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ORIGINAL RESEARCH

Metformin Mitigates Trimethyltin-Induced Cognition Impairment and Hippocampal Neurodegeneration

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Abstract

The neurotoxicant trimethyltin (TMT) triggers cognitive impairment and hippocampal neurodegeneration. TMT is a useful research tool for the study of Alzheimer's disease (AD) pathogenesis and treatment. Although the antidiabetic agent metformin has shown promising neuroprotective effects, however, its precise modes of action in neurodegenerative disorders need to be further elucidated. In this study, we investigated whether metformin can mitigate TMT cognition impairment and hippocampal neurodegeneration. To induce an AD-like phenotype, TMT was injected *i.p.* (8 mg/kg) and metformin was administered daily p.o. for 3 weeks at 200 mg/kg. Our results showed that metformin administration to the TMT group mitigated learning and memory impairment in Barnes maze, novel object recognition (NOR) task, and Y maze, attenuated hippocampal oxidative, inflammatory, and cell death/pyroptotic factors, and also reversed neurodegeneration-related proteins such as presenilin 1 and p-Tau. Hippocampal level of AMP-activated protein kinase (AMPK) as a key regulator of energy homeostasis was also improved following metformin treatment. Additionally, metformin reduced hippocampal acetylcholinesterase (AChE) activity, glial fibrillary acidic protein (GFAP)-positive reactivity, and prevented the loss of CA1 pyramidal neurons. This study showed that metformin mitigated TMT-induced neurodegeneration and this may pave the way to develop new therapeutics to combat against cognitive deficits under neurotoxic conditions.

Keywords Alzheimer's · Metformin · Trimethyltin · AMP-activated protein kinase · Neurodegeneration · Neuroprotection

Abbreviations

AChE	Acetylcholinesterase
AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
Aβ	Amyloid beta
BDNF	Brain-derived neurotrophic factor
CREB	CAMP response element-binding protein
GFAP	Glial fibrillary acidic protein
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
GSK3β	Glycogen synthase kinase 3
IL-6	Interleukin 6
MDA	Malondialdehyde
NFT	Neurofibrillary tangles
NOR	Novel object recognition
Nrf2	Nuclear factor erythroid 2-related factor 2
PI3K	Phosphoinositide 3-kinase
p-Tau	Hyperphosphorylated Tau
SOD	Superoxide dismutase
TMT	Trimethyltin
TNF	Tumor necrosis factor

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Introduction

Alzheimer's disease (AD) is presently the leading chronic neurodegenerative disorder which affects beyond six million individuals in the US and nearly 50 million people globally (Alzheimer Association 2020). AD is gradually and irrevocably associated with cognition deterioration and eventual dementia and disability in performing regular activities (Kawas and Corrada 2006). AD is associated with significant loss of neurons in the hippocampal and cortical areas, appearance of hyperphosphorylated tau-composed neurofibrillary tangles (NFT), and amyloid beta (Aβ) deposition as

senile plaques, and higher level of presenilin 1 (Anderson et al. 2024; Gholami 2023). AMP-activated protein kinase (AMPK) is a pivotal regulator of energy homeostasis with potential implication in the AD pathogenesis (Cai et al. 2012). Treatment options for AD are quite few and may be ineffective. Current drugs for this disease cannot reverse or prevent its neurodegenerative nature (Chen et al. 2021a). Trimethyltin (TMT) as an organotin and a specific neurotoxin of the hippocampus is used to induce a consistent model of neurodegeneration and cognitive impairment and is therefore a valuable tool to study AD pathogenesis (Jung et al. 2013; Rostami et al. 2022). In addition, cholinergic dysfunction which is observed in AD patients also occurs in TMT-intoxicated rats (Kang et al. 2016).

Metformin as a biguanide is one of the most commonly prescribed hypoglycemic agents (Hundal et al. 2000; Patel et al. 2023). Clinical studies have shown that metformin administration is associated with lower risk of AD and ensuing dementia in diabetic patients in addition to its improvement of cognitive performance (Campbell et al. 2018). Antidiabetic metformin can exert a therapeutic effect in intracerebroventricular streptozotocin sporadic model of AD, as shown by lower levels of memory loss, Aβ deposition, and tau phosphorylation in addition to its attenuation of brain insulin resistance (Abosharaf et al. 2024), it is capable to prevent N-nitrosodiethylamine-induced cognitive decline via attenuation of hippocampal amyloid beta deposition and inflammatory factors like tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Ponce-Lopez et al. 2023), and can also mitigate amyloid β-induced cognitive deficit through lowering oxidative and nitrosative stress as well as neuroinflammation (Khaleghi-Mehr et al. 2023; Liao et al. 2021). Furthermore, metformin can ameliorate paclitaxel neurotoxicity in neural crest cells (Khodabakhsh et al. 2024) and can alleviate sevoflurane-induced cognitive decline and neurogenesis deficit via nuclear factor erythroid 2-related factor 2 (Nrf2)/ Glucose-6-phosphate dehydrogenase (G6PD) pathway (Fan et al. 2023). Moreover, metformin due to its anti-inflammatory, antiapoptotic, and antioxidant potential can attenuate ethanol neurotoxicity (Sabzali et al. 2022) in addition to its protection against methamphetamine-induced cognition deficit, anxiety, depression, and neurodegeneration through cAMP response element-binding protein (CREB)/ brain-derived neurotrophic factor (BDNF)/protein kinase B (Akt)/glycogen synthase kinase 3 (GSK3) signaling (Keshavarzi et al. 2019). Accordingly, this study was designed to evaluate the potential effect of metformin in reversing cognitive deficits subsequent to TMT neurotoxin and to unravel some involved modes of action.

Materials and Methods

Animals

Thirty-two male Wistar rats at 11–12 weeks of age and weighing 195–230 g were purchased from Laboratory Animal House of Tehran University (Tehran) and were housed (4 rats/cage) at controlled room conditions for temperature (21–23 °C) and humidity (45–50%) and with free access to rat food and tap water.

Experimental Protocol

Rats were divided into 4 groups using random number protocol ($n = 8$/group), i.e., Control, Control+Metformin200, TMT, and TMT+Metformin200. In addition, a blinding strategy was uniformly applied for the experimenter. Number of used animals was obtained from our relevant previous study (Rostami et al. 2022). TMT groups received a single intraperitoneal injection of TMT-chloride (Cat # sc-301942, Santa Cruz Biotechnology, Inc., USA; dissolved in normal saline) at 8 mg/kg (Rostami et al. 2022). Metformin hydrochloride (Cat # PHR1084, Merck, Germany) was solubilized in Cremophor as the vehicle and administered daily via the gavage one h after TMT for 3 weeks. Dose of metformin was chosen from our earlier study on its neuroprotective effect in amyloid β phenotype of AD (Khaleghi-Mehr et al. 2023). Study experimental protocol is outline in Fig. 1.

