ABSTRACT

Objective: Brain damage occurs in many clinical conditions, including trauma, ischemia, and hypertension. Reactive oxygen products and lipid peroxidation are responsible for the brain damage that occurs in these clinical conditions. We investigated whether MCI-186 (3-methyl-1-phenyl-2-pyrazoline-5-one), a free radical binding agent on lipid peroxidation, affects malondialdehyde (MDA), glutathione (GSH), and glutathione peroxidase (GPx) levels in traumatic brain damage.

Methods: The traumatic brain damage model, modified by Feeney, was performed on 28 male Wistar rats separated into 4 groups. The MDA, GSH, and GPx levels in the brain tissues of each group were studied.

Results: MDA levels in the traumatized group were significantly higher than those in the sham and MCI-186 groups (p<0.05), while GSH levels were significantly higher in the sham group than in the trauma and solvent groups (p<0.05). No significant difference was observed between the sham and MCI-186 groups (p>0.05). Although the average GPx level was higher in the sham and MCI-186 groups, no significant difference was found between groups.

Conclusion: Reactive oxidation products significantly decreased in the MCI-186 group. Thus, MCI-186 can be used as a free radical-binding agent in traumatic brain damage.

Keywords: Traumatic brain injury; Antioxidant; Free radical; Lipid peroxidation; MCI-186

INTRODUCTION

Traumatic brain injury (TBI) is one of the important reasons for mortality and morbidity. As a result of traumatic brain damage, 300 people in 100,000 go into hospital and 9 of them die every year. The majority of the reason for TBI is traffic accidents. Cerebral trauma alters brain metabolism, ion hemostasis, and hemodynamics, which causes primary and secondary cell injury.2)

Traumatic brain damage causes tissue damage as a result of primary and secondary mechanisms. It is usually believed that the damage is the result of a series of molecular events that cause vascular and neuronal degeneration.21)
The earliest changes after trauma are the ones related to phospholipase activation and lipid peroxidation. The mechanical damage in cell membranes, the polyunsaturated fatty acids within the first minute of injury, and especially arachidonic acid and cyclooxygenase and lipoxygenase metabolites are capable of causing tissue damage. In traumatic primary injury, there is local and diffuse destruction of axons in the white matter of the brain stem and hemispheres. The primary effect, by starting the physio-pathological chain of events, provides the release of excitatory amino acids, the formation of oxygen-derived free radicals and lipid peroxidation, Ca\(^{2+}\) toxicity, and the formation of eicosanoids. This cascade causes secondary brain damage resulting from the loss of the transport function of the cell membrane.

Lipid peroxidation can be defined as a chemical event including the oxidation of unsaturated fatty acids in the cell membrane, initiated by reactive oxygen products. These reactive \(O_2\) radicals cause peroxidation of membrane lipids, increase of permeability, oxidation and cross-linking of sulfhydryl groups of enzymes and cyto-structural proteins, activation of proteolytic enzymes as a result of inhibition of antiproteases, degradation and shortening of DNA structure, and also depolymerization of mucopolysaccharides.

MCI-186, a synthetic-free radical binder agent. This agent has been shown to reduce hydroxyl-mediated in vivo peroxidative membrane disintegration. MCI-186 protects cerebral tissue from damage associated with ischemia and, in part, lipid peroxidation after recirculation.

In this study, the effect of MCI-186, which is known as free radical scavengers, on lipid peroxidation is investigated in order to define the role of oxygen-derived free radicals in secondary traumatic brain damage. A cerebral trauma was created in rats by using the weight drop method and open head trauma model. Malondialdehyde (MDA), one of the latest products of lipid peroxidation as an indicator of brain trauma, and glutathione (GSH) and glutathione peroxidase (GPx) activation as an indication of antioxidant defense were studied.

**MATERIALS AND METHODS**

All of the animals were obtained from the Animal Center of the Osmangazi University. The experimental protocols were approved by the Animal Care and Use Committee of the Osmangazi University (Permit Number: 2017-0043) and performed in accordance with the Animal Research guidelines.

