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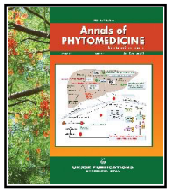

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Neurobehavioral validation of a mild cognitive impairment model using Scopolamine in C57BL6/J mice

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Abstract

Persistent neuroinflammation is one of the major characteristic feature in almost every neurodegenerative diseases. The minor cellular perturbations in the brain cells progresses towards a cumulative inflammatory state which causes delay in the transmission of synaptic signals in the process of learning and memory formation. In the early stages of cognitive impairments, there are still some chances of reversing the inflammatory status by treating with neuromodulatory agents. In order to understand the early cellular changes in the brain tissue, we developed a mild cognitive impairment model using scopolamine as an inducer of neuroinflammation. We attempted to establish the duration of impairment after a 6 day consecutive administration of scopolamine; followed by changes in object based behavioural tests and estimation of biochemical changes in the brain tissue homogenates. The results show impairments in the spatial memory, location memory and pattern separation functions in the first three weeks after scopolamine administration. In the third week and the fourth week, the mice have been able to recover to their normal behavioural function. A similar trend is seen in the biochemical changes of the brain tissue homogenates indicates a time dependant decrease in the oxidative stress from the first week to fourth week post induction. Overall, the results indicate that the scopolamine induced neuroinflammation persists for 3-4 weeks after the administration and this provides us the window to explore the any treatment options to determine the short-term changes in the brain of mice with mild cognitive impairment.

1. Introduction

Scopolamine an anticholinergic drug is most commonly used for treating postoperative nausea, and vomiting and primarily used as a treatment of motion sickness. It belongs to the family of antimuscarinic drugs which blocks the muscarinic acetylcholine receptors present both in the central nervous system and peripheral nervous system. Neuroscience-related research exploits this extended use of scopolamine to induce cognitive disorders in experimental models as it readily crosses blood-brain barrier. In Alzheimer's disease, scopolamine plays a dual role of increasing amyloid-β deposition and causing cholinergic dysfunction, both of which are the key hallmarks of the disease (Chen and Yeong, 2020). The validity of the scopolamine-induced cognitive deficit model has been studied in various ways and research have shown that male C57BL/6 mice treated with intraperitoneal scopolamine (2 mg/kg) showed delirium-like mice model with respect to the neuroinflammatory hypothesis of delirium which was assessed by neurobehavioral tests (Cheon *et al*., 2021). The study also showed in relation to delirium, treatment with scopolamine had led to a relative risk for postoperative delirium in patients undergoing orthopedic surgery (Rogers *et al*., 1990). Scopolamine based patches have also induced scopolamine-induced mental disorders, especially in the elderly (Seo *et al*., 2009; Jeurkar *et al*., 2022**)**. Animals treated with scopolamine 1 mg/kg, intraperitoneally just 30 min before assessment by step-through passive avoidance and Morris water maze (MWM) tasks showed cognitive impairment (Liao *et al*., 2020). They examined mice that were given 1 mg/kg scopolamine with or without 40 mg/kg vanillin once a day for 4 weeks. This caused mild cognitive impairment after 1 week and severe impairment 4 weeks later. Additionally, scopolamine (3 mg/kg body weight/d) was utilised to create the animal model during passive avoidance tests and MWM test in order to examine the effect of walnut oil on memory impairment in mice. In another investigation, it was discovered that transdermal scopolamine had caused mental disorientation in seven older women. Following their recovery from mental disorientation (mean time from onset to test: 66 days), they took neuropsychological testing. The findings revealed that each patient had at least one cognitive impairment. (Seo *et al*., 2009).

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In the present study, we attempt to establish an experimental model for mild cognitive impairment (MCI) in memory and learning which occur at the early onset of dementia associated with neurological illnesses. The impairment is assessed by object-based neurobehavioral tasks such as Open field test (OFT), Object location test (OLT), Novel object recognition test (NORT) and pattern separation test (PST). A vast majority of literature on scopolamine have shown the dose that causes the cognitive impairment, but there are very few studies showing the duration of impairment that persists after the treatment. The present study establishes this window period available for testing short-term interventional effects of potential therapeutics to understand the duration and efficiency.

2. Materials and Methods

2.1 Experimental animals

42 male C57BL/6J mice (8 weeks old, 25-30 g body weight) were utilised, and they were handled in accordance with IAEC regulations. The Institutional Animal Ethics Committee accepted the current study based on ethical and scientific standards with IAEC approval number: NGSMIPS/IAEC/JUNE-2021/236. The mice were kept in a 22°C, 59% humidity environment with a 12 h light/dark cycle while having access to *ad libitum* food and water.

Animals were acclimatized for 3 weeks in the animal house before being used for the study. The mice (n=42) were divided into 2 groups: naive (n=6) and disease control groups (Scopolamine induced) (n=34). The disease control mice were intraperitoneally injected with 1mg/kg scopolamine (Sigma, USA) once daily for a period of 6 days continuously. The effect of scopolamine was further validated with neurobehavioral memory and learning tests: OFT, OLT, NORT, and PST. The set of 4 tests was repeated for 4 weeks to understand the lasting effects of scopolamine whilst in comparison to the control. The mice in week 1 (n=14), week 2 (n=8), week 3 (n=8) and week 4 (n=6) after scopolamine treatment were tested with behaviour tests and were sacrificed at the end of their respective week by harvesting the brain tissue stored in -80°C for further analysis. The behavioural tests were performed with a resting period of 24 h between each behavioural test to avoid stress in animals. Only those animals which have explored a minimum of 20 sec in each training phase were considered for statistical analysis. This study enables us to understand the critical window period available to administer any drug against the cognitive deficit.

