EXPERIMENTAL STUDY

Neuroprotective effects of adrenomedullin in experimental traumatic brain injury model in rats

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ABSTRACT

BACKGROUND: Traumatic brain injuries cause damages in the brain in several ways, which include cell death because of edema, disruption of the blood–brain barrier, shear stress, and ischemia. In this study, we investigated the effects of adrenomedullin (AM) on oxidative stress and inflammation after head traumas in a rat model.

METHODS: Eighteen male adult Wistar albino rats were randomized into three groups (n=6). No traumas were applied to the control (C) group. Traumas were applied in line with Marmarau trauma model in the trauma group. The rats in the AM treatment group were treated with post-traumatic 12 μg/kg i.p. AM in addition to the trauma group. The rats were followed for 7 days in all groups and were then sacrificed. Brain tissues and blood samples were taken.

RESULTS: In the trauma group, both tissue and serum MDA, TNF-α, and IL-6 levels were significantly increased compared to the control group ($p<0.05$). In the AM-treated group, serum TNF-α levels were significantly decreased compared to the trauma group ($p<0.05$). In the trauma group, both tissue and serum GSH levels were significantly decreased compared to the control group ($p<0.05$). In the trauma group, serum Vitamin D3 levels were significantly decreased compared to the control group ($p<0.05$). In the AM-treated group, both tissue and serum GSH levels were significantly increased compared to the trauma group ($p<0.05$).

CONCLUSION: These results indicate that AM has neuroprotective effects on traumatic brain injury in a rat model.

Keywords: Adrenomedullin; inflammation; oxidative stress; traumatic brain injury.

INTRODUCTION

Traumatic brain injury (TBI) is among the main causes of mortality and morbidity in the population below the age of 45 in the entire world. Based on the mechanical effect of the trauma, several lesions occur in the brain, such as contusion, laceration, intracranial hemorrhage, and diffuse axonal damage. In addition to primary lesions, a secondary process caused by direct effect is responsible for mortality and morbidity, and therefore, sensitivity to the therapy starts.[1] The primary and secondary processes cause that cell mediators are released (e.g., pro-inflammatory cytokines, prostaglandins, free radicals, and complement system), adhesion molecules are expressed, and chemotaxis of glial and defines cells occurs.[1,2] Secretion and production of pro-inflammatory cytokines following TBI were detected in human and experimental animal models. Elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-1β, and central nervous system (CNS) cytokines indicate that they play significant roles in post-traumatic pathological processes.[2–4] Before the secondary damage starts after trauma, preventive treatments must be initiated in terms of reducing mortality and morbidity.[1]

Avoiding and reducing secondary brain injuries following head traumas have been the interest of recent studies conducted on CNS traumas. Even though the exact mechanism of delayed injuries following mechanical traumas is not clear, the previous

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researchers reported that free radical production following head traumas has important roles in secondary injuries.[5,6]

Lipid peroxidation (LP) has significant effects on tissue damage after head injuries. Reactive oxygen radicals that cause damages in cellular components have important roles in ischemic or hypoxic tissues initiating the LP process following head traumas. However, antioxidant agents might protect brain tissue against oxidative damage.[7]

The antioxidant-oxidant systems of organisms are balanced normally. If this balance is disrupted in favor of the oxidants, then, leukocytes produce inflammatory mediators (e.g., bradykinin, prostaglandin, leukotriene, platelet-activating factor, serotonin, adhesion molecule P-selectin, TNF-α, IL-1, IL-6, and IL-1β) together with free oxygen radicals. CNS consists mostly of lipids; and therefore, the LP caused by free radicals can cause severe damage.[8,9]

Adrenomedullin (AM) is a potent, long-lasting vasoactive peptide that was originally isolated from human pheochromocytoma.[10] Several studies have demonstrated that AM acts as a vasodilator, bronchodilator, neurotransmitter/modulator, inhibitor of apoptosis, anti-migratory, antimicrobial, and anti-inflammatory agent.[10,11] The level of AM is elevated in the plasma in some diseases (e.g., hypertension, renal failure, heart failure, endotoxic shock, and hemorrhagic shock.[12–14]

The inhibition of LP and inflammation by many agents, such as antioxidants or free radical scavengers, may be useful in the treatment of head injuries. In the present study, our objective was to define the possible potential neuroprotective effects of AM after head traumas in a rat model. For this purpose, the effects of AM were assessed by biochemical analyses (oxidative stress biomarkers and inflammation parameters).

