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Metformin restores cognitive dysfunction and histopathological deficits in an animal model of sporadic Alzheimer's disease

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ABSTRACT

Background: Metformin has been introduced as a neuroprotective agent in recent years. Here we evaluate the therapeutic effects of metformin in sporadic mouse model of Alzheimer's disease (SAD).
Methods: AD was induced by streptozotocin (STZ, 0.5 mg/kg) on days 1 and 3. Metformin (MET, 200 mg/kg per day) was used for two weeks. Novel objective recognition (NOR) and Barnes Maze test were used to test the learning and memory. Nissl staining was used as s histological method for counting the dying neurons in different regions of hippocampus. Immunofluorescence staining against glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1) and NeuN were used to visualize reactive astrocytes, microglia and neurons, respectively.
Results: In NOR test, the discrimination indices in the STZ group were significantly lower than the control and treatment groups. Goal sector/non-goal sector (GS/NGS) ratio index in Barnes maze was increased in metformin group compared to other groups. The number of dying neurons was increased by SAD and metformin reduced it. GFAP level was increased in CA1, CA3 and cortex of STZ group and reversed following the treatment. Iba1 level was significantly higher in STZ group in CA3 and cortex regions compared to Control and decreased by metformin in CA3 and cortex. Counting $NeuN^{+}$ cells demonstrated significant reduction of neurons in DG+CA1 and CA3 after SAD induction.
Significance: Metformin decreased inflammatory cells and reactive astrocytes as well as the dying neurons in the hippocampus region and the cortex in SAD, and improved the cognitive performance.

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1. Introduction

One of the most prevalent neurological diseases of the century is Alzheimer's disease (AD) which is characterized by progressive cognitive decline [1]. It is neuropathologically characterized by the accumulation of extracellular neuritic plaques and fibrils in the brain, mainly consisting of aggregated amyloid beta (Aβ) peptides and intracellular neurofibrillary tangles (NFT), composed of hyperphosphorylated Tau (p-Tau) [2]. Soluble Aβ peptides through binding to their receptors would be responsible for generating the neurodegenerative process. Likewise, synaptic damage is mediated by soluble Aβ oligomers, which have been shown to better correlate with the disease severity than with the accumulation of insoluble Aβ peptides into plaques triggering AD pathophysiology [2]. Familial form of AD (FAD) 2) is associated with mutations in the amyloid precursor protein (APP) and presenilin-1 and 2 PSEN 1 and PSEN genes. In sporadic AD (SAD) which accounts for more than 95% of all AD patients, the hallmarks seem somewhat different, and the inflammatory processes have been proposed as the main mechanism [3]. Most of the AD cases are sporadic which are less obviously impacted by a single gene mutation. The cause of SAD is unknown and additional factors, other than genetic and age may be involved in the neurodegenerative process pointing out that AD is a multifactorial pathology. There are a number of risk factors identified in SAD development among them obesity, type 2 diabetes mellitus (T2DM) and neuroinflammation have been identified as well-known risk factors [2].

SAD affects people without a family history of the disease. Insulin resistance is one of the most important causes of SAD [2]. As the major hallmark of AD especially in sporadic AD, the innate immune cells, microglia as well as the astrocytes mediate the neuro-inflammatory response. Synaptic dysfunction, neural death and neurogenesis suppression can be done by pro-inflammatory molecules such as tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1β, IL-18, and small-molecule messengers such as nitric oxide (NO) in response to insulin resistance and the presence of Aβ toxicity in the brain. According to previous epidemiological reports, diabetes has been linked to dementia [4]. Interventions that target astrocyte and microglial priming in the preclinical phase of the disease and control their response in the brain once the AD process has been started, show anti-inflammatory strategies as new approach for AD treatment [4]. Obesity, T2DM and associated comorbidities, all have been linked to the development of late onset AD (LOAD) [5]. LOAD has recently been labeled as a "metabolic disease" (type 3 diabetes) [6] associated with inefficient glucose utilization by the brain, insulin resistance, and chronic mild inflammation in the brain [7,8].

This association could be related to inflammation, oxidative stress, vascular involvement, increased level of brain amyloid peptides, and hyperinsulinemia [9,10]. Streptozotocin (STZ) can be utilized to induce the activation of pathophysiological processes which mimics the pathophysiology of SAD [11]. STZ is produced by *Streptomycetes achromogenes* and because of its capability to impair the pancreatic β cells and induce insulin resistance, in the systemic use, it initiates diabetes. STZ inhibits the insulin receptor function in the brain and disrupts the glucose and energy metabolism [12].

Metformin is a biguanide that contains a couple of guanidine molecules [13,14] and its chemical structure is highly hydrophilic (1, 1-dimethylbiguanide hydrochloride). This antidiabetic drug reduces liver gluconeogenesis and insulin resistance, resulting in lower plasma glucose levels [15]. It can cross the blood–brain barrier (BBB) and has been previously linked to improved cognitive performance [16]. Inhibition of hyperinsulinemia results in limited formation of amyloid plaques and the advanced glycation of end-products in the brain and decreases the inflammation and oxidative stress [17]. According to studies on transgenic mice APPswe, PS1dE9 and PDAPP (J9); and AD models induced by chemicals such as mice sporadic models of AD induced by STZ and etc., metformin prevents hippocampal insults and spatial memory decline, reduces inflammation and regulates the AMPK/mTOR/S6K/Bace1 pathway. In addition, metformin increased insulin receptor sensitivity and facilitates neuronal survival [18]. According to Shi et al. the effect of metformin on neurodegenerative diseases (ND) such as dementia, AD, Parkinson's disease (PD) and Huntington's disease (HD) has been evaluated. Based on the findings, metformin showed a significant reduction in the incidence of ND in patients with T2DM [19]. Additionally, metformin was suggested to restore the abnormal blood-brain barrier transport of amyloid-β (Aβ), improve memory, and neurogenesis by activating protein kinase C/CREB binding protein (PKC-CBP) and AMPK pathways [20,21]. In a recently published study, it was shown that administration of metformin was associated with memory and learning improvement in SAMP8 mouse models of AD with accelerated aging [22]. However, to our knowledge the restorative effect of metformin on histopathological and memory deficits in animals with developed SAD model needs further clarification.

We designed the current study to evaluate the effects of metformin on an established sporadic Alzheimer's disease (SAD) in mice. We performed behavioral evaluation of the cognition performance, as well as the neuroinflammation and gliosis levels assessments. The potential of metformin to preserve neurons from further degeneration was assessed through dying neurons counting. Total neuron counts were assessed by NeuN staining. Histopathological evaluations were assessed in both hippocampus and cerebral cortex.

2. Material & methods

2.1. Animals and the interventions

In this study, 5–6-month-old male mice (C57BL/6) were used. Mice had access to food and water ad libitum and were housed in an environment with 12/12 h light/dark cycle and temperature maintained at 22 °C ± 2 °C. Attempts were made to keep the number of animals utilized to a minimum and to alleviate their suffering. All protocols and procedures followed the ethical guidelines stablished by the European Communities Council Directive 2010/63/EU. The stereotaxic surgery protocol and animal endpoint was approved at Tarbiat Modares Ethics Committee (approval number: D52/6725).

