THE ROLE OF PI-3 KINASES/AKT PATHWAY ON THE NEUROPROTECTIVE EFFECT OF MELATONIN AFTER CEREBRAL ISCHEMIA

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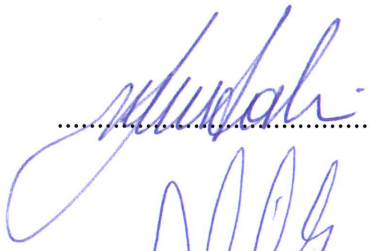
Prof. Dr. Fikrettin Şahin ..... 
(Supervisor)

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“this thesis is dedicated to
My family”

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I would like to thank; first of all my advisor Prof. Dr. Fikrettin ŞAHİN, who opened his laboratories for my studies and being a magnificent boss. I am also thankful to Assoc. Prof. Ülkan KILIÇ for teaching me molecular biology protocols and Assist. Prof. Yalçın GÜNAL for modestly sharing his excellent physiology knowledge.

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ABSTRACT

THE ROLE OF PI-3 KINASES/AKT PATHWAY ON THE NEUROPROTECTIVE EFFECT OF MELATONIN AFTER CEREBRAL ISCHEMIA

Apart from its metabolic functions, melatonin is a potent neuroprotective molecule owing to its antioxidative actions. However, the roles of melatonin receptors and signalling in the neuroprotective effects of melatonin after cerebral ischemia remain unknown. With the use of mice model of cerebral ischemia and PI-3Kinase/AKT pathway inhibitor wortmannin, I evaluated the effects of melatonin on functional recovery, brain injury, edema formation, signaling pathways resulting from ischemic brain injury.

In this study, It is shown that melatonin treatment significantly decreases the infarct volume and brain edema after middle cerebral artery occlusion and this decrease is reversed by i.c.v injection of wortmannin. It is also demonstrated that melatonin treatment increases AKT phosphorylation after cerebral ischemia and the inhibition of AKT with Wortmannin reverses neuroprotective action of melatonin after 30 and 90 min of focal cerebral ischemia. Furthermore, melatonin treatment increases the number of surviving neurons, and decreases the number of TUNEL positive cells, which is a indicator of apoptosis.

These results provide evidence that the neuroprotective effects of melatonin appear to be mediated through PI-3Kinase/Akt pathway. Furthermore, the robust functional improvement encourages proof-of-concept studies with melatonin in stroke patients.

ÖZET

SEREBRAL İSKEMİ SONRASI PI-3 KINAZ/AKT YOLAĞININ MELATONİNİN NÖROPROTEKTİF ETKİSİ ÜZERİNDEKİ ROLÜ

Metabolik fonksiyonlarının yanı sıra, antioksidatif etkileri nedeniyle melatonin kuvvetli bir nöroprotektif moleküldür. Ancak, serebral iskemi sonrasında melatoninin nöroprotektif etkileri dahilinde melatonin reseptörlerinin görevi ve sinyal iletimi hala bilinmemektedir. Farede serebral iskemi modeli ve PI-3 Kinaz/AKT yolağı inhibitörü wortmannini kullanarak, melatoninin iskemik beyin hasarından kaynaklanan fonksiyonel geri kazanım, beyin hasarı, ödem oluşumu, sinyal yolaklarına etkilerini değerlendirdik.

Bu çalışmada, orta serebral arter oklüzyonu sonrası melatonin tedavisinin beyin hasarı ve beyin ödemini anlamlı derecede azalttığı ve beyiniçi Wortmannin enjeksiyonunun bunu geriye çevirdiği gösterilmiştir. Ayrıca, melatonin tedavisinin serebral iskemi sonrasında AKT fosforilasyonunu arttırdığını ve Wortmannin ile AKT inhibisyonunun 30 ve 90 dakika fokal serebral iskemi sonrasında melatoninin nöroprotektif etkisini tersine çevirdiği gösterilmiştir. Buna ilaveten, melatonin tedavisinin, yaşayan nöron sayısını arttırdığı ve apoptoz göstergesi olan TUNEL pozitif hücre sayısını azalttığı görülmüştür.

Bu sonuçlar melatoninin nöroprotektif etkilerinin PI3K/Akt yolağı aracılığıyla gerçekleştiğine dair kanıt sunmaktadır. Ayrıca, kuvvetli fonksiyonel iyileşme felçli hastalarda melatonin ile kavram ispatı çalışmalarını desteklemektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

AGC	Protein kinase a, g, c family
BAD	Bcl-2 associated death protein
BAX	Bcl-2 associated X protein
BCL-2	B-cell lymphomal leukaemia
BIM	Bcl-2 interacting mediator of cell death
cAMP	Cyclic-adenosine monoPhosphate
FOXO1	The forkhead transcription factor
GSK3	Glycogen synthase kinase
IAP	Inhibitors of apoptosis
IKK	IkB kinase
mTOR	Mammalion target of rapamycin
NF-kB	Nuclear factor kappa b
PI-3 Kinase	Phosphatidylinositide 3 kinase
PIP2	Phosphatidylinositide (3, 4) bisphosphate
PIP3	Phosphatidylinositide (3, 4, 5) triphosphate
PDK-1	Phosphoinositide dependent kinase
PKA	Protein kinase a
PKB	Protein kinase b
PKC	Protein kinase c
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
RAS	Rat sarcoma
S6K1	Ribosomal protein kinase1
TNF	Tumor necrosis factor

1. INTRODUCTION

Melatonin is not only a circadian rhythm related hormone but also has good effects on treatments of neurological diseases. Apart from its metabolic functions, melatonin is a potent neuroprotective molecule owing to its anti-oxidative actions. However, the roles of melatonin receptors and signaling pathways in the neuroprotective effects of melatonin after cerebral ischemia remain unknown.

Cerebral ischemia is a medical condition results from blockage of the blood flow to the brain. According to the world health organization, ischemic stroke is the third most common cause of death for developing countries. Since melatonin is primarily produced by the pineal gland inside the brain and a powerful antioxidant agent, it is reasonable to hypothesize that melatonin treatment will have beneficial effects on stroke patients. Various studies have shown neuroprotective effects of numerous sorts of melatonin treatments on *in vitro* model systems and/or animal models of cerebral ischemia [2-4].

In this study, the role of PI-3 Kinases/AKT Pathway on the neuroprotective effect of melatonin treatment is examined with the use of PI-3 Kinase inhibitor wortmannin, in a mouse model of transient focal cerebral ischemia.

2. THEORETICAL BACKGROUND

In most metabolic process of eukaryotic cells, the activities of almost all enzymes, membrane dynamics, channels on the membranes, activation/deactivation of signaling pathways are reversible controlled by kinases (phosphorylation) or phosphatases (de-phosphorylation) [5].

Protein kinases are the members of the largest protein families known for more than 500 enzyme members in human being, enzymes that modify other proteins via transferring phosphate groups from mostly ATP to them.

In this chapter, general information on the PI-3 Kinases/AKT Pathway, melatonin and its receptors, antioxidative character of melatonin, stroke and transient focal cerebral ischemia is briefly explained.

2.1. PHOSPHATIDYLINOSITIDE 3 – KINASE (PI-3 K)

Non-apoptotic cell death, autophagy and programmed cell death, apoptosis affect the route of neurodegenerative disorders, including Huntington's disease, Alzheimer's disease, Parkinson's disease, Epilepsy and Cerebral Ischemia. Autophagy can help to repair and remove non-functioning organelles of a cell and apoptosis assist with tissue remodeling and regeneration during development and cell injury [6].

Modulation of apoptosis can be controlled through Phosphatidylinositide 3-kinase (PI 3-K) Pathway. PI 3-K is a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositide and phosphoinositides and regulates metabolism, cell growth and survival [7].

2.1.1. Sub-classes of PI – 3 Kinase

Based on their structure and substrate specificity, the PI 3-K family is grouped into three classes, designated as classes I, II and III.

Class I, PI 3-K consists of two subunits, a regulatory subunit (p85α, p85β, and p85γ) and a p110 catalytic subunit (p110α, p110β, p110γ, and p110δ) This kinase phosphorylates phosphoinositide (PI), phosphatidylinositide 3-phosphate (PI-3-P), and phosphatidylinositide (3,4)-bisphosphate (PI-3, 4-P2), producing PI-3-P, PI-3, 4-P2, and phosphatidylinositide (3,4,5)-triphosphate (PI-3, 4, 5-P3) respectively.

Class II, consists of only a p110 catalytic subunit that phosphorylates PI and PI-3-P, resulting in the production of PI-3-P and PI-3, 4-P2.

