



Minocycline attenuates depressive-like behaviors in mice treated with the low dose of intracerebroventricular streptozotocin; the role of mitochondrial function and neuroinflammation

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Abstract

Neuroinflammation and mitochondrial dysfunction are suggested as mechanisms which are implicated in the pathophysiology of depression. Streptozotocin (STZ) is known to produce immune-inflammatory responses and mitochondrial dysfunction in different types of animal models of disease (e.g. type-1 diabetes and Alzheimer's disease). Therefore, a single low dose of Streptozotocin (STZ; intracerebroventricular, i.c.v, 0.2 mg/mouse) was used to induce an animal model of depression. The present study aims to investigate the effects of short (24 h) and long (14 days) exposure to minocycline on STZ-induced depressive-like behaviors (n = 6–8), hippocampal oxidative state biomarkers (n = 4), and the expression of hippocampal genes related to innate immunity (n = 3) in the hippocampus of male adult mice. In addition, the protective effects of different modes of minocycline (acute pretreatment (20 mg/kg, 1 h before STZ), acute post-treatment (20 mg/kg, 24 h after STZ), chronic pretreatment (5 mg/kg/day for 14 days before STZ), and chronic post-treatment (5 mg/kg/day for 14 days after STZ) were compared with the STZ effects. As the data showed, both short and long effects of STZ were associated with the depressive-like behaviors, abnormal mitochondrial function, and upregulation of neuroinflammatory genes in the hippocampus. Different modes of minocycline treatment could attenuate the negative impact of STZ on animals. The data suggested that minocycline at a human therapeutic dose (5 mg/kg) had protective effects against acute cellular damage induced by oxidation and the consequent inflammatory responses.

Keywords Minocycline · Streptozotocin · Depression · Mitochondria · Neuroinflammation · Hippocampus

Introduction

Depression, a debilitating psychiatric disorder accompanied with some diseases such as cardiovascular disorders (CVD), is considered as one of most challenging health issues in the current century [1]. In recent decades, extensive attempts have been made to understand the underpinning

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mechanisms involved in the pathophysiology of depression. Ample evidence have revealed that immune-inflammatory responses are activated in the brain due to the depression pathogenesis [2, 3]. In addition, the increased research in this area indicates that mitochondrial dysfunction is tightly linked to immune-inflammatory responses which triggers TLR signaling via damaged-associated molecular patterns (DAMPs) [4]. Mitochondrial dysfunction is associated with massive production of reactive oxygen species (ROS), which causes oxidative damage to a variety of biomolecules (such as lipids, protein and DNA) [5]. Preclinical animal models of depression show that mitochondrial dysfunction is connected with impaired energy metabolism and initiation of cell death [6, 7]. In addition, mitochondrial dysfunction produces a variety of DAMPs (such as ROS, oxidized lipids, oxidized cardiolipin, mitochondrial DNA) and activates inflammatory signaling through different mechanisms including TLRs activation and inflammasome formation [8–10].

In another study, the authors confirmed that the intracerebroventricular (i.c.v) administration of STZ (0.2 mg/mouse) led to depressive-like behaviors, mitochondrial dysfunction and enhanced transcription of genes which was associated with TLR-4 signaling pathway after 24 h [11]. In this regard, numerous clinical and preclinical studies have revealed the antidepressant potential of many compounds. Minocycline is a long half-life, broad-spectrum antibiotic with high lipophilicity, which is able to pass blood brain barrier and enter the brain [12]. The relevant evidence indicates that minocycline exerts its neuroprotective effects through different mechanisms such as promotion of neurogenesis, upregulation of neurotrophic factors, and protection of oligodendrocytes following brain injury [13]. Therefore, we decided to examine whether the effectiveness of minocycline in depressive-like behavior is associated with mitochondrial dysfunction and consequent neuro-inflammation responses. Since STZ does not pass blood brain barrier, the mice were treated with a single i.c.v injection of a STZ (0.2 mg/mouse) to show the involvement of sterile inflammation and brain metabolic changes in the depressive-like behaviors [11]. Furthermore, to show the efficacy of minocycline in depression-like behavior, acute and chronic treatments of drug were used in present study.

Materials and methods

Animals

Male mice (25–30 g) were purchased from the Pasteur Institute, Tehran, Iran. They were housed under standard conditions protocol after acclimation with free access to food and water ad libitum. All experiments were conducted between 10:00 and 14:00 and all procedures were carried out

according to NIH Guide for the Care and Use of Laboratory Animals, which was confirmed by the Animal Ethics committee of Zanjan University of Medical Sciences (registration number: ZUMS.REC.1394.244). Great attempts were made for minimizing the use of animals and optimizing their comfort.

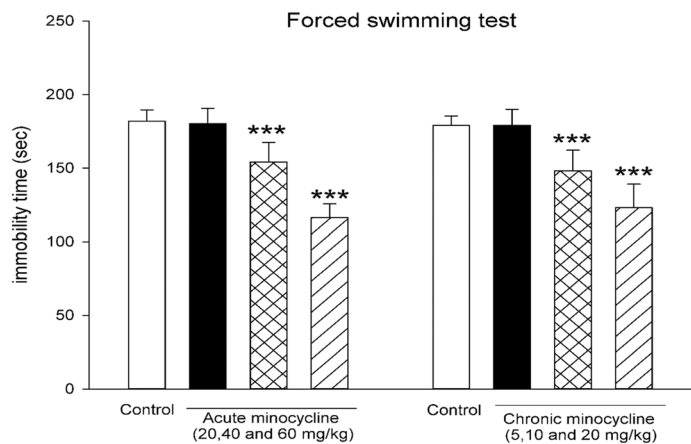
Drugs and treatments

Streptozotocin (STZ) (Sigma, St Louis, MO, USA) injected into the i.c.v at the dose of 0.2 mg/4 μ L/mouse according to previously-reported studies [11, 14]. Sterile physiological saline (0.9%; 4 μ L/per mouse, i.c.v) as the solvent of STZ were administered to the animals in the sham group to eliminate the probable effect of saline. Doses of minocycline were selected based on the pilot study as well as the previously-published works [15, 16]. Minocycline was dispersed in sterile physiological saline (0.9%) and then, it was administered (intraperitoneal; i.p.) at the dose of 20 mg/kg and 5 mg/kg to assess the acute and chronic effects of drug, respectively. After treatments, the behavioral tests including open field test (OFT), forced swimming test (FST) and splash test (n = 7–8) were performed on animals. Molecular assessments were the assessment of gene expression (n = 3), mitochondrial function (n = 4) and histopathological changes (n = 3) in the hippocampus. Different sets of animals were used for molecular assessment in the present study.

