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Experimental Research Article

Combined metformin and insulin therapy improves neurocognitive dysfunction in type 2 diabetic rat model via anti-inflammatory and antioxidant mechanisms

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ABSTRACT

Introduction: Improper glycemic control is associated with diabetic cognitive dysfunction. Several studies have confirmed the neuroprotective effects of metformin and insulin. This study aimed to investigate the effects of metformin and/or insulin therapy on neurocognitive functions in a type 2 diabetes mellitus (T2DM) rat model.

Methods: Fifty adult male Wistar rats were used in this study and had free access to water and a normal chow diet. After an acclimatization period, 10 rats were kept on a normal chow diet and considered as the control group. T2DM was induced in the other 40 rats by a high-fat diet and low-dose streptozotocin method. Then, diabetic rats were randomly allocated into 4 equal groups: Non-treated diabetic group; Metformin-treated diabetic group (treated with metformin 250 mg/kg/day for 6 weeks); Insulin-treated diabetic group (treated with NPH insulin 40 U/kg for 6 weeks); and Metformin and insulin-treated diabetic group. Neurocognitive functions were assessed by footprint assay, Y-maze, open field test, and Morris water maze. Glycaemic profile, serum levels of amyloid A, interleukin-18, and nuclear factor-kappa B were analyzed. Brain malondialdehyde and total antioxidant capacity were measured. A histopathological examination of the frontal lobe was performed.

Results: Treatment with metformin and/or insulin significantly improved the impaired neurocognitive dysfunction, brain oxidative stress, changes in biochemical parameters, and the associated histopathological changes in the frontal cortex of diabetic rats. The combined therapy showed a better effect than either monotherapy alone.

Conclusion: Metformin and insulin therapy may be valuable for the prevention of neurocognitive dysfunction in T2DM.

Keywords:
Diabetes mellitus
Memory
Metformin
NF-kB
Oxidative stress

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Introduction

Diabetes mellitus (DM) is a metabolic disorder with a growing universal prevalence. 700 million people are anticipated to have type 2 diabetes mellitus (T2DM) by the year 2045 (Magliano et al., 2021). Chronic hyperglycemia triggers several problems such as retinopathy, nephropathy, neuropathy, and cardiovascular complications (Papachristoforou et al., 2020). However, the adverse effects of DM on the nervous system are less studied.

Many studies have authorized a higher prevalence of neurocognitive dysfunction in patients with T2DM (Wium-Andersen et al., 2020). The cognitive dysfunction in T2DM ranges from slight impairment to dementia (Koekkoek et al., 2015). Neurocognitive dysfunction affects attention, memory, executive function, visuospatial and psychomotor performance, speed of information processing, and language skills (Karvani et al., 2019).

The fundamental mechanisms of T2DM-associated cognitive dysfunction are not clear. However, insulin resistance, oxidative stress, neuroinflammation, microvascular changes, abnormalities of metal ions, glymphatic dysfunction, and microbiome disturbance are implied (Luo et al., 2022). Hyperglycaemia leads to alteration in various intracellular and extracellular signaling pathways in the central nervous system, resulting in synaptic dysfunction, increased neuronal apoptosis, and structural alteration in grey and white matter microstructure. Eventually, these changes lead to neurocognitive dysfunction (Gupta et al., 2023).

T2DM is associated with low-grade systemic inflammation and increased inflammatory mediators such as interleukin-18 (IL-18) (Fischer et al., 2005). Il-18 is related to impaired cognition in Alzheimer's disease (Scarabino et al., 2020). In addition, nuclear factor-kappa B (NF-kB) is claimed to have a role in cognitive dysfunction in diabetic rats (Kumar Datusalia and Sunder Sharma 2016). A family of transcription factors known as NF-kB is implicated in inflammatory response, regulation of synaptic transmission, expression of neuronal genes, as well as, learning and memory (Bracchi-Ricard et al., 2008). Serum amyloid A (SAA) is an acute-phase protein produced by the liver during inflammation. It can cross the blood-brain barrier and activate glial cells, aggravating neuronal inflammation, which leads to depressive-like behavior and impaired memory in conditions with abundant amyloid, such as Alzheimer's disease and diabetic brain (Jang et al., 2019).

Diabetic cognitive dysfunction is often accompanied by adverse health outcomes, emphasizing the importance of effective management (Biessels and Whitmer 2020). Proper glycemic control has been suggested to play a significant role in preventing diabetic cognitive dysfunction (Zheng et al., 2021). Some antidiabetic drugs have been proven to have neuroprotective effects in aging, neurodegeneration, and T2DM-related brain injury (Luo et al., 2022). Metformin remains the 1st line therapy for T2DM (Baker et al., 2021), with numerous experimental and clinical studies confirming its protective role against diabetic cognitive dysfunction. However, the underlying mechanism remains unclear (Madhu et al., 2022). In addition, insulin has a neuroprotective effect, and insulin deficiency can lead to neurodegeneration (Evans et al., 2014).

Although the neuroprotective effect of both metformin and insulin against diabetes-induced cognitive dysfunction is well established, the effect of their combination has not been studied before. Also, there is little research about their effects on the frontal lobe in diabetic rats, the underlying mechanisms are still unclear. Thus, this study aimed to investigate the effect of metformin mono- and combined therapy with insulin on neurocognitive functions in experimentally induced T2DM rat models, and whether the combined therapy offers better protection. To elucidate some possible neuroprotective mechanisms the levels of SAA, NF-KB, IL-18, and oxidative stress markers were assessed. In addition, histopathological analysis of the frontal lobe was conducted.

Materials and methods

Animals

Fifty adult male Wistar rats weighing 150-180 g were used in this study. The rats were housed under standard conditions under a natural light-dark cycle. They had free access to water and a normal chow diet consisting of 5% fat, 20% protein, and 52% carbohydrate, as a percentage of total Kcal (Qian et al., 2015). They were acclimated to the environment for 14 days. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (National Research Council). This research was consented by the Research Ethical Committee of the institution (IRB approval No. 2/2021 PHYS25).

