



ORIGINAL RESEARCH

THE PROTECTIVE EFFECT OF MELATONIN AND AMLODIPINE AGAINST CEREBRAL ISCHEMIA/REPERFUSION-INDUCED OXIDATIVE BRAIN INJURY IN RATS

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ABSTRACT

Objective: In the present study, we investigated the putative protective effect of melatonin and amlodipine against ischemia/reperfusion (I/R)-induced brain damage.

Material and Methods: Wistar albino rats were subjected to 15 min of bilateral carotid artery occlusion followed by 24 h of reperfusion. Melatonin and amlodipine were administered in doses of either 10mg/kg ip or 50µg/rat icv just before reperfusion. After neurological examination the rats were decapitated. In the brain tissue samples, malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) and Na⁺-K⁺-ATPase activities, and luminol lucigenin chemiluminescence (CL) were determined. Brain edema was evaluated by the wet-dry weight method, and blood brain barrier (BBB) permeability was evaluated by the Evans Blue (EB) extravasation.

Results: The neurological deficit was significantly improved in the melatonin and amlodipine groups when compared with the vehicle-treated groups. Ischemia/reperfusion caused a significant decrease in the brain GSH and Na⁺-K⁺-ATPase activity, which was accompanied with significant increases in the MDA level, MPO activity, and CL levels of the brain tissues. On the other hand, both melatonin and amlodipine treatment reversed all these biochemical indices as well as brain water content alterations induced by I/R.

Conclusion: These findings suggest that both melatonin and amlodipine effectively modulate neuro-behavioural and neurochemical changes in global ischemia, most probably by virtue of their antioxidant properties.

Keywords: melatonin; amlodipine; brain; ischemia / reperfusion, lipid peroxidation

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SEREBRAL İSKEMİ/REPERFÜZYONA BAĞLI OKSİDAN BEYİN HASARINA KARŞI MELATONİN VE AMLODİPİNİN KORUYUCU ETKİLERİ

ÖZET

Amaç: Bu çalışmada melatonin ve amlodipinin iskemi/reperfüzyon (İ/R) ile oluşan beyin hasarına karşı olası koruyucu etkileri araştırıldı.

Yöntem: Wistar Albino sıçanlar 15 dakika bilateral carotid arter oklüzyonunu takiben 24 saat süreyle reperfüzyona bırakıldı. Melatonin ve amlodipin 10mg/kg ip veya 50µg/sıçan icv dozlarında reperfüzyondan hemen önce uygulandı. Nörolojik tayin sonrası hayvanlar dekapite edildi. Beyin dokularında malondialdehit (MDA) ve glutatyon (GSH) düzeyleri ile myeloperoksidaz (MPO) ve Na⁺-K⁺-ATPaz aktiviteleri tayin edildi. Dokuda serbest radikal oluşumu luminol ve lusigenin kemiluminesans (KL) yöntemi ile ölçüldü. Beyin ödemi yaş/kuru ağırlık üzerinden, kan beyin bariyer (KBB) geçirgenliği de Evans Mavisini (EM) extravazasyonu ile değerlendirildi.

Bulgular: Çözücü uygulanan grup ile karşılaştırıldığında nörolojik bozuklukların melatonin ve amlodipin gruplarında düzeldiği belirlendi. İskemi/reperfüzyon beyinde GSH ve Na⁺-K⁺-ATPaz aktivitesinde azalma ve bununla birlikte MDA, MPO ve KL düzeylerinde artışa neden oldu. Buna karşılık melatonin ve amlodipin tedavileri tüm incelenen biyokimyasal parametrelerdeki değişimi ve İ/R nin neden olduğu beyin ödemi geri çevirdi.

Sonuç: Bu bulgulara göre gerek melatonin ve gerekse amlodipin muhtemelen antioksidan özellikleri aracılığı ile global iskemiye bağlı olarak meydana gelen nörokimyasal ve davranışsal değişiklikleri module edebileceği düşünülmektedir.

