Received: 20.03.2012 / **Accepted:** 04.06.2012
DOI: 10.5137/1019-5149.JTN.6197-12.1

The Therapeutic Effects of Melatonin and Nimodipine in Rats after Cerebral Cortical Injury

Ratlarda Beyinde Kortikal Hasar Sonrası Melatonin ve Nimodipinin Etkisi

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Presented in: *21.Turkish Neurosurgery Congress, Aksu, Antalya, 20–24 April 2007.*

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ABSTRACT

AIM: Secondary brain injury starts after the initial traumatic impact and marked by an increase in the intracellular calcium concentrations.This cascadeeventually results in membrane lipid peroxidation and neuronal cell death.

MATERIAL and METHODS: We investigated the neuro-protective effects of nimodipine and melatonin in 38 rats after 6 hours of head trauma using the cortical impact injury model of Marmarou.

RESULTS: Brain water in the melatonin-given group decreased significantly comparing to that of control group the brain water in the nimodipine given group increased significantly comparing to that of trauma group. Histopathologically, brain edema was significantly low in melatonin-administered group comparing to that of control group while there were no changes in brain edema in the nimodipine given group and in the group that both nimodipine and melatonin were administered in combination. MDA levels in the brain tissues were significantly lower in the melatonin and nimodipine groups comparing to those of trauma and control group however this difference was by far significant in melatonin group comparing to nimodipine group.

CONCLUSION: Melatonin appears to have neuro-protective effects on the secondary brain damage while nimodipine and nimodipine plus melatonin combination did not show such neuro-protective effects on the secondary brain injury.

KEYWORDS: Melatonin, Nimodipine, Head trauma, Rat

ÖZ

AMAÇ: Sekonder beyin hasarı ilk travmatik etkiden sonra başlarken uzamış serebral iskemiye bağlı olarak oluşan enerji eksikliği sonucunda kalsiyum kanallarının çalışmamasıyla hücre içinde biriken artmış düzeyde kalsiyumla karakterizedir. Bu durum daha sonra araşidonik asit metabolitleri nöropeptidler ve serbest oksijen radikalleri gibi endojen maddelerin aktive olmasına neden olur ve bu kaskat sonuçta membrane lipid peroksidasyonuna ve nöronal hücre ölümüne yol açar. Malondialdehit (MDA) membran peroksidasyonu sırasında oluşan serbest oksijen radikallerinin endirekt olarak ölçülmesine olanak vermektedir.

YÖNTEM ve GEREÇLER: Çalışmamızda, Marmarou ve arkadaşlarınca önerilen kortikal çarpma travmasının şıcanlarda uygulanması sonrasında travmanın 6.saatinde nimodipin ve melatoninin nöron-koruyucu etkileri araştırıldı.

BULGULAR: 1-Melatonin grubunda beyin su miktarı kontrol grubuna gore belirgin olarak düşük bulundu. 2-Histopatolojik olarak, beyin ödemi melatonin verilen grupta kontrol grubuna gore anlamlı olarak düşüktü;bununla birlikte, nimodipine verilen grupla mimodipine artı melatonin verilen grup arasında beyin ödemi miktarı açısından bir fark saptanmadı. 3-Beyin dokusundaki MDA düzeyleri mimodipin ve melatonin grubunda travma ve control grubuna gore belirgin olarak düşük bulundu.Bununla birlikte, bu fark melatonin grubunda çok daha belirgindi.

SONUÇ: Melatonin sekonder beyin hasarını azaltmada etkiliyken nimodipin ve nimodipine ek melatonin kombinasyonu ise sekonder beyin hasarı üzerinde benzer nöron-koruyucu bir etki göstermemişlerdir.

