Quench Academy of Medical Education and Research
Annals of Medical Physiology
www.amphysiol.com

Original Research Article

Caffeine and *Camellia sinensis* enhance cognition and decrease acetylcholinesterase activity in scopolamine-induced memory loss in female Swiss mice

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Article history

Received 01 August 2022
Revised 03 October 2022
Accepted 19 October 2022
Online 17 November 2022
Print 17 November 2022

Keywords

Acetylcholinesterase
Caffeine
Green tea
Memory loss
Scopolamine

Abstract

Caffeine and *Camellia sinensis* (green tea) has been known to have positive effect on memory. The present study investigated the possible effect of caffeine and green tea co-administration on oxidative stress markers, inflammatory marker and acetylcholine esterase activity in scopolamine-induced memory loss in female Swiss mice. Memory behavioral tests using Y-maze and Morris water maze was carried out, followed by oxidative stress biomarkers, acetylcholinesterase activity and Tumor Necrosis Factor alpha (TNF-α) evaluation from the mice brain tissues after caffeine and green tea administration. Scopolamine administered intraperitoneally at a dose of 1mg/kg Body Weight (BW) for 7 days significantly reduced the percent alternation of the mice in Y-maze thus, increased acetylcholinesterase activity and increased TNF-α level. However, caffeine administered orally at a dose 50mg/kg BW and green tea administered orally at a dose of 60mg/kg BW increased the percent alternation significantly, reduced acetylcholinesterase activity and reduced the TNF alpha level significantly. Oxidative stress markers evaluated GSH and MDA, showed no significant difference across all groups. These findings showed scopolamine has a deteriorating effect on cognition by increasing acetylcholinesterase activities thus mopping out acetylcholine at a faster rate. However, caffeine and green tea singly and in combination restored cognition, reduced acetylcholinesterase activity and restored TNF-α level. The neuroprotective effect of caffeine and green tea was compared to that of Donepezil, an established cognition enhancing drug and the effect was agonistic. The ability of caffeine and green tea to reduce acetylcholinesterase activity could be the mechanism for the ability to enhance memory. The ability of these compounds in restoring TNF alpha level also further potentiates their neuroprotective capability.

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DOI: https://doi.org/10.23921/amp.2022v6i2.00062
Print ISSN: XXXX-XXXX
Online ISSN: 2456-8422

Introduction

Spatial memory is a form of memory responsible for the recording and recovery of information needed to plan a course to a location and to recall the location of the object or the occurrence of an event **[1]**. A person's spatial memory is needed to navigate around a familiar object. Various part of the brain is involved with memory including the hippocampus, posterior parietal lobe, entorhinal cortex, prefrontal cortex, retrosplenial cortex, and perirhinal cortex. Memory loss occurs when the stored memory is disturbed making storing and retrieval difficult; this may be due to a ton of facts or; either physical, mental, emotional disturbances, or even a neurological disorder **[2]**.

Scopolamine also known as hyoscine hydrobromide with the trade name buscopan is an antimuscarinic, anticholinergic medication used to treat abdominal cramping and pain, esophageal spasms, renal colic, and bladder spasms **[3]**. It is a standard drug used for inducing cognitive deficits in animals. It confers impairment of learning acquisition and short-term memory and elevates acetylcholinesterase (AchE) levels in the cortex and hippocampus reducing acetylcholine level, and consequently impairing memory functions in animals **[4]**. It is regarded as a non-muscarinic receptor antagonist and at higher concentrations, it blocks nicotinic acetylcholine receptors **[4,5]**. Recent studies have reported that scopolamine increases the accumulation of reactive oxygen species (ROS) that induces oxidative stress leading to memory impairment **[6]**.

Donepezil is a specific non-competitive and reversible inhibitor of acetylcholinesterase. It is an anticholinesterase agent thus inhibiting hydrolysis of acetylcholine leading to an increased concentration of acetylcholine and choline in cholinergic synapses **[7]**. Donepezil enhance spatial memory via different mechanism such as, acetylcholinesterase inhibition, enhancement of cholinergic neurotransmission, putative modulation of other neurotransmitter systems, activation of neurotrophic mechanisms, promotion of non-amyloidogenic pathways leading to reduced corticohippocampal atrophy **[8]**.

