

Neurobehavioural, Biochemical and Histological Effects of Metformin in a Scopolamine-Induced Cognitive Impairment Model in Male Wistar Rats

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Abstract

Alzheimer's disease (AD) causes progressive memory loss and cognitive decline. Current treatments, including donepezil, provide mainly symptomatic relief, prompting interest in drug repurposing. Metformin, an antidiabetic drug, has demonstrated promising neuroprotective effects beyond glycaemic control. The present study assessed the effects of metformin on behavioural, systemic biochemical, and histological alterations in a scopolamine-induced cognitive impairment model in male Wistar rats. Fifty rats weighing 200–240 g were randomly assigned to five groups (n=10). Group A received normal saline and distilled water; Group B received scopolamine (1 mg/kg); Group C received scopolamine (1 mg/kg) and donepezil (3 mg/kg); Groups D and E received scopolamine (1 mg/kg) with metformin at 100 and 200 mg/kg, respectively. Scopolamine was administered intraperitoneally for 7 days, while metformin, donepezil, and vehicle treatments were administered orally for 21 days. Behavioural assessments were conducted on day 28. Scopolamine administration was associated with alterations in behavioural performance, systemic biochemical indices, and neuronal morphology. Metformin treatment was associated with improvements in locomotor activity, spatial working memory, and recognition memory. Serum biochemical analyses demonstrated treatment-related alterations in malondialdehyde, total antioxidant capacity, tumour necrosis factor- α , interleukin-1 β , interleukin-10, and acetylcholinesterase activity relative to the scopolamine group. Histological examination showed preservation of neuronal architecture in the prefrontal cortex and CA1 region of the hippocampus following metformin treatment. Overall, metformin attenuated selected behavioural, systemic biochemical, and histological alterations associated with scopolamine-induced cognitive impairment. These findings support further investigation of metformin in experimental models of cognitive dysfunction.

KEYWORDS: Scopolamine, Metformin, Donepezil, Cognitive Impairment, Neurodegeneration

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory impairment, cognitive decline, and neuronal loss, particularly in the hippocampus and prefrontal cortex. Oxidative stress, cholinergic dysfunction, and neuroinflammation are central to its pathogenesis [1]. Projections indicate that by 2050, over 150 million individuals worldwide will be personally living with AD, an impending crisis made worse by the absence of curative therapies. With an

extensive economic and emotional impact, Alzheimer's disease is no longer merely an illness; it is a worldwide societal issue [2]. Current pharmacological treatments provide only symptomatic relief and do not halt or reverse the underlying disease pathology [3,4]. This emphasizes the urgent need for novel or repurposed agents with neuroprotective potential. One widely used experimental approach to model cognitive impairment is the administration of scopolamine.

Scopolamine is an anticholinergic and antispasmodic medication that has been licensed in over 80 countries to reduce

motion sickness-induced nausea and vomiting, as well as postoperative nausea and vomiting [5]. It functions as a non-selective muscarinic antagonist and competitively inhibits G-protein-coupled postganglionic muscarinic receptors for acetylcholine, resulting in central sedative, antiemetic, and amnestic actions in addition to peripheral antimuscarinic effects [6]. Also, blocking these muscarinic receptors disrupts the normal function of the central cholinergic system, a critical pathway for memory and learning [7]. Scopolamine administration has also been associated with increased oxidative stress, which may contribute to impairments in learning and memory [8]. This disruption of cholinergic neurotransmission is relevant to the cognitive dysfunction, in which cholinergic deficits have been implicated. Therefore, the cholinergic hypothesis can be modelled through the injection of Scopolamine, which induces cognitive deficits.

Increasing attention has been directed toward drug repurposing strategies for the treatment of cognitive impairments as seen in Alzheimer's disease. Metformin, which is a biguanide used in the management of diabetes mellitus, has been demonstrated to have neuroprotective properties [9]. Preclinical research suggests that metformin may improve mitochondrial function, regulate oxidative stress, and modulate inflammatory pathways [10]. However, the effects of metformin in scopolamine-induced cognitive impairment warrant further investigation. Although previous studies have reported beneficial effects of metformin on scopolamine-induced learning and memory impairment, the available evidence has largely focused on behavioural outcomes (using only the Morris water maze and Y-maze test) and selected mechanistic pathways.

The present study sought to extend this literature by providing an integrated assessment of behavioural, systemic biochemical, and histological outcomes. Specifically, the study combined multiple behavioural assessments with systemic (serum) oxidative and inflammatory biomarkers and histological evaluation of the prefrontal cortex and CA1 region of the hippocampus. In addition, two metformin doses were evaluated, with donepezil included as a reference treatment, allowing the effects of metformin to be compared with those of a standard cholinesterase inhibitor. This multidimensional approach provides a broader assessment of the effects of metformin on behavioural performance, systemic biochemical status, and neuronal morphology in a scopolamine-induced cognitive impairment model.

The objectives of this study were to assess the effect of metformin on body weight, food intake, open-field locomotion and exploratory behaviours, spatial working memory in the Y-maze, and recognition memory in rats with scopolamine-induced cognitive impairment. Additionally, the study aimed to investigate the effects of metformin on lipid peroxidation, total antioxidant capacity, inflammatory cytokines, specifically

serum TNF- α , IL-10, and IL-1 β , and acetylcholinesterase activity. Furthermore, the study examined histoarchitectural changes in the prefrontal cortex and CA1 region of the hippocampus using haematoxylin and eosin and Cresyl Fast Violet staining.

2. Materials and Methods

2.1. Chemicals and Drugs

Metformin hydrochloride, Scopolamine hydrobromide, Donepezil hydrochloride, and Normal saline were purchased from Sumther Pharmacy, Ogbomoso, Oyo State, Nigeria. Assay kits for malondialdehyde (MDA), tumour necrosis factor- α , total antioxidant capacity, acetylcholinesterase (AChE), and pro-inflammatory cytokines (TNF- α , IL-1 β) (Biovision Inc., Milpitas, CA, USA).

2.2 Experimental Animals

Fifty healthy Wistar rats used in this study were obtained from Empire Breeders, Osogbo, Osun State, and housed in wooden cages at room temperature ($25^{\circ}\text{C} \pm 2.5^{\circ}\text{C}$) with a 12-hour light cycle. Rats were acclimatised to the experimental conditions for one week and had free access to food and water throughout the experimental period.

All experimental procedures were conducted in accordance with the approved protocols of the Faculty of Clinical Sciences, Ladoke Akintola University of Technology, and in accordance with the European Council Directive 2010/63/EU on the protection of animals used for scientific purposes. At the end of the experiment, animals were euthanised by cervical dislocation performed by trained and experienced personnel, in accordance with the approved animal-care and euthanasia procedures. The study was conducted and reported in accordance with the ARRIVE 2.0 guidelines for reporting animal research.

