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ORIGINAL RESEARCH

Effects of Melatonin and Memantine Administration on The Learning and Memory Performances of Hypoxic Juvenile Rat Pups

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Abstract

Objective: Herein, we aimed to investigate the long-term effects of neonatal hypoxia and the potential protective role of melatonin and memantine on the learning and memory.

Methods: Seven-day-old rat underwent right carotid ligation, followed by hypoxia. Rat received Melatonin (MLT) (4 mg/kg), Memantine (MEM) (20 mg/kg), and MLT+MEM combination after hypoxia. We tested these rats for anxiety by elevated O-maze and for spatial learning and memory by Morris water maze (MWM) at postnatal day 45.

Results: Hypoxia increased the level of anxiety compared to the control group (p=0.05) while treatment of MLT, MEM, and MLT+MEM ameliorated this effect. In addition, hypoxia produced significant decrease in spatial learning of the rats on the fourth day of training (P≤0.05) and the percent time spent in the platform quadrant and the entrance frequencies to the platform quadrant compared to the control group (P=0.049 and P=0.023). Treatment of MLT, MEM, and MLT+MEM after hypoxia improved the performance of the rats at the third (P=0.686, P=0.876, P=0.977, respectively) and fourth day (P=0.738, P=0.553, P=0.789, respectively) of MWM training. The decrease in the percent time spent was ameliorated by the treatment of MLT (P=0.239), MEM (P=0.289), and MLT+MEM (P=0.567) compared to the control group. In addition, MLT treatment significantly increased the entrance frequency to the platform quadrant compared to the hypoxia group (P=0.020).

Conclusion: Our data suggested that the MLT was more effective in the release of memory deficits from hypoxia-related damage. MLT might have a therapeutic value in improving hypoxic damage in the developing brain.

Keywords: Neonatal Hypoxia, Learning and Memory, Melatonin, Memantine, Morris Water Maze

INTRODUCTION

Hypoxia-ischemia is a type of brain damage that occurs due to insufficient blood flow to cells with a lower blood oxygen concentration. In addition, this condition produces cognitive impairment and synaptic dysfunction which results in neurodegeneration (1). Perinatal hypoxia can produce developmental delays in cognitive issues and motor skills in addition to neurodegenerative conditions such as epilepsy, cerebral palsy, and severe sensorimotor abnormalities (2,3). There are several causes of hypoxia-ischemia such as drug and alcohol use, severe fetal anemia, excessive bleeding from the placenta and umbilical cord accidents. The neuronal death in the brain due to hypoxia-ischemia occurs through selected excitatory neuronal circuits (4). Inefficient anaerobic metabolism during brain ischemia results in rapid depletion of ATP and accumulation of lactate in the cells due to membrane disruption. Excitotoxicity due to excess glutamate-related NMDA receptor activation which results in calcium accumulation in the cell also

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damages cell membranes and internal constituents such as mitochondria. All of these processes cause energy depletion which ends with necrotic/apoptotic cell death in the brain (5,6). Cognitive impairments such as disorders of consciousness, impairments of attention and memory impairment, and executive dysfunction follow these pathophysiological alterations. The vulnerability of post-hypoxic cognitive impairments depends on according to the affected region such as the upper brainstem, thalamus, cerebellum, basal ganglia, medial temporal structures, cortical layers 3, 5, and 6.

Classical treatment strategies for hypoxia include mechanical ventilation, hyperbaric oxygen treatment or medications for controlling seizures. While substantial or complete cognitive recoveries occur over the first 1-2 years post-injury, some individuals are often functionally disabling (7). The effectiveness of nonpharmacological and pharmacotherapeutic approaches to treat hypoxia is not well established. Therefore, potential neuroprotective agents can be used to enhance the functional and cognitive recoveries from the hypoxic brain injury. For example some pharmaceutical and phytotherapeutical agents like harmane, agmatine or melatonin can be a potential molecules to improve the learning and memory impairments after hypoxia (8-10). Melatonin (MLT) and memantine (MEM) are one of these potential neuroprotective agents whose action was previously revealed by us and other investigators after brain injury in both mice and rats (11-18). MLT (N-acetyl-5-methoxytryptamine), being an anti-apoptotic, anti-inflammatory, anti-oxidative, and analgesic neuroprotectant, has been widely known to inhibit neuronal apoptosis (19-23). Because of its protective effect on neurons, the previous investigations show that MLT may be a beneficial hormone against neurodegenerative and psychiatric diseases and cognitive disorders (24-28). MEM, a non-competitive NMDA receptor blocker, protects neurons from hypoxic injury and prevents memory impairment after cerebral ischemia (29-32).