A total of 28 male rats, weighing between 250 and 320 g were used. All rats were allowed to eat and drink freely and reside in a room with optimum temperature, humidity, and 12-hour day and night cycle. All rats were separated into 4 groups (n=7): Group 1: sham, Group 2: control, Group 3: 3 mg/kg intravenous (IV) sodium hydroxide 8 (solvent) administered before the trauma, and Group 4: MCI-186 (3 mg/kg IV) administered before the trauma.

The 50–60 mg/kg ketamine hydrochloride, 10–12 mg/kg xylazine hydrochloride anesthesia is applied on the rats. In order to inhibit the secretion, 0.1 mg/kg atropine sulfate was given.
intraperitoneally. The rats were intubated endotracheally with an angiocatheter following the anesthesia and they were ventilated with 70% O$_2$, 30% atmospheric air with a tidal volume of 2 mL. After anesthesia and ventilation, blood gas measurements were performed by using the blood taken from the left heart intracardially and respiration rates were kept under control as over PaCO$_2$ 35–45 mmHg, PaO$_2$ 100 mmHg. Their body temperatures were kept stable between 36.5–37.5°C by placing a rectal probe. A TBI was created with the modified Feeney model by Kochanek et al.$^{15}$ Dura is protected and a significant traumatic contusion occurs by this method. The head was shaved in a prone position and fixed with nails on the trauma device. The scalp was opened with midline vertical incision. After retraction of skin, an effort was made not to damage the dura during craniectomy to the right parietal bone by taking the sagittal and coronal sutures as a guide with a high-speed dental drill and thin-tip clamp. A plastic guide tube, with a length of 12.5 cm, inner diameter of 3.5 mm and outer diameter of 5.5 mm, was placed vertically to the dura on the craniectomy site. A lesion was created by dropping a brass rod with a length of 15.2, diameter of 3.5 mm and weight of 10 g from a height of 5 cm into the guide tube (FIGURE 1). The rats were allowed to wake up following the tracheal extubating. The rats were decapitated after 12 hours, and brains were collected. The cortex, hippocampal gyrus and amygdala of the right hemisphere was separated by dissection under a magnifying glass. Cerebellum left hemisphere and basal ganglia were not included into the study. All the materials were stored at −70°C to study MDA, GSH, GPx. The coronal sections taken from the lesion area were stained with hematoxylin-eosin and the histopathological lesion caused by the trauma was shown.

The comparison between 2 groups was checked by Mann-Whitney U test and Kruskal-Wallis test. The p-values <0.05 were considered to indicate statistical significance.

**RESULTS**

All trauma was created while the ventilation was set to obtain the blood gas values as PCO$_2$ 35–45 mmHg and 100 mmHg in PO$_2$ (TABLE 1).
In rats with traumatic damage, while the median MDA values of sham group are 1.56, they were 2.72 in trauma group, 2.09 in solvent group and 1.59 in MCI-186 group. It is observed that the lowest median value within brain tissue MDA values belongs to the MCI-186 group. MDA values of the solvent group were significantly higher than the sham and MCI-186 group (\(p=0.039\)). The average values of the group were determined as lower than the trauma group but no statistical difference was found (\(p=0.041\)). The median values of MCI-186 group were determined as lower than the sham group but no statistical difference was found (\(p=0.03\)) (FIGURE 2).

The brain tissue GSH values of the rats which were exposed to traumatic brain damage by the modified Feeney model were found as 68.12 in control group, 45.56 in the solvent group, and 62.29 in the MCI-186 group. GSH levels of both the trauma and solvent group were found lower than the sham group statistically (\(p=0.038\)). There was no significant difference between sham and MCI-186 groups (FIGURE 3).

| Group                 | PCO\(_2\) and PO\(_2\) levels during head injury |
|-----------------------|-----------------------------------------------|
|                       | Sham (\(n=7\)) | Trauma (\(n=7\)) | Solvent (\(n=7\)) | MCI-186 (\(n=7\)) |
| PCO\(_2\)             | 40.53±3.22     | 40.00±2.03       | 40.71±3.54       | 39.52±3.99         |
| PO\(_2\)              | 138.93±24.35   | 131.8±30.38     | 117.15±21.83    | 142.71±27.63       |

Values are presented as average ± standard deviation.