Caffeine is an antagonist of adenosine receptors. The blocking of these receptors modulates glutaminergic, cholinergic, dopaminergic, serotoninergic, and noradrenergic neurotransmission **[9,10]**. If taken, it can help to modulate the aforementioned pathways including the cholinergic pathway responsible for the neurotransmission pathways involved in memory and cognitive mechanisms. Previous research has shown that caffeine has beneficial effects on cognitive impairment and neurodegenerative diseases including Alzheimer's and Parkinson's disease because of its neuroprotective effects **[11,12]**.

Green tea is made from *Camella Sinensis* leaves and buds which have not undergone the same withering and oxidation process used to make oolong teas and black teas. Green tea is considered to have originated in China and has since then spread to other countries including Nigeria. It contains bioactive substances such as polyphenols, flavonoids, quercetin, and myricetin. Polyphenols found in green tea include epigallocatechin gallate (EGCG), epicatechin gallate, epicatechins and flavanols **[13]**. Catechins are dietary polyphenolic compounds associated with a wide variety of beneficial health effects *in vitro*, and *in vivo*. These therapeutic properties have long been attributed to the catechins' antioxidant and free radical scavenging effects. Catechins' actions of attenuating oxidative stress and the inflammatory response may, in part, account for their confirmed neuroprotective capabilities. The versatility of the mechanism of action of green tea increases its therapeutic potential for numerous clinical disorders **[14]**.

Therefore, this study aimed to evaluate the effect of green tea and caffeine singly and in combination on scopolamine-induced spatial memory loss.

Materials and methods

Drug, supplement and chemicals

Scopolamine known by the trade name Buscopan and Donepezil was obtained from Tuyil pharmaceutical Industry in Ilorin, Nigeria. The green tea used for this study is a product of Biogenic Zhejiang, China. Caffeine and chemicals used for biochemical assays were purchased from Sigma-Aldrich, St. Louis, USA. Assay kits were obtained from Elabscience, Texas, USA.

Experimental animals

Thirty-six (36) female Swiss mice, seven (7) weeks old weighing between 20 and 25g was obtained from Dezalem Rat Enterprise Osogbo, Nigeria, and was adequately checked to ascertain their gender. The animals were housed by animal ethics in an environment with an ambient temperature of about 25^{o}C, under a cycle of 12 hours of light and 12 hours of darkness at the animal house of the Faculty of Basic Medical Science, College of Health Sciences, University of Ilorin. Ethical approval was obtained from the Ethical committee of the Faculty of Basic Medical Sciences, University of Ilorin with approval number: UERC/ASN/2016/487. The mice were acclimatized for 2 weeks and provided with adequate food and clean water *ad libitum.* At the

beginning of the third week, the animals were trained in Morris water and Y-maze.

Experimental design

Thirty-six (36) Swiss mice were randomly grouped into six with six mice in each group (n=6).

- Group 1 (control) were healthy mice, given distilled water orally.
- Group 2 (scopolamine) was administered 1mg/kg BW of scopolamine (i.p.) for 7 days.
- Group 3 (scopolamine + donepezil); Mice were administered 1mg/kg BW of scopolamine 30 minutes after pretreatment with 1mg/kg BW of a standard drug, donepezil orally.
- Group 4 (scopolamine + caffeine) was administered 1mg/kg BW 30 minutes after pretreatment with 50mg/kg of caffeine orally.
- Group 5 (scopolamine+ green tea) was administered 1mg/kg BW of scopolamine 30 minutes after pretreatment with 60mg/kg of green tea orally.
- Group 6 (scopolamine + caffeine+ green tea) was administered 1mg/kg BW, 30 minutes after pre-treatment, 50mg/kg BW of caffeine, and 60mg/kg BW of green tea orally.

Behavioral tests were carried out on the seventh day to access their spatial memory and the animals were euthanized to get their organs for biochemical assays.

Induction of spatial memory loss using Scopolamine

Scopolamine (1mg/kg BW) was given intraperitoneally (i.p.) to the experimental mice for seven (7) days (following the administration of either donepezil, caffeine, green tea, or their combination as pretreatment) to induce memory loss based on previous research literature **[15]**. Memory loss was confirmed after 7 days using Morris water maze and Y-Maze.