Animals were initially divided to 2 main groups: 1) eleven mice without any treatment as Control; 2) eighteen mice treated with STZ as SAD model. These mice were treated with intracerebroventricular (ICV) injections of STZ in aCSF (0.5 mg/kg) at the first and

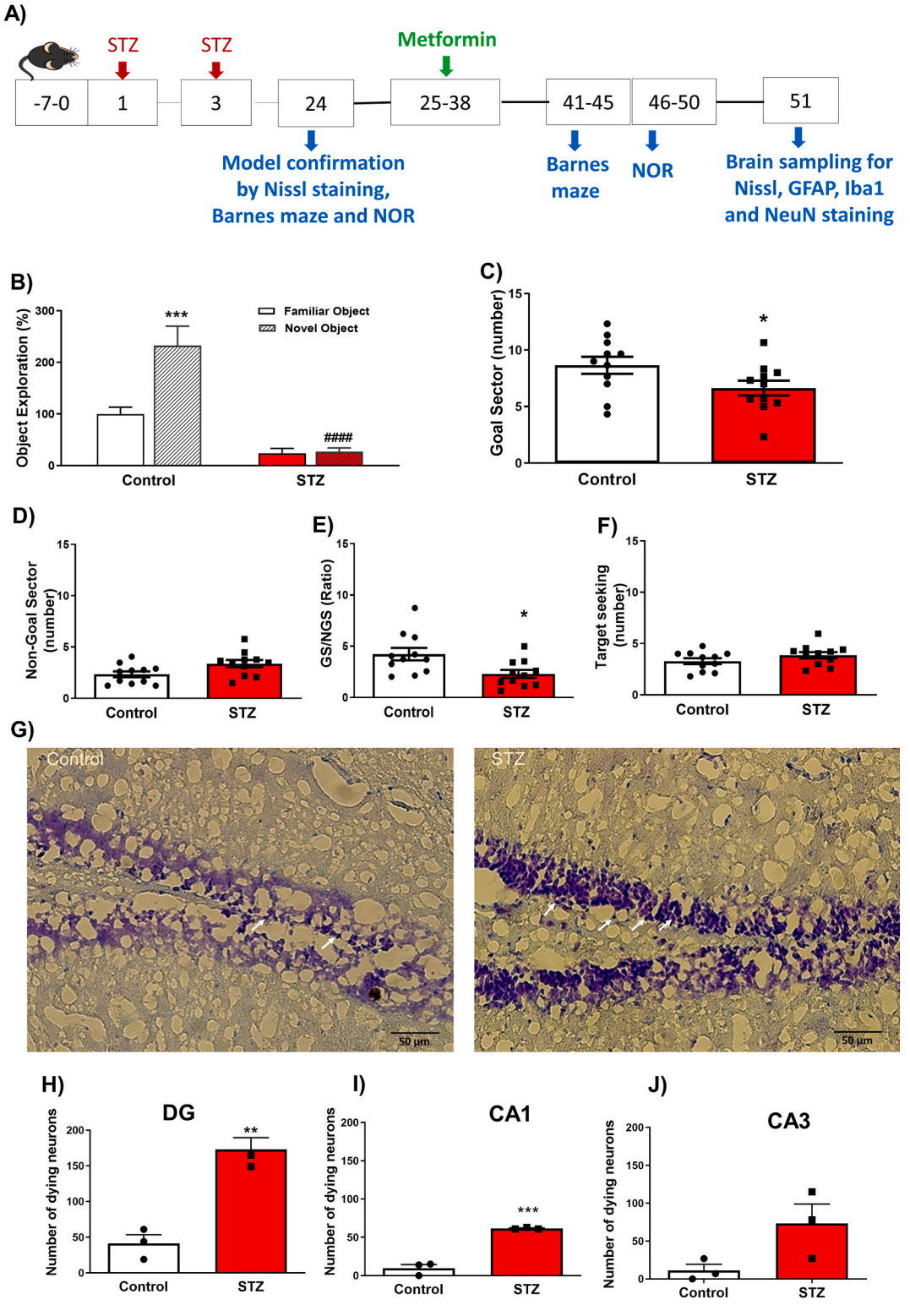

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Fig. 1. Streptozotocin (STZ) injection into mice ventricles caused cognitive impairment and neural damage as assessed 3 weeks post STZ injection. A) Schematic representation of experimental timeline. B) Novel Object Recognition (NOR) test demonstrated a significant memory loss in SAD mice compared with control group. Object exploration percentage was analyzed using two-way ANOVA. C–F) Barnes Maze test results. C: Goal Sector exploration number data showed significant differences between control and STZ model group. D: Non-Goal Sector exploration number and E: GS/NGS ratio data demonstrated significant differences between the Control and STZ model group. F: Target seeking analysis showed no significant differentiation. G-J) Nissl staining sections obtained micrographs as representatives for Control and STZ groups and the quantitative analysis data. G: Nissl-stained micrographs images of dentate gyrus represents samples of dying neurons in two experimental groups. H–J: the average number of dying neurons in DG, CA1 & CA3, and the mean differences between the groups. *P < 0.05, **P < 0.01 and ***P < 0.001 vs Control, and $^{\#\#\#\#}$P < 0.0001 vs novel object in Control.

third day of experiment [23]. First, animals were anesthetized using administration of ketamine/xylazine and placed in a stereotaxic device. The injections were done bilaterally at 0.9 mm lateral to the midline, 0.02 anteroposterior (AP) from bregma and 2.4 mm depth from dura, using two stereotaxic surgeries for each animal at days1 and 3. STZ powder (0.5 mg) was dissolved in aCSF (Ingredients of artificial cerebrospinal fluid: 147 mM NaCl, 2.9 mM KCL, 1.6 mM MgCl2, 1.7 mM CaCl2 & 2.2 mM dextrose) and injected in the volume of 2 μl in each side (total volume: 4 μl). After 21 days, mice which showed SAD phenotype, underwent the behavioral tests, then divided to 2 subgroups; 11 mice were kept as SAD group with no further treatment, and 7 SAD mice received metformin as treatment (STZ + MET group). Metformin 200 mg/kg dissolved in PBS, was injected every day (100 μl at morning and 100 μl at afternoon (every 12 h)) for 2 weeks (days 22–35) (Fig. 1). Metformin dose was selected base of previous studies which reported its neuroprotective/neuro-restorative effects [24]. Animals underwent Barnes maze test and novel object recognition (NOR) test to assess their learning, memory and cognitive performance prior to sacrifice for histopathological evaluations as mentioned in the timeline presented in Fig. 1A.

2.2. Cognitive assessment using barnes maze test

The Barnes maze was used to measure the spatial memory and learning skills in animal groups on days 38–42. Barnes maze test was performed on a circular table with a diameter of 90 cm and a height of 90 cm from the floor. Twenty holes, each with a diameter of 5 cm, were regularly spaced around the table's circumference. The target hole (main hole) was the only one that led to an escape chamber where the animal could hide. To enhance the animal's motivation to hunt for the target hole, illumination was increased and at the middle of the table, was kept at 1350 lux. The animal was introduced into the start box placed in the maze center for the habituation phase, at the beginning of each trial. The start box was removed 5 s later, and mouse was given 300 s to explore the area. The procedure included four training days (every day 4 trials, each 5 min) and one test day. In all stages, the Barnes maze area and the start and escape boxes were cleaned with 96% ethanol prior to introducing the animal to remove the interfering smell. Movement strategy to find the escape box (target hole) after moving out the start box at the center of Barnes maze has been classified as direct, serial and random. When radially moved toward the escape box, the movement was considered as direct strategy. If animal moved from one hole to the next one and continued until finding the target hole, the strategy was named serial. The strategy was named random when animals moved randomly, without any plan, till finding the escape box [25]. On the test day, the escape box was removed, and the animal was placed in the center of Barnes maze and observed for 5 min. The video tracking system Ethovision XT 11 (Noldus Company, Netherland) was used to collect and evaluate the behavior [26,27].

2.3. Cognitive assessment using novel objective recognition

NOR test was used to assess the cognitive impairment in mice. There were three steps in the task procedure: habituation, familiarization and testing. During the habituation phase, mice investigated a square open-field area with 39 × 39 × 20 cm inner dimensions without an item for three days, 10 min per session. Each mouse was placed in the area with two identical items (A + A) in the middle of the field for 10 min on the fourth day (familiarization phase). For the test phase, mice were reintroduced to the area 24 h later with two different objects; one that was identical to those presented the day before (A) and the other that was novel (B) for 10 min. In all stages, the area and objects were cleaned with 96% ethanol prior to animal introduction to remove smell signals. Exploration was described as an animal's snout pointing toward an object, smelling, or touching it. The percentage of discriminating index (DI) was used to determine the difference in exploration time between familiar (A) and unfamiliar (B) objects (equation I). As a result, each object's exploration time was divided by the total exploration time, and expressed as a percentage [28]. The Ethovision XT 11 (Noldus Company, Netherland) software was used for NOR test evaluation.

