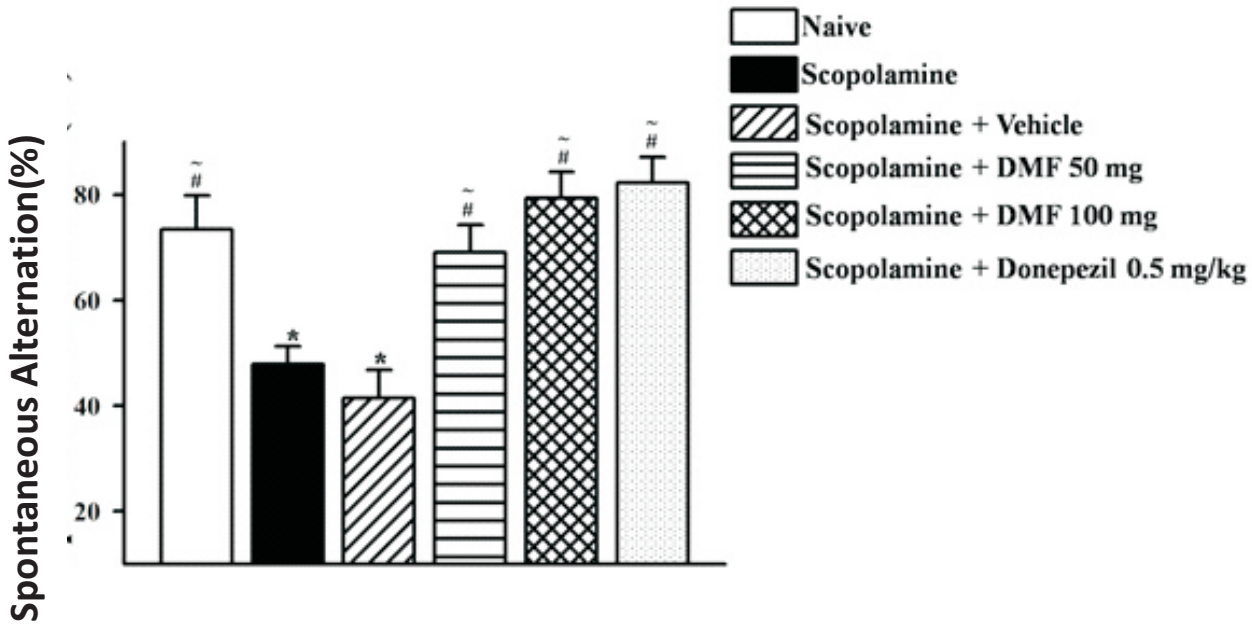

Figure 1. Statistical analysis of spontaneous alternation in the Y-maze test following acute drug administration. Both doses of DMF improved spontaneous alternation performance in the Y-maze test. Data is expressed as mean ± SEM. *$p<0.05$ compared to naive, #$p<0.05$ compared to scopolamine, ~ compared to vehicle; n=6 animals per group

Evaluations after Chronic Administration
Y-Maze Spontaneous Alternation Test

There was no significant difference in baseline spontaneous alternation performance across all the groups prior to drug intervention (Figure 2).