2.3 Experimental Methodology

Fifty Male Wistar rats, each weighing 200-240 g, were randomly divided into 5 groups of ten ($n=10$) animals each, in accordance with the laboratory's established animal-study protocol. Random allocation was performed by randomly selecting animals and assigning them to the five treatment groups until each group contained 10 animals. Animals were labelled according to their assigned treatment groups and housed together with other animals from the same treatment group in designated cages. Group A (control group) received 4 mL/kg of Normal saline during the 7 days, while they received 5 mL/kg of distilled water during the 21 days of post-treatment; Group B (Scopolamine Control) received scopolamine intraperitoneally at 1mg/kg for 7 days after acclimatization and 5mL/kg of distilled water during the 21 days post-treatment. Group C received scopolamine intraperitoneally at 1mg/kg and was post-treated with Donepezil orally at 3mg/kg for 21days [11]. Group D received scopolamine at 1mg/kg for 7 days and was post-treated with metformin orally at 100mg/kg for 21 days; Group E received scopolamine at

1mg/kg for 7 days and was post-treated with metformin orally at 200mg/kg for 21 days [3, 12]. 4 mL/kg of normal saline was the vehicle used for scopolamine administration in Groups B, C, D and E, which is why Group A received normal saline in the first 7 days. The 5 mL/kg was the vehicle used for the donepezil and metformin administration in Groups C, D, and E, which is why Group A received distilled water. Administration volumes were standardized across groups. At the end of the experimental period, animals were exposed to behavioural paradigms (Open Field, Y-maze, and Novel Object Recognition Test). Twenty-four hours after the behavioural tests, animals were sacrificed by cervical dislocation, and blood samples were collected through an intracardiac puncture for assessment of Interleukin (IL) 10, Interleukin 1B, Tumor Necrosis Factor (TNF), Malonaldehyde (MDA) levels, Acetylcholinesterase, and total antioxidant capacity (TAC). The brain was excised and fixed in 10% neutral buffer formalin-calcium. Sections of the hippocampus were then processed for paraffin embedding and stained for histological analysis.

2.4 Behavioural Testing

2.4.1 Open Field Behaviours

Experimental Animals were exposed to the open field arena for 10 minutes, during which the following behaviours were observed and recorded: horizontal locomotion, rearing, and grooming. The open field test took place in a square enclosure with a rigid floor, measuring 36×36×26 cm. The wood structure was painted white, and the floor was divided into 16 equal squares using permanent blue lines. Each rat was placed near the centre of the enclosure. During 10 minutes, the following behaviours were recorded: total horizontal and vertical movements (line crossing), rearing frequency (number of times the rat stood on its hind legs, either with forelimbs against the enclosure walls or freely in the air), and grooming frequency (number of body-cleaning actions, including use of paws, licking of the body and pubic area, and face washing behaviours indicative of stereotypic activity) [13].

2.4.2 Y-Maze Test (Spatial Working Memory)

The Y-maze test was used to assess spatial working memory. The Y-maze is composed of three equally spaced arms (120 degrees, 41cm long, and 15cm high). The floor of each arm is made of Perspex and is 5cm wide. Spatial working memory is measured by monitoring spontaneous alternation behaviours, specifically the propensity of rodents to alternate between conventionally non-reinforced choices in the Y maze on successive opportunities. Each rat was placed in one of the arm compartments and was allowed to move freely until its tail completely entered another arm. The sequence of arm entries was manually recorded, the arms being labelled A, B, or C. An alternation is defined as entry into all three arms consecutively; for instance, if the animal makes the following arm entries: A, C, B, A, A, C, A, B, C, A, C, B, A, B, C, in this example, the

animal made 15 entries, 4 of which are correct alternations. The maximum number of spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation is calculated as Percentage spontaneous alternation = (number of actual alternations / total arm entries – 2) × 100. For each animal, the test was done for 5 mins. The apparatus was cleaned with 5% alcohol and allowed to dry between sessions [14].

2.4.3 Novel Object Recognition (NOR) Test

The NOR test is highly sensitive to scopolamine-induced cognitive deficits and was used to assess recognition memory. The test was carried out as described by Hashmi *et al.* (2014). Briefly, animals were allowed to acclimatize to the box (40cm × 40cm × 40cm) for 5 mins, followed by a familiarization session and test session (10 mins each, with a 20-min interval between sessions). In the familiarization session, two objects were placed in two opposite corners of the box, 5cm away from the respective corners, and the test animal was allowed to explore the box and interact with both objects. In the test session, one of the objects was replaced with a novel object, and the test animal was allowed to interact with and attempt to recognize it. The time when the animal was physically touching or sniffing the object was considered exploration time [30]. Discrimination scores were also calculated using the following equation:

$$DI = \frac{T_{\text{novel}} - T_{\text{familiar}}}{T_{\text{novel}} + T_{\text{familiar}}}$$

where T_{novel} and T_{familiar} represent the time spent exploring the novel and familiar objects, respectively. Higher discrimination scores indicate higher recognition of novel objects [30].

2.5 Biochemical Test

2.5.1 Lipid Peroxidation

Lipid peroxidation levels were evaluated by measuring malondialdehyde content, assessed as levels of thiobarbituric acid reactive substance in the serum. A combination of free malondialdehyde and thiobarbituric acid reactive substance forms a coloured complex which can be measured spectrophotometrically. The concentration is expressed in μmol , following the kit manufacturer's instructions.

2.5.2 Antioxidant Status

Total antioxidant capacity was assayed using the Trolox Equivalent Antioxidant Capacity Assay, which assesses the ability of antioxidants in a given sample to react with oxidised products, as previously described by [15].

2.5.3 Tumour Necrosis Factor- α , Interleukin (IL) -10, Interleukin (IL)-1 β and Acetylcholinesterase

Serum concentrations of interleukin-1 β (IL-1 β), interleukin-10 (IL-10), and tumour necrosis factor-alpha (TNF- α), and acetylcholinesterase were determined using commercial assay kits in accordance with the manufacturers' instructions.

2.6 Tissue Histology

Rat brains were dissected and fixed in neutral buffered formalin-calcium. The prefrontal cortex and CA1 region of the hippocampus were processed for paraffin embedding. Tissue sections were cut at a thickness of 5 μ m and stained with haematoxylin and eosin (H&E) and Cresyl Fast Violet for histological assessment. Two animals from each experimental group were selected for histological assessment, with two consecutive sections, each 5 μ m thick, examined per animal. The prefrontal cortex and CA1 region were assessed qualitatively for general tissue architecture, cellular organisation, neuronal morphology, and Nissl staining characteristics. Particular attention was given to the apparent morphology and organisation of pyramidal cells, granule cells, neuroglial cells, and Nissl substance. Histological assessment was performed under blinded conditions with respect to treatment identity; laboratory personnel involved in tissue processing and assessment were provided with group designations but were not informed of the specific treatments administered to the groups. No quantitative neuronal counts or histomorphometric measurements were performed.

2.7 Photomicrography

Histological sections of the prefrontal cortex and CA1 region of the hippocampus were examined using an Olympus binocular light microscope at $\times 400$ magnification. Images were captured using a Canon PowerShot 2500 digital camera. Representative photomicrographs were selected to illustrate the general histological appearance and key cellular features observed in the respective brain regions. The images were selected to visually demonstrate the predominant morphological features observed during the qualitative assessment and were not used for quantitative image analysis.

