**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 myeloperoxidaz (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 Mavisi (EM) extravazasyonu ile değerlendirilidi.

**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 ödemini 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 değişikleri 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 vasculature[1]. 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 mediators[1]. 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 damage[6].

$Ca^{2+}$ 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, dihydropiridine (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 reoxygenation[9]. Futhermore, chronic administration of amlodipine in spontaneously hypertensive rats has been shown to reduce the oxidative stress in brain[10]. 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 mice[11].

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 ± 2ºC) 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; 50µg/rat)-treated I/R group, 4- melatonin (ip; 10mg/kg)-treated I/R group, 5- amlodipine (icv; 50µg/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 50µg/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 56ºC 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 peroxidation[14] and the results are expressed as nmol MDA/g tissue. GSH was determined by the spectrophotometric method, based on the use of Ellman's reagent[15] and the results are expressed as µmol 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 % hexadecyl-trimethylammonium 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 $H_2O_2$ 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 hydolysis 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 $MgCl_2$, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4), while the $Mg^{2+}$-ATPase activity was determined in the presence of 1mM ouabain. The difference between the total and the $Mg^{2+}$-ATPase activities was taken as a measure of the $Na^+$-$K^+$-ATPase activity[17]. The reaction was initiated with the addition of the homogenate (0.1 ml) and a 5-min preincubation period. at 37º C was allowed. Following the addition of $Na_2ATP$ 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 Subarrow[17]. 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 method[18].

**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, $H_2O_2$, HOCl radicals and lucigenin is selective for $O_{-2}$ . 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 extravasation[12]. 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 μg/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 brain[12]. 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 ºC. 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]×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 ± 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 amlodipin 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.

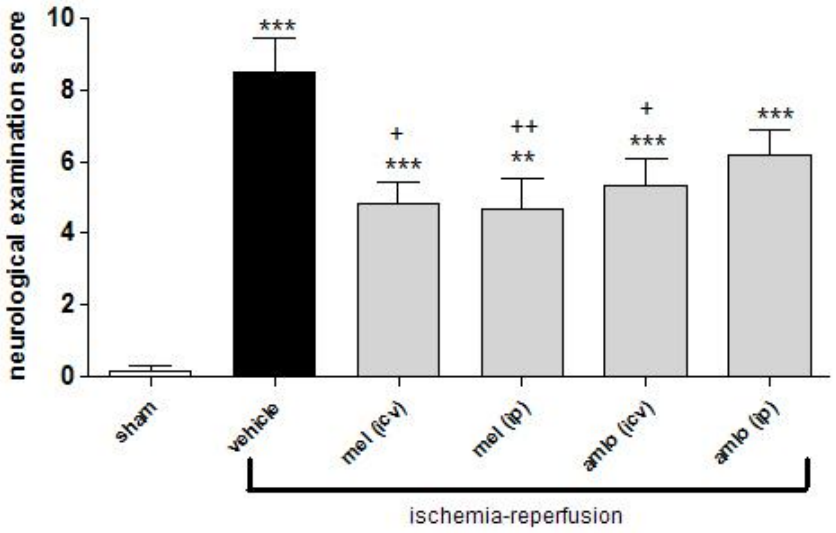


**Figure 1:** Bederson's modified neurological examination scores at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. ** p<0.01, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated I/R group. Values are represented as mean ± SEM.

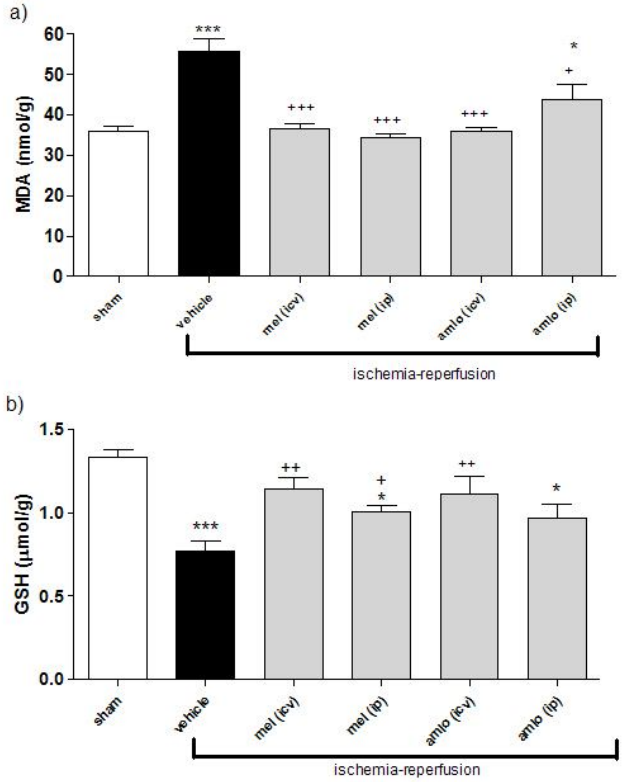


**Figure 2:** a) Malondialdehyde (MDA) and b) Glutathione (GSH) levels in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, *** p<0.001 vs sham group; + p<0.05, ++ p<0.01, +++ p<0.001 vs vehicle-treated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

