THE EFFECTS OF MELATONIN AND MEMANTINE ON BRAIN INJURY AFTER STROKE

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“this thesis is dedicated to
Serhat Sevli and
İsmail Sayın”

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ABSTRACT

THE EFFECTS OF MELATONIN AND MEMANTINE ON BRAIN INJURY AFTER STROKE

Melatonin and memantine are clinically used agents that have neuroprotective effects against ischemic injury via antioxidative mechanisms and preventing excitotoxicity, respectively. In this study, a combination therapy including both melatonin and memantine was used upon 90 minutes of transient focal cerebral ischemia and 24 hours reperfusion in mice. Twenty-four hours after cerebral ischemia, neurological deficit, infarct volume and brain swelling were evaluated. Melatonin, memantine and melatonin/memantine combination reduced the infarct volume significantly compared to vehicle treated control group. In addition, melatonin and melatonin/memantine combination treatments decreased brain swelling upon ischemia significantly. Neurological deficit scores were significantly lower than the control group only in melatonin treated group. These results indicate that melatonin/memantine combination is a relevant and useful treatment option against stroke.

ÖZET

MELATONİN VE MEMANTİNİN BEYİN FELCİ SONRASI OLUŞAN BEYİN HASARINA OLAN ETKİLERİ

Melatonin ve memantin klinik olarak kullanılan, sırasıyla antioksidatif işleyişler ve eksitotoksisiteyi engelleme yollarıyla serebral iskemiye karşı nöroprotektif etkileri olan ajanlardır. Bu çalışmada melatonin ve memantin içeren bir kombinasyon terapisi farelerde 90 dakikalık geçici bölgesel serebral iskemi sonrası kullanıldı. 24 saat sonra, nörolojik hasar puanları, infarkt hacmi ve beyin ödemi değerleri hesaplandı. Taşıyıcı uygulanan kontrol grubuna kıyasla melatonin, memantin ve mealtonin/memantin kombinasyonu infarkt hacmini anlamlı olarak azalttılar. Ayrıca melatonin ve melatonin/memantine grupları beyin felci sonrası beyin ödemini anlamlı şekilde azalttılar. Nörolojik hasar puanları yalnızca melatonin uygulanan grupta kontrol grubundan anlamlı olarak daha azdı. Bu sonuçlar melatonin/memantin kombinasyonunun beyin felcine karşı ilgili ve faydalı bir tedavi seçeneği olduğunu göstermektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

AFMK	N^1-acetyl-N^2-formyl-5-methoxykynuramine
AMK	N^1-acetyl-5-methoxykynuramine
BBB	Blood-Brain Barrier
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central Nervous System
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
eNOS	Endothelial nitric oxide synthase
I/R	Ischemia/reperfusion
iNOS	Induced nitric oxide synthase
K_D	Dissociation constant
LDF	Laser Doppler flowmetry
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MT_1	Melatonin receptor 1
MT_2	Melatonin receptor 2
mtDNA	Mitochondrial DNA
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
OGD	Oxygen-glucose deprivation
OGSD	Oxygen-glucose-serum deprivation
PARP	Poly-adenosine diphosphate-ribose polymerase
rCBF	Regional cerebral blood flow
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCN	Suprachiasmatic nucleus
SOD	Superoxide dismutase
t-PA	Tissue plasminogen activator

1. INTRODUCTION

Although the scientific adventure of pineal gland and melatonin has started in 1960s, its philosophical/spiritual adventure has started long ago in 17th century when René Descartes called the pineal gland the “seat of the soul”, a place where our thoughts are formed. Although melatonin is originally isolated as a hormone related to the circadian rhythm of the body, with the accumulation of experimental evidences about the beneficial effects of melatonin on various diseases and/or conditions, melatonin is started to be considered as a miraculous molecule. In 1990s, together with the high public interest, due to its great therapeutic potential melatonin has become the “holy-grail” of medicine, which will cure all diseases, even aging [1]! While melatonin treatments were not able to satisfy all the high expectations clinically, melatonin still holds promising therapeutic potentials, especially in central nervous system (CNS) diseases, such as cerebral ischemia, including stroke.

Cerebral ischemia is a medical condition results from the impairment of the blood flow to the brain. Since melatonin is primarily produced by the pineal gland inside the brain and melatonin is a powerful antioxidant agent, it is reasonable to hypothesize that melatonin treatment will have beneficial effects on stroke patients. Various *in vitro* and *in vivo* studies have shown neuroprotective effects of various sorts of melatonin treatments on *in vitro* model systems and/or animal models of cerebral ischemia [2-4].

Memantine is a derivative of anti-influenza agent adamantane and it is originally synthesized to be a drug to decrease blood sugar levels, which is unfortunately failed to have such activity. Fortunately, it is discovered to be a glutamate receptor antagonist and now it is a clinically approved drug. Memantine has been shown to decrease the damage caused by over-activation of glutamate receptors in various types of acute and chronic neurological diseases including stroke [5, 6].

In this study, protective effects of post-ischemic melatonin and memantine treatments, alone or combined, are examined in a mouse model of transient focal cerebral ischemia.

2. THEORETICAL BACKGROUND

In this chapter, general information on melatonin hormone, melatonin receptors and interacting proteins, antioxidative properties of melatonin, memantine, cerebral ischemia (stroke), melatonin and memantine treatment in cerebral ischemia is explained and presented briefly.

2.1. MELATONIN AS A DARK-SIGNALING HORMONE

Melatonin is a naturally occurring methoxyindole with the chemical name as *N*-acetyl-5-methoxytryptamine which is first isolated and characterized by Aaron Lerner in 1958 from bovine pineal gland [7]. From an early and simplistic point of view, melatonin is accepted as a hormone produced by the pineal gland and transported through bloodstream to other parts of the body where it exerts its effects. As a signaling molecule, melatonin can be defined as a dark-signaling molecule due to its high plasma levels at nights and low plasma levels during the daytime [7-9]. With its rhythmic production depending on the dark and light conditions in the environment, melatonin plasma concentrations reflects the information about the time of the day. This unique 24-hours profile of melatonin production by the pineal gland is an excellent example of circadian rhythm. Moreover, since melatonin is transported to various tissues, it is reasonable to think that melatonin is involved in circadian rhythm signaling of many other tissues as a synchronizer. Besides carrying information about the time of the day, plasma melatonin concentrations can also be interpreted by certain tissues to gain information about the time of the year since the nights will be long in winters and short in summers [10]. Another interesting aspect of rhythmic melatonin production is the fact that the highest production is at nights in both diurnal, which are active in daytime, and nocturnal, which are active at nights, animals [7].

Although most of the studies about melatonin were made about mammalian species, it has been shown that melatonin exists virtually in all animals [11]. Moreover, melatonin is also produced by several plants and identified in many parts of them as roots, stems, leaves and seeds [12]. Finally, melatonin production in unicellular eukaryotes and

bacteria has also been demonstrated [11]. Despite the fact that melatonin exists in plants and unicellular organisms, it seems that production of melatonin in these organisms is independent of dark and light signals. These observations implies that besides being an important dark-signaling molecule, melatonin has other important properties that caused this molecule to be used by nature starting from the relatively early days of evolution and to be conserved through evolution [8].

The circadian production of melatonin from the pineal gland is regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. SCN is the center of the body's endogenous clock to regulate circadian cycles such as sleep-wake, temperature, drinking and feeding. The information about the light in the outside environment is received by the SCN through specialized melanopsin receptor containing retinal ganglion cells in the retina. After the information about the dark-light conditions is interpreted in SCN, the command to synthesize melatonin is send to the pineal gland through superior cervical ganglia [7, 8, 13]. The primary neurotransmitter used to control the activity of the pineal gland is norepinephrine. Norepinephrine released at the nerve terminals binds to β-adrenergic receptors on the pinealocytes, cells in the pineal gland responsible for melatonin synthesis, by causing the expression enzymes necessary for melatonin production [7, 8].

