Metformin Protective Effects in LPS-Induced Alzheimer's Disease Mice Model: NO-cGMP-K_{ATP} Pathway Involvement

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Received: Sep. 2024; Accepted: Dec. 2024

Abstract- This project has studied the effects of metformin on cognition impairment, depression, hyperalgesia, stress oxidative and neuroinflammation in a rodent Alzheimer's disease (AD) model created via LPS (lipopolysaccharide). For defining possible mechanisms, the NO/cGMP/K_{ATP} pathway roleplay was considered . Mice model was created via LPS treatment. Open field forced swimming and hot plate tests were done. Shuttle-box test and Y-maze test were used to assay Learning-memory. Biochemical assay compromised malondialdehyde (MDA) and TNF-alpha concentration and superoxide dismutase (SOD) activity measurement in hippocampus samples. NO-cGMP-KATP pathway contribution was assessed by its agonists/antagonist pre-treatment, 15 min before metformin (150, 200, 250 mg/kg). Initial latency (IL) was increased by LPS injection while it was reduced by metformin (250 mg), in shuttle-box test. Pretreatment with methylene blue, L-NAME and glibenclamide before metformin augmented IL, although it was diminished by L- arginine and sildenafil pretreatment. Also, metformin increased the LPS induced step through latency (STL) reduction. L-NAME, methylene blue and glibenclamide decreased the STL, but it was increased by L-arginine and sildenafil. In Y-maze test, metformin increased the LPS-induced spontaneous alternation reduction. L-NAME, methylene blue and glibenclamide decreased it. LPS added immobility time in forced swimming trial, whereas it was reduced thru metformin. L-NAME, methylene blue and glibenclamide increased the anti-depressive effect of metformin while it was attenuated via L-arginine, sildenafil and diazoxide. LPS treatment diminished the threshold of pain perception in hot-plate test, while metformin didn't have any significant effect. Metformin reduced the LPS-induced lipid peroxidation (MDA level). But, L-NAME, methylene blue and glibenclamide worsen the lipid peroxidation, whereas it was improved by L-arginine and sildenafil. Metformin improved LPS-induced reduction in SOD activity. SOD activity was reduced by L-NAME, methylene blue and glibenclamide pre-treatment. LPS enhanced TNF-alpha amount that decreased by metformin. Pre-injection with methylene blue, L-NAME and glibenclamide increased TNF-alpha concentrations while L-arginine, sildenafil and diazoxide reduced it. Conclusions: Metformin can improve learning-memory loss, depression, hyperalgesia, neuroinflammation and oxidative stress produced by LPS and NO/cGMP/K_{ATP} pathway maybe has a roleplay.

Acta Med Iran 2025;63(January-February):7-18.
https://doi.org/10.18502/acta.v63i1.18589

Keywords: Metformin; Lipopolysaccharides (LPS); Cognition; Oxidative stress; Depression; Hyperalgesia; Neuroinflammation; Mice

Introduction

There are several evidence indicating relations between diabetes and insulin resistance with mental

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deficiencies and memorial damage. So, Alzheimer's disease (AD) can be considered as "type 3 diabetes" (1). Similar to AD and dementia, diabetes is mentioned as a risk factor for cognitive loss and perhaps causes cognitive deficit. Based on previous reports, some antidiabetic drugs, for example metformin have shown some advantage and might contribute to cognition improvement. So, diabetic patients are at risk for cognitive impairment. Therefore, metformin can be a new researching target that beyond glucose-lowering outcomes, can be considered in neurodegenerative diseases including AD (2). Also, other reports confirm the anti-oxidative and anti-inflammatory effects of metformin can improve cognition loss. But the mechanisms of metformin effects are unknown (3).

Since, a biguanide-dependent mechanism via stimulating the NO/cGMP/K^+ channel pathway can cause neural effects such as antinociception, maybe this pathway involves in the metformin effects on neurodegenerative disorders (4). For instance, findings have displayed that nitric oxide (NO) is effective in the colitis (5). Moreover, NO protects cells biochemical enzymes via picking up the oxidative factors. And also, NO-induced cGMP production in turn, can cause anti-inflammatory and regulatory effects (6). Furthermore, based on the previous studies, K_{ATP} channels opening inhibits the reactive oxygen species release from the cell organelles. So, it can produce anti-inflammatory and anti-oxidative outcomes (7).

This research project was designed to realize the improving properties of metformin on some AD complications such as cognition loss, depression, hyperalgesia, neuroinflammation and stress oxidative on AD model caused by LPS. The possible contribution of NO-cGMP-K_{ATP} pathway was surveyed for defining possible mechanism.

Materials and Methods

Mice

This study was done on NMRI male mice with a full food/water accessibility (weighting 25-30 g). The mice shelter had a normal glory cycle, temperature and humidity. This project was accepted by Dezful University of Medical Sciences ethics committee (IR.DUMS.REC.1399.003) and all proceedings were in accordance with NIH Guide and ARRIVE guidelines. Just 6 mice per group were considered for finding trustworthy scientific data.

Prescriptions

Metformin, LPS (Lipopolysaccharide), sildenafil (a phosphodiesterase type 5 [PDE5] blocker), diazoxide for K^+_{ATP} channel opening, Glibenclamide for K^+_{ATP} channel inhibition, L-arginine (a NO forerunner) and L-NAME (a nitric oxide synthase [NOS] blocker) were achieved from Sigma–Aldrich (USA). Methylene blue as a guanylyl cyclase inhibitor was purchased from Merck (Germany). These mentioned medicines were freshly dissolved by normal-saline but glibenclamide was dissolved in DMSO (dimethyl sulfoxide 1%, Fermentas Life Sciences, Lithuania). After that, these drugs were diluted by normal Salin up to 10 times of the first quantity. All said medicines were inoculated (10 ml/kg) to animals via intraperitoneal space (i.p.). The study method and drugs dose were conducted based on previous reports (8,9).