In our previous studies, we combined MLT with MEM, which is a well-known neuroprotective agents, to investigate the signaling mechanism after ischemic injury in mice and we noted that melatonin/memantine combination reduced ischemic injury and IgG extravasation, indicating vascular leakage in the brain through the MAP kinase pathway (15). In addition, we also observed that combination therapy also reduced brain injury and the density of DNA fragmentation after traumatic brain injury (13). Furthermore, the neuroprotective effect of MLT and MEM in cognitive function in adult life also investigated in both mice and rats with hypoxia (33-37). However, there is no behavioral study showing the effects of MLT and MEM combination in the learning and memory capacity of rats. In addition, the most used model of neonatal hypoxia is Levine-Rice model of rat rather than using mice because this model in the rat mimics the human brain lesion and the following sensory motor deficits and cognitive disabilities (38,39). Therefore, in the present study, we aimed to investigate the effect of MLT, MEM and their combination in hypoxic-ischemic rat in response to behavioral alterations in their adult life.

METHODS

Animals

Experiments were performed using male 7-day-old Sprague Dawley rats (11-15 g) in accordance to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approval of the Animal Care and Use Committee at Yeditepe University (27/08/2010-135). All animals were maintained under a constant 12:12-h light-dark cycle with ad libitum access to food and water. Rat pups were randomly divided into five groups; control (n=9), hypoxia (vehicle administration, n=8), hypoxia treated with MLT (4 mg/kg, n=7), (M5250, Sigma Chemicals, St Louis, MO, USA), hypoxia treated with MEM (20 mg/kg, n=7), (M9292, Sigma Chemicals, St Louis, MO, USA) and hypoxia treated with MLT/ MEM combination (n=8).

Construction of Rat Model of Newborn Hypoxia Ischemia

In the present study, we used Levine-Rice model of neonatal hypoxia because this model in rats leads to deficient in anxiety-related behavior and cognitive impairment in early and late development due to lesions in hippocampus, striatum and cortex (38,40). Briefly, neonatal male rat pups were anesthetized via inhalation of isoflurane, then, a midline neck incision was made, the left common and external carotid artery was isolated and ligated, and then the incision was sutured. After operation, pups were revived at room temperature, normally fed with breast milk and treated under hypoxia: Then, pups were placed into a closed chamber at 37°C injected with nitrogen gas containing 5% CO_2 and 8% O_2. After 120 min, pups were taken and continued to be normally fed with breast milk. The injections of MLT and MEM done immediately after and 2 hours after surgery.

Behavioral Analysis

At the juvenile age, pups were subjected to behavioral tests to analyze their levels of anxiety and capacity of learning and memory.

The elevated O maze was used to assess the level of anxiety of rat pups. The test apparatus is a round 5.5 cm wide polyvinyl-chloride runway which was placed 40 cm above the floor. The elevated O maze had two opposing 90° closed parts which were protected by polyvinyl-chloride walls and two 90° open parts which were not protected by walls. Each pup was released in one of the open parts and observed for 10 min. The time spent in the unprotected sector was measured whenever the animal entered this sector with all four paws.

Morris water maze (MWM) was used for determination of learning and memory capacities. Circular MWM tank (150 cm X 60 cm) was filled with nontoxic paint containing water and the temperature of water maintained at 23 ^{0}C by an automatic heater. Computerized video tracking system was used to observe the rat pup in the pool while it was finding hidden platform (11 cm X 11 cm) according to extra-maze cues. During place learning, rats were given four daily trials, for 4 consecutive days. The trial was stopped the platform was found by the rat pup or 60 s passed. All the movements of pups (swim velocity, distance and trajectory and escape latency) were recorded by the video-tracking system. On the probe trial which measure the strength of the acquired place preference, the pups were swam for 60-s in the pool without platform. The percentage of the distance swum, and time spent in the platform quadrant and in an imaginary circle (40 cm in diameter) around the platform called annulus 40 were recorded.