Behavioral studies

Morris water maze

The Mice were subjected to Morris water maze test following drug administration according to the method of Morris in 1984. During the memory test, the animals were allowed to swim for a maximum of 60 seconds and the time spent to reach the escape platform was recorded.

Y-maze

Y-maze was carried out based on published protocol with modifications to adapt the system to mice **[16]**. The mice were placed in the maze and allowed to freely explore all arms of the maze. The number of entries into arms and correct alternations between arms was recorded to determine the percentage alternation. The test was carried out for a maximum duration of 5 minutes and the maze was cleaned with 5% alcohol and cotton swap and allowed to dry between sessions **[17]**.

$$\%Spontaneous\ alternation = \frac{actual\ alternations}{maximum\ alternations} \times 100$$

Biochemical analysis

At the end of the experiment, the animals were anesthetized using ketamine, and euthanized. The brain was harvested into sample bottles containing phosphate buffer saline (PBS) 7.4 and placed in ice packs. The tissues were minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces are weighed and then homogenized in PBS (PBS) with a glass homogenizer on ice.

Estimation of Acetylcholinesterase activity

The Acetylcholinesterase Activity Assay kit provides a simple and direct procedure for measuring AChE levels in a variety of samples such as blood, serum and plasma. This assay is an optimized version of the Ellman method in which thiocholine, produced by AChE, reacts with 5,5'-dithiobis (2-nitrobenzoic acid) to form a colorimetric (412 nm) product, proportional to the AChE activity present. One unit of AChE is the amount of enzyme that catalyses the production of 1.0 mmole of thiocholine per minute at pH 7.5 at room temperature.

Estimation of Gluthathione level

These spectrophotometric procedures are based on the method of Ellman **[18]**, who reported that 5,5'-dithiobis- (2, -nitrobenzoic acid) is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. Brain tissue was rinsed with PBS. Homogenized in 2.5ml protein precipitation reagent. Centrifuged at 3000rpm for 10 minutes. The clear supernatant was used for the assay. The nitromercaptobenzoic acid anion has an intense yellow color which when measured at a wavelength of 412nm was used to measure SH groups.

Estimation of Malondialdehyde (MDA) level

This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm. Materials used include; Indicator 2-Thiobarbituric Acid, Acid Reagent 10% Acid

Solution in Dimethylsulfoxide, MDA Standard 10 mM Malondialdehyde Tetrabutylammonium Salt, Microplate, they were stored at a temperature of 4°C.

Estimation of TNF-α level

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Mice TNF-α. Samples (or Standards) are added to the micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Mice TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Mice TNF-α, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat TNF-α. Calculation of the concentration of brain's TNF-α in the samples were done by comparing the OD of the samples to the standard curve.

Statistical analysis

All data are presented as mean±S.E.M. and were analyzed using GraphPad prism software version 8.0. Statistical analysis was done using ANOVA. Comparison between the groups was considered statistically significant at $p<0.05$.

Results

Behavioral studies

Morris water

Effect of donepezil, caffeine, green tea and their combination on Morris water maze

The untreated scopolamine-induced group significantly increased in time to locate the escape platform compared to control and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea). Groups administered donepezil, caffeine, green tea and caffeine combined with green tea significantly decreased when compared to scopolamine group but not significantly different from control (**Figure 1**).

Y-maze

Effect of donepezil, caffeine, green tea and their combination on percentage alternation in Y-maze

The untreated scopolamine-induced group significantly reduce in percent alternation compared to control and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea). Groups administered donepezil, caffeine, green tea and caffeine combined with green tea significantly increased when compared to scopolamine group but not significantly different from control (**Figure 2**).

