



Research article

Effects of atorvastatin and metformin on development of pentylenetetrazole-induced seizure in mice



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ABSTRACT

Recent studies have shown that statins and Metformin may have beneficial effects on seizure through different mechanisms. In the current study, we investigated whether Metformin, Atorvastatin, and concomitant uses of them have beneficial effects on pentylenetetrazole (PTZ)-induced kindling. Adult male C57BL/6 mice were randomly divided into four experimental groups with seven mice in each group. Group 1, control group; group 2, received Metformin (200 mg/kg, i.p.); group 3, received Atorvastatin (10 mg/kg, i.p.); group 4, received Atorvastatin (10 mg/kg, i.p.) plus Metformin (200 mg/kg, i.p.). Twenty minutes after injection of the mentioned drugs, the experimented mice received 37/5 mg/kg of PTZ intraperitoneally on alternating days. Then the convulsive behavior signs were evaluated for 20 min after each PTZ injection. There were significant differences in the stage 2 latency parameter among group 2 ($p = 0.033$, $F = 8.46$)/group 3 ($p = 0.032$, $F = 10.42$)/group 4 ($p = 0.008$, $F = 24.57$) as compared to the control group, while no significant differences were found comparing only group 2,3, and 4 with each other excluding the control group. Pretreatment with Atorvastatin ($p = 0.002$, $F = 33$), Atorvastatin + Metformin ($p = 0.006$, $F = 20.77$), and Metformin alone increased stage 5 latency as compared to the PTZ group, significantly. Also, our results have shown that pretreatment with Atorvastatin ($p = 0.013$, $F = 14.48$), Metformin ($p = 0.015$, $F = 16.67$), and concomitant usage of them significantly decreased stage 5 duration as compared to the control group. Our findings clearly demonstrate that concomitant use of Metformin and Atorvastatin has no more protective effect against the development of kindling as compare to these drugs alone. Thus, we concluded that, these drugs may inhibit kindling via a similar mechanism and we suggested that it is probably through regulation of autophagy.

1. Introduction

Epilepsy is a common chronic neurological disorder that annually, about 50,000 to 100,000 new cases of it are reported and there are an estimated at least sixty-five million worldwide people living with this disease [1]. Approximately 30–40% of epileptic patients have shown refractoriness to medications [2].

Large body of evidence shows that statins are very effective in decreasing neuroinflammation, oxidative stress, neurotoxicity, and declining nitric oxide (NO). Neuroprotective effect of statins is really

efficient in improving various life threatening conditions including brain injury, stroke, and cerebral ischemia [3]. Recent studies have shown that Atorvastatin has a protective role in seizure and this effect is independent from cholesterol-lowering properties [4, 5, 6]. Lee et al. have indicated that Atorvastatin inhibits kainic acid-induced seizure, and hippocampal cell death [7]. Several mechanisms and pathways have been proposed for anticonvulsant effects of statins, such as; regulating the glycogen synthase kinase-3 β (GSK-3 β) pathway [8], inhibition of neuroinflammation [9], modulating hippocampal levels of dopamine, glutamate, and gamma-aminobutyric acid (GABA) [10].

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Metformin, an oral antidiabetic drug, is another medicine that has a protective effect against seizures, memory, learning injuries, and oxidative damage [11]. It has been reported that reactive oxygen species (ROS) may have a vital function in the development and progression of epilepsy [12], hence, antioxidant agents have been recommended as new therapeutic medications for the treatment of epilepsy. The previous study revealed that Metformin could decrease ROS [13], and inflammation [14]. In addition, Metformin has a beneficial effect on the antioxidant defense system by inducing Nrf2 [nuclear factor (erythroid-derived 2)-like 2; NFE2L2] target gene activation [15], upregulating the uncoupled proteins 2 (UCP2) [16], and improving blood-brain barrier (BBB) function by activating AMP-activated protein kinase (AMPK) [14, 17]. The previous study indicated that BBB leakage occurs during epileptogenesis and lead to the progression of epilepsy [18], and an increase in the BBB permeability was observed in mice with generalized convulsive seizures induced by acute pentylentetrazole (PTZ) injection [19].