MATERIALS AND METHODS

The permission for this experimental study was received from the "Animal Ethics Committee" of Saki Yenilli Experimental Animals Reproduction Laboratory (October 02, 2020, March 06, 2022), and the study was completed in line with the dictates of the Research Committee at the Experimental Research Centre of Saki Yenilli Experimental Animals Reproduction Laboratory.

Eighteen male adult Wistar albino rats weighing between 180 and 220 g (mean weight: 210±10 g) were included in the study. All animals were housed under standard temperature and humidity with light and dark cycles of 12 and 12 h, respectively. No water, food, and light restrictions were applied to animals. During the experiments, humane care was given to all animals according to "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" by NHS. The animals were anesthetized using xylazine hydrochloride (5 mg/kg) (Alfazyne, Ege Vet, Turkey) and ketamine hydrochloride (40 mg/kg) (Ketalar, Eczacibasi, Turkey), and every surgical procedure was performed under sterile conditions by the surgeon of the team (AP).

To arrange three study groups, 18 rats were randomly allocated:

Control group (n=6): No trauma models were applied to the rats. They were followed up in their cages for 7 days without being disturbed.

Trauma group (n=6): The rats were injected with intramuscular 70 mg/kg ketamine and 5 mg/kg xylazine anesthetic agent, and the traumatic brain damage was induced with Marmarau drop weight model.[15]

To do this;
After midline scalp incision under local anesthesia and sterile conditions, the periosteal that covered the vertex was removed with a dissector. A 3 mm high and 10 mm diameter metal disk were glued using bone wax to the vertex between the coronal and lambdoid sutures to provide a wider cranial contact level and to induce diffuse cranial damage.

The rats were placed on a foam bed in a prone position. The lower end of the metal tube was placed directly on the metal disk in the skull of the rat. Closed head trauma was induced by releasing 250 g of weight from a height of 1 m.

The rats were followed in their cages without disturbing for 7 days after this procedure.

AM-treated group (n=6): A single dose of 12 µg/kg i.p. AM (Sigma Chemical Co., St. Louis, MO, USA) was administered right after laminectomy and acute trauma to the six rats. The rats were followed in their cages for 7 days without disturbing.[16]

The routine care of the animals in the three groups was performed with 12 h light (08–20), 12 h dark photoperiods, at 21–23°C room temperature, in cages with six rats, with standard pellet feed and tap water.

The rats were sacrificed with the aspiration of cardiac blood, and the blood samples were placed in gel tubes. Serum samples were separated by centrifugation of 3.000×g for 10 min at 4°C and were frozen to −80°C. The excised brain was washed in cold 0.9% NaCl, wiped, and weighed. To perform biochemical analysis, they were then fixed in liquid nitrogen and kept frozen at −80°C.

Biochemical Analysis

Determination of Tissue and Serum MDA Levels

MDA (LP end-products) levels were determined using the thiobarbituric acid (TBA) test based on the spectrophotometric measurement of the concentration obtained from the end-product of the reaction between lipid peroxides and TBA.[17]

Determination of Tissue and Serum GSH Levels

The Ellman reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) was used to quantify the number of aliphatic thiol groups in the sample. In mild alkali pH, tissues and serum reacted with the Ellman reagent; and p-nitrophenol anion per every thiol group was calculated with spectrophotometry.[18]

Determination of Tissue Protein Levels

Tissue protein levels were determined using the Lowry method with bovine serum albumin as the standard.[19]

Determination of Tissue and Serum TNF-α and IL-6 Levels

Tissue and serum TNF-α and IL-6 levels were also determined by a solid-phase sandwich enzyme-linked immunosorbent assay.