Study design

First, a dose–response study was conducted to specify an appropriate sub-effective dose for minocycline so that it produced no effect on depressive-like behaviors of animals. Then, control mice were treated with different acute doses of minocycline (20, 40, 60 mg/kg, i.p.) 45 min prior to behavioral tests in order to assess depressive-like behaviors. The dose of 20 mg/kg was also chosen as the sub-effective dose of minocycline (n = 18–24) for acute treatment. To determine the sub-effective dose of minocycline for chronic treatments, control mice were treated with different chronic doses of minocycline (5, 10, 20 mg/kg, i.p.), and animals were subjected to behavioral tests in order to assess depressive-like behaviors. At the end, the dose of 5 mg/kg was chosen as the sub-effective dose of minocycline (n = 18–24) for chronic treatment. To investigate the effects of minocycline on STZ-induced depressive-like behaviors, animals were exposed to different modes of minocycline treatment. To do so, animals were divided into 10 groups as follows (please see Fig. 1): *Group 1*: mice which received sterile physiological saline (0.9%) as vehicle of STZ or minocycline and served as the sham (control) group; *Group 2*: mice which were treated with a single dose of minocycline alone (20 mg/kg, i.p.) and were

Fig. 1 Effects of several doses of minocycline in acute administration (5, 10 and 20 mg/kg) and chronic administration (5, 10 and 20 mg/kg) on depressive-like behaviors in the FST. Values are shown as the mean \pm SD from 6 to 8 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control group



subjected to all tests after 24 h [acute minocycline, AM (20 mg/kg)]; *Group 3*: mice which took a single dose of STZ (4 μ L/ mouse, i.c.v) and after 24 h, were exposed to all tests [STZ-1 day]; *Group 4*: mice which were administered with a single dose of minocycline (20 mg/kg, i.p.) 24 h after STZ (i.c.v) injection, and then, were exposed to all tests 45 min later [STZ + AM (24 h)]; *Group 5*: mice which received a single dose of minocycline (20 mg/kg, i.p.) 1 h before STZ (i.c.v) injection, and after 24 h, were exposed to all tests [AM (1 h) + STZ]; *Group 6*: mice which were treated with sterile physiological saline (0.9%) as a vehicle of STZ or minocycline for 14 days and served as the sham (control) group; *Group 7*: mice which were given a daily dose of minocycline alone (5 mg/kg, i.p.) for 14 days and in the last day, were exposed to all tests [chronic minocycline, CM (5 mg/kg/day for 14 days)]; *Group 8*: mice which received a single dose of STZ (4 μ L/ mouse, i.c.v) and after 14 days, were exposed to all tests [STZ-14 day]; *Group 9*: mice which were given a daily dose of minocycline (5 mg/kg, i.p.) for 14 days after a single STZ (i.c.v) injection, and then were exposed to all tests in the last day [STZ + CM]; *Group 10*: mice which were administered with a daily dose of minocycline (5 mg/kg, i.p.) for 14 days followed by a single dose of STZ (i.c.v), and after 24 h, were exposed to all tests [CM + STZ]. Behavioral tests including OFT, Splash test, and FST (n = 6–8) were performed on animals in all experimental groups.

For cellular and molecular studies, animals from all groups (n = 10 for each group) were euthanized using pentobarbital (60 mg/kg, i.p.). After the collection of blood samples, tubes were placed at 37 °C for 30 min to allow the blood to be coagulated. Then, serum was separated by centrifugation (10 min, 3500 rpm) to measure glucose levels. At the same time, hippocampi were dissected on ice-cold surface and all samples (except those used for microscopic evaluations) were stored at – 80 °C for further analysis. Hippocampal samples (obtained from all experimental groups) were divided into two groups, one for measuring oxidative

stress parameters (n = 4) and the other for total RNA extraction and gene expression studies (n = 3).

Behavioral assessments

Forced swimming test (FST)

In the FST, the despair behavior in rodents are reflected in increased immobility time when facing an inescapable and unavoidable challenge [17]. Then, according to previous studies, FST **was used** to assess the behavioral despair in animals [17]. Therefore, mice were individually put in glass cylinders (10 \times 25 cm, diameter \times height) which contained 19 cm of water at 23 \pm 1 °C. They were allowed to swim for 6 min and then, throughout the last 4 min of the test, the immobility time was recorded. The mice which stopped struggling to be floated on the water and just made movements to keep their heads above water were regarded to be immobile mice.

Splash test

Splash test was conducted to evaluate the motivational and self-care behaviors according to previously-described methods [18]. Grooming behavior in response to the sprinkling of 10% sucrose solution on the dorsal coat of the mice is considered as an indirect measure of palatable solution intake and the total grooming activity time was recorded by videotape for 5 min. Grooming activity consisted of nose/face/body grooming and head washing.

Open field test (OFT)

To assure that motor activity alterations did not change the duration of immobility time, locomotor activity of mice was measured using OFT based on the author's previously-published study [19]. The OFT apparatus was composed of dimly-illuminated Plexiglas box (50 cm \times 50 cm \times 40 cm).

Mice were gently placed separately in the center of the apparatus and the distance moved (horizontal activity) and the number of rearing (vertical activity) were videotaped and reported within a 5-min session. After each test, the apparatus was cleaned with 10% ethanol solution to remove animal clues.