In contrast, **class III** consists of vacuolar protein - sorting defective 34 (Vps34) catalytic subunit. It only functions through PI to produce PI-3-P.

2.1.2. Activation of PI-3 Kinases

PI-3 Kinases are regulated via different sub-classes. First of all, for the activation of PI-3 Kinases, tyrosine kinase and G protein-coupled receptors should be stimulated by growth factors or cytokines, this recruit PI-3 Kinase to the plasma membrane.

Tyrosine-phosphorylated proteins, which are not receptors, have function as adaptors linking PI-3 Kinase class I, to membrane-associated proteins co-localizing the enzyme with its substrate. The association with tyrosine phosphorylated proteins increases the activity of the enzyme. For activation of PI-3 Kinase class I, binding of its p85 SH3 domains to proline-rich regions of diverse proteins or association of its p110 catalytic subunit to GTP loaded Ras are required [8].

After activation, PI-3 Kinases phosphorylate phosphatidylinositide (3,4)-bisphosphate (PI(3,4)P2, PIP2), producing phosphatidylinositide (3,4,5)-triphosphate (PI(3,4,5)P3, PIP3). Increased number of PIP3 on the cell membrane stimulates Akt, mediates transition of Akt to cell membrane. It also causes colocalization of phosphoinositide dependent kinase (PDK-1), and finally Akt is phosphorylated by PDK-1 on the serine 473 and threonine 308 residues.

2.1.3. Inhibition of PI-3 Kinases

PI -3 Kinases have two well-known inhibitors; wortmannin, irreversible inhibitor and LY294002 Hydrochloride (2-(4-Morpholino)-8-phenyl-4*H*-1-benzopyran-4-one), reversible inhibitor that are a potent and specific for PI-3 Kinases.

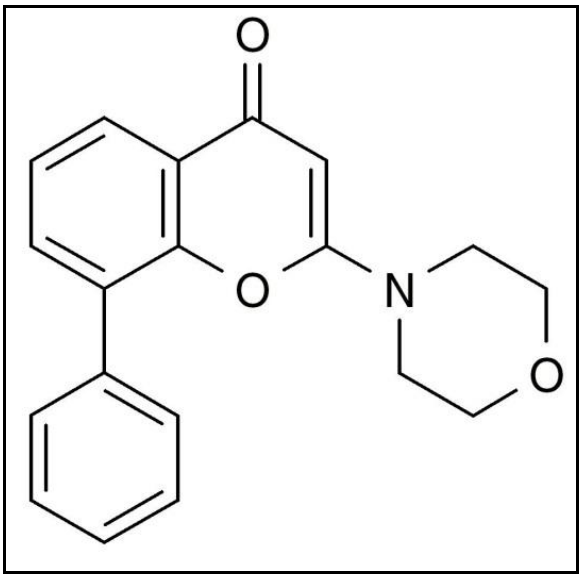

Figure 2.1. Chemical structure of PI-3 Kinase inhibitor: LY294002

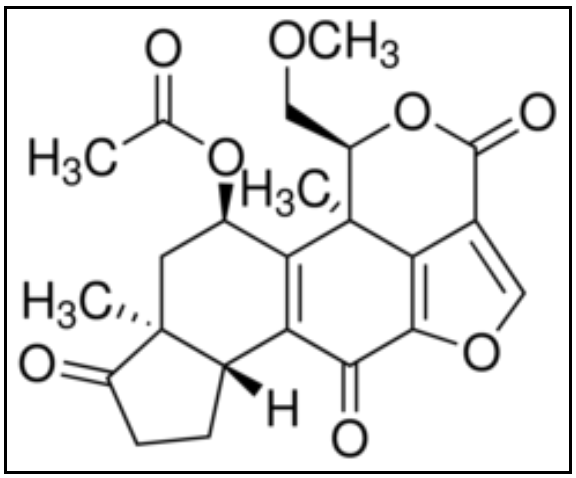

Figure 2.2. Chemical structure of PI-3 Kinase inhibitor: Wortmannin

2.1.4. PI-3 Kinase/AKT and Other Signaling Pathways

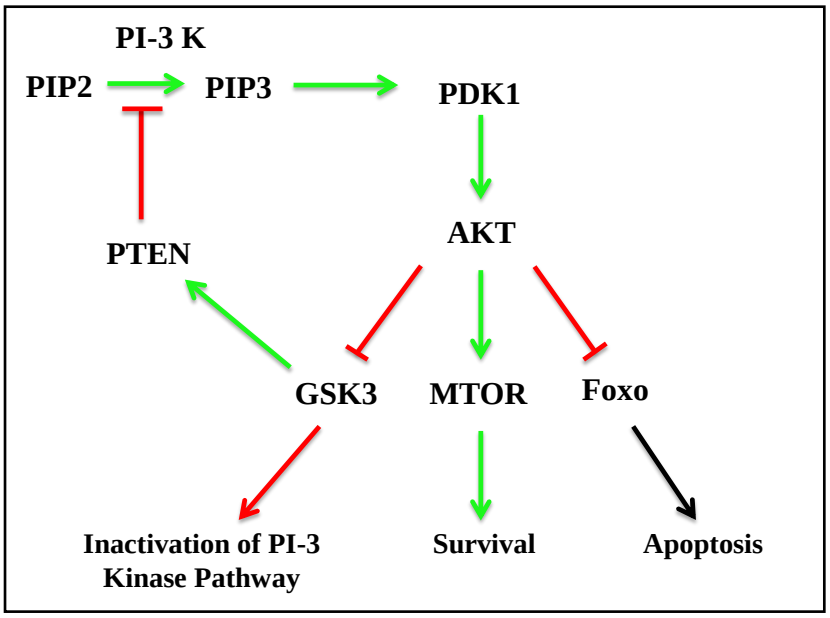

Figure 2.3. Schematic design of PI-3 Kinase/Akt and other signaling pathways

The cellular signaling pathways of PI 3-Kinase/Akt, consist of kinases and phosphatases, works in consistency. Mammalian target of rapamycin (mTOR), glycogen synthase kinase (GSK3) and phosphatase and tensin homolog deleted from chromosome 10 (PTEN), are tightly linked in this pathway and all together, they modulate cellular growth, survival, proliferation or apoptosis.

In physiological conditions, PIP2 is phosphorylated by PI-3 Kinase, this accumulates PDK1 to plasma membrane and PDK1 phosphorylates (activates) Akt. Phosphorylated Akt, 1-) phosphorylates GSK3 which makes it inactive. 2-) phosphorylates mTOR which makes it active. 3-) phosphorylates Foxo which makes it inactive. PI 3-Kinase/Akt can regulate multiple targets, including -inhibiting the activity of pro-apoptotic BAD protein, and GSK3, the forkhead transcription factor (FOXO1) which is responsible for cell cycle arrest and associated with 14-3-3 proteins and pro-apoptotic BIM protein and activating mTOR which is central modulator of cellular metabolism.

As a major negative regulator of PI-3Kinase/Akt pathway, active GSK (non-phosphorylated) phosphorylates PTEN, phosphatase, which makes it active, dephosphorylates PIP3 to PIP2, resulting non-phosphorylation of Akt and inactive Akt.

2.2. AKT, PROTEIN KINASE B

In mammals, protein kinase B (PKB), also known as Akt (60 kDa) is a cytoplasmic serine/threonine kinase and belongs to the cyclic-Adenosine MonoPhosphate (cAMP) - dependent kinase and AGC kinase superfamily of protein kinases and consists of three functional domains: The N-terminal Plectrin Homology (PH), Catalytic domain and The C-terminal hydrophobic motif [7].

After identification of the AKT8 retrovirus that is responsible for spontaneous thymoma in AKR strain of mice, it was found that there exists a serine/threonine kinase which is similar to protein kinase A (PKA), responsible for regulation of glycogen, sugar and lipid metabolism as cAMP-dependent manner, and protein kinase C (PKC), responsible for regulation of transcription, cell growth and several signal transduction cascades and it has been termed as protein kinase B (PKB) or AKT, where AK stands for strain of mice, and T stands for thymoma. Up today, AKT have three family members, Akt1 or PKB alpha, Akt2 or PKB beta, Akt3 or PKB gamma.

Akt, protein kinase B has a major role in numerous physiological and pathophysiological processes as multiple cellular response, including signalling from PDK1, activation of Akt, to downstream targets such as GSK-3, mTOR, Bad, Bim, the forkhead family members (FOXO) and GLUT4 translocation. Akt is not only involved in apoptosis, but also its role in glucose metabolism, cytoskeleton regulation, protein synthesis and many other cellular processes had been presented [8, 9].