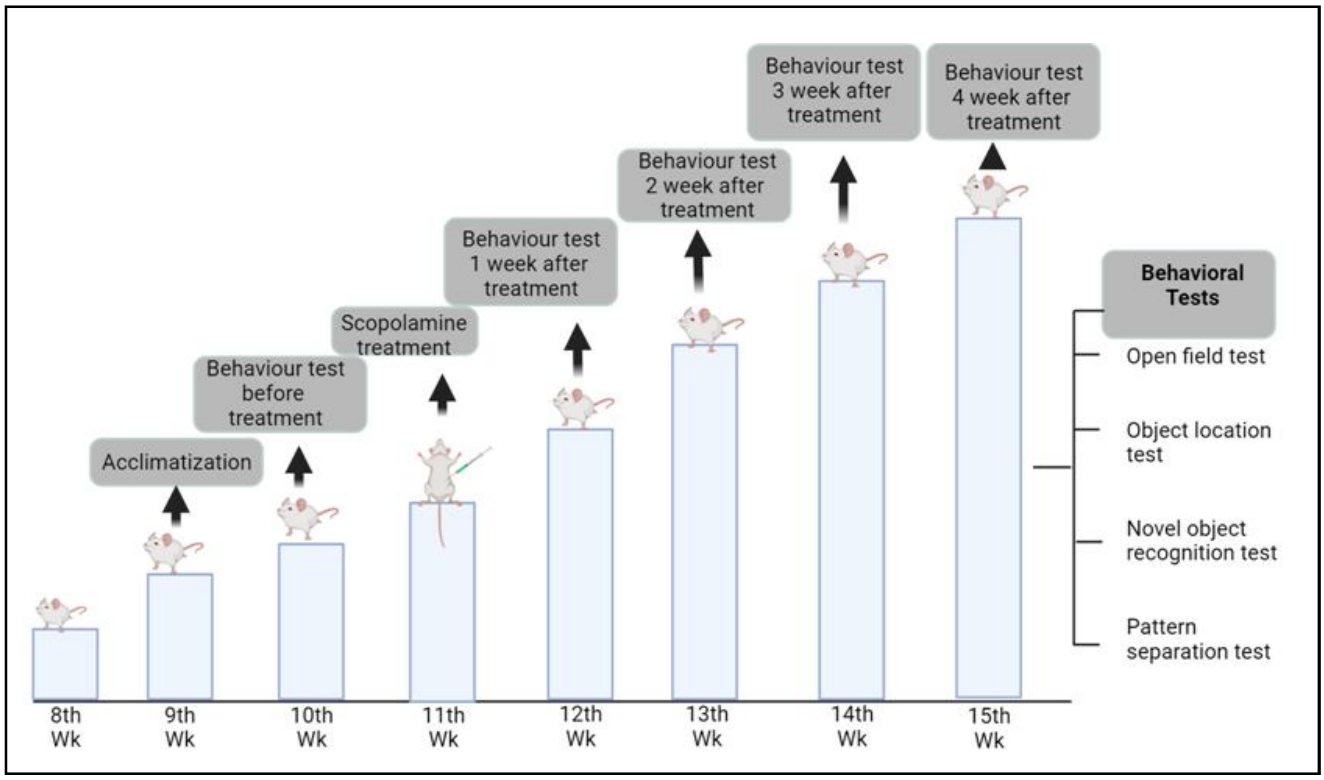

Figure 1: Study design to induce cognitive deficits after scopolamine treatment and the behavioural parameters used to determine the integrity of the hippocampus and different regions of the cortex.

2.1.1 Open field test

Open field test (OFT) analyses of general motor performance and anxiety-like behaviour. In this behavioural evaluation, the overall motor function and relative levels of anxiety of control and scopolamine-treated mice were assessed (Injamuri, *et al*., 2022). On the computer tracking system (All maze video tracking system), the testing arena (a 100 × 100 cm open field box) was divided into 9 squares: 4 in the corners, 4 on the sides and 1 in the centre. Figure 2 shows how the generated grid appeared on the computer screen. The four squares at each corner were referred to as corners (C), the remaining squares in the perimeter were referred to as sides (S), and the central square was referred to as the central zone (CZ). Each mouse was placed in a corner of an open field that was well-lit, and the movement of each mouse was video tracked for 5 min using the All maze video tracking system. Before each mouse's experiment began, the apparatus was cleaned with 70% alcohol and allowed to

dry by air. The following variables were measured: (i) dwell time in sides; (ii) dwell time in corners; and (iii) overall distance travelled (Kodali *et al*., 2023).