Mitochondrial function

Preparation of mitochondria

All the mice were fasted overnight and then, sacrificed. Hippocampi were immediately dissected out, soaked in the liquid nitrogen and placed at $-80\text{ }^{\circ}\text{C}$ freezer until the assays were performed. Homogenization was conducted using a cold mannitol solution medium at $4\text{ }^{\circ}\text{C}$. The homogenate was centrifuged ($1000 \times g$, 10 min at $4\text{ }^{\circ}\text{C}$) and then, the obtained supernatant was centrifuged again ($10,000 \times g$, 10 min, at $4\text{ }^{\circ}\text{C}$) as a source of mitochondrial fraction based on the previously-described method [20]. The obtained pellet (P_2 fraction) which included both synaptic and non-synaptic mitochondria was re-suspended in the desired buffer on the basis of oxidative stress markers consisting of ROS, glutathione (GSH), and ATP. To normalize experimental condition in each sample, similar mitochondrial protein levels ($100\text{ }\mu\text{g/ml}$ mitochondrial protein) were employed in all treated groups based on Bradford test [21].

ROS formation

To measure ROS level, mitochondrial suspension obtained from hippocampi were incubated with 2', 7'-dichlorofluorescein diacetate (DCFH-DA; $10\text{ }\mu\text{M}$) in the respiration buffer containing KCl (130 mM), MgCl_2 (5 mM), NaH_2PO_4 (20 mM), ADP (1.7 mM), β -NADPH (0.1 mM), and FeCl_3 (0.1 mM) ($\text{pH}=7.4$). The mitochondrial H_2O_2 was measured by spectrophotometric method according to the author's previous work [22].

ATP levels

When 0.5 mL of mitochondrial fraction (in TCA 6%) was mixed with 0.5 mL of KOH 0.05 M (on ice), 1 mL deionized water was added to the mixture. Then, after 2 min, $650\text{ }\mu\text{L}$ of KH_2PO_4 (0.05 M) was added and vortexed. After filtering, ATP level in each sample was evaluated using luciferase enzyme as explained in the authors' previous study [11, 22]. Sirius tube luminometer (Berthold Detection System, Germany) were also used to measure Bioluminescence intensity.

Glutathione (GSH) levels

Briefly, 0.1 mL of supernatant was added to 0.1 mol l^{-1} of phosphate buffer and 0.04% DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) as GSH reagent in a total volume of 3.0 mL ($\text{pH} 7.4$). Using a spectrophotometer (UV-1601 PC, Shimadzu, Japan), the developed color was measured at 412 nm and GSH content was represented as $\mu\text{g mg}^{-1}$ protein [23].

Quantitative RT-PCR (qRT-PCR)

Isolated RNA was purified from hippocampi by TRIzol (Invitrogen) and mRNA levels of the selected genes were evaluated using qRT-PCR. cDNA was synthesized using PrimeScript™ RT Master Mix (Takara Bio, Inc., Japan) from $1\text{ }\mu\text{g}$ of total RNA as the template. qRT-PCR was performed on real time PCR (Roche Diagnostics, Germany) using SYBR Premix Ex Taq technology (Takara Bio, Japan). Thermal cycling conditions included respectively, an initial activation step lasting for 30 s at $95\text{ }^{\circ}\text{C}$, 45 cycles consisting of a denaturation step for 5 s at $95\text{ }^{\circ}\text{C}$ and a combined annealing/extension step for 20 s at $60\text{ }^{\circ}\text{C}$. To show whether all primers yielded a single PCR product, melting curve analysis was implemented. Table 1 indicates the genes and the primers used by them. Hypoxanthine phosphoribosyl transferase 1 (*Hprt1*) was extended as the housekeeping gene due to its stably expressed reference genes in our target tissue. The fold change of each target mRNA relative to *Hprt1*

Table 1 Primer sequences used for Real-time PCR assay

Gene	Sequence (5' → 3')	
	Forward	Reverse
<i>Il6</i>	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
<i>Il1β</i>	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
<i>Tnfa</i>	CTGAACTTCGGGGTGATCGG	GGCTTGCTCACTCGAATTTGAGA
<i>Hprt1</i>	TGCTCGAGATGTGATGAAGG	AAGCAGATGGCCACAGAAGT
<i>Myd88</i>	ATCGCTGTTCTTGAACCTCTCG	CTCACGGTCTAACAAGGCCAG
<i>Tlr2</i>	CTCTTCAGCAAACGCTGTTCT	GGCGTCTCCCTCTATTGTATTG
<i>Tlr4</i>	ATGGCATGGCTTACACCACC	GAGGCAATTTTGTCTCCACA
<i>Nlrp3</i>	ATCAACAGGCGAGACCTCTG	GTCTCTGGCATAACCATAGA

was measured based on $2^{-\Delta\Delta ct}$ relative expression formulas [11].

Serum glucose levels

It was measured either before the injection of STZ or 24 h after STZ injections in animals. The animal was decapitated using pentobarbital (60 mg/kg, i.p.) under mild anesthesia and their blood was collected. Then, Serum glucose concentrations were evaluated using the glucose oxidase method (Glucose Analyzer II, Beck-man).

Statistics

Results were expressed as mean \pm SD and SPSS 17 software was used for statistical analyses. Comparison between the groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests by using the Graph-Pad Prism software (version 6). $P < 0.05$ was considered statistically significant.