FIGURE 2. Brain tissue median MDA values (nmol/mg protein).
MDA: malondialdehyde.
*Comparing with sham (\(p<0.05\)); †Comparing with MCI-186 (\(p<0.05\)).

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FIGURE 3. Graphical display of median GSH values of brain tissue (ng/mg protein).
GSH: glutathione.
While GPx levels are 18.17 in sham group, 14.26 in trauma group, 15.36 in solvent applied group and 16.92 in MCI-186 group. Although median GPx levels are determined as high in the sham and MCI-186 groups, no significant difference was found (FIGURE 4). All GSH, GPx, and MDA results were summarized in TABLE 2.  

DISCUSSION

Traumatic brain damage is an important reason for mortality and morbidity. Recent publications show that lipid peroxidation induced by free oxygen radicals and related neuronal damage plays a key role in the primary and secondary stages of TBI. The increased level of oxygen free radicals after the brain damage causes loss of microvascular autoregulation, ischemia, peroxidation of membrane phospholipids, and excessive calcium release which plays a major role in posttraumatic cell death. The brain is notably susceptible to lipid peroxidation due to its high amount of polyunsaturated fatty acids, low antioxidant capacity, and high O\textsubscript{2} consumption. Brain tissue includes high amounts of Fe and Cu. These are the substances that promote the formation of free radicals.

Kontos et al. showed that free oxygen radicals (liquid pressure) were formed after experimental brain trauma in the cat brain. Iron injection into the rabbit cortex causes the creation of superoxide radicals, brain edema, lipid peroxidation, and cortical damage on the injected site. Kontos et al. showed that arachidonic acid vasodilates pial arteries dose-dependently in the post-traumatic brain and explained this phenomenon with the formation of free oxygen radicals. This made people think that usage of free radical scavengers can be effective for the treatment of trauma.
Lipid peroxidation is a complex phenomenon in which polyunsaturated fatty acids in phospholipids of cellular membranes react to create lipid hydroperoxides. It starts with the formation of free radicals. The most important indicator of oxidative stress during trauma is MDA. The reaction of hydrogen peroxide formed during oxidative stress and unsaturated fatty acids of all reactive oxygen products initiates lipid peroxidation.

Lipid peroxidation is the most popular and the easiest method to measure free radical activity in biological samples. In animal experiments, it has been shown that antioxidants prevent oxidative damage caused by free radicals. Antioxidants that scavenge free radicals are used during this phenomenon and the level of antioxidants are used indirectly to determine free radical activity.

MDA level increases right after the traumatic brain damage and this increase continues for 36 hours depending on the severity of the trauma. Inci et al. declared that the effects of vitamin E on lipid peroxidation in terms of time and dose by causing severe and mild traumatic brain damage with the Feeney model. They reported that lipid peroxidation was found significantly higher than the control group at the beginning of the trauma in rats subjected to mild trauma and decreased to the normal values compared to the control group after 36 hours. They clarified that lipid peroxidation increased rapidly in the severe trauma group and was 100% higher than the control values at the end of 26 hours. They explained this with the depletion of antioxidant defense systems in the severe trauma group.

Barut et al. investigated lipid peroxidation after the experimental spinal cord damage in 60 rats and presented that MDA levels increased after 15 minutes and reached a maximum level at the 1. Hour of the trauma and then started to decrease. This is the indication of the presence of a defense system in the organism, superoxide dismutase (SOD) and catalase step in to reduce lipid peroxidation and action. Chan et al. measured MDA, GSH, and GPx levels during reperfusion following bilateral carotid artery occlusion in 1992. They found that MDA, GSH, and GPx levels were significantly higher than the controls after the first hour of reperfusion. They reported that MDA, GSH, and GPx levels increased after the fourth hour of reperfusion and reached 180% of the sham group and 223% of the control group after the 12th hour of reperfusion and decreased to the level of the sham group in 24 hours. Shivakumar et al. reported that there was a significant decrease of total GSH levels in the brain after 1-hour reperfusion following a 30-minute carotid artery occlusion and this decrease was 85% compared to the sham group, brain GSH levels increased after a 4 hours of reperfusion and reached 91% of the sham group, and GSH levels returned to normal completely after 8 hours of reperfusion. They also observed GPx activity in the same experiment and reported that there was no significant change. They said that reperfusion following a 50% decrease in blood flow to the brain can protect itself against oxidative damage which is determined by increased MDA levels and changes between GSH/oxidized GSH.