2.8 Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA) to assess differences among the experimental groups. For analyses yielding a significant overall group effect, Tukey's honestly significant difference (HSD) post-hoc test was used for multiple pairwise comparisons. Data were expressed as mean $\pm$ standard error of the mean (SEM). The assumptions of were assessed before performing ANOVA. Statistical significance was set at $p < 0.05$. For each outcome, the F-statistic, degrees of freedom, exact p -value, and effect size (η^2) were reported where applicable. Ninety-five percent confidence intervals (95% CIs) were reported where appropriate.

However, weekly body weight and feed intake were analysed using two-way repeated-measures analysis of variance (ANOVA), with treatment group as the between-subject factor and week as the within-subject factor. The main effects of treatment and week, as well as the treatment $\times$ week interaction, were assessed.

3 Result

3.1 Effect of Metformin on Body Weight

Figure 1a shows the effect of Metformin on the relative change in body weight in scopolamine-induced rats. One-way ANOVA showed a significant effect of treatment on body weight, $F(4,45) = 27.30$, $p < 0.000001$, $\eta^2 = 0.708$. There was no significant decrease ($p < 0.05$) in weight gain across the SCOP and SCOP/DON groups, while there was a significant decrease in weight with SCOP/MET100 ($p < 0.001$) and SCOP/MET200 ($p < 0.001$) compared with the control group. Compared with the scopolamine group, the SCOP/MET100 ($p < 0.0104$) and SCOP/MET200 ($p < 0.0001$) groups showed a significant decrease in body weight.

Figure 1b shows the effect of Metformin on the weekly change in body weight in scopolamine-induced rats. There was no significant decrease in weight gain across the SCOP and SCOP/DON groups, while there was a significant decrease in the weight of the groups SCOP/MET100 and SCOP/MET200 compared with the control group. Compared with the scopolamine group, the SCOP/MET100 and SCOP/MET200 groups showed a significant decrease in body weight.

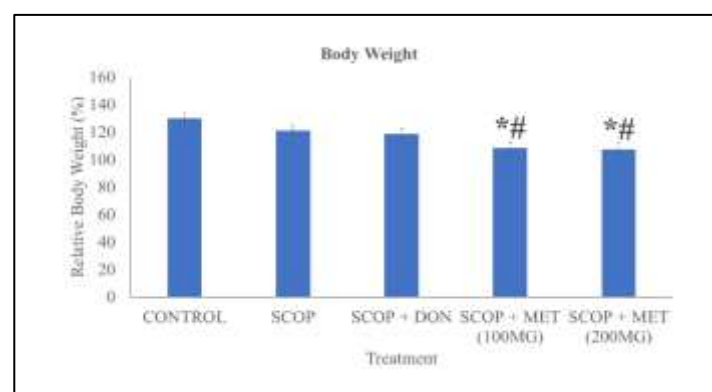


Figure 1a Effect of Metformin on the relative change in body weight in scopolamine-induced rats. Each bar represents Mean $\pm$ S.E.M. * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. Number of rats per treatment group = 10. SCOP: Scopolamine. Tukey's HSD post-hoc test showed that the SCOP+MET100 mg/kg and SCOP+MET200 mg/kg groups differed significantly from the control and scopolamine groups.

3.2 Effect of Metformin on feed intake in Scopolamine-induced rats

Figure 2a shows the effect of Metformin on relative feed intake in scopolamine-induced rats. One-way ANOVA showed a significant effect of treatment on feed intake, $F(4,45) = 52.0$, $p < 0.001$, $\eta^2 = 0.822$. There was no significant decrease in feed intake in the SCOP group compared with the control group, while the SCOP/MET100 and SCOP/MET200 showed a significant decrease ($p < 0.05$) in feed intake. Compared with the scopolamine group, the groups administered SCOP/MET100 ($p < 0.0018$) and SCOP/MET200 ($p < 0.05$) showed no significant decrease in feed intake.

Figure 2b shows the effect of Metformin on weekly feed intake in scopolamine-induced rats. There was no significant decrease in feed intake in the SCOP group compared with the control group ($p < 0.05$), while the SCOP/MET100mg/kg and SCOP/MET200mg/kg showed a significant decrease in feed intake. Compared with the scopolamine group, the groups administered SCOP/MET100mg/kg and SCOP/MET200mg/kg showed no significant decrease in feed intake.

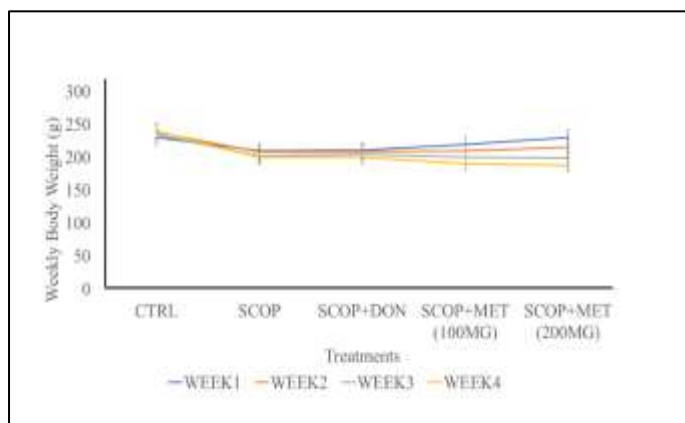


Figure 1b. Effect of Metformin on the weekly change in body weight in scopolamine-induced rats. Data are presented as mean $\pm$ SEM ($n = 10$ per group). Body weight was assessed weekly over the experimental period. Statistical significance was set at $p < 0.05$.

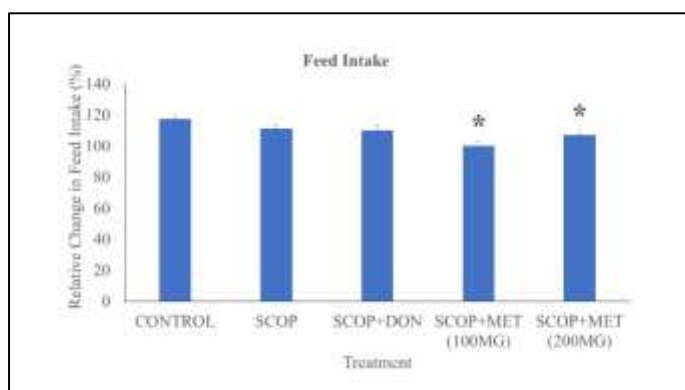


Figure 2a Effect of Metformin on relative change in feed intake. Each bar represents Mean $\pm$ S.E.M. * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. Number of rats per treatment group = 10. SCOP: Scopolamine. Tukey's HSD post-hoc test showed that the SCOP+MET100 mg/kg and SCOP+MET200 mg/kg groups differed significantly from the control group.