Anahtar Kelimeler: melatonin, amlodipin, beyin, iskemi / reperfüzyon, lipid peroksidasyonu

INTRODUCTION

Ischemic stroke is the third cause of death and the most common cause of neurological disability. The majority of strokes result from embolic or thrombotic obstruction of the cerebral vasculature¹. Ischemic brain damage was regarded simply as the outcome of reducing the oxygen supply of neurons and other cells below the threshold for adequate energy production to allow their survival and early reperfusion has been believed to be beneficial to reduce infarct extension and neurological damage. There is a large body of evidence to indicate that much of the ischemic damage is mediated by active processes, many of which lead to or result from the production of free radicals and other highly reactive oxidizing chemical species. Reperfusion, on the other hand, is more detrimental to the ischemic tissues where leukocytes play an important role in the development of ischemia/ reperfusion (I/R) injury by releasing various chemical mediators such as proteases, reactive oxygen species and lipid-derived mediators¹. Thus free radical ablation for the treatment of reperfusion injury has found its first clinical application in the prevention of postischemic tissue injury and agents proposed to be useful

in the clinical settings of I/R damage include free radical scavengers.

Melatonin, the chief indolamine produced by the pineal gland, has been shown to be an effective antioxidant and free radical scavenger^{2,3}. There is a substantial body of evidence for the protective effect of melatonin and its metabolites against DNA, lipids, and proteins, which are the result of a number of endogenous and exogenous free radical generating processes^{4,5}. In addition, besides directly neutralizing a number of free radicals and reactive oxygen and nitrogen species, melatonin stimulates several antioxidative enzymes (e.g., superoxide dismutase, glutathione peroxidase and glutathione reductase), which increase its efficiency as an antioxidant. The marked protective effects of melatonin against oxidative stress are aided by its ability to cross all biological membranes. That is, melatonin, may reach to its highest concentrations in the nucleus of the cell where it, protects DNA from free radical damage⁶.

Ca²⁺ channel blockers are widely used agents in the treatment of a variety of cardiovascular diseases. They could also be effective pharmacotherapeutic agents for some types of



brain dysfunction by regulating neuronal Ca^{2+} homeostasis. Among Ca^{2+} channel blockers, dihydropyridine (DHP) derivatives play an important part and are usually used for the treatment of hypertension. However, research of the non-hypotension effects of DHP derivative Ca^{2+} channel blockers has attracted more attention in recent years, especially for their effect on neuroprotection. Amlodipine, besides being a Ca^{2+} channel blocker, has also antiinflammatory-antioxidant and antiapoptotic activity^{7,8}. It has been shown that pretreatment of isolated cortical neurons with amlodipine prevented cell death during hypoxia and reoxygenation⁹. Furthermore, chronic administration of amlodipine in spontaneously hypertensive rats has been shown to reduce the oxidative stress in brain¹⁰. In a recent study amlodipine treatment was also found to be effective both in reducing the size of the ischemic lesion and in improving the neurological score in apolipoprotein E-deficient mice¹¹.

Based on these findings, the present study was conducted to demonstrate the beneficial effects of amlodipine given icv or ip against I/R-induced brain tissue damage and also to compare these effects with those of melatonin.

MATERIAL AND METHOD

Surgical procedure and treatment

Male Sprague Dawley rats weighing 250 to 300 g, were fasted for 12 h, but allowed free access to water before the experiments. The animals were kept in individual wire-bottom cages, in a room at a constant temperature ($22 \pm 2^{\circ}C$) with 12-h light and dark cycles, and fed with standard rat chow. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee.

The rats were divided into three sets of groups of 18 each: 1- vehicle-treated sham operated control (C) group, 2- vehicle-treated ischemia-reperfusion (I/R) group 3- melatonin (icv; $50\mu g/rat$)-treated I/R group, 4- melatonin (ip; $10mg/kg$)-treated I/R group, 5- amlodipine (icv; $50\mu g/rat$)-treated I/R group

and 6- amlodipine (ip; $10mg/kg$)-treated I/R group.

Under anesthesia ($100 mg kg^{-1}$ ketamine and $0.75 mg kg^{-1}$ chlorpromazine; intraperitoneally), the bilateral carotid arteries were occluded for 15 min to induce ischemia and then subjected to reperfusion for 24 h. Melatonin and amlodipine were administered in a dose of either $10mg/kg$ ip or $50\mu g/rat$ icv just before reperfusion. 24h after the reperfusion period, neurological examination scores were recorded and the first set of rats were decapitated. Brain tissue samples were taken for the determination of malondialdehyde (MDA) and glutathione (GSH) levels and myeloperoxidase (MPO) and $Na^{+}-K^{+}$ -ATPase activities. The formation of reactive oxygen species in the brain tissue samples was monitored by using the chemiluminescence (CL) technique with luminol and lucigenin probes. In the second set, blood brain barrier (BBB) permeability was studied by the Evans blue (EB) method, and in the third set, brain water content was assessed.