MDA, GSH, and GPx levels of the brain tissue were measured in our study. Tissue MDA levels were found significantly higher in the trauma group than the sham group (p<0.05). Likewise, the trauma group was found significantly higher than the MCI-186 group (p<0.05). In the literature, it has been shown that MDA levels reach a maximum level at the first hour after the trauma and this increase continues at the 36th hour depending on the degree of the trauma. In our study, MDA levels at the 12th hour after the trauma were examined. As the MDA levels at the 12th hour after the trauma were significantly higher than the sham group and this increase continued at the 36th hour, too, it was considered to be consistent with
the literature. The tissue GSH levels in the sham group were found significantly higher than the trauma and solvent group ($p<0.05$). A significant difference couldn’t be found between the sham group and the MCI-186 group. In the literature, it has been shown that GSH levels decrease significantly within 1 hour especially after ischemia and reperfusion, and return to normal after 4 hours. The fact that the GSH level was found to be significantly lower in the sham group within 12 hours after the trauma in our study can be explained by the excessive use of antioxidant defense depending on the severity of the trauma. Although the average GPx level was higher in the sham group and MCI-186 group, the fact that this is not statistically significant can be explained by the fact that this enzyme is not found high enough in the brain.

In the literature, it is seen that free oxygen radicals which cause lipid peroxidation, play an important role in brain edema. Accordingly, in the year of 1992, Long et al.\(^7\) claimed that antioxidant substances can be used in the treatment of posttraumatic brain edema. SOD, an endogenous free radical scavenger, was the first substance to be tried.\(^7\) However, Chan and Ikeda indicated that this enzyme does not reduce free radical levels sufficiently.\(^3,11\) The reason for this is that the molecular weight of SOD is 31 thousand, so it does not exceed the BBB under normal conditions. In addition, SOD has a very short biological half-life that lasts 6 minutes. This limits the clinical use of this enzyme. Therefore, SOD was conjugated with polyethylene glycol. As a result of this, the half-life of SOD extended to 5 days. Thus, successful results were obtained in clinical and experimental studies of polyethylene glycol-SOD compounds. In 1996, Young et al.\(^27\) indicated that there were no statistical differences in neurological functions or mortality rates of 463 patients with severe head trauma who were treated with polyethylene glycol-SOD and given a placebo.

A second treatment type is to prevent the occurrence of the Haber-Weiss reaction, which plays an important role at the beginning steps of lipid peroxidation. In order to prevent this reaction, deferoxamine was used to bind free iron and some successful results were obtained from experimental studies.\(^9\)

In recent years, there are many studies in which free radical binding agents are used. There are a lot of publications on the use of the free radical binding agent called MCI-186 in ischemic neural damage. On the contrary, no publication has been found in the literature on the use of MCI-186 in traumatic neural damage.

It is reported that 15-HPETE, the intermediate product of arachidonic acid metabolism, causes damage in cultured aortic endothelial cells and MCI-186 prevents all harmful effects of 15-HPETE.\(^26\) MCI-186 protects cerebral tissue from the damage related to lipid peroxidation formed after ischemia and recirculation partly, and it does this by binding OH radical.\(^14,18,26\) MCI-186 also has an inhibitory effect on iron-dependent lipid peroxidation.