3.3 Effect of Metformin on horizontal locomotor activity in Scopolamine-induced rats

Figure 3 shows the effect of Scopolamine on horizontal locomotor activity measured as the number of line crossings during 10 minutes. One-way ANOVA showed a significant effect of treatment on line crossing, $F(4,45) = 35.6$, $p < 0.001$, $\eta^2 = 0.760$. There was a significant decrease in locomotor activity with SCOP/DON ($p < 0.0001$), SCOP/MET100

($p < 0.0001$), and SCOP/MET200 ($p < 0.0002$), and a significant ($p < 0.001$) decrease with SCOP compared to control. In comparison to SCOP, horizontal locomotor activity increased with SCOP/DON ($p < 0.0012$), SCOP/MET100 ($p < 0.0041$), and SCOP/MET200 ($p < 0.0001$). Compared with the SCOP/DON group, the SCOP/MET200MG group showed a significant ($p < 0.0183$) increase.

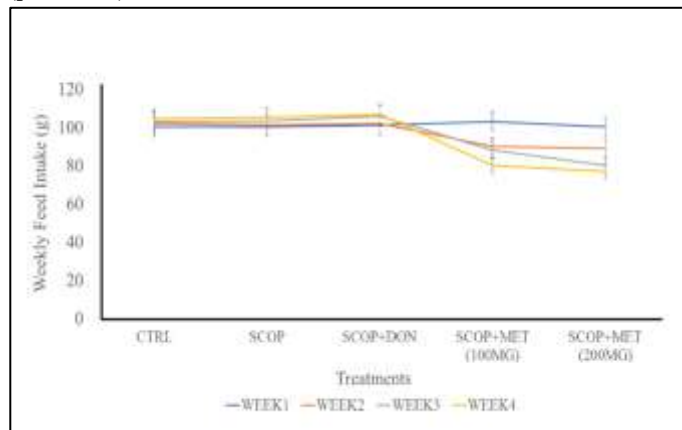


Figure 2b Effect of Metformin on weekly change in feed intake. Data are presented as mean $\pm$ SEM ($n = 10$ per group). Body weight was assessed weekly over the experimental period. Statistical significance was set at $p < 0.05$.

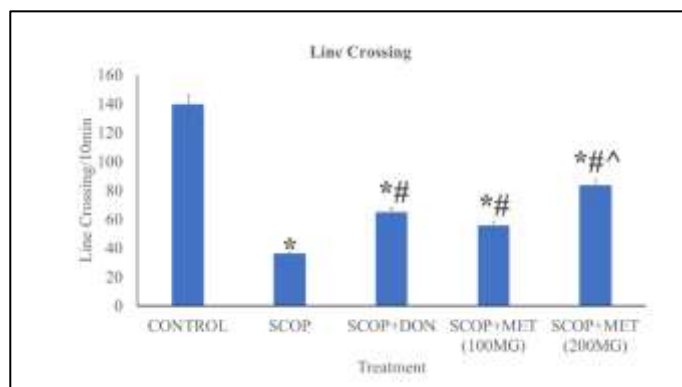


Figure 3 Effect of Metformin on Horizontal Locomotor Activity (Line Crossing). Each bar represents Mean $\pm$ S.E.M. * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. ^ $p < 0.05$ significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

3.4 Effect of Metformin on Rearing Activity

Figure 4 shows the effect of Scopolamine on rearing activity. One-way ANOVA showed a significant effect of treatment on rearing, $F(4,45) = 22.2$, $p < 0.001$, $\eta^2 = 0.664$. There was a significant decrease in vertical locomotor activity (Rearing) with SCOP/DON, SCOP/MET100, and SCOP/MET200, and a significant ($p < 0.0001$) decrease with SCOP compared to control. In comparison to SCOP, vertical locomotor activity increased with SCOP/DON, SCOP/MET100, and SCOP/MET200. Compared with the SCOP/DON group, the SCOP/MET100 ($p < 0.001$) group showed a significant decrease.

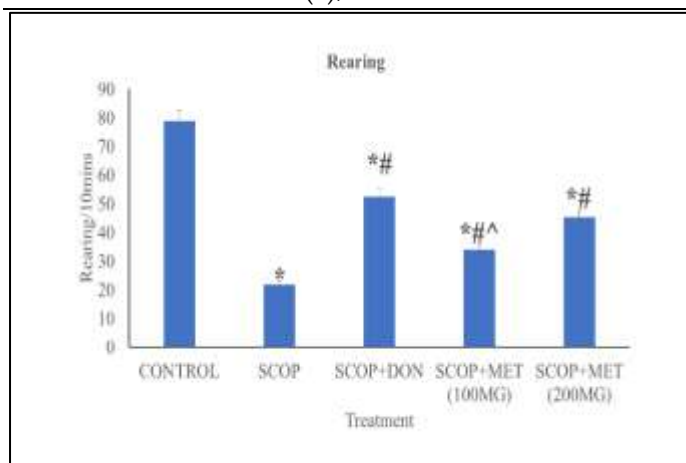


Figure 4: Effect of Metformin on Rearing Activity. Each bar represents Mean $\pm$ S.E.M, * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. ^ $p < 0.05$ significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

3.5 Effect of Metformin on Self-Grooming Activity

Figure 5 shows the effect of Metformin on grooming behaviour. One-way ANOVA showed a significant effect of treatment on grooming, $F(4,45) = 5.05$, $p < 0.001$, $\eta^2 = 0.310$. There was a significant decrease in self-grooming activity with SCOP/DON ($p = 0.0018$), SCOP/MET100 ($p = 0.0249$), and SCOP/MET200 ($p = 0.03$), when compared with the SCOP group. There was a significant increase in self-grooming activity in the SCOP group when compared with the control. Compared with the SCOP/DON group, the SCOP/MET200MG group showed a significant increase.

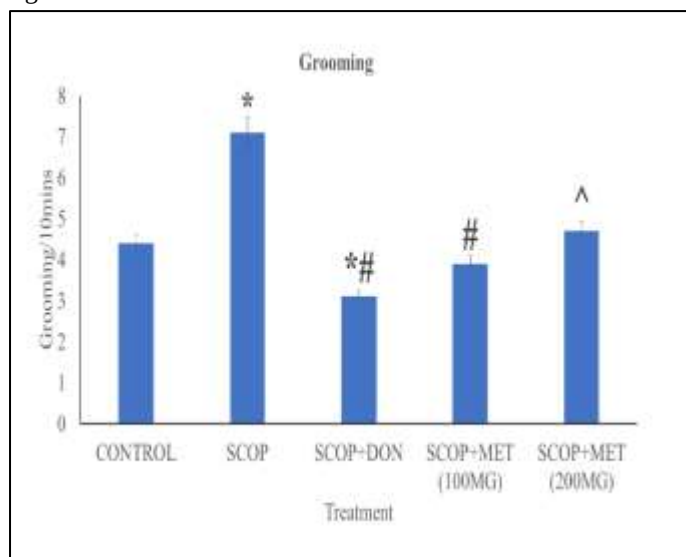


Figure 5. Effect of Metformin on Self-grooming Activity in Scopolamine-induced rats. Each bar represents Mean $\pm$ S.E.M, * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. ^ $p < 0.05$ significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

3.6 Effect of Metformin on Spontaneous Alternation in the Y-Maze

Figure 6 shows the effect of Metformin on spontaneous alternation in the Y-Maze. One-way ANOVA showed a significant effect of treatment on spontaneous alternation, $F(4,45) = 4.75$, $p < 0.001$, $\eta^2 = 0.297$. There was a significant decrease in spontaneous alternation in the SCOP ($p < 0.0002$), SCOP/DON ($p < 0.0160$), and SCOP/MET100MG ($p < 0.0222$), groups compared to the control. In comparison to SCOP and SCOP/DON, spontaneous alternation increased significantly in the SCOP/MET200MG ($p = 0.0016$) group.