Melatonin (*N*-acetyl-5-methoxytryptamine) is basically synthesized from serotonin (5-Hydroxytryptamine) which is a neurotransmitter produced from L-tryptophan. Tryptophan is converted into 5-hydroxy-L-tryptophan by tryptophan hydroxylase enzyme and than 5-hydroxy-L-tryptophan is converted into serotonin by aromatic L-amino acid decarboxylase enzyme. After serotonin is synthesized, melatonin is produced from a two step pathway as shown in Figure 2.1. Firstly serotonin is acetylated by arylakylamine *N*-acetyltransferase into *N*-acetylserotonin. This step is the rate limiting step of melatonin synthesis from serotonin. Then, finally, *N*-acetylserotonin is methylated by hydroxyindole *O*-methyltransferase enzyme to produce melatonin [9, 14, 15]. Circulating melatonin is primarily catabolyzed by the liver. In the liver melatonin is firstly hydroxylated to form 6-Hydroxymelatonin and excreted as primarily sulfate conjugates. Also there is evidence that melatonin can be deacetylated to form 5-methoxytryptamine by melatonin specific deacetylases or aryl acylamidases in cells carrying these enzymes [7, 8]. At the cellular level, melatonin can also be converted into other metabolites which will be discussed in the

later chapters.

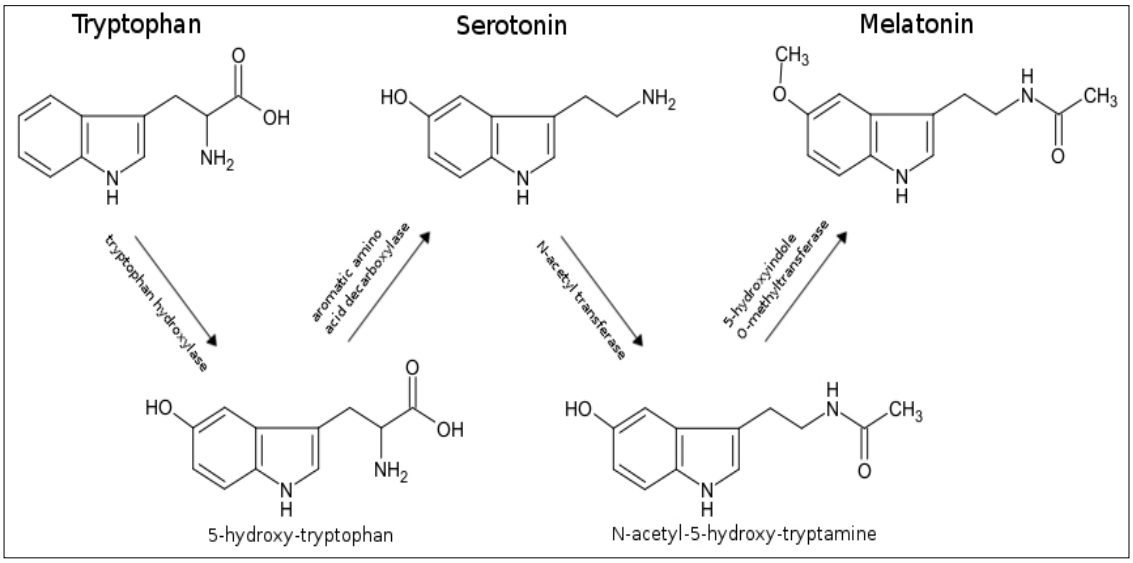

Figure 2.1. Melatonin synthesis from tryptophan via serotonin [8]

Melatonin is generally considered as a hormone and it is the primary product of the pineal gland but it is not the only site where melatonin is produced. Melatonin can be synthesized by many other parts of the body to exert autocrine and paracrine functions. Gastrointestinal tract [16], skin [17], bone marrow [18], retina [19], lymphocytes and platelets [20] are the body parts and cells reported to be able to synthesize melatonin other than the pineal gland in mammals [21]. However, the systemic contribution of these production sites to plasma melatonin levels is insignificant.

Although melatonin is a circulating hormone, its concentration in the cerebrospinal fluid (CSF) is several orders of magnitude higher than its plasma level. It has been shown in sheep that melatonin from the pineal gland is released into the third ventricle, and thereby into CSF, through the pineal recess [22, 23]. Since the structures around the pineal gland and the pineal recess in humans and sheep are very similar, it is reasonable to assume that CSF melatonin concentrations in humans are also very high when compared to the plasma levels [22, 23].

Besides being a dark-signaling molecule and a general synchronizer of daily and seasonal rhythms for the body, melatonin has been reported to have various functions.

Among these the most interesting and may be the most crucial one is the fact that melatonin is a highly effective antioxidant compound [24, 25]. Melatonin is also, to some extent, shown to have oncostatic [26] and immunomodulatory [27] features. Furthermore, melatonin can also affect cell survival, cardiovascular and reproductive systems [7, 8]. In the context of this work, melatonin's antioxidative and immunomodulatory properties and effects on cell survival will be discussed.

2.2. MELATONIN RECEPTORS AND INTERACTING PROTEINS

There are two mammalian high affinity membrane receptors designated as MT_1 and MT_2, previously known as Mel_{1a} and Mel_{1b}, respectively. MT_1 has a slightly higher affinity to 2-^{125}I-melatonin, which is the radiolabel used in melatonin receptor studies, in picomoles region than MT_2 but both receptors have a similar pharmacological profile with K_D values changing between 25-160 pM [28-30]. Mel_{1c} is another high affinity membrane melatonin receptor which is not present in mammalian species [31]. There is also another low affinity melatonin receptor formerly called MT_3 which is identified as quinone reductase 2 enzyme [28-30]. Moreover, due to its small and lipophilic structure melatonin can also easily pass through biological membranes such as cell membrane and blood-brain barrier (BBB). Therefore, it is a natural ligand for the retinoid related orphan nuclear hormone receptor RZR/RORα superfamily. Among the members of this family RORα1 and RORα2 can be functional intracellular melatonin receptors in immune cells, while RZRβ might be the one in CNS [8, 29, 30]. Furthermore, melatonin is also shown to interact with intracellular proteins such as calreticulin, calmodulin and tubulin [7, 8, 30]. Of note, GPR50 is an orphan G-protein coupled receptor which has 45% amino acid homology to human MT_1 and MT_2 receptors but GPR50 does not bind to melatonin although it is shown to interact with MT_1 and MT_2.[32].

Melatonin receptors MT_1 and MT_2 are expressed in various tissues to exert their diverse physiological effects such as SCN , pars tuberalis, hippocampus, cerebellum and various other regions in CNS, ventricular walls, coronary arteries, cerebral arteries, aorta and other arteries in cardiovascular system, prostate, breast, myometrium, ovary cells, gallbladder, duodenum, skin, various immune cells, adipocytes and possibly many other tissue and cell types [29, 30].

MT_1 and MT_2 receptors are members of seven transmembrane G-protein coupled receptor family. MT_1 has 350 and MT_2 has 362 amino acids and they have more than 90% amino acid homology with each other. MT_1 and MT_2 have a unique NRY motif in their intracellular loop II which separates them from the rest of the G-protein coupled receptor family which have a DRY or ERY motif instead [28]. Both of these receptors can interact with various types of G-proteins depending on the tissue and cell type. As a general rule MT_1 activation with melatonin binding causes decrease in cyclic adenosine monophosphate (cAMP) levels, protein kinase A activity and phosphorylation of the transcription factor CREB (cAMP-responsive element binding) protein whereas it also causes increase in phosphorylation of mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2), extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and Jun N-terminal kinase (JNK). Similar to MT_1 activation, MT_2 activation also causes decrease in cAMP levels. In addition to this, MT_1 and MT_2 activation also inhibits soluble guanyl cyclase activity and, thereby; causes a decrease in cyclic guanosine monophosphate (cGMP) levels, too [28-30, 32]. Melatonin receptor dependent and independent effects melatonin will be discussed in more detail in the forthcoming chapters.