Neurological Examination

The neurological examination scores were conducted according to Bederson's modified neurological examination test^{12,13}. We used a twenty-point neuro-score to assess motor and behavioural deficits. Briefly, the consciousness, performance in a smooth climbing platform, extremity tonus, walking and postural reflexes, circling and response to the nociceptive stimuli were assessed. For walking and posture tests, rats were allowed to move about freely on the floor and were observed. In the circling test, the rats were held gently by the tail, suspended one meter above the floor, and observed for forelimb flexion. Normal rats extend both forelimbs towards the floor. The rotation degree and time was measured. Finally the response to the nociceptive stimuli was assessed by the tail-immersion test in $56^{\circ}C$ water. All behavioural tests were conducted by a 'blinded' investigator. The sequence of testing animals by a given task was randomized for the animals.



MDA and GSH assays

The tissue samples were homogenized with ice-cold 150 mM KCl for the determination of the MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation¹⁴ and the results are expressed as nmol MDA/g tissue. GSH was determined by the spectrophotometric method, based on the use of Ellman's reagent¹⁵ and the results are expressed as μ mol GSH/g tissue.

MPO activity

MPO activity in tissues was measured by a procedure similar to that described by Hillegas et al.¹⁶. Samples of brain tissues were homogenized in 50 mM potassium phosphate buffer (PB), with pH 6.0, and centrifuged at 41,400 g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw-cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance, measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Na⁺-K⁺-ATPase activity

Since the activity of Na⁺-K⁺-ATPase, a membrane-bound enzyme required for cellular transport, is very sensitive to free radical reactions and lipid peroxidation, reductions in this activity can indirectly indicate membrane damage. The measurement of Na⁺-K⁺-ATPase activity is based on the measurement of inorganic phosphate released by ATP hydrolysis during the incubation of homogenates with an appropriate medium containing 3 mM ATP as a substrate. The total ATPase activity was determined in the presence of 100 mM NaCl, 5 mM KCl, 6 mM MgCl₂, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4), while the Mg²⁺-ATPase activity was determined in the presence of 1mM ouabain. The difference between the total and the Mg²⁺-ATPase activities was taken as a measure of the Na⁺-

K⁺-ATPase activity¹⁷. The reaction was initiated with the addition of the homogenate (0.1 ml) and a 5-min preincubation period. at 37° C was allowed. Following the addition of Na₂ATP and a 10- min re-incubation period, the reaction was terminated by the addition of ice-cold 6 % perchloric acid. The mixture was then centrifuged at 3500 g, and Pi in the supernatant fraction was determined by the method of Fiske and Subarrow¹⁷. The specific activity of the enzyme was expressed as nmol Pi mg⁻¹ protein h⁻¹. The protein concentration of the supernatant was measured by the Lowry method¹⁸.

Chemiluminescence (CL) assay

To assess the role of reactive oxygen species in CLP-induced tissue damage, luminol and lucigenin CL were measured as indicators of radical formation. Measurements were made at room temperature using Junior LB 9509 luminometer (EG&G Berthold, Germany). Brain homogenates were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers such as lucigenin or luminol for a final concentration of 0.2 mM. Luminol detects a group of reactive species, i.e. .OH, H₂O₂, HOCl radicals and lucigenin is selective for O₂ . Counts were obtained at 1 min intervals and the results were given as the area under curve for a counting period of 5 minutes. Counts were corrected for wet tissue weight (rlu/mg tissue)^{19,20}.

Evans Blue (EB) assay for blood brain barrier (BBB) permeability

To evaluate the BBB integrity, EB dye was used as a marker of albumin extravasation¹². Briefly, EB dye (2% in saline, 4 ml/kg) was injected via the jugular vein 24h after the induction of reperfusion and it was allowed to remain in circulation for 30 min. At the end of the experiments, the chest was opened and rats were perfused transcardially with 250 ml of saline at a pressure of 110 mm Hg for approximately 15 min until the fluid from the right atrium became colourless. After decapitation, the brain was removed. Then, each brain was weighed for quantitative measurement of EB-albumin extravasation.



The brain samples were homogenized in 2.5 ml phosphate-buffered saline and mixed by vortexing for 2 min after the addition of 2.5 ml of 60% trichloroacetic acid (TCA), to precipitate the protein. The samples were cooled and then centrifuged for 30 min at 1000 g. The supernatant was measured at 620 nm for absorbance of EB using a spectrophotometer (Shimadzu UV1208, Japan). EB was expressed as $\mu\text{g}/\text{mg}$ of brain tissue against a standard curve.