Y Maze

The Y maze was made of black Plexiglas and composed of three arms and a central area. Each rat was tested for 8 min. The Y maze allows the animals to freely explore the three arms of the maze, with a natural tendency to explore an arm that was not visited immediately before. A high percentage of alternation between the three arms is indicative of good spatial memory. The maze was wiped with diluted ethanol between sessions and the percentage of alternation was calculated as described in a past study (Rostami et al. 2022).

Novel Object Discrimination

This test was done in 1 day in two 5-min sessions, with an interval of 4 h. In the first session, the rats were exposed to two identical objects. Four hours later, one of the objects was randomly replaced with another one. In both stages,

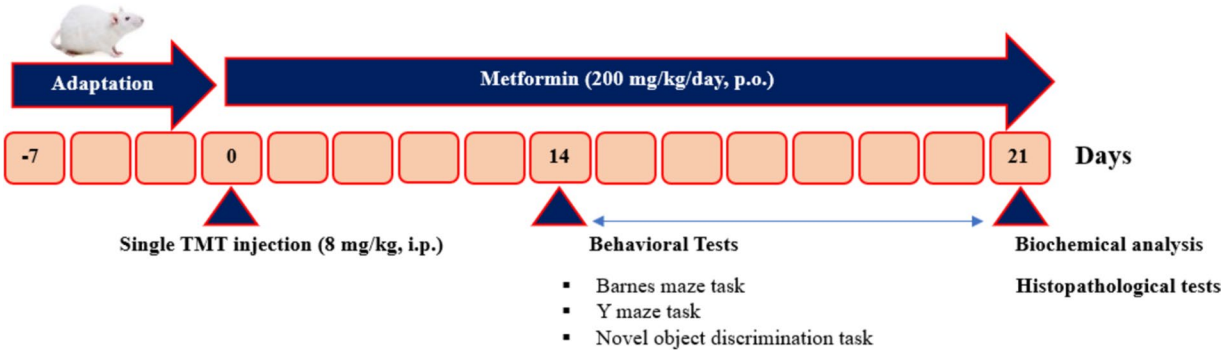

Fig. 1 Design of the study. Trimethyltin (TMT) was intraperitoneally given to generate model of cognitive dysfunction and neuronal degeneration. Metformin was orally given at 200 mg/kg for 3 weeks

exploration of each object was recorded. Discrimination index was defined as the time spent exploring the novel object relative to the total time, as reported before (Rostami et al. 2022).

Barnes Maze

This maze is a spatial memory task. Maze had a flat circular field with a distance of 100 cm above the floor and a diameter of 120 cm, and had 20 holes at a diameter of 10 cm in its periphery. In addition, a white light bulb was placed in the center to create an aversive stimulus. This experiment had two sessions: training and probe. Each rat had 2-min exploration in each session. Escape response from the lit arena and learning the position of escape box was trained. In the probe test, latency to find the escape box and the number of errors (number of explorations of holes different from the escape box) were recorded, with its protocol reported before (Rostami et al. 2022).

Tissue Homogenate Preparation

Rats were anesthetized and euthanized with ketamine (100 mg/kg) (Cat # 36408/3000, Alfasan, Netherlands) and xylazine (15 mg/kg) (Cat # 36408/3007, Alfasan, Netherlands). The brain was separated for homogenate preparation (right side) and histochemical assessment (left side in 10% formalin solution). Hippocampal tissue was homogenized in cold 150 mM Tris buffer (pH 7.4). The obtained homogenate was centrifuged at 7826 × g for 15 min (4 °C) to obtain supernatant. All biochemical tests and histological analyses were conducted in duplicate and their average was taken for each sample. For biochemical tests, inter-assay CV was less than 12% and intra-assay CV was less than 10%. For standard curves and formulae, if any, we used Microsoft Excel 2016.

Total protein concentration was determined using the bicinchoninic acid kit, Kiazist, Iran (Cat # KBCA96), in which proteins reduce bivalent Cu into monovalent Cu at 55 °C for 30 min and absorbance was taken at 560 nm and with albumin as the standard.

Quantification of Presenilin 1, *p*-Tau, Nrf2, TNF, Il-10, and AMPK

ELISA method was applied for the measurement of TNF (Cat # sc-52746; RRID # AB_630341), presenilin 1 (Cat # sc-365495; RRID # AB_10844473), p-Tau (Cat # sc-32275; RRID # AB_628325), interleukin-10 (IL-10) (Cat # sc-365858; RRID # AB_10859554), and AMP-activated protein kinase (AMPK) (Cat # sc-398861) and detection horseradish peroxidase (HRP)-conjugated (Cat # sc-516102; RRID # AB_2687626) antibodies, all from Santa Cruz Biotechnology, Inc. (USA) and results were brought in pg/mg of protein. For each parameter, ODs were determined at 450 nm and these were converted into actual concentration using relevant standard curves. Specificity of the used antibodies in this study had been determined by our supplier, i.e., Santa Cruz Biotechnology, Inc. (USA).

Measurement of Oxidative Stress and Cell Death-Related Factors

Malondialdehyde (MDA) level was measured using a commercial kit from KiaZist, Iran (Cat # KMDA96) using thiobarbituric acid in a diluted solution of glacial acetic acid and heating at 95 °C for 20 min and with tetraethoxypropane as the standard. Absorption was read at 535 nm and data were converted into nmol/mg of protein.

For measuring superoxide dismutase (SOD) activity, a commercial kit (Cat # 706002, Cayman Chemical, USA) was used utilizing a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. In this test, sample and radical detector solution were reacted with xanthine oxidase and with standard as erythrocyte SOD. After 30 min, absorbance was taken at 445 nm and SOD activity was calculated from an equation obtained

from the linear regression of the standard curve. One unit of SOD activity was defined as the amount of enzyme needed for exhibiting 50% dismutation of superoxide radicals. Final data were reported as SOD activity/mg of protein.

For measuring catalase activity, a commercial kit (Cat # KCAT96, KiaZist, Iran) was used using methanol, hydrogen peroxide, potassium hydroxide solution, purpald, and potassium periodate and absorbance was read at 550 nm. Final data were presented as catalase activity/mg of protein and with formaldehyde as the standard.

To determine caspase 1 as a pyroptosis index, a commercial assay kit from Abcam, USA (Cat # ab273268) was used with YVAD-p-NA as the substrate and final production of chromophore p-nitroanilide and detection at 405 nm and data were presented as ODs of samples.

For determining caspase 3 as an indicator of cell death, DEVD-p-NA (Abcam, USA; Cat # ab39401) was used as the substrate and final production of chromophore p-nitroaniline and absorbance was detected at 405 nm and data were presented as ODs of samples.

Acetylcholinesterase (AChE) Activity Determination

For this test, AChE kit from Abcam, USA (Cat # ab138871) was used with its reagent containing DTNB and acetylthiocholine and after 30 min, absorbance was taken at 408 nm. To exclude butyrylcholinesterase, we also used in parallel donepezil as a specific inhibitor of AChE. Final data were presented in nmol/min/mg of protein.