Equation I:

$$DI\% = \frac{new\ object\ exploration\ time}{total\ exploration\ time} \times 100$$

2.4. Nissl staining

In this study 0.1% Cresyl violet acetate (IHC world, USA) was used to stain the coronal hippocampus 10 μm-sections. In the first step, the brain samples were passaged in ethanol gradient 96%, 80% and 70% each for 1 min. After washing in tap water for 2–3 min, samples were stained in Cresyl fast violet for 3 min. After washing in water, samples entered to ethanol 70% and 80% each for 15 s and

ethanol 90%, 96%, 100% each for 2 min. Finally, samples were placed in Xylol for 5 min. Three mice per group and 3 sections per mouse were stained and photographed under an Olympus BX-51 microscope and DP72 camera. The number of dying neurons in DG, CA1 and CA3 regions was measured. Dying neurons were those with dark cytoplasmic Nissl staining, and not-prominent nucleoli [29, 30]. Neurons with abnormal morphologies of shrunken and hyperbasophilic appearance were distinguished as dying neurons.

2.5. Immunofluorescence staining

For immunofluorescence staining, mice were sacrificed under deep anesthesia and then perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer (PB). The brains were extracted and post-fixed for an additional night; then cryoprotected in a 30% sucrose-PFA-PB solution at 4 °C. The samples were kept frozen at −80 °C. A cryostat apparatus was used to make 10-μm thick coronal sections. Fixed sections collected on the positive charged slides were washed for 3 times each for 5 min with PBS 1% in room temperature. In next step, sections were incubated with Triton 0.3% for 20 min. Then samples were incubated with blocking solution (NGS 10% + 10 μl Triton100 x) for 1 h. Afterward, the slides were washed with PBS 3 times, each 5 min, and incubated with polyclonal rabbit anti-GFAP (1/500), anti- (1/1000) and mouse anti-NeuN (1/1000) primary antibodies at 4 °C, overnight. Sections were washed 3 times for 5 min each, in PBS, before been incubated for 1 h in dark at room temperature with red goat anti-rabbit and anti-mouse IgG secondary antibody (1:500; Invitrogen, Eugene, OR, USA) [28,31,32]. The images were obtained by fluorescence microscope, Olympus BX-51 and DP72 camera, with magnifications of 100 or 200. Three sections with ~10-μm thickness were selected, then photographed, and quantified using Fiji Software (NIH, USA) for each animal and averaged. This average value was entered to group mean calculation.

2.6. Statistical analysis

GraphPad Prism V8.0 was used for statistical analysis and the graphs preparation. All data was presented as means ± SEM and the minimum significance level of mean differences was set at $p<0.05$. The normal distribution was assessed using Shapiro-Wilk test. Student's *t*-test was used to compare control and STZ groups in model conformation experiments. One-way ANOVA with Tukey's post-hoc were used to compare behavioral and IHC data of Control, treated and non-treated groups. Two-way ANOVA was used to compare animal performance in NOR and Barnes maze during trial days.

3. Results

3.1. AD phenotype was developed following STZ treatment

Evaluation using NOR test showed that STZ caused significantly lower performance in exploring the novel object. The significant difference was observed between Control and STZ groups ($p < 0.0001$) (Fig. 1B).

The Barnes maze results showed that STZ-treated animals stayed in the goal sector less than Control group (Fig. 1C). The results also show that STZ animals appeared more frequently in non-target holes compared to Controls and spent more time in non-goal sectors

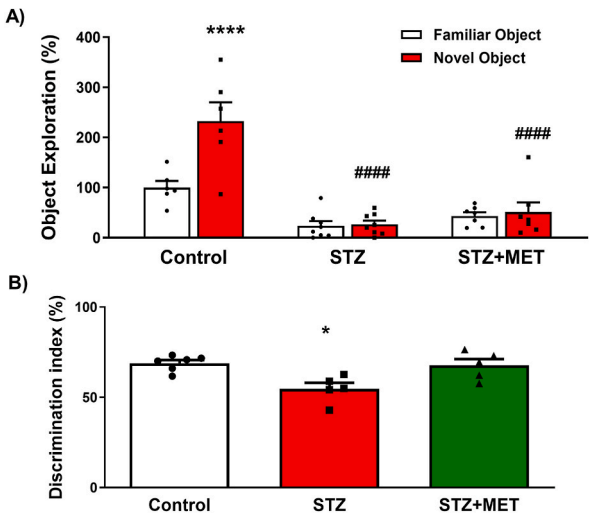

Fig. 2. Novel Object Recognition (NOR) test in control, STZ and STZ + MET groups demonstrated a significant memory loss in SAD mice compared to control group and a relative improvement in metformin-treated group. A) Object exploration percentage was analyzed using two-way ANOVA, significant differences were demonstrated between Novel and Familiar object in control group. B) Discrimination index percentage showed the positive effect of metformin on cognitive performance. *$p < 0.05$ and ****$p < 0.0001$ vs. control, ####l < 0.0001 vs. novel object in Control group.

(Fig. 1D). The GS/NGS ratio was significantly lower in STZ group (Fig. 1E), while the ratio of touching the entire holes/20 was not significantly different between the groups (Fig. 1F).

Fig. 1G demonstrated Nissl-stained dentate gyrus of control and STZ groups. The average number of dying neurons in dentate gyrus