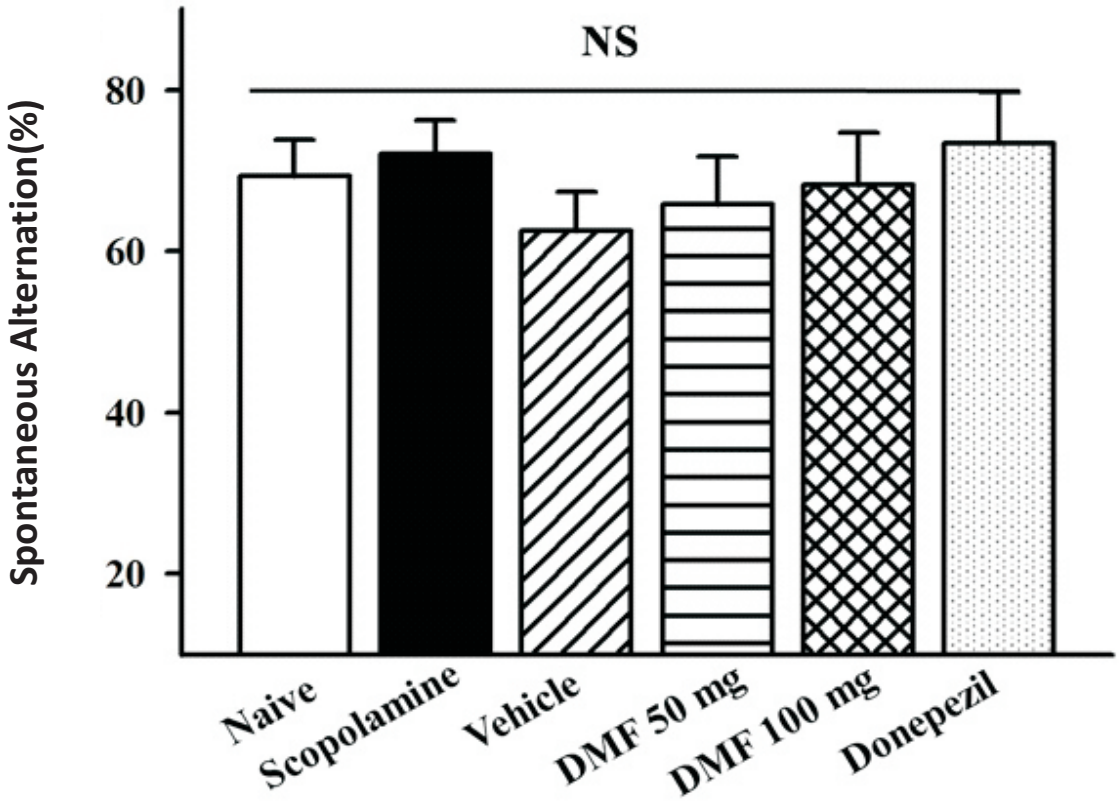

Figure 2. Statistical analysis of spontaneous alternation in the Y-maze test analysis of prior to drug treatment. There was no significant difference in spontaneous alternation performance in the Y-maze test in all test groups. Data is expressed as mean ± SEM. NS means no significant difference. n=8 animals per group

Following treatment with scopolamine for 1 week, percentage spontaneous alternation performance in naive animals was significantly different from all scopolamine treated animals as shown in Figure 3.