Histological Studies

Hippocampal blocks were processed and embedded in paraffin and sectioned on a rotary microtome (DidSabz, Urmia, Iran) and 5-µm coronal sections were taken. Alternate sections were stained with Cresyl violet acetate (Cat # C5042, SigmaAldrich, USA). CA1 pyramidal neurons irrespective of their size were counted at planes 3.3–3.8 mm behind the bregma and at 1.2–2.4 mm from the midline in an area of 0.1 mm^2 in accordance to the rat stereotaxic atlas. For counting, at least five sections and 50 µm apart for each sample were assessed twice and their average was obtained. Cells with visible boundary and nucleolus were counted. ImageJ 1.49 (NIH) was used for this analysis.

For glial fibrillary acidic protein (GFAP) immunohistochemistry (IHC), sections were exposed to primary monoclonal antibody against GFAP (Cat # sc-166481, RRID # AB_2294569; 1:65), then with secondary horseradish peroxidase (HRP)-conjugated antibody (Cat # sc-51s6102, RRID # AB_2687626; 1:80), both from Santa Cruz Biotechnology, USA, and final reaction with 3,3′ -diaminobenzidine (Cat # sc-209686, Santa Cruz Biotechnology, USA) and hydrogen peroxide. Sections were counterstained with Hematoxylin (15 s) and IRA for GFAP-reacted astrocytes was determined at planes 3.3–3.8 mm behind the bregma and at 1.2–2.4 mm from the midline in an area of 0.1 mm^2 in accordance to the rat stereotaxic atlas. For assessment of GFAP immunoreactivity (IRA), at least four sections from each animal and 50 µm apart, were evaluated twice and their average was obtained. GFAP IRA was assessed in the stratum radiatum area which is rich in GFAP-immunoreactive astrocytes. The GFAP-positive area, marked in brown, was calculated with the exclusion of background staining from the measurement. All histologic assessments were done employing OBN 141 microscope (KERN & SOHN GmbH, Germany) and using ImageJ 1.49, NIH.

Statistical Tests

Statistical tests were done in GraphPad Prism 9.5 and data were presented as means ± SD. After ascertaining normal distribution of data for each factor in Shapiro–Wilk test and testing for outliers by Grubbs' method, the statistical tests two-way ANOVA and Tukey post-test were used with p less than 0.05 as significant. To assess variance homogeneity, we used Bartlett test. Results for data normality using Shapiro–Wilk test and variance homogeneity using Bartlett test have been brought in the Supplement 1. For evaluation of relationship between the designated factors, Pearson linear correlation analysis was used.

Results

This research work was conducted to show the effect of metformin in reversing cognitive deficits following TMT neurotoxic effect with employment of different behavioral tests besides a multitude of biochemical and histochemical analyses.

Metformin Mitigated TMT Behavioral Deficits

To analyze short-term spatial and working memory, the Y-maze test was used (Kraeuter et al. 2019). TMT reduced the percentage of Y maze alterations when administered alone ($p < 0.001$), but this reduction was significantly less when administered together with metformin. Metformin alone had no effect (Fig. 2A, two-way ANOVA, interaction TMT x metformin: $F1, 28 = 10.52$, $p = 0.003$).

Novel object discrimination test was used to assess the animals' capacity to memorize previously-visited objects and to explore novel objects (Hawiset et al. 2023), as shown by the discrimination index (Fig. 2B). TMT group demonstrated a significantly lower discrimination score ($p < 0.001$) as compared with the control data and this deficit was less in metformin-treated TMT group. Metformin alone did not have a

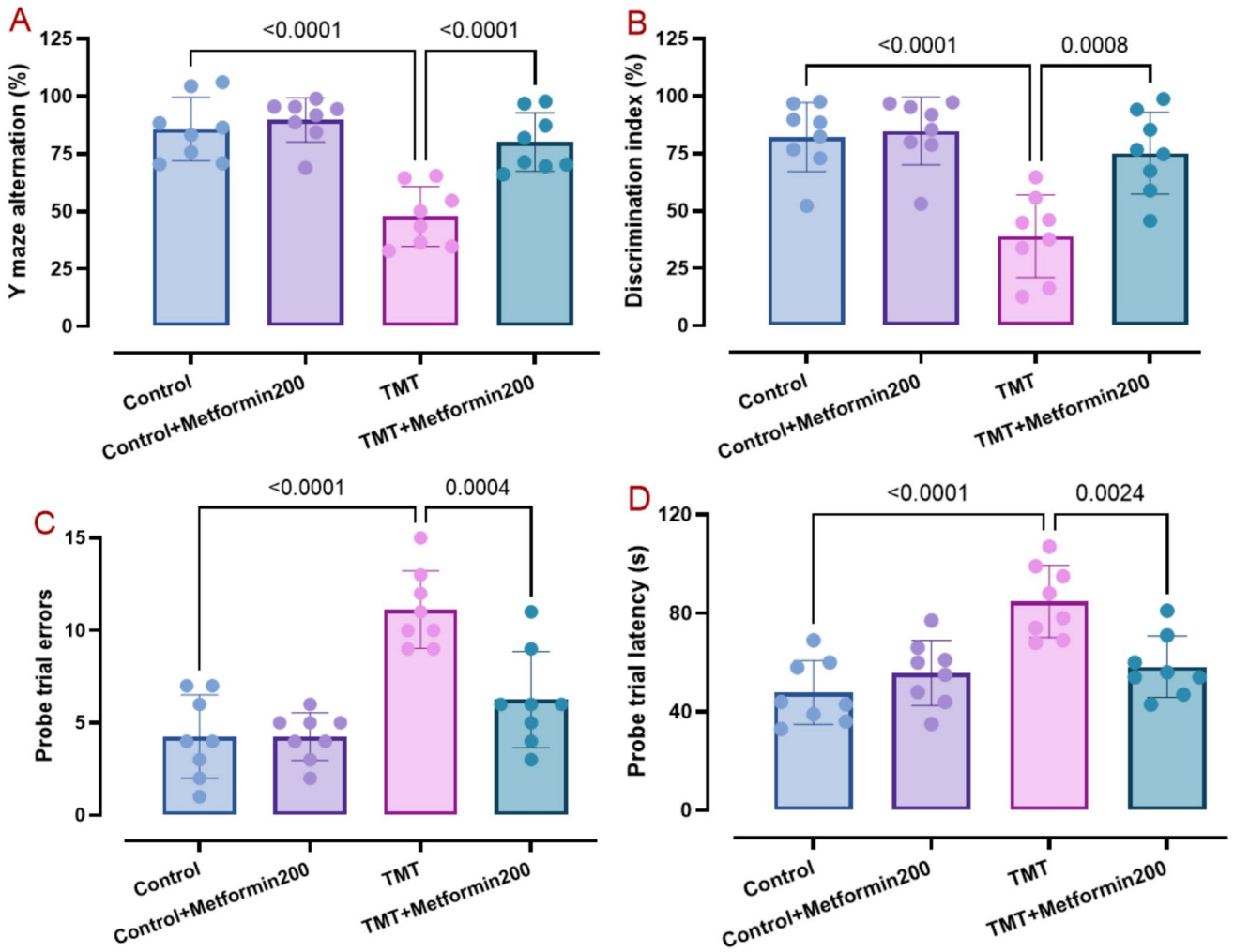

Fig. 2 Data of behavioral tests comprising Y maze (pane **A**), novel object discrimination (pane **B**), and Barnes maze (panes **C** and **D**). Metformin mitigated TMT behavioral deficits in these tasks, as analyzed by two-way ANOVA and Tukey tests. (means ± SD, $n = 8$/group)

significant effect (Fig. 2B, two-way ANOVA, interaction TMT x metformin: F1, 28 = 8.33, $p = 0.007$).