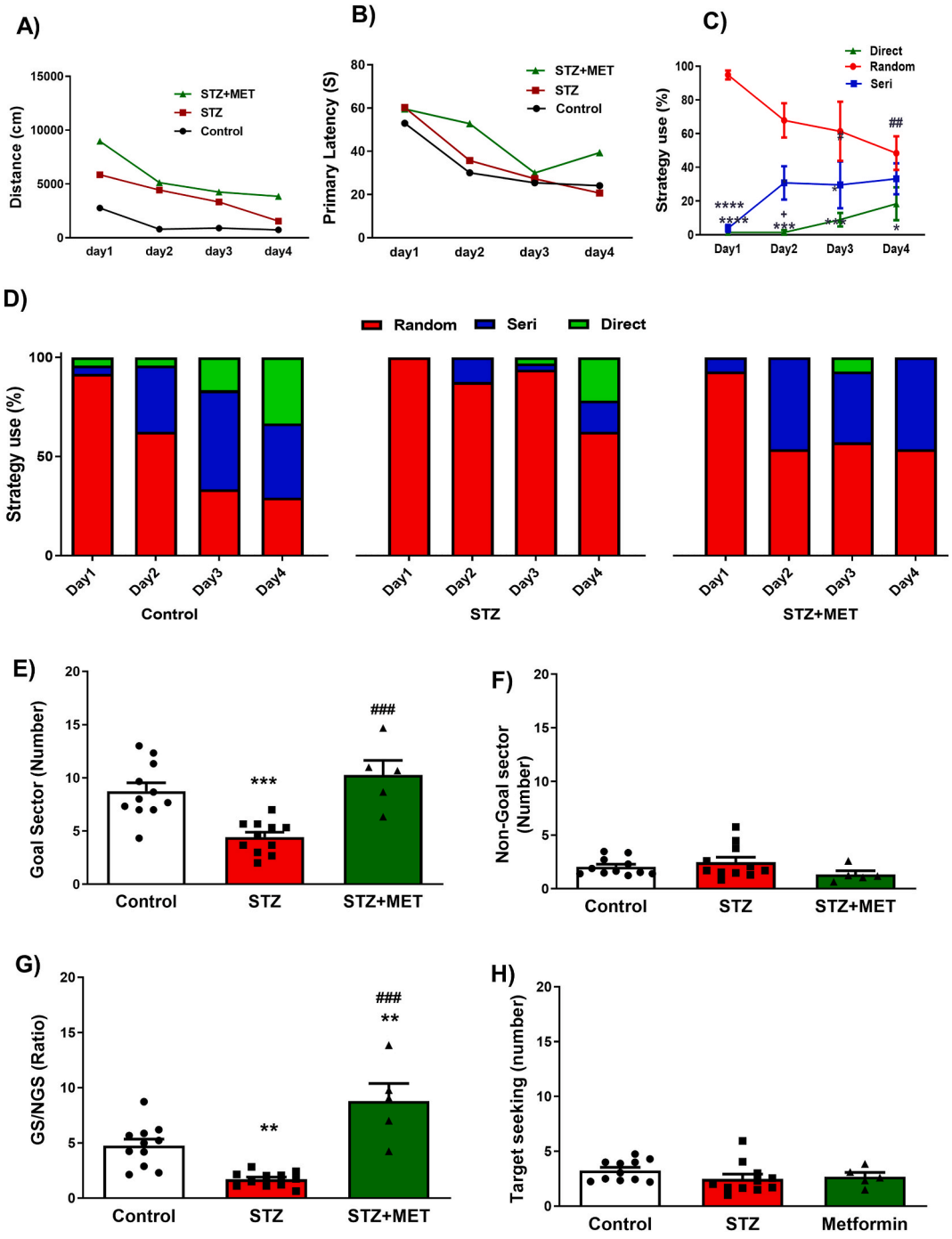

Fig. 3. Barnes Maze test performance in trial and the test day. A) Distance measures in 4 trial days demonstrated no significant difference between 3 groups. B) Primary latency calculated as duration to enter the escape box showed no significant difference between 3 groups. C-D) movement strategy to find the escape box in trial days. E) Goal sector exploring number at the test day showed remarkable difference between Control, STZ and STZ + MET. F) Non-Goal Sector exploration number showed no significant difference between 3 groups. G) GS/NGS ratio demonstrated significantly difference between Control and STZ and control and treatment group (STZ + MET). H) Target seeking number showed no difference between the groups. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group, $^{\#\#\#}p < 0.0001$ vs STZ group.

(DG) and CA1 regions was significantly increased in STZ group (Fig. 1H and I; $p < 0.01$ and $p < 0.001$, respectively). Number of dying neurons observed in CA3 was not statistically different (Fig. 1J).

3.2. Metformin improved cognitive performance in SAD mice

Results obtained from NOR test showed that the total time spent to explore the familiar and novel objects was significantly higher in the Control group (Fig. 2A). Animals treated with metformin (STZ + MET) in novel object exploration percentage demonstrated a significantly elevated index ($p < 0.0001$). The percentage of discrimination index was significantly higher in Control group and was the lowest one in STZ group (Fig. 2B). No significant difference was observed between Control and treated group.

Barnes maze data on trial days (days 1–4) showed that the distance travelled to find the target hole was reduced in all animal groups during the trial days (Fig. 3A), although the total distance was lower for Control. Primary latency in the trial days of Barnes maze test was decreased (Fig. 3B).

The movement strategy of animals was analyzed as direct, serial and random types. At the initial trials animals in different groups mostly used the random strategy while it was gradually reduced in the next trials. Control animals showed the highest amount of reduction in random strategy and replaced it mainly by serial strategy and in lower extent by the direct strategy. STZ showed lower amount of reduction in random strategy. STZ + MET group showed a prominent decline in using random strategy by replacing it mainly with the serial strategy. Direct strategy to find the escape box mainly observed in Control by time (Fig. 3C–D). Compared to

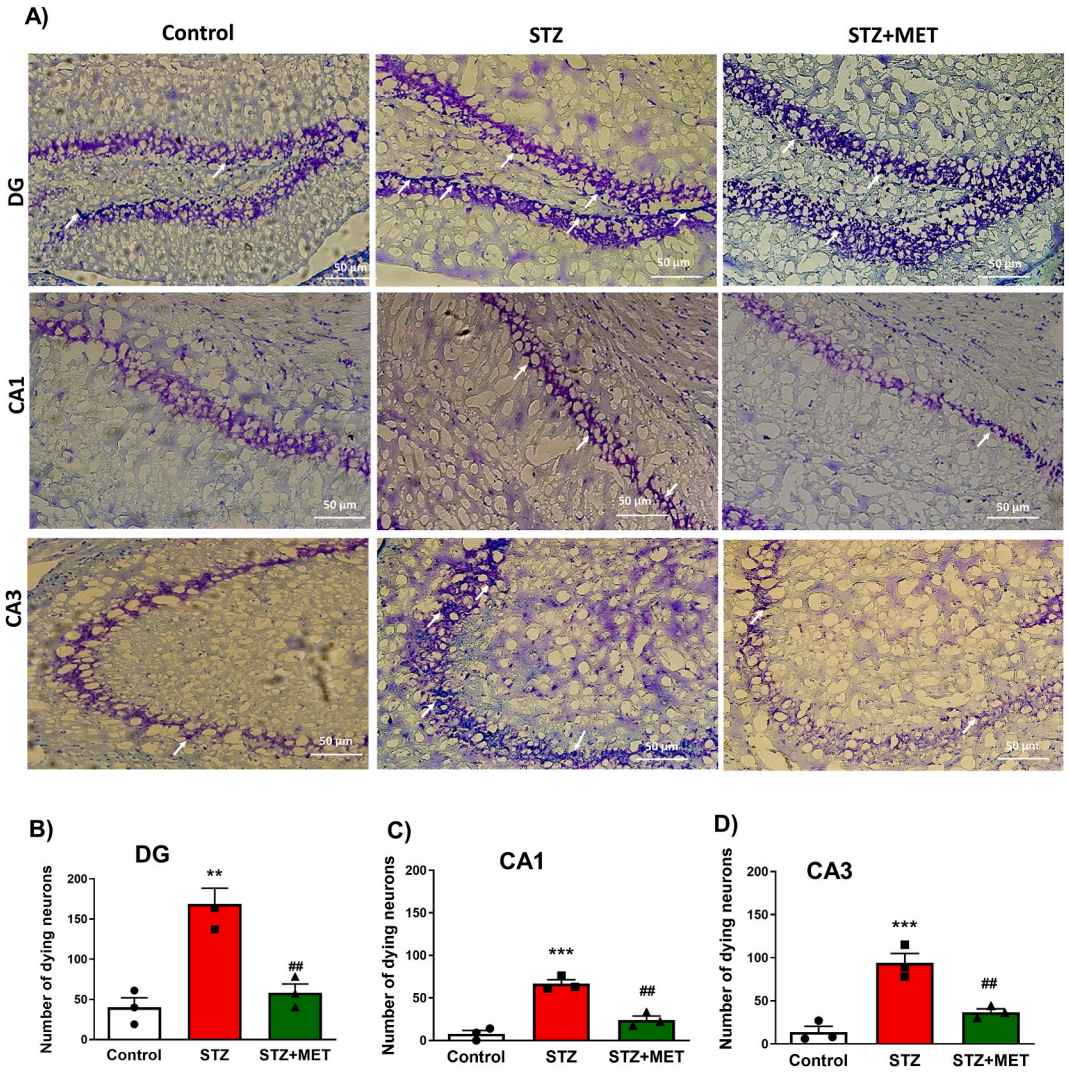

Fig. 4. Nissl Staining demonstration of average number of dying neurons in the hippocampus in three experimental groups (Control, STZ and STZ + MET). A) Representative Nissl-stained images of DG, CA1 and CA3. B) The average number of dying neurons in DG of Control, STZ and STZ + MET mice. C) The average number of dying neurons in CA1 of Control, STZ and STZ + MET mice. D) The average number of dying neurons in CA3 in different groups. $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control group, $^{\#\#}p < 0.01$ vs STZ group.

STZ, STZ + MET group mice were more successful in substituting random to serial strategy as it was done in Control.

On the test day, (day 5), poking the goal sector (number of pokes of correct holes (including main, right & left holes)/3 within 5 min was at the highest level in the treatment group (STZ + MET) and showed significant difference with STZ group ($p < 0.001$). GS number in Control group was 9.0 vs. STZ group that was 4.5 ($p < 0.001$, Fig. 3E). Non-goal Sector (the number of poking of incorrect holes (the other 17 holes)/17), was the highest in STZ animals and no significant difference was observed between the experimental groups (Fig. 3F). GS/NGS ratio in test day in Barnes Maze was significantly the highest one in the treatment group (STZ + MET) as compared with Control and STZ groups ($p < 0.01$ and $p < 0.0001$, respectively). The lowest one presented in STZ group with significant difference between control vs. STZ ($p < 0.01$, Fig. 3G). Target seeking parameter (the number of poking of both correct and incorrect holes/total holes (20)), in the test day was not significantly different between the groups (Fig. 3H) [27].