ANAHTAR SÖZCÜKLER: Melatonin, Nimodipin, Kafa travması, Sıçan

INTRODUCTION

Deaths due to head trauma are currently the third most common cause of the mortalities worldwide. Traumatic brain injury (TBI) occurs through primary and secondary mechanisms. Primary brain injury is generally associated with cerebral contusions, hematomas and diffuse axonal injuries at the time of the traumatic insult (23,30). Secondary brain injury occurs due to the excess release of the excitatory aminoacids and neuromediators aspartate and glutamate, increased intracellular calcium, the activation of arachidonic acid cascade, and eventually the induction of lipid peroxidation via the formation of free oxygen radicals (3,14,19,34). Glutamate and aspartate release increase significantly from the presynaptic membranes following traumatic cerebral ischemia due to the depletion of energy at the cellular level. Glutamate and aspartate in turn stimulates post-synaptic N-methyl-D-Aspartate (NMDA) receptors, resulting in the activation of Gproteins and opening of the receptor-dependent Ca2+. Additionally, G-protein activates phoslipase-C which degrades phosphatidyl inositol diphosphate (PIP2) into inositol triphosphate and diacyl glycerol (DAG)[7]. While PIP2 facilitates the transport of Ca2+ into the cytoplasm from the endoplasmic reticulum, DAG activates protein kinase C (PKC). Another way of intracellular influx of Ca2+ is the opening of voltage-gated Ca2+ channels and increased extracellular K+ due to ion-pump insufficiency[8]. In conclusion, increased intracellular Ca2+ following head trauma is the key event of the whole intracellular cascade which leads to formation of free-oxygen radicals and membrane peroxidation and neuronal cell death. Numerous pharmaceutical agents have been tried to reverse the post-traumatic intracellular cascade, leading to neuronal cell death. Melatonin is a pineal-gland hormone and is found in all animals from the most primitive to the most evolved organisms. It is synthesized from the amino acid tryptophan or is formed as the major metabolic end product of serotonin in the pineal gland. It has strong anti-oxidant and free-radical reducing effects thereby detoxifying reactive oxygen products (8,9,15). Additionally, it also inhibits the pro-oxidative enzyme nitric oxide synthase upon stimulating the glutathione peroxidase, superoxide dismutase, and G-6-P dihydrogenase. Since melatonin is a lipophilic enzyme, it does not need a specific binding site or a receptor on the cell membrane. Nimodipine is a calcium-channel blocker and since it is highly-lipophilic, it can easily penetrate into the central nervous system in considerable amount (30,37). Nimodipine has more influence on voltage-gated Ca2+ channels and these particular channels are more abundant in brain. Consequently, nimodipine appears to have anti-vasospasm and antiischemic effects blocking Ca2+ channels in brain. There are numerous studies in the literature reporting anti-vasospasm and indirect anti-oxidant effects of nimodipine (18,20,23,33). However, to the best of our knowledge, the effects of melatonin and nimodipine on traumatic brain injury were not investigated in combination.

MATERIAL and METHODS

This experimental study was carried out at the Animal laboratory of the Medical Faculty of Hacettepe University, in Ankara after the consent of the related ethnic committee. We used 38 Spraque-Dawley male rats each weighting 250 grams. We choose the impact acceleration model of Marmarou in order to produce diffuse-head trauma. In this model, iron-made balls each weighting 350 grams were allowed 1-meter free-fall through a cylindrical tube with the inner diameter of 19 mm. Steel discs (10mm x 3mm) were placed midline in between the coronal and lambdoid sutures in order to prevent the linear and depressed fractures after trauma. Rats were positioned prone following the administration of anesthetic ketamine (50 mg/kg) intraperitoneally. Melatonin and nimodipine were also administered in solution through intraperitoneal route immediately after the trauma. Rats were categorized into six groups as follows; Group (1); the control group with no trauma, Group (2); the trauma group with no treatment, Group (3); the melatonin group (100 mg/kg of melatonin) after the trauma, Group (4); the nimodipine group (2 mg/kg of nimodipine) after the trauma, Group (5); the group which was administered both melatonin 100 mg/kg and nimodipine 2 mg/kg in combination, Group (6); the control group which was given only 1cc solutions without melatonin and nimodipine itself. All experiment sessions were performed day time between 9 am and 4 pm and rats were decapitized under anesthesia. All solutions in which melatonine and nimodipine were dissolved were prepared fresh and stored in dark environment in order to prevent exposition of light until injection. These solutions were composed of ethyl alcohol 20%, poly ethylene glycol 400 17%, sodium citrate, and citric acid 0.03% in distilled water.