Determination of Serum Vitamin D Levels

Determination of serum Vitamin D3 levels was performed using the immune analysis method. Serum Vitamin D3 levels were calculated using Beckman Coulter assay kits.

Statistical Analysis

Statistical package SPSS for Windows, version 22.0 (SPSS, Chicago, Illinois, USA) was used for analyses with the descriptive statistics presented as mean±SD. Kruskal–Wallis test and Mann–Whitney U test were used for statistical analyses. P<0.05 was considered to be statistically significant.

RESULTS

Tissue Changes

In the trauma group, tissue MDA, TNF-α, and IL-6 levels were significantly increased compared to the control group (p<0.05). In the trauma group, tissue GSH levels were significantly decreased compared to the control group (p<0.05). In the AM-treated group, tissue MDA levels were significantly decreased compared to the trauma group (p<0.05). In the AM-treated group, tissue TNF-α and IL-6 levels were decreased compared to the trauma group. However, this difference was not significant (p>0.05). In the AM-treated group, tissue GSH levels were significantly increased compared to the trauma group (p<0.05). A comparison of the AM-treated and control groups demonstrated no significant difference (p>0.05) (Table 1).

Serum Changes

In the trauma group, serum MDA, TNF-α, and IL-6 levels were significantly increased compared to the control group (p<0.05). In the trauma group, serum GSH and Vitamin D3 levels were significantly decreased compared to the control group (p<0.05). In the AM-treated group, serum TNF-α levels were significantly decreased compared to the trauma group (p<0.05). In the AM-treated group, serum MDA and IL-6 levels were decreased compared to the trauma group. However, this difference was not significant (p>0.05). In the AM-treated group, serum GSH levels were significantly increased

Table 1. Tissue MDA, TNF-α, IL-6, and GSH levels of all the groups (Mean±SD)

	Control (n=6)	Trauma (n=6)	AM-treated (n=6)
MDA (μmol/g protein)	6.74±2.17[a]	12.33±4.56[a,b]	6.63±1.92[b]
TNF-α (pg/mg protein)	111.25±5.33[a]	150.21±27.47[a]	116.83±22.06
IL-6 (pg/mg protein)	1.37±0.56[a]	2.08±0.34[a]	1.57±0.64
GSH (μmol/g protein)	0.18±0.04[a]	0.09±0.04[a,b]	0.17±0.05[b]

[a]MDA, TNF-α, IL-6, and GSH between the trauma group and the control group (p<0.05). [b]MDA and GSH between the trauma group and the AM-treated group (p<0.05). MDA: Malondialdehyde; TNF-α: tumour necrosis factor-alpha; IL: Interleukin; GSH: Glutathione; AM: Adrenomedullin; SD: Standard deviation.

Table 2. Serum MDA, TNF-α, IL-6, GSH, and Vit D levels of all the groups (Mean±SD)

	Control (n=6)	Trauma (n=6)	AM-treated (n=6)
MDA (μM)	8.29±0.76[a]	10.27±0.98[a]	9.13±0.64
TNF-α (pg/mL)	107.73±0.92[a]	124.31±5.89[a,b]	107.74±0.57[b]
IL-6 (pg/mL)	1.4±0.32[a]	1.76±0.18[a]	1.57±0.64
GSH (μM)	0.45±0.15[a]	0.22±0.11[a,b]	0.42±0.06[b]
Vit D3 (ng/mL)	75.05±7.09[a]	61.55±2.67[a]	69.36±8.64

[a]MDA, TNF-α, IL-6, GSH, and Vit D3 between the trauma group and the control group (p<0.05). [b]TNF-α and GSH between the trauma group and the AM-treated group (p<0.05). MDA: Malondialdehyde; TNF-α: tumour necrosis factor-alpha; IL: Interleukin; GSH: Glutathione; AM: Adrenomedullin; SD: Standard deviation.

compared to the trauma group ($p<0.05$). In the AM-treated group, serum Vitamin D3 levels were increased compared to the trauma group. However, this difference was not significant ($p>0.05$). A comparison of the AM-treated and control group demonstrated no significant difference ($p>0.05$) (Table 2).