Except from physiological role, Akt has a critical and central role in a variety of pathways that is related to cell survival and inflammatory processes such as phosphorylation of proteins IKKa, responsible for inflammation response, NFkB, responsible for controlling the DNA transcription, GSK-3, responsible for phosphorylation of PTEN which resulting inactivation of Akt.

2.2.1. Activation of Akt

Activation of Akt, protein kinase B, is necessary for cell survival and repairing cellular injury which is shown by a number of different studies. Activation of Akt requires phosphorylation of PIP3 from PIP2, which activates PDK1, translocation of Akt from cytosol to plasma membrane, binding through its PH domain, finally Akt becomes ready for phosphorylation at serine473 and threonine308 by phosphoinositide dependent kinase (PDK) 1 and PDK2 respectively [9, 10].

2.2.2. Downstream targets of Akt, Protein Kinase B

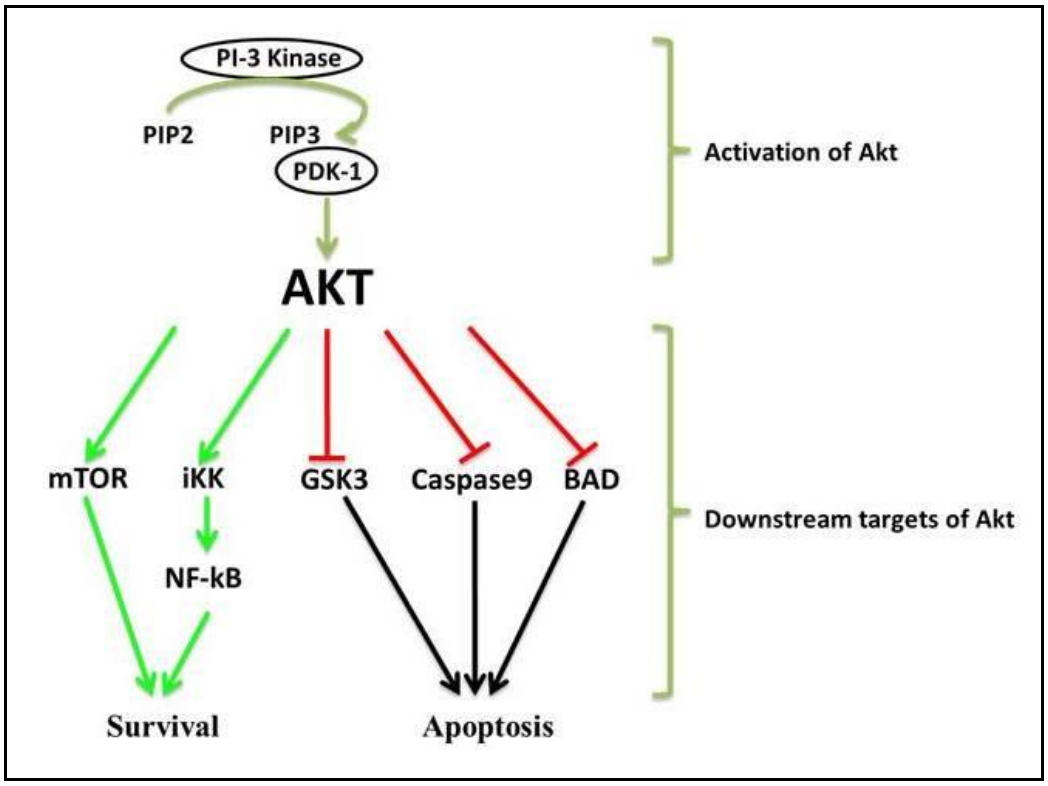

Figure 2.4. Downstream targets of Akt pathway

The downstream targets of the Akt, protein kinase B include BAD (Bcl-2 associated death protein), caspase 9, mTOR, GSK3, CREB, iKK, NF-kB and the forkhead transcription factor (FOXO). Akt phosphorylates pro-apoptotic protein BAD at serine136 and caspase-9 at ser196 which results inactivation of BAD and blocking caspase 9 activation. BAX is critical for permeabilization of mitochondria and let it release cytochrome c. Phosphorylation of BAD by Akt results in inhibiting the activity of BAX,

and increases the integrity of mitochondria that protects it from releasing cytochrome c. Release of cytochrome c from mitochondria activates cleavage of procaspase 9 to caspase 9 and also formation of the apoptosome complex.

For physiological conditions, the role of GSK3 is to synthase glycogen starting from the activation of insulin receptor pathway. In pathophysiological conditions, GSK3β is known to inactivate survival factors via phosphorylation at thr216 and induce caspase 3, BCL-2 (B-cell lymphomal leukaemia) and BAX. Akt can also phosphorylate GSK-3β at serine9 and inactivate this enzyme, thus preventing GSK-3β from initiating an apoptotic pathway via caspase 3 activation and release of cytochrome c from mitochondria.

mTOR, similar to Akt, is a member of the PI-3 Kinase related serine/threonine superfamily, which is responsible for transcription, cytoskeleton organization, and cell growth in physiological conditions. when, phosphorylation and activation of mTOR at ser2448 by Akt, causes it to phosphorylate its downstream protein ribosomal protein kinase1 (S6K1) that modulate protein translation and cell survival [11, 12].

Akt also prevent apoptosis through Nuclear Factor kappa B (NF-kB) and IkB kinase (IKK) pathway. Phosphorylation of IKK at thr23 site by Akt, activates it, then it regulates NF-kB activity. Then, NF-kB inhibits the activity of caspase 3, 7 and 9 by stimulating the expression of the inhibitors of apoptosis (IAP) protein family and also supresses the impact of the Tumor Necrosis Factor (TNF) alpha related apoptosis.

2.2.3. Akt; Survival or Apoptosis

Akt may also rescue cells from apoptosis by inhibiting the Bax-independent apoptosis pathway through a forkhead box transcription factor (65 – 67). Other death genes such as Fas, Fas ligand, and Bim are upregulated in response to apoptotic stimuli during brain ischemia (52, 54). Fas and Fas ligand trigger activation of caspase 8 through the adaptor molecule FADD (Fas-associated via death domain) and in turn caspase 8 either directly activates caspase 3 or induces the translocation of Bid, another apoptotic Bcl-2 family member, to the mitochondria. The most compelling targets for Akt include Bad, caspase 9, members of the Forkhead family of transcription factors (FOXO), CREB and NF- B. The Bcl-2 family comprises proapoptotic and antiapoptotic proteins implicated in the process of death signaling in mitochondria [13, 14].

Evidence has been presented that apoptosis appears in the peripheral penumbra of ischemia [4, 5] and PI3K/Akt pathwaymediates neuronal survival after cerebral ischemia and reperfusion [6–8]. Phosphorylation of Akt promotes cell survival against cerebral ischemic insult by phosphorylation and subsequent inactivation of many proapoptotic proteins, such as glycogen synthase kinase 3 beta (GSK3 beta) [7], Bad [9], and Forkhead transcription factors [10]. Although there is growing evidence that PI3K/Akt pathway and neuronal survival following cerebral ischemia are closely correlated, few studies further clarify whether PI3K/Akt pathway contributes to the protection of NVU after cerebral ischemia and reperfusion.

2.3. MELATONIN

Melatonin is a well-studied signaling molecule which is produced by pineal gland and travels to the other body parts via the blood circulation. The amount of melatonin that is produced by the pineal gland fluctuates and is increased at night, while its amount in the plasma drops during daytime [15, 16].

The production of melatonin by pineal gland is shown to be under the control of the suprachiasmatic nucleus (SCN) of the hypothalamus. Melatonin is synthesized from a neurotransmitter which is called serotonin through a two-step pathway. Initially, serotonin is acetylated by arylakylamine *N*-acetyltransferase into *N*-acetylserotonin. Next, *N*-acetylserotonin is methylated by hydroxyindole *O*-methyltransferase so as to synthesize melatonin [18, 19, 20]. Moreover, majority of melatonin in the circulation is catabolyzed in the liver.

Even though pineal gland is the main producer of melatonin in the body, it is also produced in several other tissues, such as gastrointestinal tract [21], skin [22], bone marrow [23], retina [24], lymphocytes or platelets [25], however when the plasma melatonin levels are considered, the amount of melatonin which is produced from these sites is not significant.

Although it is thought to act as a synchronizer for the body, melatonin is also associated with several other functions. For instance, it is one of the most powerful antioxidant compounds in the body [8]. It has been reported that melatonin harbors oncostatic [30] and immunomodulatory [31] features. In several studies, it has been associated with increased cell survival in cardiovascular and reproductive systems [16, 17, 26, 27, 28].