2.3. MELATONIN AS AN ANTIOXIDATIVE AGENT

Besides being an important signaling molecule, melatonin is a very powerful antioxidant molecule, which is an agent that can neutralize the dangerous effects of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Melatonin is even more powerful than vitamin E, which is a natural reference molecule to measure the antioxidative capacity, in direct free radical scavenging. This high antioxidant capacity of melatonin comes from the fact that the products occurring from the reactions of melatonin with ROS/RNS and some natural metabolites of melatonin also have antioxidative capacity. Furthermore, melatonin is not only a direct free radical scavenger like most of the antioxidant agents but it is also a molecule that can induce expression of antioxidant enzymes and inhibit pro-oxidative enzymes. Melatonin has been shown to increase the levels of free radical scavenger enzymes in the cell including mitochondrial and cytosolic superoxide dismutase (SOD) enzymes, glutathione peroxidase and glutathione reductase. In addition, melatonin decreases neural and inducible nitric oxide synthase (NOS) and 5- and 12-lipoxygenases, playing roles in cell death [24, 33, 34].

Melatonin can directly scavenge the hydroxyl radical (•OH), carbon trioxide ($CO_3^{\bullet-}$), superoxide ($O_2^{\bullet-}$), nitric oxide (•NO), nitrogen dioxide ($\bullet NO_2$) and peroxynitrite ($ONOO^-$). With these ROS/RNS scavenging reactions and several enzymatic and nonenzymatic reactions melatonin can be converted into cyclic 3-hydroxymelatonin and kynuramine derivatives N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) as shown in Figure 2.2. Kynuramine derivative melatonin metabolitesAFMK and AMK has also been shown to be antioxidant molecules. Furthermore, melatonin and its metabolite AMK can also act as a electron donor in single-electron transfer reactions in mitochondria which prevents the formation of free radicals in the first place and increases the efficacy of ATP production [8, 24, 33, 34].

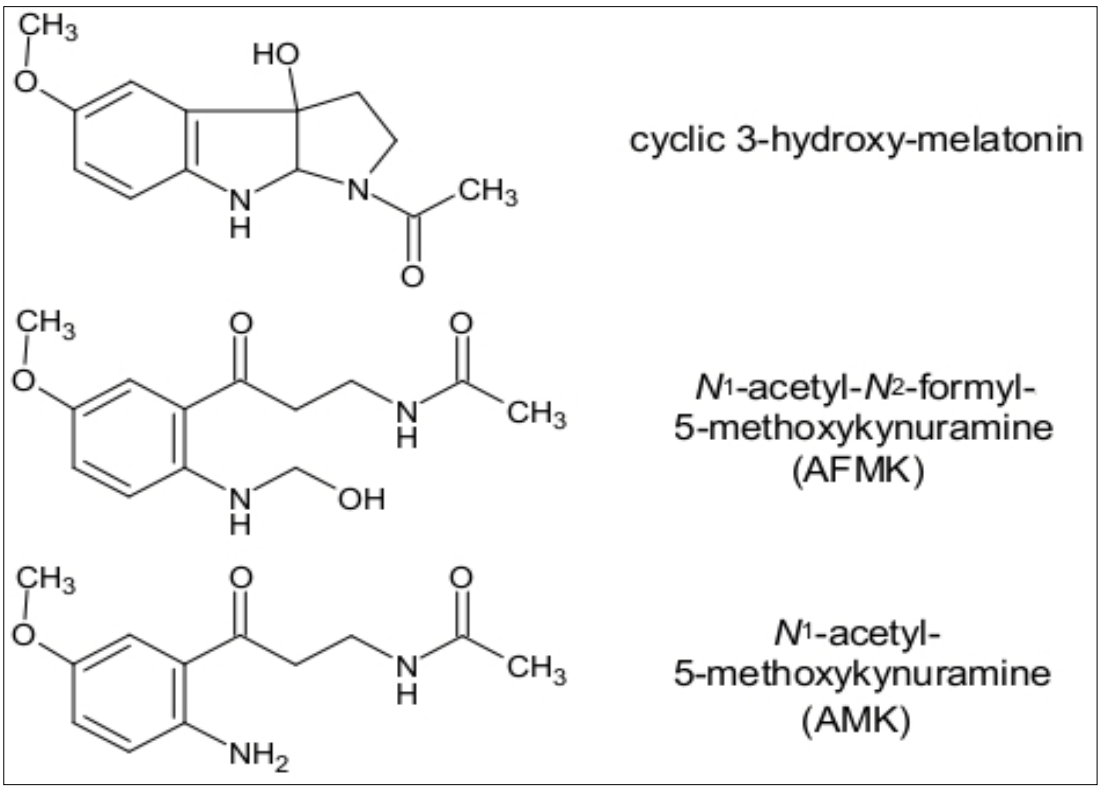

Figure 2.2. Metabolites of melatonin as an antioxidant [8]

2.4. MEMANTINE AS A CALCIUM CHANNEL BLOCKER

Memantine (1-amino-3,5-dimethyl-adamantane) is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist which has been recently approved in United States (US) and European Union (EU) for the treatment of moderate to severe dementia of

Alzheimer's disease [5, 6, 35] as shown in Figure 2.3. NMDA receptors are the primary glutamate-gated ion channels in the nervous system, together with the less common ones as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors that allow the influx of cations, mostly calcium [36]. NMDA-type glutamate receptors are extremely important in several chronic and acute neurological diseases that have excitotoxicity, which is the over-activation of these receptors, and will be discussed in more detail in forthcoming chapters, as a factor in the progression of cellular damage.

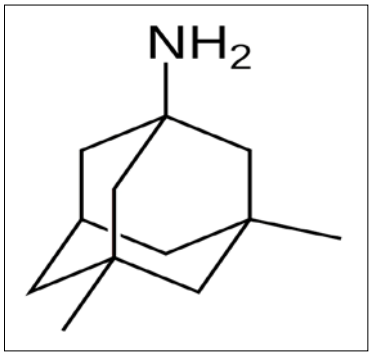

Figure 2.3. Chemical structure of memantine [6]

In previous attempts, many NMDA antagonists failed to pass clinical trials, mainly due to unacceptable side effects. The side effects of NMDA antagonists were especially due to their unfavorable effects on autonomic controls of physiological conditions, in which NMDA receptors play functional roles. This inability of the NMDA antagonists to differentiate between physiological and pathophysiological activation of glutamate receptors had almost extinguished the NMDA antagonists concept until the discovery of memantine as a novel antagonist. Voltage-dependent blockage of the receptor channel, uncompetitive antagonism, moderate affinity and fast on/off kinetics are the properties of memantine that separates it from other NMDA antagonists and decrease the unfavorable side effects [5, 6, 35, 37].

2.5. CEREBRAL ISCHEMIA (STROKE)

Cerebral ischemia or stroke is a medical condition in which blood supply to an organ or a tissue is reduced permanently or transiently. In transient cerebral ischemia,

blood circulation to the ischemic region is reestablished by itself or fibrinolytic drugs, such as recombinant tissue plasminogen activator (t-PA). It is called reperfusion. Due to high oxygen and glucose consumption of brain, it is highly susceptible to ischemia/reperfusion (I/R) injuries where blood supply is interrupted or cut off for some time. In such cases while short supply of oxygen and glucose cause damage to neural tissue by changing their cellular metabolism, reoxygenation of the tissue result in further damage. Stroke is the third leading cause of death in developed countries and stroke survivors suffer serious disabilities. Stroke is caused by an embolus or thrombosis that can plug arteries that supplies the brain with blood or overall circulation problems such as heart failures [38, 39].