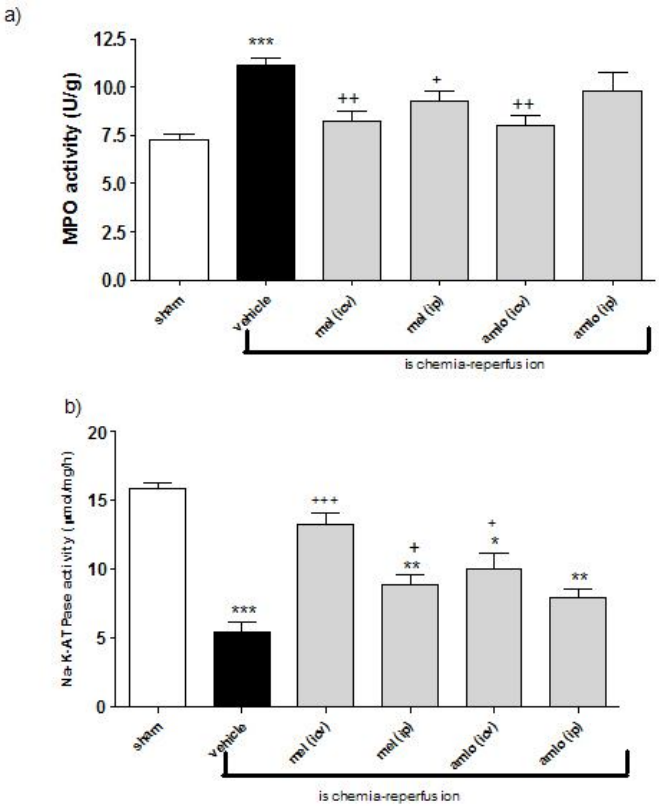


**Figure 3:** a) Myeloperoxidase (MPO) and b) Na+-K+-ATPase activities in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, ** p<0.01, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated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

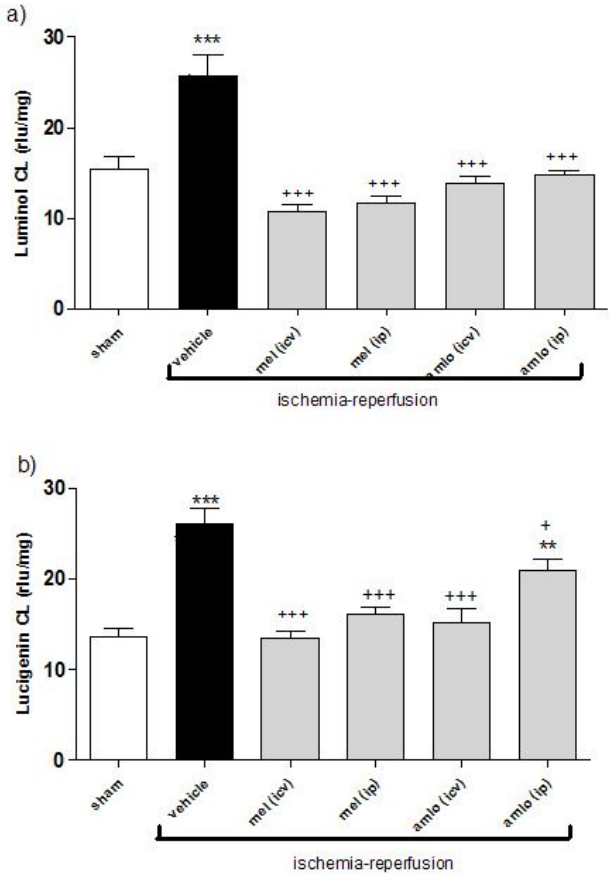


**Figure 4:** a) Luminol and b) lucigenin chemiluminescence levels in the brain tissue of vehicle-, melatonin- or amlodipine-treated I/R groups at 24h following cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, *** p<0.001 vs sham group; + p<0.05, +++ p<0.001 vs vehicle-treated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