Despite of the potential benefits of Metformin and Atorvastatin, only a few studies have been conducted on the anticonvulsant effect of these drugs. Therefore, to further investigate the impact of Metformin and Atorvastatin on seizure activity, in the current study, we investigated whether Metformin and Atorvastatin treatment has any favourable impression on seizure induced by PTZ in the most widely-accepted animal model. This way we tried to understand the process of epileptogenesis and discover novel compounds with anticonvulsant activity.

2. Materials and methods

2.1. Animals, drugs, and chemicals

Adult male C57BL/6 mice, weighing 20 ± 2 g (6–8 weeks old) were used in this study. They were kept under controlled light and condition (12:12 h light/dark cycle, $25 \pm 1^\circ\text{C}$, 55% relative humidity) with free access to water and food. All experimental procedures were approved by the Ethics Committee of the Golestan University of Medical Sciences (No. IR.GOUMS.REC.1394.89).

Atorvastatin, Metformin, and PTZ were purchased from Sigma-Aldrich, Germany. Metformin and PTZ were dissolved in physiological saline and Atorvastatin was dissolved in 40% dimethyl sulfoxide (DMSO), and 60 % physiological saline. All drugs were prepared freshly prior to the injections.

2.2. Induction of kindling and design of the experiment

For induction of kindling a subconvulsive dose of PTZ (40 mg/kg, i.p.) was injected intraperitoneally (i.p.) every 48 h. The injections were repeated until all of the mice showed the full kindling state [20]. After PTZ injection, the convulsive behavior was observed for a period of 20 minutes [20, 21]. Seizure stage was evaluated using the following scale: stage 0, no response; stage 1, ear and facial twitching; stage 2, convulsive waves axially through the body; stage 3, myoclonic jerks and rearing; stage 4, clonic convulsions with the animal falling on its sides; and stage 5, repeated severe tonic-clonic or lethal convulsions [11, 22, 23]. Stage 2 and 5 latencies (S2L, S5L), stage 5 duration (S5D), and seizure stage (SS) were evaluated during the experiment.

The animals were randomly divided into four experimental groups with seven mice in each group. Group 1, control group, received PTZ (40 mg/kg, i.p.) [20]; group 2, received Metformin (200 mg/kg, i.p.) [11]; group 3, received Atorvastatin (10 mg/kg, i.p.) [6]; group 4, received Atorvastatin (10 mg/kg, i.p.) + Metformin (200 mg/kg, i.p.). Mice in the last three groups received PTZ twenty minutes after pretreatment.

2.3. Statistical analysis

Statistical significance was carried out using repeated-measures ANOVA and one-way ANOVA for multi-group comparisons. Tukey test

was used for the post hoc comparison. Differences were considered significant where $p < 0.05$.

3. Results

According to the present study, Metformin, Atorvastatin, and concomitant use of them showed conspicuous anticonvulsant and anti-epileptogenic effects, while there are no significant differences between the experimented groups.

As shown in Figure 1, there were significant differences in S2L between group 2 ($p = 0.033$, $F = 8.46$)/group 3 ($p = 0.032$, $F = 10.42$)/group 4 ($p = 0.008$, $F = 24.57$) as compared to the control group, however, no considerable differences were seen among groups 2, 3, and 4 excluding the control group.

As shown in Figure 2, pretreatment with Atorvastatin ($p = 0.002$, $F = 33$), Metformin, or combination of them ($p = 0.006$, $F = 20.77$) increased S5L as compared to the control group, significantly.

Similarly, pretreatment with Atorvastatin ($p = 0.013$, $F = 14.48$), Metformin ($p = 0.015$, $F = 16.67$) and concomitant use of them significantly decreased S5D as compared to the control group (Figure 3). Despite of the previous mentioned results, our studies showed pretreatment with these drugs has no significant effect on SS (Figure 4). We need to mention that our pretreated groups received more injections to become full-kindled as compared to the control group (11 vs 7).