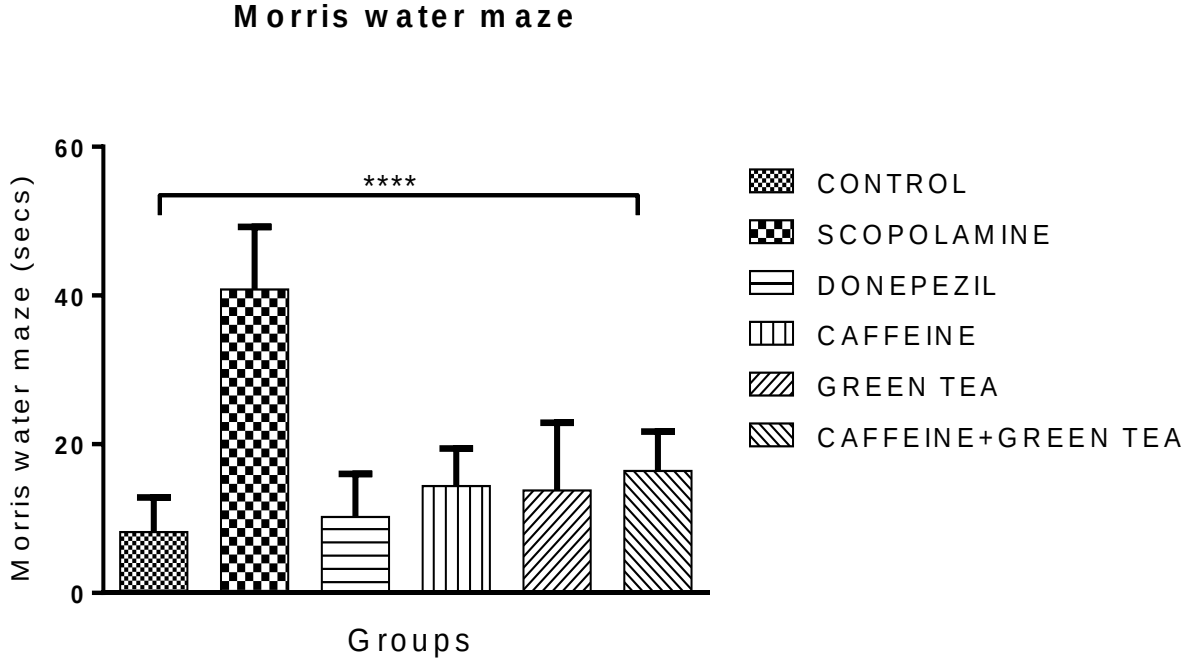

Figure 1. Effect of administration of scopolamine, donepezil, caffeine, green tea and caffeine + green tea on memory function in female Swiss mice. The results obtained after the 7 days of administration revealed that; there was a statistically significant increased time to locate the escape platform due to the memory loss in the mice in group 2 (scopolamine group) compared to group 1 (control group). Each point is the Mean± SEM. $p< 0.05$ indicating the significant differences from all the other groups.