Brain-tissue evaluation

Brain edema: Rats were decapitized, and fresh brains were removed. Brains were weighted at the time of removal and after 24 hours. Brain-water content (BWC) was calculated as below.

BWC % = (wet weight- dry weight) / wet weight X 100

MDA-determination of lipid peroxidation

Brain tissue samples were stored at -20°C. Lipid peroxidation was calculated as nanomole per gram of wet brain-tissue. This calculation was based on the absorptivity of the color generated by the malondialdehyde mixed with thiobarbituric acid.

Light-microscopic examination

Brain tissue samples were fixed in 10% buffered neutral formalin solution for one week. After fixation tissue samples were processed according to routine light microscopic tissue preparation method and were embedded in paraffin. Sections cut in 5 mm were stained with Hematoxylin & Eosin and Luxol fast blue were examined and photographed by Leica DMR-RCM microscope (reflection contrast microscope, Wetzlar-Germany).

RESULTS

Brain water content (BWC) and malonyldialdehyde (MDA) levels:

Brain water content (BWC) of each group was compared using Kruskal-Wallis analysis and the nimodipine and melatonin administered groups were compared to control groups using Mann-Whitney U test (Figure 1).

The differences of BWC % in each group was statistically significant (p=0.01). The BWC % in trauma group increased significantly comparing to that of control (p=0.004). The BWC % in nimodipine group was found higher than that of trauma group (p=0.02) while there was no statistically significant difference in BWC% between the melatonin and the trauma groups (p=0.17). Melatonin had no effect on increasing BWC % unlike nimodipine. Furthermore, there was no significant change in BWC% between the melatonin+nimodipine group comparing to trauma group (p=0.44). When MDA levels were compared, there were significant differences between all groups (p=0.001) (Figure 2).

When MDA levels of other groups were compared to that of the control group, only the trauma group had a significantly elevated MDA levels (p=0.004). MDA values were significantly lower in both nimodipine and only nimodipine solution administered groups comparing to that of trauma group (p=0.01 and p=0.03, respectively). However, there was no significant difference in MDA levels between the nimodipine and the solution group (p=0.35). On the contrary, we found that MDA levels were significantly lower in melatonin group than those of both trauma group and solution groups (p=0.001 and p=0.001, respectively). Even though MDA levels were found lower in melatonin+nimodipine given group than that of only melatonin-given group, this difference was not significant (p=0.15).

Histological findings

Control group; the neurons and glial cell were in normal architecture and the neuropil in between them preserved normal organization. There was no stasis in the vessels (Figure 3A).

Trauma group; neurons and neuropil appeared normal without any obvious edema in the areas distant to trauma while there were edematous foci in the vicinity of the trauma. We observed extensive congestion and stasis in the vessels which was particularly obvious in the vicinity of the trauma. In higher magnification, neuropil appeared blurry due to edema. In addition, we observed that axons and dendrites have lost their organization with disperse edematous areas of different sizes (Figure 3B). Extensive vacuolization of neuronal nuclei and disappearance of nuclear membrane were other prominent findings. Furthermore, edema around the necrotic/apoptotic cells and capillaries was of considerable significance.

Solution group; there were focal areas of edema in neuropil in vacuolar pattern. We observed that neuropil and neurons appeared in normal architecture in the non-edematous areas. However, there was extensive vasodilatation and statis in the blood vessels in the entire brain tissue samples (Figure 3C).

