Iranian Journal of Pharmaceutical Sciences
2022: 18 (2): 138-147
www.ijps.ir

Original Article

Protective Effects of Memantine, an NMDA Receptor Antagonist, Against Cerebral Ischemia/Reperfusion Induced Injuries in Rats with Transient Congenital Hypothyroidism

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Abstract

Stroke is one of the major causes of mortality worldwide. Memantine, an NMDA receptor antagonist, has protective effects on neuronal cells and is important candidate as a neuroprotective agent in cerebral ischemia. On the other hand, thyroid hormones are one of the important factors in the development of the central nervous system (CNS) and its activity and the long-term adverse effects of transient thyroid function abnormalities at birth on intellectual development has been proven. Therefore, the aim of the present study was to evaluate the effects of memantine on cerebral ischemia/reperfusion (I/R) induced injuries in transient congenital hypothyroidism (TCH). The adult male Wistar TCH rats (240±20 g) were underwent forebrain ischemia by bilateral common carotid artery occlusion for 17 min. Memantine (20 mg/kg) alone or in combination with vitamin C (200 mg/kg) were administered intraperitoneally (ip) for 7 days after cerebral ischemia. Then, histopathology, cerebral infarct size and malondialdehyde level were evaluated. Histopathological analysis showed that memantine significantly decreased leukocyte infiltration in comparison to I/R group ($p<0.01$). Memantine also reduced infarct size and malondialdehyde level compared with I/R group ($p<0.01$). Memantine and vitamin C combination group had no significant effects on leukocyte infiltration, infarct size and malondialdehyde level. Our results showed that memantine through reduction in leukocyte infiltration, lipid peroxidation and infarct size could reduce cerebral ischemia/reperfusion induced injuries in transient congenital hypothyroidism. Hence, memantine might be considered as a neuroprotective agent in hypothyroidism.

Keywords: Brain ischemia, Cerebral infarction, Hypothyroidism, Inflammation, Memantine, Oxidative stress.

1. Introduction

Stroke is a serious and common pathological condition, which is the second most common cause of death [1, 2]. There are three types of stroke: subarachnoid hemorrhage, intracerebral hemorrhage, and ischemic stroke [3]. Ischemic stroke causes about 83% of all strokes and is the major cause of mortality worldwide [4]. Ischemic cerebrovascular diseases usually leads to irreversible brain damage, which ultimately reduce the quality of life in these patients [5]. Oxidative stress and excessive inflammatory responses have already been identified to

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Cite this article as: Ayadi S, Abbaszadeh S, Derafshpour L, Saboory E, Karimipour M, Soraya H., Protective Effects of Memantine, an NMDA Receptor Antagonist, Against Cerebral Ischemia/Reperfusion Induced Injuries in Rats with Transient Congenital Hypothyroidism, Iran. J. Pharm. Sci., 2022, 18 (2): 138-147.

contribute in the pathogenesis of ischemia reperfusion injury in many organs, including the brain [6]. Within hours of the ischemia insult, neutrophilic infiltration promotes the release of pro-inflammatory mediators such as cytokines and chemokines and free radicals, all of which contribute in tissue damage [7]. The biochemical alterations after cerebral ischemia starts with ion homeostasis disruption, which include an increase in the level of extracellular potassium and glutamate and over the time result in over-activation of N-methyl-D-aspartate (NMDA) receptors, intracellular Ca^{2+} overload and ultimately cell death [8]. NMDA receptors are primarily found on the postsynaptic membranes at the synaptic and also extrasynaptic positions [9] and play an important role in the cellular processes triggered by cerebral ischemia that lead to neuronal cell death [10]. By over-activation of NMDA receptors, excessive amounts of Ca^{2+} influx into the cytoplasm and mitochondria which ultimately activates enzymes such as phospholipase A_2 and cyclooxygenase causing free radical generation that interfere with the innate free radical scavenging mechanisms [11]. Therefore, it seems that free radicals can play an important role in the pathophysiological conditions of the central nervous system (CNS) [9, 12].

Memantine is an uncompetitive NMDA receptor antagonist and has a neuroprotective and therapeutic effect in several neuropsychiatric diseases [13]. Memantine blocks the entry of excessive calcium through the channel and thus has neuroprotective effects in *in vivo* and *in vitro* models [14]. A study by Kilic *et al.* showed that memantine reduced infarct size and improved neuronal function after focal cerebral ischemia in mice [9]. There are some studies that demonstrated the neuroprotective effects of memantine on a rat model of stroke [14, 15]. Considering the important role of oxidative stress in the brain ischemia, vitamin C as an antioxidant demonstrated neuroprotective effects [16, 17]. Furthermore, according to studies in stroke patients the supply of vitamin E in combination with vitamin C reduced lipid peroxidation and inflammation [18].