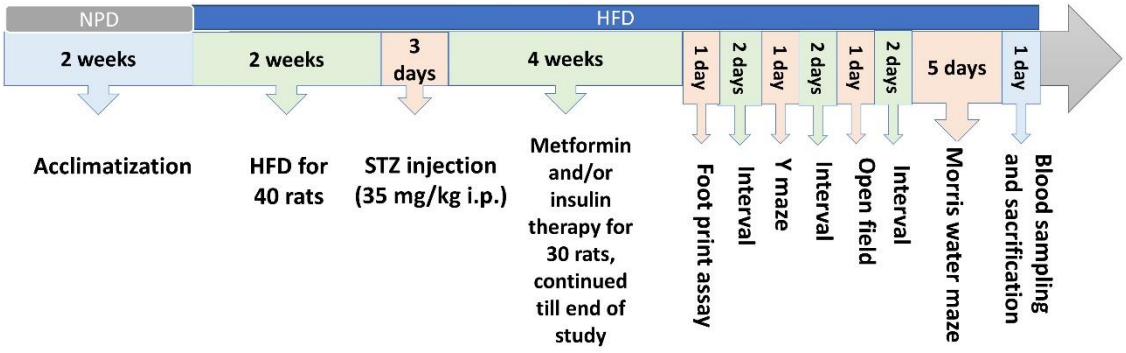

FIGURE 1. Timeline for the experiment. NPD: normal pellet diet; STZ: streptozotocin; HFD: high fat diet.

Chemicals and drugs

Streptozotocin (STZ) was purchased from Sigma-Aldrich Company, USA. Metformin (500 mg/tablet) was obtained from Amoun Company, Egypt. NPH insulin (Novolin N, 100 U/ml) was sourced from Novo Nordisk, Bagsværd, Denmark. All other chemicals were obtained from commercial sources.

Experimental design

After an acclimatization period, 10 rats were kept on a normal chow diet and considered the control (C) group. T2DM was induced in 40 rats using a high-fat diet (HFD) and low-dose streptozotocin (STZ) method. Subsequently, the diabetic rats were randomly allocated into 4 equal groups: 1) Non-treated diabetic (DM) group. 2) Metformin-treated diabetic (Met) group: treated with metformin (250 mg/kg/day) via oral gavage for 6 weeks (Ren et al., 2020). 3) Insulin-treated diabetic (Ins) group: treated with NPH insulin for 6 weeks. The preliminary dose was 40 U/kg given subcutaneously daily. Monitoring of blood glucose was done daily. Proper glycaemic control was considered if rat plasma glucose decreased below 200 mg/dl. Subcutaneous insulin doses were administered as necessary (Grover et al., 2002). 4) Metformin and insulin-treated diabetic (met+Ins) group: treated with both metformin and insulin following the same regimen as in the former groups. All diabetic rats continued to be fed the HFD until the end of the experiment.

After 59 days from the start of the study period, neurobehavioral tests were conducted while maintaining the HFD. Metformin and/or insulin therapy were continued till the day of sacrification. After completion of neurobehavioral tests, retroorbital blood samples were collected following a 12-hour fast using two tubes: the first contained EDTA to measure glycated hemoglobin (HbA1C) and the second was plain to separate serum. The samples were stored at -80ºC for biochemical analysis. Lastly, the rats were euthanized by inhalation of anaesthetic overdose followed by decapitation. Brain tissues were isolated after perfusion from cranial cavities. One hemisphere was homogenized for the measurement of oxidative stress markers, while the other hemisphere was prepared for histological study. The timeline for the experiment is illustrated in Figure 1.

Induction of type 2 diabetes mellitus

T2DM was experimentally induced according to the method by (Srinivasan et al., 2005)**.** Rats were fed a HFD ad libitum for two weeks. As a percentage of total kcal, the HFD consisted of 58% fat, 25% protein, and 17% carbohydrate. The composition of HFD in g/kg was as follows: 365 normal pellet diet, 310 lard, 250 casein, 10 cholesterol, 60 vitamin and mineral, three DL-methionine, one yeast powder, and one NaCl. Then, rats were injected with STZ (35 mg/kg i.p.) dissolved in cold 0.1 M citrate buffer. A 5% glucose solution was administered post-injection to prevent hypoglycemia-related mortality (Ghasemi and Jeddi 2023). Fasting blood glucose (FBG) was measured 72 hours after STZ injection. Rats were deemed to have diabetes if their fasting glucose was >150 mg/dl (Furman 2015).

Neurobehavioral tests

All tests were performed at a fixed time each day. Rats were left two days to recover from handling and the testing procedure before doing the next test (Paylor et al., 2006).

Footprint assay

The footprint assay was conducted to evaluate motor coordination (Brooks et al., 2012). Forepaws were painted with red ink, while hind paws were inked blue. Rats were allowed to walk in a tubular tunnel covered with paper. Stride length was calculated by measuring the distance between the soles of two paw prints for the same limb. For each rat, three consecutive gait cycles were analyzed, and the average was estimated (Hurlock et al., 2009).

Y-maze

The Y-maze test, as previously reported, was used to evaluate the spatial working memory. The maze consisted of three identical wooden arms (60 cm long, 25 cm high, and 12 cm wide) divergent at an angle of 120˚ from each other, forming an equilateral central triangular area. Before the test, rats were habituated to handling for 15 min/day for three successive days. Each rat was positioned in the central area, and exploration of the arms was allowed for 8 minutes. The sequences of arm entries were recorded and analyzed manually. A correct choice was counted for every three successive entries into three dissimilar arms. Spontaneous alternation was calculated as follows = [number of correct choices / (total number of arm entries - 2)] x 100 (Chavoshinezhad et al., 2019).

Open field test

This test was used to assess anxiety-like and exploratory behavior in rodents (Kremer et al., 2021). Rats were placed individually in the center of an unfamiliar arena of 100 x 100 cm with 40 cm height. The test room was dimly illuminated. The animals were left to explore the arena for five minutes. The field was cleaned with 70% ethanol between trials to eradicate scent clues. All movements were recorded and analyzed (Ke et al., 2020). The following parameters were assessed: 1) Central time (the time spent at the centre of the field); 2) Total distance moved (total number of floor units entered with all paws); 3) Centre square entries (number of entries with all paws in the central square); 4) Freezing time (the time spent without any movements); 5) Number of rearing (number of times the rat stood on its rear paws); 6) Number of Grooming (number of body cleaning with paws, body picking with the mouth and face washing actions) (Gould et al., 2009).