The neural tissue damaged by ischemia can be roughly separated into two regions as the core in which the blood supply is below 20% of the normal values and the penumbra surrounding the core in which the blood supply is partially restored. There are several spatiotemporal aspects of cellular damage caused by cerebral ischemia which results in apoptotic or necrotic cell death depending on the type and severity of the damage as shown in Figure 2.4. First of all, decrease in oxygen and glucose supply results in energy failure in neurons which would mean the loss of ionic gradients across cell membranes and membrane potentials which heavily depend on active transport systems. This causes the opening of voltage-gated calcium channels and release of glutamate, which is an excitatory neurotransmitter. Since reuptake mechanisms also fails due to low cellular energy, glutamate accumulates in the extracellular environment causing more cells to become excited and release glutamate and this overall process is called excitotoxicity. Loss of membrane potential due to overactivation also causes anoxic depolarization with the efflux of potassium ions and the influx of sodium and chloride ions inside the cells. This will result in cell swelling and edema because of water diffusion following these ion gradients. Another consequence of uncontrolled depolarization of the cells and accumulation of glutamate causes neighboring cells to depolarize which is called peri-infarct depolarization. Due to the fact that membrane potential can not be restored, calcium ions starts to accumulate in the cytosol which would lead to activation of proteases such as calpains that will degrade proteins such as cytoskeletal proteins in an uncontrolled manner. Calcium overload also results in the activation of phospholipase A_2 and cyclooxygenase enzymes which will produce free radicals via lipid metabolism. Together with the unregulated protein degradation because of calcium accumulation, free radicals damage the

mitochondrial membranes and this triggers production of more free radicals. Mitochondrial damage and DNA damage caused by free radicals induces apoptotic pathways in cells which do not die due to membrane failure by necrosis [38, 39].

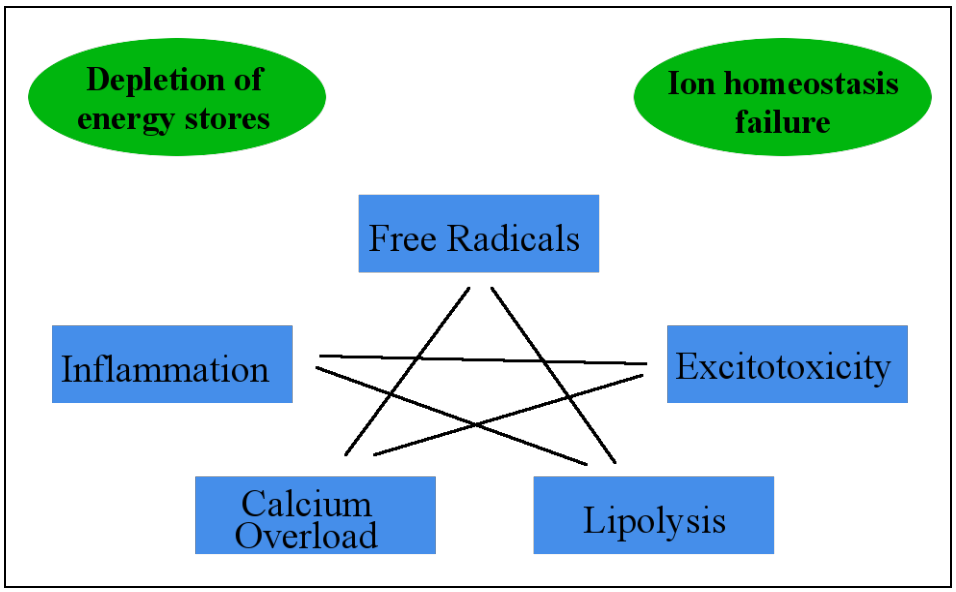

Figure 2.4. Aspects of tissue damage in cerebral ischemia

As a response to low oxygen levels the active form of the transcription factor hypoxia-inducible factor-1 (HIF-1) also starts to accumulate in cerebral ischemia as early as 1 hour, which will result in expression of genes that will help the cells to cope with the hypoxic stress such as erythropoietin and vascular endothelial growth factor (VEGF) [40]. Moreover, single strand DNA damage caused by I/R injury activates poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP) enzyme which is an enzyme functions in DNA repair. However, PARP activation results in further depletion of, already low, cellular energy stores [41].

Another important aspect of the ischemic damage to the neural tissue is the inflammation in the damaged area. Microglia cells, which are resident macrophages of the CNS, become activated within 24 hours after the onset of ischemia and start secreting inflammatory cytokines. Moreover, vessels around the infarct zone express adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) in the very early phases of ischemia [39, 42].

Furthermore, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α in the core area of the ischemia damage [43]. These adhesion molecules cause the infiltration of neutrophils, macrophages and monocytes into the damaged area causing more damage and inflammation. Nitric oxide produced by these cells also contributes to the formation of free radicals exacerbating the damage [39].

There are several models to study I/R injuries in brain both in animals or *in vitro* systems. Two major animal models of brain I/R injuries are global, in which blood supply to all of the brain has been cut off, and focal, in which blood supply to some parts of the brain has been cut off, cerebral ischemia models. Also there are permanent ischemia models in which blood supply is not restored when compared to transient ischemia models. Hypoxia/ischemia is another model which employs 8% oxygen inhalation together with surgical intervention to brain blood supply which produces similar damage to cerebral ischemia. Certain chemicals can also be used to simulate certain aspects of I/R injuries in animal models, including endothelin. Moreover, *in vitro* models of primary neuronal cultures or cell lines subjected to oxygen glucose deprivation (OGD), hypoxic conditions and certain chemicals can also be used as I/R brain injury models [38]. Specific animal models used in this study will be explained in detail in later chapters.

Despite several attempts to develop a neuroprotective treatment for stroke, so far all clinical trials have failed to reproduce the neuroprotective effects observed in animal models. Timing of the treatment, the concentrations of drugs in circulation and brain, possible side effects of the drugs and differences between animal models and human cases might be the reason for the failures of the clinical treatments. Today, the only clinical treatment against stroke is the t-PA which aims to restore the blood supply to the brain. Another issue about neuroprotective drugs against cerebral ischemia is the presence of the BBB. BBB separates the CSF and the brain from the circulation with the tight junctions around capillaries allowing the diffusion of only small and lipophilic molecules and; thus, limits the diffusion of certain potential neuroprotective agents [2, 38, 39].

2.6. MELATONIN IN TREATMENT OF ISCHEMIA/REPERFUSION BRAIN INJURY

Melatonin has high lipid and water solubility, high efficiency in passing the blood-brain barrier, effective half life values in plasma and brain after intravenous injections, relatively good intracellular distribution, and being non-toxic to humans makes melatonin a promising neuroprotectant candidate in diseases such as stroke [2, 44]. Since melatonin is naturally produced in the body, primarily by pineal gland, effect of physiological melatonin on I/R damage is investigated on *in vivo* models [45, 46]. In these experiments stroke models in which pineal glands are removed via pinealectomy are used and this resulted in greater neurodegeneration than the control groups. Effect of melatonin treatment on brain I/R is investigated in different model organisms as rats [46, 47], Mongolian gerbils [48], mice [49] and cats [50] and resulted in decreased infarct volumes. Various types of melatonin treatments in ischemia models are reported with concentrations changing from 2.5 mg/kg to 40 mg/kg, as a bolus injection or continuous administration, timing of the treatment changing from one hour before ischemia onset to two hours after the ischemia onset [2-4]. In a systematic analysis, effectiveness of melatonin administration against brain I/R injuries in experimental animal models is found to be 42.8% improvement in the outcome and the effective concentrations of melatonin are found to be the ones higher than 5 mg/kg [3]. Besides having an effective neuroprotectant activity in short time interval before and after treatments [3, 4, 51], melatonin is also shown to have beneficial neuroprotectant activity when administered prophylactically before stroke [52] or 24 hours after stroke and continued in a regular fashion [53].

2.6.1. Effects of melatonin on *in vitro* models of ischemia/reperfusion brain injury

Melatonin's protective effects are also investigated on neural cells under different conditions *in vitro*. I/R injury effect on neural cells are usually simulated by using oxygen-deprived medium and air with low glucose medium and/or serum deprivation or by chemical induction [38, 51]. In hippocampal slices, melatonin treatment improved the synaptic potential recovery after hypoxia/reoxygenation stress [54]. In primary cultures of rat cortical neurons, melatonin treatment protected the neurons against excitotoxic stress mediated by NMDA receptors and hypoxia/reoxygenation [55]

Astrocytes are star shaped glia cells in CNS which have supporting functions for neurons and melatonin increased cell survival in primary astrocyte cultures against RNS stress inducing chemicals and serum deprivation [56]. Also it is shown that melatonin is able to protect primary neural cultures more effectively than NOS and caspase inhibitors against oxidative stress [57]. In NMDA and OGD stress on primary neural cultures melatonin increases mitochondrial membrane integrity by preventing formation of mitochondrial permeability transition pores which would otherwise cause release of calcium and cytochrome-c that will result in cell death [58].