Figure 2C and D present the impact of treatments on the Barnes maze performance to evaluate in more detail spatial learning and memory (Tancheva et al. 2023). Our data analysis showed that TMT group had significantly higher errors ($p < 0.001$) and greater latency ($p < 0.001$) and metformin treatment of the TMT group significantly reduced errors ($p < 0.001$) (Fig. 2C, two-way ANOVA, interaction TMT x metformin F1, 28 = 10.62, $p = 0.002$) and latency ($p = 0.002$) (Fig. 2D, two-way ANOVA, interaction TMT x metformin: F1, 28 = 13.35, $p = 0.001$). In addition, metformin alone did not have a significant effect on these factors.

Metformin Attenuated Cell Death, Inflammation, Pyroptosis, and Oxidative Stress, and Markers of AD Pathology

AD phenotype is typified with elevated brain levels of oxidative, apoptotic, and inflammatory burden (Zhang et al. 2023) as well as enhancement of presenilin 1 (Martinez-Feduchi et al. 2024) and *p*-Tau (Pradeepkiran et al. 2024). Accordingly, we measured hippocampal levels of relevant factors. Data analysis showed elevated levels of MDA (Fig. 3A, two-way ANOVA, $p < 0.001$; interaction TMT x metformin: $F_{1, 24} = 5.90$, $p = 0.02$), TNF (Fig. 4A, two-way ANOVA, $p < 0.001$, interaction TMT × metformin:

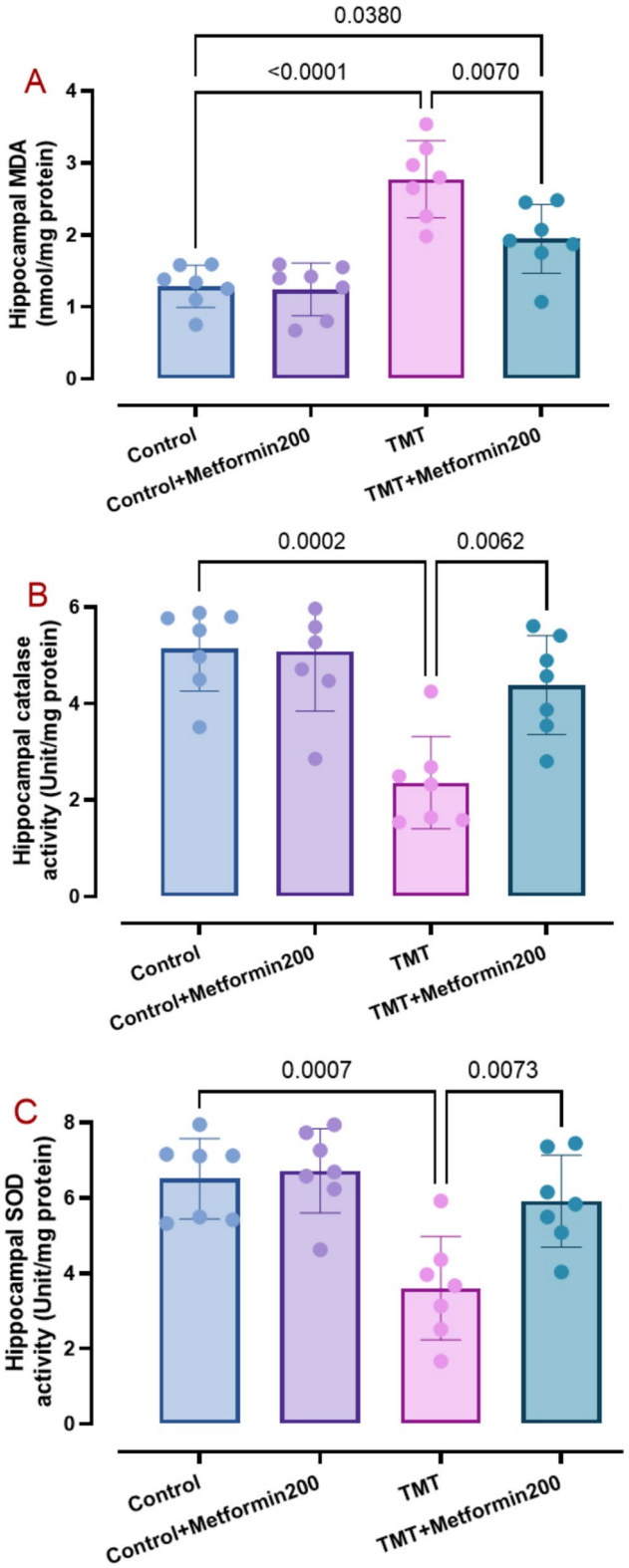

Fig. 3 Data of oxidative stress factors comprising MDA (pane **A**), catalase activity (pane **B**), and SOD activity (pane **C**). Metformin properly attenuated TMT oxidative burden and improved antioxidants, as analyzed by two-way ANOVA and Tukey tests. (means ± SD, $n = 7$/group)

$F_{1, 24} = 5.54$, $p = 0.02$), presenilin 1 (Fig. 4C, two-way ANOVA, $p = 0.003$, interaction TMT × metformin: $F_{1, 24} = 4.55$, $p = 0.04$), p-Tau (Fig. 4D, two-way ANOVA, $p < 0.001$, interaction TMT x metformin: $F_{1, 24} = 7.45$, $p = 0.01$), caspase 1 (Fig. 5A, two-way ANOVA, $p = 0.002$, interaction TMT × metformin: $F_{1, 24} = 8.21$, $p = 0.008$), and caspase 3 (Fig. 5B, two-way ANOVA, $p < 0.001$, interaction TMT × metformin: $F_{1, 24} = 8.83$, $p = 0.006$) and lower levels of catalase activity (Fig. 3B, two-way ANOVA, $p < 0.001$, interaction TMT × metformin: $F_{1, 24} = 7.10$, $p = 0.01$) and SOD activity (Fig. 3C, two-way ANOVA, $p < 0.001$, interaction TMT × metformin: $F_{1, 24} = 5.36$, $p = 0.02$) in the TMT group. On the other hand, metformin significantly reversed MDA ($p = 0.007$), SOD ($p = 0.007$), catalase ($p = 0.006$), TNF ($p = 0.04$), presenilin 1 ($p = 0.005$), *p*-Tau ($p = 0.02$), caspase 1 ($p = 0.03$), and caspase 3 ($p = 0.01$) besides significant elevation of IL-10 (Fig. 4B, two-way ANOVA, $p < 0.001$, interaction TMT x metformin: $F_{1, 24} = 13.87$, $p = 0.001$). In addition, metformin alone did not have a significant effect on these parameters.