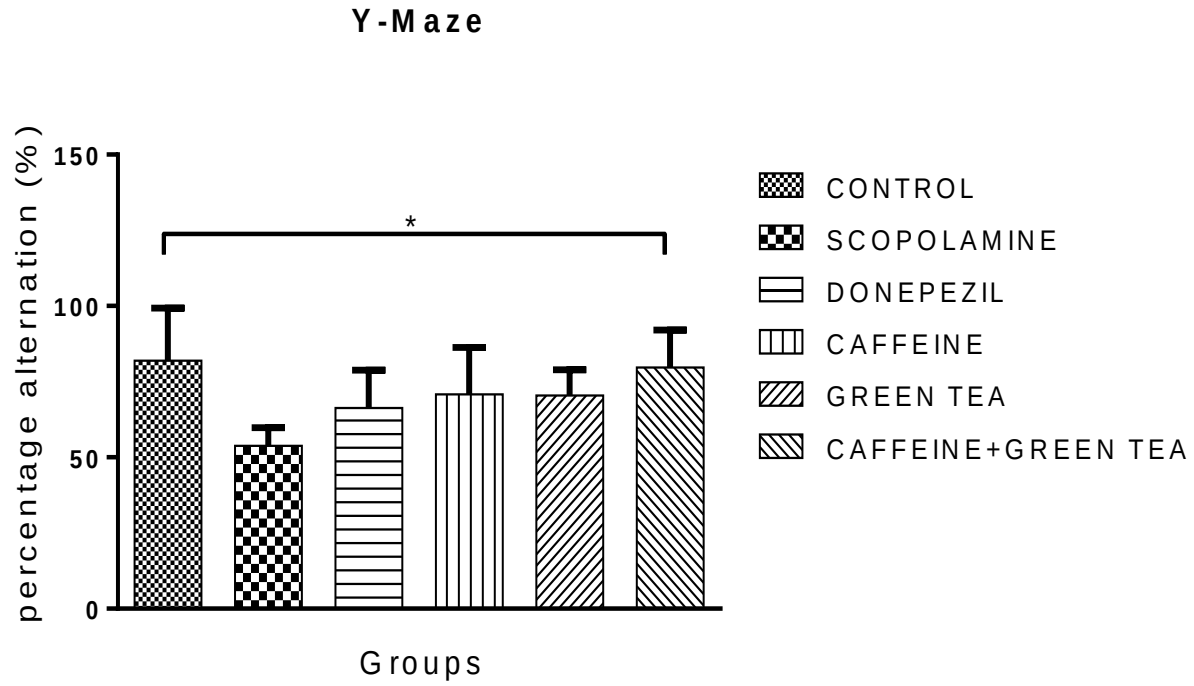

Figure 2. Effect of administration of scopolamine, donepezil, caffeine, green tea and caffeine + green tea on memory function in female Swiss mice. The results obtained after the 7 days of administration revealed that; group 2 (scopolamine) has a reduced number of alternation compared to control group due to the memory loss. Group 6 (scopolamine + caffeine + green tea) shows an increased in percentage alternation compared to group 2 (scopolamine) which confirms the ameliorating function of caffeine and green tea. Each point is the Mean± SEM. p< 0.05 indicating the significant differences from all the other groups.

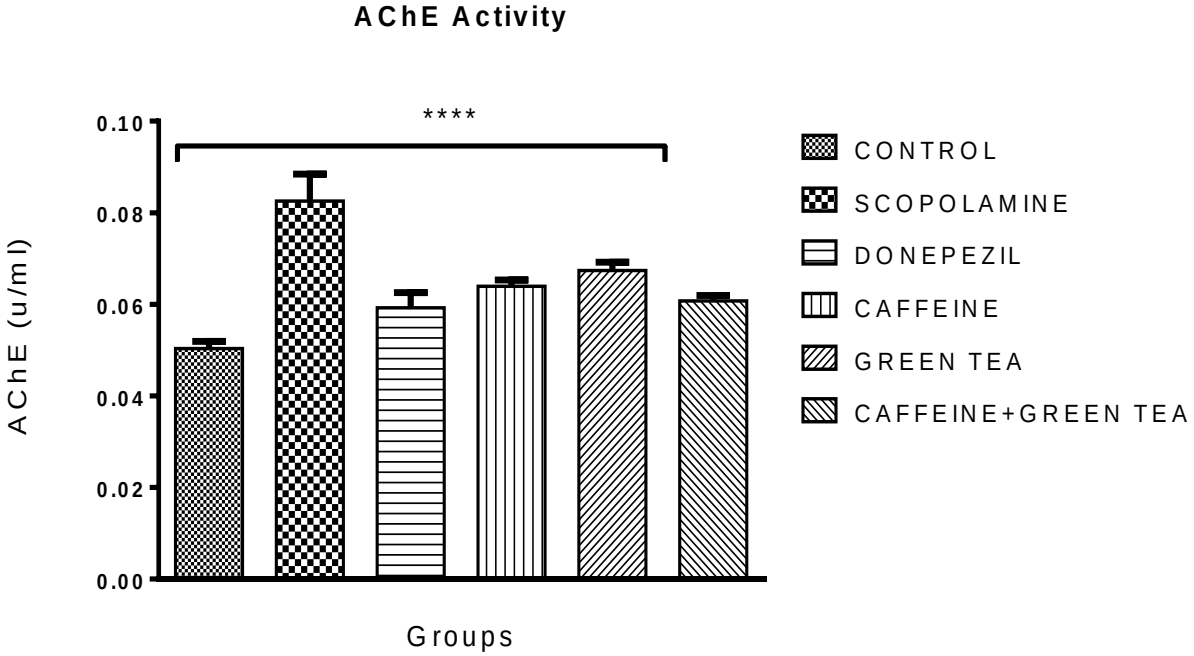

Figure 3. Effect of administration of scopolamine, donepezil, caffeine, green tea and caffeine + green tea on Acetylcholinesterase (AChE) activity in the brain of female Swiss mice. The results obtained after the 7 days of administration revealed that; there was a significant increase in AChE activity in the Scopolamine (Group 2) when compared to Control (group 1), Donepezil (group 3), Caffeine (group 4), Green tea (group 5) and Caffeine + Green tea (group 6). Each point is the Mean ± SEM. p< 0.05 indicating the significant differences from all the other groups.