MCI-186 has no effects on oxygen. Briefly, it has been reported that MCI-186 inhibits both non-enzymatic lipid peroxidation and lipoxygenase activity. No inhibitory effect on cyclooxygenase activity was observed in vitro.\(^26\)

Oishi et al.\(^19\) investigated the effects of MCI-186 on monoamine metabolism in brains of both normal and ischemic rats and found that while a single dose of 3 mg/kg IV MCI-186 prevented ischemic brain edema, it had no significant effect on the concentrations of dopamine, norepinephrine, and 5-hydroxytryptamine. They explained this by its free radical binding function.
Nishi et al. [10] researched the effects of MCI-186 on brain edema. They created a permanent embolism by injecting polyvinyl acetate into the left internal carotid artery and induced ipsilateral cerebral edema. They assessed brain edema by measuring brain water content, NA⁺ and K⁺ concentrations after 24 hours. In this study, it was reported that MCI-186 was given as 1–3 mg/kg IV. Inhibited cerebral edema. In the same study, it was indicated that while 1.0 mg/kg IV. Dexamethasone prevented cerebral edema, 4.0 mg/kg indomethacin (cyclooxygenase inhibitor) didn’t reduce cerebral edema. This study was conducted to show that arachidonic acid cyclooxygenase products that released from neural membrane phospholipids are not likely to be related to the pathogenesis of persistent brain edema induced by polyvinyl acetate. It has been submitted that MCI-186 reduces cerebral edema by suppressing the production of free radicals or lipoxygenase metabolites including lipid peroxidation.

In our study, MCI-186’s significantly lowering MDA, which is an indicator of lipid peroxidation after trauma, compared to the trauma group can be explained by the fact that the drug iron-mediated lipid peroxidation. In the presence of trauma, iron is released from hemoglobin in the brain. The superoxide anion, which is also released after trauma, combines with iron to form the hydroxyl radical and this causes lipid peroxidation. MCI-186 might have decreased MDA values by also preventing this nonenzymatic lipid peroxidation. The significantly higher GSH levels in the sham group compared to the trauma group can be an indirect indicator of free radical-mediated damage in the model we used. The absence of a significant difference in GSH levels between the sham group and the MCI-186 group may give rise to the thought that MCI-186 has a positive effect on antioxidant defense systems by preventing lipid peroxidation. However, the increase of GSH in the MCI-186 group did not fully compensate for the decrease of GSH in the trauma group.

According to the literature studies, MCI-186 is ineffective on oxygen and cyclooxygenase enzymes as in vitro. Maybe MCI-186 can also act on cyclooxygenase enzyme as in vivo. In the light of these results, it can be said that free radical binders will be beneficial in the damage caused by brain trauma and MCI-186 can be used for this purpose.

CONCLUSION

The effects of the free radical binding agent MCI-186 on the prevention of lipid peroxidation, MDA levels were found to be significantly lower in the group administered the drug compared to the trauma group. As a result, MCI-186 prevents lipid peroxidation on the traumatic brain tissue. This effect has been attached to the effect of MCI-186 by inhibiting iron-related lipid peroxidation.

REFERENCES

1. Barut S, Canbolat A, Bilge T, Aydin Y, Cokneşeli B, Kaya U. Lipid peroxidation in experimental spinal cord injury: time-level relationship. *Neurosurg Rev* 16:53-59, 1993
2. Chan PH. Role of oxidants in ischemic brain damage. *Stroke* 27:1124-1129, 1996
3. Chan PH, Longar S, Fishman RA. Protective effects of liposome-entrapped superoxide dismutase on posttraumatic brain edema. *Ann Neurol* 21:540-547, 1987
4. Faden AI. Pharmacological treatment of central nervous system trauma. *Pharmacol Toxicol* 78:1247, 1996
   [PUBMED] [CROSSREF]

5. Faden AI, Salzman S. Pharmacological strategies in CNS trauma. *Trends Pharmacol Sci* 13:29-35, 1992
   [PUBMED] [CROSSREF]

6. Hall ED, Braughler JM. Free radicals in CNS injury. *Res Publ Assoc Res Nerv Ment Dis* 71:81-105, 1993
   [PUBMED]

7. Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem* 59:1609-1623, 1992
   [PUBMED] [CROSSREF]

8. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging* 18:685-716, 2001
   [PUBMED] [CROSSREF]

9. Halliwell B, Gutteridge JM. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* 246:501-514, 1986
   [PUBMED] [CROSSREF]