2.4. MELATONIN RECEPTORS AND INTERACTING PROTEINS

There are two mammalian high affinity membrane receptors that are known to bind to melatonin; MT_1 and MT_2. These two receptors belong to the seven transmembrane G-protein coupled receptor family of proteins and share more than 90% similarity in their amino acid sequences. Although these two receptors have similar pharmacological profiles, MT_1 exerts a slightly higher affinity to melatonin [32-34]. In addition, MT_3 is another low affinity melatonin receptor which has been identified as a quinone reductase 2 enzyme [32-34].

Interestingly, MT_1 and MT_2 both contain a unique NRY motif in their intracellular loop II, unlike the other members of the G-protein coupled receptor family which are known to contain a DRY or ERY motif [32]. MT_1 and MT_2 are shown to communicate with several G-proteins based on the tissue and cell that they are located in. Upon binding of melatonin, MT_1 is activated and causes a reduction in the cAMP levels, protein kinase A activity and phosphorylation of the transcription factor CREB. Activated MT_1 also amplifies the phosphorylation levels of MEK1 and 2, ERK1 and 2 and JNK. Activation of MT_2 is also associated with a decrease in cAMP levels.

Melatonin receptors MT_1 and MT_2 are found in various tissues, including 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 [33, 34].

2.5. MELATONIN AS AN ANTIOXIDATIVE AGENT

Melatonin is able to neutralize the dangerous effects of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). It has been shown that melatonin is even more powerful than vitamin E in neutralizing the effects of free radicals. This is mainly because not only melatonin but also the products of its reaction with ROS/RNS as well as other metabolites of melatonin contain high antioxidant capacity as well. On top of that, melatonin induces the expression of other antioxidant enzymes, while inhibiting the pro-oxidative enzymes. It has been shown that melatonin can alter the levels of mitochondrial and cytosolic superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase. It can further cause a decrease in the levels of neural and inducible nitric oxide synthase (NOS) [29, 35, 36].

Melatonin can scavenge the hydroxyl radical (•OH), carbon trioxide ($CO_3^{\bullet-}$), superoxide ($O_2^{\bullet-}$), nitric oxide (•NO), nitrogen dioxide ($\bullet NO_2$) and peroxynitrite ($ONOO^-$) and can be converted into AFMK (N^1-acetyl-N^2-formyl-5-methoxykynuramine) and AMK (N^1-acetyl-5-methoxykynuramine) via ROS/RNS scavenging reactions and through several other enzymatic and non-enzymatic reactions. Perhaps not surprisingly, AFMK and AMK also possess antioxidant properties. [17, 29, 35-39].

2.6. 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. It is also 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 [40, 41].

3. MATERIALS & METHODS

3.1. EXPERIMENTAL SETUP AND GROUPS

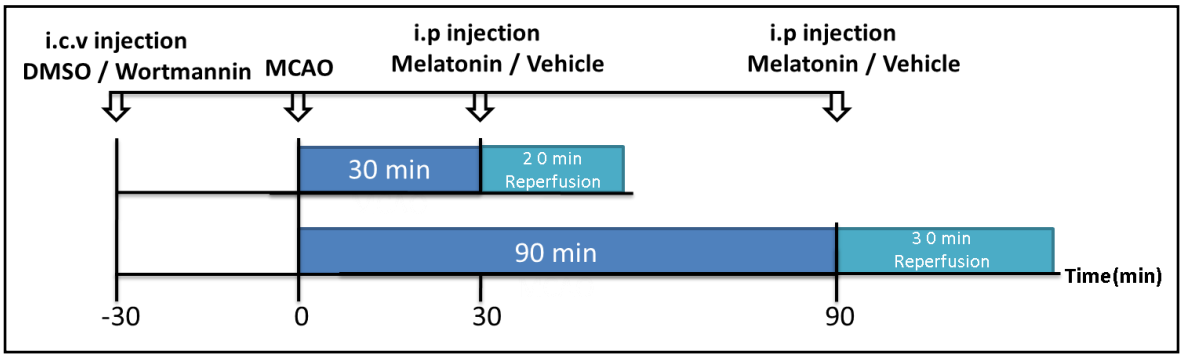

Figure 3.1. Representation of experimental setup

All experimental procedures were carried out 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 (20-25 g) randomly assigned to the following experimental groups for 30 min MCAO 72 h reperfusion or 90 min MCAO 24 h reperfusion: DMSO (Sigma-Aldrich, Germany), (n = 7); received i.c.v injection of DMSO 30 min prior to cerebral ischemia and i.p injection of 0.9% saline immediately at the reperfusion onset, DMSO and Melatonin (n = 7); received i.c.v injection of DMSO 30 min prior to cerebral ischemia and i.p injection of 4 mg/kg melatonin (Sigma-Aldrich, Germany) dissolved in 0.9% saline immediately at the reperfusion onset, Wortmannin (Sigma-Aldrich, Germany) (n =7); received i.c.v injection of Wortmannin (dissolved in 100% DMSO) 30 min prior to cerebral ischemia and i.p injection of 0.9% saline immediately at the reperfusion onset, Wortmannin and Melatonin (n = 7); received i.c.v injection of Wortmannin 30 min prior to cerebral ischemia and i.p injection of 4 mg/kg melatonin dissolved in 0.9% saline immediately at the reperfusion onset.

3.2. INDUCTION OF CEREBRAL ISCHEMIA & REPERFUSION

Animals were anesthesized with 1.5% 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). During the experiments, 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 MCA occlusion (MCAO) was induced using a intraluminal filament technique [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 7-0 nylon monofilament (Doğsan, Turkey) 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.

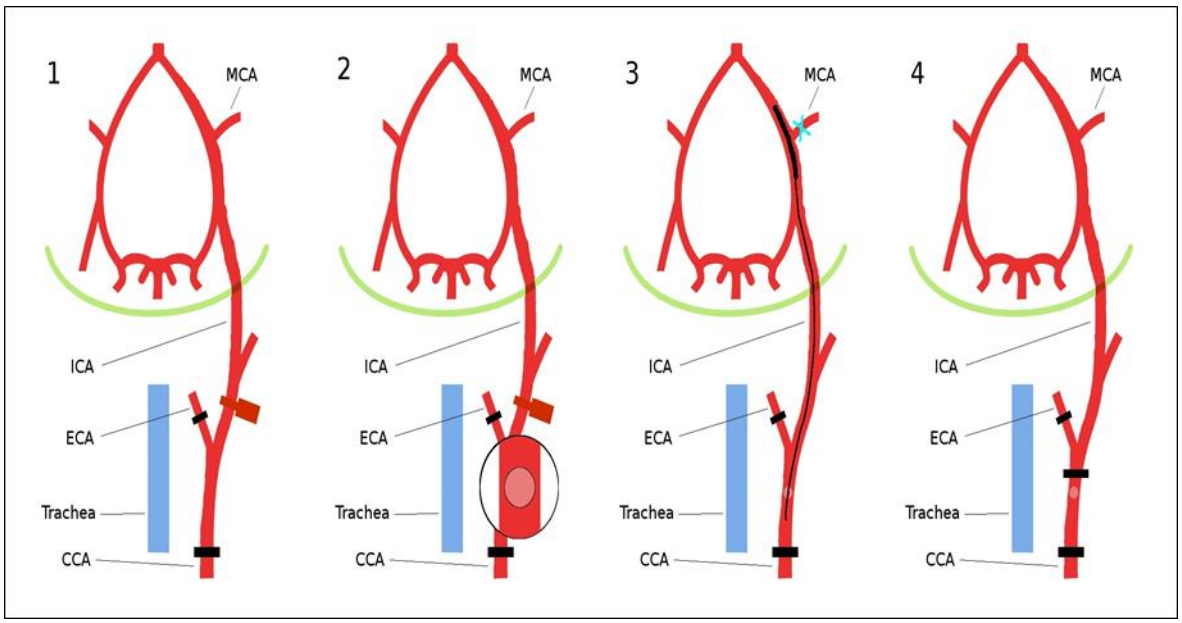

Figure 3.2. Graphical representation of the surgical operation of cerebral ischemia

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

72 h after 30 min MCAO or 24 h after 90 min MCAO, animals were reanesthesized 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. Briefly sections were fixed with 4% Paraformaldehyde (PFA), washed with Phosphate Buffered Saline (PBS), stained with cresyl violet, alcohol series, mounted with Entellan.

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.