DISCUSSION

In this study, we demonstrated that AM treatment significantly neurologic function and its neuroprotective or repair effect are exerted through anti-apoptotic and anti-inflammatory effects on the acute-phase brain changes following head traumas, preventing and reducing secondary brain damage. In addition, biochemical analysis of the serum and brain tissue verified its systemic and local effects.

Following TBI, morbidity and mortality depend on primary damage (e.g., contusion, laceration, diffuse axonal injury, and intracerebral hematoma) and secondary damage that occurs at the time of injury with elevated intracranial pressure, swelling, and hypoxia/ischemia. Following head traumas, preventing and reducing secondary brain damage have been the focus of recent studies on CNS trauma. It was speculated in the previous studies that an important factor precipitating post-traumatic degeneration in the brain is free oxygen radical-induced LP.[7]

Today, no surgical and medical treatments are available for primary damage; however, it is possible to influence the injury caused by the cascade of biochemical events occurring secondarily.[7]

Sufficient experimental supports are available regarding the importance of early appearance and pathophysiology of oxygen radical formation and cell membrane LP in the injured CNS. LP is a process that progresses geometrically and spreading over the surface of the cell membrane, which causes impairment on the phospholipid-dependent enzyme, disruption of ionic gradients. The membrane lysis MDA is the main breakdown product of LP in CNS.[6]

Phospholipids of neural membranes are damaged by free oxygen radicals. It was proposed that in situ myelin proteins are highly susceptible to the attack of ROS in the membrane.[20]

A great number of physiological and pathologic processes are associated with AM (e.g., vasodilation, angiogenesis, cancer promotion, apoptosis, cell growth regulation, and differentiation). It was speculated that AM protects several cells against oxidative stress, which is induced by stressors, such as hypoxia, ischemia-reperfusion (I/R), and hydrogen peroxide (H_2O_2).[21,22] The aim of this study was to evaluate the efficacy of AM on the damage associated with oxidative stress and inflammation created by head trauma in an experimental animal model.

It was demonstrated that AM protects myocardial cells against ischemia/reperfusion injury through suppression of oxidative stress-induced activation of the pro-apoptotic factor Bax and activation of the antiapoptotic Akt-Bad-Bcl-2 signaling pathway.[23] It was demonstrated that AM gene delivery significantly reduced myocardial infarction, apoptosis, and superoxide (SOD) production in the rat model with myocardial I/R injury. To improve spinal cord protection by pharmacological means, we aimed to investigate the protective effects of AM on the spinal cord during ischemia in an experimental model.[24]

The mechanism by which AM protects from I/R injury is unclear. I/R injury is a complex process; calcium overload and excessive production of oxygen-free radicals are the main mechanisms involved in I/R injury. AM can antagonize myocardial injury induced by I/R through inhibiting oxidative stress and reducing calcium overload.[24]

MDA is an indicator of oxidative stress within the cell and is a relatively stable end-product of LP. Some studies have shown an increase in lipid peroxidase in inflammatory diseases.[25] In our study, in the trauma group, both tissue and serum MDA levels were significantly increased compared to the control group. In the AM-treated group, tissue MDA levels decreased significantly compared to the trauma group. In the AM-treated group, serum MDA levels were decreased compared to the trauma group. However, this difference was not significant.