2.1.2 Object location test

This evaluation consists of three consecutive trials with a 30 min break between them. The first trial involved placing the mouse in the middle of an empty open field box that measured 100 cm (L) by 100 cm (W) by 60 cm (H) and letting the mice explore it at their leisure for five minutes (the habituation phase, Figure 3). The second trial began 30 min after the first trial and involved putting the mice in the middle of an open field box with two identical objects on either side and letting them examine the objects at their own pace for 5 min (*i.e*., the sample phase, Prior to the start of every mouse's experiment, the equipment was cleaned with 70% alcohol and allowed to dry by air. The amount of time spent examining an object when it is in its original location, after it is relocated to a new one, and overall were all measured. The time spent with the object transferred to a novel place/total time spent investigating both the object moved to a novel place and the object remaining in the familiar place 100 was also used to construct the place discrimination index. After that, within each group, the percentages of object exploration time spent with the object transferred to a novel location in comparison to the object remaining in the familiar location were compared.

The unique place discrimination index between untreated mice and those given scopolamine was also directly compared. In order to determine whether sadness (or lack of motivation) interfered with the spatial recognition memory testing, the velocity and the overall distance moved during the third trial (test phase) were also looked at and compared between the two groups. The mice's inclination to investigate the object that has been relocated demonstrates their capacity for place recognition memory (Hattiangady *et al*., 2014).

2.1.3 Novel object recognition test

Each mouse underwent three consecutive trials in this test, with a 30 min break between each trial. The first trial involved placing the mice in the middle of an empty open field box and letting them roam around freely for 5 min (the habituation phase). The second trial involved placing the mice in the middle of an open field box with two identical objects on either side and letting them roam around freely for 5 min (the sample phase). The mice were allowed to explore objects for five minutes in the same open field box during the second trial (the object recognition memory testing phase), which started 30 min later.

When a mouse's nose is 2 cm or closer from an object, it is deemed to be exploring it. The third trial's mouse movement was continually monitored and videotaped using the All maze video tracking system. Prior to the start of each mouse's experiment, the apparatus was cleaned with 70% alcohol and allowed to dry by air. Data on the total amount of time spent investigating all of the objects-the novel object, the familiar object, and both-was gathered. The time spent with the novel object divided by the overall time spent exploring the object was multiplied by 100 to create the novel object discrimination index.

After that, within each group, the percentages of item exploration time spent with the novel object in comparison to the familiar object were compared. Additionally, animals treated with scopolamine and control mice were directly compared using the novel object discrimination index. To ascertain whether sadness (or lack of desire) interfered with the object recognition memory testing, the velocity and the total distance moved during the third trial (test phase) were also gathered and compared between the two groups. The decision to spend more time exploring the unfamiliar thing than the familiar object demonstrates the utilisation of memory and learning processes (Madhu *et al*., 2019).

2.1.4 Pattern separation test

The hippocampus, a region of the brain involved in creating new memories, was used in the pattern separation test. The hippocampus CA1 region of the brain, which is crucial for aiding the brain in representing and remembering spaces, was tested on object location. The perirhinal cortex, which is involved in the establishment of episodic memory, and the hippocampus were tested for novel object recognition.

The PST used in this investigation consisted of four consecutive trials (T1-T4), each lasting 5 min, with a 30 min ITI in between. The movement of the animals in T2-T4 was captured by the All maze video-tracking system. The ability for pattern separation in T4 requires the memory formation for objects and floor patterns in T2 and T3, and such competence requires exploration of various objects and floor patterns for significant periods of time in T2 and T3. Therefore, the animals that explored objects for less than 20 sec in T2 and T3 were included for data analysis. Results were calculated and compared, including the percentage of time spent exploring NO on P2 compared to FO on P2, the TOETs, the total distance travelled, and the velocities of movement in various trials (Kodali *et al*., 2021).

2.1.5 Tissue harvest and biochemical measurements

After the completion of neuro behavioural tests animals from each group were euthanized by decapitation under deep anaesthesia as detailed in our previous study. Blood was quickly collected by direct cardiac puncture. The serum was extracted after centrifugation and stored at -80°C until further use. The fresh brains from these animals were quickly removed, snap-frozen on dry ice, and stored at -80°C until further analysis. These brain tissues were further used for biochemical studies and neurotransmitter analysis. Furthermore, few animals from each group were anesthetized and stored in 4% paraformaldehyde. These fixed brain tissues were used for various immunofluorescence studies.

2.2 Biochemical analysis

Measurement of oxidative stress markers from brain tissue lysates and the serum were performed.

2.2.1 Lysate preparation

Each isolated brain tissue sample was weighed and lysed in ice-cold phosphate buffer of pH 7.4 with protease inhibitor and using sonication for 15 sec. The lysed solution was centrifuged for 20 min at 3000 rpm at 4ºC. The aliquots of the supernatant solution were stored at -80ºC until further use. The protein concentration was measured in different samples using Lowry's method. Malondial-dehyde (MDA), superoxide dismutase assay (SOD), catalase (CAT), and glutathione.

2.2.2 Lipid peroxidation (MDA)

By dilution 0.1 ml of sample with 0.4 ml of distilled water and 1 ml of TCA-TBA reagent (TCA-TBA reagent), the production of malondialdehyde was calculated. For 15 min, the reaction mixture was heated in a water bath. The malondialdehyde standard curve was used to derive the endpoint, which was measured at 535 nm (Tenkanidiyoor *et al*., 2016).