3.4. ANALYSIS OF DNA FRAGMENTATION

From animals subjected to 30 min MCAO 72 h reperfusion, brain section from mid-striatum level were stained by Terminal transferase biotinylated-dUTP nick End Labeling (TUNEL) using a in situ cell death detection kit (Roche, Switzerland). Briefly, sections were fixed with 4% PFA, washed with PBS, antigen retrieval with citrate, blocked with Normal Goat Serum (NGS), reacted with TUNEL reaction mixture and finally counterstained with DAPI. In stained sections, TUNEL positive cells, DNA-fragmented cells, were counted in nine random regions of interest (ROI) in the striatum. Mean values were calculated for all areas ipsilateral (ischemic) to stroke.

3.5. ANALYSIS OF SURVIVING NEURONS

From animals subjected to 30 min MCAO 72 h reperfusion, cresyl violet stained sections from bregma level were microscopically evaluated by counting the density of surviving medium to large sized neurons in the striatum in the same nine random ROI in the striatum. Mean values were calculated for all areas both ipsilateral (ischemic) and contralateral (non-ischemic) to the stroke. Finally, the percentage of surviving neurons was calculated.

3.6. ANALYSIS OF SERUM IGG EXTRAVASATION

From animals subjected to 90 min MCAO 24 h reperfusion, brain sections obtained from the mid-striatum were stained by using Universal Elite ABC-Peroxidase kit(pk 7100; Vector Lab. USA) via standard peroxidase staining protocol. Briefly, sections were rinsed for 10 min at room temperature in shaker in PBS, fixed in 4% PFA. Endogenous peroxidase were blocked with methanol/0.3% H2O2. Sections were immersed in 0.1 M PBS containing 2% normal horse serum, were incubated for 1 h in biotinylated anti-mouse IgG (Santa Cruz, Switzerland) and stained with an avidin peroxidase kit (Vectostain Elite) and diaminobenzidine (pk-4100; Vector Lab, USA). Sections were scanned, converted into gray values, and densitometrically analyzed by subtracting optical densities in the contralateral nonischemic from those in the ischemic cortex and striatum, thus evaluating IgG extravasation in the core of the MCA territory.

3.7. PROTEIN QUANTIFICATION BY WESTERN BLOT

From animals subjected to 90 min MCAO 24 h reperfusion, brain tissue samples were harvested from ischemic and non-ischemic hemispheres individually by using cryostat. Tissue samples from the same groups were pooled, homogenized and treated with protease inhibitor cocktail and phosphatase inhibitor cocktail. Protein concentration was measured by using Qubit Protein Assay Kit via Qubit 2.0 Fluoremeter according to manufacturers protocol (Invitrogen, life technologies, USA).

At first day, equal amounts of protein (20 μg) were loaded into 4-12 % NuPAGE gel, run for 1 h at 150 V, then transferred to PolyVinylidene Fluoride Membrane (PVDF) via iBlot Dry Blotting System (Invitrogen, life technologies, USA).

After transfer, membranes were blocked in 5% non-fat milk in 50 mm Tris-buffered saline containing 0.1% Tween twenty (TBS-T), for 1 h at room temperature on shaker. Membranes were washed in TBS-T and incubated overnight with rabbit monoclonal anti phospho-akt (4060; Cell Signaling Technology, USA) or rabbit monoclonal anti phospho-GSK 3 Alpha/Beta (8566; Cell Signaling Technology, USA) or rabbit polyclonal anti phospho-PTEN (9554; Cell Signaling Technology, USA) at 1:1000 dilution.

At second day, membranes were washed in TBS-T and goat anti-rabbit (sc-2004; Santa Cruz Biotechnology, USA) diluted 1:5000 in blocking solution for 1 h at room temperature on shaker. Blots were performed at least three times. Protein loading was controlled by stripping and reprobing the blots with rabbit polyclonal anti-b-actin antibody (4967; Cell Signaling). The blots were developed using ECL-Advanced Western Blotting Detection kit (Amersham, GE Health Care UK Limited) and visualized by the fusion FX7 (DNR, Medsantek, Istanbul, Turkey). Protein levels were analyzed densitometrically using the ImageJ program and corrected with values determined on b-actin blots and expressed as relative values compared to control.

3.8. 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.D. with n values indicating the number of different animals analyzed. P values < 0.05 are considered statistically significant.

4. RESULTS

4.1. LASER DOPPLER FLOWMETRY

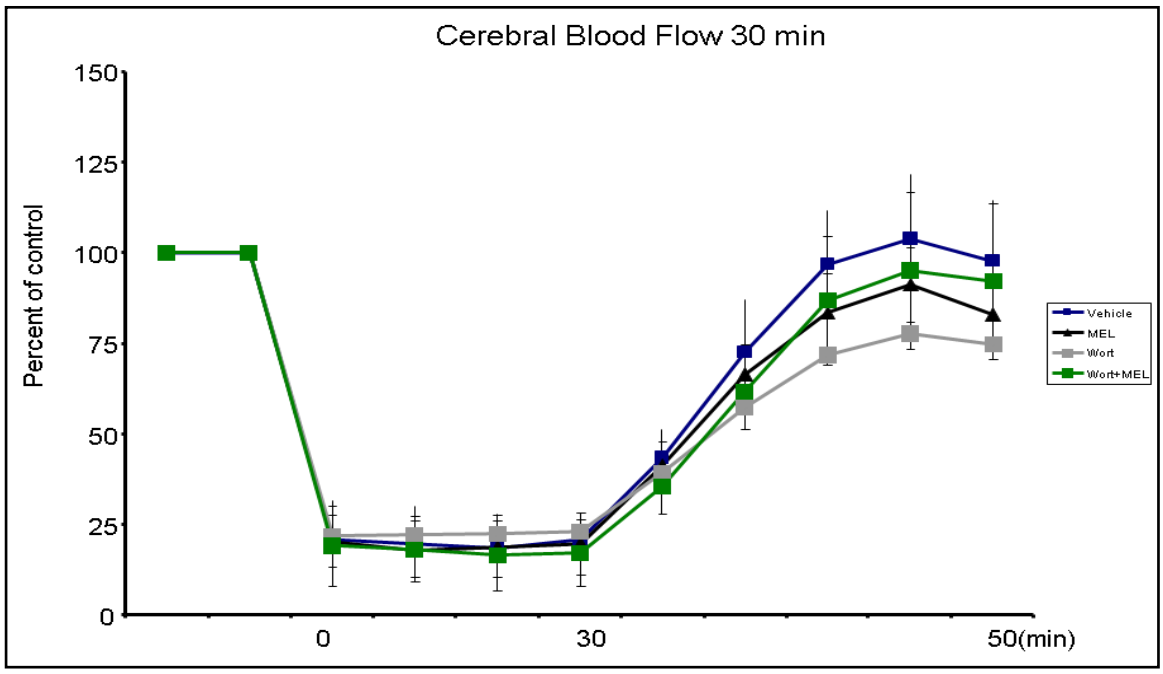

Figure 4.1.1. Laser Doppler flow (LDF) during and after ischemia for 30 min MCAO + 72 h reperfusion groups

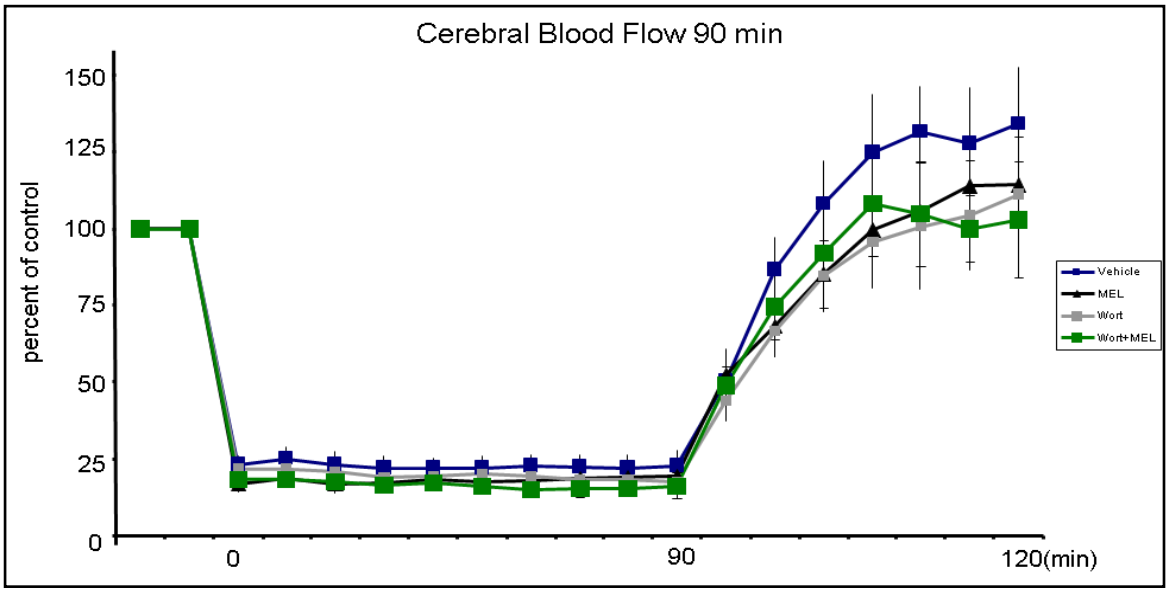

Figure 4.1.2. Laser Doppler flow (LDF) during and after ischemia for 90 min MCAO + 24 h reperfusion groups

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 51].