Melatonin group; neurons and neuropil have preserved their normal histological patterns. However, congestion and stasis in the blood vessels were prominent. There were only few focal edematous areas in neuropil (Figure 3D).

Nimodipine group; neuropil appeared blurry as in trauma group, but the edema was more diffuse than melatonin group (Figure 3E). However, there were congestion and stasis in some of the capillaries comparing to trauma and melatonin group.

Melatonin+Nimodipine group; intensive edema was observed in this group. Edema in the neuropil was more diffuse comparing to melatonin group. Peri-cellular edema and cellular degeneration were also more prominent in the areas where neuropil appeared edematous (Figure 3F). Furthermore, while there were significant vasodilatation and stasis in the large-sized arteries, no stasis was present in nimodipine group.

DISCUSSION

In the literature we reviewed, the therapeutic efficacy of nimodipine in TBI is stil debatable (17,18,32,33,38). However, there are experimental studies reporting the potential therapeutic use of melatonin in preventing secondary brain injury in head traumas. In previous studies, melatonin has been shown to be effective as a free radical scavenger and as an antioxidant. However, there are no studies designed to investigate the effects of combination of nimodipine and melatonin on TBI. Therefore, in our study, we also planned to investigate the combinative effects of these two agents to clarify if there is any synergistic effect of nimodipine and melatonin when administered together. In recent years, it was shown that Ca++ channel blockers have decreased ischemic brain injury by inhibiting vascular resistance and increasing regional blood flow. Thus, nimodipine has been in clinical use for the treatment of ischemic cerebrovascular diseases, subarachnoid hemorrhages, and TBI's. However, there is still controversy regarding the usefulness of nimodipine in TBI's. Recent studies have demonstrated that Ca++ channel blockers have increased blood flow through selective influence on small-sized pial arteries (2,27,28).

In our study, we also have shown that there were arteriolar dilatation and loss of stasis histopathologically after the administration of nimodipine. However, we did not observe any neuro-protective effect of nimodipine on brain-tissue. Takayasu et al. (31) did investigate the sensitivity of cerebral arteries and arterioles of rats to nimodipine in vitro. In this study, authors were able to demonstrate that nimodipine had more influence on cerebral arterioles than angiographically visible arteries. The double-blinded follow up study Pickard et al. (24) have shown that there was no difference in arteriolar dilatation of patients with subarachnoid hemorrhages between the nimodipine given group and control group.

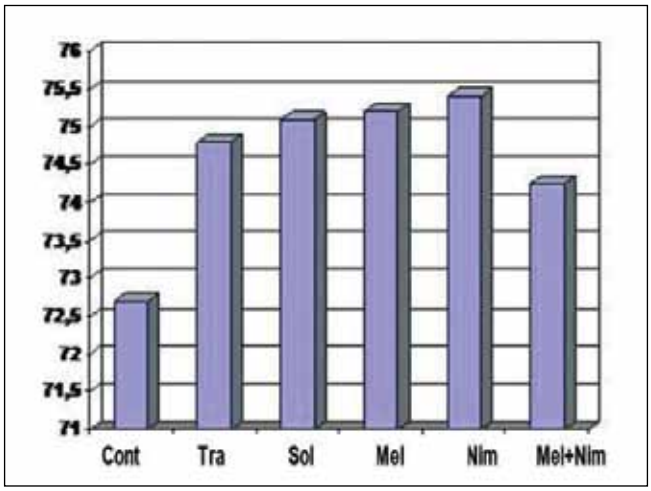

Figure 1: Brain water content (BWC) of each group was compared using Kruskal-Wallis analysis and the nimodipine and melatonin administered groups were compared to control groups using Mann-Whitney U test.