As the first step in this study, the sub-effective dose of minocycline (both acute and chronic treatments) was determined by using FST. Different doses of minocycline were utilized for acute treatment (20, 40 and 60 mg/kg) and chronic treatment (5, 10 and 20 mg/kg). No significant difference was observed in the immobility time of mice treated with the doses of 20 (acute) and 5 mg/kg (chronic) of minocycline (Fig. 1a, $p > 0.05$) and that of the untreated animals. However, higher doses of minocycline (40 and 60 mg/kg, $***p < 0.001$ for both and 10 and 20 mg/kg, $***p < 0.001$ for both) resulted in a significant decrease in the immobility time of the mice in the FST compared with the untreated animals [$F(3,27) = 66.46$; $p < 0.001$ in acute treatment and $F(3,27) = 36.23$; $p < 0.001$ in chronic treatment; Fig. 1]. In addition, treatments, except for the high dose of minocycline (60 mg/kg), which slightly decreased locomotor activity of mice in comparison with untreated groups (data are not shown), had no significant effect on locomotor activity measures in the OFT. Moreover, treating animals with STZ (i.c.v)/minocycline/ combination of STZ and minocycline did not significantly change the serum glucose levels in animals (between 108 and 117 mg/dl; data not shown).

After determining the sub-effective doses of minocycline for acute and chronic studies, the acute and chronic effects of STZ on mice were assessed. In comparison with control mice, STZ treatment significantly raised the immobility time in the FST after 24 h (Fig. 2a, $*p < 0.05$), and 14 days (Fig. 2a, $*p < 0.05$). In the splash test, the same treatment remarkably reduced the grooming activity time of mice after 24 h (Fig. 2b, $**p < 0.01$) and 14 days (Fig. 2b, $***p < 0.001$) in comparison with control counterparts. Furthermore, STZ treatment had no significant effect on the OFT factors (number of rearing and distance moved)

in animals after 24 h and 14 days compared with control counterparts (Fig. 2c, d, $p > 0.05$).

To study the effects of minocycline on STZ-induced depressive-like behaviors, animals were treated with different modes of minocycline treatment as mentioned in the study design. Results revealed that acute administration of minocycline considerably decreased the immobility time in STZ-treated mice in the FST [$F(4,35) = 7.203$; $p < 0.01$]. Post-hoc analysis showed that both pre-treatment (Fig. 2a, $\#p < 0.05$) and post-treatment (Fig. 2a, $\#p < 0.05$) with acute minocycline led to a significant decline in the immobility time in STZ-treated mice in the FST. In the same way, acute administration of minocycline significantly raised the grooming activity time in STZ-treated mice in the splash test [$F(4,35) = 11.738$; $p < 0.001$]. Post-hoc analysis showed that both pre-treatment (Fig. 2b, $\#\#p < 0.01$) and post-treatment (Fig. 2b, $\#\#\#p < 0.001$) with acute minocycline significantly increased the grooming activity time in STZ-treated mice in the splash test. Furthermore, treatments had no significant effect on OFT measures (the distance moved, [$F(4, 35) = 0.685$; $p > 0.05$ and the number of rearing [$F(4,35) = 3.066$; $p > 0.05$] in all groups (Fig. 2c, d).

As the Results indicated, chronic administration of minocycline significantly decreased immobility time of STZ-treated mice in the FST [$F(4,31) = 6.498$; $p < 0.01$]. Post-hoc analysis showed that both pre-treatment (Fig. 2a, $\#p < 0.05$) and post-treatment (Fig. 2a, $\#p < 0.05$) with acute minocycline significantly decreased the immobility time in STZ-treated mice in the FST. Likewise, chronic administration of minocycline led to a remarkable increase in the grooming activity time in STZ-treated mice in the splash test [$F(4,31) = 9.54$; $p < 0.001$]. Post-hoc analysis demonstrated that both pre-treatment (Fig. 2b, $\#p < 0.05$) and post-treatment (Fig. 2b, $\#\#p < 0.01$) with chronic minocycline significantly increased grooming activity time in STZ-treated mice in the splash test. In comparison with STZ-treated mice, chronic treatment with minocycline significantly changed the number of rearing [$F(4,30) = 8.532$; $p < 0.001$], but not the distance moved [$F(4,31) = 2.002$; $p > 0.05$, Fig. 2c] by mice in the OFT. As the results of Post-hoc analysis revealed, pre-treatment with chronic minocycline increased the number of rearing in mice (Fig. 2d, $***p < 0.001$), while post-treatment with chronic minocycline had no significant effect on the number of rearing compared with the STZ-treated mice (Fig. 2d, $p > 0.05$).

The effects of STZ and minocycline on mitochondrial factors

One-way ANOVA analysis demonstrated that experimental groups were significantly different in mitochondrial ROS formation in the hippocampus following the administration of acute minocycline [$F(4,15) = 28.091$, $p < 0.001$] and