Brain water content

Brain edema was evaluated by the drying-weighing method based on the measurement of the water content of the brain¹². The rats were decapitated at 24h and the whole of the brains were taken. Immediately after being removed, the brain tissue was placed on filter paper for the removal of excess water. The porcelain capsule was dried in the incubator. Then the tare was calculated. Afterwards, the brain tissue was placed in the porcelain capsule and re-weighed. Next, the brain tissue in the porcelain capsule was put in an incubator with a constant temperature and humidity to be dried for 24 h at 100 °C. 24 h later the dried brain in the porcelain capsule was re-weighed. The percentage of water was calculated according to the following formula: $\%H_2O = [(wet\ weight - dry\ weight) / wet\ weight] \times 100$.

Statistical Analysis

Statistical analysis was done using a GraphPad Prism 3.0 (GraphPad Software, San Diego; CA; USA). All data are expressed as means \pm S.E.M. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were considered as significant.

RESULTS

Neurological Examination

The neurological examination scores were significantly higher in the ischemia-reperfusion group showing deterioration of

neurological signs when compared with the sham group. However, melatonin and amlodipine when given icv, improved the neurological signs while ip treatment was effective only with melatonin (Fig. 1).

MDA and GSH assays

The brain MDA, which is an index of tissue lipid peroxidation, was found to be significantly higher in the I/R group than that measured in the sham-operated control group. Treatment with melatonin and amlodipine decreased the elevated MDA level significantly and brought back to control level (Fig. 2a). However, ip amlodipine treatment did not go back to control level.

The endogenous antioxidant, GSH level in the brain tissue, was decreased significantly due to I/R as compared to the levels measured in the sham-operated control group, while both melatonin and amlodipine treatment significantly reversed this GSH reduction (Fig. 2b).

MPO activity

MPO activity, an index for neutrophil infiltration, showed an increase as a result of I/R. All the treatments, except ip amlodipine, significantly reduced MPO activity (Fig. 3a).

Na⁺-K⁺-ATPase activity

The Na⁺-K⁺-ATPase activity was significantly reduced in the I/R group. The pump activity was preserved by the icv treatment of melatonin and amlodipine (Fig. 3b).

Chemiluminescence (CL) assay

The luminol and lucigenin CL significantly increased in the I/R group. All the treatments significantly reduced the luminol CL almost to control levels (Fig. 4a), whereas lucigenin CL was significantly reduced in ip and icv melatonin and also icv amlodipine (Fig. 4b). Although there was a reduction in the lucigenin CL of the ip amlodipine group, it was still significantly higher than the control.

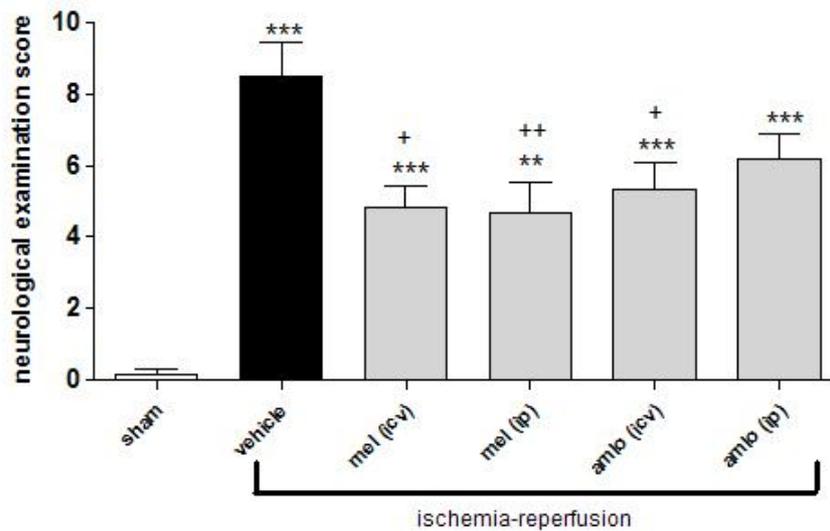


Figure 1: Bederson's modified neurological examination scores at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. ** p<0.01, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated I/R group. Values are represented as mean ± SEM.