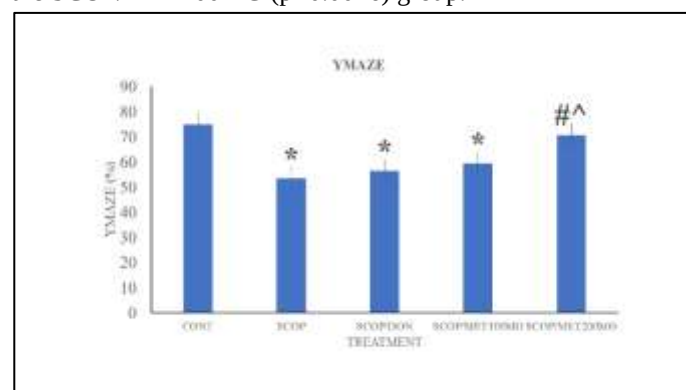


Figure 6 Effect of Metformin on Spontaneous Alternation in Scopolamine-induced rats. Each bar represents Mean $\pm$ S.E.M, * $p < 0.05$ significant difference from control. # $p < 0.05$ significant difference from SCOP. ^ $p < 0.05$ significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

3.7 Effect of Metformin on Novel Object Exploration Time

Figure 7 shows the effect of metformin on novel object exploration time. One-way ANOVA showed a significant effect of treatment on novel object exploration time, $F(4,45) = 12.1$, $p < 0.001$, $\eta^2 = 0.519$. There was no significant decrease in novel object exploration time across the SCOP/DON and SCOP/MET100MG groups, whereas there was a significant decrease in the SCOP group and a significant increase in the SCOP/MET200 ($p = 0.0103$) group when compared to the control. Compared to SCOP, novel object exploration time increased ($p = 0.0001$) across all groups. Compared with the SCOP/DON, there was a significant decrease in the time spent on familiar object exploration time in SCOP/MET100 ($p < 0.0001$) and SCOP/MET200 ($p = 0.0306$).

3.8 Effect of Metformin on Novel Object Discrimination Ratio

Figure 3.8 shows the effect of metformin on novel object discrimination ratio. One-way ANOVA showed a significant effect of treatment on novel object exploration time, $F(4,45) = 47.8$, $p < 0.001$, $\eta^2 = 0.810$. There was a significant increase ($p = 0.0001$) in the novel object discrimination ratio in the groups treated with SCOP/DON, SCOP/MET100,

SCOP/MET200, and a significant decrease with SCOP when compared with the control group.

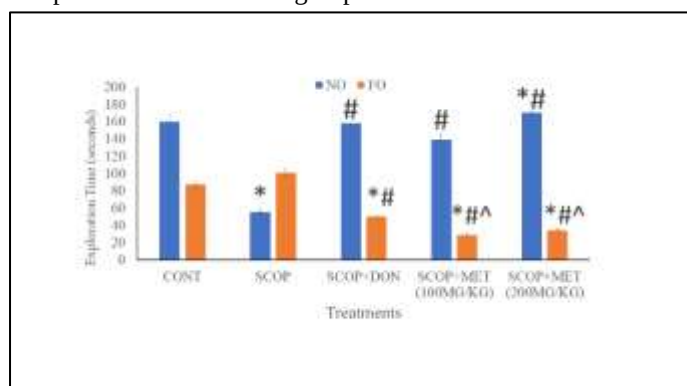


Figure 7. Effect of Metformin on Novel Object Exploration Time. Each bar represents Mean $\pm$ S.E.M. * $p<0.05$ significant difference from control. # $p<0.05$ significant difference from SCOP. ^ $p<0.05$ significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

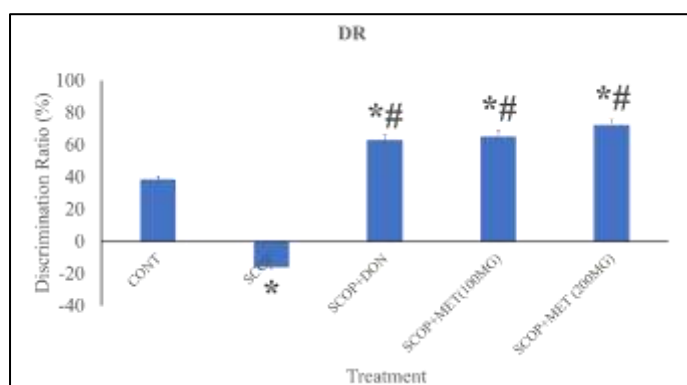


Figure 8. Effect of Metformin on Novel Object Discrimination Ratio. Each bar represents mean $\pm$ S.E.M. * $p<0.05$ indicates a significant difference from control. # $p<0.05$ indicates a significant difference from SCOP. Number of rats per treatment group = 10. SCOP: Scopolamine.

3.9 Effect of Metformin on the oxidative stress markers, inflammatory cytokines, and acetylcholinesterase activity

Table 1 shows the effects of metformin on Malonaldehyde (MDA), Total Antioxidant Capacity (TAC), Tumour Necrosis Factor (TNF- α), Interleukin-10, Interleukin-1 β , and Acetylcholinesterase activity. There was a significant [F (4, 25) = 5.98, $\eta^2 = 0.590$, $p=0.0016$] increase in Malonaldehyde (MDA) in the SCOP group as compared to the control group. Compared to the SCOP group, there was a significant decrease in groups administered SCOP/DON ($p=0.0441$), SCOP/MET100 ($p=0.0321$), and SCOP/MET200 ($p=0.041$).

Total Antioxidant Capacity (TAC) decreased significantly [F(4,25)=5.82, $\eta^2 = 0.482$, $p=0.0001$] in the SCOP and SCOP/MET100 groups compared to the control. Compared to SCOP, there was a significant increase ($p=0.0001$) in TAC in the

group administered SCOP/DON, SCOP/MET100, and SCOP/MET200. When compared with the SCOP+DON group, there was a significant reduction ($p=0.0001$) in TAC in the group administered SCOP/MET 100.