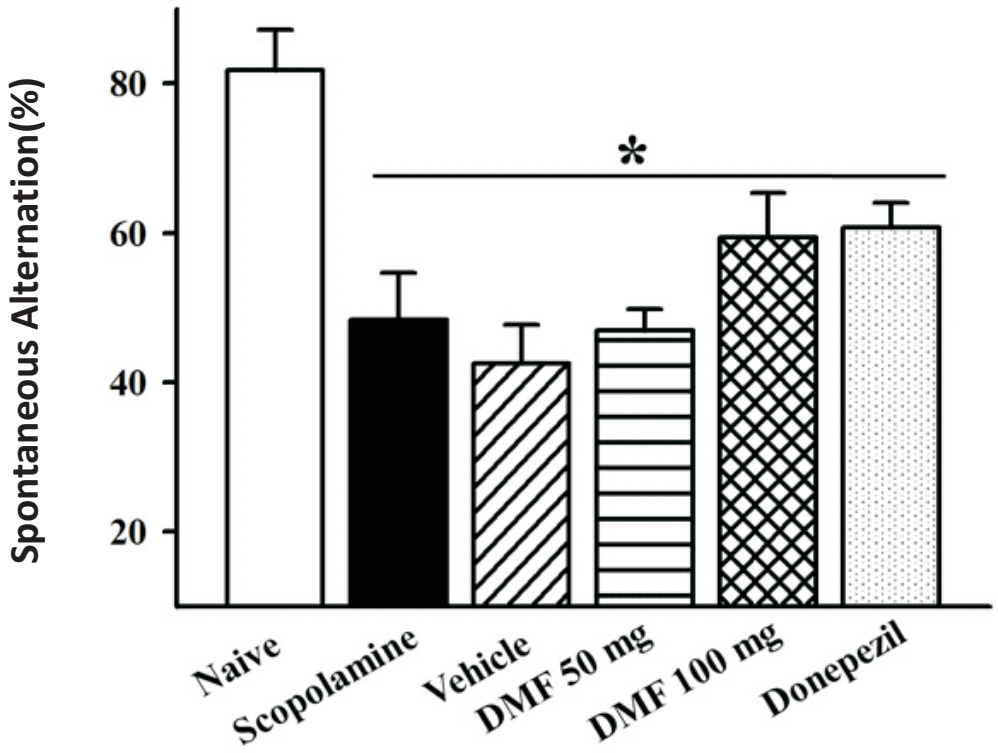

Figure 3. Statistical analysis of spontaneous alternation in the Y-maze test following treatment with scopolamine. Treatment with scopolamine reduced spontaneous alternation performance in all test groups. Data is expressed as mean ± SEM. *p<0.05 compared to naive; n=8 animals per group.

Treatment with 50 and 100 mg/kg DMF improved spontaneous alternation performance though not statistically significant when compared to vehicle and scopolamine treated animals. There was also no significant difference between naive, donepezil and both dose levels of DMF treated animals. Data is presented in Figure 4.

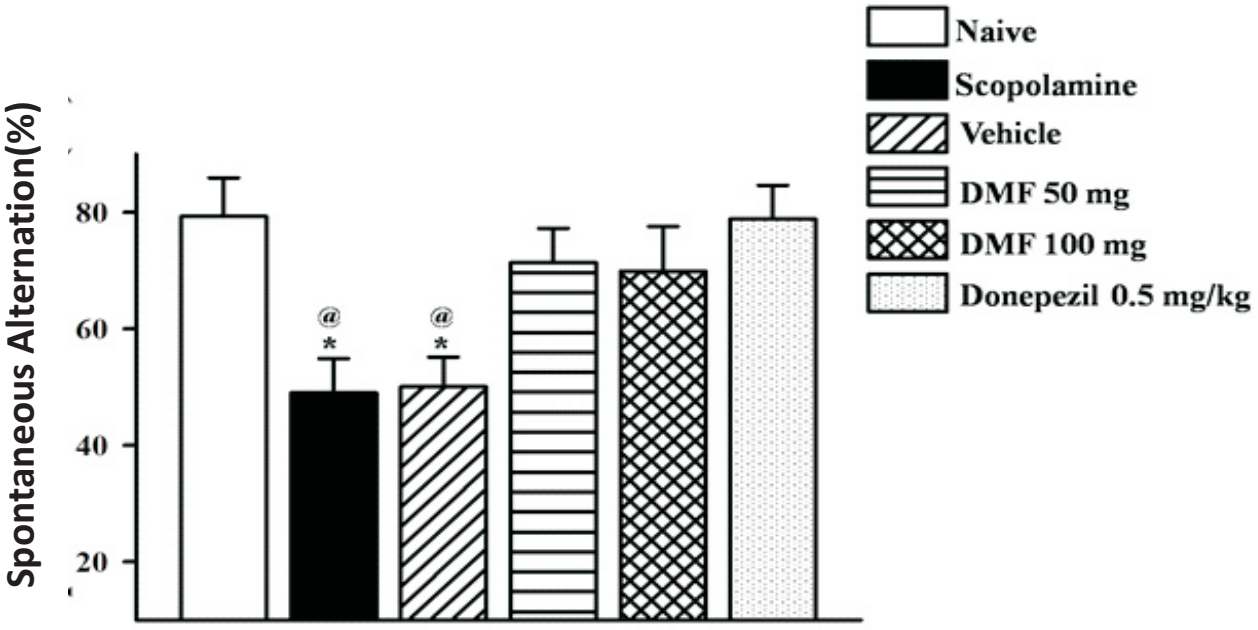

Figure 4. Statistical analysis of spontaneous alternation in the Y-maze test following treatment with the various test drugs. Treatment with DMF (50 and 100 mg/kg) and donepezil improved spontaneous alternation performance in all test groups. Data is expressed as mean ± SEM. *p<0.05 compared to naive; @ compared to donepezil; n=8 animals per group.

Post treatment with scopolamine and the various test drugs, 100 mg/kg DMF significantly increased (p<0.05) spontaneous alternation performance, this was not significantly different from donepezil and naive animals. Low dose DMF -50 mg/kg did not significantly improve spontaneous alternation performance when compared to vehicle and scopolamine treated animals. The results obtained are depicted in Figure 5.