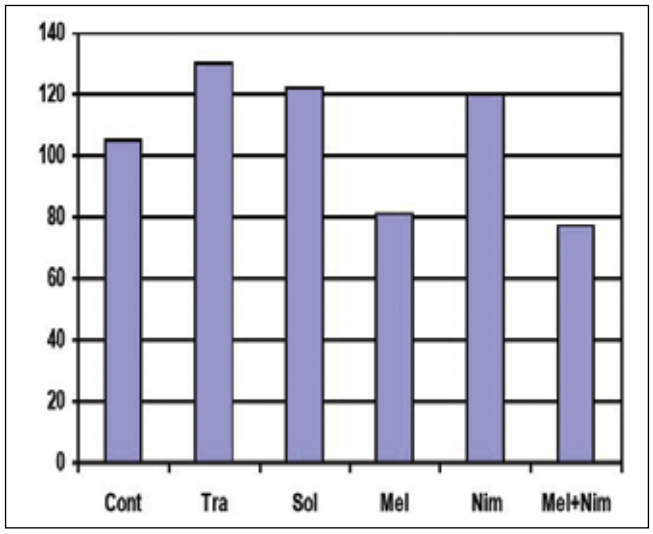

Figure 2: When MDA levels were compared, there were significant differences between all groups (p=0.001).