In vitro effects of melatonin against oxidative stress [59-61] and exicotoxicity [62] is also examined in neural cell lines and protective effects are observed as increase in cell survival. In chemically induced oxidative mitochondrial DNA (mtDNA) damage conditions on SHSY5Y neural cells, melatonin also has neuroprotective effects by preserving mitochondrial potentials, decreasing mitochondrial oxygen free radicals and protecting mtDNA against oxidative damage [59]. Melatonin administration decreases the apoptosis level of N2a neuroblastoma cells against oxygen-glucose-serum deprivation (OGSD) and decrease the amount of intracellular ROS, caspase 3 activity and cytochrome C release from mitochondria [60]. Moreover, melatonin decreased nuclear factor κB (NF-κB) activation and Bax induction upon hydrogen peroxide treatment in SHSY5Y neural cells [61]. In a study, it is also shown that melatonin specifically prevented the mitochondrial ROS production against glutamate-induced oxytosis in HT22 mouse hippocampal cells line [62].

2.6.2. Effects of melatonin on *in vivo* models of ischemia/reperfusion brain injury

Besides reducing infarct volume and increasing neural survival, different physiological effects of melatonin administration as orally, intravenously (i.v.) and intraperitoneally (i.p.) are investigated in different animal models of I/R brain injury to elucidate the exact molecular mechanisms behind this neuroprotectant effects [2-4]. In mongolian gerbils, melatonin prevented the increase in nitric oxide (NO) and cGMP levels after transient brain I/R injury [63] as well as decrease in nitrotyrosine, indicator of peroxynitrite production, PARP activity, indicator of DNA damage and ATP depletion and malondialdehyde, indicator of lipid peroxidation [48], in cerebral ischemia models.

Reduced levels of malondialdehyde and reduced glutathione are also observed in melatonin treated rats in middle cerebral artery occlusion (MCAO) [64]. In global ischemic rats melatonin administration reversed the decreasing effect of ischemia on SOD and glutathione reductase levels in brain [65]. Moreover, in transient ischemia, melatonin enhanced the induction of Bcl-2, a pro-survival protein, and ERCC6, a protein that functions in DNA repair mechanisms [66]. Both prophylactic and acute melatonin administrations are shown to increase phosphporylated Akt levels whereas only prophylactic treatment increased phosphprylated mitogen-activated protein kinase/extracellular regulated kinase (ERK)-1/-2 and Jun kinase (JUK)-1/2 levels [67]. Furthermore, melatonin administration reversed the negative effects of t-PA, which is used to treat patients with stroke, by decreasing levels of induced NOS (iNOS) levels and increasing phosphprylated Akt [68]. In another study melatonin treatment also decreased iNOS levels while preventing the injury-induced decrease in endothelial NOS (eNOS) levels upon MCAO [69]. Recently, by two-dimensional gel electrophoresis certain cell differentiation and stabilization proteins that are differentially expressed in melatonin and vehicle-treated rats upon MCAO are identified [70].

2.6.3. Effects of Melatonin on Blood-Brain Barrier

Effects of melatonin are also examined on BBB permeability during the reperfusion phase of I/R injuries. Although melatonin administration 30 minutes prior to ischemia onset did not protected against BBB breakdown [71], administration at the reperfusion onset is shown to protect BBB permeability and decrease hemorrhagic transformation of t-PA therapy [72, 73]. Prophylactic and acute treatments of melatonin also resulted in decreased levels of endothelin converting enzyme-1 (ECE-1) levels, which produces endogenous vasoconstructor endothelin-1 [52]. Furthermore, melatonin is shown to reduce the elevated levels of matrix metalloproteinase-9 (MMP-9), which is member of an enzyme family responsible from the pathogenesis of brain edema and hemorrhagic transformation in stroke [74].

2.6.4. Effects of Melatonin on Inflammation in Ischemic Brain

Regarding the inflammation aspect of I/R injuries, it is also reported that melatonin

administration in stroke models decreased the injury-increased levels of myeloperoxidase, indicator of leukocyte infiltration, and cyclooxygenase-2 (COX-2), indicator of inflammation and oxidative stress [48, 75]. Moreover, it is observed that in COX-1 knock-out mice stroke model melatonin's neuroprotective effects are decreased when compared to wild type ones [76]. Melatonin also inhibited microglial activation against kainic-acid-induced excitotoxicity in rats but not astroglial activation indicated by glial fibrillary acidic protein (GFAP) [77]. Furthermore, melatonin treatment decreased neutrophil and macrophage/activated microglia infiltration in transient focal cerebral ischemia, verified with flow cytometry analysis [78].

2.6.5. Behavioral Aspects of Melatonin Treatment in Stroke

Neuropretective effects of melatonin have also been verified by behavioral recovery after stroke. Additional experiments showed that aged pinealectomized rats committed more working memory errors upon I/R injury in tactile radial maze training [79]. Almost all of the cases, neuroprotectivity of melatonin administration in stroke models are accompanied with improvement in behavioral examinations on sensory-motor grading scales [4, 80, 81]. Long term behavioral improvement upon melatonin treatment in stroke is also documented as increased performance in learning and working memory tests 90 days after ischemia [82]. Chronic melatonin treatment is also shown to reduce ischemia-induced hyperactivity in rats both in short term and long term evaluations [53, 83].

2.7. MEMANTINE IN TREATMENT OF ISCHEMIA/REPERFUSION BRAIN INJURY

As an NMDA receptor antagonist memantine has been shown to be useful in treatment of several neurological disorders with excitotoxicity, including stroke. Although several other NMDA antagonists have been shown to reduce the infarct volume in animal models of I/R brain injury, memantine has a clinical advantage over others with much less side effects. In recent studies, memantine was administered either before or up to two hours after stroke, with bolus injections and/or continuous administrations. Memantine is used in focal and global brain ischemia models with concentrations between 10-20 mg/kg, which is higher that its use in chronic neurological disease in accordance with the more

severe nature of ischemia models. Another important aspect of memantine treatment is that it does not interfere with the normal learning and memory functions of animals which are consistent with its somewhat lower side effects as a NMDA antagonist [5, 6].

20 mg/kg intraperitoneal (i.p.) memantine administration is shown to reduce the infarct volume more than 35 %, compared to control groups, when given 15 minutes before the onset of ischemia on neonatal rats [84]. 10 mg/kg i.p. memantine injection also resulted in decreased infarct areas in adult rats upon focal ischemia when administered 15 minutes after the onset of ischemia [85]. Moreover, 10 mg/kg and 20 mg/kg concentrations of memantine caused a dose-dependent reduction in cerebral damage in rat ischemia models when applied one hour before ischemia [86]. Memantine is also shown to be protective in cultured neurons upon chemically induced hypoxia [86]. Furthermore, memantine treatment positively affected the behavioral and learning effects of global ischemia when administered 20 minutes before ischemia onset [87].

3. MATERIALS & METHODS

3.1. EXPERIMENTAL SETUP AND GROUPS

All experimental procedures were conducted with governmental approval according to local guidelines for the care and use of laboratory animals. All animals were kept under regular lighting conditions as 12 hours darkness and 12 hours light. Adult male C57BL/6j mice (22-26 g) randomly assigned to the following experimental groups: Control (n = 7); received 5% ethanol in 0.9% saline immediately at the reperfusion onset and 20 minutes after the reperfusion onset, Melatonin (n = 7); received a full dose of 4 mg/kg melatonin (Sigma-Aldrich, Germany) dissolved in 0.9% saline immediately at the reperfusion onset and and an additional half dose of 2 mg/kg 20 minutes after the reperfusion onset, Memantine (n =7); received a full dose of 20 mg/kg memantine (Sigma-Aldrich, Germany) dissolved in 0.9% saline immediately at the reperfusion onset and an additional half dose of 10 mg/kg 20 minutes after the reperfusion onset, Melatonin/Memantine (n = 7); received both of the treatments applied to the Melatonin and Memantine groups as shown in Figure 3.1.