4. Discussion

Earlier studies demonstrated that Metformin can cross the BBB and have anti-inflammatory, antioxidant [24], and neuroprotective [25] properties. Besides, it is reported that Metformin has beneficial effects on neurological disorders [24, 26, 27]. Metformin could directly and indirectly modulate some signaling pathways like Sonic hedgehog (Shh), bone morphogenetic protein (BMP), and atypical protein kinase C (aPKC) [28, 29, 30]. In a research done by Wing and his colleagues, it was shown that aPKCs ζ and ι play a critical role in the development of embryonic precursors. They also showed that PKCs-CBP is essential for human forebrain neurogenesis. Their *in vivo* study demonstrated that Metformin enhances neurogenesis in a CBP-dependent fashion in adult mice [24]. In another study, Dadwal et al. developed a cell replacement strategy. They found that, administration of Metformin in hypoxia-ischemia (H/I) injury model activates endogenous neural progenitor cells (NPCs) [31]. It is proven that, Shh and BMP signaling pathways have a very important role in neurogenesis [32, 33]. Gajera and his team have stated that, LRP2 receptor in BMP signaling pathway is so vital to coordinate the specification of proliferative and non-proliferative cells fates and Loss of LRP2 activity suppresses progenitor cell proliferation, hence, neurogenic output is decreased in subependymal zone (SEZ) of the brain which is considered as the stem cell niche [34]. In another paper, Ding et al. have concluded that Shh pathway contributes to brain plasticity and following functional recovery after treating the stroke by bone marrow stromal cells in mice [35]. Consequently, it could be elucidated that Metformin which is used as an efficient drug in modulating signaling pathways such as BMP, Shh, and aPKC can induce neurogenesis.

Since free radicals and reactive oxygen species are supposed to mediate the seizure development, researches on antiepileptic compounds modulating signaling pathways such as BMP, Shh, and aPKC with antioxidant and neuroprotective effects have been considered [11].

In the present study, we found that Metformin significantly increased S2L and S5L parameters as compared to the control group. Moreover, the pretreatment with Metformin elevated the number of injections to reach full kindling. Therefore, anticonvulsant activity of Metformin in epilepsy progression by these results was proven. Previous studies demonstrated that BBB dysfunction occurs during epileptogenesis and contributes to the development of epilepsy [18], and it is known that BBB permeability was increased in mice with generalized convulsive seizures induced by acute PTZ [19]; meanwhile, it has been reported that Metformin can have

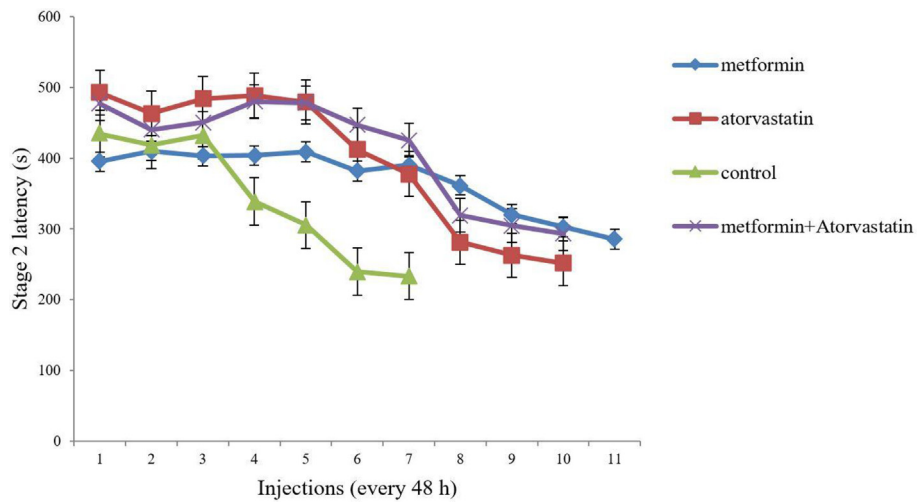


Figure 1. Comparison of the effect of ‘Metformin’, ‘Atorvastatin’ and ‘Metformin + Atorvastatin’ treatments on stage 2 latency in PTZ kindling mice. Values are expressed as mean ± SD (n = 7 in each group), and are shown for each injection ($p < 0.05, \#p < 0.01$).