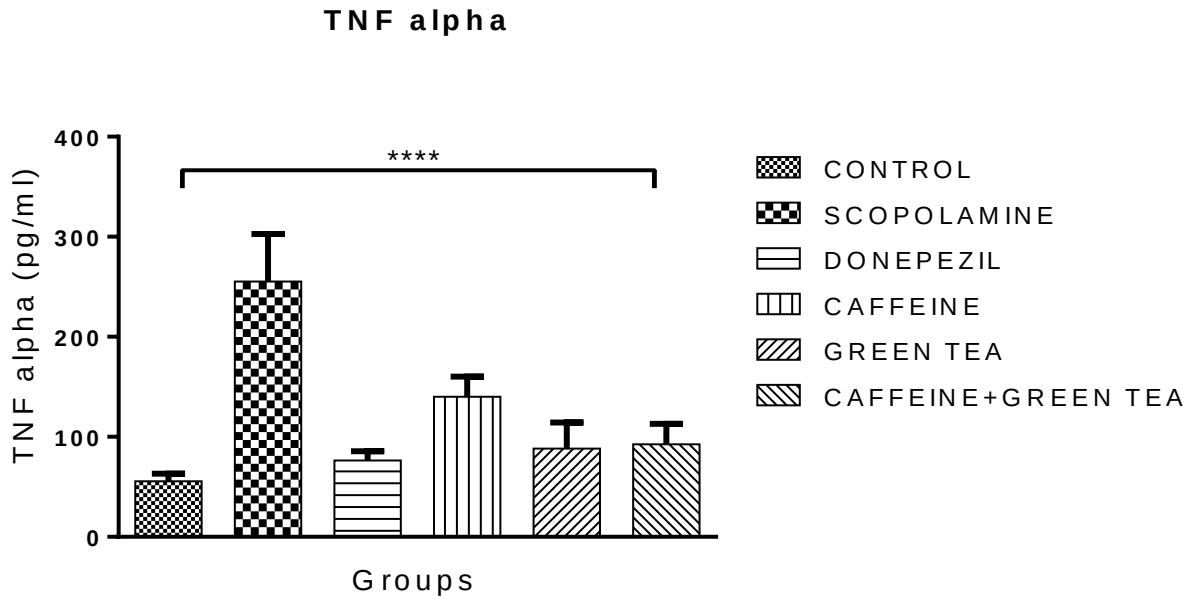

Figure 4. Effect of administration of scopolamine, donepezil, caffeine, green tea and caffeine + green tea on TNF-α level in the brain of female Swiss mice. The results obtained after the 7 days of administration revealed that; there was a significant increase in the level of the inflammatory marker TNF Alpha in the Scopolamine (Group 2) when compared to Control (group 1), Donepezil (group 3), Caffeine (group 4), Green tea (group 5) and Caffeine + Green tea (group 6). Each point is the Mean ± SEM. $p < 0.05$ indicating the significant differences from all the other groups.

Biochemical analysis

Glutathione (GSH) level: Effect of donepezil, caffeine, green tea and their combination on GSH level in scopolamine-induced memory impaired mice brain

The control, scopolamine-induced group and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea) have no significant difference.

Malondialdehyde (MDA) level

The control, scopolamine-induced group and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea) have no significant difference in MDA activity.

Acetylcholinesterase activity

The untreated scopolamine-induced group significantly increased AChE activity compared to control and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea). Groups administered donepezil, caffeine, green tea and caffeine combined with green tea significantly decreased when compared to scopolamine group but not significantly different from control (**Figure 3**).

TNF-α level of brain tissue

The untreated scopolamine-induced group significantly increased Inflammatory activity compared to control and the treated groups (donepezil, caffeine, green tea, caffeine combined with green tea). Groups administered donepezil, caffeine, green tea and caffeine combined with green tea significantly decreased when compared to scopolamine group but not significantly different from control (**Figure 4**).