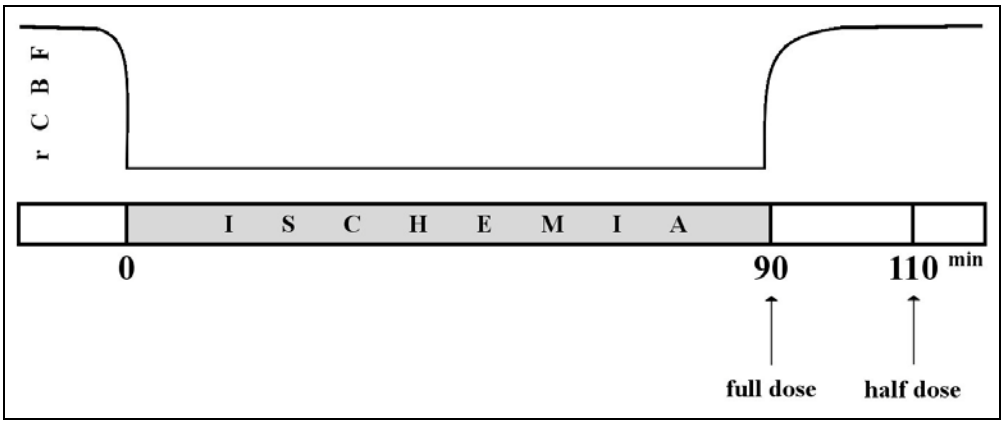

Figure 3.1. Representation of experimental setup

3.2. INDUCTION OF CEREBRAL ISCHEMIA & REPERFUSION

Animals were anesthesized with 1% isofluorane (30% O_2, remainder N_2O) and

rectal temperature was maintained between 36.5 and 37.0°C using a feed-back controlled heating system (MAY instruments, Ankara, Turkey). 15 minutes before the ischemia onset, during ischemia and 20 minutes after the reperfusion onset, blood flow was measured by laser Doppler flowmetry (LDF) using a flexible 0.5 mm fiber optic probe (Perimed, Sweden), which was attached to the intact skull overlying the middle cerebral artery (MCA) territory (2 mm posterior/6 mm lateral from bregma). Focal ischemia due to MCAO was induced using a intraluminal filament technique as shown in Figure 3.2 [52, 53]. Briefly, a midline neck incision was made and the left common carotid artery and external carotid artery were isolated and ligated. A microvascular clip (FE691; Aesculap, Germany) was temporarily placed on the left internal carotid artery. A 8-0 nylon monofilament (Ethilon, Ethicon, Germany) coated with silicon resin (Xantopren, Bayer Dental, Japan), the diameter of the coated thread being 180-190 µm, was introduced through a small incision into the left common carotid artery and advanced 9 mm distal to the carotid bifurcation for MCAO. After 90 minutes, reperfusion was initiated by withdrawal of the thread. After the treatments, the wound are closed with sutures, anesthesia discontinued and animals are returned to their home cages.

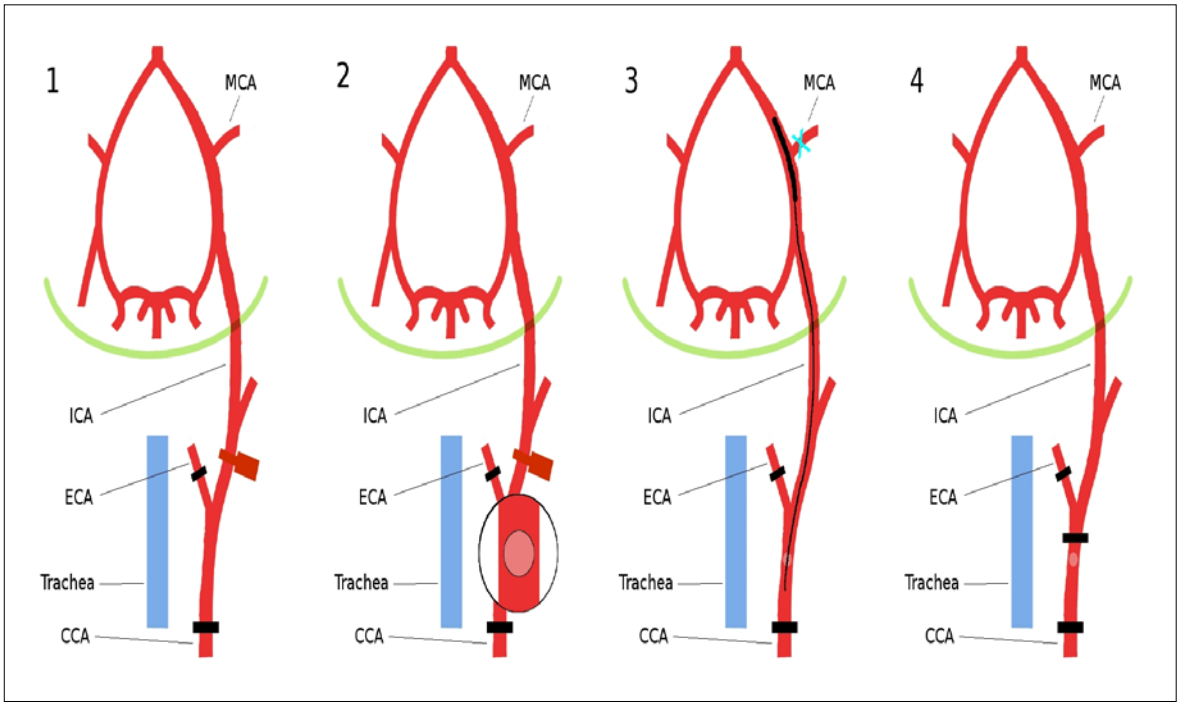

Figure 3.2. Graphical representation of the surgical operation for the induction of cerebral ischemia and reperfusion [52]

3.3. NEUROLOGICAL DEFICIT SCORES

24 hours after MCAO, neurological deficits were examined and scored using the following scores: 0: normal function; 1: flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail; 2: circling to the contralateral side but normal posture at rest; 3: reclination to the contralateral side at rest; 4: absence of spontaneous motor activity [88].

3.4. CRESYL VIOLET STAINING, INFARCT VOLUME & BRAIN SWELLING CALCULATIONS

24 hours after MCAO, animals were re-anesthetized with isofluorane and decapitated. Brains were removed and frozen on dry ice. Subsequently, brains were cut on a cryostate into 18 μm coronal sections. Sections from defined rostrocaudal levels, 2 mm apart, were stained with cresyl violet stain according to standard histological staining procedures. The stained sections were analyzed using the ImageJ software (NIH, US) to outline infarct zones. The area of infarction was assessed by subtracting the nonlesioned area of the ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of these lesion areas. Brain swelling (edema) was calculated as the volume difference between the ischemic and the nonischemic hemisphere, and expressed as a percentage of the intact hemisphere.

3.5. STATISTICS

For statistical data comparisons, a standart software package (SPSS for Windows; SPSS Inc., Chicago, IL, USA) was used. Differences between groups were calculated by one-way ANOVA, followed by least significant differences tests. All values are given as mean ± S.E.M. with n values indicating the number of different animals analyzed. P values < 0.05 are considered significant.