Thyroid hormones are one of the most important factors in the development of CNS [19]. The necessity of these hormones is clearly seen in maturation and the formation and function of the CNS (20). The maternal thyroid hormone that cross through the placenta is the most important source of supply of thyroid hormone during the first trimester in the fetus [20, 21]. Hypothyroidism during the fetal and early postnatal period impaired learning and memory and also reduced hippocampal brain-derived neurotrophic factor (BDNF) in rat pups [22] and decreased long-term potentiation in hippocampus of TCH rats [23]. Congenital hypothyroidism is associated with decreased glutamate uptake with subsequent influx of Ca^{2+} through NMDA receptors in rat hippocampus. Over-activation of NMDA

receptors lead to an overload of Ca^{2+} within the cells, which trigger glutamate excitotoxicity and oxidative stress [24]. Considering these issues blocking NMDA receptors with memantine, a NMDA receptor antagonist, could reduce the brain injuries. To the best of our knowledge, there is no study conducted on the neuroprotective effects of memantine in hypothyroidism. However, the present study was aimed to evaluate the effects of memantine alone and in combination with vitamin C on cerebral ischemia/reperfusion induced injuries in rats with TCH.

2. Materials and Methods

2.1. Animals

Female Wistar rats (n=10) weighing 240 ±20 g were mated and then housed individually. The rats were given food and water ad libitum and were housed in the Animal House of Urmia University of Medical Sciences at a controlled ambient temperature of 22±2 °C with 40-60% relative humidity and a normal 12-h light/12-h dark cycle. This study was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and was approved by the Ethics Committee of Urmia University of Medical sciences (Code: IR.UMSU.REC.1396.309).

2.2. Experimental protocol

Beginning on gestational day 6 (GD6) and continuing until postnatal day (PND) 21, hypothyroidism was induced in rats by the addition of 100 ppm of propylthiouracil (PTU), the thyroid hormone synthesis inhibitor, to the drinking water. Exposure to PTU terminated when offspring were weaned on PND21 [22, 23]. The male rats (8-10 weeks old, weighing 240±20 g) with transient congenital hypothyroidism (TCH) were randomly divided into 4 groups (n = 8 each group). Rats in group 1 (control) received intraperitoneally (*ip*) saline injection (0.5 ml) for 7 days. Rats in group 2 (I/R), underwent on bilateral common carotid ligation for 17 min for induction of cerebral ischemia and then were injected *ip* with saline for 7 days. Rats in groups 3 were treated *ip* with memantine (20 mg/kg) [8] for 7 days after cerebral ischemia and rats in group 4 were treated *ip* with combination of memantine (20 mg/kg) and vitamin C (200 mg/kg) [25] after ischemia for 7 days. Memantine and vitamin C was dissolved in saline and was injected based on body weight.

2.3. Surgery procedure

The rats were anesthetized by an *ip* injection of a mixture of ketamin (60 mg/kg) and xylazin (10 mg/kg). Then, placed in a supine position and when the rats no longer responded to external stimuli a midline neck incision was made and the common carotid artery was carefully dissected. The rats were subjected to bilateral common carotid occlusion for 17 min. Then clamps were removed and the skin was sutured [4].

2.4. Histopathological Examination

At the end of the experiment (on day 8), the rats were euthanized with pentobarbital (200 mg/kg; *ip*) and the brains were quickly removed and fixed in 10% formalin. After that, tissues were stained by hematoxylin and eosin

(H&E). The leukocyte infiltration into the brain and necrosis were evaluated in each section of the brain tissue using a morphometric point-counting procedure. Two persons (one pathologist, one trained person) graded the histopathological changes as 1, 2, 3, and 4 for low, moderate (focal cerebral damage or small multifocal degeneration with slight degree leukocyte infiltration), high (extensive degeneration and/or diffuse leukocyte infiltration), and intensive (necrosis with diffuse leukocyte infiltration) pathological changes, respectively [4, 26].

2.5. Determination of lipid peroxidation

The level of serum MDA, as a marker of lipid peroxidation, was measured spectrophotometrically and expressed as a nanomole/ml after cerebral ischemia/reperfusion using the standard procedure [4, 27].