Behavioral tests

Behavior evaluation contained shuttle box (passive avoidance memory analysis), Y-maze (spatial memory analysis), open-field, forced-swimming and hot plate tests. Afterward, for lipid peroxidation and oxidative stress status evaluation, TNF-alpha and MDA (malondialdehyde) level also SOD (superoxide dismutase) performance was assayed in hippocampi tissue samples.

Shuttle box test

After some modifications, the Tamburella's method was done for the passive avoidance task valuation. This tool has two nearby Plexiglas light/dark rooms of similar sizes (27×14.5×14 cm). Their floor was covered by separate stainless-steel bars. The bright room had a 5 W lamp. The test protocol had three days of try. On the first day, mice had 10 minutes to get familiar with apparatus. Next day, animals were located in the brilliant part and the gate was opened, 10 secs later. The delay of entry to the dim part was noted as cognition parameter, initial-latency (IL). Afterwards, the gate was fastened. So, when all paws touched the floor bars for 3 secs, a little electrical 0.3 mA shock was done. On the last day, they were located in the brilliant box and 10 seconds later the gate was opened, with no shock. The dim box entrance delay was measured by way of step-through latency (STL), as memory/learning index. Trial cut-off was designed for 300 sec (9).

Y-maze test

A device consists of 3 parts (A, B, C) by identical 30 cm length, width (5 cm) and height (12 cm) and 120° angle between arms was used for short-term memory

assay. All mice were observed for eight min. Primary, every animal was located within a separate part. Each stayed part's name was noted. In addition, the count of entrance to each part was noted. Then alternation-percentage index for all animals was estimated. For this, the ratio of the actual number of alterations to the probable number (the total number of arm entries minus two) multiplied in 100 was calculated. The total quantity of parts visit was assumed locomotion parameter (9).

Open field test (OFT)

Animal activity was examined with an open field test just before other behavioral evaluations. This device had a woody box to the dimensions of 40×60×50 cm. The tool ground had twelve same quadrangles. Animals were placed in the left angle separately and could travel freely. The squares number crossing with all paws were recorded (during six min). In order to minimize animal signs, after every mouse test, the tool was cleaned by 10% ethanol solution (8).

Forced-swimming test (FST)

This trial was designed to assess depressive-like behavior. Each animal was released in a cylinder-shaped water chamber, to the dimensions of 10 cm diameter and 25 cm tallness contains 19 cm water (25±1° C). Each mouse had enough time (six min) to swim freely. Then, the immovability period in the final four minutes of the trial was noted. End of effort (being motionless) except critical activities to hold the head out of water mentioned; immobility time (8).

Hot-plate test

The instrument had a graded 52±0.2° C hot metal plate with a Plexiglas cylinder for mouse placement. Licking back foot or jumping out of the cylinder were considered as pain perception threshold. The test had two days, at first day mice had 2 minutes to know device. The second day was trial day. To prevent tissue injury, 40 second cut-off time was chosen. After every mouse test, the tool was cleaned by 10% ethanol solution (10).

TNF-α, MDA level and SOD activity assay

Mice hippocampal samples were dissected immediately after anesthesia. Samples were rinsed by sodium chloride 0.9% and were placed at –80° C, up to ELISA test. After shaking (for 90 min) and centrifuging (for 15 min at 4° C, 4000×g), their supernatants were separated (11). ELISA kits for SOD, MDA and TNF-α assay achieved from the LDN Immunoassays Company (Germany). The assays were conducted based on company instruction.

Medications

To make an Alzheimer's disease model; first day, all groups received LPS injection for 7 consecutive days (0.25 mg/kg, 0.1 mL/10 g). Instead, the control group received same quantity of vehicle (12). During the first set of the trials, to find the effective dose of metformin; 150, 200 and 250 mg/kg or 10 ml/kg solvent were injected thirty minutes before behavioral tests (for 14 days).

During the second set, for finding potential NO/cGMP/K_{ATP} roleplay in metformin outcomes, groups were pretreated by agonist/antagonist for each case, fifty minutes earlier than efficient dose of metformin (250 mg/kg). NO involvement was examined via L-arginine (750 mg/kg) and L-NAME (10 mg/kg) or their vehicle pre-treatment, 15 min before metformin. cGMP roleplay was assessed via methylene blue (20 mg/kg) and sildenafil (5 mg/kg) or their vehicles pre-treatment, 15 min before metformin. K^+ channels involvement was evaluated via glibenclamide (1 mg/kg) and diazoxide (5 mg/kg) or their vehicles pre-treatment, fifty minutes before metformin. These agonist/antagonist treatment protocol/doses were planned based on our group's previous studies (9).

Statistics

Results was expressed as means±SEM. Normality was assessed through Kolmogorov–Smirnov assay. Statistical analysis was done by version 22 of SPSS and one-way ANOVA followed by Tukey's post-hoc trial. A *P* below 0.05 was assumed as significancy between groups.

Results

Metformin effects in the shuttle-box test

LPS enhanced ($^{**}P<0.01$) IL and decreased ($^{***}P<0.001$) STL versus control group. Metformin (250 mg/kg) reduced IL ($^{\#}P<0.05$) and enhanced STL ($^{\#}P<0.05$) significantly at the doses of 200 and 250 mg/kg (Figure 1A). So as for assessment of probable role of NO/cGMP/K_{ATP}, mice were pre-received suitable agonists/antagonists while these have no significant influence on IL and STL, when have used lonely.