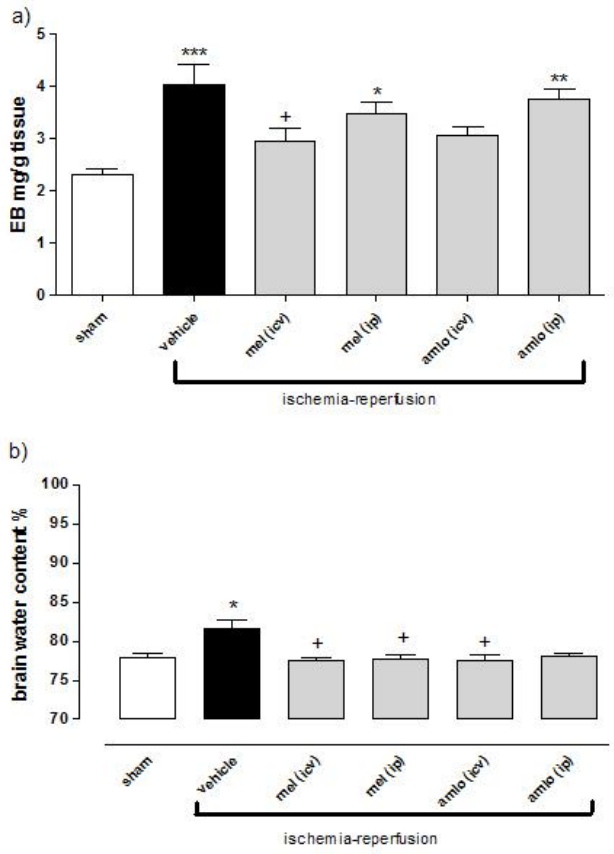


**Figure 5:** a) Evans blue (EB) and b) water content of the brain tissues of the rats at 24h following the cerebral ischemia-reperfusion. Each group consists of 6 rats. * p<0.05, ** p<0.01, *** p<0.001 vs sham group; + p<0.05 vs vehicle-treated I/R group. Values are represented as mean ± SEM.. *p<0.05 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 understood[21]. 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 DNA[6]. 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 $H_2O_2$, OH-, hypochlorite, peroxynitrite, and lipid peroxyl radicals, whereas lucigenin is particularly sensitive to superoxide radical[20]. 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 stress[24]. Because of their exposed sulfhydryl groups, non-protein sulphydryls 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 $Ca^{2+}$ 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. Futhermore, chronic administration of amlodipine in spontaneously hypertensive rats has been shown to reduce the oxidative stress in brain[10].

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 neutrophils[27]. 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+, exitotoxicity, altered arachidonic acid release and metabolism, mitochondrial dysfunction, acidosis, and edema, predispose the brain to the formation of free radicals during cerebral ischemia[28]. 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 radicals[29]. Since this membrane-bound enzyme requires phospholipids for maintenance of its activity and is susceptible to structural changes due to lipid peroxidation[30,] assessment of the $Na^+$-$K^+$-ATPase activity is also used as an index for oxidant-induced tissue injury and lipid peroxidation[31]. 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 al[32] 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 nature[34]. 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 injury[35]. 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 improvements[11]. 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 phospholipids[36]. 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 channels[36]. 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 barrier[37].

On the otherhand, melatonin readily penetrates the blood–brain barrier and diffuses into neurons and glia equally[38,] 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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## REFERENCES

1. Matsuo Y, Onodera H, Shiga Y, et al. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. Brain Res 1994;656:344-352.
2. Tan DX, Manchester LC, Reiter RJ, et al. Significance of melatonin in antioxidative defense system: reactions and products. Biol Signals Recept 2000;9:137-159.
3. Chauvin G, Vannier G, Gilad E, et al. Melatonin is a scavenger of peroxynitrite. Life Sci 1997;60:169-174.
4. Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007;42:28-42.
5. Manda K, Ueno M, Anzai K. AFMK, a melatonin metabolite, attenuates X-ray-induced oxidative damage to DNA, proteins and lipids in mice. J Pineal Res 2007;42:386-393.
6. Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. A review of the evidence. Cell Biochem Biophys 2001;34:237-256.
7. Yoshii T, Iwai M, Li Z, et al. Regression of atherosclerosis by amlodipine via anti-inflammatory and anti-oxidative stress actions. Hypertens Res 2006;29:457-466.