2.2.3 Superoxide dismutase (SOD)

By treating 0.1 ml of sample with a combination of methionine, riboflavin, and nitro blue tetrazolium chloride, the superoxide dismutase activity was measured. Under fluorescent lighting, this combination was allowed to stand for 10 min. At 560 nm, the blue-green solution was measured. For lysates, superoxide dismutase activity was reported in units/mg of protein (Suchetha Kumari *et al*., 2014; Raviraj *et al*., 2023).

2.2.4 Catalase

By treating 10 µl of material with 3 ml of 60 mM hydrogen peroxide, the catalase activity was measured. For 2 min, the kinetic measurement was taken at 240 nm with a 30 sec delay. The activity was measured in units per milligram of protein for tissue homogenates (Tenkanidiyoor *et al*., 2016).

2.2.5 Glutathione

Ellman's method (Merghem *et al*., 2020) was used to determine reduced glutathione. The assay is based on the oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), in which DTNB and glutathione (GSH) react to form 2-nitro-5-thiobenzoic acid (TNB), which is yellow in colour. As a result, GSH concentration can be calculated by measuring absorbance at 412 nm. 50 µl of tissue homogenate was diluted in 10 ml of phosphate buffer (0.1 M, pH 8). 20 µl of DTNB (0.01 M) was added to 3 ml of the dilution mixture. At 412 nm, absorbance is measured against a blank made under the same conditions (Merghem and Dahamna, 2020).

2.3 Statistical analysis

For the behavioural studies, the results were expressed as mean percentage object exploration ± standard error of mean (SEM) within groups were compared using the unpaired 't' test with the p values <0.05 was considered to be statistically significant. The results between the groups were expressed as in the ability to discriminate between the novel and familiar objects expressed as discrimination index and the groups were subjected to Grubb's outlier test and Shapiro-Wilks normality test. If all the groups passed the normality test, then they were then subjected to ANOVA with Tukey's multiple comparisons and if the groups did not pass the normality test, then they were compared using ANOVA with non-parametric Kruskal Wallis test. For the biochemical analyses the results are expressed as mean ± standard deviation (SD) and the groups were subjected to Grubb's outlier test, Shapiro-Wilks normality test and then ANOVA with Tukey's multiple comparison or Kruskal Wallis tests.

3. Results

From the present study, we observed a significant impairment in the object based behavioral tests after scopolamine treatment for six consecutive days (ip; 1mg/kg bd wt).

In the open field test, there were no significant changes in the duration of exploration at any time point. This indicates that there was no significant locomotory functional changes among the mice (Figure 2).

There was a significant decrease in the spatial recognition memory after scopolamine treatment in the first week after treatment. This was indicated by decrease in the percent novel object exploration time in the P2 after getting acclimatized to the surrounding environment. Further, these changes were persistent in week 2 and 3 post-scopolamine treatment. In the fourth week, these observations were reversed indicating near-normalization of scopolamine-mediated memory inhibition (Figure 3).

The hippocampal dependant object location memory was impaired in mice after scopolamine treatment in the 1st week post-treatment. This was persistently similar upto 3 weeks post-treatment and after 4 weeks post-treatment, these results were normalized and the mice were again exploring novel locations in the testing phase (Figure 4).

The complex function of distinguishing between similar but non-identical pattern of events without any overlap was tested using PST. Interestingly, the mice showed impairment in identifying differing objects with pattern cues post-scopolamine treatment after the 1st week. This behaviour persisted after 3 weeks post-scopolamine treatment but was found to be normalised after 4 weeks post-treatment (Figure 5).

The biochemical functions indicated significant oxidative stress induced by scopolamine treatment. There was a significant increase in the SOD, CAT, GSH and MDA levels in the scopolamine treated groups after 1st week of treatment which persisted after 3 weeks of scopolamine post-treatment. These values were near normalised after 4 weeks of post-treatment indicating a normalised oxidative stress and the action of natural defence mechanism mediating its role (Figure 6).

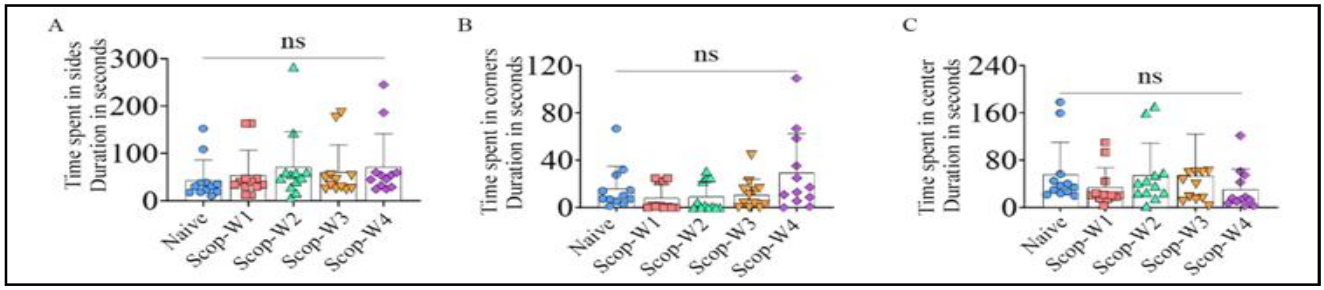

Figure 2: Summarises the results of open field test (OFT) between different groups with 2A: shows the duration spent in the sides; 2B: shows duration in the corners and 2C: showing duration spent in the centre. [The results are expressed as mean values ± SEM, p<0.05 was considered significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001].