Statistical Analysis

The repeated measures ANOVA with group as independent factor, and sessions as repeated measures were used for MWM learning data. For the multiple comparisons post hoc test (Fisher's least significant difference (LSD)) was used. Additionally, one-way ANOVA with group as an independent factor was applied to the all experimental data. The statistical analyses were performed using the statistical package SPSS and a P-value ≤ 0.05 was considered as statistically significant.

RESULTS

Elevated O Maze

According to the one-way ANOVA performed for time spent on the open parts of the O maze yielded insignificant treatment effect ($F_{(4,35)}$=1.262, p=0.303). Post hoc LSD test showed that the time spent in the open part was significantly decreased in the pups of the hypoxia group compared to the that of the control group rats (p=0.050). However, the treatment of melatonin, memantine and their combinations reduced the anxiety levels of the hypoxic rats (Fig. 1).

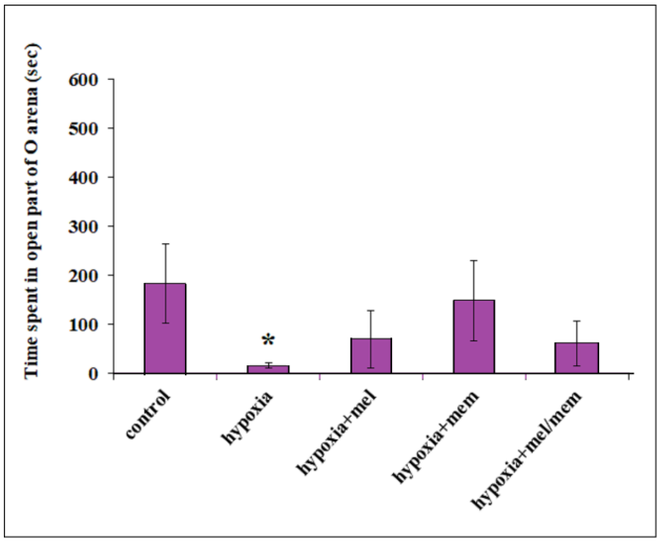

Figure 1. Mean time spent in the open parts of the elevate O-maze for 10 min. Error bars denote SEM. * indicates significant difference at P ≤ 0.05 compared to control group.

Classical MWM Training

According to the two-way repeated measures ANOVA (group x day) performed for escape latency and swim distance yielded a significant day effect ($F_{(3,84)}$=35.748, p≤0.001 and $F_{(3,84)}$=40.811, p≤0.001, respectively) showing a general decrease in overall latency and distance throughout the training period. The day X group interaction for both latency and distance were insignificant ($F_{(12,84)}$=1.244, p=0.268 and $F_{(12,84)}$=1.339, p=0.213, respectively). In addition, no between-group differences were revealed for latency and distance ($F_{(4,28)}$=1.044, p=0.402 and $F_{(4,28)}$=0.552, p=0.699, respectively).

On the fourth day of the acquisition training, the pups in the hypoxia group had the worst performance compared to the other groups both in the escape latency and swam distance (control: P=0.018, MLT:P=0.031, MEM:P=0.050, MLT+MEM:P=0.026). (Fig. 2A and 2B). Treatment of MLT, MEM and MLT+MEM combinations after hypoxia caught up the performance of the control pups at the third (P=0.686, P=0.876, P=0.977, respectively) and fourth (P=0.738, P=0.553, P=0.789, respectively) day of training while the

performances of MEM and MLT+MEM were poor on the second day of training compared to the control group (P=0.056 and P=0.064, respectively) (Fig. 2A and 2B).