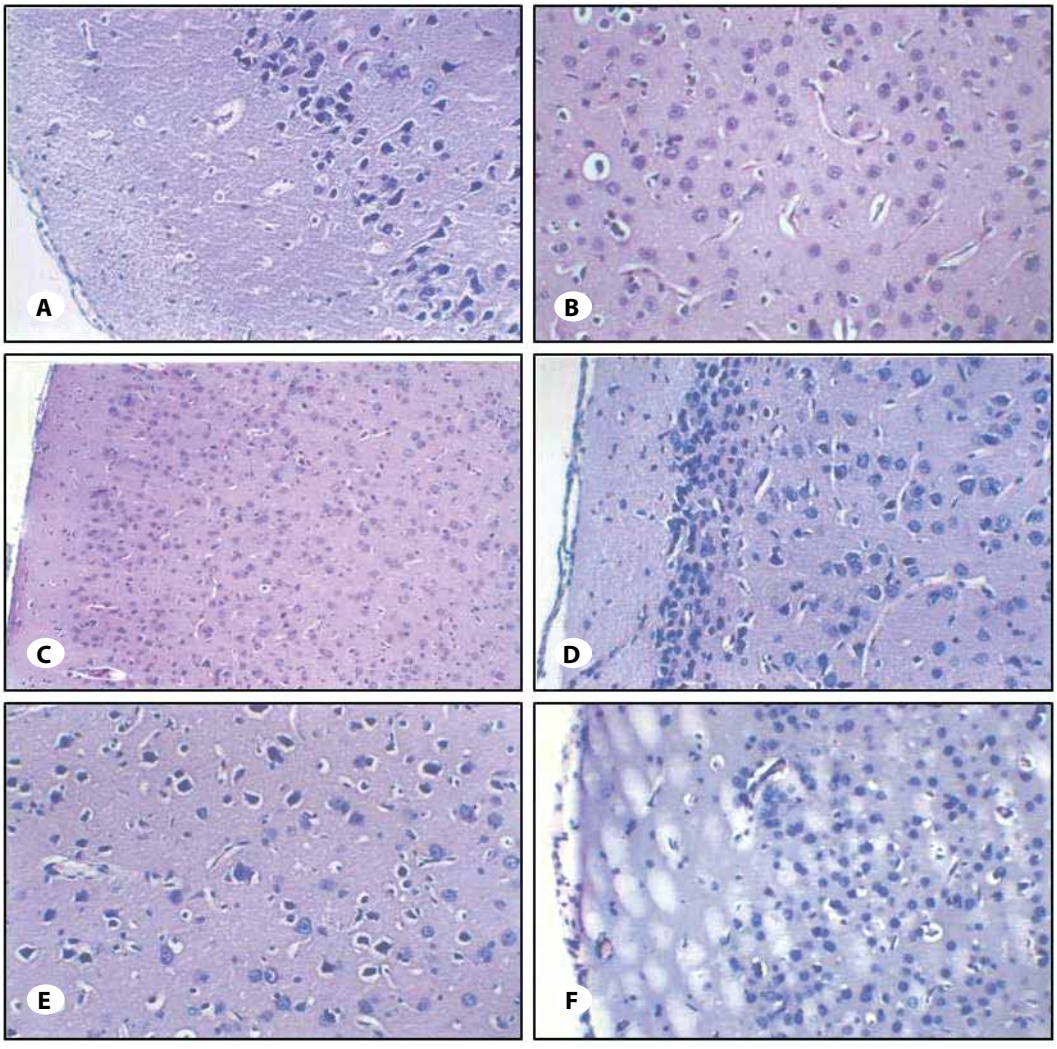

Figure 3: A: Control, **B:** Trauma, **C:** Solution, **D:** Melatonin, **E:** Nimodipine, **F:** Melatonin+Nimodipine group; Healthy neurons and neuropil (**A,** HEx200), edema in the neuropil and degeneration in some neurons; (**B,** HEx200), vasodilatation and stasis in vessels between the normal neurons; (**C,** HEx100), edema in the neuropil and necrotic changes in some neurons; (**D,** HEx200), local edematous areas in the neuropil; (**E,** HEx200), diffuse edema in the neuropil and many necrotic neurons; (**F,** HEx200).