NO roleplay in the metformin effects in the shuttle-box test

L-NAME (10 mg/kg) increased ($^{**}P<0.01$) IL and decreased ($^{*}P<0.05$) STL against LPS+ metformin (250 mg/kg) received group. While L-arginine (750 mg/kg)

pre-treatment reduced ($^{*}P$<0.05) IL and enhanced ($^{*}P$< 0.05) STL against LPS+ metformin (250 mg/kg) received group (Figure 1B).

cGMP roleplay in the metformin effects in the shuttle-box test

Methylene blue (20 mg/kg) enhanced ($^{**}P$<0.01) IL and reduced ($^{*}P$<0.05) STL against LPS+ metformin (250 mg/kg) received group. While sildenafil (5 mg/ kg) pre-treatment reduced ($^{*}P$<0.05) IL and enhanced ($^{*}P$<0.05) STL against LPS+ metformin (250 mg/kg) received group (Figure 1C).

K_{ATP} channels roleplay in the metformin effects in the shuttle box test

Glibenclamide (1 mg/kg) enhanced ($^{*}P$<0.05) IL and reduced ($^{**}P$<0.01) STL comparing LPS+ metformin (250 mg/kg) received group. But diazoxide (10 mg/kg) has no significant effect on IL and STL against LPS+metformin (250 mg/kg) received group (Figure 1D).

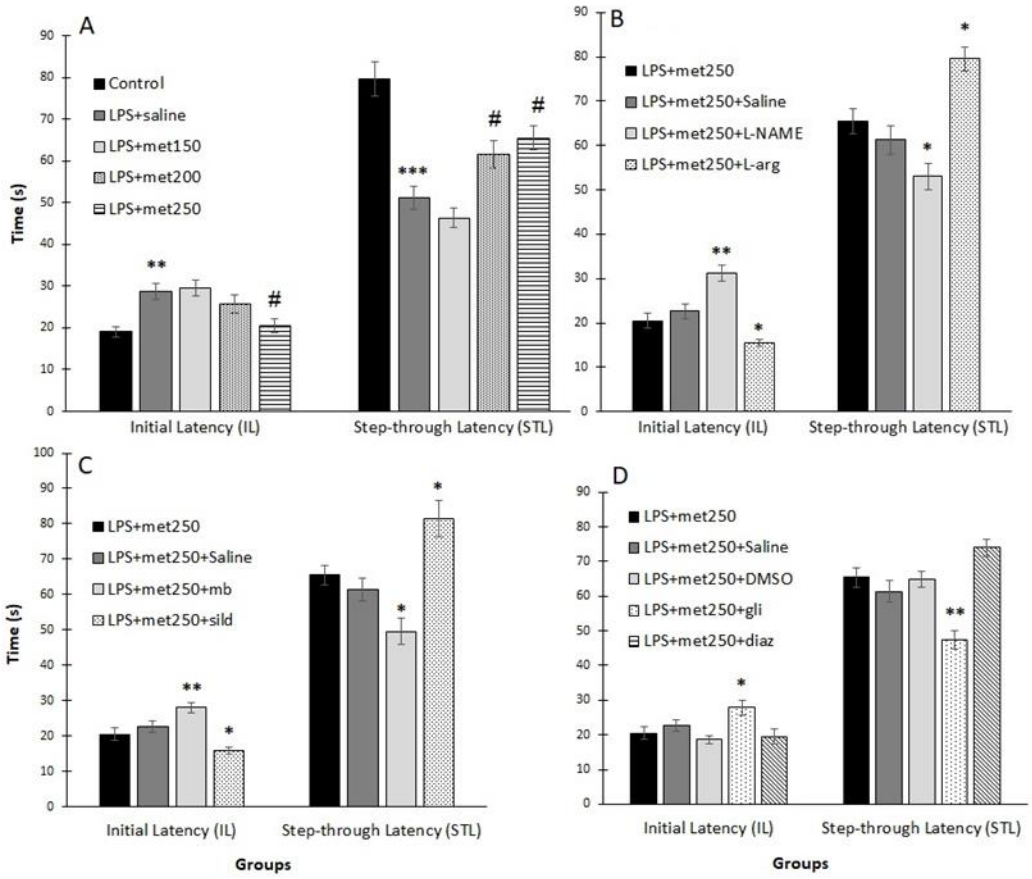

Figure 1. Metformin effects (150, 200 and 250 mg/kg) in IL/STL in shuttle box test (A), $^{**}P$< 0.01 and $^{***}P$< 0.001 versus solvent-received control group. #P<0.05 versus LPS+solvent received group. NO/cGMP/K_{ATP} roleplay in metformin effects are shown in sections B, C and D one-to-one, $^{*}P$<0.05, $^{**}P$<0.01 versus LPS+metformin (250 mg/kg) received group

Metformin effects in the Y-maze test

The overall number of arm entrances had no difference between groups. LPS decreased spontaneous alternation percentage (SAP) against control group ($^{*}P$<0.05). While metformin (250 mg/kg) enhanced SAP ($^{\#}P$<0.05) (Figure 2A). For assessment of potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on SAP, when have used lonely.

NO roleplay in the metformin effects in Y-maze test

L-NAME (10 mg/kg) pre-treatment decreased ($^{**}P$<0.01) SAP against LPS+ metformin (250 mg/kg) received group. While L-arginine (750 mg/kg) pre-treatment had no significant effect (Figure 2B).

cGMP roleplay in the metformin effects in Y-maze test

Methylene blue (20 mg/kg) pre-treatment decreased (***P<0.001) SAP against LPS+ metformin (250 mg/kg) received group. While sildenafil (5 mg/kg) pre-treatment had no significant effect (Figure 2C).