Morris water maze

MWM was used to assess spatial learning and retention memory in rats, as designated formerly by (Leo et al., 2019). A circular pool measuring 120 cm in diameter was filled with water to a depth of 30 cm. The maze was divided into 4 quadrants. A platform was fixed in the middle of the south-west quadrant for all trials. The platform was hidden 2 cm under the water surface. A spatial acquisition trial was performed first, with 4 tests per day for four successive days. In each test, the rat was located in one quadrant and allowed to find the platform within one minute. The starting position was changed randomly. If the rat failed to reach the platform within 60 seconds, it was gently directed to the platform and allowed to stay on it for 20 seconds. On the 5th day, the probe trial was performed to assess memory retention by removing the platform from the maze. The latency time to reach the target quadrant and the time spent in it were recorded (Chavoshinezhad et al., 2019).

Biochemical analysis

FBG and HA1C levels were measured using kits purchased from Spectrum Diagnostics, Egypt (Catalog No: 250-001 and 255-000, respectively) according to the manufacturer´s instructions. Serum insulin, SAA, and IL-18 were measured using rat ELISA kits purchased from Abcam, Cambridge, UK); Catalog No: ab273188, ab215090 and ab213909, respectively). Serum NF-kB was measured using rat ELISA kits (MBS287521, MyBioSource, San Diego, USA). Malondialdehyde (MDA) and total antioxidant capacity (TAC) were measured in brain homogenate using colorimetric kits (Biodiagnostic Company, Giza, Egypt).

HOMA-IR index was calculated as follows: HOMA-IR index = [Fasting serum insulin (μU/ml) X fasting serum glucose (mg/dl)] / 405 (Matthews et al., 1985). QUICKI index was calculated as follows: QUICKI = 1/ (logI0 + logG0) (Chen et al., 2003).

Histopathological examination

One hemisphere from each animal in all groups (n=10) was fixed immediately in a 10% neutral-buffered formalin solution. Then, the samples were processed for preparation of 5-μm thick paraffin slices. Paraffin sections were stained with haematoxylin and Eosin (H&E) for examination by light microscope (Suvarna et al., 2018).

TABLE 1: Effect of metformin and/or insulin therapy on glycaemic state in the studied groups.

Parameters	Experimental Groups					F_{welch} test	P value for ANOVA
	C	DM	Met	Ins	Met+Ins		
Fasting blood glucose (mg/dl)	128.33±12.9	466.14±62**	213.29±28.5**+	190.71±22.8*+	185±21.08*++	=99.997	<0.001
Fasting serum Insulin (µIU/ml)	8.48±1.69	3.05±0.42**	5.22±0.66**	7.825±0.87**++	8.49±1.0$^{4++\$}$	=39.558	<0.001
HbA1C (%)	2.27±0.25	7.36±0.75**	5.36±0.32**++	4.42±0.24**++$^{\$\$}$	3.99±0.30**++$^{\$\$}$	=255.79	<0.001
HOMA-IR Index	2.68±0.56	3.53±1.04*	2.73±0.33+	3.65±0.24*$	3.86±0.52*$	=7.596	<0.001
Quick-IR index	0.33±0.01	0.32±0.1*	0.33±0.01	0.32±0.01*$	0.31±0.01*$	=7.511	<0.001

Data are expressed as mean ± S.D. (n=10). HA1C: glycated hemoglobin; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance Index; Quicki-IR: Quantitative Insulin Sensitivity Check Index. C: control group; DM: diabetes mellitus group; Met: metformin-treated group; Ins: insulin-treated group; Met+Ins: metformin and insulin-treated group. *P< 0.05 vs. C group. **P< 0.001 vs. C group. ++P< 0.001 vs. DM group. $P< 0.05 vs. Met group. $$ P< 0.001 vs. Met group.

Statistical analysis

Data were analyzed by SPSS version 26.0 [SPSS Inc., Chicago, IL, USA]. The normality of distribution was conducted by the Shapiro-Wilk test. The significance between groups was determined by One-way ANOVA followed by post-hoc Tukey's multiple comparison test. All results were expressed as mean ± standard deviation (SD). Significant *P*-value was set at ≤ 0.05.

Results

Biochemical results

Glycaemic state assessment

Fasting blood glucose: The mean value of FBG in the DM group was significantly higher than in the C group. All treated groups (Met, Ins, and Met+Ins) presented significantly lower FBG values compared with the DM group. The FBG values in the Met, Ins, and Met+Ins groups were significantly higher than in the C group (Table 1).

Fasting serum insulin: The mean values of serum insulin in the DM and Met groups were significantly lower than in the C group. Ins and Met+Ins groups showed significantly higher serum insulin values compared with the DM group. Also, the Met+Ins group showed significantly higher serum insulin compared with the Met group (Table 1).

HbA1C: The mean values of HbA1C in DM, Met, Ins, and Met+Ins groups were significantly higher than in the C group. The values of HbA1C in all treated groups were significantly decreased compared with the DM group. Also, the values of HbA1C in the Ins and Met+Ins groups were significantly decreased compared with the Met group (Table 1).

HOMA-IR index: The mean values of the HOMA-IR index in DM, Ins, and Met+Ins groups were significantly higher than in the C group. In the Met group, it was significantly decreased when compared with the DM group and insignificantly changed when compared with the C group. The values of the HOMA-IR index in the Ins and Met+Ins groups were significantly higher than those of the Met group (Table 1).

Quicki-IR index: The mean values of the Quicki-IR index in DM, Ins, and Met+Ins groups were significantly lower than in the C group. In the Met group, it was insignificantly changed when compared with both the C and DM groups. The Ins and Met+Ins groups showed significantly lower Quicki-IR index than the Met group (Table 1).

Inflammatory and oxidative stress markers

Serum amyloid A

The mean values of SAA in DM, Met, Ins, and Met +Ins groups were significantly higher than in the C group. All treated groups showed significantly lower

TABLE 2: Effect of metformin and/or insulin therapy on serum levels of amyloid A, nuclear factor-kappa beta (NF-kβ), interleukin-18 (IL-18), and total antioxidant capacity (TAC), and on malondialdehyde (MDA) level in the brain tissue in the studied groups.