It was shown that GSH is an important cellular antioxidant and protects cells from damaging effects of oxidation products, such as H_2O_2, SOD, and hydroxyl radicals), which are normally produced and destroyed by the cell in metabolism. The GSH supply may have critical roles in antioxidant defines. The redox state of the cell might become oxidized under oxidant stress, which results in insufficient antioxidant defines to avoid irreversible damage (e.g., LP). Peroxynitrite might indirectly potentiate damage by reacting with GSH, which disables important protective antioxidant mechanisms.[26] In our study, both tissue and serum GSH levels were significantly decreased in the trauma group compared to the control group. In the AM-treated group, both tissue and serum GSH levels were significantly increased compared to the trauma group.

Menku et al.[7] demonstrated that tissue MDA levels were significantly lower in the propofol and citicoline combination group than in the control and propofol groups. Regarding the activities of SOD, increases were detected in the combination and propofol groups. No statistically significant differences were detected among the groups in terms of glutathione peroxidase activities.

In our previous study, tissue MDA levels were significantly decreased in the alemtuzumab-treated group compared to the trauma group. In the alemtuzumab-treated group, tissue GSH

levels were significantly increased compared to the trauma group.[27]

Vitamin D is a steroid hormone, which is produced in the epidermis photochemically. Vitamin D is known to be involved in bone mineralization regulation and calcium-phosphorus balance, but recent research speculates that it may have significant effects on cell proliferation, differentiation, neurotransmission, neuroplasticity, neurotropic, and neuroprotective effects in CNS.[28]

It was demonstrated that Vitamin D deficiency is common in patients after TBI with 46.5% of patients being Vitamin D deficient, and a further 33.7% being insufficient, with an overall 80.2% having low concentrations.[29]

Vitamin D deficiency leads to greater open-field behavioral deficits in TBI rat models, attenuating the useful effects of progesterone administration.[30]

In our study, serum Vitamin D3 levels were significantly decreased in the trauma group compared to the control group. In the AM-treated group, serum Vitamin D3 levels were increased compared to the trauma group. However, this difference was not significant.

TNF-α and pro-inflammatory cytokines (e.g., IL-6 and IL-1β) were reported to be elevated after spinal cord injury within hours. Recent studies have argued that these cytokines, which increase in serum, are related directly to persistent motor dysfunction and histopathological damage.[31–33]

It was shown that plasma TNF-α, IL-6, and IL-1β levels were significantly increased in the trauma group compared to the control group. The concurrent administration of adalimumab and methylprednisolone created plasma TNF-α, IL-6, and IL-1β levels that were significantly decreased.[34]

It was thought that anti-inflammatory actions would be relevant but there was no change in the levels of IL-1 or IL-6 over time. TNF-α levels increased. In their study, they found that TBI AM treatment significantly increased brain tissue MPO and MDA levels and decreased GSH levels. Hence, they have seen that AM increases these inflammatory markers in TBI at the dose of 12 μg/100 g in rats.[35]

AM has also been demonstrated to inhibit the secretion of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α.[35] Furthermore, it was found that AM provokes endothelial PI3K/Akt activation, promotes vascular regeneration, reducing the increased endothelial permeability that is induced by cytokines, endotoxins, or reactive oxygen species, and thus limiting the formation of inflammatory exudates.[36]

In our study, both tissue and serum TNF-α and IL-6 levels were significantly increased in the trauma group compared to the control group. In the AM-treated group, tissue TNF-α, tissue, and serum IL-6 levels were decreased compared to the trauma group. However, this difference was not significant. In the AM-treated group, serum TNF-α levels were significantly decreased compared to the trauma group.

The results of findings suggested that AM can improve neurologic function, and its neuroprotective or repair effect is exerted through anti-apoptotic and anti-inflammatory effects.