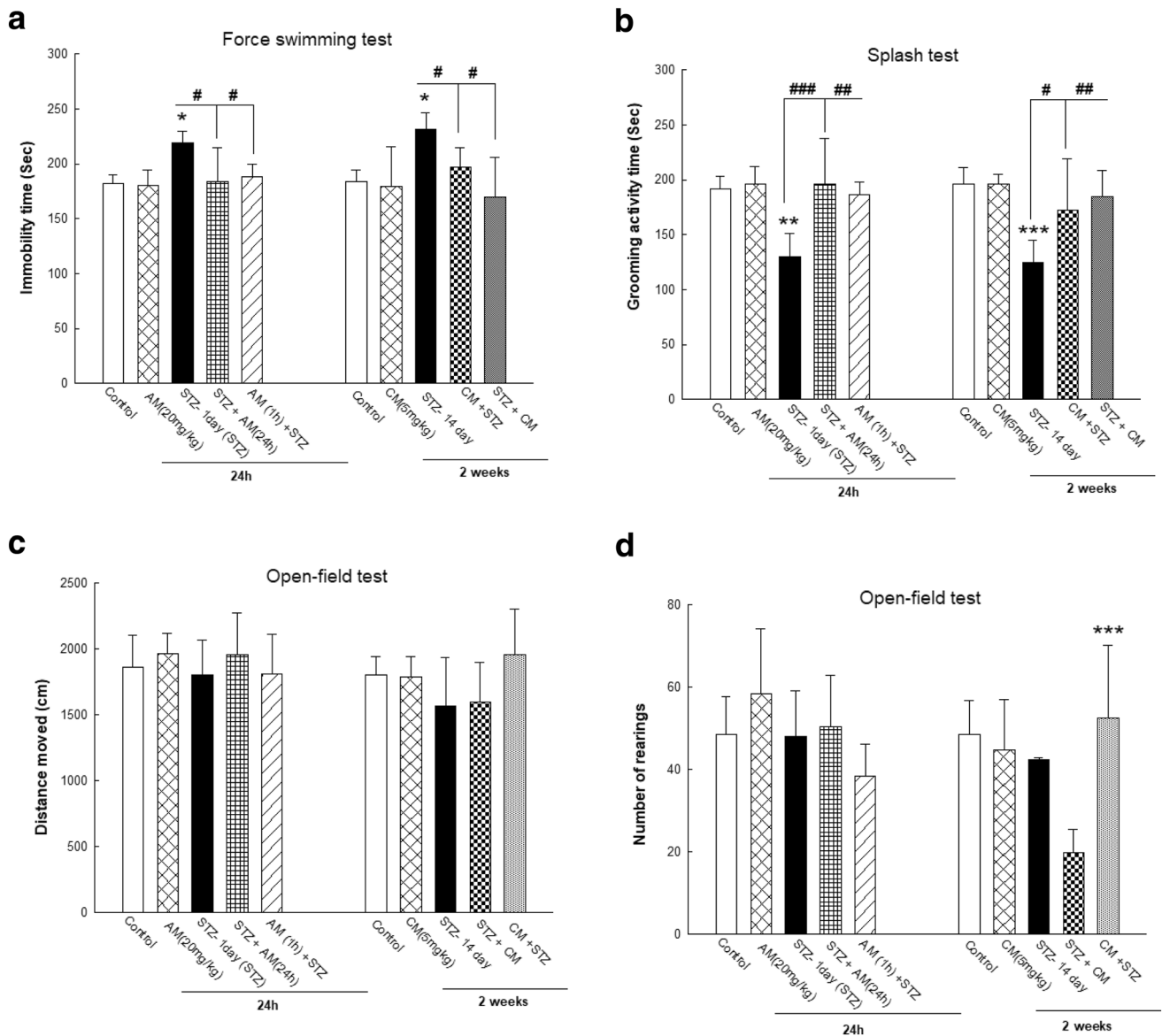


Fig. 2 Effects of treatment with minocycline (acute and chronic administration) on despair behavior in the FST (a), self-care behavior in the splash test (b), distance moved in the OFT (c) and number of rearings in the OFT (d). Values are expressed as the mean \pm SD and

were analyzed using one-way ANOVA followed by Tukey's post hoc test. AM acute minocycline and CM chronic minocycline. * $P < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control group. # $P < 0.001$ and ## $p < 0.01$ compared with STZ-received mice

chronic minocycline [$F(4,15) = 30.018$, $p < 0.001$]. Post-hoc analysis showed a significant rise in mitochondrial ROS levels following STZ treatment in the hippocampus of animals 24 h (Fig. 3a, *** $p < 0.001$) in comparison with the control group. The results indicated that both pre-treatment (Fig. 3a, ## $p < 0.01$) and post-treatment (Fig. 3a, ## $p < 0.01$) with acute minocycline significantly decreased ROS levels in the hippocampus of animals compared with STZ-treated group. Post-hoc analysis demonstrated a significant increase in mitochondrial ROS levels in the hippocampus of animals 14 days following STZ treatment (Fig. 3a, *** $p < 0.001$) in comparison with the control group. Furthermore, both

pre-treatment (Fig. 3a, ### $p < 0.001$) and post-treatment (Fig. 3a, ### $p < 0.001$) with chronic minocycline significantly decreased ROS levels in the hippocampus of animals in comparison with STZ-treated group. Furthermore, administration of minocycline did not induce significant changes in ROS level in all groups in comparison with normal animals (Fig. 3a, $p > 0.05$).

The results of one-way ANOVA analysis showed the significant differences of experimental groups in mitochondrial GSH levels in the hippocampus after acute [$F(4,15) = 4.414$, $p < 0.01$] and chronic [$F(4,15) = 8.122$, $p < 0.01$] treatment with minocycline. Post-hoc analysis indicated a significant

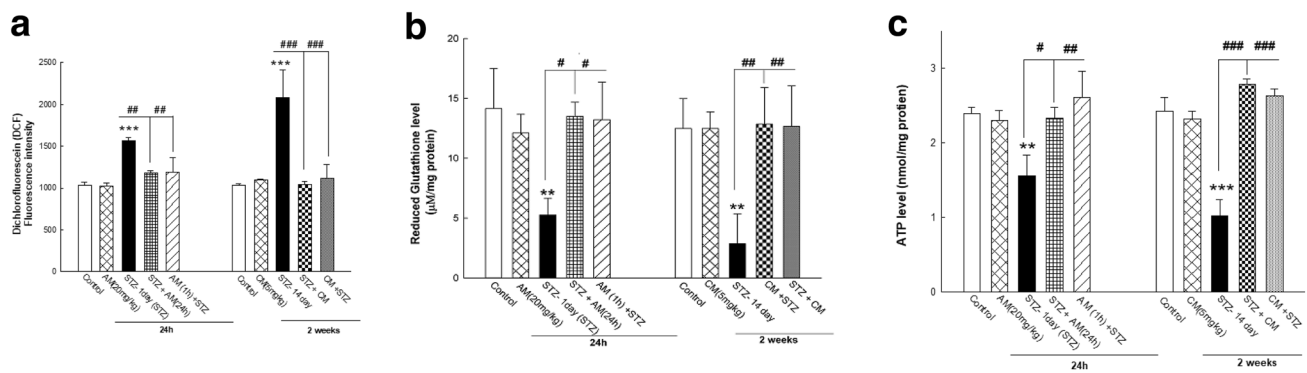


Fig. 3 Effects of treatment with minocycline (acute and chronic administration) on **(a)** ROS production, **(b)** glutathione level and **(c)** ATP level in the hippocampus. Values are expressed as the mean \pm SD and were analyzed using one-way ANOVA followed by