Metformin Improved the Energy Homeostasis Marker AMPK

The energy homeostasis marker AMPK in association with mitochondrial biogenesis play important roles in AD-related pathogenesis (Cai et al. 2012). Thus, we investigated how metformin affects its hippocampal levels following TMT injury. Statistical analysis of data for AMPK (Fig. 5C, two-way ANOVA, interaction TMT x metformin: $F_{1, 24} = 6.82$, $p = 0.01$) showed that hippocampal level of AMPK is lower in the TMT group ($p = 0.003$) when compared to the control data and metformin at 200 mg/kg caused its significant improvement ($p = 0.02$) in relation to the TMT group. Additionally, metformin alone did not have a significant impact on this factor.

Metformin Decreased Hippocampal Activity of AChE

AD-related pathology is associated with cholinergic dysfunction (Tripathi et al. 2024). Thus, the impact of TMT injection and oral metformin on the hippocampal activity of AChE was assessed in different groups. Our data analysis (Fig. 5D, two-way ANOVA, interaction TMT x metformin: $F_{1, 24} = 4.67$, $p = 0.04$) showed that TMT group had pronounced enhancement of AChE ($p < 0.001$, an increase by 136.2%) in relation to the control data. Conversely, metformin at 200 mg/kg was capable to reduce AChE activity in the TMT group ($p = 0.01$). However, metformin alone did not cause a significant change of AChE activity.

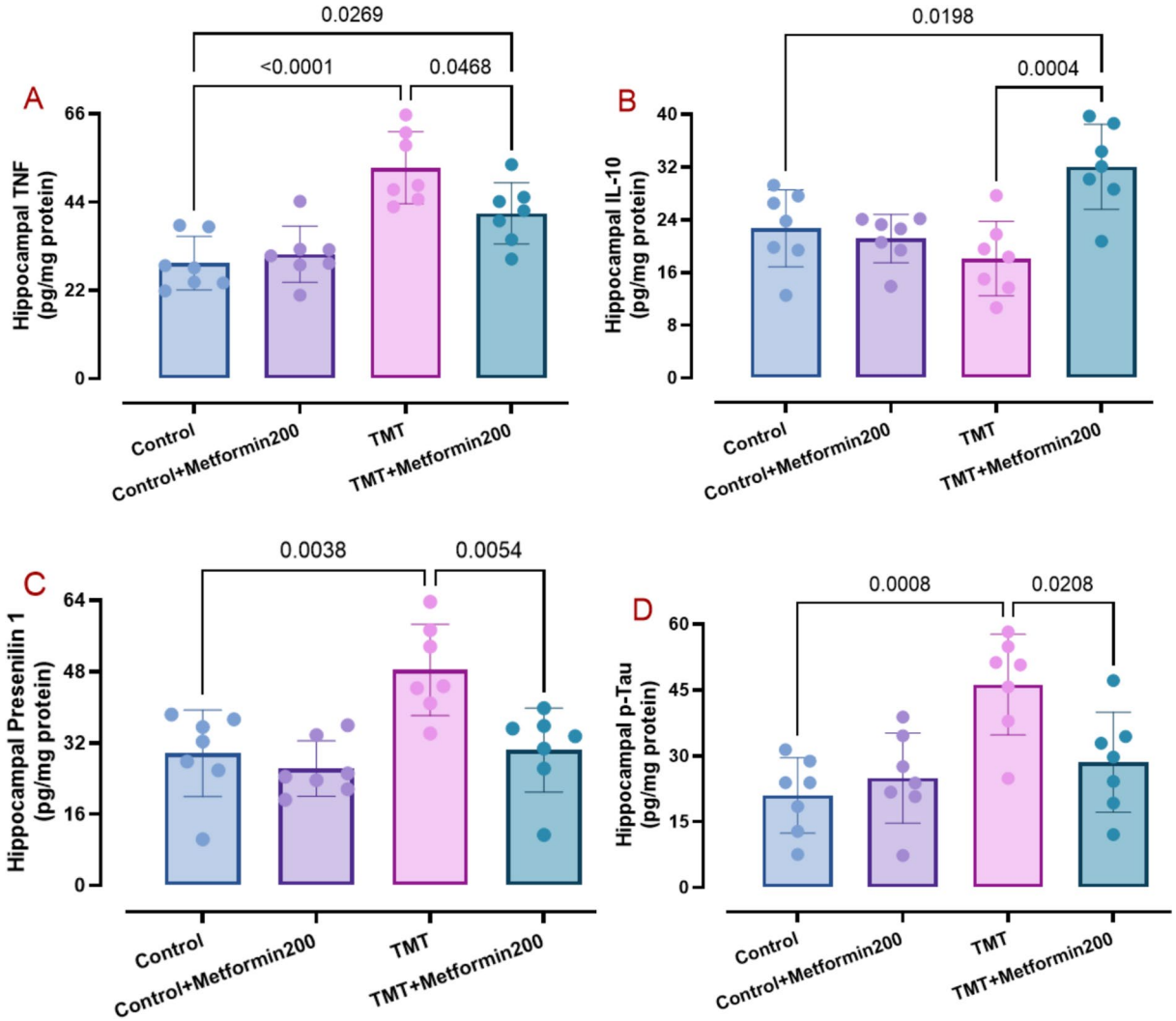

Fig. 4 Data of inflammation-associated factors including TNF (pane **A**) and IL-10 (pane **B**) in addition to AD pathology markers consisting of presenilin 1 (pane **C**) and p-Tau (pane **D**), as analyzed by two-way ANOVA and Tukey tests. Metformin reversed TMT-induced changes regarding these factors. (means ± SD, $n = 7$/group)

Metformin Prevented Hippocampal CA1 Astrogliosis and Neurodegeneration

Robust neurodegenerative changes (Tripathi et al. 2024) and reactive astrogliosis (Fontana et al. 2023) are notably observed in brain of AD patients. Upon Nissl staining (Fig. 6A), we noted karyorrhexis and pyknosis, necrotic changes, and even cell membrane disintegrity in the TMT-injected rats in the hippocampal CA1 subfield and these improper changes were less frequent in the TMT group treated with metformin at 200 mg/kg. Our data analysis (two-way ANOVA, interaction TMT x metformin: $F_{1, 20} = 6.20$, $p = 0.02$) showed that TMT injection notably reduced cell density ($p < 0.001$) and metformin at 200 mg/kg significantly attenuated this decline ($p = 0.02$). Additionally, metformin treatment alone did not significantly change number of Nissl-stained neurons.