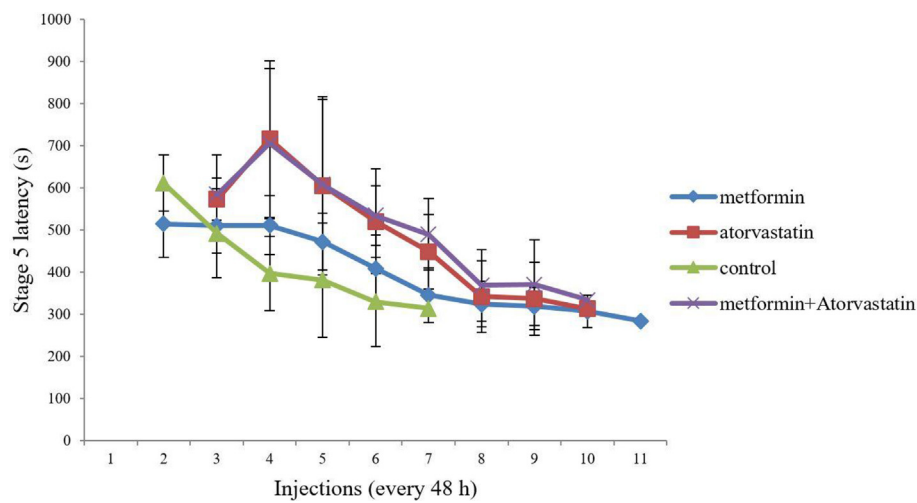


Figure 2. Comparison of the effect of ‘Metformin’, ‘Atorvastatin’ and ‘Metformin + Atorvastatin’ treatments on stage 5 latency in PTZ kindling mice. Values are expressed as mean ± SD (n = 7 in each group), and are shown for each injection ($p < 0.05, \#p < 0.01$).

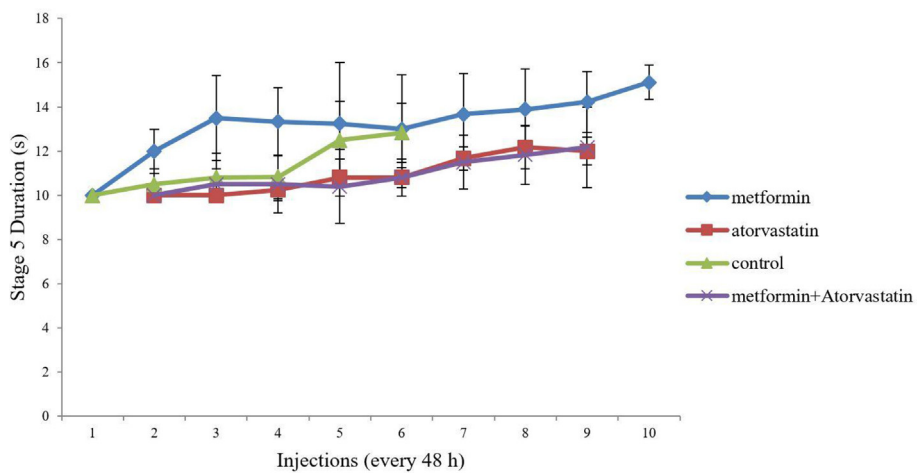


Figure 3. Comparison of the effect of ‘Metformin’, ‘Atorvastatin’ and ‘Metformin + Atorvastatin’ treatments on stage 5 duration in PTZ kindling mice. Values are expressed as mean ± SD (n = 7 in each group), and are shown for each injection ($p < 0.05, \#p < 0.01$).

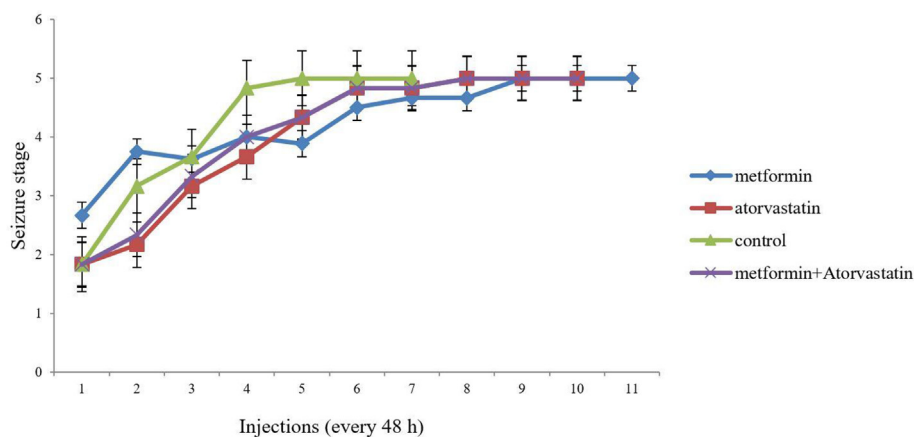


Figure 4. Comparison of the effect of 'Metformin', 'Atorvastatin' and 'Metformin + Atorvastatin' treatments on seizure stage in different injections in PTZ kindling mice. Values are expressed as mean \pm SD ($n = 7$ in each group), and are shown for each injection ($p < 0.05$, $p < 0.01$).