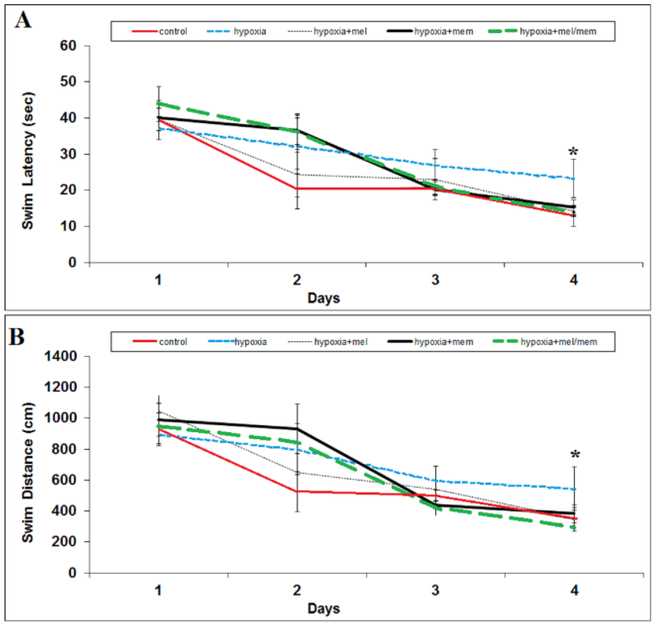

Figure 2. Mean swim latency (A) and mean swim distance calculated for the first 4 days of MWM training. Error bars denote SEM. * indicates significant difference at P ≤ 0.05 compared to control group.

Probe Trial of MWM

Pups' performance in the MWM on the one day 60-s probe trial was assessed by the percentage of time spent in the platform quadrant (Fig. 3); time in the annulus 40 (Fig. 4); and the entering frequency to platform quadrant (Fig. 5). In the memory retention test, the pups in the hypoxia group showed a worse performance compared to the control group (p=0.049) (Fig. 3). Treatment hypoxia with MLT (P=0.239), MEM (P=0.289) or MLT+MEM (P=0.567) combination improved the memory performance compared to the control group.

In addition, we measured the time spent in the annulus 40 because two rats may sometimes spent the same time and swim the same distance in the platform quadrant during the probe trial when their swim trajectories are compared one of the rats appear to swim at much shorter distance to the previous platform location. In this parameter the performance of the hypoxia group was worse than the control group, however, it did not reach the accepted significance level (p=0.117) (Fig. 4).

As seen from the Figure 4, the frequency of entering the platform quadrant from other quadrants were determined for all of the groups. Post hoc LSD test confirmed that the pups in the hypoxia group had significantly lower frequency to enter the platform quadrant than pups in the control group (p=0.023). We found that there was a significantly higher number of entrances in the hypoxia+MLT group compared to the hypoxia group (p=0.020). In addition, there was a marginally significant difference in the hypoxia+MEM group compared with the hypoxia group (p=0.068). There was no significant difference in the frequency to the platform quadrant of the all treatment groups compared to the control group. (Fig. 5).

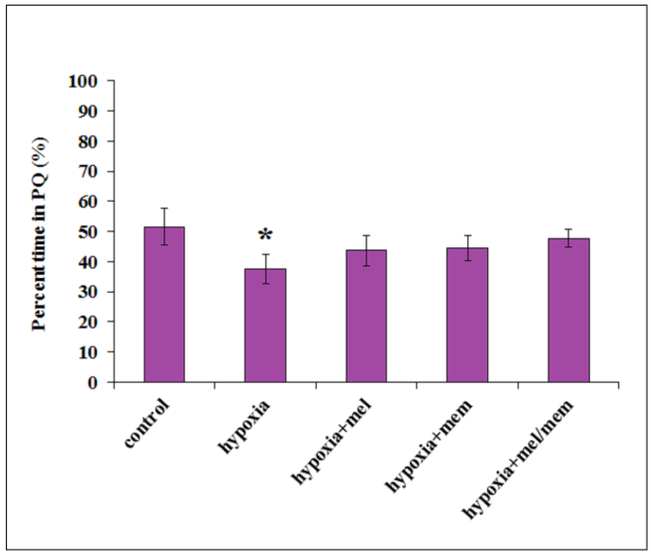

Figure 3. Mean percent time spent in the platform quadrant on the probe trial. Error bars denote SEM. * indicates significant difference at P ≤ 0.05 compared to the control group.