3.3. Metformin reduced dying neurons in SAD mice

Number of dying neurons was evaluated within the DG, CA1 and CA3 using Nissl staining (Fig. 4A). According to the quantified data presented in Fig. 4 (B-D), the average number of dying neurons in DG was significantly increased following the SAD induction compared to Control group ($P < 0.01$). Metformin treatment reduced the number of dark cells (dying neurons) to the control level with

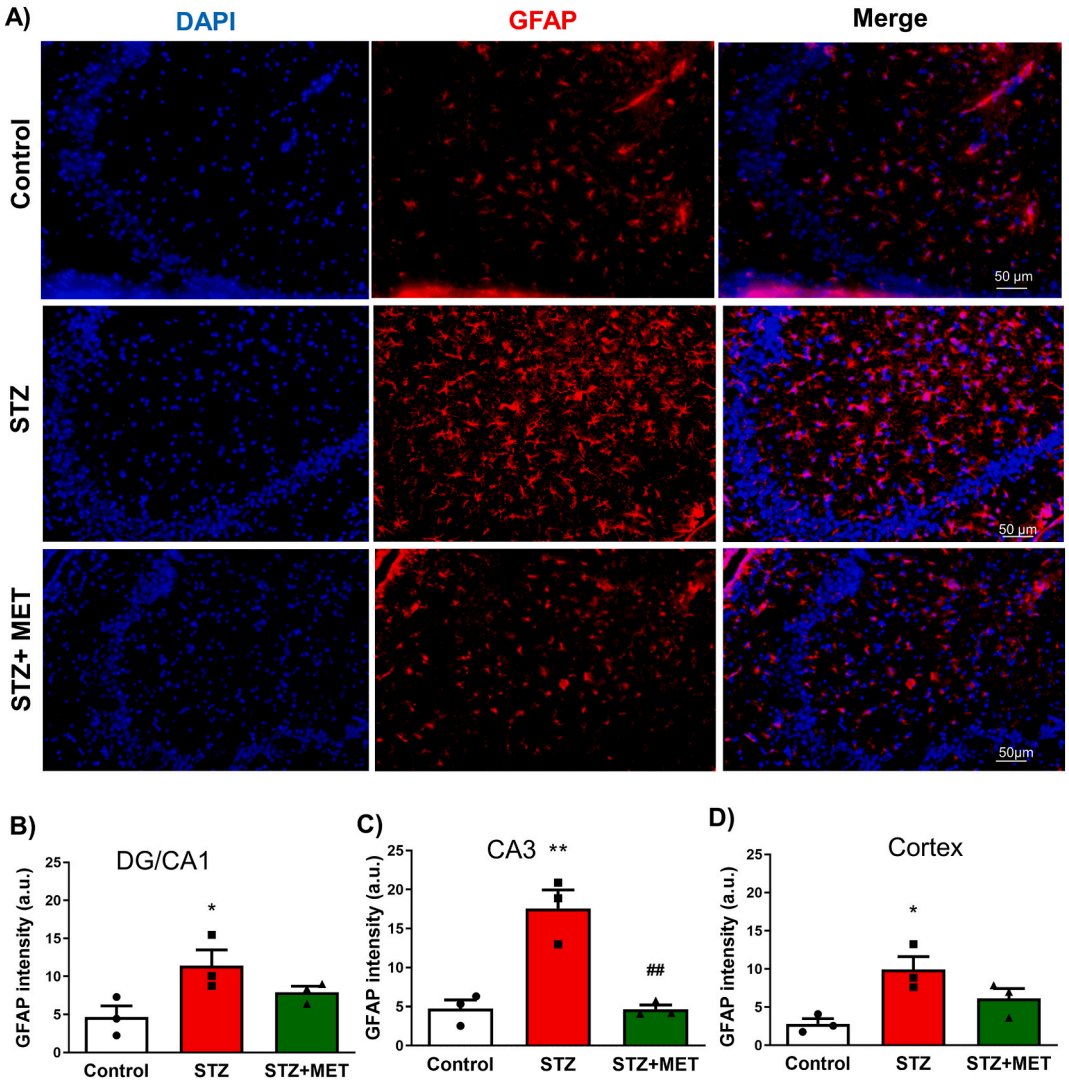

Fig. 5. A) Representative micrograph for GFAP staining in CA3. B) Quantification of GFAP immunofluorescence of DG/CA1 regions of hippocampus showed significantly difference between control and STZ groups. C) Quantification of GFAP intensity of CA3 region of hippocampus in three experimental groups demonstrated significant difference between control and SAD groups and also between SAD model and treatment groups. D) Quantification s of GFAP intensity of Cortex in three groups of study showed significantly difference in control group Vs. STZ group. *P < 0.05, **P < 0.01 vs Control group, $^{\#\#}p < 0.01$ vs STZ group.

significant difference Vs. STZ group, ($P < 0.01$, Fig. 4B). In CA1, number of dying neurons was significantly higher in STZ group as compared to Control ($P < 0.001$) and the number of dying neurons significantly decreased in treated group (STZ + MET) Vs. STZ group ($P < 0.01$, Fig. 4C). In CA3, the population of dying neurons was significantly increased in STZ group compare with control group ($P < 0.001$), metformin treatment significantly reduced the dying neuron in CA3 Vs. STZ group ($P < 0.01$) (Fig. 4D).

3.4. Metformin ameliorated gliosis in SAD mice

To measure the extent of reactive astrocyte, following SAD induction and the treatment, the intensity of GFAP staining was measured in different areas of brain section including DG/CA1, CA3 and the adjacent brain cortex in same sections. Based on immunofluorescence studies, GFAP intensity in DG/CA1, CA3 and cortex regions were significantly increased in STZ group compared to Control group, in addition treatment with metformin was effective to reduce the intensity of GFAP in three selected area of brain compared to STZ group (Fig. 5A–D). In DG/CA1 hippocampal area significant difference was observed between control and STZ groups ($p < 0.05$, Fig. 5B). Mean fluorescence GFAP intensity was highest in CA3 of STZ group in compare with Control and treated groups (P