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 [50, 51], intraluminal MCA thread occlusion resulted in a sharp decrease of cerebral blood flow to ~20% of the pre-ischemic control values in the MCA territory

4.2. INFARCT VOLUMES

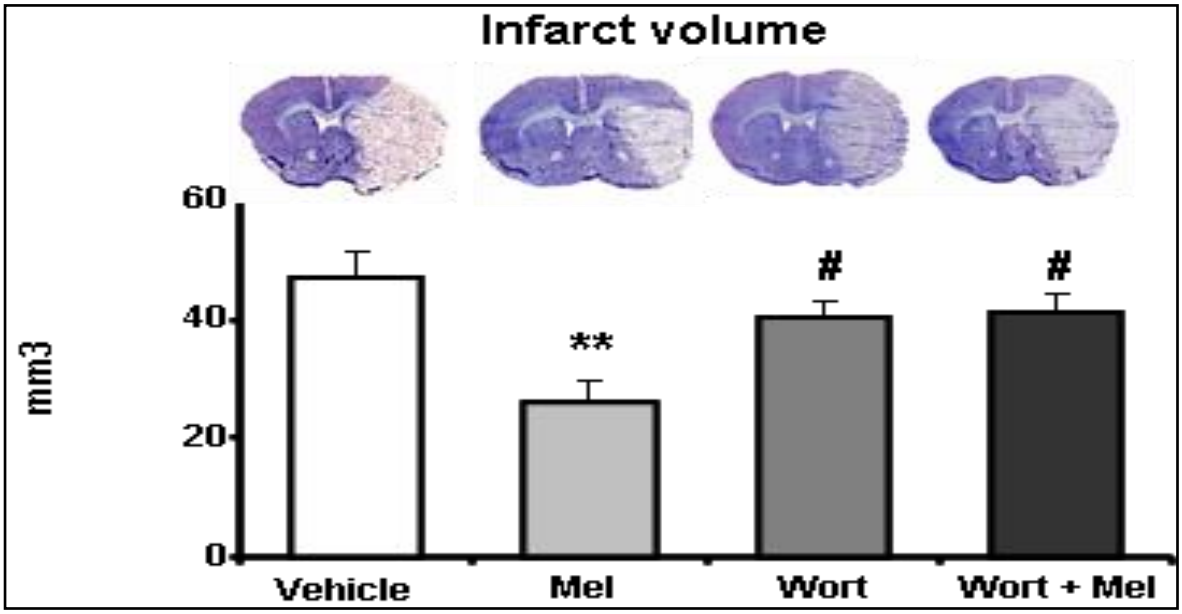

Figure 4.2. Cresyl violet staining and schematic representation of the changes in infarct volume after MCAO

For 90 min MCAO + 24 h reperfusion groups, infarct volume analyses were done after cresyl violet staining on frozen sections. For all groups, after cerebral ischemia reproducible brain infarct was detected and noted.

Melatonin treatment was statistically significant when compared to both vehicle-treated ($p<0.01$) and wortmannin groups ($p<0.05$). Although melatonin was administered following the inhibition of PI-3 Kinase/Akt pathway with wortmannin, melatonin treatment did not decrease the infarct volume as much as melatonin-only treatment, which leads us to the point that reduction in the infarct volume is directly related to the PI-3 Kinase/Akt pathway.

Decreases of infarct volume caused by melatonin treatment were reversed by wortmannin, thus PI-3 Kinase/Akt pathway plays a critical protective role in the reduction of infarct size after cerebral ischemia.

4.3. BRAIN SWELLING

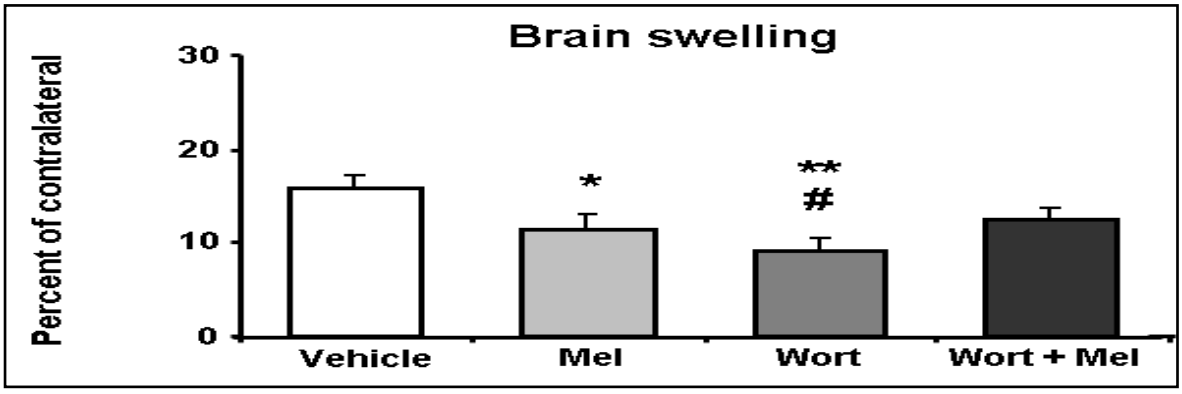

Figure 4.3. Changes in brain swelling after 90 min MCAO + 24 h reperfusion

For 90 min MCAO + 24 h reperfusion groups, brain swelling analyses were done after cresyl violet staining on frozen brain sections.

Melatonin treatment was statistically significant when compared to the vehicle-treatment ($p<0.05$). Surprisingly, only i.c.v. injection of wortmannin significantly decreased the brain swelling when compared to vehicle-treated ($p<0.01$) and melatonin-only treatment groups ($p<0.05$).

Brain swelling as a result of injury may result from several causes, and elucidating

the precise reason behind this phenomenon is rather difficult. Lower levels of brain swelling in wortmannin groups might be associated with the elevated levels of BBB integrity that has been observed in these groups.

4.4. DNA FRAGMENTATION

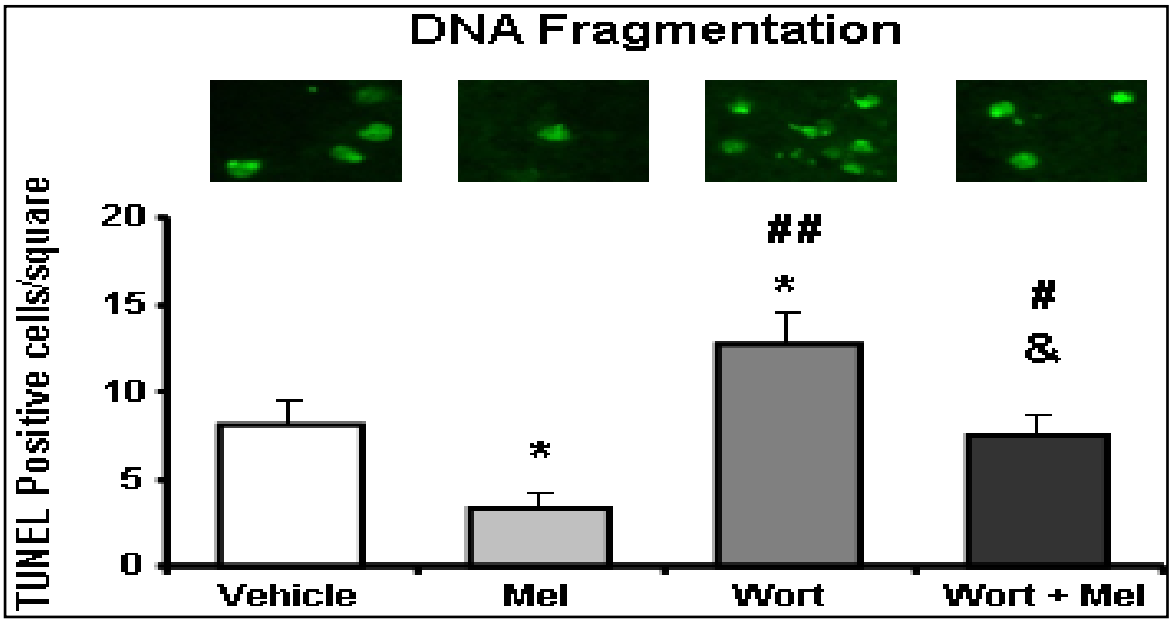

Figure 4.4. Results of DNA Fragmentation after TUNEL staining

DNA fragmentation was evaluated on brain sections of animals that were subjected to 30 min MCAO + 72 h reperfusion which were stained with terminal transferase dUTP nick-end labeling (TUNEL). One sample from each of the animals in the 30 min MCAO + 72 h reperfusion group was stained, nine random ROI were analyzed for each sample and the mean values of the samples in the same group were calculated.