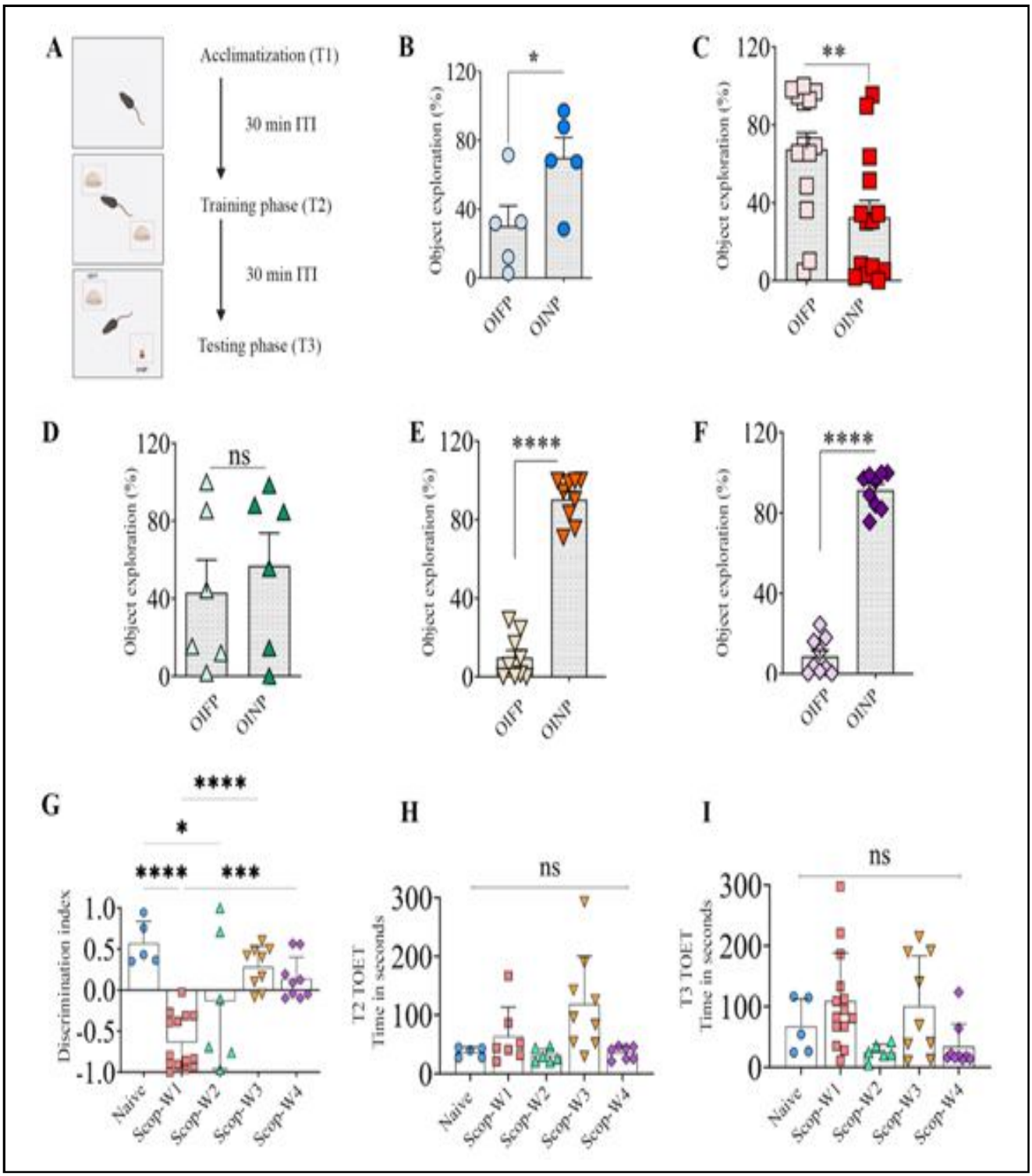

Figure 3: The behavioural procedure performed during the study is summarized in 3A. The behaviour results of NORT showing the within group comparisons between per cent novel object recognition and familiar object recognition time as indicated by 3B: naïve. 3C: 1week after scopolamine treatment. 3D: 2 week after scopolamine treatment. 3E: 3 week after scopolamine treatment. 3F: 4 week after scopolamine treatment. The figure 3G: compares the discrimination index which is a between group comparison to show the novel object recognition ability across the groups. 3H and 3I: The total object exploration times (TOETs) (phase 2 and phase 3). [The results are expressed as mean values ± SEM, $p<0.05$ was considered significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$].