Measurement of GFAP immunoreactivity (IRA) (Fig. 6B) around the CA1 subfield (stratum radiatum) as an index of astrocytic response to toxic insult (two-way ANOVA, interaction TMT x metformin: $F_{1, 20} = 21.88$, $p < 0.001$) showed higher IRA in the TMT-injected rats ($p < 0.001$) (an increase of 227.3%) and GFAP IRA was less noted in the TMT group treated with metformin at 200 mg/kg ($p < 0.001$). Moreover, metformin treatment alone did not produce significant change of GFAP IRA.

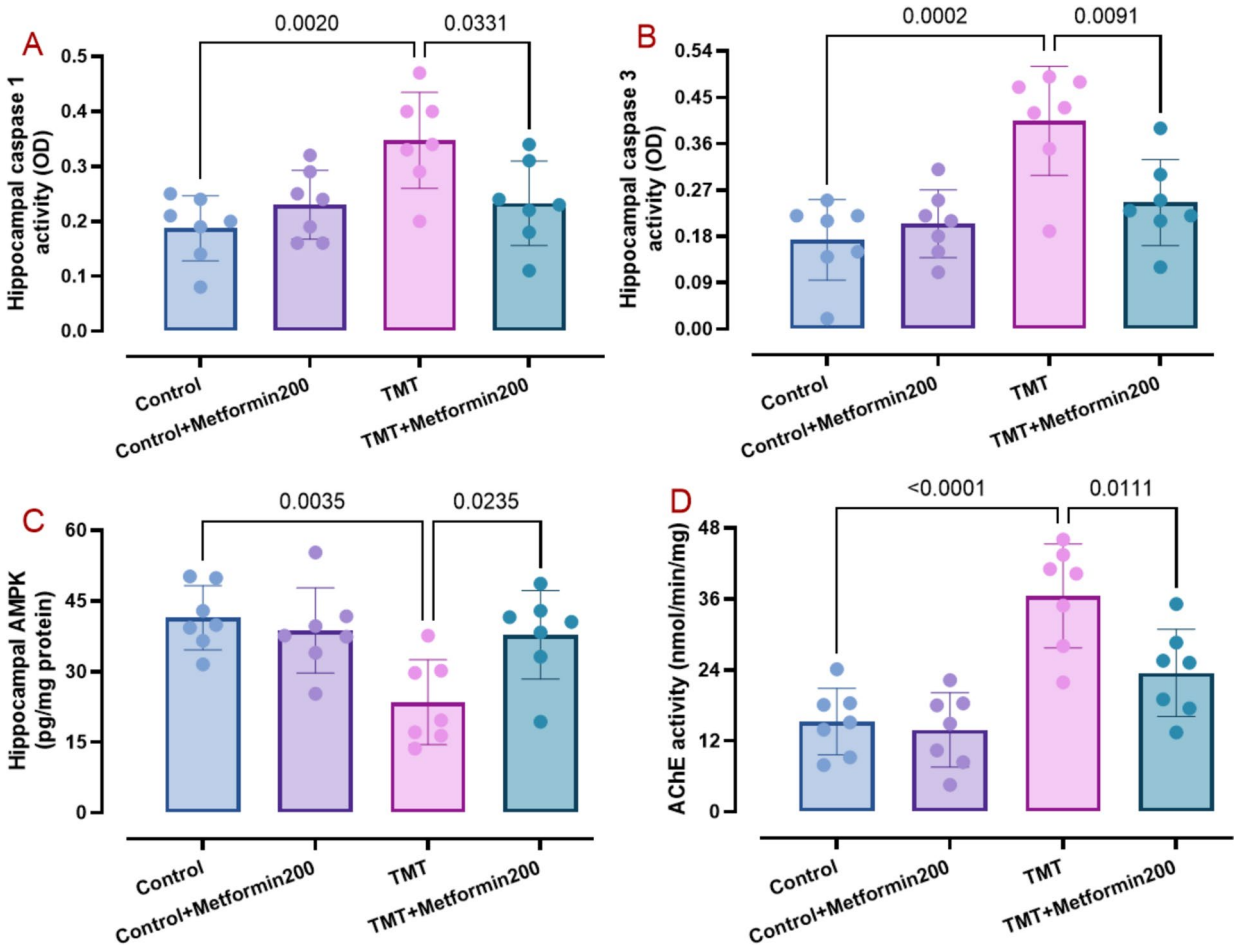

Fig. 5 Data for hippocampal level of caspase 1 (pane **A**), caspase 3 (pane **B**), AMPK (pane **C**), and AChE activity (pane **D**), as analyzed by two-way ANOVA and Tukey tests. Metformin was capable to reverse TMT-induced changes regarding these factors. (means ± SD, $n = 7$/group)

Correlation Analysis for Oxidative and Neurodegenerative Changes

To explore the relationship between oxidative and neurodegenerative changes for TMT and metformin-treated groups, we conducted Pearson correlation analysis for MDA as a specific oxidative factor and the number of CA1 pyramidal neurons as a relevant factor for neurodegeneration. Such analysis showed a significantly negative relationship for the TMT group ($r = -0.92$, $p = 0.007$) and the metformin-treated TMT group ($r = -0.90$, $p = 0.01$).

Discussion

In the current work, protective impact of metformin against TMT-instigated neurotoxicity and neurodegeneration was investigated. We observed beneficial effects of antidiabetic metformin in the mitigation of hippocampal CA1 neurodegeneration alongside its reduction of deficits in various behavioral tests. Spatial memory deficits correlate well with hippocampal neuronal injury (Geloso et al. 2011; Thong-asa et al. 2020). TMT neurotoxin causes profound damage to the hippocampal CA1 subfield (Thong-asa et al. 2020), as observed in our study. This high vulnerability of CA1 area to TMT is related to its lower level of calcium-binding protein calretinin as a key protein to combat TMT-provoked calcium overload (Geloso et al. 1998; Thong-asa et al. 2020).