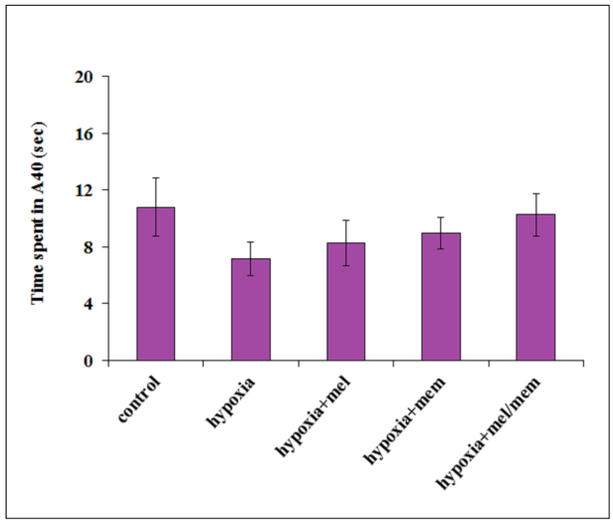

Figure 4. Mean time spent in the annulus 40 on the probe trial. Error bars denote SEM. * indicates significant difference at P ≤ 0.05 compared to the control group.

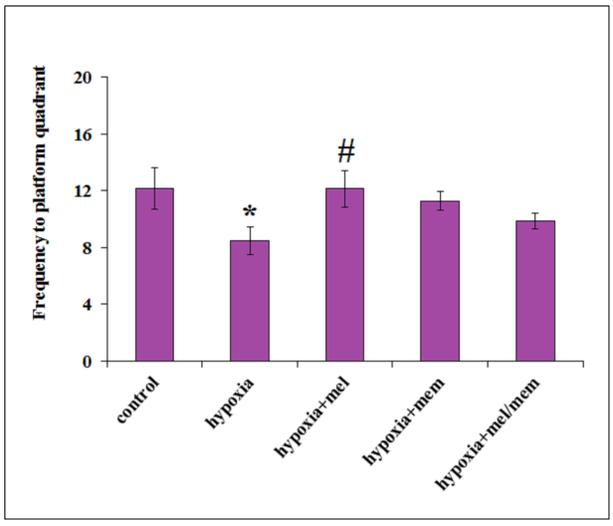

Figure 5. Mean entrance frequency to platform quadrant on the probe trial. Error bars denote SEM. * indicates significant difference at P ≤ 0.05 compared to the control group. # indicates significant difference at P ≤ 0.05 compared to the hypoxia group.

Lastly, we determined the ratio of time spent in the platform quadrant to the time spent in the opposite quadrant as a level of memory retention. The ratio was the lowest at the hypoxia group, however, it did not reach an accepted significance level (P=0.131) (Fig. 6).

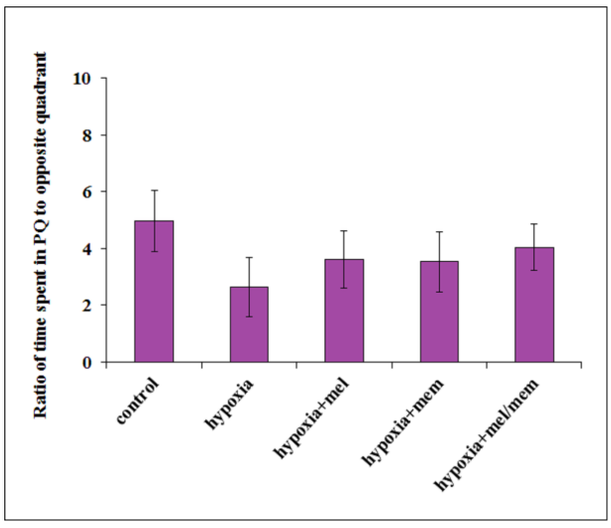

Figure 6. Mean ratio of time spent in the platform quadrant to the opposite quadrant on the probe trial. Error bars denote SEM.

DISCUSSION

Spatial long-term learning and memory is one of the cognitive disabilities that are led by hypoxia (41). It requires the reoxygenation after hypoxia for the functional recovery, however, restoration of blood flow is associated with increasing the rate of tissue damage and might profound the inflammatory response (42). Therefore, potential neuroprotective agents that have anti-inflammatory and anti-oxidative effects may be used during reperfusion for amelioration of functional and cognitive disabilities. In the present study, MLT, MEM and their combination were used to investigate their potential effects on the learning and memory impairments of the hypoxic rat pups.