8. Liu LL, Li QX, Xia L, Li J, Shao L. Differential effects of dihydropyridine calcium antagonists on doxorubicin-induced nephrotoxicity in rats. Toxicology 2007;231:81-90.
9. Yamagata K, Ichinose S, Tagami M. Amlodipine and carvedilol prevent cytotoxicity in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. Hypertens Res 2004;27:271-282.
10. Hirooka Y, Kimura Y, Nozoe M, et al. Amlodipine-induced reduction of oxidative stress in the brain is associated with sympatho-inhibitory effects in stroke-prone spontaneously hypertensive rats. Hypertens Res 2006;29:49-56.
11. Mogi M, Iwai M, Chen R, et al. Amlodipine treatment reduces stroke size in apolipoprotein E-deficient mice. Am J Hypertens 2006;19:1144-1149.
12. Toklu HZ, Keyer Uysal M, Kabasakal L, et al. The effects of riluzole on neurological, biochemical and histological changes in early and late term of sepsis in rats. J Surg Res 2008; Doi:10.1016/j.jss.2008.03.013.
13. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL. Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurological examination. Stroke 1986;17:472-476.
14. Beuge JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–311.
15. Beutler E. Glutathione in red blood cell metabolism. A Manuel of Biochemical Methods, Grune and Stratton, New York, NY: 1975:112–114.
16. Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990; 24: 285-295.
17. Reading HW, Isbir T. The role of cation activated ATPase in transmitter release from the art iris. Q J Exp Physiol Cogn Med Sci 1980;65:105-116.
18. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. J Biol Chem 1951;193:265-75.
19. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;92:2546-2551.
20. Davies GR, Simmonds NJ, Stevens TR, et al. Helicobacter pylori stimulates antral mucosal reactive oxygen metabolite production in vivo. Gut 1994;35:179–185.
21. Read JS, Hirano T, Davis SM, Donnan GA. Limiting neurological damage after stroke. Drugs Aging 1999;14:11–39.
22. Candelario-Jalil E, Alvarez D, Merino N, León OS. Delayed treatment with nimesulide reduces measures of oxidative stress following global ischemic brain injury in gerbils. Neurosci Res 2003;47:245-253.
23. Ozkul A, Akyol A, Yenisey C, et al. Oxidative stress in acute ischemic stroke. J Clin Neurosci 2007;14:1062-1066.
24. Ross D. Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther 1988;37:231-249.
25. Gilgun-Sherki Y, Melamed E, Offen D. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacol 2001;40:959-975.
26. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 2004;134:489-492.
27. Hendriks JJ, Teunissen CE, de Vries HE, Dijkstra CD. Macrophages and neurodegeneration. Brain Res Rev 2005;48:185-195.
28. Homi HM, Freitas JJ, Curi R, Velasco IT, Junior BA. Changes in superoxide dismutase and catalase activities of rat brain regions during early global transient ischemia/reperfusion. Neurosci Lett 2002;333:37-40.
29. Kim GW, Kondo T, Noshita N, Chan PH. Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral ischemia/reperfusion in mice: implications for the production and role of superoxide radicals. Stroke 2002;33:809-815.
30. Koner BC, Banerjee BD, Ray A. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Ind J Exp Biol 1998;36:395-398.
31. Tuna M, Polat S, Erman T, et al. Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5\'-triphosphatase and superoxide dismutase activation, and ultrastructural changes. J Neurosurg 2001;95:64-73.
32. Mrsic-Pelcic J, Pelcic G, Vitezic D, et al. Hyperbaric oxygen treatment: the influence on the hippocampal superoxide dismutase and Na+,K+-ATPase activities in global cerebral ischemia-exposed rats. Neurochem Int 2004;44:585-594.
33. Kempski O. Cerebral edema. Semin Nephrol 2001; 21: 303-307.
34. Unterberg AW, Stover J, Kress B, Kiening KL. Edema and brain trauma. Neuroscience 2004;129:1021-1029.
35. Kaptanoglu E, Okutan O, Akbiyik F, et al. Correlation of injury severity and tissue Evans blue content, lipid peroxidation and clinical evaluation in acute spinal cord injury in rats. J Clin Neurosci 2004;11:879-885.
36. Mason RP, Walter MF, Trumbore MW, Olmstead Jr. EG, Mason PE. Membrane antioxidant effects of the charged dihydropyridine calcium antagonist amlodipine. J Mol Cell Cardiol 1999;31:275–281.
37. Uchida S, Yamada S, Nagai K, Deguchi Y, Kimura R. Brain pharmacokinetics and in vivo receptor binding of 1,4-dihydropyridine calcium channel antagonists. Life Sci 1997;61:2083-2090.
38. Reiter RJ. Oxidative damage in the central nervous system: protection by melatonin. Prog Neurobiol 1998;56:359–384.