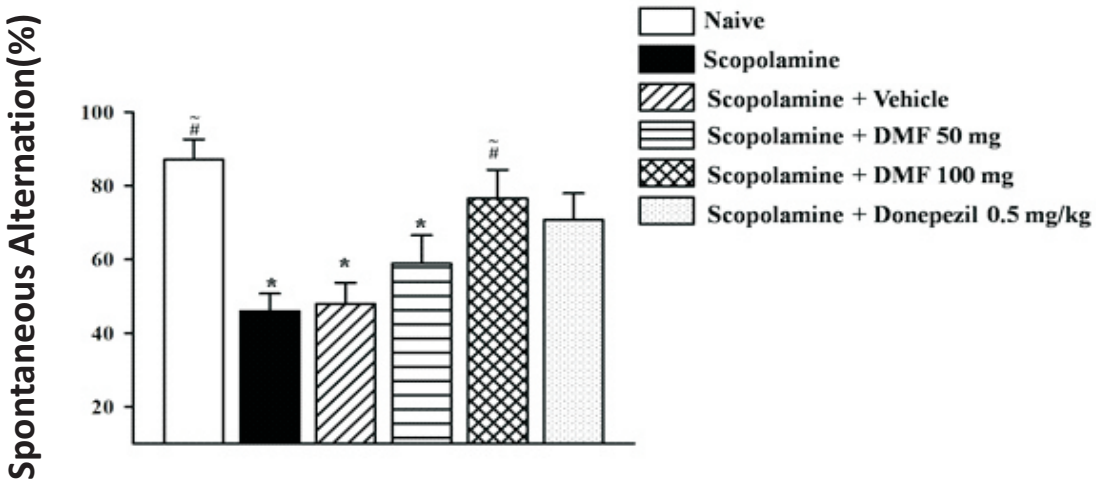

Figure 5. Statistical analysis of spontaneous alternation in the Y-maze test following treatment with scopolamine and test drugs Treatment with scopolamine reduced spontaneous alternation performance which was reversed by high dose DMF. Data is expressed as mean ± SEM. *p<0.05 compared to naive, #p<0.05 compared to scopolamine, ~ compared to vehicle; n=8 animals per group

Biochemical Evaluations

Brain catalase levels

Brain catalase levels were different in the various intervention groups; naive and donepezil treated animals gave the highest expression, followed by DMF 100 mg/kg and DMF 50 mg/kg treated mice. These were significantly different ($p<0.05$) from scopolamine and vehicle treated animals. This data is presented in Figure 6.

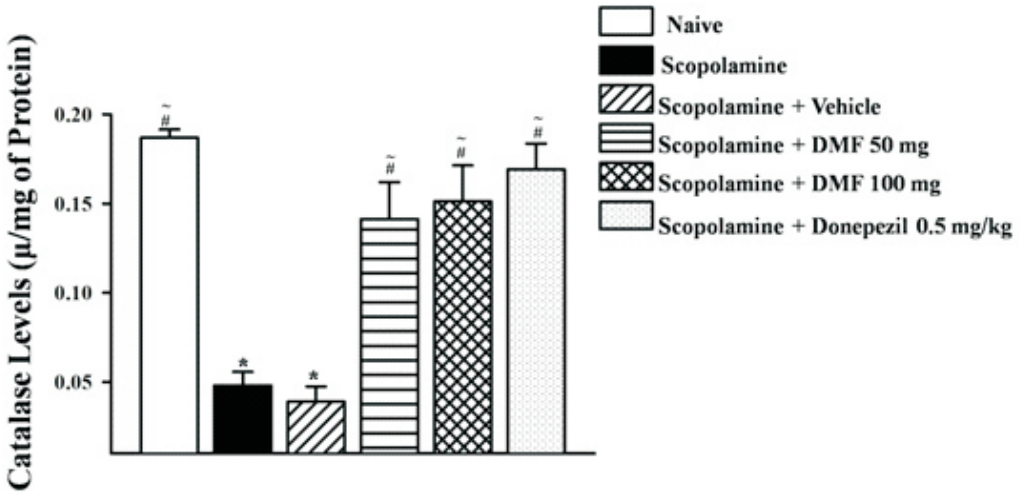

Figure 6. Statistical analysis of brain catalase levels following scopolamine and chronic drug administration. DMF and donepezil increased brain catalase Data is expressed as mean ± SEM. *p<0.05 compared to naive, ~ p<0.05 compared to vehicle, #p<0.05 compared to scopolamine, n=8 animals per group.