Tukey's post hoc test. *AM* acute minocycline and *CM* chronic minocycline. *** $P < 0.001$ compared with control group. ## $P < 0.01$ and ### $p < 0.001$ compared with STZ-received mice

decrease in mitochondrial GSH levels in the hippocampus of animals 24 h after STZ treatment (Fig. 3b, ** $p < 0.01$) in comparison with control group. In addition, both pre-treatment (Fig. 3b, # $p < 0.05$) and post-treatment (Fig. 3b, # $p < 0.05$) with acute minocycline showed a significant increase in GSH levels compared with STZ-treated group. Post-hoc analysis as well indicated a significant decrease in mitochondrial GSH levels in the hippocampus of animals 14 days following STZ treatment (Fig. 3b, ** $p < 0.01$) in comparison with control group. Results showed that both pre-treatment (Fig. 3b, ## $p < 0.01$) and post-treatment (Fig. 3b, ## $p < 0.01$) with chronic minocycline significantly increased GSH levels compared with STZ-treated group. Furthermore, administration of minocycline did not induce significant changes in mitochondrial GSH levels in all groups in comparison with control animals (Fig. 3b, $p > 0.05$).

The data showed significant differences in mitochondrial ATP levels in the hippocampus among experimental groups after acute [$F(4,15) = 12.936$, $p < 0.01$] and chronic treatment with minocycline [$F(4,15) = 97.754$, $p < 0.001$]. Post-hoc analysis indicated that there was significant decline in mitochondrial ATP levels in the hippocampus of animals 24 h after STZ treatment (Fig. 3c, ** $p < 0.01$). Furthermore, both pre-treatment (Fig. 3c, ## $p < 0.01$) and post-treatment (Fig. 3c, # $p < 0.05$) with acute minocycline significantly increased mitochondrial ATP levels compared with STZ-treated group. Post-hoc analysis also demonstrated a significant decrease in mitochondrial GSH amounts in the hippocampus of animals 14 days after STZ treatment in comparison to the control group (Fig. 3c, *** $p < 0.001$). In addition, both pre-treatment (Fig. 3c, ### $p < 0.001$) and post-treatment (Fig. 3c, ### $p < 0.001$) with chronic minocycline significantly increased GSH levels in comparison with STZ-treated group. However, administration of minocycline did not cause significant changes in mitochondrial

ATP levels in all groups compared with the control animals (Fig. 3c, $p > 0.05$).

The effects of STZ and minocycline on the expression of genes relevant to neuroinflammation

The results indicated that acute minocycline treatment significantly changed the expression of genes relevant to neuroinflammation except for *Il1 β* [$F(4,10) = 3.117$, $p > 0.05$, Fig. 4a] and *Tnfa* [$F(4,10) = 0.155$, $p > 0.05$, Fig. 4g]. Statistical analysis revealed significant differences between experimental groups in the expression of *Myd88* [$F(4,10) = 18.736$, $p < 0.001$], *Il6* [$F(4,10) = 18.736$, $p < 0.001$], *Nlrp3* [$F(4,10) = 88.513$, $p < 0.001$], *Tlr2* [$F(4,10) = 11.742$, $p < 0.001$] and *Tlr4* [$F(4,10) = 6.89$, $p < 0.01$] in treated mice after acute minocycline treatment. The expression of *Il6* (Fig. 4b, *** $p < 0.001$), *Myd88* (Fig. 4c, * $p < 0.05$), *Nlrp3* (Fig. 4d, *** $p < 0.001$), *Tlr2* (Fig. 4e, *** $p < 0.001$), and *Tlr4* (Fig. 4f, * $p < 0.05$) in the hippocampus of animals increased 24 h after STZ treatment in comparison with the control group. Post-hoc analysis revealed that pre-treatment of STZ-treated animals with acute minocycline significantly declined the expression of *Il6* (Fig. 4b, ### $p < 0.001$), *Nlrp3* (Fig. 4d, ### $p < 0.001$), *Tlr2* (Fig. 4e, ### $p < 0.001$), and *Tlr4* (Fig. 4f, # $p < 0.05$) in comparison with STZ-treated animals. Pre-treatment with minocycline caused no significant change in the expression of *Il1 β* (Fig. 4a, $p > 0.05$), *Myd88* (Fig. 4c, $p > 0.05$), and *Tnfa* (Fig. 4g, $p > 0.05$) genes compared with STZ-treated groups. Post-treatment of STZ-treated animals with acute minocycline significantly decreased the expression of *Il6* (Fig. 4b, ### $p < 0.001$), *Nlrp3* (Fig. 4d, ### $p < 0.001$), and *Tlr2* (Fig. 4e, ### $p < 0.001$) in the hippocampus in comparison with STZ-treated animals. Post-treatment with minocycline did not significantly change the expression of *Il1 β*