2.6. Cerebral Infarct Size

For measurement of infarct size, the brains were carefully removed and freezed at -20 °C for 15 min and then cut into slices of 2 mm thickness and completely immersed in a 2% solution of 2,3,5 Triphenyltetrazolium chloride (TTC) for 20 min at a temperature of 37°C in a darkroom. After staining, brain slices were fixed with 10% formaldehyde for 24 h. The stained sections were digitally photographed and measured using Image j software. Normal areas stain red while infarcted areas remain pale white [28].

2.7. Statistical analysis

All results were presented as mean ± SEM. One-way ANOVA was used to make comparisons between the groups. If the ANOVA analysis demonstrated significant differences, a Student-Newman-Keuls *posttest* was employed to compare the mean values between the groups. $P<0.05$ was considered statistically significant.

3. Results and Discussion

3.1. The effects of memantine on histopathology of the brain tissue in TCH male rats

Neutrophils had homogeneity dispersion in the control group and there was no evidence of inflammatory cells accumulation in the control group. The histological sections of the brains obtained from I/R group demonstrated the infiltration of neutrophils into the brain tissue (Fig 1). Memantine treatment significantly prevented inflammatory responses and reduced the infiltration of neutrophils after cerebral ischemia reperfusion ($P<0.01$). However, in combination with vitamin C, there was no significant reduction in inflammatory cells (Fig 1, 2).

3.2. The effects of memantine on cerebral infarct size in TCH rats

As shown in Fig 3A, the infarcted site was significantly higher in I/R group compared to control: however, it was decreased in memantine treated group. Moreover, the quantitative data confirmed that the brain infarction in the I/R group was significantly higher than that of the control group ($P <0.001$), which indicated a high rate of injury (Fig 3B). Treatment with memantine 20 mg/kg, significantly reduced brain

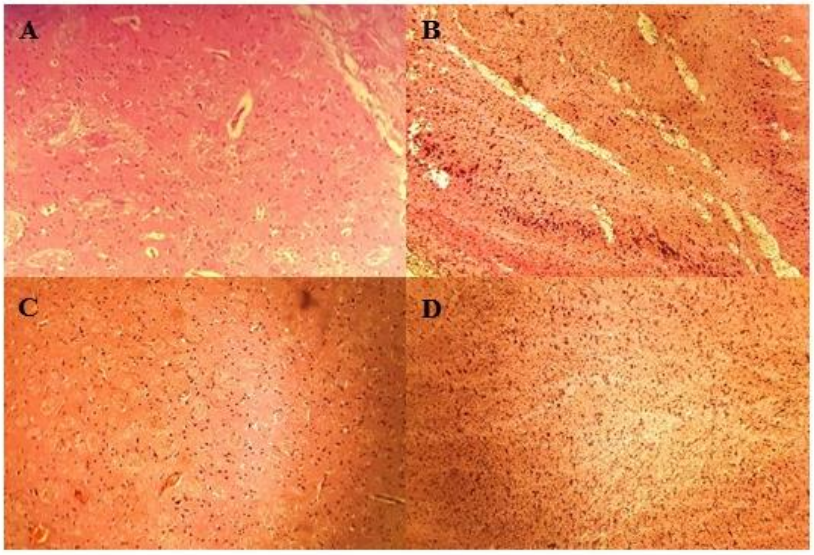

Figure 1. Photomicrographs of brain stained with H&E. Sections from brain tissues of (A) Control group showing homogeneity dispersion of leukocytes and no evidence of inflammatory cells accumulation. (B) I/R group showing leukocyte accumulation. (C) Memantine at dose of 20 mg/kg showing reduction in leukocyte infiltration after cerebral ischemia reperfusion. (D) Combination of memantine 20 mg/kg and vitamin C 200 mg/kg did not reduce leukocyte infiltration into the brain tissue after cerebral ischemia reperfusion.

infarct size in comparison to the I/R group (P<0.01). However, memantine and vitamin C combination group had no significant effect in comparison to I/R group.

3.3. The effects of memantine on lipid peroxidation in TCH rats

To determine the lipid peroxidation, malondialdehyde (MDA) level, as a marker for oxidative stress was measured in the serum. As shown in fig 4, serum levels of MDA in the I/R group showed a significant increase compared with the control group (P<0.05) and treatment with memantine significantly decreased MDA