Parameters	Experimental Groups					F_{welch} test	P value for ANOVA
	C	DM	Met	Ins	Met+Ins		
Amyloid A (pg/ml)	1.03±0.10	2.85±0.08*	2.66±0.16*$^{+}$	2.28±0.11*$^{++}$$	1.30±0.09*$^{+\$\bullet}$	=349.76	<0.001
NF-kB (pg/ml)	0.53±0.08	1.61±0.11*	1.61±0.14*	1.16±0.16*$^{++}$$	0.82±0.26*$^{+\$\bullet}$	=57.40	<0.001
IL-18 (pg/ml)	8.70±0.99	10.94±0.85**	8.07±0.94^{++}	8.50±0.43^{++}	7.50±0.97^{++}	=16.08	<0.001
MDA (nmol/g.tissue)	20.31±1.43	49.17±2.61*	45.44±2.57*	40.41±3.30*$^{+\$}$	24.42±3.08*$^{+\$\bullet}$	=161.13	<0.001
TAC (μM/g.tissue)	22.49±1.41	14.79±1.14**	18.69±1.15**$^{++}$	16.87±1.13**$^{+}$	20.71±0.96$^{+\bullet\bullet}$	=46.861	<0.001

Data are expressed as mean ± S.D. (n=10). C: control group; DM: diabetes mellitus group; Met: metformin-treated group; Ins: insulin-treated group; Met+Ins: metformin and insulin-treated group. *P < 0.05 vs. C group. ** P < 0.001 vs. C group. $^{+}$P < 0.05 vs. DM group. $^{++}$ P < 0.001 vs. DM group. $^{\$}$ P < 0.05 vs. Met group. $^{\bullet}$ P < 0.05 vs. Ins group. $^{\bullet\bullet}$ P < 0.001 vs. Ins group.

SAA values compared with the DM group. The values of SAA in the Ins and Met+Ins groups were significantly lower than those in the Met group. Also, the Met+Ins group showed significantly lower SA A than that of the Ins group (Table 2).

Serum NF-kB

The mean values of NF-kB in DM, Met, Ins, and Met+Ins groups were significantly higher than in the C group. Ins and Met+Ins groups showed significantly lower serum NF-kB compared with both DM and Met groups. Also, the Met+Ins group showed significantly lower serum NF-kB than that of the Ins group (Table 2).

Serum IL-18

The mean value of serum IL-18 in the DM group was significantly higher than in the C group. All treated groups showed significantly lower serum IL-18 values compared with the DM group (Table 2).

Brain MDA

The mean values of MDA in DM, Met, Ins, and Met+Ins groups were significantly higher than in the C group. The Ins and Met+Ins groups showed significantly lower MDA values than those of the DM and Met groups. Also, the Met+Ins group showed a significantly lower MDA value than that of the Ins group (Table 2).

Brain TAC

The mean values of TAC in the DM, Met, and Ins groups were significantly lower than in the C group. The Met, Ins, and Met+Ins groups showed significantly higher TAC values than the corresponding value of the DM group. Also, the Met+Ins group showed a significantly higher TAC value compared with the Ins group. There was an insignificant change between Met+Ins and C groups (Table 2).

Neurobehavioral tests

Footprint assay

The stride length in DM, Met, Ins, and Met+Ins groups (10.24 ± 0.39, 10.02 ± 0.60, 11.24 ± 0.51, and 11.79 ± 0.72 cm, respectively) was significantly lower than the corresponding value of the C group (13.06±0.59 cm). Ins and Met+Ins groups showed significantly higher stride length values than those of both DM and Met groups (Figure 2-A).

Y maze

The total number of arm entries was significantly lower in DM, Met, Ins, and Met+Ins groups than in the C group (21.90±1.92, 25.10±1.91, 28.81±2.13 and 26.77±1.78 vs 31.32±2.34, respectively). The total number of arm entries in the Met, Ins, and Met+Ins groups was significantly higher than those of the DM group. The Met+Ins group showed significantly higher values than the Met group (Figure 2-B). The percentage of spontaneous alternation was significantly lower in the DM group than in the C group (32.65±4.08 vs 62.37±2.65 %, respectively). The values of the percentage of spontaneous alternation in Met, Ins, and Met+Ins groups (51.23±7.25, 60.12±8.0, and 67.38±14.12 %, respectively) were significantly higher than those of the DM group. Also, the Met+Ins group showed a significantly higher value of percentage of spontaneous alternation compared with the Met group (Figure 2-C).