10. Holley AE, Cheeseman KH. Measuring free radical reactions in vivo. *Br Med Bull* 49:494-505, 1993
    [PUBMED] [CROSSREF]

11. Ikeda Y, Anderson JH, Long DM. Oxygen free radicals in the genesis of traumatic and peritumoral brain edema. *Neurosurgery* 24:673-685, 1989
    [PUBMED] [CROSSREF]

12. Ikeda Y, Long DM. The molecular basis of brain injury and brain edema: the role of oxygen free radicals. *Neurosurgery* 27:1-11, 1990
    [PUBMED] [CROSSREF]

13. Inci S, Ozcan OE, Kiliç K. Time-level relationship for lipid peroxidation and the protective effect of α-tocopherol in experimental mild and severe brain injury. *Neurosurgery* 43:330-335, 1998
    [PUBMED] [CROSSREF]

14. Kawai H, Nakai H, Suga M, Yuki S, Watanabe T, Saito KI. Effects of a novel free radical scavenger, MCl-186, on ischemic brain damage in the rat distal middle cerebral artery occlusion model. *J Pharmacol Exp Ther* 281:921-927, 1997
    [PUBMED]

15. Kochanek PK, Wallisch JS, Bayır H, Clark RS. Pre-clinical models in pediatric traumatic brain injury—challenges and lessons learned. *Childs Nerv Syst* 33:1693-1701, 2017
    [PUBMED] [CROSSREF]

16. Kontos HA, Wei EP. Superoxide production in experimental brain injury. *J Neurosurg* 64:803-807, 1986
    [PUBMED] [CROSSREF]

17. Long DM, Maxwell RE, Choi KS, Cole HO, French LA. Multiple therapeutic approaches in the treatment of brain edema induced by a standard cold lesion in Reulen HJ, Schürmann K (eds): Steroids and brain edema. Heidelberg: Springer Berlin, pp87-94, 1972

18. Nishi H, Watanabe T, Sakurai H, Yuki S, Ishibashi A. Effect of MCI-186 on brain edema in rats. *Stroke* 20:1236-1240, 1989
    [PUBMED] [CROSSREF]

19. Oishi R, Itoh Y, Nishibori M, Watanabe T, Nishi H, Saeki K. Effect of MCI-186 on ischemia-induced changes in monoamine metabolism in rat brain. *Stroke* 20:1557-1564, 1989
    [PUBMED] [CROSSREF]

20. Petry MA, Poulet P, Haas A, Namer IJ, Wagner J. Reduction of traumatic brain injury-induced cerebral oedema by a free radical scavenger. *Eur J Pharmacol* 307:149-155, 1996
    [PUBMED] [CROSSREF]

21. Prius M, Greco T, Alexander D, Giza CC. The pathophysiology of traumatic brain injury at a glance. *Dis Model Mech* 6:1307-1315, 2013
    [PUBMED] [CROSSREF]

22. Regan RF, Choi DW. Excitotoxicity and central nervous system trauma. New York, NY: Oxford University Press, 1994

23. Shivakumar BR, Kohli SV, Ravindranath V. Glutathione homeostasis in brain during reperfusion following bilateral carotid artery occlusion in the rat. *Mol Cell Biochem* 111:125-129, 1992
    [PUBMED] [CROSSREF]

24. Uysal M. Free radicals, lipid peroxides and conditions affecting the prooxidant-antioxidant balance in the organism. *Klinik Gelişim* 11:336-341, 1998

25. Watanabe T, Egawa M. Effects of an antistroke agent MCI-186 on cerebral arachidonate cascade. *J Pharmacol Exp Ther* 271:1624-1629, 1994
26. Watanabe T, Yuki S, Egawa M, Nishi H. Protective effects of MCI-186 on cerebral ischemia: possible involvement of free radical scavenging and antioxidant actions. J Pharmacol Exp Ther 268:1597-1604, 1994

27. Young B, Runge JW, Waxman KS, Harrington T, Wilberger J, Muizelaar JP, et al. Effects of pegorgotein on neurologic outcome of patients with severe head injury. A multicenter, randomized controlled trial. JAMA 276:538-543, 1996