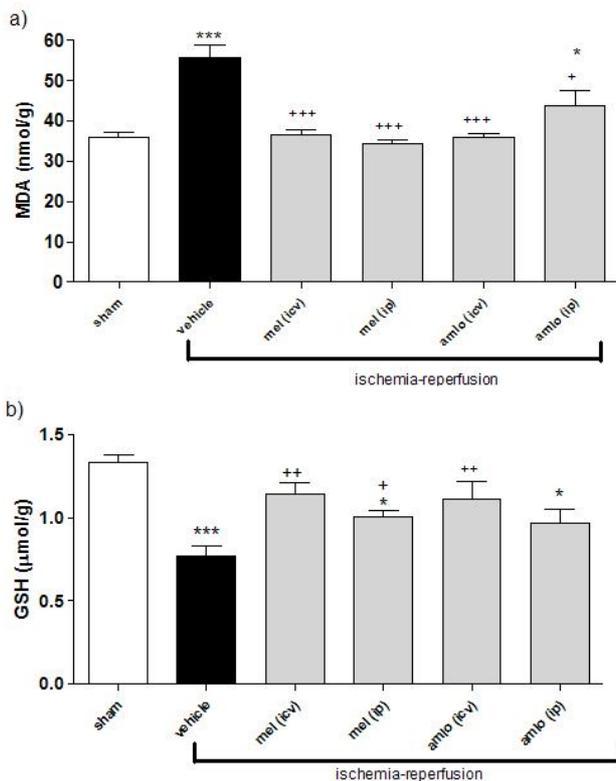


Figure 2: a) Malondialdehyde (MDA) and b) Glutathione (GSH) levels in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, *** p<0.001 vs sham group; + p<0.05, ++ p<0.01, +++ p<0.001 vs vehicle-treated I/R group. Values are represented as mean ± SEM.

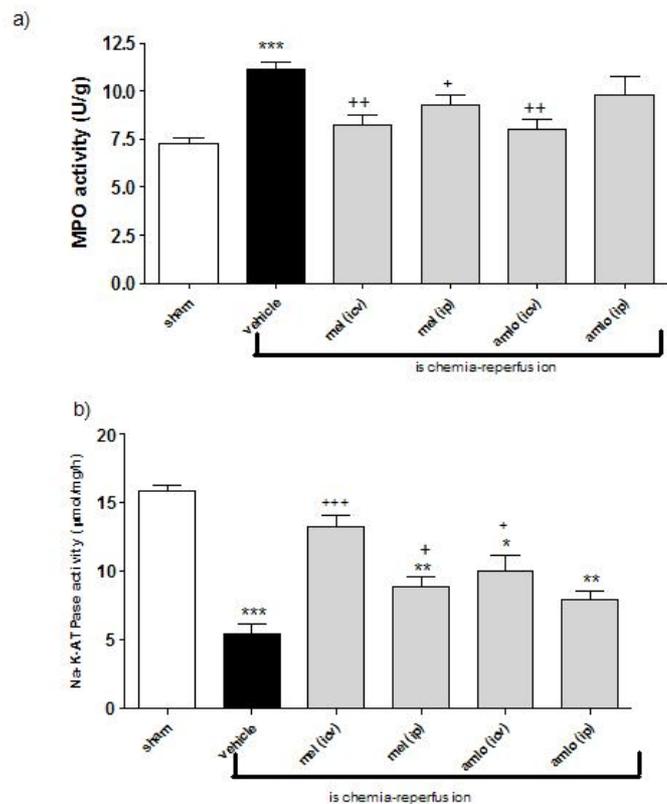


Figure 3: a) Myeloperoxidase (MPO) and b) Na⁺-K⁺-ATPase activities in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, ** p<0.01, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated I/R group. Values are represented as mean ± SEM.



EB assay for BBB permeability

The EB content of the brain significantly increased in the vehicle-treated I/R group, indicating the change in BBB integrity. Only icv melatonin treatment could significantly reverse EB extravasation. The other

treatments failed to protect BBB integrity (Fig. 5a).

Brain water content

Brain edema was significantly higher in vehicle-treated I/R group. The increased brain water content was significantly reversed by all treatments except ip amlodipine (Fig. 5b).