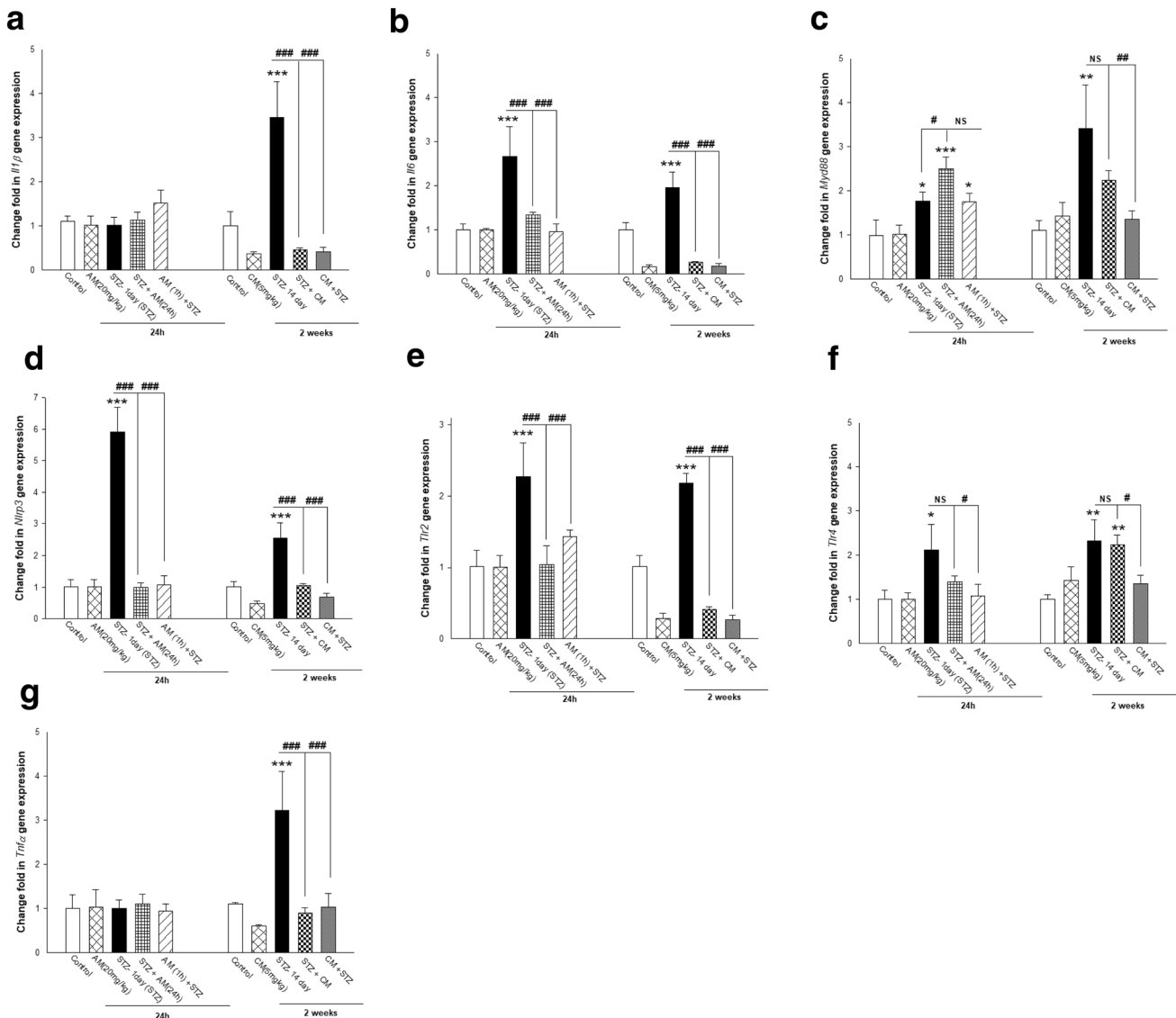


Fig. 4 Effects of treatment with minocycline (acute and chronic administration) on expression of genes related to neuroinflammation in the hippocampus **a** *Il1β*, **b** *Il6*, **c** *Myd88*, **d** *Nlrp3*, **e** *Tlr2*, **f** *Tlr4* and **g** *Tnfa* in the hippocampus. Values are expressed as the mean \pm SD

(Fig. 4a, $p > 0.05$), *Tlr4* (Fig. 4f, $p > 0.05$), and *Tnfa* (Fig. 4g, $p > 0.05$) genes compared with STZ-treated groups. In addition, post-treatment with acute minocycline significantly increased the expression of *Myd88* (Fig. 4c, $\#p < 0.05$) in the hippocampus in comparison with STZ-treated animals. In addition, acute treatment with minocycline did not revealed significant differences in the expression of genes in control animals (Fig. 4, $p > 0.05$ for all).

Based on One-way ANOVA analysis, experimental groups significantly differed in the expression of *Il1β* [$F(4,10) = 33.971$, $p < 0.001$], *Il6* [$F(4,10) = 58.964$, $p < 0.001$], *Myd88* [$F(4,10) = 11.019$, $p < 0.01$], *Nlrp3* [$F(4,10) = 36.591$, $p < 0.01$], *Tlr2* [$F(4,10) = 175.742$,

and were analyzed using one-way ANOVA followed by Tukey's post hoc test. AM acute minocycline and CM chronic minocycline. *** $P < 0.001$ compared with control group. # $P < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared with STZ-received mice

$p < 0.001$], *Tlr4* [$F(4,10) = 12.193$, $p < 0.001$], and *Tnfa* [$F(4,10) = 18.908$, $p < 0.05$] genes in the hippocampus after chronic minocycline treatment. The expression of *Il1β* (Fig. 4a, *** $p < 0.001$), *Il6* (Fig. 4b, *** $p < 0.001$), *Myd88* (Fig. 4c, ** $p < 0.01$), *Nlrp3* (Fig. 4d, *** $p < 0.001$), *Tlr2* (Fig. 4e, *** $p < 0.001$), *Tlr4* (Fig. 4f, ** $p < 0.01$), and *Tnfa* (Fig. 4g, *** $p < 0.001$) in the hippocampus of animals increased 14 days after STZ treatment in comparison with control group. Post-hoc analysis also revealed that pre-treatment of STZ-treated animals with chronic minocycline significantly decreased the expression of *Il1β* (Fig. 4a, ### $p < 0.001$), *Il6* (Fig. 4b, ### $p < 0.001$), *Myd88* (Fig. 4c, ## $p < 0.01$), *Nlrp3* (Fig. 4d, ### $p < 0.001$),

Tlr2 (Fig. 4e, ### $p < 0.001$), and *Tlr4* (Fig. 4f, # $p < 0.05$), and *Tnfa* (Fig. 4g, ### $p < 0.001$) genes in the hippocampus in comparison with STZ-treated animals. Post-treatment of STZ-treated animals with chronic minocycline for 14 days significantly reduced the expression of *Il1 β* (Fig. 4a, ### $p < 0.001$), *Il6* (Fig. 4b, ### $p < 0.001$), *Nlrp3* (Fig. 4d, ### $p < 0.001$), *Tlr2* (Fig. 4e, ### $p < 0.001$), and *Tnfa* (Fig. 4g, ### $p < 0.001$) in the hippocampus compared with STZ-treated animals. Post-treatment with minocycline caused no significant difference in the expression of *Myd88* (Fig. 4c, $p > 0.05$) and *Tlr4* (Fig. 4f, $p > 0.05$) genes in comparison with STZ-treated groups. In addition, chronic treatment with minocycline did not indicate any significant difference in the expression of genes in control animals (Fig. 4a–g, $p > 0.05$).