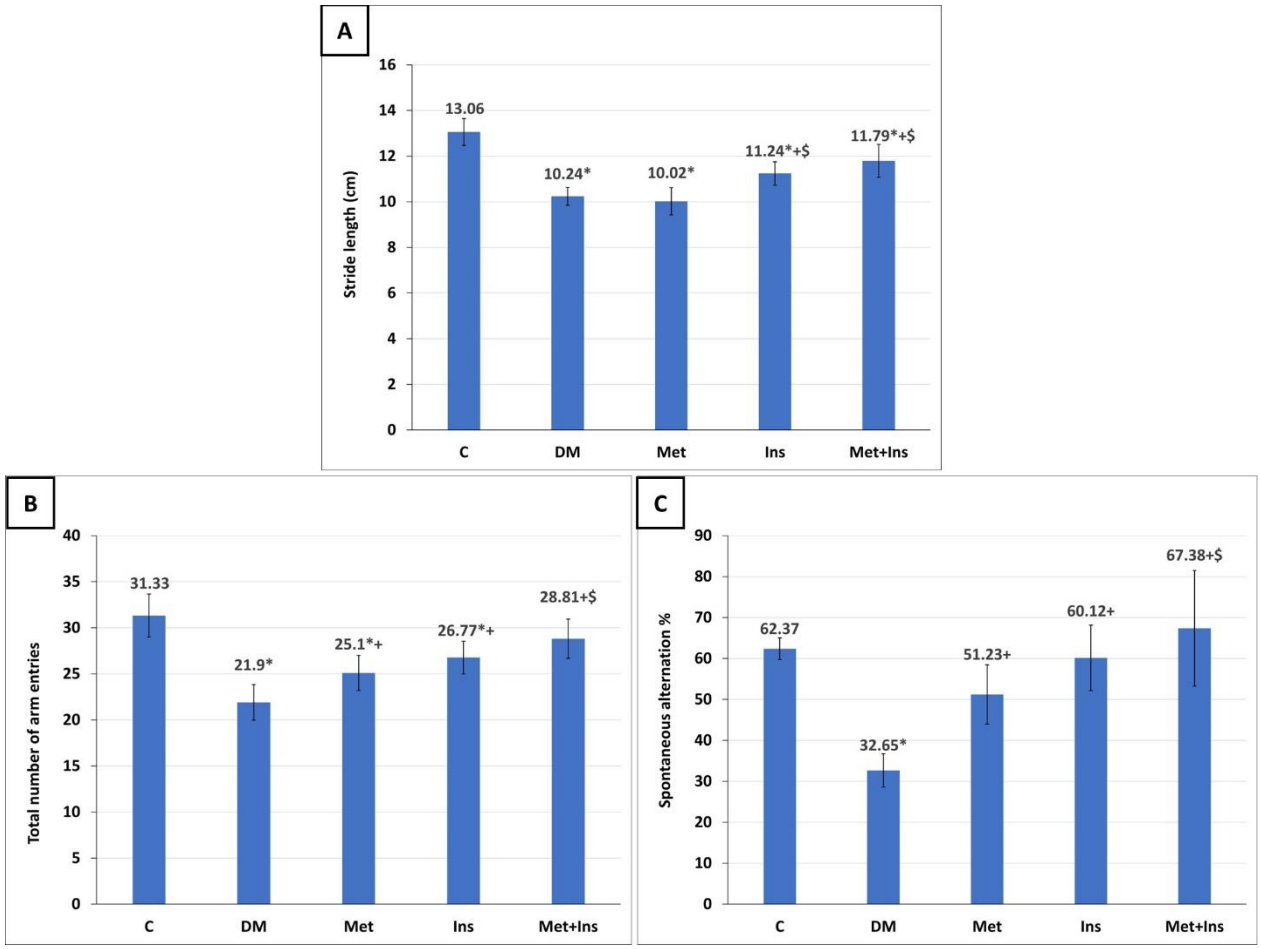

FIGURE 2. Effect of metformin and/or insulin therapy on footprint assay (A), total number of arm entries in Y-maze (B), and spontaneous alternation in Y-maze (C) in type 2 diabetic rats. Results are expressed as mean ± S.D. (n=10). C: control group; DM: diabetes mellitus group; Met: metformin-treated group; Ins: insulin-treated group; Met+Ins: metformin and insulin-treated group. $^{*}P < 0.05$ vs. C group. $^{+}P < 0.05$ vs. DM group. $^{\$}P < 0.05$ vs. Met group.

Open field test

The central time in DM, Met, and Ins groups (3.9±1.6, 16.0±4.9 and 22.9±3.7 sec, respectively) was significantly lower than in the C group (49.4±9.2 sec). All treated groups showed significantly higher values of central time compared with the DM group. Also, the Met+Ins group showed a significantly higher value of central time (40.3±7.4 sec) than the corresponding values of the Met and Ins groups. There was an insignificant change between Met+Ins and C groups (Figure 3-A).

The freezing time in DM, Met, and Ins groups (169.0±35.9, 84.1±10.4, and 40.4±5.2 sec, respectively) was significantly higher than the corresponding value of the C group. The Met and Ins groups showed significantly lower freezing time values than the DM group. There were significantly lower freezing time values in the Ins group than in the Met group. Also, the Met+Ins group showed a significantly lower value (12.1±2.1 sec) compared with the Met, Ins, and C groups. There was an insignificant change between Met+Ins and C groups (Figure 3-B).

The distance moved in DM, Met and Ins groups (93.7±6.8, 119.4±9.5 and 179.1±8.5 cm, respectively) was significantly lower than the corresponding value of the C group (442.9±14.57 cm). Both Met and Ins groups showed significantly higher values of the distance moved than the DM group and significantly lower values than the C group. There were significantly higher values of the distance moved in the Ins group than in the Met group. Also, the Met+Ins group showed significantly higher distance moved values than the corresponding values of the D, Met, and Ins groups. There was an insignificant change between Met+Ins and C groups (Figure 3-C).

The number of central entries in the DM group was significantly lower than in the C group (1.4±0.9 vs