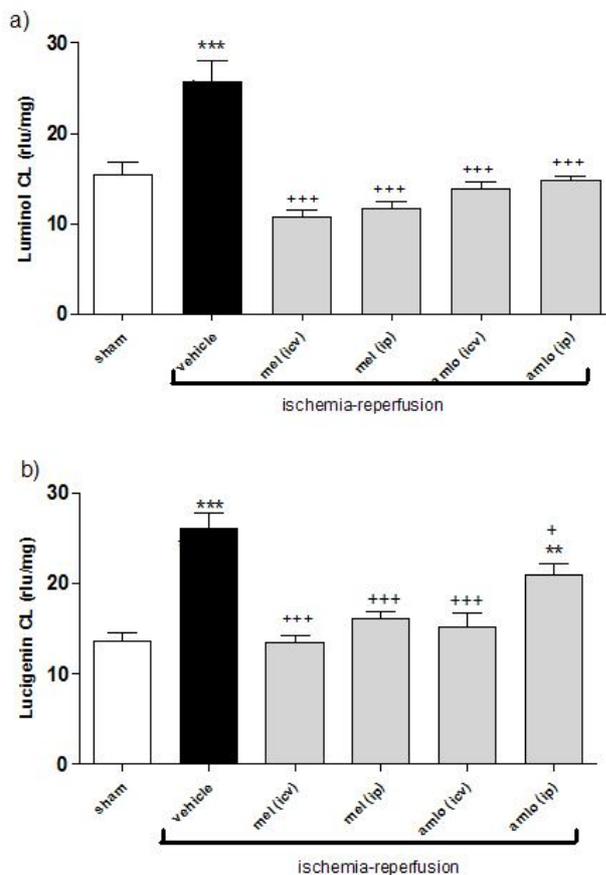


Figure 4: a) Luminol and b) lucigenin chemiluminescence levels in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated I/R group. Values are represented as mean ± SEM.

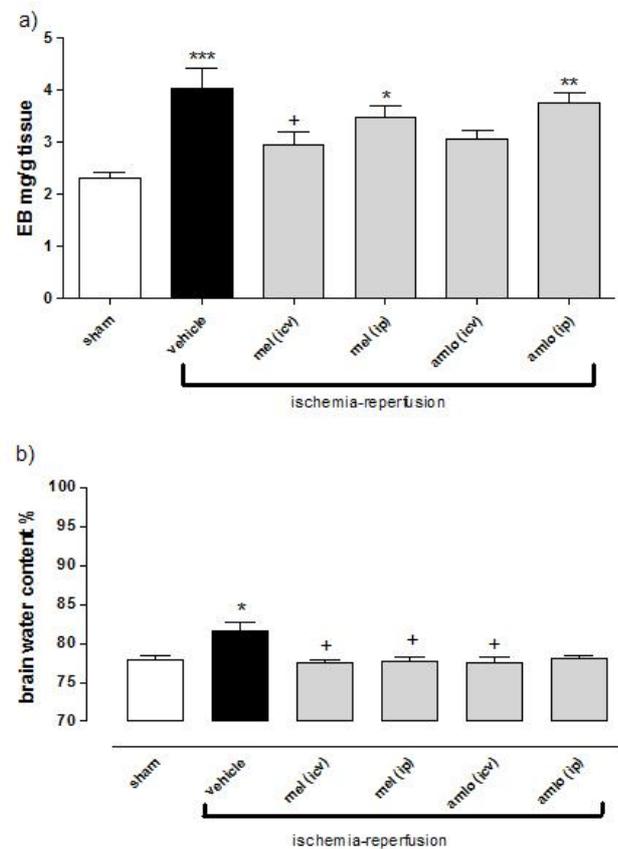


Figure 5: a) Evans blue (EB) and b) water content of the brain tissues of the rats at 24h following the cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, ** p<0.01, *** p<0.001 vs sham group; + p<0.05 vs vehicle-treated I/R group. Values are represented as mean ± SEM. *p<0.05 vs sham; + p<0.05 vs vehicle. Values are represented as mean ± SEM.



DISCUSSION

Acute ischemic stroke is a leading cause of death and long-term disability in adults. Treatment strategies for limiting neuronal injury after a stroke are difficult to develop, primarily because the pathophysiology involved is not yet well understood²¹. Several mechanisms of neuronal injury in strokes have been proposed, including increased excitotoxicity, calcium overload, protein inhibition and the formation of free radicals. Preclinical and clinical studies have demonstrated that free radical scavengers have neuroprotective effects on ischemic stroke. The results of the present study demonstrate that amlodipine, like melatonin, has protective effects on I/R-induced brain injury. The increased neurological scores indicating impaired neurological symptoms are reversed by this treatment. As the oxidative injury on cellular structures is reduced by both treatments, the intracellular antioxidant glutathione, which is otherwise oxidized during the process of inactivating free radicals, is not changed. Moreover I/R-induced elevation in MPO activity and luminol and lucigenin CL values are inhibited by these treatments, indicating that the neuroprotective effects of melatonin and amlodipine are neutrophil-dependent and also due to their antioxidant action.