4. RESULTS & DISCUSSION

4.1. LASER DOPPLER FLOWMETRY

LDF recordings were performed for the left MCA territory during ischemia, as well as 15 minutes before and 20 minutes after the start of reperfusion. Regional cerebral blood flow (rCBF) results indicated that the MCAO was highly reproducible and no statistically significant difference was observed between groups by using repeated- and on-way-ANOVA analysis. As in previous studies [52, 53, 88], intraluminal MCA thread occlusion resulted in a sharp decrease of cerebral blood flow to ~15% of the pre-ischemic control values in the MCA territory. In all groups thread retraction after 90 minutes was followed by a rapid restoration of blood flow. In vehicle treated Control group and Melatonin/Memantine group the cerebral blood flow during the first 20 minutes of reperfusion were ~85% of pre-ischemic levels whereas in only Melatonin and only Memantine group the values were ~110% although these differences were not statistically significant as shown in Figure 4.1.

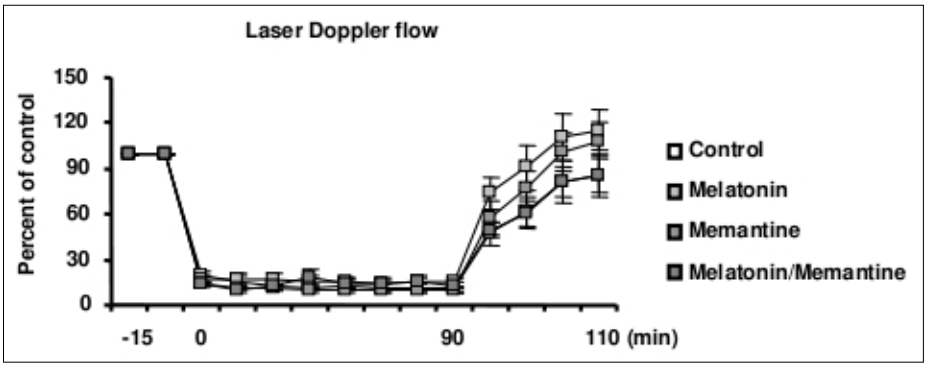

Figure 4.1. Laser Doppler flowmetry results of the operations (Mean ± S.E.M.)

LDF measurements have recently become a necessity for the studies involving cerebral I/R injuries to ensure the reproducibility and success of the operations. Although rCBF measured by LDF does not reflect the exact blood flow values, it shows the relative changes in rCBF perfectly. Moreover, since LDF is an instantaneous, continuous and relatively non-invasive method it is highly advantageous to monitor hemodynamic changes

[89]. No direct effect of memantine has been reported previously to effect rCBF, which is consistent with our results. Melatonin, on the other hand, has been reported to have some cardiovascular effects but the major affects seem to be indirect through SCN such as blood pressure regulation in a circadian manner. Furthermore, melatonin is shown to induce vasodilation via MT_1 receptor but to induce vasoconstriction via MT_2 and the overall response depends on the expression levels of these receptors, but in cerebral vessels vasoconstriction seems to be the main result [8, 28]. Also melatonin administration is shown to cause a decrease in rCBF in a mouse MCAO model [2]. However, direct cardiovascular effects of melatonin is rather complex and minor in terms of MCAO model and in our study no significant difference was observed between experimental groups during the LDF recordings.

4.2. INFARCT VOLUMES

In vehicle treated animals, reproducible brain infarcts were observed 24 hours after reperfusion. In the vehicle treated Control group, infarct volume was 64±5 mm3 and all other treatment groups caused about 50% reduction in infarct volume. Infarct volume was 31±7 mm3 in Melatonin group, 35±8 mm3 in Memantine group and 20±4 mm3 in Melatonin/Memantine group. Although the difference between treatment groups and control groups was statistically significant ($p<0.01$), there were no significant difference between Melatonin, Memantine and Melatonin/Memantine groups as shown in Figure 4.2 & Figure 4.3. Reduced infarct volumes for both Melatonin and Memantine only groups were consistent with the previously reported neuroprotective effects of these agents in stoke models [2-6]. Although Melatonin/Memantine group decreased the infarct volume more than that of either treatment alone, no cumulative effect was observed.

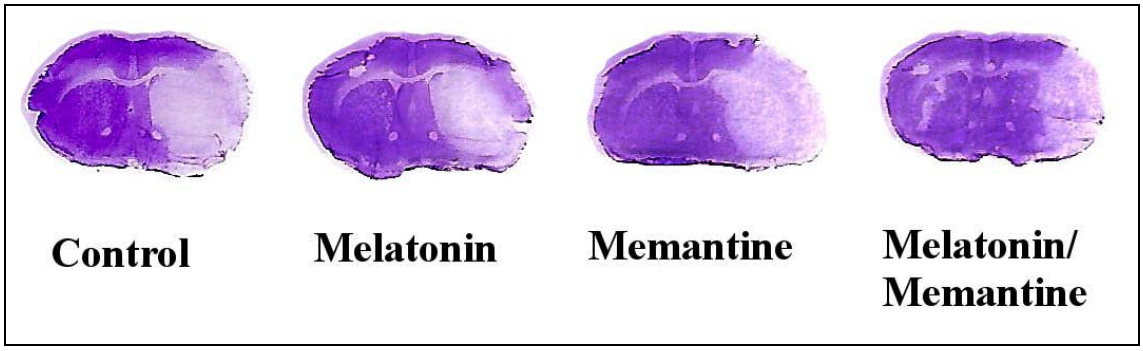

Figure 4.2. Representative figures of brain sections after cresyl violet staining

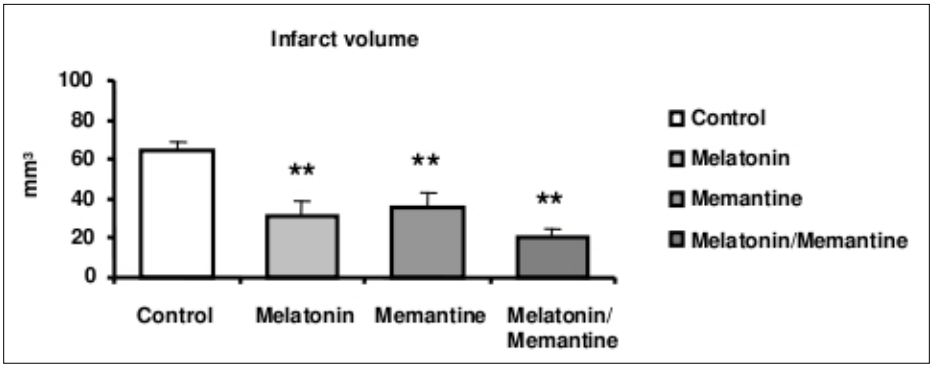

Figure 4.3. Infarct volumes of the experimental groups (Mean ± S.E.M., ** = $p<0.01$)

One important aspect of this study is the time of administration of the agents with respect to ischemia onset. In clinical aspect, it is almost impossible to treat a stroke victim before the ischemia and medical treatment usually occurs after the onset. In this work, treatments are done 90 and 110 minutes after the occlusion, therefore; this study can be a clinically relevant model. Moreover, both melatonin and memantine are clinically approved drugs for treatments of generally sleeping disorders and dementia, respectively [6, 7].

Furthermore, these agents have relatively wide therapeutic windows in I/R brain injury treatments. Melatonin is shown to be neuroprotective with its various protective effects starting from one hour before ischemia onset to two hours after the onset together with prophylactic use and longer term treatments [2-4]. Memantine also has a similar neuroprotection profile from 15 minutes before ischemia onset to two hours after the onset

[5]. This information is highly relevant when the neuroprotective mechanisms of these agents against ischemic damage are considered. Melatonin can protect the cells from free radicals in the short term and melatonin can also decrease apoptosis and inflammation which continues for longer periods after ischemic injury whereas memantine is effective only against exicitotoxity and peri-infarct depolarization which start immediately with the energetic failure and continues for hours [5-8, 39].

Another important aspect of this study is the add-on application of the agents, as a full dose at the start of reperfusion and a half dose 20 minutes after the first dose. Although memantine is known to have a half life of almost 3 hours after i.p. injections, it is not the case for melatonin since melatonin has a half life of 20 minutes [2, 90]. Also both melatonin and memantine can cross the BBB rapidly after i.p. Injections [2-4, 5, 6, 90]. Moreover, multiple doses of melatonin treatments resulted in higher efficacy in neuroprotectivity [3].