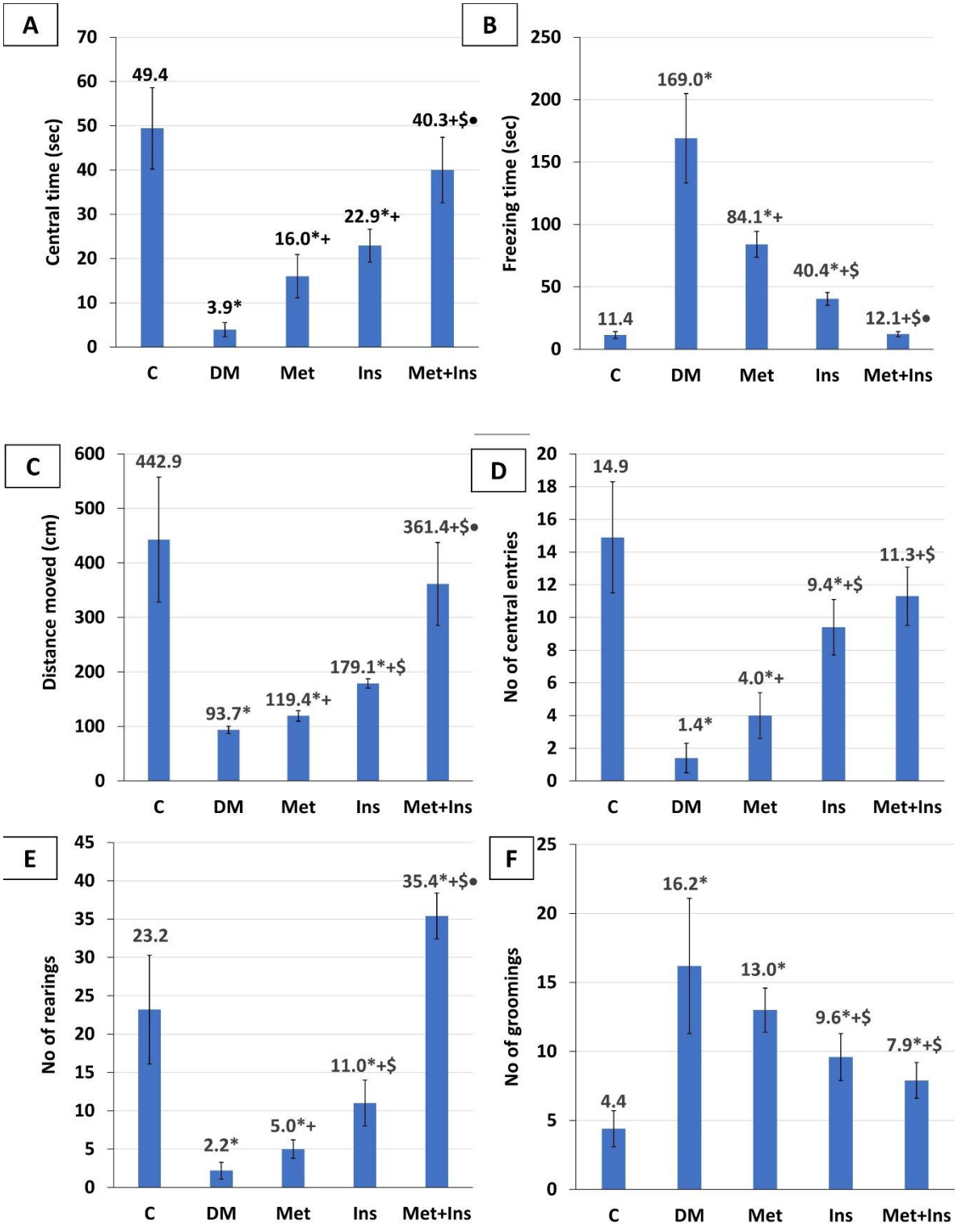

FIGURE 3. Effect of metformin and/or insulin therapy on open field results in type 2 diabetic rats. Results are expressed as mean ± S.D. (n=10). C: control group; DM: diabetes mellitus group; Met: metformin-treated group; Ins: insulin-treated group; Met+Ins: metformin and insulin-treated group. $^{*}P < 0.05$ vs. C group. $^{+}P < 0.05$ vs. DM group. $^{\$}P < 0.05$ vs. Met group. $\bullet P < 0.05$ vs. Ins group.

14.9±3.4, respectively). The number of central entries in the Met and Ins groups (4.0±1.4 and 9.4±1.7, respectively) was significantly higher than in the DM group and significantly lower than in the C group. There were significantly higher values of the number of central entries in the Ins group than in the Met group. Also, the Met+Ins group showed a significantly higher number of central entries (11.3±1.8 sec) compared with the DM

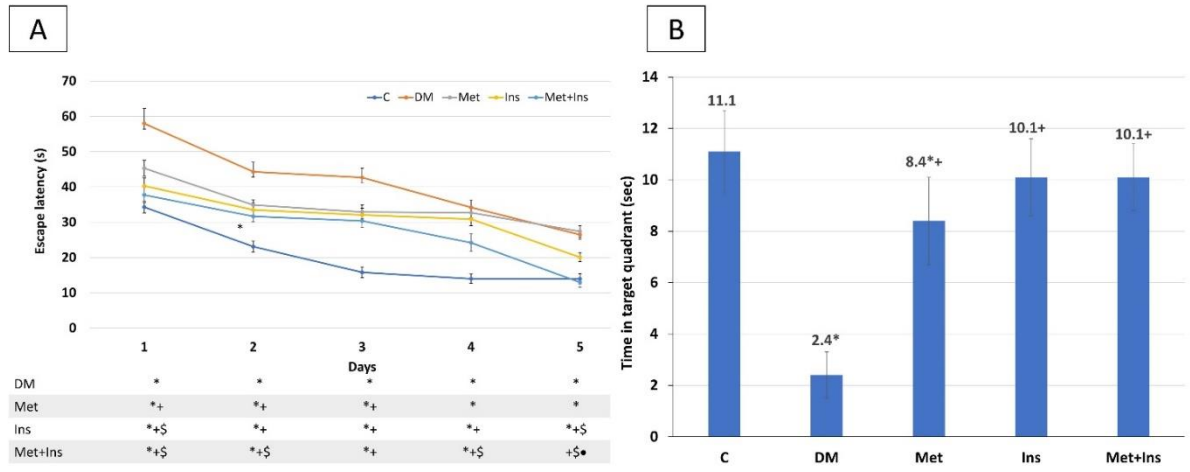

FIGURE 4. Effect of metformin and/or insulin therapy on the spatial learning and retention memory evaluated by Morris water maze test in type 2 diabetic rats. Results are expressed as mean ± S.D. (n=10). C: control group; DM: diabetes mellitus group; Met: metformin-treated group; Ins: insulin-treated group; Met+Ins: metformin and insulin-treated group. $^{*}P < 0.05$ vs. C group. $^{+}P < 0.05$ vs. DM group. $^{\$}P < 0.05$ vs. Met group. •$P < 0.05$ vs. Ins group.