Our histochemical findings indicated that most of CA1 pyramidal neurons in the TMT group are degenerated and exhibiting robust pyknosis and shrinkage as signs of cell death besides their disorganized appearance, as reported before (Thong-Asa et al. 2020). Hippocampal CA1 subfield plays pivotal role in spatial cognition (O'Keefe 1993). Significant injury of CA1 area coexisted with deficits of spatial learning and memory in the TMT-intoxicated group in this study. In contrast, oral metformin at 200 mg/kg was associated with better cognition as well as preservation of CA1 neuronal organization, indicating its neuroprotective effect. In support of these data, metformin can prevent

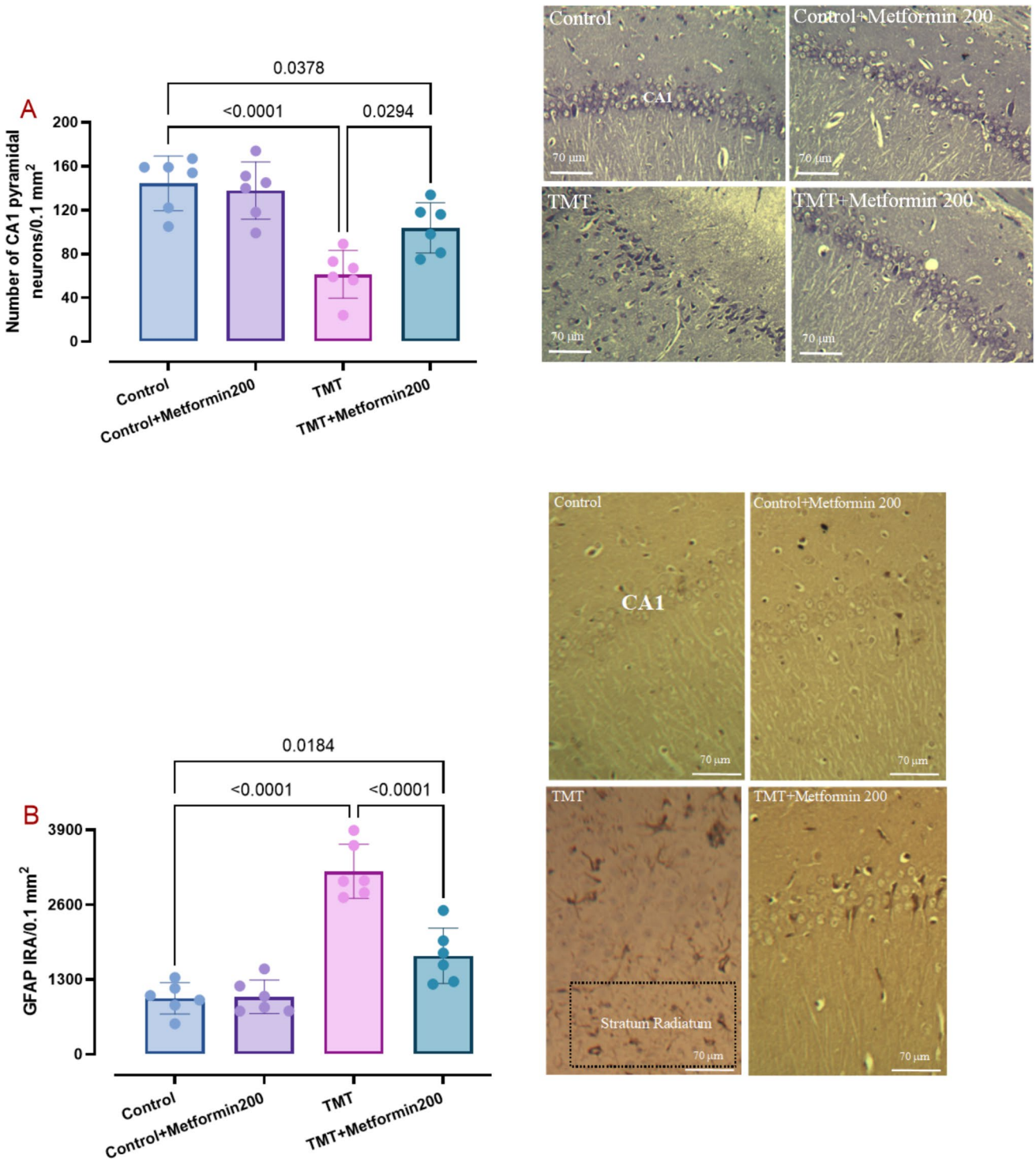

Fig. 6 Density of CA1 Nissl-stained neurons (pane **A**) and immunoreactivity for GFAP as a marker of astrocytes (pane **B**) and related photomicrograph. Metformin at 200 mg/kg was able to attenuate CA1 neurodegeneration and astrogliosis. Dotted-border rectangle shows stratum radiatum area for assessment of GFAP immunoreactivity. Two-way ANOVA and Tukey tests. (means ± SD, $n = 6$/group)

cognitive dysfunction and histopathological changes in a model of sporadic AD (Rabieipoor et al. 2023) and can ameliorate sevoflurane-instigated neurogenesis damage and cognitive decline linked to its enhancement of Nrf2 and glucose-6-phosphate dehydrogenase expression and its attenuation of cell death (Fan et al. 2023), which was also shown in this study for cell death and pyroptotic factors including caspases 1 and 3.

TMT neuronal injury is attributed to various pathologic mechanisms including oxidativestress, loss of mitochondrial integrity, apoptotic events, calcium overload, and neuroinflammation (Geloso et al. 2011; Go et al. 2023). In this study, higher hippocampal levels of oxidative factors such as MDA, the pro-inflammatory factor TNF, cell death factors consisting of caspases 1 and 3 and concomitant lower activity of catalase and SOD were shown in the rats from the TMT group. Conversely, metformin treatment at 200 mg/kg was successful to significantly reverse these abnormal alterations and accordingly protects hippocampus against TMT toxicity. In support of these data, metformin can exert neuroprotective effect via activating AMPK pathway in 3-acetylpyridine model of cerebellar ataxia which was associated with lower levels of pro-inflammatory cytokines (Atella et al. 2024), it is capable to protect hippocampal slices against methylglyoxal glutamatergic toxicity and to lower neuroinflammation (Vizuete et al. 2023), and can also exert neuroprotective effect and lower oxidative stress linked to GSK3β cascade and enhancement of antioxidants following glutamate neurotoxicity (Oruc et al. 2024).

There is convincing evidence that NFT tau phosphorylation and Aβ plaques as typical pathological markers of AD increase in the brain of AD patients (Xia et al. 2021; Yu and Wu 2021) and also in TMT-induced AD phenotype (Park et al. 2019). Higher level of hyperphosphorylated tau is associated with cognition impairment through prompting neuronal damage and apoptosis and with final appearance of neurofibrillary tangles (NFTs) (Park et al. 2019). Higher hippocampal levels of presenilin 1 and p-tau correlated with TMT-induced neurotoxicity, neuroinflammation, and culminant apoptosis and cell death (Brown et al. 2019; Nurmasitoh et al. 2023), as was similarly shown in the present work. Conversely, oral metformin was able to prevent such alterations regarding p-tau and presenilin 1. Validating this finding, antidiabetic metformin could attenuate hippocampal and cortical tau pathology and amyloid-β level through enhancement of microglial autophagy, increasing number of microglial cells around Aβ plaques, and promotion of phagocytosis of tau in APP/PS1 model of AD (Chen et al. 2021b). In our study, metformin administration at a dose of 200 mg/kg to the TMT-injured rats was associated with significantly lower hippocampal level of presenilin 1. However, Picone et al. (2015) have shown elevated level of presenilin 1 in the brain of normal mouse following metformin administration for 7 days via drinking water (2 mg/ml) which was comparable to a dose of 300 mg/kg/day of metformin (Picone et al. 2015). Interestingly, we did not find significant change of presenilin 1 in the control group treated with metformin at a dose of 200 mg/kg. A substantial volume of recent researches supports the beneficial impact of metformin on reducing the risk of AD through reducing its pathogenic factors (Ale Mahmoud Mehraban et al. 2024; Khaleghi-Mehr et al. 2023; Ou et al. 2018; Pilipenko et al. 2020). These reports clearly show that further research works are still required to evaluate the exact effect of the antidiabetic drug metformin on the AD-related factors.