AM is produced by a great number of cell populations (e.g., neurons, glial cells, fibroblast, epithelial cells, adrenocortical cells, and macrophages) as well as several neoplastic cells. AM's vasoactive effects are well-established because it is already known to increase blood flow to the brain, heart, and kidneys, causing hypotension after intravenous administration.[37]

In neurological diseases, the involvement of AM is investigated, and it was shown that AM functions as an inflammatory agent, a neuromodulator, a neurotransmitter, and a neurohormone through autocrine or paracrine mechanisms. AM and its receptors are widely distributed throughout and play an extensive role in the nervous system.[38]

Some progress has also been made regarding AM in the study of proliferation and differentiation of the nervous system. The application of AM for the regulation of regeneration of various types of neuronal cells is a promising therapeutic strategy for the treatment of many neurodegenerative diseases. AM regulates the proportions of different types of neurons, astrocytes, and oligodendrocytes produced from progenitor cells (neural stem cells).[39]

Another important role of AM in cerebral vascular biology is the maintenance of autoregulation. It is ensured through autoregulation that cerebral perfusion is maintained in cerebral blood flow disturbances such as hypovolemia and cerebral ischemia. AM may also contribute to brain repair after damage by stimulating angiogenesis and neurogenesis.[38]

In summary, AM individually exerts neuroprotective effects in animal models of acute brain injury. However, extensive preclinical testing remains to be done to determine mechanisms of action and the appropriate doses. A comprehensive assessment of the effect of AM treatment on the systemic immune response and anti-inflammatory cascade following an acute brain injury will also be useful in establishing the mechanisms involved in tissue protection and the overall disease outcome in TBI.

Conclusion

The results of our study have shown that AM might prevent brain trauma injury and is a biochemically strong anti-inflammatory, antioxidant, and neuroprotective agent. We suggest

that the mechanisms of these effects involve the reduction of oxidative stress, LP, and inflammation, and cytoprotective effect on brain cells. More studies with different dosage strategies and time protocols are required to evaluate the roles of this agent in TBI to consider their potential clinical uses. It is expected that this study will guide future researches on this topic. There is a need for further studies on this subject.

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DENEYSEL ÇALIŞMA - *ÖZ*

Sıçanlarda deneysel travmatik beyin hasarı modelinde adrenomedullin'in nöroprotektif etkileri

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AMAÇ: Travmatik beyin hasarı beyne; hücre ölümü, kan-beyin bariyerinin bozulması ve iskemi dahil olmak üzere çeşitli şekillerde zarar verir. Bu çalışmada, sıçan modellerinde kafa travması sonrası adrenomedüllinin (AM) oksidatif stres ve enflamasyon üzerindeki etkilerini araştırdık.
GEREÇ VE YÖNTEM: On sekiz erkek yetişkin Wistar-Albino sıçan rastgele üç gruba (n=6) ayrıldı. Kontrol (C) grubuna herhangi bir travma uygulanmadı. Travma grubundaki sıçanlara Marmarau travma modeline uygun travma uygulandı. Adrenomedüllin tedavi grubundaki sıçanlara ise travma grubuna ek olarak travma sonrası 12 µg/kg ip adrenomedüllin uygulandı. Tüm gruplardaki sıçanlar yedi gün boyunca takip edildi ve sonrasında sakrifiye edilip beyin dokuları ve kan örnekleri alındı.
BULGULAR: Travma grubunda hem doku hem de serum MDA, TNF-α ve IL-6 seviyeleri kontrol grubuna göre anlamlı olarak arttı ($p<0.05$). AM ile tedavi edilen grupta, serum TNF-α seviyeleri travma grubuna göre anlamlı olarak azaldı ($p<0.05$). Travma grubunda hem doku hem de serum GSH düzeyleri kontrol grubuna göre anlamlı olarak azaldı ($p<0.05$). Travma grubunda serum vit D3 seviyeleri kontrol grubuna göre anlamlı olarak azaldı ($p<0.05$). AM ile tedavi edilen grupta, hem doku hem de serum GSH seviyeleri, travma grubuna göre anlamlı olarak arttı ($p<0.05$).
TARTIŞMA: Bu sonuçlar AM'nin bir sıçan modelinde travmatik beyin hasarı üzerinde nöroprotektif etkilere sahip olduğunu göstermektedir.
Anahtar sözcükler: Adrenomedüllin; antioksidan; enflamasyon; oksidatif stres; travmatik beyin hasarı.

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