considerable therapeutic benefits to prevent the development and formation of BBB disruption [17]. Furthermore, studies have proved that Metformin could activate AMPK, an important sensor of energy balance [36]. Metformin could promote neurogenesis and improve spatial memory function, by activation of AMPK pathway [28]. Therefore, the beneficial effect of Metformin on epilepsy could be modulated by AMPK pathway and it could prevent the increment of BBB disruption.

Atorvastatin is categorized as a class of Hypolipidemic statins. Several studies have shown neurogenesis effect and protective role of atorvastatin in the seizure. Statins function as neuroprotective agent in various life compromising conditions such as stroke and traumatic brain injury [3]. Several reports are available on the anticonvulsant effect of statins and a few of them contain disputable report of atorvastatin impact on experimental seizure models [37, 38].

In some studies, statins have demonstrated a neuroprotective effect on an animal model for Alzheimer's disease and ischemic brain injury through NO-dependent pathways. It reveals the role of NO as a second messenger system of statins. It functions via various mechanisms like upregulation of endothelial NO synthase, antiapoptotic effects, reduction in oxidative stress and inducible nitric oxide synthase [3, 37, 39, 40]. In a paper, Mahmood et al. investigated the impact of a combination therapy of marrow stromal cells (MSCs) and statins (atorvastatin) on brain injury caused by trauma in rats. When they administered statins orally, after the traumatic brain injury, functional outcome and neuroplasticity were improved [41].

In the present study, we also demonstrated that Atorvastatin, and Atorvastatin plus Metformin treatments increased the latency to PTZ-induced seizure. Studies have revealed that statins, particularly atorvastatin, have beneficial effects on several excitotoxic disorders, including stroke [42], traumatic brain injury [39], glutamate exposure [43], and cerebral ischemia [44]. In agreement with our study results, Lee and colleagues showed that atorvastatin treatment (10 mg/kg/day for 7 days) significantly decreases the severity of kainate-induced seizures [7]. In addition, Piermartiri et al. reported that atorvastatin treatment prevents the incidence of tonic and/or clonic seizures induced by quinolinic acid in around 33% of tested mice [5]. However, Lee et al. claimed that the seizure score does not change with a single injection of atorvastatin (30 min after kainate administration) [7]. It has also been reported that atorvastatin withdrawal facilitated the occurrence of PTZ-induced seizures, as evidenced by a decline in the latency to clonic and generalized seizures [6].

Increasing evidence indicates that BBB disruption is an important etiologic factor in seizure disorders [45, 46]. Interestingly, several studies have revealed that atorvastatin inhibits BBB breakdown [47, 48]. On the other hand, Van Vliet et al. have demonstrated that atorvastatin treatment did not affect BBB leakage [49]. Moreover, the anti-epileptic

effects of atorvastatin might be due to reduced cholesterol levels. Therefore, it seems that the molecular mechanisms of atorvastatin are not necessarily limited to its neuroprotective properties, and further studies are needed. Furthermore, several studies have shown that inhibition of mTOR pathway can prevent seizure [50, 51]. Indeed, Atorvastatin, and Metformin could effectively promote autophagy through mTOR inhibition [52, 53], and studies displayed that, suitable activation of autophagy has antiepileptic effects [54]. Our findings clearly indicate that Metformin, Atorvastatin, and the combination of these two drugs have anti-convulsant effects which could be through regulation of autophagy.

Declarations

Author contribution statement

Mohammad Ali Zeyghami, Ebrahim Hesam: Conceived and designed the experiments; Performed the experiments.

Parand Khadivar, Halimeh Khaton Hesam: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ali Ahmadnia: Analyzed and interpreted the data; Wrote the paper.

Abolfazl Amini: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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