Brain Glutathione Levels

Levels of glutathione in the brain was significantly higher in donepezil, naive and DMF 100 and 50 mg/kg treated animals when compared to scopolamine and vehicle treated animals. These values were significantly different for scopolamine and vehicle treated animals (Figure 7).

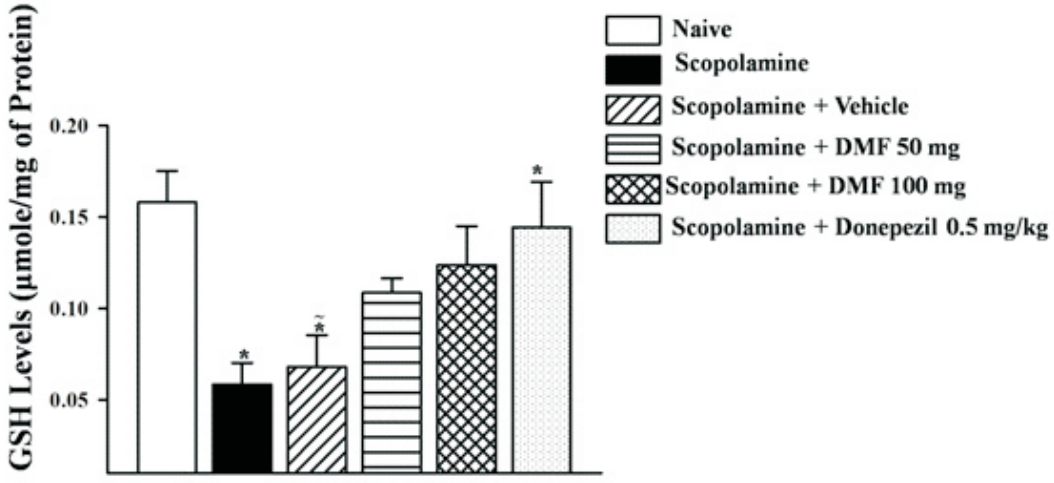

Figure 7. Statistical analysis of brain glutathione levels following scopolamine and chronic drug administration. DMF and donepezil increased brain glutathione. Data is expressed as mean ± SEM. *p<0.05 compared to naive, ~ p<0.05 compared to vehicle; n=8 animals per group.

Malondialdehyde levels

Malondialdehyde, a product of lipid peroxidation estimated as thiobarbituric reactive acid substances was significantly higher ($p<0.05$) in scopolamine and vehicle treated animals when compared to naive, donepezil, DMF 50 mg/kg and DMF 100 mg/kg. Data is presented in Figure