Memantine is a NMDA antagonist with much less side effects than other antagonists such as MK-801, in animal stroke models, however; no difference in their neuroprotective capacity is observed, at least in terms of infarct volumes [5, 84]. Although there were some attempts to prove that memantine does not interfere with normal physiological functions of glutamate receptors by using behavioral tests, the doses used in these studies were too high to determine side effects and the timing of the tests were inconvenient to deduct a clear conclusion [5, 6].

There are several combination therapies reported in I/R brain injuries either with melatonin or memantine but a melatonin and memantine combination in relevant stroke models was not found in literature search. With this combination, targeting of almost all aspects of cerebral ischemic damage, namely; free radicals, inflammation and apoptosis by melatonin and excitotoxicity by memantine, is tried.

PARP inhibitors nicotinamide and 3-aminobenzamide are tested in combination with melatonin upon focal ischemia in rats and the combination therapies caused reduced volume of cerebral infarction than the individual treatments [91]. In another study in MCAO model in rats, meloxicam, an inhibitor of COX-2, has resulted in enhanced

neuroprotection when combined with melatonin in the treatments [92]. Moreover, in a mild ischemia model in mice, neuroprotective effects of melatonin administration have been reported when combined with t-PA, which is the only agent that can be clinically used in stroke victims [49]. These reports indicate that melatonin can be used as an add-on agent in existing therapies for cerebral disorders considering its tolerability at even high doses [2, 3].

Memantine in combination with ß2-adrenoceptor antogonist clenbuterol treatment resulted in reduced infarct size than the individual treatments both in a mouse model of cerebral ischemia and hippocampal neurons exposed to glutamate. Furthermore, addition of memantine to the treatment with clenbuterol prolonged the therapeutic window of clenbuterol upto 2 hours [93]. In hypoxic-ischemic brain injury, an AMPA/KA receptor blocker topiramate improved the pathological outcome and performance when combined with NMDA receptor blocker memantine [94]. Moreover, in excitotoxic injury model memantine combined with tea polyphenol, which is an antioxidant and anti-inflammatory agent, were able to improve the locomotor activity while none of the alone treatment were not [95]. Together with the combination therapies reported with melatonin, these results imply that melatonin and memantine combination therapy can have synergistic effects in stroke treatment.

4.3. BRAIN SWELLING

Upon MCAO, moderate brain swelling is observed in all experimental groups. Ischemic brain edema, expressed as percentage swelling of the ipsilateral hemisphere, was 11±1%, 6±2%, 8±2%, and 5±1% in Control, Melatonin, Memantine and Melatonin/Memantine treated groups, respectively. Compared with vehicle treated animals, brain swelling was statistically significant in Melatonin and Melatonin/Memantine treated animal group ($p<0.05$) as shown in Figure 4.4.

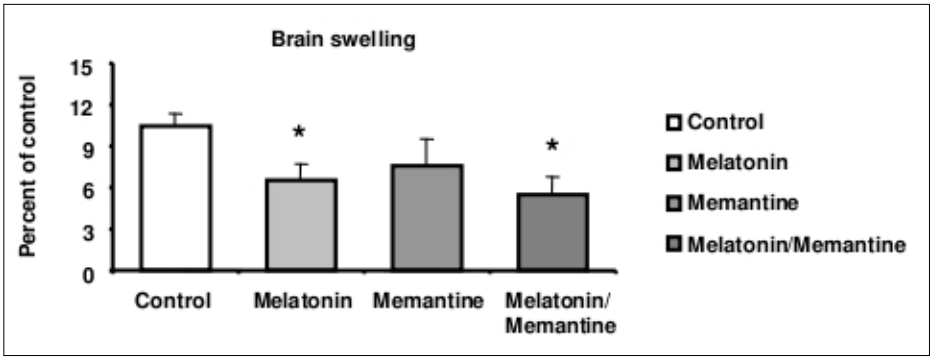

Figure 4.4. Brain swelling values of the experimental values (Mean ± S.E.M., * = $p<0.05$)

Interestingly, memantine-only treatment decreased brain swelling but the decrease was not significant. However, memantine and other NMDA antagonists have been reported to reduce brain swelling [5, 6, 85]. This difference is probably about the timing of the memantine administration since in studies where memantine reduced edema it is administered close to ischemia onset whereas in this study memantine is administered 90 minutes after ischemia onset. This implies that memantine's effects on early mechanisms responsible from edema formation such as ionic balance and excitotoxicity.

4.4. NEUROLOGICAL DEFICIT SCORES

24 hours after MCAO, neurological deficits associated with ischemia is observed in all groups and the improvement of ischemic injury by melatonin, memantine or melatonin/memantine treatments was accompanied by an improvement of neurological deficits. In the vehicle treated Control group mean neurological deficit scores was 1.8±0.2. This scores were significantly reduced to 1.1±0.2 in the melatonin treated animals ($p<0.05$), Neurological deficit scores were 1.4±0.2 and 1.3±0.2 in memantine and melatonin/memantine treated animals, respectively as shown in Figure 4.5.

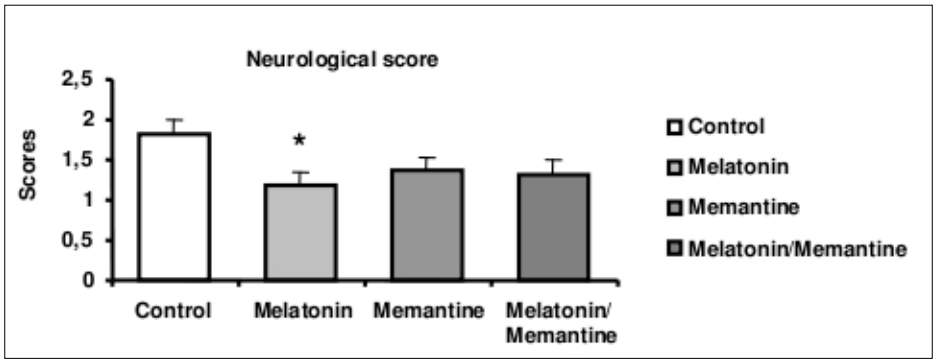

Figure 4.5. Neurological deficit scores of the experimental groups (Mean ± S.E.M., * = $p<0.05$)

Decrease in neurological deficit scores of ischemic animals in melatonin treated groups were consistent with the literature indicating various positive effects of melatonin in improved behavioral and performance aspects after cerebral ischemia [2-4]. Although NMDA antagonists are known to have certain side effects due to intervention with normal physiological activities of NMDA receptors, however; in our experimental settings it is not possible to come into conclusions about the neurological effects of memantine after cerebral ischemia.

5. CONCLUSION and RECOMMENDATIONS

5.1. CONCLUSION

In this study, effects of melatonin and memantine treatments as alone or in combination were analyzed after MCAO model of stroke in mice. Blood flow over the ischemia area was monitored with LDF throughout the experiments. After 24 hours neurological deficits were scored and animals were anesthetized and decapitated. Infarct volumes and brain swelling values calculated after cresyl violet staining of brain sections. All melatonin, memantine and melatonin/memantine treated groups resulted in statistically significant reduced infarct volumes when compared to vehicle treated control group. Brain swelling also decreased in all treatment groups when compared to control group but the differences were significant in melatonin only and melatonin/memantine groups. Neurological deficits were also decreased in all treatments groups when compared to control group but the differences were only significant in melatonin group. These results prove that combination therapy including melatonin and memantine is a relevant and beneficial option for clinical treatment of stroke.

5.2. RECOMMENDATIONS

As a future work, the same melatonin/memantine combination therapy can be used with different concentrations and administration timing profiles can be used in experimental stroke models. Also different combinations including melatonin or memantine, or even a triple combination therapy including both, can be tried in cerebral ischemia treatment to cover all aspects of ischemic damage. Moreover, long term effects or behavioral aspects of this combination therapy can be studied in milder versions of cerebral ischemia models.

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