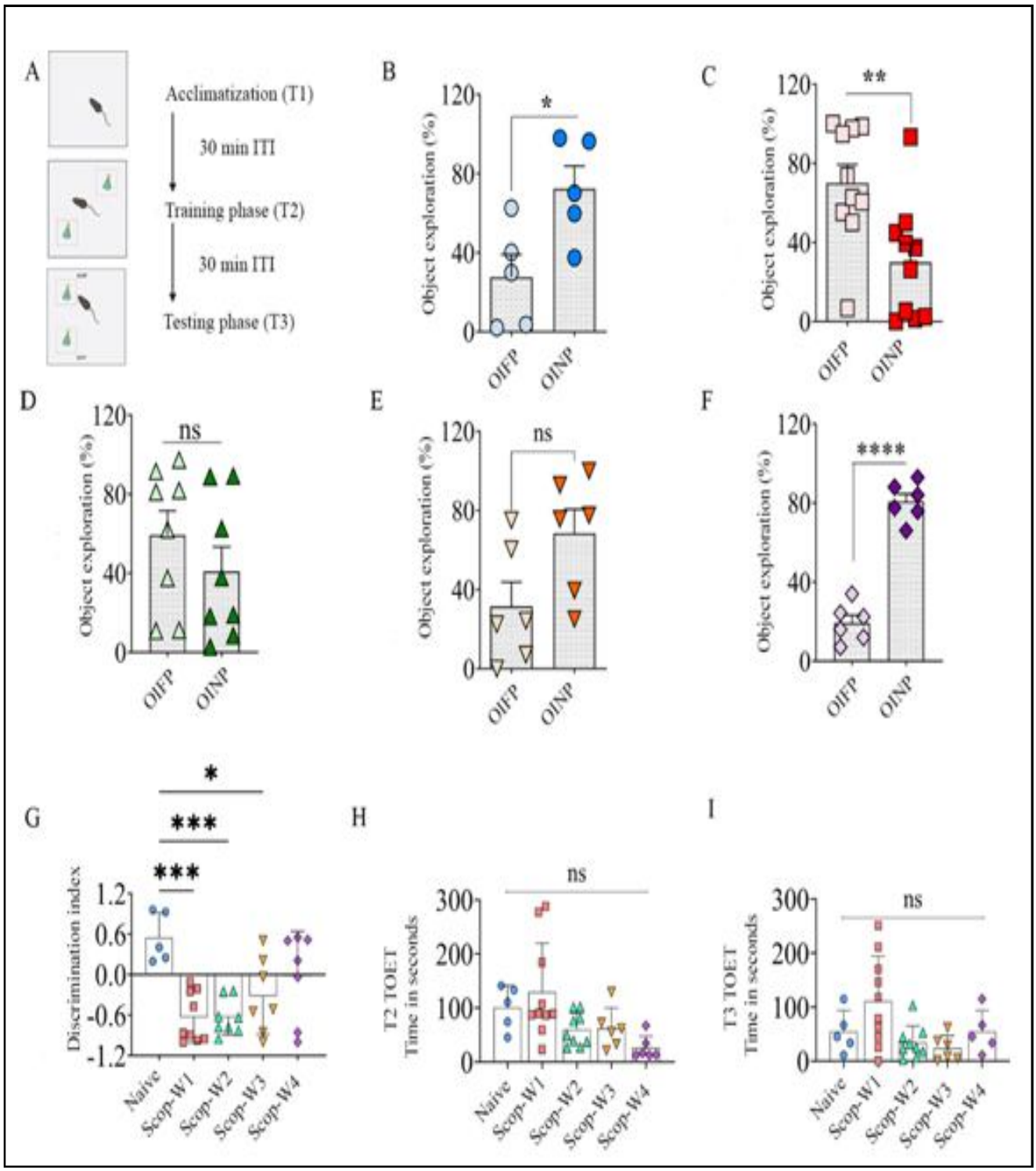

Figure 4: The behavioural procedure performed during the study is summarized in 4A. The behaviour results of object location test (OLT) showing the within group comparisons between per cent novel object location and familiar object location time as indicated by 4B: naive. 4C: 1week after scopolamine treatment. 4D: 2 week after scopolamine treatment. 4E: 3 week after scopolamine treatment. 4F: 4 week after scopolamine treatment. 4G: compares the discrimination index which is a between group comparison to show the novel object location ability across the groups. 4H and 4I: The total object exploration times (TOETs) (phase 2 and phase 3). [The results are expressed as mean values ± SEM, $p<0.05$ was considered significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$].