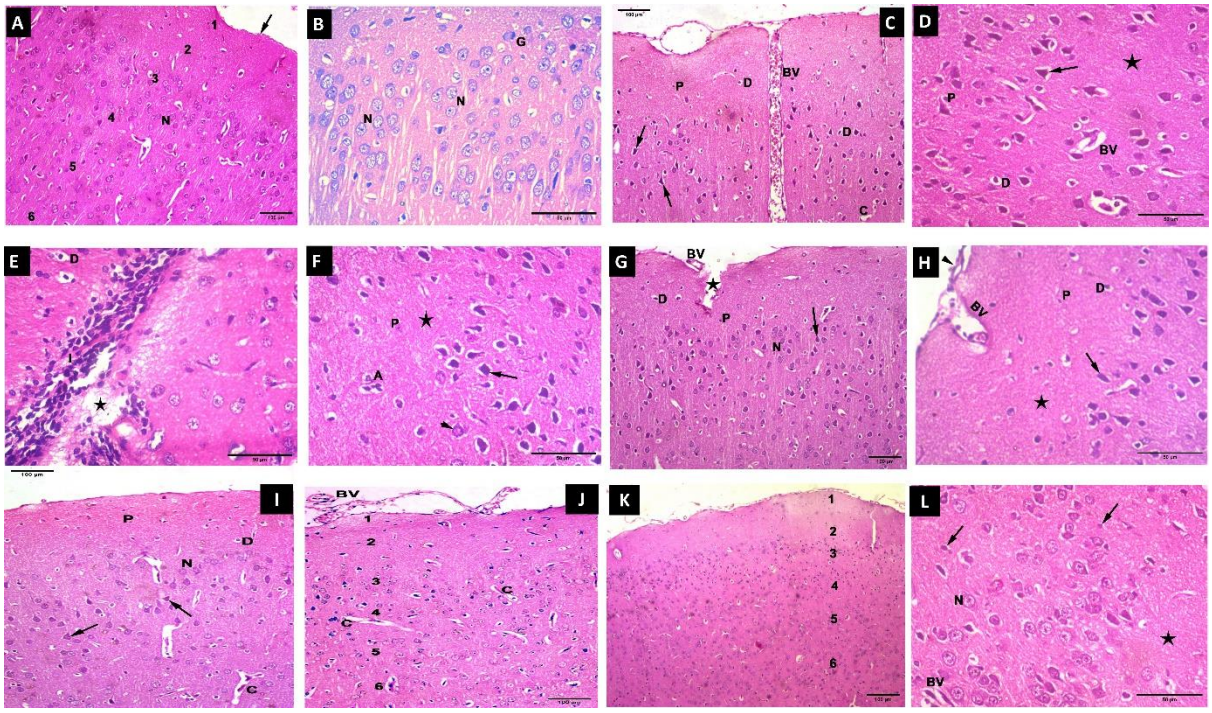

FIGURE 5. Effect of metformin and/or insulin therapy on histopathological changes of frontal cortex in type 2 diabetic rats examined by Haematoxylin and Eosin (H&E) staining. (A): Control group showing well-organized neuronal cells regularly arranged in six layers (H&EX100). (B): Control group showing normal neuronal pyramidal cells (N) and granular cells (G), (H&EX400). (C-F): Diabetic group showing disorganization of the 6 layers and congested blood vessels (BV). The molecular layer displays haloed malformed neurons (D), granule cells with deeply stained pyknotic nuclei (P), distorted neurons with cell bodies of varying shapes and surrounded by haloes (arrow), depletion of the cellular element of cortical layers (star), inflammatory cell infiltrate (I), degenerated ghost-like cell (arrowhead) bizarre shaped pyramidal cells (A) (C: H&E X100; D, E and F; H&E X400). (G): Metformin-treated group showing some pyramidal cells (N) are normal, deformed neurons with irregularly shaped cell bodies and surrounded by haloes (arrow), granule cells with deeply stained pyknotic nuclei (P), deformed neurons surrounded by haloes (D) are still seen, congested dilated blood vessels (BV) and loss of tissue is seen (star), (H&EX100). (H): Metformin-treated group showing depletion of the cellular element of cortical layers (star), (H&E X400). (I&J): Insulin-treated group showing normal 6 layers of the cerebral cortex. but congested blood vessels (BV) and blood capillaries are still seen (C). Many normal pyramidal cells (N), few deformed neurons with irregularly shaped cell bodies (arrow), few granule cells with deeply stained pyknotic nuclei (P), and few deformed neurons surrounded by haloes (D) are still seen, (H&EX100). (K): Metformin and insulin-treated group showing normal all layers of cerebral cortex more or less like control, (H&EX100). (L): Metformin and insulin-treated group showing normal pyramidal cells (N) and granular cells (arrows) more or less like control wide space between cells is still seen (star), (H&EX400).

and Met groups. There was an insignificant change between Met+Ins and C groups (Figure 3-D).

The number of rearing in the DM group was significantly lower than in the C group (2.2±1.1 vs 23.2±7.1, respectively). The values of number of rearing in Met, Ins, and Met+Ins groups (5.0±1.2, 11.0±3.0, and 35.4±3.0, respectively) were significantly higher compared with the DM group. The values of the number of rearing in the Ins and Met+Ins groups were significantly higher than in the Met group. Also, the Met+Ins group showed significantly higher values of number of rearing than the Ins group. There were significant changes between the treated groups and the C group (Figure 3-E).

The number of grooming in DM, Met, Ins, and Met+Ins groups (16.2±4.9, 13.0±1.6, 9.6±1.7 and 7.9±1.3, respectively) were significantly higher than the corresponding value of the C group (4.4±1.3). The values of number of grooming in Ins and Met+Ins groups were significantly lower than their corresponding in both DM and Met groups (Figure 3-F).

Morris water maze

During the probe trial, the time of escape latency in DM, Met, and Ins groups (26.5±2.6, 27.4±1.7, 20.1±1.3 sec, respectively) was significantly higher than that of the C group (14.0±1.4 sec). The values of the Ins and Met+Ins groups (20.1±1 and 13.0±1.4 sec, respectively) were significantly lower than the corresponding values of the DM and Met groups. Also, the Met+Ins group showed significantly lower time than the Ins group (Figure 4-A).

In addition, the time in the target quadrant during the probe trial in the DM group was significantly higher than in the C group (2.4±0.9 vs 11.1±1.6 sec, respectively). The treated groups Met, Ins, and Met+Ins showed significantly higher values (8.4±1.7, 10.1±1.5, 10.1±1.3 sec, respectively) compared with the D group. The Met group showed significantly lower values than the C group. There were insignificant changes between the Ins and Met+Ins groups compared with the C group (Figure 4-B).

Histological results

H&E-stained slices of the frontal cortex in the C group showed well-organized neuronal cells regularly arranged in six layers. The diabetic rats showed histopathological features like disorganization of the layers, deformed neurons, depletion of the cellular elements, inflammatory cell infiltration, and dilated congested blood vessels. Treatment with metformin or insulin partially alleviated the histopathological features. While the combined therapy with metformin and insulin improved the histopathological features almost to the normal (Figure 5).

Discussion

Improper glycaemic control is associated with diabetic cognitive dysfunction (Lin et al., 2023). So, proper glycaemic control is expected to ameliorate DM-associated cognitive dysfunction. Several studies approved the neuroprotective effect of metformin and insulin (Evans et al., 2014; Madhu et al., 2022). Thus, this study aimed to investigate the effect of glycaemic control by metformin and/or insulin therapy on neurocognitive functions in an experimentally-induced T2DM rat model. Also, we aimed to elucidate some possible neuroprotective mechanisms for metformin and insulin.

In the current study, T2DM was induced by HFD and low-dose STZ. It is a well-established model simulating T2DM and its accompanying complications in humans (Wickramasinghe et al., 2022). Diabetic rats showed hyperglycemia, increased HbA1C, hypoinsulinemia, insulin resistance, and decreased insulin sensitivity compared with the C group (Chao et al., 2018). HFD induces insulin resistance, while STZ induces ß-cell impairment. The level of insulin depends on the residual functional ß-cell. In the early stages of T2DM, there is a normal or increased insulin level. While, at the late stages, there is impaired insulin secretion, which is in accordance with our results (Skovsø 2014). The combined metformin and insulin therapy exhibited better glycaemic control than metformin monotherapy.

In accordance with previous studies, behavioral tests revealed impaired spatial memory and learning in diabetic rats evaluated by Y-maze and MWM (Liu et al., 2020). Also, diabetic rats showed impaired locomotor activity and anxiety-like behavior tested by footprint assay and open field test. These results agree with previous ones (Bădescu et al., 2016; Dong et al., 2023; Mehta et al., 2017). Impaired gait in DM is attributed to diabetic peripheral neuropathy (Henderson et al., 2019).