Experimental and clinical studies have shown that any harmful tissue event (infection, trauma, hypoxia) is perceived by macrophages and monocytes, which in turn secrete cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF- α). These cytokines activate inflammatory cells (neutrophils, macrophages/monocytes, platelets, mastocytes) which release large amounts of toxic oxidizing substances, namely reactive oxygen species (ROS), and cause cellular injury via several mechanisms including peroxidation of membrane lipids, as well as oxidative damage of proteins and DNA⁶. It is difficult to quantitate oxygen radicals because of their reactive nature and short lives; however, luminol- and lucigenin-enhanced CL is a simple and reproducible

tool indicating the involvement of free radicals. The two CL probes, luminol and lucigenin, differ in selectivity. Luminol detects H₂O₂, OH⁻, hypochlorite, peroxy nitrite, and lipid peroxy radicals, whereas lucigenin is particularly sensitive to superoxide radical²⁰. In the present study, increases in luminol- and lucigenin- CL levels support the notion that brain injury induced by I/R involves toxic oxygen metabolites and melatonin treatment decreases these elevations by its antioxidant action. Furthermore amlodipine treatment also decreased the CL, indicating that besides calcium antagonism, amlodipine also has an antioxidant effect. In accordance with the increases in ROS, the brain MDA level was significantly increased, indicating the presence of enhanced lipid peroxidation due to I/R injury, while the levels of tissue glutathione declined, demonstrating the depletion of the antioxidant pool. Several studies have demonstrated that I/R in the brain is associated with lipid peroxidation, which is an autocatalytic mechanism leading to the oxidative destruction of cellular membranes^{22,23}. Glutathione, on the other hand, is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress²⁴. Because of their exposed sulfhydryl groups, non-protein sulphhydryls bind a variety of electrophilic radicals and metabolites that may be damaging to cells. It has been proposed that antioxidants, which maintain the concentration of reduced GSH, may restore the cellular defense mechanisms, block lipid peroxidation and thus protect against the oxidative tissue damage^{25,26}. In the present study, melatonin as well as amlodipine when given icv or ip, decreased the MDA levels and restored the I/R-induced depletion of GSH levels, indicating that neuroprotective effects are due to their antioxidant properties. It has been demonstrated that amlodipine besides its Ca²⁺ antagonistic activity also has an anti-inflammatory and antioxidant effect⁷. In the study of Yamagata et al.⁹, pretreatment of



isolated cortical neurons with amlodipine prevented cell death during hypoxia and reoxygenation. Furthermore, chronic administration of amlodipine in spontaneously hypertensive rats has been shown to reduce the oxidative stress in brain¹⁰.

The neutrophils seem to act as an amplifier of the initial reperfusion reaction and are considered as an important factor in the damaging cascade that takes place upon reperfusion. It is well documented that I/R elicits an acute inflammatory response characterized by the activation of neutrophils²⁷. Since activation of neutrophils might lead to the generation of reactive oxygen metabolites, prevention of neutrophil accumulation might also result in reduced lipid peroxidation. In the present study, elevated levels of MPO activity, which is an index of tissue neutrophil infiltration suggest that I/R induced brain damage is neutrophil-dependent while melatonin and amlodipine significantly reduced the I/R induced augmentation in tissue MPO activity, which resulted in decreased lipid peroxidation and MDA levels as given above.

Na⁺-K⁺-ATPase, a maintainer of electrolyte and fluid balance in cells, organs and whole body, is an integral membrane protein that couples the hydrolysis of ATP to the vectorial transport of Na ions K ions across the plasma membranes. A number of events, like decrease in ATP levels, increase in intracellular Ca²⁺, excitotoxicity, altered arachidonic acid release and metabolism, mitochondrial dysfunction, acidosis, and edema, predispose the brain to the formation of free radicals during cerebral ischemia²⁸. After reperfusion, these events can set off a cascade of other biochemical and molecular sequela such as the xanthine-xanthine oxidase reaction and phospholipase activation, leading to the overproduction of free radicals²⁹. Since this membrane-bound enzyme requires phospholipids for maintenance of its activity and is susceptible to structural changes due to lipid peroxidation³⁰, assessment of the Na⁺-K⁺-ATPase activity is also used as an index for oxidant-induced tissue injury and lipid