Discussion

This study was undertaken to investigate whether caffeine and green tea could improve memory impairment via the cholinergic pathways. Medicinal plants are playing a significant role in the management of memory deficit and Alzheimer's disease. In this study we evaluated the effect of caffeine and green tea on the memory function of amnesic mice in the Y-maze, Morris water maze and biochemical assays. Scopolamine promoted amnesia in the animals through impaired memory by blocking the muscarinic cholinergic receptors in the brain as earlier reported **[19]**. In the present study, chronic administration of Scopolamine (Group 2) to mice decreased the percentage alternation in the arms of the Y-maze when compared to the Group 1 (control), Group 3 (donepezil), Group 4 (caffeine), Group 5 (green tea) and Group 6 (caffeine + green tea). Administration of caffeine and green tea increases the percentage alternation in the three (3) arms of the Y-maze. The increase in the percentage alternation in the Y-maze indicated an improvement of memory. The significant increase in the number of entries and the time spent in the preferred arm reflects a good functioning of the memory. These results showed the antagonistic effects of caffeine and green tea on the action of

Scopolamine, which could be owing to the presence of bioactive substances such as polyphenols, flavonoids, quercetin, myricetin of green tea and binding of caffeine to adenosine receptor subtypes, that may inhibit the effects of Scopolamine and thereby improve memory loss. Many studies reported that polyphenols have antioxidant capacity neutralizing free-radicals by crossing the blood brain barrier to protect the brain and nervous system. The main function of polyphenols include improvement in memory **[6]**. In addition, the increase in the number of entries and percentage alternations of the three (3) arms of the Y-maze, suggests the increase of the exploration behavior and thus the memory faculties **[20]**.

The results obtained from the Morris water test showed significant increase in time to locate the escape platform in the Group 2 administered scopolamine only when compared to the Group 1 (control), Group 3 (donepezil), Group 4 (caffeine), Group 5 (green tea) and Group 6 (caffeine + green tea). There was also a decrease in time the Group 6 administered green tea and caffeine located the escape platform compared to the Group 2 (scopolamine). This shows that caffeine and green tea improved the memory of the animals.

The central cholinergic system plays a vital role in the processes of memory **[21]**. A dysfunction of neurons containing acetylcholine in the elderly presents cognitive deficiencies. Scopolamine produces severe cholinergic deficits and increased activity of acetylcholinesterase in the hippocampus, thereby reinforcing neurodegeneration in the brain. Treatment with caffeine and green tea significantly reduced the activity of acetylcholinesterase compared to the Group 2 (scopolamine) according to this study. This study suggests that the memory enhancing effects of caffeine and green tea can be explained by the inhibition of acetylcholinesterase activity and increased release of acetylcholine into the synaptic gap and its fixation on the postsynaptic receptors.

In the biochemical assays, result shows a decrease in the level of TNF-α activity in the brain in Group 6 (caffeine + green tea) when compared to Group 2 (scopolamine). Although there was decrease in brain glutathione (GSH) in Group 6 (caffeine + green tea) when compared to the Group 2 (scopolamine), and decrease in the level of malondialdehyde (MDA) in Group 6 (caffeine + green tea) when compared to the Group 2 (scopolamine) but no significant difference was observed. These showed that the combination of caffeine and green tea has anti-inflammatory effect. The group administered with standard drug donepezil (Group 3) also showed close similarity in the behavioral and biochemical assays of the animals when compared to the Group 1 (control group).

Conclusion

The under seek of this study shows the beneficial potential of caffeine and green tea in ameliorating spatial memory loss. Scopolamine which is a muscarinic receptor agonist and also when given in chronic amounts can block nicotinic receptors was studied against an antagonist caffeine (owing to it binding to the adenosine receptor subtypes) and green tea (owing to the presence of bioactive substances such as polyphenols, flavonoids, quercetin, myricetin). Caffeine and green tea improved and reduced the adverse effects of scopolamine in female Swiss mice. These beneficial effects were confirmed in the cholinergic neurotransmission. Also confirmed in this study was the anti-inflammatory properties of these compounds when co-administered. However, their antioxidant properties may be further established in future studies with extended duration of treatment.

Acknowledgments

I acknowledge my mentor, Professor Bamidele Victor Owoyele for his enormous contribution and mentorship that led to the success of this research.

Source of funding

Self-funded.

Disclosure of financial and non-financial relationships and activities, and conflicts of interest

The author(s) declare(s) that they have no conflict of interest to disclose.

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