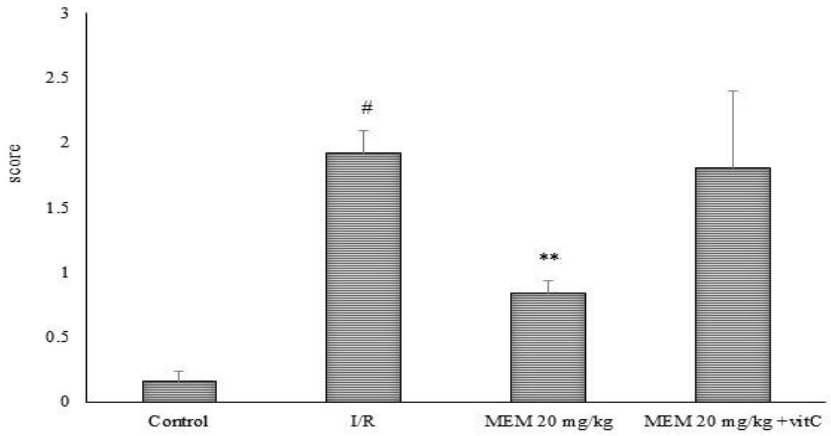

Figure 2. Grading of leukocyte infiltration in the brain tissues. Data are the mean ± SEM (n = 4). # $p < 0.01$ from respective control value; ** $p < 0.01$ as compared with I/R group using one way ANOVA with Student-Newman-Keuls post hoc test. I/R: Ischemia reperfusion; Mem: Memantine; vit C: Vitamin C.

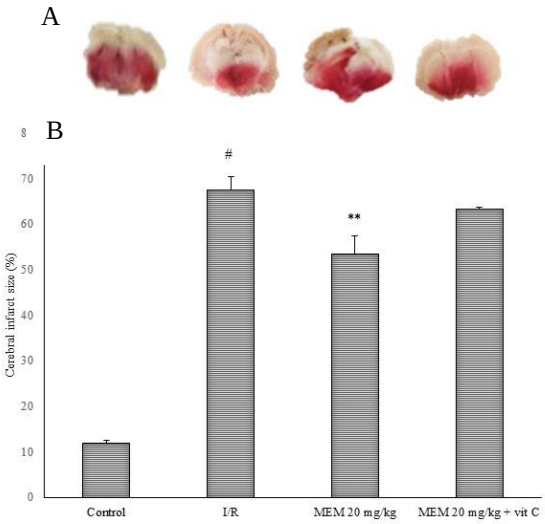

Figure 3. A) Representative images of TTC stained sections of brain after cerebral ischemia/reperfusion. B) Graphs show analysis of infarct size. Treatment with memantine (Mem) significantly reduced infarct size. Data are mean ± SEM. n = 4 in each group. # $p < 0.001$ from respective control value; ** $p < 0.01$ as compared with I/R group using one way ANOVA with Student-Newman-Keuls post hoc test. I/R: Ischemia reperfusion; Mem: Memantine; vit C: Vitamin C.

level in comparison to the I/R group ($p<0.001$). However, there was no significant reduction in the combination of memantine and vitamin C group in comparison to I/R group.

Nowadays, stroke is one of the leading causes of mortality and morbidity worldwide [29]. In this study, we evaluated the effects of memantine on cerebral ischemia/reperfusion (I/R) induced injuries in transient congenital hypothyroidism (TCH) in rats. The results of this study showed that treatment with memantine reduced leukocyte accumulation, cerebral infarct size and lipid peroxidation. However, addition of vitamin C, as an antioxidant, to memantine treated

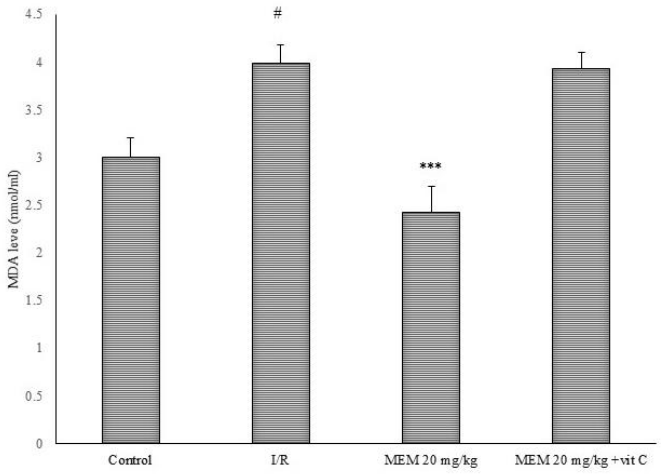

Figure 4. The effect of treatment with memantine (Mem) on malondialdehyde (MDA) levels after cerebral ischemia/reperfusion. Values are mean ± SEM (n = 8). # $p < 0.05$ from respective control value, ***$p < 0.001$, as compared with I/R group using one way ANOVA with Student-Newman-Keuls post hoc test. I/R: Ischemia reperfusion; Mem: Memantine; vit C: Vitamin C.

group did not show any significant protective effect on cerebral I/R induced injury.