K_{ATP} channels roleplay in the metformin effects in Y-maze test

Glibenclamide (1 mg/kg) pre-treatment decreased (**P<0.01) SAP against LPS+ metformin (250 mg/kg) received group. Whereas diazoxide (10 mg/kg) pre-treatment had no significant effect (Figure 2D).

Metformin Effects in the open-field and forced-swimming tests

The locomotion had no significance among study groups, in open-field test (P>0.05). LPS enhanced the immovability period against control group (***P<0.001) in FST. Metformin (250 mg/kg) reduced the immovability period (##P<0.01; Figure 3A). For assessment of potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on FST data, when have used lonely.

NO roleplay in the metformin antidepressant effects in FST

L-NAME (10 mg/kg) pre-treatment reduced (**P<0.01) the immovability. While L-arginine pretreatment (750 mg/kg) enhanced (*P<0.05) it (Figure 3B).

cGMP roleplay in the metformin antidepressant effects in FST

Pre-treatment by methylene blue (20 mg/kg) reduced the immovability (**P<0.01). While sildenafil (5 mg/ kg) pre-treatment increased immovability (**P<0.01) (Figure 3C).

KATP channels roleplay in the metformin antidepressant effects in FST

Glibenclamide (1 mg/kg) pre-treatment reduced (*P<0.05) immovability. While diazoxide pre-treatment (10 mg/kg) enhanced (*P<0.05) the immovability (Figure 3D).

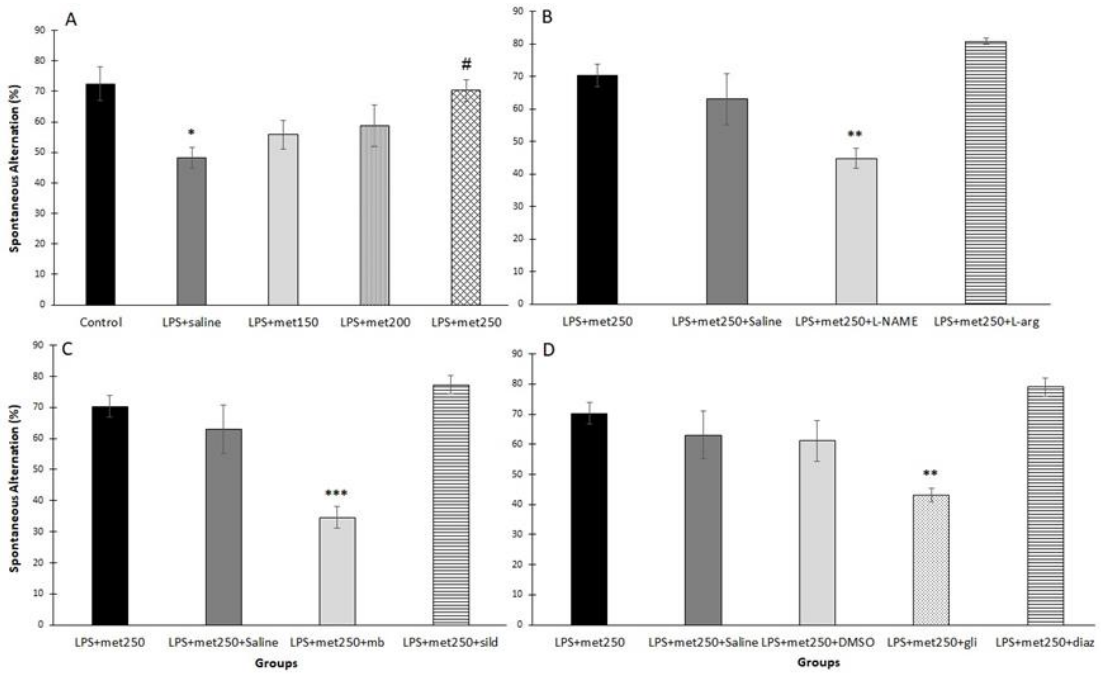

Figure 2. Metformin (150, 200 and 250 mg/ kg) Effects in Y-maze test (A), *P<0.05 against vehicle- received control group. #P<0.05 against LPS+vehicle received group. NO/cGMP/K_{ATP} roleplay in metformin effects are displayed in sections B, C and D one-to-one, ***P<0.001, **P<0.01 against LPS+metformin (250 mg/kg) received group.

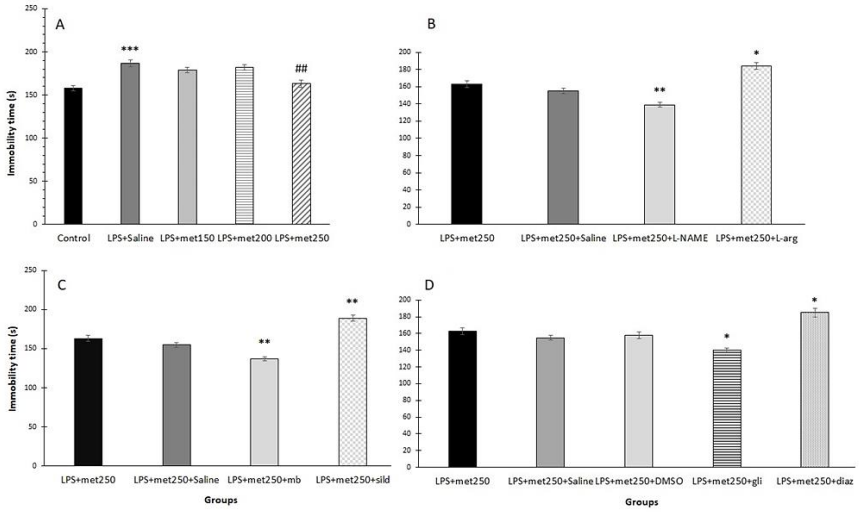

Figure 3. Metformin effects (150, 200 and 250 mg/kg) in FST (A), ***P<0.001 against solvent-received control group. ##P<0.01 against LPS+solvent received group. NO/cGMP/K_{ATP} roleplay in metformin effects are displayed in sections B, C and D one-to-one, *P<0.05, **P<0.01 against LPS+metformin (250 mg/kg) received group