Following experimental neonatal hypoxia, death in the pyramidal neurons in the hippocampus and a decrease of dendritic spine density may cause learning impairments (43-45). Based on the present data, we observed that neonatal hypoxia which was performed by left common carotid artery ligation at 7-day-old rat resulted in impaired performance of behavioral abilities as a decline in learning capacity. In agreement with our study, Halis and her colleagues (46) found that 7-day-old rat pups with hypoxic ischemia showed a significantly impaired performance of spatial learning and memory in a hidden platform task. In addition, similar studies also proved the hypoxia-related learning impairments in the rat pups (47-50). In addition, neonatal hypoxia also increased the anxiety levels of rat pups as showed previous experiments (51,52).

It is suggested that the exact basis of behavioral deficiencies resulting from hypoxia may occur due to a series of events beginning with excitotoxicity and following with inflammation and oxidative stress due to reperfusion to finish with extensive cell death (34,53-55). As previously mentioned, MLT is usually known its anti-oxidative and anti-inflammatory action (56,57). Furthermore, MLT's anxiolytic effect was also shown previously (58). In addition, MLT also promotes neurogenesis which makes it a good candidate for neuroprotection (59). On the other hand, MEM prevents excitotoxicity because acting as an NMDA open-channel blocker in the presence of prolonged elevation of glutamate concentration suggesting an increase in the learning and memory abilities and decrease in the anxiety level (60,61). In the present study, we observed that both MLT and MEM treatments improved the anxiety levels of hypoxic rat pups. In addition, the pups in the hypoxia+MLT group showed similar performance with control pups in the learning the place of hidden platform in the second, third and fourth day of training. This result may suggest that melatonin administration

is more effective in the amelioration of hypoxia-related learning impairments. In addition, MEM treatment and both MLT/MEM combination treatment had positive effects on the capacity of learning abilities after hypoxia. The *per se* MLT and the *per se* MEM effect on the hypoxia-related learning impairment also noted by other authors in a limited number of studies (34,36,62). In previous studies, it was suggested that MLT administration improve the hypoxia-induced neurobehavioral dysfunction due to its role in the inhibition of microglial activation (34). In addition, prevention of the translocation of NF-κB to the nucleus and thus reduce the up-regulation of pro-inflammatory cytokines may be another molecular correlate of curative effect of MLT on the learning performance (63). On the other hand, the dose used in the present study is effective for ameliorative effect of MEM on the learning performance because it did not block long-term potentiation as previously mentioned (64).

It was also established that neonatal hypoxia produced significant long-term behavioral impairments of spatial memory in the Water maze in adult rats (65). The memory performance in the pups of hypoxia group were worse than the performance of control pups while there was not a total amnesia in the hypoxic pups. This was also consistent with previous studies (34,36,46,62). In all our treatment groups, we observed a slight increase in the percent time spent in the platform quadrant as a memory retention parameter. However, the *per se* MLT treatment was more effective in the rescue of memory from hypoxia-related damage when we compared to the entrance frequencies to platform quadrant of the hypoxia group and the MLT-treated group. The effectiveness of melatonin treatment on the memory was also proved in the pathologies of hippocampus such as Alzheimer's disease (66-69). The memory enhancing capacity of MLT being as a factor for hippocampal neurogenesis was also postulated in a previous experiment which was performed in animals whose memories were suppressed by hypoxia (34). However, in one of the previous studies, they showed that MEM at doses which do not block long term potentiation may show neuroprotective effects rather than memory-enhancing effects such as MLT (64).

In conclusion, administration of both MLT and MEM significantly improved hypoxia-induced learning and memory deficits. However, the low effectiveness of combination therapy in response to the individual MLT or MEM treatments need further investigation to obtain the optimum dose for combination therapy against to neonatal hypoxia. In agreement with the previous data, MLT may be more effective than memantine in the amelioration of neurobehavioral deficiencies after hypoxic conditions. These data suggest that MLT may be of therapeutic value in ameliorating hypoxic damage in the developing brain.

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