Tumour Necrosis Factor- α (TNF- α) increased significantly [F (4, 25) = 4.69, $\eta^2 = 0.429$, $p=0.005$] in the SCOP, SCOP/MET100 and SCOP/MET200 groups compared to the control. Compared to the SCOP, TNF- α levels showed a significant decrease ($p=0.0001$) in the SCOP/MET100 and SCOP/MET200 groups. Interleukin-10 levels decreased significantly [F(4,25)=5.68, $\eta^2 = 0.990$, $p=0.0001$] in the SCOP, SCOP/DON, SCOP/MET100, and SCOP/MET200 groups compared to control. Compared to SCOP, there was a significant increase ($p=0.0001$) with SCOP/DON, SCOP/MET100, and SCOP/MET200. Compared to the SCOP/DON group, there was a significant increase in IL-10 ($p=0.0001$) in the SCOP/MET100 group and a significant reduction in the SCOP/MET200 group.

Interleukin-1 β increased significantly [F(4,25)=10.7, $\eta^2 = 0.63$, $p=0.0001$] with SCOP, SCOP/DON, SCOP/MET100, and SCOP/MET200, compared to the control group.

Acetylcholinesterase increased significantly with SCOP, SCOP+DON, SCOP+MET100, and SCOP/MET200, compared to control. Compared to SCOP, there was a significant decrease ($p=0.0001$) in the groups administered SCOP+DON, SCOP+MET100, and SCOP/MET200. Relative to the SCOP/DON group, there was a significant increase in the SCOP/MET100 ($p=0.0108$) and SCOP/MET200 ($p=0.008$) groups.

3.10Effect of Metformin on the prefrontal cortex histomorphology

Figures 9A–E and 10A–E show representative haematoxylin and eosin (H&E) and Cresyl Fast Violet-stained sections of the prefrontal cortex, respectively. In the control group (Fig. 9A), H&E staining showed distinct granule cells (GC), numerous well-distributed multipolar pyramidal cells (PC), and neuroglial cells (NG). Cresyl Fast Violet staining of the control group (Fig. 10A) showed well-distributed multipolar pyramidal cells (PC), deeply stained granule cells (GC), neuroglial cells (NG), and prominent Nissl staining (N).

In the scopolamine group (Figs. 9B and 10B), apparent alterations in neuronal morphology, including karyolysis, vacuolation, pale-staining nuclei, and reduced Nissl staining, were observed. In contrast, the metformin-treated groups (Figs. 9D–E and 10D–E) showed comparatively preserved neuronal morphology and Nissl staining relative to the scopolamine group.

3.11Effect of Metformin on the CA1 of the hippocampus histomorphology

Figure 11 (A-E) and Fig 12 (A-E) show slides of the haematoxylin and eosin and cresyl fast violet-stained sections of

the rats' CA1 of the hippocampus. The H&E-stained section of Figure 11A revealed distinct layers of the CA1, with numerous pyramidal neurons (PNs) and neuroglial (NG) cells. In the Cresyl Fast Violet stain, a well-defined pyramidal neuron (PN), neuroglial cells (NG), and noticeable Nissl bodies (N) were observed.

In the scopolamine group (Figure 11B and 12B), apparent chromatolytic changes in pyramidal neurons, alterations in cellular organisation, and reduced Nissl substance were observed. The metformin-treated groups (Figure 11D and 11E) showed comparatively preserved pyramidal neuronal morphology and Nissl substance relative to the scopolamine group.

Table 1: showing the effect of Metformin on Biochemical Assays

GROUPS	MDA (μM)	TAC (mM TE)	TNF (pg/ml)	IL-10 (pg/ml)	IL-1β (pg/ml)	AchE (mM)
Normal Control	0.69± 0.01	11.98± 1.35	20.19± 1.45	6.65± 0.03	4.57± 0.5	0.45± 0.01
SCOP	0.94± 0.11*	2.4± 0.62*	35.28± 1.6*	1.03± 0.28*	27.33± 3.55*	1.36± 0.01*
SCOP+DON	0.69± 0.02#	12.35± 0.94#	27.97± 4.96	2.28± 0.09*#	20.49± 2.09*	0.87± 0.04*#
SCOP+MET100	0.67± 0.03#	8.84± 0.26*#^	32.06± 0.61*#	3.94± 0.01*#^	26.24±4 .16*	1.21± 0.11*#^
SCOP+MET200	0.65± 0.01#	13.67± 0.44#	27.17± 2.8*#	1.67± 0.05*#^	27.08± 3.07*	1.17± 0.08*#^

Data represented as Mean ± S.E.M, *p<0.05 significant difference from control, #p<0.05 significant difference from SCOP, ^p<0.05 significant difference from SCOP/DON. Number of rats per treatment group = 10. SCOP: Scopolamine.

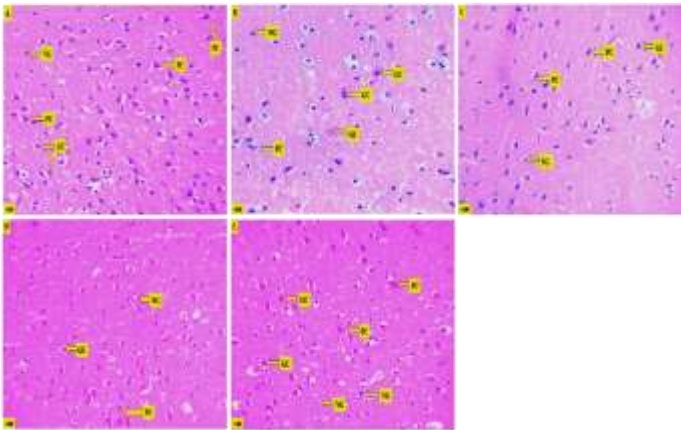


Figure 9: Photomicrographs of H&E-stained sections of the prefrontal cortex of rats. Stained sections of the prefrontal cortex showed distinct granule cells (GC), with numerous pyramidal cells (PC) and neuroglial cells (NG) in the control group (Fig. 9A). The scopolamine group (Fig. 9B) showed apparent karyolysis and pale staining of pyramidal and granule cells. The donepezil-treated group (Fig. 9C) showed some preservation of pyramidal and granule cell morphology, although alterations remained apparent. In contrast, the

metformin-treated groups (Figs. 9D and 9E) showed comparatively preserved pyramidal and granule cell morphology relative to the scopolamine group. Mag × 400.