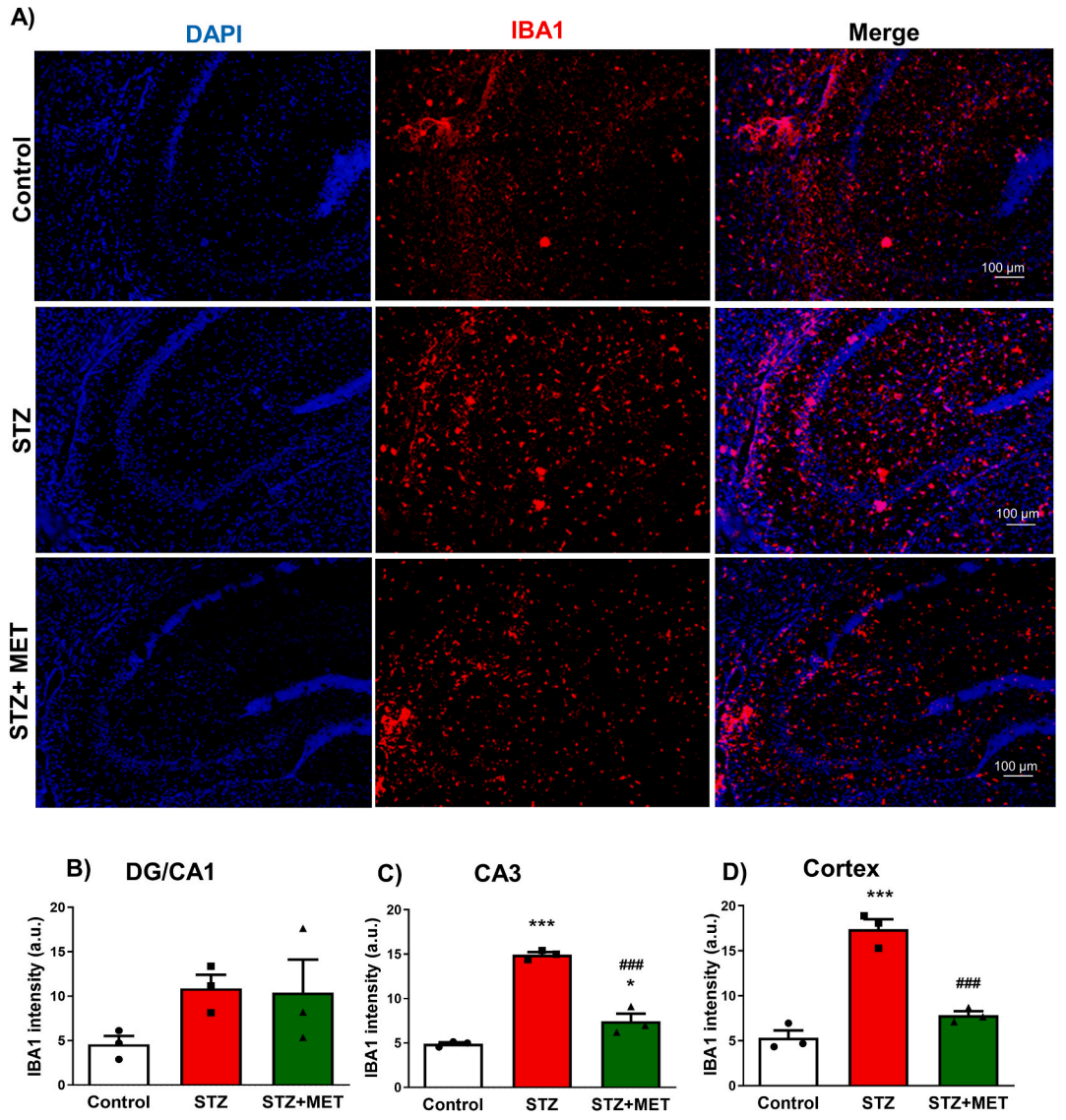

Fig. 6. A) Representative micrographs for IBA1staining in CA3. B) Quantification of IBA1 immunofluorescence of DG/CA1 regions of hippocampus showed no significantly differences between experimental groups. C) Quantification of IBA1 intensity of CA3 region of hippocampus in three experimental groups demonstrated significant differences between control and two other experimental groups such as STZ and treatment group and also between significant difference can be seen between STZ and STZ + MET groups. D) Quantification s of IBA1 intensity of Cortex in three groups of study demonstrated significantly differences in control Vs. STZ groups and in STZ Vs. treatment groups. ***$P < 0.001$ vs Control, $^{###}p < 0.001$ vs STZ.

< 0.01) Vs. control group and ($P < 0.01$) Vs. STZ + MET group (Fig. 5C). In cortex evaluation of Mean fluorescence GFAP intensity demonstrated significant difference between STZ Vs. control group ($P < 0.05$) (Fig. 5D).

To evaluate the neuroinflammation extent, we measured the intensity of IBA1 staining in the selected areas. Fig. 6A–D shows the intensity of IBA1 in DG/CA1, CA3 and the adjacent cortex. In DG/CA1 region, the IBA1 intensity was higher in STZ group compared to Control in view of mean but no significant difference observed among three experimental groups (Fig. 6B). Compared to Control, Intensity of IBA1 in CA3 was significantly increased by STZ induction ($p < 0.0001$) and reversed in STZ + MET group ($P < 0.05$). Based on the measurement of IBA1 intensity and the evaluation of reactivated microglia in the CA3, metformin was able to reduce and improve the inflammation in CA3 and significant difference observed in STZ + MET Vs. STZ group ($P < 0.01$) (Fig. 6C). The intensity of IBA1 in cortex was significantly increased in STZ group as compared to Control ($P < 0.0001$). According to measurement of Mean fluorescence IBA1 intensity, metformin treatment can be affective for reduction the neuroinflammation in cortex. Between STZ and STZ + MET groups significant difference was observed ($P < 0.001$, Fig. 6D).

NeuN as a mature neuronal marker stains the nuclei and was used to evaluate changes in the number of neurons in different areas of brain such as DG/CA1, CA3 of hippocampus and adjacent cortex (cortex area in same sections studied for hippocampal changes). Fig. 7A shows the sample micrographs obtained from DG/CA1. Immunofluorescence findings and $NeuN^+$ cells counting in DG/CA1

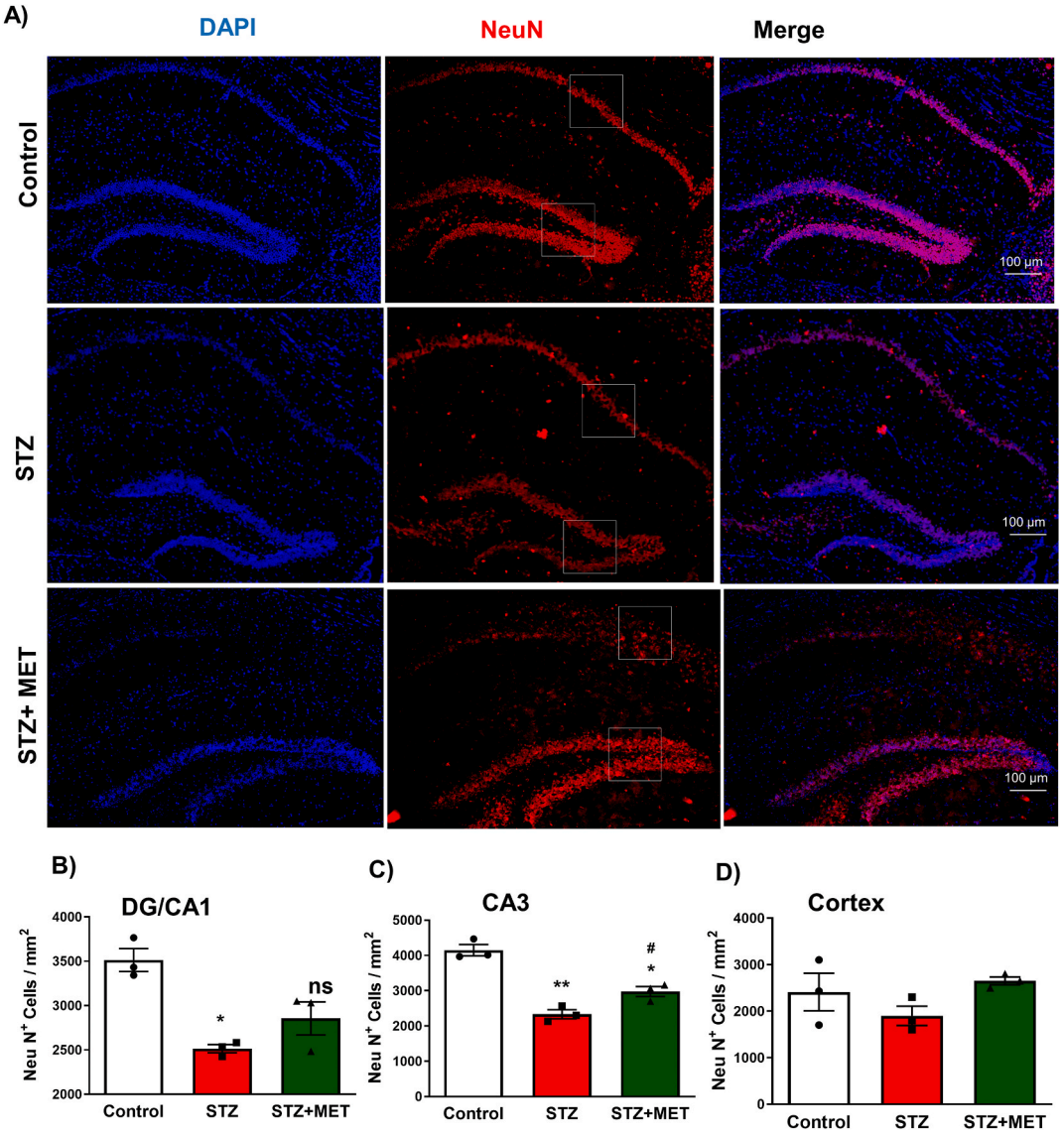

Fig. 7. A) Representative micrographs for $NeuN^+$ cells in DG/CA1. B) the number of NeuN positive cells in DG/CA1. This graph showed significant difference between control and STZ groups. C) the number of NeuN positive cells in CA3 that demonstrated significant differences between three experimental groups. D) the number of NeuN positive cells in cortex. There is no significant difference between three experimental groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs Control, $^{\#}p < 0.051$ vs STZ.