The results of TUNEL positive cells/square were; Vehicle 8.1 ± 3.76, Melatonin 3.24 ± 2.33, Wortmannin 12.72 ± 4.66 and Wortmannin + Melatonin 7.50 ± 3.14.

Melatonin treatment significantly decreased the number of TUNEL positive cells ($p<0.05$), and only i.c.v injection of Wortmannin significantly increased the number of TUNEL positive cells ($p<0.05$). When Melatonin was administered after i.c.v injection of Wortmannin, Wortmannin significantly reduced the favorable effect of Melatonin ($p<0.05$).

Together with cresyl violet staining and the results of DNA fragmentation, these data indicate that inhibition of PI-3 Kinase/Akt pathway renders the brain susceptible to ischemic damage and irreversible cell injury predominantly in striatum.

4.5. NEURONAL SURVIVAL

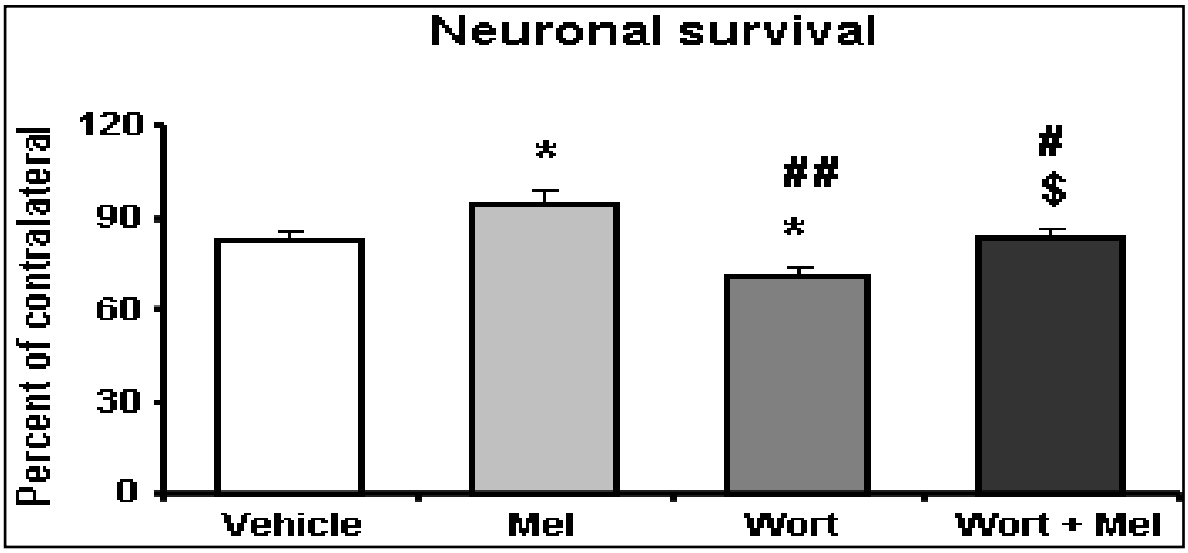

Figure 4.5. Results of neuronal survival after cresyl violet staining

Neuronal survival analysis was evaluated on brain sections of animals that were subjected to 30 min MCAO + 72 h reperfusion which were stained with cresyl violet dye. One sample per each animal in the 30 min MCAO + 72 h reperfusion group was stained, nine random ROI were analyzed for each sample and the mean values of the samples in the same group were calculated. With these data, percentage of surviving neurons was determined for each structure.

The results of neuronal survival were; vehicle 82.66 ± 6.55, melatonin 94.07 ± 12.83, wortmannin 71.17 ± 5.94 and wortmannin + melatonin 83.31 ± 5.88.

Melatonin treatment significantly increased neuronal survival, whereas only i.c.v injection of wortmannin significantly decreased neuronal survival ($p<0.05$). When melatonin was administered after i.c.v injection of wortmannin, wortmannin significantly reduced the favorable effect of Melatonin ($p<0.05$).

Treatment effect of melatonin on surviving neurons was reversed by wortmannin

which indicates that the PI-3 Kinase/Akt pathway plays a critical role in neuronal survival.

4.6. SERUM IGG EXTRAVASATION

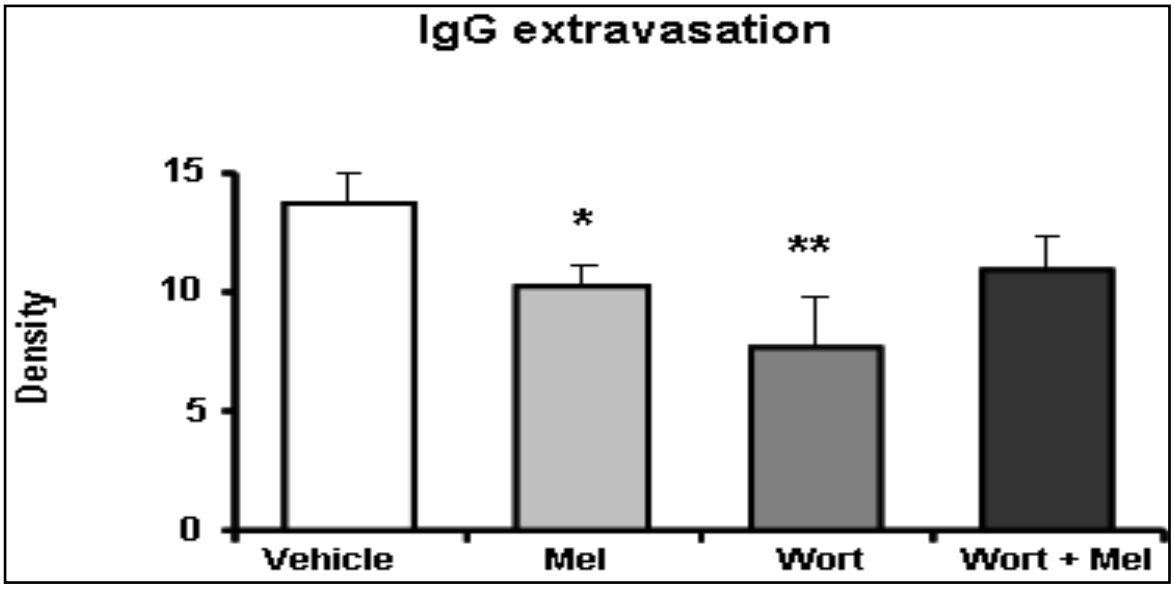

Figure 4.6: Results of serum IgG extravasation after peroxidase staining

For the purposes of serum IgG extravasation analyses, 9 different range of interests of 90 min + 24 h reperfusion groups, that cover the whole striatum of ischemic hemisphere were photographed and on these photographs, mean optical densities for the whole striatum were measured using Image J software (NIH, USA), from which background densities in corresponding non-ischemic areas were subtracted.

BBB integrity was evaluated by serum IgG extravasation into the brain parenchyma. Serum IgG extravasation was significantly reduced in melatonin ($p<0.05$) and only i.c.v. injection of wortmannin groups.

After brain injuries, blood brain barrier (BBB) integrity can be degraded, and increased BBB permeability after cerebral ischemia, can cause edema formation, excitotoxicity and inflammation, eventually resulting in apoptosis.