Thyroid hormones play an important role in the development of the CNS [20]. However, there are conflicting results with the effects of hypothyroidism on brain ischemia. It has been reported that hypothyroidism has been associated with poor functional outcome after acute ischemic stroke and also has been considered a risk factor for the development of acute ischemic stroke [30]. Another study demonstrated that congenital hypothyroidism is associated with increment of glutamate level and excessive influx of ca^{2+} through NMDA receptors, which lead to glutamate excitotoxicity and oxidative stress [25]. In contrast, a study reported neuroprotective effects of hypothyroidism in cerebral ischemia reperfusion through reduction in oxidative stress and cell death [31].

Ischemic stroke causes glutamate efflux and excessive activation of NMDA receptors that possibly leads to neuronal toxicity and death and considered as one of the main pathogenic mechanisms of ischemia-induced brain injury [29, 32]. Over-activation of NMDA receptors can lead to calcium overload in neurons and increases the production of reactive oxygen species (ROS) that ultimately cause cell necrosis and apoptosis [33, 34]. Additionally, NMDA receptor-mediated signaling can activate the expression of inflammatory genes independently from the onset of the neuronal cell death [35]. It has been reported that NMDA receptor antagonists reduce cell death and thus improve stroke in animal models [29]. However, little is known about the neuroprotective effects of NMDA receptor antagonists in rats born from mothers with hypothyroidism. A study was conducted to investigate the effects of memantine, an NMDA receptor antagonist, on edema and infarct size after focal cerebral I/R in rats. This study showed that memantine can reduce the severity of neurological loss and infarct size, therefore can have neuroprotective effects [36]. It was reported that memantine, decreased the mean infarct size in treated group compared to the control group and has positive effects on cerebral ischemia in rats [37]. The results of the present study also showed that treatment with memantine reduced the infarct size after global cerebral ischemia/reperfusion in TCH rats. Cattani D *et al.* described the role of congenital hypothyroidism and NMDA receptor activity on ROS generation and oxidative stress [24]. Ozsüer *et al*. showed the beneficial effects of memantine on closed head trauma in rats through reduction in lipid peroxidation [38]. In line with these studies, our results show that memantine reduces lipid peroxidation after stroke in TCH rats. There are studies also suggesting that inflammatory processes might be related to the NMDA receptor signaling [39, 40]. Wu Q *et al.* reported that propofol via NMDA receptor inhibition, reduces pro-inflammatory enzyme expression [41]. Memantine can also play a neuroprotective role by reducing the release of proinflammatory factors from over-activated microglia [42]. Our study also showed that memantine reduced leukocyte infiltration after cerebral ischemia/reperfusion in TCH rats. On the other hand, there are numerous studies about the antioxidant effects of vitamin C, which suggest it's neuroprotective role by reducing lipid peroxidation [18]. However, the results of the present study indicated that treatment with memantine in combination with vitamin C had no

significant effect on the cerebral I/R induced injury in TCH rats. Vitamin C as an antioxidant, is known to have a variety of effects on the central nervous system (CNS). Evidence suggested that there is a link between vitamin C and glutamate, the major excitatory CNS neurotransmitter, in the CNS. It has been reported that vitamin C, through inhibition of glutamate uptake promote an accumulation of extracellular glutamate in the synaptic cleft. Moreover, vitamin C enhances NMDA receptor activity and decreases MK801, which antagonizes NMDA binding to NMDA receptors. Furthermore, vitamin C promotes the internalization of GluN1 NMDA receptor subunits [43, 44]. Therefore, the above indicated points lead us to believe that vitamin C may antagonize the effects of memantine and reduce the effectiveness of the drug in the case of cerebral ischemia reperfusion. This study, is although significantly revealing the neuroprotective effects of memantine in transient congenital hypothyroidism, but still has some limitations. We have shown that treatment with memantine in combination with vitamin C had no significant effect on the cerebral I/R induced injury in TCH rats but we did not try to clarify the effects of vitamin C alone in this model.

4. Conclusion

Our results show that memantine reduces leukocyte infiltration as well as lipid peroxidation and can also reduce infarct size in cerebral ischemia/reperfusion induced injuries, in transient congenital hypothyroidism rats. Hence, memantine might be considered as a neuroprotective agent in hypothyroidism.

Acknowledgments

The present study was financially supported by the Research Vice Chancellors Office of the Urmia University of Medical Sciences, Urmia, Iran.

Conflict of interest

The authors declare to have no conflict of interest.

Funding

None.

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