showed significant reduction following the SAD induction compared to Control group ($P < 0.01$) and declined from 3500/mm^2 to 2500/mm^2. Compared to STZ group, the number of NeuN$^+$ cells were increased in treatment group (STZ + MET), showing that metformin was able to ameliorate neuronal loss, however NeuN counts in STZ + MET group were still lower than the Control group ($P < 0.05$, Fig. 7B). Neuronal loss was affected by metformin in CA3 area and significantly restored, compared to the STZ group ($p < 0.05$). The number of NeuN$^+$ cells in CA3 was significantly decreased in both STZ and STZ+MET groups Vs. Control ($P < 0.001$ and $P < 0.01$, respectively, Fig. 7C). Counting of NeuN$^+$ cells in the adjacent cortices demonstrated no significant differences between the experimental groups (Fig. 7D).

4. Discussion

In this study, STZ affected healthy mice and induced sporadic AD-like pathology after 21 days. Treatment with metformin improved the animal behavioral performance including learning, and memory, and played a restoring effect on histopathological deficits induced by STZ administration.

Like diabetes, AD hires multiple pathophysiological mechanisms that impair insulin sensitivity, cognition and glucose metabolism [21]. Anti-diabetic agent, metformin hydrochloride, which acts through activation of AMPK, has showed positive effects on the survival of the neurons, neurogenesis and the formation of spatial memory [33,34]. Accordingly, it was quite rational to observe anti-AD effect following metformin administration in mice that had already developed AD-like pathology.

In behavioral evaluations, including NOR and Barnes maze tests, two I.C.V. injections of STZ led to induction of SAD –like pathology in adult healthy mice (SAD mice). According to results of NOR, memory impairment in SAD mice was shown in and the percentage of discrimination index was significantly decreased in SAD group as well as novel object exploration percentage that decreased considerably in SAD group compared with Control. According to the NOR results, the time spent for exploring the novel object was decreased significantly in SAD mice. However, the percentage of exploration for novel object in Control mice was about twice the SAD group. In Barnes maze test, the ratio of goal sector to non-goal sector time in the SAD mice was significantly decreased that may imply for impairment of spatial memory and learning in the STZ group. In histology, SAD mice showed increased number of dying neurons in DG and CA1 which confirmed the results of behavioral tests. These finding showed successful development of SAD–like pathology in our experimental setting. In the next step, we tried to mention the effect of metformin on the disease parameter in a condition that the pathology is already established. Therefore, our outcomes have the potential to be extrapolated to the possible effect of metformin in patients which are already diagnosed with AD.

Based on NOR, metformin treatment in a good extent reversed the cognitive deficits. In Barnes maze test, the time spent in GS area was significantly increased by metformin. Same findings were observed in other parameters like ratio of GS/NGS. These findings indicate that metformin administration improves spatial memory in mice. Metformin treatment was reported to protect against STZ-induced impairments in spatial learning and memory [34]. In the trial days, Pilipenko et al. found that rats which received metformin as a protective drug spent 43% more time in the target quadrant and crossed the platform zone twice more in Morris water maze test. Metformin enhanced learning and memory impairment after 14 days administration [35]. Mostafa and colleagues found that administration of 100 mg/kg metformin for two weeks would protect spatial learning and memory in a rat model of scopolamine induced learning/memory impairment [1]. In another study, A previous report investigated that metformin could improve learning and memory dysfunction while applied during the AD developing phase in transgenic mice [1]. Another report administered that 100 mg/kg metformin for two weeks in a rat model of scopolamine-induced learning/memory impairment, and reported spatial learning and memory preservation [36].

In this study, all groups (control, STZ and STZ + MET) had similar searching patterns at the beginning of the training. Random search strategies were used in more than half of the trials and the rest were divided between the direct and serial strategies. However, there was a significant difference between the groups in using the direct and serial strategies. According to Harrison FE et al. reports, it is expected that control mice will be able to find the escape box quickly by using serial and direct search strategies [34]. Learning and memory impairment, as well as cognitive deficits in SAD mice, prevent to find the escape box location as well as remember the signs that used as an escape box guide. These mice are mostly using random search strategy to find the escape box location. However, in metformin-treated mice, because cognitive memory and learning were somewhat improved, most of these mice used the serial search strategy to find the escape box. Preferred strategy for metformin-treated mice was serial after the first trial days. The Lack of direct strategy in metformin-treated mice compared to control mice indicates that metformin was able to improve spatial learning and memory somewhat over a 14-day period of treatment, but this improvement may not be as elaborate as in Control mice. Here, after developing SAD we started the metformin administration for two additional weeks. Therefore, in addition to protection, metformin possessed restoring effect in the context of an established SAD model which imply for the possibility of administrating this FDA approved drug in patients with progressed AD.

We also performed histological studies to know the neuronal cell number in hippocampus areas. The results of the Nissl staining performed in the present study indicated a decline in the number of neurons and alterations in their morphology in hippocampus. According to the results of counting the number of dying neurons of DG, CA1 and CA3 regions of hippocampus in Nissl-stained sections, treatment with metformin was able to reduce the rate of dying neurons a factor that may contribute to the memory restoration following metformin treatment.

Reactive gliosis and neuroinflammation are known as the neural tissue consequences of neurodegenerative disorders and can be monitored through the increased activity of astrocytes and microglial cells. Hippocampal astrogliosis was remarked by increased GFAP staining in CA1 and DG regions. The results of this study demonstrated that GFAP level was significantly increased in STZ group and decreased after administration of metformin. The results come to an agreement with Pilipenko et al. report [37]. In their study GFAP

level was lower in animals treated with 100 mg metformin during the disease progression. Intensity of reactive astrocytes (inflammation) in CA1 was reduced following administration of metformin 100 mg/kg, while at the dose of 75 mg/kg, decreased astrogliosis observed only in CA1 and DG. Oliveira et al. reported significant decreased level of GFAP in diabetic mice that received metformin 200 mg/kg [38]. They also found that astrocyte activity was reversed to the control level. It was suggested that metformin up regulates AMPK expression in glial cells and results in decreased Aβ deposition [37].

Iba-1 protein is a marker of microglia and macrophages. Olivia et al. found higher activation level of microglia in diabetic mice and metformin decreased this activity [37]. In current study, we found that Iba-1 level was significantly higher in CA3 and cortex in STZ group and metformin was effective in reducing the level of this protein reactivity, indicating that inflammation response in STZ group was inhibited by metformin.

Quantifying the NeuN in three different regions of hippocampus and cortex demonstrated that neuronal loss was accrued in SAD mice. Significant decrease in the neuronal count was shown in hippocampus. Treatment with metformin to some extent prevented the neuronal death in different parts of the hippocampus and cortex or restored it. In CA3 of treatment group, significant increase observed in the number of neurons compared to STZ group.

To reduce the number of animals used in this study, same samples were used for both immunofluorescence and Nissl staining. Using frozen sections reduced the quality of images in Nissl staining in some extents. As another limiting point, the high density of neurons in DG, may have been caused underestimation of dying neurons count, especially in STZ group. This underestimation may not interfere with our interpretation on the effects of metformin.

5. Conclusion

Our results demonstrated the potential neuroprotective effects of metformin in SAD mice as mentioned by restoring the cognitive performance and NeuN positive cells. Additionally, metformin decreased the neuroinflammation in the hippocampal region of the brain as well as the gliosis and dying neurons. While the previous reports on the effects of metformin on preclinical AD animal models mainly reported the neuroprotection during the disease settlement, our data showed that metformin had both protective and restorative effects on established AD. Relevant clinical trials seems reasonable to check its possible administration in AD management.