Consequently, authors suggested that nimodipine had no anti-vasospasmic effect but it may have some effect on angiographically occult arterioles. Likewise, in 1990, Gaab et al. (11) demonstrated that there were increase in brain edema, dysfunction of cerebral autoregulation and disruption of blood brain barrier in nimodipine-administered rats. These finding were consistent with those of our study. We also observed that BWC increased significantly after 6 hours following trauma in nimodipine-given group. Furthermore, we found that there was far more extensive brain tissue damage in nimodipine group comparing to melatonin group. Kaynar et al. (16) investigated the effects of nimodipine on neural- tissue lipid peroxidation in rats. In this study, authors created spinal cord injury using clip-compression method and applied single dose of nimodipine of 0.05 µg/kg in the early phase of the spinal cord injury. Authors have concluded that nimodipine had no lowering-effect on MDA levels; thus this finding was consistent with that of our study regarding the effects of nimodipine on MDA levels after head trauma. Likewise, Ercan et al. (10) also investigated the MDA levels in rats following head trauma and they applied single dose of nimodipine of 1.5 µg/kg via carotid arteries or juguler veins. They then calculated MDA levels in traumatic brain tissue 1 hr after the trauma and they found MDA levels were significantly lower in nimodipine applied group in the acute phase of the trauma. On the contrary, Ak et al. (1) found that MDA levels did not decrease after the infusion of nimodipine of 2 µg/kg via jugular vein in 30 min in the same head trauma model. In our study, MDA levels were found lower than that of trauma group 6 hr after trauma but this decrease was far below than that of the group given melatonin. Furthermore, there was increase in BWC and no decrease in brain edema after the trauma in the group given melatonin. Taken together, nimodipine seems to have no neuroprotective effect in the early phases of trauma but it appeared to induce vasodilatation and reversing the stasis on particularly cerebral capillaries. When melatonin and nimodipine were administered in combination, we observed that melatonin improved the worsening effect of BWC of nimodipine comparing to that of trauma group, however this difference was not statistically significant. Yang Shu et al. [20] used the same trauma model of Marmarou et al. (22) that we chose in our study and they infused nimodipine over 24 hours intravenously immediately after the head trauma at the dose of 50 µg/kg. Authors then investigated the BWC, neuronal cytoplasmic free calcium levels, and the histopathological findings at the 0.5, 6, 24, 48, and 72 hr after the trauma. The authors found that neuronal cytoplasmic free calcium levels were significantly decreased in the group treated with nimodipine and there was less spasm in middle cerebral artery as well as less neuronal damage ultrastructurally under the electron microscopic examination. Thus, the authors concluded that nimodipine exerted its anti-vasospasmic effects by blocking numerous Ca2+ channels in brain. In our study, vascular stasis was observed only in large-size arteries while there was no stasis in capillaries. Yang et al. (38) also reported that they observed extensive neuropil edema and capillary vasodilatation that are consistent with our findings. However, we did not observe any healing effect of nimodipine on brain edema unlike Yang et al. (38) did report such an effect of nimodipine in their study. However, our study was restricted by the findings up to 6 hours after the trauma. However, the findings of the study of Pillai et al (26) have revealed that there was no improvement in Glascow coma scale (GCS) of patients with GCS less than 8 and treated with nimodipine through oral or nasogastric route at the dose of 30 mg/6 hr for 3 weeks. Thus, anti-edematous effect of nimodipine suggested by Yang et al. (38) was not supported by the clinical trial of Pillai. Melatonin, a hormonal product of pineal gland is a very potent free radical scavenger and is not receptor-dependent thus it can penetrate all cellular membranes. Additionally, melatonin is non-toxic and both hydrophilic and lipophilic. The role of free oxygen radicals in TBI's was first described by Long et al. (21) Pieri et al. (25) have also shown that melatonin was a much more potent free radical scavenger compared to vitamin E and C. Gonca Akbulut et al. (12) also investigated the role of melatonin on free oxygen radical in rats after head trauma. In this study, melatonin appeared to lower the MDA levels when injected immediately after the trauma. However, interestingly MDA levels were found higher in melatonin group than that of the trauma group when melatonin was injected 2-hours after the trauma. In their experimental study, Sarrafzadeh et al. (29) reported that melatonin decreased contusion volume in cerebral hemispheres of rats when it was injected at night time intra-peritoneally. However, this improvement in contusions was not observed in the group melatonin was administered to during the daytime. The authors also found that brain edema was less in the group which melatonin was administered during daytime; however, there was no difference in BWC between the melatonin group and the trauma group. In our study, we also did not observe any decrease in BWC in melatonin group comparing to trauma group. However, we found that melatonin decreased brain edema and MDA levels significantly comparing to trauma group. Gorgulu et al. (13) also demonstrated that melatonin decreased the infarct area and brain edema in rats when they administered melatonin intraperitoneally 15 min after cold-induced traumatic injury in the brain. In their clinical study, Benloucif et al. (5) have shown that nimodipine augmented the suppression effect of day light on plasma melatonin levels. Interestingly, we demonstrated that when nimodipine and melatonin were administered in combination, brain edema was more diffuse than both those of the groups which nimodipine and melatonin were given separately. In this regard, we thought that the findings of Benloucif et al. (5) support our results in that nimodipine may have increased the suppression effect of day light on the plasma melatonin level thus anti-edematous effect of melatonin was lowered by the counteraction of nimodipine when they are given in combination. However, MDA levels in nimodipine plus melatonin group was not significantly different than that of melatonin group.

Our experimental study demonstrated that melatonin appeared to have anti-edematous and free oxygen radical

lowering effects after cerebral cortical injury in rats. These therapeutic effects were not observed in nimodipine given group. Nimodipine only reversed the stasis and congestion in capillaries but not in large-size arteries. As a novel contribution to the literature, we have found that nimodipine counteracted against the anti-edematous and free oxygen radical lowering effects of melatonin possibly by augmenting the suppression effect of light on plasma melatonin levels as suggested previously in clinical trials. In addition, we need to discuss the limitation of the this study which was restricted by the findings up to 6 hours after the trauma; wherein the secondary effects of head injury begin to predominate. For this reason, there should be more randomised multicentric trials to investigate the role of melatonin and nimodipine in neuroprotection.

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