Metformin effects on the hot-plate test

LPS reduced the pain threshold against control group (*P<0.05). Metformin didn't have significant effect on the pain threshold by any doses (Figure 4A). For assessment of potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on the pain threshold, when have used lonely.

NO roleplay in the effects of metformin in hot-plate test

L-NAME (10 mg/kg) Pre-treatment diminished (*P<0.05) the thermal pain threshold versus LPS+metformin (250 mg/kg) received group. While L-arginine (750 mg/kg) pre-treatment enhanced (*P<0.05) the pain threshold versus LPS+ metformin (250 mg/kg) treated group (Figure 4B).

cGMP roleplay in the metformin effects in hot-plate test

Methylene blue (20 mg/kg) reduced (*P<0.05) the thermal pain perception versus LPS+metformin (250 mg/kg) treated group. But, sildenafil (5 mg/ kg) had no significant influence (Figure 4C).

K_{ATP} channels roleplay in the metformin effects in hot-plate test

Glibenclamide (1 mg/k) and diazoxide (10 mg/kg) pre-treatment had no effect versus LPS+ metformin (250 mg/kg) treated group (Figure 4D).

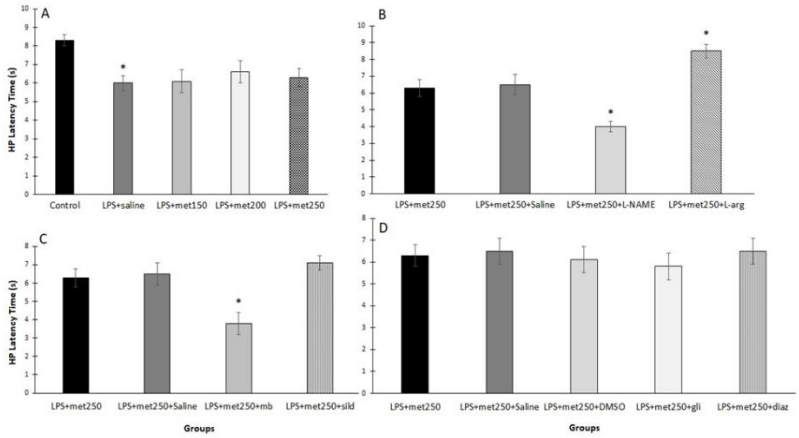

Figure 4. Effects of metformin (150, 200 and 250 mg/kg) on pain threshold (A), *P< 0.05 against solvent-received control group. Involvement of NO-cGMP-K_{ATP} pathway is shown in sections B, C and D one-to-one, *P< 0.05 against LPS+metformin (250 mg/kg) treated group

Metformin effects on the brain tissue TNFα quantities

Brain tissue TNFα quantities (pg/ml) was assayed as inflammation indicator, where it was enhanced by LPS against control (***P<0.001). Metformin (250 mg/kg) diminished TNF-α quantities ($^{\#\#}P$<0.01; Figure 5A). To assay potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on the TNFα quantities, lonely.

NO roleplay in the metformin effects on TNFα quantities

L-NAME (10 mg/kg) pre-treatment enhanced (*P<0.05) the TNFα quantities. But L-arginine (750 mg/kg) reduced (*P<0.05) it versus LPS+metformin (250 mg/kg) treated group (Figure 5B).

cGMP roleplay in the metformin effects on TNFα quantities

Methylene blue (20 mg/kg) pre-treatment enhanced (*P<0.05) the TNFα quantities. But, sildenafil (5 mg/kg) reduced (*P<0.05) the TNFα quantities against LPS+metformin (250 mg/kg) treated animals (Figure 5C).

K_{ATP} channels roleplay in the metformin effects on TNFα quantities

Glibenclamide (1 mg/kg) pre-treatment enhanced (**P<0.01) the TNF-α quantities. But, diazoxide (10 mg/kg) pre-treatment reduced (*P<0.05) the TNFα quantities against LPS+metformin (250 mg/kg) treated animals (Figure 5D).

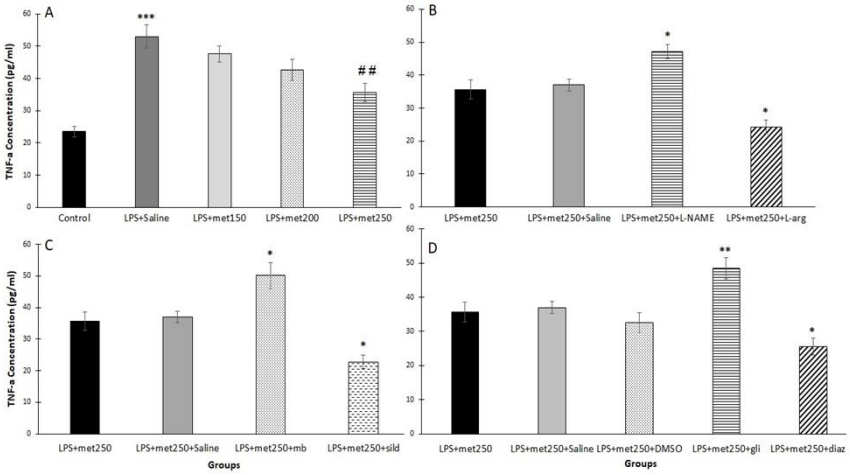

Figure 5. Metformin (150, 200 and 250 mg/kg) effects on TNFα quantities in the hippocampi samples (A), ***P< 0.001 versus solvent-received control group. ##P< 0.01 versus LPS+solvent received group. NO-cGMP-K_{ATP} roleplay in metformin effects are displayed in sections B, C and D one-to-one, *P< 0.05, **P< 0.01 versus LPS+metformin (250 mg/kg) treated group.