Discussion

The present study investigated the short- and long-term effects of a single intracerebroventricular injection of low doses of STZ on adult mice. STZ triggered oxidative stress and innate immune responses in the hippocampi and provoked depressive-like behaviors in animals. The effects of acute and chronic minocycline treatment were also compared with the negative impact of STZ, here. The results showed that minocycline treatment alleviates depressive-like behaviors in animals via mitigating oxidative stress and inflammatory responses in the hippocampus.

Numerous studies have indicated that oxidative stress [3, 5] and immune-inflammatory pathways are involved in the pathophysiology of depression [24, 25]. The current study utilized an animal model of depression, in which mitochondrial dysfunction and sterile inflammation are the major players. According to the authors' previous studies, STZ-treated animals exhibit depressive-like behaviors along with hippocampal mitochondrial dysfunction and immune-inflammatory responses 24 h after the administration of a single low dose of STZ [11]. Results of the current study not only confirmed our previous results (short-term effects after 24 h), but also revealed that STZ-induced effects are present in animals 2 weeks (long-term effects) after the single administration of STZ. Here, both short- and long-term effects of STZ on the hippocampal redox balance were associated with oxidative stress (increased ROS formation), impaired antioxidant capacity (decreased mitochondrial GSH), and impaired energy metabolism (decreased mitochondrial ATP levels). The results indicated that low doses of STZ could severely affect mitochondrial function and redox system in the hippocampus.

As mentioned earlier, mitochondrial dysfunction is known to produce a variety of DAMPs (such as ROS, oxidized lipids, oxidized cardiolipin, mitochondrial DNA) and

activate inflammatory signaling through different mechanisms including TLRs activation and inflammasome formation [8, 9]. Based on the results, ROS overproduction and impaired redox system were accompanied with overexpression of genes related to innate immunity after short- and long-term exposure to STZ. After short term exposure to STZ, expression pattern of some genes was different from the pattern observed 14 days after STZ treatment. Expression of both *Il1 β* and *Tnfa* were not affected after short term exposure to STZ, but increased significantly after 14 days. The data indicated that chronic effects of STZ-induced damage were different from early responses to STZ, and lack of treatment worsened the inflammatory response by the involvement of *Il1 β* and *Tnfa* and neuroprotection. On the other hand, STZ exposure increased the expression of *Il6* in both conditions, suggesting the important role of IL-6 in early response to oxidative damage and consequent behavioral abnormalities. Clinical studies showed that IL-6 increases in the brain of patients with depression [26]. Further evidence demonstrated that transgenic mice with the overexpression of IL-6 in the brain exhibited depressive-like behaviors in the FST just like the animals that received intracranial administration of IL-6 [27]. Therefore, ROS overproduction and inflammatory responses after STZ treatment in the hippocampus was supported.

The protective effects of different modes of minocycline were compared here with the negative impact of STZ on hippocampal oxidative state, inflammatory responses and behavior of animals. Previous studies provided evidence that minocycline had various therapeutic effects ranging from anti-inflammatory and antioxidant to neuroprotective and antidepressant effects [12, 28–30]. It was also shown that both pre- and post-STZ treatments with (acute and chronic) minocycline led to the attenuation of STZ-induced effects on depressive-like behaviors, oxidative state, and immune-inflammatory gene expression.

Recent evidence indicates that a single dose STZ (very low dose, i.c.v) is used to induce an animal model of sterile inflammation-induced depression [11, 31]. Interestingly, evidence indicated that there is comorbid depression and diabetes in patients with AD, and similar bi-directional comorbidity is observed in patients with depression and diabetes [32–35]. It is supposed that mitochondrial dysfunction, (neuro) inflammation, cognitive and behavioral abnormalities, and oxidative stress were involved in the pathophysiology of depression [3, 36]. Based on our findings, minocycline treatment attenuated STZ-induced alterations in oxidative state, immune-inflammatory status, and behavior of animals. Previous studies on rodents revealed that minocycline exerted antidepressant-like effects through improving the behaviors associated with the core symptoms of depression [30]. In addition, minocycline was shown to have neuroprotective effects against STZ-induced retinopathy [37], neuropathy

[38], and neuropathic pain through attenuating the inflammatory responses and oxidative stress [39]. Similar to STZ-induced effects, there are pathological conditions such as acute stroke and traumatic brain injury (TBI), in which mitochondrial dysfunction and neuroinflammation play key roles in their pathology. The evidence indicates their liable and efficient therapeutic effects of minocycline in both TBI [40, 41], and acute stroke [29].

Overall, an animal model of depression induced by oxidative stress and neuroinflammation was used in the present study, showing that different modes of minocycline treatment were able to alleviate the impact of STZ at molecular and behavioral levels. Results of this study may be useful for studies on the comorbid depression in AD, type 2 diabetes or following TBI and stroke.

Author contributions Conceived and designed the experiments and the study protocol: MJH & SEM; Performed the experiments: HM, SA, MM & SB; Analyzed the data: MJH, SA & HAK; Interpretation of the data: MJH & SEM; Wrote the paper: MJH, SA & SEM. Critical review of the manuscript: MJH, SA & SEM.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving human participants and/or animals All procedures were performed in line with the NIH Guide for the Care and Use of Laboratory Animals, which were approved by the Animal Ethics committee of Zanjan University of Medical Sciences (registration number: ZUMS.REC.1394.244).

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