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Production notes

Author contribution statement

Saghar Rabieipoor: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Meysam Zare: Performed the experiments; Analyzed and interpreted the data.
Miren Ettcheto: Analyzed and interpreted the data; Wrote the paper.
Antoni Camins; Mohammad Javan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antoni Camins reports financial support was provided by Center for Networked Biomedical Research on Neurodegenerative Diseases. Antoni Camins reports financial support was provided by European Regional Development Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17873.

References

[1] X.-Y. Lu, et al., Metformin Ameliorates Aβ pathology by insulin-degrading enzyme in a transgenic mouse model of Alzheimer's disease, Oxid. Med. Cell. Longev. 2020 (2020), 2315106.
[2] F.G. De Felice, R.A. Gonçalves, S.T. Ferreira, Impaired insulin signalling and allostatic load in Alzheimer disease, Nat. Rev. Neurosci. (2022) 1–16.
[3] W. Zhong, et al., *Pathogenesis Of Sporadic Alzheimer's Disease by Deficiency of NMDA Receptor Subunit GluN3A*. Alzheimer's & Dementia, 2021.
[4] F. Leng, P.J.N.R.N. Edison, Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? Nat. Rev. Neurol. 17 (3) (2021) 157–172.
[5] F. Xue, H.J.C. Du, TREM2 mediates microglial anti-inflammatory activations in Alzheimer's disease: lessons learned from transcriptomics, Cells 10 (2) (2021) 321.
[6] M. Michailidis, et al., Alzheimer's disease as type 3 diabetes: common pathophysiological mechanisms between Alzheimer's disease and type 2 diabetes, Int. J. Mol. Sci. 23 (5) (2022) 2687.
[7] L. Li, et al., Diabetes mellitus increases risk of incident dementia in APOE ϵ4 carriers: a meta-analysis, J. Alzheim. Dis. 74 (4) (2020) 1295–1308.
[8] M.J.E.N. Suzanne, Type 3 diabetes is sporadic Alzheimer' s disease: mini-review, Eur. Neuropsychopharmacol 24 (12) (2014) 1954–1960.
[9] S. Ahtiluoto, et al., Diabetes, Alzheimer disease, and vascular dementia: a population-based neuropathologic study, Neurology 75 (13) (2010) 1195–1202.
[10] J.M. Haus, et al., Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance, Diabetes 58 (2) (2009) 337–343.
[11] P.K. Kamat, Streptozotocin induced Alzheimer's disease like changes and the underlying neural degeneration and regeneration mechanism, Neur. Regener. Res. 10 (7) (2015) 1050.
[12] K.G. Ravelli, et al., Intracerebroventricular streptozotocin as a model of Alzheimer's disease: neurochemical and behavioral characterization in mice, Neurotox. Res. 31 (3) (2017) 327–333.
[13] G. Rena, D.G. Hardie, E.R.J.D. Pearson, The mechanisms of action of metformin, Diabetologia 60 (9) (2017) 1577–1585.
[14] K. Chaudhari, C.D. Reynolds, S.-H.J.G. Yang, Metformin and cognition from the perspectives of sex, age, and disease, Geroscience 42 (1) (2020) 97–116.
[15] M. Markowicz-Piasecka, et al., Metformin–a future therapy for neurodegenerative diseases, Pharmaceut. Res. 34 (12) (2017) 2614–2627.
[16] W.-s. Lv, et al., The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats, Brain Res. 1444 (2012) 11–19.
[17] A.R. Cameron, et al., Anti-inflammatory effects of metformin irrespective of diabetes status, Circ. Res. 119 (5) (2016) 652–665.
[18] S.R. Poor, et al., Metformin a potential pharmacological strategy in late onset Alzheimer's disease treatment, Pharmaceuticals 14 (9) (2021) 890.
[19] Q. Shi, et al., Effect of metformin on neurodegenerative disease among elderly adult US veterans with type 2 diabetes mellitus, BMJ Open 9 (7) (2019), e024954.
[20] K.A. DiTacchio, S.F. Heinemann, G.J.J.o.A.s.D. Dziewczapolski, Metformin treatment alters memory function in a mouse model of Alzheimer's disease, J. Alzheim. Dis. 44 (1) (2015) 43–48.
[21] J. Wang, et al., Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation, Cell Stem Cell 11 (1) (2012) 23–35.
[22] S.A. Farr, et al., Metformin improves learning and memory in the SAMP8 mouse model of Alzheimer's disease, J. Alzheim. Dis. 68 (4) (2019) 1699–1710.
[23] F. Rostami, et al., Streptozotocin-induced hippocampal astrogliosis and insulin signaling malfunction as experimental scales for subclinical sporadic Alzheimer model, Life Sci. 188 (2017) 172–185.
[24] S. Esmaeilnejad, S. Semnanian, M. Javan, Metformin protects myelin from degeneration in A mouse model of iysophosphatidylcholine-induced demyelination in the optic chiasm, Cell J. 23 (1) (2021) 119.
[25] M.W. Pitts, Barnes maze procedure for spatial learning and memory in mice, Bio-protocol 8 (5) (2018).
[26] A. Cano, et al., Dual-drug loaded nanoparticles of Epigallocatechin-3-gallate (EGCG)/Ascorbic acid enhance therapeutic efficacy of EGCG in a APPswe/PS1dE9 Alzheimer's disease mice model, J. Contr. Release 301 (2019) 62–75.
[27] G.R. Morel, et al., Cognitive impairment and morphological changes in the dorsal hippocampus of very old female rats, Neuroscience 303 (2015) 189–199.
[28] M. Ettcheto, et al., Evaluation of neuropathological effects of a high-fat diet in a presymptomatic Alzheimer's disease stage in APP/PS1 mice, J. Alzheim. Dis. 54 (1) (2016) 233–251.
[29] K. Lin, et al., 20 (S)-protopanaxadiol and oleanolic acid ameliorate cognitive deficits in APP/PS1 transgenic mice by enhancing hippocampal neurogenesis, J. Gins. Res. 45 (2) (2021) 325–333.
[30] H. Ooigawa, et al., The fate of Nissl-stained dark neurons following traumatic brain injury in rats: difference between neocortex and hippocampus regarding survival rate, Acta Neuropathol. 112 (4) (2006) 471–481.
[31] A. Mokhtarzadeh Khanghahi, et al., In vivo conversion of astrocytes into oligodendrocyte lineage cells with transcription factor Sox10; Promise for myelin repair in multiple sclerosis, PLoS One 13 (9) (2018), e0203785.
[32] M.S. Seyedsadr, et al., Inactivation of sphingosine-1-phosphate receptor 2 (S1PR2) decreases demyelination and enhances remyelination in animal models of multiple sclerosis, Neurobiol. Dis. 124 (2019) 189–201.
[33] S. Amato, H.-Y.J.C.C. Man, Bioenergy sensing in the brain: the role of AMP-activated protein kinase in neuronal metabolism, development and neurological diseases, Cell Cycle 10 (20) (2011) 3452–3460.
[34] V. Pilipenko, et al., Neuroprotective potential of antihyperglycemic drug metformin in streptozocin-induced rat model of sporadic Alzheimer's disease, Eur. J. Pharmacol. 881 (2020), 173290.
[35] D.K. Mostafa, C.A. Ismail, D.A.J.P. Ghareeb, Differential metformin dose-dependent effects on cognition in rats: role of Akt, Psychopharmacology 233 (13) (2016) 2513–2524.
[36] F.E. Harrison, et al., Spatial and nonspatial escape strategies in the Barnes maze, Learn. Mem. 13 (6) (2006) 809–819.
[37] W.H. Oliveira, et al., Effects of metformin on inflammation and short-term memory in streptozotocin-induced diabetic mice, Brain Res. 1644 (2016) 149–160.
[38] Z. Ou, et al., Metformin treatment prevents amyloid plaque deposition and memory impairment in APP/PS1 mice, Brain Behav. Immun. 69 (2018) 351–363.