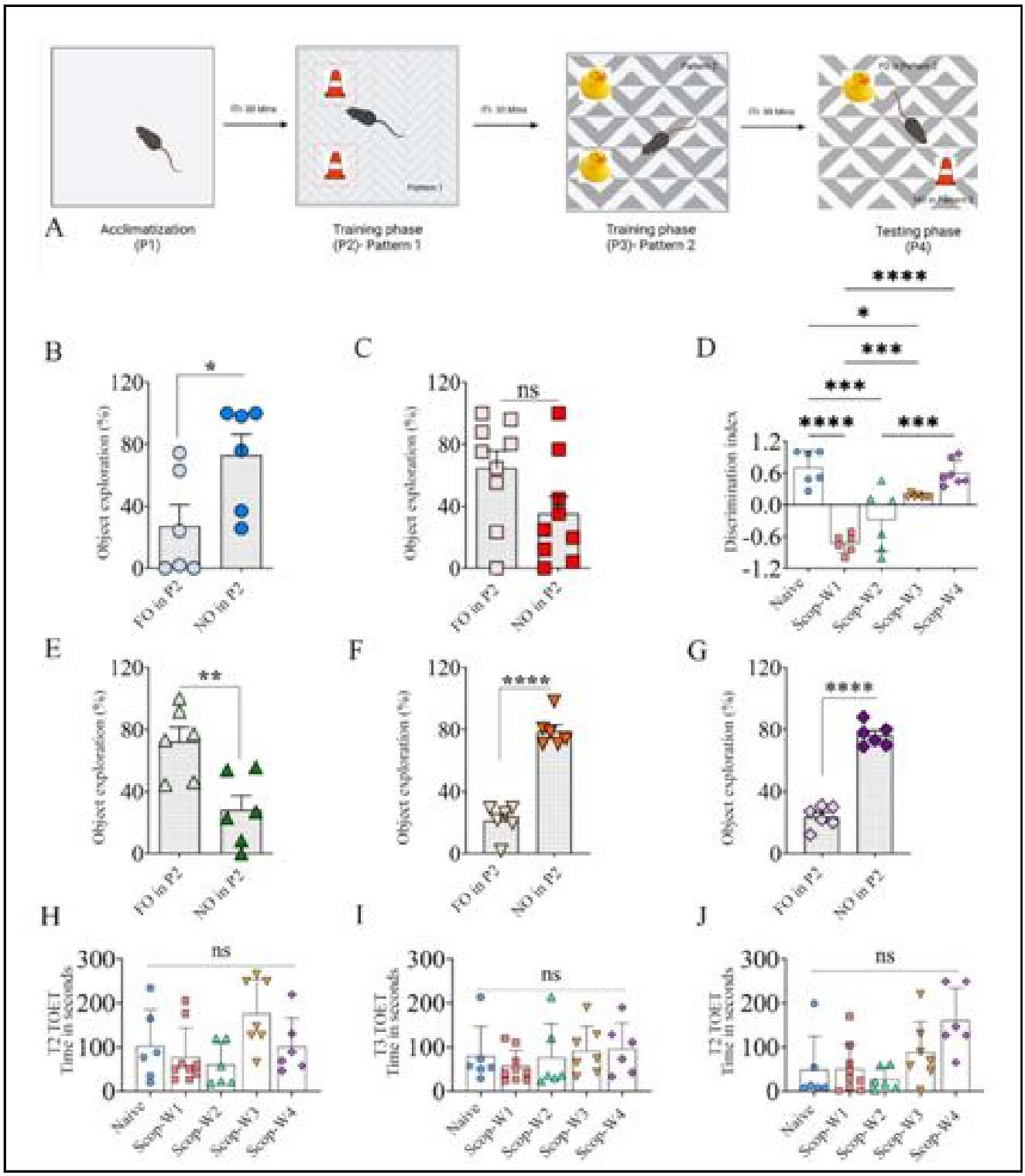

Figure 5: The behavioural procedure performed during the study is summarised in 5A. The behaviour results of pattern separation test (PST) showing the within group comparisons between per cent previously explored object in pattern 2 and recently explored object in pattern 2 as indicated by 5B: naive. 5C: 1week after scopolamine treatment. 5D: 2 week after scopolamine treatment. 5E: 3 week after scopolamine treatment. 5F: 4 week after scopolamine treatment. 5G: compares the discrimination index which is a between group comparison to show the pattern separation function across the groups. 5H, 5I, 5J: The total object exploration times (TOETs) at (phase 2, phase 3 and phase 4). [The results are expressed as mean values ± SEM, $p<0.05$ was considered significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$].