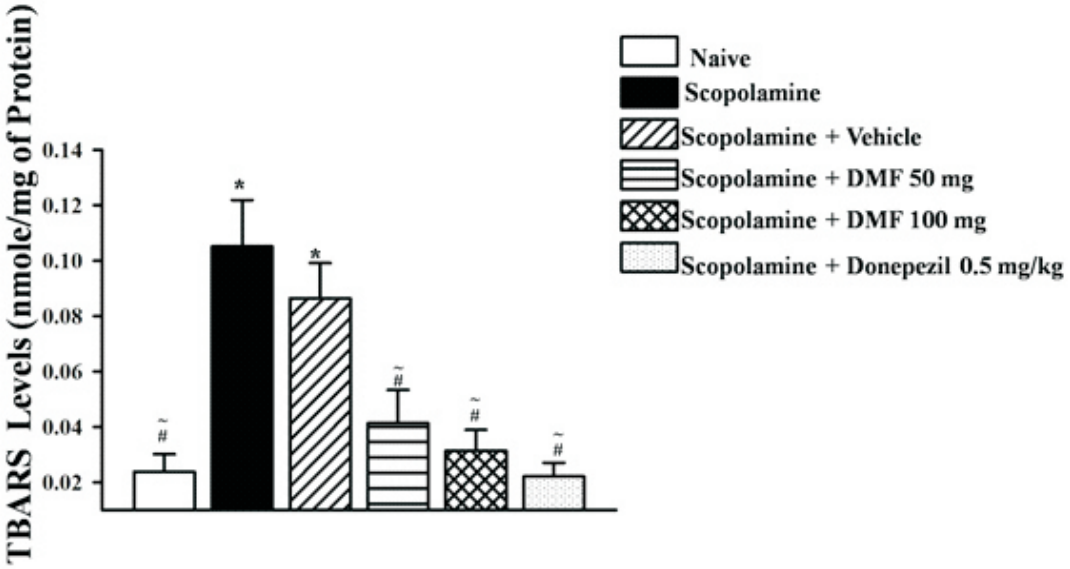

Figure 7. Statistical analysis of brain thiobarbituric reactive acid substances levels following scopolamine and chronic drug administration. DMF and donepezil reduced levels of thiobarbituric reactive acid substances in the brains of animals in this study. Data is expressed as mean ± SEM. *$p<0.05$ compared to naive, ~ $p<0.05$ compared to vehicle; n=8 animals per group.

DISCUSSION

In this study we investigated the potential ameliorative effects of dimethyl fumarate in scopolamine-induced cognitive impairment and oxidative stress in mice. The results show that treatment with DMF, (i) improved behavioural parameters following acute and chronic drug treatment in the Y-maze test (ii) improved the antioxidant profiles and reduced lipid peroxidation products in brain tissues.

Spontaneous alternation in the Y-maze test which is a measure of working and spatial memory is based on the ability of rodents to remember the sequence of arms visited and avoid revisits. Typically, a rodent usually remembers the least recently visited arm in order to alternate the arm choice, thus animals with cognitive deficits have low spontaneous alternation performance percentage values.[27, 34] Treatment with scopolamine induced cognitive deficits which was reversed by high and low doses of DMF as shown by the spontaneous alternation values in the Y-maze test.

In order to assess the effect of DMF on cholinergic activity which is indicative of cognitive function,[7] acetylcholinesterase levels in the brain of the different intervention groups were measured. Scopolamine has been shown to induce cognitive deficits and oxidative stress similar to what is observed in patients with memory impairments and cognitive dysfunction.[35, 36]

Reduction in antioxidant enzymes is a characteristic of neurodegenerative disorders and agents which reverse this free radical-induced oxidative stress have been demonstrated to be neuroprotective.[11,37] Scopolamine significantly increased acetylcholinesterase levels but this increase was inhibited by high dose of DMF. Reduction in levels of acetylcholinesterase by DMF could be suggestive of improved cholinergic neurotransmission and possible inhibition of acetylcholine by neuronal degradation. However, more mechanistic studies will be required to confirm this hypothesis.

We also evaluated the antioxidant profiles and levels of lipid peroxidation products of animals treated with DMF following daily exposure to scopolamine treatment. Levels of catalase in scopolamine and vehicle treated animals were significantly lower than those of naive,

DMF and donepezil treated animals. Scopolamine which has been shown to induce cognitive deficits is also associated with oxidative stress.[35-37] Catalase is an important antioxidant enzyme responsible for detoxification of hydrogen peroxide which contribute to oxidative stress, hence augmenting its endogenous function would also be neuroprotective.[38] Levels of GSH were also significantly higher in DMF and donepezil treated animals. GSH is considered the first line of defense against oxidative stress and protects neurones from hydrogen peroxide induced cytotoxicity.[39,40]

Lipid peroxidation which is enhanced by GSH depletion is another indicator of brain oxidative stress.[26,39] Unlike other membranes, there is a high percentage of long chained poly unsaturated fatty acids in neuronal membranes and these are the targets of free radical induced lipid peroxidation especially in neurodegenerative disorders.[4, 41] Results obtained showed a high amount of monodialdehyde (measured in the form of thiobarbituric reactive substance)- which is the most abundant aldehyde resulting from lipid peroxidation- in scopolamine and vehicle treated animals with significantly lower values in DMF and donepezil treated animals.

Despite the findings obtained, this study has some limitations which authors were unable to resolve in the resource limited settings in which the study was carried out. Other biochemical and molecular assays such as Western blotting, immuno-histochemistry and polymerase chain reaction assays for specific protein biomarkers could not be performed.

CONCLUSION*:*

Taken together, our results show that DMF ameliorates scopolamine induced cognitive deficits and attenuates scopolamine induced oxidative stress in mice.

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