4.7. WESTERN BLOT ANALYSES

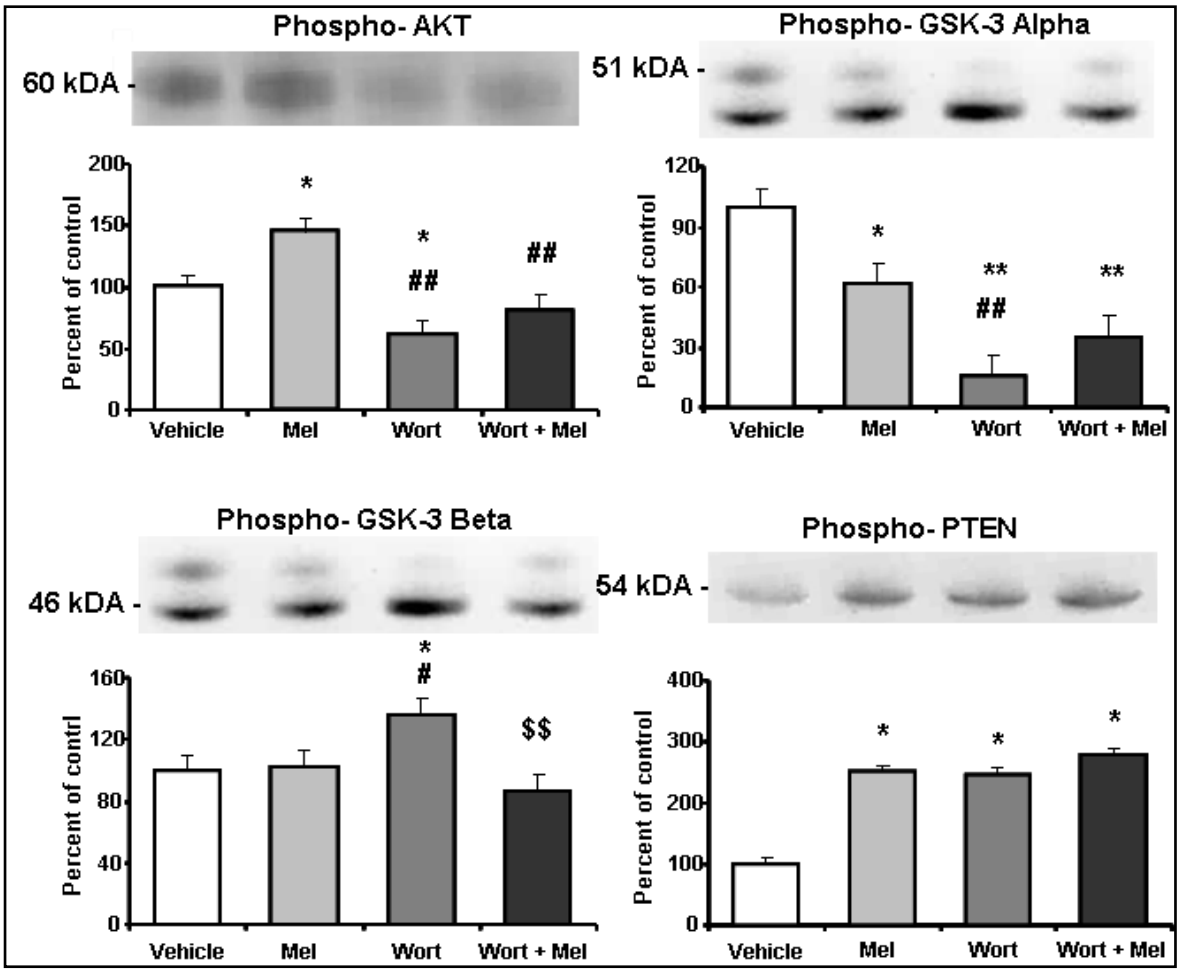

Figure 4.7. Western Blot analyses of phospho-Akt, phospho-GSK-3 alpha and beta, and phospho-PTEN.

To characterize the effects of PI-3 kinase/Akt pathway on melatonin treatment, tissue lysates from ischemic (ipsilateral) hemispheres were analyzed. In the ischemic hemisphere, melatonin treatment significantly increased the phosphorylation and activation of Akt ($p<0.05$) and the phosphorylation and inactivation of GSK-3 Alpha, which is significantly reversed by i.c.v. injection of wortmannin.

GSK-3 Beta is related to pro-apoptotic pathways. While melatonin treatment kept the expression of it as physiological level, i.c.v. injection of wortmannin significantly increased the expression of pro-apoptotic GSK-3 Beta.

When compared to the vehicle groups, melatonin treatment and i.c.v. injection of wortmannin were significantly increased the phosphorylation of PTEN. PTEN inhibits phosphorylation of PIP2 to PIP3, and remotely block phosphorylation of Akt.

It has been shown that PI-3 Kinase/Akt pathway prevents apoptosis by enhancing mitochondrial stability under conditions in which tissue oxygen levels are low cause of cerebral ischemia.

Together with the rest of the data, these results indicated that decrease in infarct volume and DNA fragmentation, and increase in neuronal survival is regulated by PI-3 Kinase/Akt pathway. Moreover in this pathway, phosphorylation and activation of Akt and phosphorylation and inactivation of pro-apoptotic GSK-3 Beta play an important role.

5. DISCUSSION

Brain tissue requires a relatively high consumption of oxygen and glucose and depends exclusively on oxidative phosphorylation for energy production. Focal and global impairments of cerebral blood flow block the delivery of oxygen and glucose and impair the energetics required to maintain ionic gradients through the plasma membranes (2). High mitochondrial calcium accumulation also alters the permeability of the mitochondrial membrane, inhibits mitochondrial ATP production and promotes necrosis. In addition, selective permeability of the outer membrane releases cytochrome c, which activates caspases. Caspases, in turn cleave specific cytoplasmic and nuclear protein substrates to coordinate apoptosis.

The PI-3 Kinase/Akt signal pathway plays an important role in the activation of cell survival and the suppression of cell death. Moreover, neuroprotective effects of melatonin against ischemic brain injury could be mediated through the activation of PI3- K/Akt pathway.

To evaluate how PI-3 Kinase/Akt pathway deactivation influences histopathological injury, the density of injured, i.e., TUNEL + cells and the percentage of surviving neurons in the ischemic striatum, wortmannin, irreversible PI-3 Kinase inhibitor was used.

Significant decrease in infarct volume and the number of DNA fragmentation and significant increase in the number of surviving neurons when treated with melatonin that reversed by wortmannin inhibition, these data showed that PI-3 Kinase/Akt pathway has a wide range of role in neuroprotective effect of melatonin.

Western blot analysis demonstrated that phosphorylation-activation of Akt and phosphorylation-inactivation of GSK-3 Beta levels were altered by the inhibition of PI-3 Kinase/Akt pathway. Although melatonin did not decrease the levels of GSK-3 Beta, it kept the levels of GSK-3 Beta at physiological levels and did not lead to an increase in the level of GSK-3 Beta.

Although melatonin stabilized BBB integrity as seen in the serum IgG extravasation and decreased edema as seen in the brain swelling, these characteristic of melatonin were not reversed by wortmannin. Edema formation and protection from edema are not only related with PI-3 Kinase/Akt pathway which may be related to other signaling pathways.

6. CONCLUSION and RECOMMENDATIONS

6.1. CONCLUSION

In this study, the role of PI-3 Kinase/Akt pathway in the neuroprotective effect of melatonin was analyzed in a mouse MCAO model. Blood flow over the ischemia area was monitored with LDF throughout the experiments. After 24 hours for 90 min MCAO or 72 hours for 30 min MCAO, animals were anesthetized and decapitated. Infarct volumes and brain swelling values were calculated after cresyl violet staining of brain sections. Melatonin treatment significantly decreased infarct volume, brain swelling and DNA fragmentation when compared to the vehicle-treated control groups, while wortmannin reverses the effect of melatonin suggesting a crucial role of PI-3Kinase/Akt pathway in the neuroprotective effect of melatonin.

These results provide evidence that PI-3 Kinase/Akt pathway has an important role in the neuroprotective effect of melatonin, through phosphorylation-activation of Akt which is associated with an increase in neuronal cell survival and phosphorylation-inactivation of GSK-3 Beta which is involved in apoptosis. Beside melatonin, combination therapy including drugs that inhibit GSK-3 Beta or cause an activation of Akt is beneficial for clinical treatment of stroke or possibly of other neurological disorders.

6.2. RECOMMENDATIONS

As a future work, the PI-3 Kinase/Akt pathway can be further targeted. In addition, combination therapies with melatonin can be used in experimental stroke models. Moreover, in order to characterize each member of the downstream targets of PI-3 Kinase/Akt pathway, they should be individually investigated to be able to see the whole map. Therefore, drugs that target these pathways or combination therapy with melatonin can be a clinical treatment option for cerebral ischemia.

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