Metformin effects on the brain tissue MDA quantities

malondialdehyde as a lipid peroxidation indicator was evaluated in hippocampus samples. LPS enhanced the MDA quantities comparing control group (***P<0.001). Metformin reduced the MDA quantities at doses of 200 and 250 mg/kg ($^{\#}P$<0.05, $^{\#\#}P$<0.01) (Figure 6A). To assay potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on the MDA quantities, lonely.

NO roleplay in the metformin effects on MDA quantities

L-NAME (10 mg/kg) pre-treatment enhanced (*P<0.05) the MDA quantities. Whereas L-arginine (750 mg/kg) pre-treatment reduced (**P<0.01) it against LPS+metformin (250 mg/kg) received group (Figure 6B).

cGMP roleplay in the metformin effects on MDA quantities

Methylene blue (20 mg/kg) pre-treatment enhanced (*P<0.05) the MDA quantities. But, sildenafil (5 mg/ kg) pre-treatment reduced (*P<0.05) it against LPS+metformin (250 mg/kg) received group (Figure 6C).

K_{ATP} channels roleplay in the metformin effects on MDA quantities

Glibenclamide (1 mg/kg) pre-treatment enhanced (*P<0.05) MDA quantities against LPS+metformin (250 mg/kg) received group. But, diazoxide (10 mg/kg) pre-treatment had no significant effect (Figure 6D).

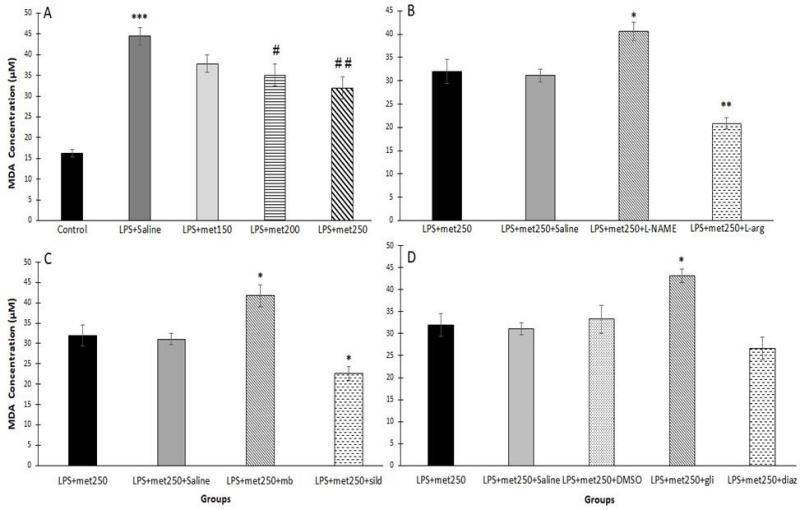

Figure 6. Effects of metformin (150, 200 and 250 mg/kg) on the MDA quantities in the hippocampaus samples (A), ***P<0.001 against solvent-received control group. #P<0.05, ##P<0.01 against LPS+solvent received group. NO/cGMP/K_{ATP} roleplay was displayed in sections B, C and D one-to-one, *P<0.05, **P<0.01 against LPS+metformin (250 mg/kg) received group

Metformin effects on SOD activity

Superoxide dismutase activity (u/ml) as an antioxidant indicator was evaluated in hippocampus tissue samples. SOD activity was reduced by LPS, comparing control group (***P<0.001). Metformin (250 mg/kg) enhanced the SOD activity ($^{\#\#}P$<0.01) (Figure 7A). To assay potential NO/cGMP/K_{ATP} roleplay, mice were pre-received suitable agonists/antagonists while these have no significant influence on the SOD activity, lonely.

NO roleplay in the metformin effects on SOD activity

L-NAME (10 mg/kg) pre-treatment diminished (***P<0.001) SOD activity against LPS+metformin (250 mg/kg) received group. but, L-arginine (750 mg/kg) pre-treatment had no significant influence (Figure 7B).

cGMP roleplay in the metformin effects on SOD activity

Methylene blue (20 mg/kg) pre-treatment diminished (**P<0.01) the SOD activity against LPS+metformin (250 mg/kg) received group. But, sildenafil (5 mg/ kg) pre-treatment had no significant effect (Figure 7C).

K_{ATP} channels roleplay in the metformin effects on SOD activity

Glibenclamide (1 mg/kg) pretreatment reduced (*P<0.05) the SOD activity against LPS+metformin (250 mg/kg) received group. But, diazoxide (10 mg/kg) pretreatment had no significant effect (Figure 7D).