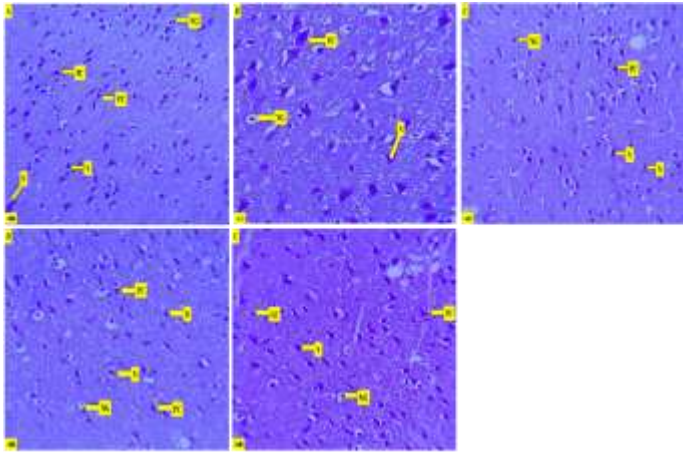


Figure 10: Photomicrographs of Cresyl Fast Violet-stained sections of prefrontal cortex of rats. The staining revealed well-distributed multipolar pyramidal cells (PC), deeply stained granule cells (GC), neuroglial (NG), and noticeable Nissl bodies (N) in Fig 10A. Fig 10B exhibited vacuolations, pale-staining nuclei, and Nissl bodies, whereas Fig 10C, 10D, and 10E showed greater retention of Nissl bodies and protection against neuronal damage. Mag. ×400

Discussion

This study evaluated the effects of metformin on neurobehavioural performance, biochemical markers, and histomorphological features in a scopolamine-induced cognitive impairment model. A donepezil-treated group was included as a positive control and reference treatment. Overall, metformin treatment was associated with improvements in several behavioural and biochemical parameters altered by scopolamine, alongside apparent preservation of neuronal morphology in the prefrontal cortex and CA1 region of the hippocampus.

Scopolamine did not significantly reduce body weight or feed intake. However, metformin treatment resulted in significant reductions in body weight in the SCOP+MET100 mg/kg and SCOP+MET200 mg/kg groups. This observation may be related to the metabolic effects of metformin, which have been associated with increased fatty acid oxidation and reduced lipogenesis, thereby contributing to weight regulation [17,18]. A reduction in feed intake was also observed in the metformin-treated groups. Metformin has been reported to influence metabolic pathways involved in energy balance and appetite regulation [31]. Compared with the SCOP+DON group, no significant differences in body weight or feed intake were observed. These findings suggest that the metabolic effects observed in the present study were more evident following metformin treatment.

Administration of scopolamine was associated with alterations in behavioural performance, antioxidant status and inflammatory markers, characterised by reduced locomotor and exploratory activities, impaired spatial working and recognition

memory, increased serum MDA and TNF- α levels, and reduced TAC and IL-10. Metformin attenuated several of these alterations, with improvements observed in behavioural performance, antioxidant status and inflammatory markers. These findings are consistent with the reported antioxidant and anti-inflammatory properties of metformin [10,26].

Serum AChE activity was also altered following scopolamine administration. Treatment with donepezil reduced AChE activity, consistent with its established cholinesterase-inhibitory action [20]. Metformin similarly reduced serum AChE activity compared with the scopolamine group, although the reduction was less pronounced than that observed with donepezil. The improvement in AChE activity following metformin treatment was accompanied by improvements in behavioural performance, particularly at the 200 mg/kg dose. This pattern may suggest an association between improved cholinergic status and the behavioural effects observed following metformin treatment. Previous studies have also suggested that metformin may influence cholinergic signalling and cognitive function [19,26]. The oxidative stress findings further demonstrated beneficial effects of metformin. Scopolamine administration was associated with increased MDA and reduced TAC, indicating an altered systemic oxidative balance. Metformin treatment attenuated these changes, with the 200 mg/kg dose showing a more favourable antioxidant profile. The reduction in MDA alongside improved TAC suggests that metformin may help restore the balance between oxidative damage and antioxidant capacity. These findings are consistent with previous reports describing the ability of metformin to modulate oxidative stress and improve cellular antioxidant defence [10].

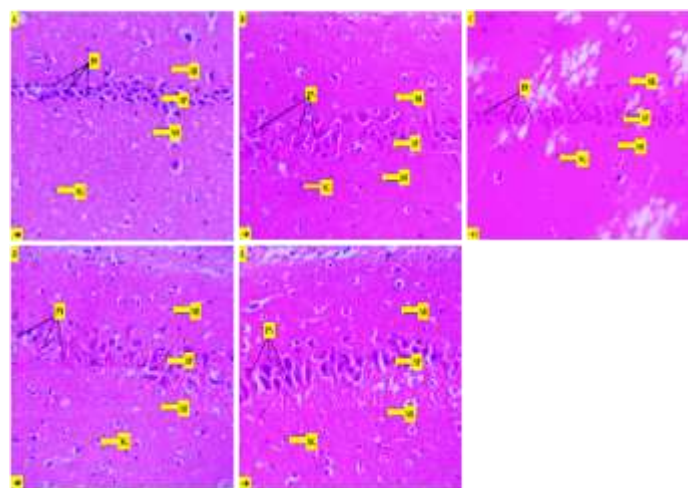


Fig. 11: Photomicrographs of H&E-stained sections of the Cornu Ammonis 1 (CA1) of the hippocampus Cresyl Fast Violet-stained sections of the CA1 region showed distinct cellular organisation, with numerous pyramidal neurons (PNs) and neuroglial (NG) cells in the control group (Fig. 11A). In the scopolamine group (Fig. 11B), apparent alterations in pyramidal neuronal morphology, characterised by pale staining, were observed. The donepezil- and metformin-treated groups (Fig. 11C–E) exhibited relatively preserved neuronal morphology

compared with the scopolamine group. SR: Stratum Radiata, SP: Stratum Pyramidalis, SO: Stratum Oriens Mag $\times$ 400

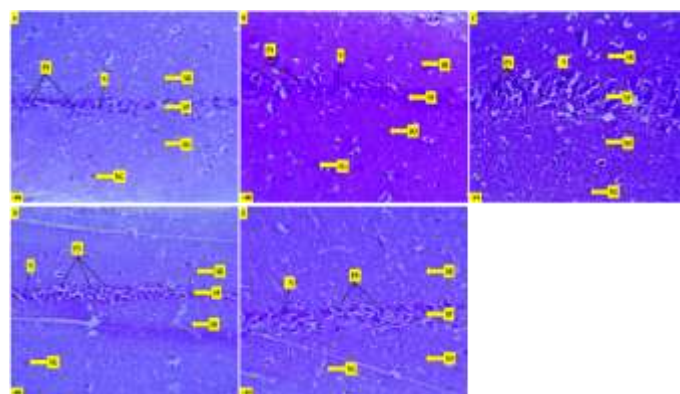


Figure 12: Photomicrographs of Cresyl Fast Violet-stained sections of the Cornu Ammonis 1 (CA1) of the hippocampus. Cresyl Fast Violet staining of the CA1 region showed well-defined pyramidal neurons (PNs), neuroglial cells (NG), and prominent Nissl substance (N) in the control group (Fig. 12A). In the scopolamine group (Fig. 12B), apparent alterations in pyramidal neuronal morphology, including reduced Nissl staining and cellular disorganisation, were observed. In contrast, the donepezil and metformin-treated groups (Fig. 12C–E) showed comparatively preserved pyramidal neuronal morphology and Nissl staining relative to the scopolamine group. SR: Stratum Radiata, SP: Stratum Pyramidalis, SO: Stratum Oriens Mag $\times$ 400.