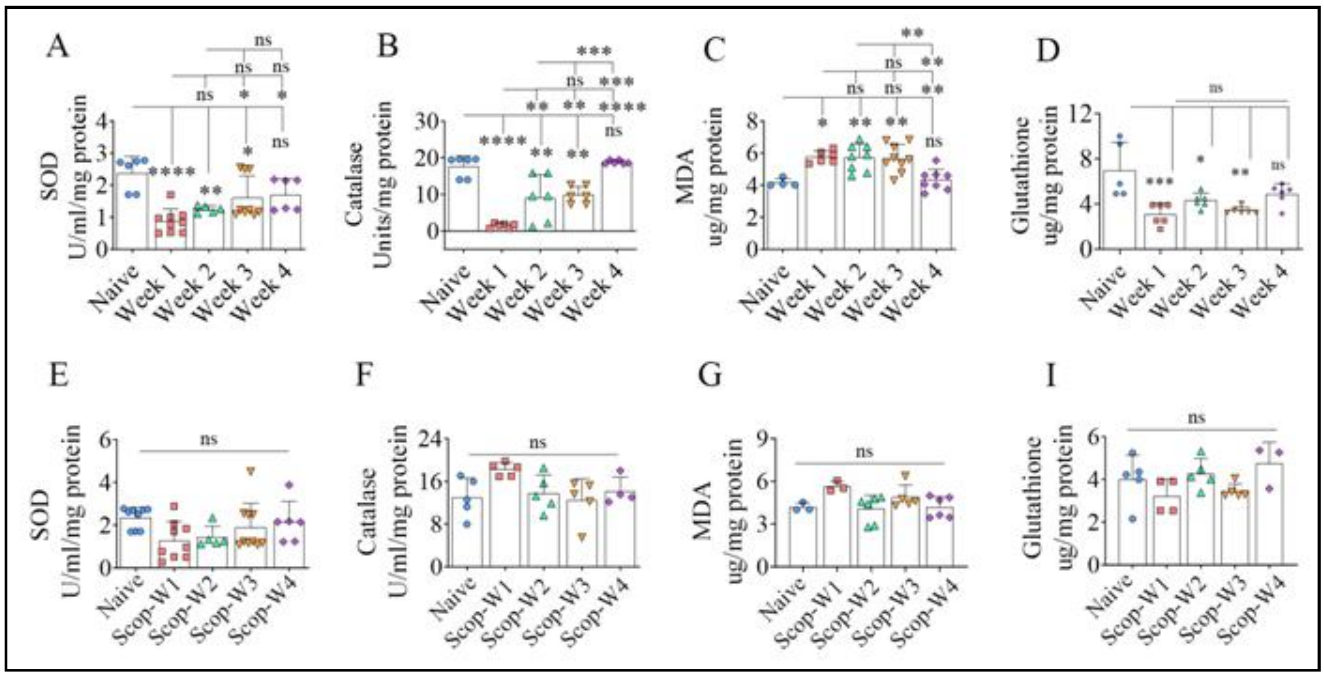

Figure 6: The biochemical changes among the different groups. The results of 5A: superoxide dismutase. 5B: catalase. 5C: malondialdehyde (MDA). 5D: reduced glutathione, 5E: serum superoxide dismutase. 5F: serum catalase. 5G: serum MDA. 5I: serum glutathione. [The results are expressed as mean values ± SD, $p<0.05$ was considered significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$].

4. Discussion

In the present study, we attempted to establish a mild model of cognitive impairment using scopolamine as a mediator of neuroinflammation. This would enable us to understand the minor perturbations in behavioural and biochemical functions. It is important to understand these changes as it would help us in learning about the early stages of dementia which are often ignored. Identifying at the early stages would enable early intervention and also the critical treatment window available to introduce the treatment.

The study was based on numerous studies published earlier which used scopolamine as a mediator of brain oxidative stress when administered intra peritoneally. Many studies used scopolamine and simultaneously introduced their candidate intervention to study immediate changes (Karim *et al*., 2023; Flood and Cherkin, 1986). However, there were lot of inconsistencies with the introduction of scopolamine and simultaneous intervention with any candidate drug compounds. As the exact duration of cognitive impairment varies because of the poor bioavailability of scopolamine in different tissues (Karim *et al*., 2023) and especially in the brain (Flood and Cherkin, 1986). This leads to inaccurate and irreproducible outcomes in terms of behavioural and biochemical measurements.

The present study enables us to understand the exact duration of impairment after consecutive administration of scopolamine. This provides a consistent levels of impairment observed as a certain level of scopolamine maintained in circulation causes changes which could replicate the mild cognitive impairment seen in the early stages of dementia. Further, the biochemical changes provide a clear duration of persistent oxidative stress during this period.

The object based behavioural parameters provide a clear evidence about the hippocampal and cortical regions of the brain. These tests indicate minor perturbations in the behaviour associated with object recognition, location and pattern separation function. There could be temporary inhibition of neurogenesis indicated by reduced pattern separation function. The elevated oxidative stress could also interfere with the synapse formation and transmission of inputs into the memory cells (Kandlur *et al*., 2020).

Scopolamine is a muscarinic antagonist, which induces a blockade in the central cholinergic system that critically impairs the ability of to maintain attention, process information and acquire new knowledge (Jeong *et al.,* 2008). These cognitive deteriorations observed resemble very closely with the memory disturbances in the Alzheimer's disease (AD) in humans. Further, it has also been established that oxidative stress induced tissue alterations in the brain serve as an early event which may gradually lead to the cognitive disturbances and the pathological features seen in AD (Ding *et al.,* 2007). Hence, the present study provides a critical information related to the oxidative changes in conjunction with the behavioural alterations seen after scopolamine administration.

The alterations in the levels of oxidative stress markers in the brain tissue homogenates with no significant changes in the serum indicate a brain specific elevation in the hippocampal/ cortical oxidative stress (Supplementary Figure 2S). Interestingly the transient elevation in these oxidative stress markers normalise in the groups at 4 weeks post-scopolamine treatment (Manju and Pushpa, 2020; Raut and Shaji, 2021). This could be due to the innate antioxidative system balancing the oxidative changes and restoring the antioxidative function in the brain tissue.

Overall, the present study provides an evidence about the degree of cognitive impairment mediated by scopolamine treatment. The critical window of opportunity to introduce an intervention is anywhere between the 1st and the 3rd week post scopolamine treatment as there is a natural reversal of behaviour and biochemical parameters in the 4th week post-treatment.

However, the study has a few limitations. We did not show any histopathological evidence of neuroinflammation in the brain cortex or hippocampus. As this would have been more convincing to establish specific type of brain cell such as neurons or microglia or astrocytes that could have been affected by scopolamine treatment. Further, we did not establish the exact molecular pathway involved. Scopolamine may be involved in inducing certain genes associated directly or indirectly in the modulation of oxidative stress. The present results provide a clear foundation and further scope to validate these changes at histopathological and molecular levels.

5. Conclusion

The study helps us to understand the pharmacological effects of scopolamine in the early phases of cognitive dysfunction. The present study establishes the critical window period available for introducing the drug intervention. Based on the behavioural and the biochemical changes, the week 1 to week 4 post-scopolamine intervention is the critical window available to introduce the drug intervention in this model of scopolamine-induced mild cognitive impairment. This will enable to study the early onset of dementia and introduce a specific intervention of drug treatment.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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