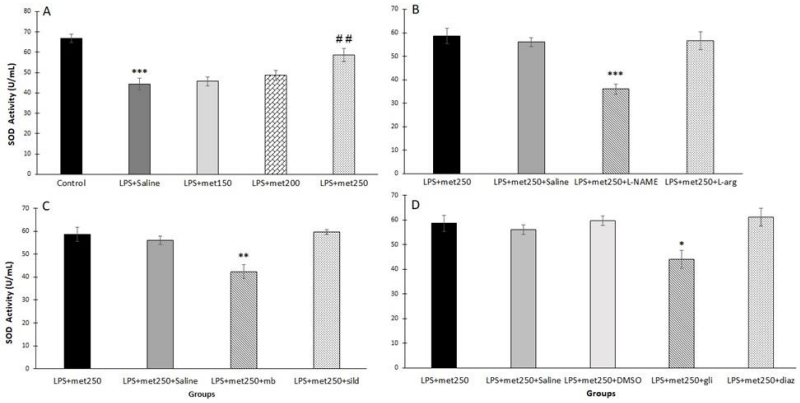

Figure 7. Metformin (150, 200 and 250 mg/kg) effects on the SOD activity (A), ***P< 0.001 against solvent-received control group. ##P< 0.01 against LPS+solvent received group. NO/cGMP/K_{ATP} roleplay in the metformin effects are displayed in sections B, C and D one-to-one, *P< 0.05, **P< 0.01 and ***P< 0.001 against LPS+metformin (250 mg/kg) received group

Discussion

In this study, Lipopolysaccharide (LPS) shot enhanced the initial latency time (IL) as a cognitive injury and decreased step through latency time (STL) as a memory-learning loss in the passive avoidance test (shuttle box). Metformin treatment caused IL reduction and STL increase. So, it can be concluded that metformin can recover the LPS-induced cognition deficit and learning-memory loss. Studies show that NO-cGMP pathways have a critical role in cognitive losses (13). Based on the results of the current study, pre-medication by a nitric oxide synthase blocker (L-NAME) added IL and decreased STL which shows the reducing influence of L-NAME on the recovering influence of metformin. But L-arginine (a NO precursor) reduced IL and added STL, which shows the rising influence of L-arginine on the metformin helpful effects. Thus, it can be concluded NO-cGMP pathway perhaps involve in the metformin helpful effects in the LPS-caused cognition deficit and memory- learning losses. In this regard, studies approve that NO/cGMP pathway has a roleplay in the learning-memory activity. By the way, methylene blue (a NOS and guanylyl cyclase inhibitor) enhanced IL and reduced STL, showing the reducing effect of methylene blue on the recovering influence of metformin. But a PDE-5 inhibitor (sildenafil) caused IL decrease and STL increase, so it enhances the metformin helpful effects. Thus, perhaps cGMP contributes in the metformin refining effect on LPS-caused cognition loss and memory-learning deficiencies. Previous studies show NO has a crucial role in the learning-memory processes, indicating that NO could provide a beneficial chance in Alzheimer's disease through variation of related NOS stimulation (14). Several reports show that many cellular tasks are regulated by K_{ATP} channels throw creating a relationship among metabolic roles and cell membrane electrical roles. So that, opening of these channels keeps a neuroprotective function through exciting synaptic connections that can decrease the neural impairments and improve learning and memory (15). Here, pretreatment by glibenclamide caused IL increase and STL decrease which means it reduces metformin helpful effect. Consequently, maybe KATP channels have a responsibility in improving effect of metformin on cognition deficit and learning-memory losses .

In a similar way, LPS made a learning-memory loss in Y-maze test, by decreasing the spontaneous alternation percentage (SAP). Metformin increased this percentage which shows improving effect of metformin on LPS-induced learning-memory loss. Since, total arm entries didn't have significant differences amid groups. So, the animals didn't have movement weakening. Neuronal nitric oxide synthase activation causes NO level increase which activates guanylate cyclase that in turn rise cGMP level. So, the fault of NO-cGMP pathway decreases Y-maze task learning ability (16). Similarly, here L-NAME decreased the spontaneous alternation percentage. Hence, it decreased the metformin recovering influence on LPS-caused memory-learning deficiencies. But, L-arginine enlarged the percentage that indicates an enhancement in metformin recovering influence. Consequently, it can be inferred; maybe NO has a role in the metformin recovering influence on the LPS-induced memory-learning losses. Also, methylene blue decreased the SAP. So, it decreased the metformin refining effect. Thus, perhaps cGMP has a roleplay in the metformin recovering influence on LPS-induced memory-learning losses, since earlier findings have been showed that extracellular cGMP regulates the Y-maze behavior learning capability (17). SAP was reduced by glibenclamide, showing a decrease in the metformin refining effect. Similarly, several earlier reports display that glibenclamide enhance SAP, proposing that KATP channels perhaps contribute to the cognitive function (18). Overall, perhaps KATP channels serves a role in the metformin recovering influence on LPS-induced memory-learning damages.

Pieces of evidence have shown that lipopolysaccharides are commonly used to create an animal model for depression like behavior (19). Similarly, in this study LPS increased the immobility time in FST, inducing a kind of depression like behavior. While metformin diminished this LPS created immobility, representing an anti-depressive outcome of metformin. Thus, metformin is able to recover LPS made depression. NO/cGMP can show an act in several neural cases for example, neurodegenerative diseases and depression (20). Similarly, here L-NAME enhanced the metformin anti-depressant outcome. But, L-arginine prevented metformin anti-depressant outcome. So, NO can diminish anti-depressant outcome of metformin. Therefore, NO plays a role in the anti-depressant outcome of metformin. The locomotion had no significance among study groups, deducing the metformin anti-depressant outcome. And also, methylene blue added the metformin anti-depressant outcome by lessening the immobility time. Whereas sildenafil avoided metformin anti-depressant outcome by enhancing that, demonstrating

control of cGMP on the anti-depressant outcome of metformin. These evidences confirm cGMP roleplay in the anti-depressant outcome of metformin. Consequently, mentioned outcome might be arbitrated by the cGMP decrease, owing to NOS decrease. So, NO/cGMP has a roleplay in the anti-depressive outcome of metformin. Furthermore, Glibenclamide in combination with metformin caused an anti-sedentary result in FST. So, it amplified the anti-depressant outcome of metformin. But diazoxide decreased the anti-depressant outcome of metformin via rising the immovability period in FST. It's showing a control of K_{ATP} channels on the anti-depressant outcome of metformin. So, the inhibition of NO/cGMP pathway maybe cause blockage of K_{ATP} channels, leading an anti-depressant outcome (20).