The behavioural findings demonstrated that scopolamine altered locomotor activity, exploratory behaviour and spatial working memory. Reduced locomotion and rearing, together with increased grooming, were observed following scopolamine administration. These behavioural changes are consistent with the effects associated with scopolamine-induced cholinergic dysfunction [21]. Donepezil improved several of these behavioural parameters, supporting its established cognitive-enhancing effects [22]. Metformin treatment also modified behavioural performance, particularly at 200 mg/kg, where line crossing and Y-maze performance were increased relative to the scopolamine group. Although the increased Y-maze performance may indicate improved spatial working memory following the higher metformin dose, this interpretation was considered alongside the observed changes in locomotor activity. Locomotor activity can influence performance in exploration-based behavioural paradigms; reduced movement may limit arm entries and exploration, whereas increased locomotion may facilitate exploration and exposure to task stimuli independent of cognitive ability. Therefore, the improvement in Y-maze performance observed with metformin, particularly at 200 mg/kg, may reflect a combination of improved cognitive performance and locomotor/exploratory activity. The lower rearing activity observed with the 100 mg/kg dose compared with donepezil further suggests that the behavioural effects of metformin may vary according to dose.

Recognition memory was similarly affected by scopolamine, as demonstrated by alterations in novel-object exploration and a reduced discrimination ratio. This finding is consistent with the role of cholinergic signalling in attention, learning and memory [24]. Donepezil improved the discrimination ratio, in agreement with previous reports of its beneficial effects in scopolamine-induced memory impairment [25]. Metformin also significantly improved the discrimination ratio compared with the scopolamine group, suggesting improved recognition memory. However, because novel-object recognition involves exploratory behaviour, differences in locomotor activity may contribute to differences in object exploration and, consequently, discrimination performance. Thus, the improved discrimination ratio following metformin treatment may reflect enhanced recognition memory. This consideration is particularly relevant to the 200 mg/kg group, which also exhibited increased locomotor activity. Accordingly, the behavioural findings are consistent with an improvement in cognitive performance following metformin treatment. The observed behavioural effects may be compatible with the changes in oxidative status, inflammatory markers and acetylcholinesterase activity observed following metformin treatment [10,26]; however, the present study does not establish a causal relationship between these biochemical changes and the behavioural outcomes.

Scopolamine treatment increased serum TNF- α and IL-1 β levels, while metformin reduced these pro-inflammatory cytokines. Conversely, IL-10, an anti-inflammatory cytokine, was reduced following scopolamine administration and increased following metformin treatment. These findings indicate that metformin influenced the systemic inflammatory profile in the present model. The reduction in TNF- α and IL-1 β together with increased IL-10 may reflect a shift towards a more balanced inflammatory state following metformin treatment. Previous studies have reported anti-inflammatory effects of metformin and its ability to regulate inflammatory signalling [27,28]. Interestingly, the response was greater at 100 mg/kg than at 200 mg/kg, indicating that the effect on IL-10 was not strictly dose-related. This finding may reflect differences in dose-related pharmacodynamic responses to metformin. Alternatively, biological variability may have contributed to the observed difference between doses. The possibility that lower-dose metformin may promote a greater anti-inflammatory response is therefore a hypothesis that warrants further investigation rather than a demonstrated mechanism. [32].

The histological findings provided additional morphological evidence of changes associated with scopolamine administration and their apparent attenuation following treatment. Cresyl Fast Violet-stained sections of the prefrontal cortex and CA1 region showed apparent alterations in neuronal morphology and Nissl staining in the scopolamine group, whereas the metformin-treated groups showed comparatively preserved neuronal

architecture. These observations are consistent with the behavioural and biochemical findings and suggest that the improved functional outcomes observed following metformin treatment were accompanied by preservation of neuronal morphology.

Overall, the findings demonstrate that metformin attenuated several behavioural, oxidative and inflammatory alterations associated with scopolamine-induced cognitive impairment. The effects were particularly evident at the 200 mg/kg dose, which showed improvements across several behavioural and biochemical parameters. The concurrent changes in behavioural performance, oxidative status, inflammatory markers, serum AChE activity and qualitative neuronal morphology suggest that metformin may have a broad modulatory effect on the alterations associated with scopolamine-induced cognitive impairment. The present findings extend previous investigations of metformin in scopolamine-induced cognitive impairment by providing an integrated assessment of behavioural performance, systemic biochemical indices and histological alterations. While earlier studies have reported beneficial effects of metformin on scopolamine-associated learning and memory impairment, the present study additionally evaluated neuronal morphology in the prefrontal cortex and CA1 region of the hippocampus alongside systemic oxidative and inflammatory markers and acetylcholinesterase activity. The inclusion of donepezil as a reference treatment further provided a pharmacological comparator for interpreting the effects of metformin. The findings indicate the potential of metformin in attenuating behavioural, systemic biochemical, and histological alterations associated with scopolamine-induced cognitive impairment, warranting further investigation of its potential relevance to Alzheimer's disease.

Conclusion

Metformin attenuated selected behavioural, systemic biochemical and histological alterations in a scopolamine-induced cognitive impairment model. However, Donepezil showed better improvement in acetylcholinesterase activity, IL-10, and IL-1 β , which reveals that it aids anti-inflammation and reduction in pro-inflammatory cytokines more than the Metformin treatment. The potential of metformin treatment was seen in the improved locomotor and rearing activities, recognition memory, and spatial working memory, as well as preservation of cortical histoarchitecture of the prefrontal cortex and Cornu ammonis 1 of the hippocampus. Metformin also showed a dose-related difference with the groups administered at 200mg/kg, demonstrating a better reversal of the cholinergic dysfunction and neurodegenerative changes caused by scopolamine. These effects suggest metformin's mechanisms can exert effects beyond glycaemic control. Overall, the findings provide preliminary evidence supporting further investigation of metformin in experimental models of cognitive impairment.

Limitation of Study

A limitation of the study is that no formal a priori sample-size calculation based on a predefined effect size and statistical power was performed. The sample size of 10 animals per group was selected in accordance with the laboratory's established experimental protocol. However, because the sample size was not determined through a formal power analysis, the possibility of insufficient power to detect some group differences cannot be excluded. Also, scopolamine-induced cognitive impairment is an acute pharmacological model that primarily reproduces selected behavioural and cholinergic features of cognitive dysfunction and does not recapitulate the complex, progressive neuropathology of Alzheimer's disease, including amyloid- β accumulation and tau pathology.

Additionally, only adult male Wistar rats were included, which limits the generalisability of the findings across sex and other populations. The biochemical assessments were performed using serum samples and therefore may not directly reflect biochemical processes occurring within specific brain regions. Although histological assessment provided evidence of tissue-level changes, the assessment was descriptive rather than quantitatively histomorphometric, limiting the extent to which structural changes can be objectively quantified. The relatively short duration of the experimental protocol limits conclusions regarding the longer-term effects of metformin.

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Availability of data and materials

Data generated during and analysed during the course of this study are available from the corresponding author on request.

Competing interests

All authors of this paper declare that there is no conflict of interest related to the content of this manuscript.

Declarations Ethics approval

Ethical approval was obtained from the Faculty of Clinical Sciences, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria (approval number: LCHS/REC-0001/26).

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