Cholinergic dysfunction is observed in parallel to the cognitive impairment following TMT administration (Rostami et al. 2022; Tu et al. 2017). In this respect, a similar condition may also occur in animal models of AD with enhanced activity and/or production of AChE (Elseweidy et al. 2023; Gu et al. 2023; Khaleghi-Mehr et al. 2023). For this reason, AChE inhibitors have beneficial effects in clinical attenuation of AD symptoms (Moss and Perez 2021). AChE activity increases in some regions of the brain in association with amyloid beta plaques and even in blood samples of AD patients (Carvajal and Inestrosa 2011). It has been reported that increased AChE is associated with acceleration of Aβ peptides deposition in AD and it could increase Aβ neurotoxicity (Carvajal and Inestrosa 2011). Degeneration of cholinergic system and cognitive deficits are enhanced in neurodegenerative disorders like AD (Chen et al. 2022). The brains from AD patients exhibit marked neurodegeneration in parallel to reduction of cholinergic neurons and a severe deficiency of the neurotransmitter ACh (Bowen et al. 1976). AChE directly binds to presenilin 1 as an important enzyme in the processing of amyloid beta and also increases its expression and in this way increases the level of amyloid beta which aggravates cognitive functions (Campanari et al. 2014; Ramos-Rodriguez et al. 2013). In addition, abnormal changes of brain cholinergic system can provoke tau phosphorylation, neuroinflammation, and apoptosis (Chen et al. 2022). Therefore, higher hippocampal activity of AChE following TMT challenge in this study may be attributed to its interaction with presenilin 1, amyloid beta, and tau protein. In this regard, it has been indicated that both amyloid beta and hyperphosphorylated tau can influence AChE expression (García-Ayllón et al. 2011). Meanwhile, TMT-induced neurodegeneration is associated with higher AChE activity (Loullis et al. 1985). In addition, it is also possible that prevailing AChE molecules released from degenerated cholinergic neurons may be another explanation for enhanced activity of AChE. All of these suggestions besides controversies regarding changes of AChE under cognitive decline conditions have merit for further research studies (García-Ayllón et al. 2011). Conversely, metformin administration at 200 mg/kg was associated with lower hippocampal activity of AChE. Corroborating this evidence, it has been shown that metformin can lessen amyloid β-instigated cognitive impairment via mitigation of hippocampal oxidative/nitrosative stress and inflammation which is associated with lower activity of AChE (Khaleghi-Mehr et al. 2023) and can inhibit cardiometabolic-associated cognitive impairment in rats under high fat diet, partly linked to its downregulation

AChE activity and monoamine oxidase (Chellammal et al. 2022).

It has been reported that Aβ overproduction by sequential cleavage of amyloid precursor protein (APP) is linked to neuronal dysfunction and demise (Assefa et al. 2020; Selkoe and Hardy 2016). AMPK activation affects Aβ metabolism and is accordingly involved in AD pathogenesis (Assefa et al. 2020). However, there exists controversial reports in this field. For instance, contrary to our findings, Liu et al. (2021) have shown higher hippocampal expression of p-AMPK after 1–6 days in mice injected with TMT (Liu et al. 2021). This discrepancy may be due to time of assessment of AMPK, which has been after 21 days in our work. It has been even claimed that AMPK activation may not have neuroprotective effect and may even have detrimental outcomes such as Aβ production and tau phosphorylation (Cai et al. 2012). Hence, it is still unknown whether AMPK could be postulated as a promising therapeutic target for AD and further researches will be needed to elucidate the role of AMPK in AD pathology. In contrast, metformin at 200 mg/kg improved p-AMPK in the TMT-intoxicated group. In agreement with this finding, neuroprotective effects of metformin may be principally ascribed to its activation of AMPK signaling (Jinpiao et al. 2020) which causes upregulation of brain-derived neurotrophic factor (BDNF) expression which has pivotal roles in synaptic neurotransmission and memory consolidation processes (Miranda et al. 2019). Additionally, metformin can alleviate cognitive impairments in D-galactose model of aging through enhancement of AMPK/BDNF/phosphoinositide 3-kinase (PI3K) pathway (Ameen et al. 2022).

Although considerable development has been gained to unravel the involved pathogenic mechanisms for AD in addition to finding novel therapeutics, it still remains an incurable disorder. Widely used animal models for AD are not able to satisfactorily represent its pathological events (Chen and Zhang 2022). One of the established animal models for testing efficacy of promising agents against AD-like neurodegeneration and cognitive deficit is through intraperitoneal injection of TMT (Chvojkova et al. 2021). However, TMT like other neurotoxicants could not mimic all pathophysiological aspects of AD and it might be considered more a useful research tool for evaluation of hippocampus-specific neurodegeneration (Lee et al. 2016). In other words, TMT is not an AD-specific neurotoxin to precisely simulate AD-associated cognitive, pathological, and biochemical alterations and it induces other behavioral changes including seizure-like phenotype, irritability, body weight loss, hypothermia, tremor, and even tail mutilation, as reviewed before (More et al. 2016).

Lack of a priori sample size calculation, Western blotting experiments for the analyzed factors, and verification of the specificity of the used antibodies employing relevant scientific methods such as Knockout Validation protocol was some limitations of the present study. In addition, since we did not perform experiments on the involvement of AMPK pathway in the beneficial effect of metformin after its inhibition and/or blockade, this limitation should also be taken into account in the future pertinent works.

Conclusions

To conclude, this study provided essential insight into how metformin exerts its positive preclinical effects in TMT-induced neurodegeneration. This may pave the way to develop new therapeutics to combat against cognitive deficits in AD-like pathologies and type 2 diabetes. However, exact mechanism of metformin effect to counteract TMT neurotoxicity remains to be explored.

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Author Contributions MT performed experiments and helped in manuscript writing, MR designed the study and protocol of experiments, supervised conductance of experiments and wrote the manuscript, and RS helped in study design, data analysis, and writing the manuscript.

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Data Availability Data generated and analyzed during the present study will be available from the corresponding author on reasonable request.

Declarations

Conflict of interest There is no conflict of interest to express.

Ethical Approval All experimental procedures of this study were conducted under ethics committee supervision of Shahed University (# IR.SHAHED.REC.1401.131) that was in accordance to NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize number of animals and to minimize their sufferings.

Informed Consent Not applicable.

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