peroxidation³¹. Our results showed that I/R caused a significant inhibition on the enzyme activity which is accompanied with membrane damage due to increased lipid peroxidation. In agreement with our findings, Mrsic et al³² have previously shown that global cerebral ischemia induced a statistically significant decrease of the hippocampal Na⁺-K⁺-ATPase activity starting from 1 to 168 h of reperfusion. Furthermore, maximal enzymatic inhibition was obtained 24 h after the ischemic damage. As an antioxidant, melatonin and also calcium antagonist amlodipine decreased the peroxidation of membrane lipids, Na⁺-K⁺-ATPase activity was found to be restored.

Cerebral ischemia causes cell swelling and breakdown of the blood-brain barrier. Generation of ROS contributes to endothelial and cellular dysfunction, resulting in increased BBB permeability and life-threatening cerebral edema^{33,34}. In the case of ischemia, irreversible tissue damage and BBB breakdown leads to the extravasation of serum proteins and vasogenic brain edema development. Thus ischemic brain edema is initially cytotoxic and later vasogenic in nature³⁴. Cerebral ischaemia and post-ischaemic reperfusion cause cerebral capillary dysfunction, resulting in edema formation and haemorrhagic conversion. Cytotoxic edema results from energy failure, and vasogenic edema occurs when the blood vessels are damaged. Proteases and free radicals are the end result of a molecular injury cascade. The tissue EB content, which is used for the evaluation of altered BBB permeability, is accepted by some researchers as a parameter that indicates the intensity of the injury³⁵. We have found that cerebral I/R caused a significant increase in the BBB permeability at 24h after the reperfusion. Although, all of the treatments seem to decrease the altered BBB permeability, only icv administration of melatonin caused a statistically significant reduction. As mentioned before, the BBB breakdown permits extravasation of plasma-like fluid into the extracellular space. Besides these, the failure of the Na/K pumps, and the altered electrolyte balance of the cell, may



also contribute to brain edema, and pathological changes in the cellular function^{33,34}. In the present study, brain edema was significantly higher in the vehicle-treated I/R group parallel to the inhibition of Na⁺-K⁺-ATPase activity. The increased brain water content was significantly reversed by all treatments except ip amlodipine treatment. Since brain edema causes an increase in intracranial pressure and leads to neurological deficits, we have assessed neurological examination scores at 24h. The neurological examination scores which were significantly higher in the ischemia-reperfusion groups, indicate that I/R was the cause when compared with the sham group. However, melatonin and amlodipine when given icv, ameliorated the neurological signs while ip treatment was effective only with melatonin.

In the previous reports, accumulating data suggested that Ca⁺ channel antagonists may show neuroprotection via beneficial effects in endothelial function and reduction in ischemic lesion size in addition to the neurological improvements¹¹. However, in this study, ip administration of amlodipine was ineffective while icv administration was more efficient. This may probably be due to the chemical characteristics of amlodipine. Amlodipine has an amino 3-oxygen group on the dihydropyridine ring. This is an alkaline group and presents itself in ionization state under physiological conditions; this special group of amlodipine can engender electrostatic interaction between amlodipine and membrane phospholipids³⁶. High lipid solubility and the chemical structure of amlodipine has been suggested to inhibit membrane lipid-peroxidative damage. Furthermore, it has been reported that this effect of amlodipine does not depend on modulation of the calcium channels³⁶. Furthermore, Uchida et al.³⁷ reported that [3H]-amlodipine showed high affinity specific binding in the mouse brain in vitro, and its binding parameters were comparable to those of [3H]-nimodipine. However, the failure of amlodipine to produce CNS effects in vivo is considered to be mainly ascribed to

poor transport through the blood brain barrier³⁷.

On the otherhand, melatonin readily penetrates the blood-brain barrier and diffuses into neurons and glia equally³⁸, it was considered worthwhile to investigate whether melatonin affords protection against ischemic reperfusion injury in rats. Moreover, in the present study, the effects of systemic versus local amlodipine were evaluated in regard to a well-known potent antioxidant, melatonin.

In conclusion, the present study demonstrates that melatonin (icv, ip) and amlodipine (icv) neuroprotective effects against cerebral ischemia-reperfusion injury, which may be attributed to their activities on preserving the oxidant-antioxidant status and electrolyte balance, improve the neurological scores.

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