Glycaemic control by metformin and/or insulin therapy significantly improved the impaired performance in neurobehavioral tests. The combined therapy had a

better effect than metformin monotherapy in all tests. Also, the combined therapy showed a better effect than insulin alone in open field tests and the escape latency in MWM. In accordance with our results, a previous study by (Janthakhin et al., 2023) confirmed the protective role of metformin against diabetic cognitive dysfunction evaluated by a novel object recognition test. Another study reported that metformin could boost recovery of locomotor function in a spinal cord injury rat model (Chen et al., 2021). In agreement with our results, insulin therapy improved spatial memory in type 1 diabetic rats tested by T-maze (Sanna et al., 2019). Moreover, insulin therapy improved the impaired memory retention evaluated by MWM (Song et al., 2018).

The underlying neuroprotective mechanisms of metformin and insulin are still ambiguous. −This study aimed to explore the possible suggested mechanisms of metformin and insulin beyond their role in glycaemic control. We assessed NF-kB, inflammatory and oxidative stress markers, as well as histological study of the frontal cortex was done.

In the current study, the impaired neurocognitive function in diabetic rats was associated with insulin resistance as indicated by a significant increase in the HOMA-IR index. Brain oxidative stress was evident in diabetic rats by a significant increase of MDA and a significant decrease of TAC in brain homogenate. Also, diabetic rats exhibited higher SAA, IL-18, and NF-kB than the C group. Treatment with metformin and/or insulin significantly alleviated the changes in biochemical parameters. The combined therapy had a better effect than either metformin or insulin monotherapy in improving SAA, NF-kB, and brain oxidative stress.

Insulin resistance, a main feature of T2DM, is a risk factor for cognitive dysfunction (Cui et al., 2022). Till now, it is not clear whether peripheral and central insulin resistance can occur independently. It is believed that long-term insulin dysregulation not only affects diabetes development but also induces further impaired brain metabolism and neural activity (Kellar and Craft 2020). Further studies are recommended to assess central insulin resistance.

Metformin significantly improved insulin resistance as indicated by a significantly lower HOMA-IR index in the D group than Met group. This agrees with the previous report (Zhang et al., 2021). Metformin improves insulin sensitivity by stimulating the activity of insulin receptor tyrosine kinase and by upregulating glucose transporter 4 expression (Herman et al., 2022).

In addition, chronic hyperglycemia, indicated by elevated HbA1C, induces oxidative stress and activation of NF-kB, which induces expression of proinflammatory cytokines leading eventually to nerve inflammation and diabetic neuropathy (Suryavanshi and Kulkarni 2017). The brain is very liable to oxidative damage. Brain oxidative stress has a principal role in diabetic cognitive dysfunction (Muriach et al., 2014). Cellular oxidative stress stimulates mitochondrial oxidative damage, resulting in apoptosis of neurons, which is consistent with the histological study (Kermer et al., 2004).

There is an association between diabetic cognitive dysfunction and stimulation of inflammatory pathways (Piatkowska-Chmiel et al., 2021). IL-18, an inflammatory cytokine, increases in T2DM which agrees with our results (Fischer et al., 2005). In the brain, IL-18 activates microglia leading to neuronal loss. Thus, it affects cognitive function (Bossu et al., 2008). The inflammatory condition in diabetic rats was confirmed by the significant increase in SAA, which agrees with the previous study (Rosyadi et al., 2019). Il-18 had been shown to stimulate NF-kB in different cell types (Neurath et al., 1998; Wang et al., 2019). NF-kB induces transcription of proinflammatory cytokines, regulation of synaptic transmission, expression of neuronal genes, as well as, learning and memory processes (Bracchi-Ricard et al., 2008). Suppression of NF-kB was reported to improve learning and memory deficit in rats with T2DM confirming its role in diabetic cognitive dysfunction, which supports our results (Kumar Datusalia and Sunder Sharma 2016).

The nootropic effect of metformin and insulin extends beyond their effect on glycaemic control. They have antioxidant and anti-inflammatory actions as shown in this study. Consistent with our results, (Correia et al., 2008) reported that metformin could improve T2DM-associated brain oxidative stress in rats. Also, metformin can cross the blood-brain barrier and employ direct anti-inflammatory action (Łabuzek et al., 2010). The anti-inflammatory effect of metformin in this study was consistent with (Alzamily et al., 2021), who reported a significant decrease in IL-18 in patients with T2DM treated with metformin. In support of our results, a previous study reported the anti-inflammatory effect of insulin by inhibiting NF-kB in mononuclear cells in obese

patients (Dandona et al., 2001).

In the brain, insulin does not only regulate metabolism but also is implied in learning and memory processes (Zhao et al., 2004). Consistent with our results, insulin therapy provided antioxidant properties for the alleviation of diabetic encephalopathy through the Nrf2 signaling pathway (Song et al., 2018).

DM affects the morphology of dendrites in the hippocampus, prefrontal cortex, and occipital cortex (Martínez-Tellez et al., 2005). The frontal cortex has a role in motor, cognition, mood, and neuroendocrine functions (Stuss and Knight 2013). So, the histopathological study of the frontal cortex of diabetic rats was done, which was consistent with the biochemical and behavioral tests. The diabetic group showed morphological changes indicating nerve cell injury, which agrees with previous studies (Baptista et al., 2021; Ertas et al., 2023). Hyperglycemia-induced neuroinflammation and oxidative stress are suggested mechanisms for diabetic neuronal degeneration (Ola et al., 2014). Furthermore, NF-kB increases neuronal injury in the brain (Nijboer et al., 2009). Treatment with either metformin or insulin partially alleviated the histopathological changes. In the frontal lobe, insulin has a neuroprotective effect, and insulin deficiency can lead to neurodegeneration (Evans et al., 2014). The nootropic effect of insulin on the prefrontal cortex is mediated by the downregulation of BAX, an apoptotic protein (Sanna et al., 2019). The combined therapy was able to reverse these changes to apparently normal architecture supporting the importance of good glycaemic control and nootropic effects of metformin and insulin.

Conclusion

The current study emphasized the importance of good glycaemic control in alleviating diabetic neurocognitive dysfunction. It suggests that combined therapy with metformin and insulin may be valuable for the prevention of DM-associated neurocognitive dysfunction.

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

This research was consented by the Research Ethical Committee, Faculty of Medicine, Menoufia University (IRB approval No. 2/2021 PHYS25).

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