LPS can cause inflammation and pain receptor sensitization (21). Relatedly, the results of this study show that LPS has reduced the pain threshold. It means LPS induced a sort of hyperalgesia in this model. But, metformin treatment had no significant effect on the pain threshold. Nevertheless, other's reports show that NO/cGMP pathway is involved in pain sensitivity process (22). Similarly, in this study L-NAME and methylene blue reduced the pain threshold, whereas it was increased by L-arginine. It confirms the NO/cGMP pathway probable contribution in the pain process (23).

The results of this study confirm that neuroinflammation has a major responsibility in the brain illnesses, specially learning-memory losses. LPS induced neuroinflammation lead to mental damage and metabolic complaints in some brain area such as hippocampus (24). This study data displayed; LPS injection enhanced the brain tissue TNF-alpha. So, LPS can cause neural inflammatory responses and tauopathy-associated disorders (25). Although, metformin reduced the TNF-α level, indicating reduction in the LPS-induced neuroinflammation. L-NAME and methylene blue pre-treatment added the TNF-α level, showing a decrease in the recovering result of metformin on LPS-induced neural inflammation. but, L-arginine and sildenafil decreased TNF-α level, displaying a reinforcement in metformin anti-inflammatory outcome. So, perhaps NO/cGMP have a roleplay in metformin anti-inflammatory outcome. It approves earlier statements that cGMP can show an anti-inflammatory role (26). Previous studies have shown that KATP channels opening maybe has an anti-inflammatory and analgesic outcome (27). In the present study, glibenclamide rised TNF-α quantities, decreasing the metformin recovering impact on LPS-induced neuroinflammation. But diazoxide had a reverse effect. So, perhaps one can consider a roleplay for KATP channels in the metformin recovering outcome on LPS–made brain tissue inflammation. Therefore, LPS-made KATP Channels obstruction can lead to neural inflammation (28).

Lipid peroxidation is a notable pathological change in the AD. So that malondialdehyde as a final products of lipid peroxidation, can increase in the AD patients brain (29). LPS-treatment can reduce antioxidant capacity through decrease in superoxide dismutase (SOD) activity and elevation in MDA and cytokines quantities (30). In the present study, LPS added the malondialdehyde quantity as a marker of lipid peroxidation. Metformin treatment decreased the MDA levels, showing a recovering outcome on the LPS-made lipid peroxidation. Also, L-NAME and methylene blue pre-treatment enhanced the MDA quantities and therefore has reduced the metformin recovering outcome on the LPS-made lipid peroxidation. While, sildenafil and L-arginine pre-treatment diminished the MDA concentrations, indicates that it can increase the metformin recovering outcome on LPS-made lipid peroxidation. Therefore, NO and cGMP perhaps contribute to the metformin recovering outcome on LPS-made lipid peroxidation. Based on previous studies, KATP channels opening have a contribution in the protection against lipid peroxidation. So glibenclamide reduced the improving effects of simvastatin on MDA contents, by K_{ATP} channel blockage (31). Here, glibenclamide increased the levels of MDA. So, it has decreased the metformin recovering outcome on the LPS made lipid peroxidation. Therefore, K_{ATP} channels perhaps have a probable contribution in the metformin refining effect on the LPS-induced lipid peroxidation.

Studies have shown LPS can cause oxidative stress and neuroinflammation, through increasing MDA and IL-1β levels and decreasing the SOD enzyme act. IL-1β knock-down in the hippocampi better the stress oxidative, inflammation, memory losses and depression-like behavior induced by LPS (32). Similarly, in this project LPS lessened the SOD act (u/ml) in the hippocampi, inducing a kind of oxidative stress. Metformin treatment increased the SOD activity. So, metformin can recover the LPS-made SOD activity loss. L-NAME, methylene blue and glibenclamide Pre-treatment decreased the SOD activity, showing a reduction in metformin recovering outcome on LPS-made weakening in SOD activity. While L-arginine, sildenafil and diazoxide pre-treatment had no significant results in the SOD activity. So, it can be concluded that maybe NO/cGMP/K_{ATP} cell signaling path has a possible roleplay in the metformin recovering outcomes on LPS made deficiency in anti-oxidative

capability of the hippocampi samples .

And also, these antagonist/agonist drugs when used alone didn't exert significant effect on the mentioned behavioral tests and biochemical assays. So, the related results indicate the possible roleplay of NO/cGMP/K_{ATP} path in the metformin recovering outcomes .

In conclusion, it can be concluded that metformin can improve the memory-learning losses, depression like behavior, hyperalgesia, stress oxidative and neuroinflammation produced via LPS in alzeheimer's disease mouse model and NO/cGMP/K_{ATP} pathway maybe are involved in this effect.

Acknowledgments

This paper is issued from the medical thesis A.K. and financial support was provided by the Vice Chancellor of Research, Dezful University of Medical Sciences (IR.DUMS.REC.1399.003), Dezful, Iran.

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