THE ROLES OF PHOSPHOINOSITIDE-3 KINASES/AKT PHOSPHORYLATION IN THE NEUROPROTECTIVE EFFECTS OF MELATONIN ON NEURONAL SURVIVAL *IN-VITRO* AND *IN-VIVO*

by

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Submitted to the Institute of Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science
in
Biotechnology

Yeditepe University
2014

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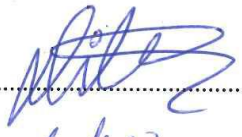
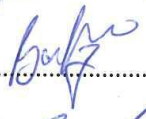
Assoc. Prof.Dilek TELCİ ..... 

Assist. Prof. Fatma Burcu ŞEKER ..... 

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DATE OF APPROVAL: 26/08/14

"This thesis is dedicated to

My parents"

ACKNOWLEDGEMENTS

I take this opportunity firstly to express my profound gratitude and deep regards to my supervisor, Prof. Fikrettin SAHİN who opened his laboratories for my studies and supported the project until the end.

At this level I feel deeply honoured in expressing my sincere thanks to Prof. Ertugrul KILIC, who I have learned many things from since I became his student. It was a great chance to be a student of such excellent scientist's. His exemplary constant encouragement helped me throughout the course of this thesis. The blessing, help and guidance given by him is not only restricted with this project but also carry me a long way in the journey of life which I am about to board.

Furthermore, I express my deepest thanks to Berrak CAGLAYAN and Ahmet Burak CAGLAYAN for being my laboratory advisers in the areas of cell culture and animal experiments, respectively. I have learned inestimable amount of experiences since I became their assistant. They took part in giving extinct necessary advices and guidance to all facilities for making life easier. I choose this moment to acknowledge their contribution gratefully.

Being students of the same supervisor, I hereby, want to express my great appreciation to Mustafa Caglar BEKER, Esra YALCIN and Taha KELESTEMUR. Each of them helped me at different levels of my project by being there when I needed it and showing their honest attention without doubt.

I am also obliged to staff members of both Medipol University research centre (REMER) and Yeditepe University Genetics and Bioengineering Department for the valuable information provided by them in their respective fields. I am grateful for their cooperation during the period of my master project thesis.

I would like to thank my best friend Ayse Nazlı KOSE YUCEL who emotionally supported

me in each turn of my life and each part of this master thesis.
Moreover, I express my grate thanks to my friends who I met throughout this journey and made my life colourful. Dear friends of Aysun DILDEN, Ipek VARTURK, Pınar GERCEK, Kubra AYDIN SELVI and Fatma OZEN. I appreciate your endless support and I hereby feel really glad that I have gained your friendship.

The last and most meaningful appreciation is there for my family. I thank almighty to my dear mum and dad, and, furthermore, to my brothers and sisters for their constant encouragement in all areas of my life and this project. Specifically to my cousin Mehmet GUNDOGDU who supported me during the preparation step, I appreciate his help a lot. It would be impossible to complete this assignment without their grate support.

This work was financially supported by Yeditepe University, European Molecular Biology Organization (EMBO), Turkish Academy of Science (TUBA).

ABSTRACT

THE ROLES OF PHOSPHOINOSITIDE-3 KINASES/AKT PHOSPHORYLATION IN THE NEUROPROTECTIVE EFFECTS OF MELATONIN ON NEURONAL SURVIVAL I*N-VITRO* AND *IN-VIVO*

Melatonin is a well-known circadian rhythm regulatory molecule found to be conserved from lower to higher organisms. Melatonin possesses several functions mainly in the biological mechanisms of aging, antioxidant scavenging and cellular survival. Depending on the background of this study, specifically, the attention was drawn into the neuro-survival abilities of melatonin. In our previous studies, we have observed that melatonin increases the phosphorylation of Akt. However, the significance of increased Akt phosphorylation was not known. In the present study, the role of Phosphoinositide-3 kinase (PI-3K) /Akt signalling pathway in the neuro-protective effects of melatonin was tested on primary cortical neuron culture, as *in vitro* model, and mice cerebral ischemia *in vivo* model through the use of Wortmannin as a PI-3K/Akt pathway inhibitor. Primary cortical neuron culture was arrived from new born (DIV 1-3) Balb/c mice and the hypoxia-OGD experiment was applied on day 7 of culturing. Melatonin and Wortmannin treatments were introduced during hypoxia-OGD and reperfusion. 30 mins of Middle Cerebral Artery Occlusion (MCAO) was applied on adult male C57BL mice for the *in vivo* experiment model. Wortmannin was administered before MCAO and melatonin was injected after 30 mins of MCAO. The cell survival counting analysis was performed from the pictures taken with confocal microscopy (20X, DAPI). Neuronal survival analysis and DNA Fragmentation analyses were found by applying Cresyl violet staining and TUNEL assay on cross sectioned *in vivo* experimental group mice brains, respectively. The results of this study indicate that specifically in the melatonin group of *in vitro* experiments the axonal extensions was protected after cortical cells were exposed to the hypoxic and OGD conditions. Similarly, melatonin significantly increased neuronal survival with the percentage of 94.1 % *in vivo*. In this study, we provide evidence that melatonin increases neuronal survival from hypoxic cell death both *in vivo* and *in vitro*. We further reveal that the inhibition of Akt phosphorylation reverses the neuro-protective effect of melatonin.

ÖZET

FOSFOİNOSİTİD-3 KİNAZ/AKT FOSFORİLASYONUNUN MELATONİNİN NÖROPROTEKTİF ETKİLERİ ÜZERİNDEKİ ROLÜNÜN *IN VITRO* VE *IN VIVO* ETKİLERİ

Melatonin az ve çok gelişmiş organizmaların tümünde bulunan ve çok bilinen sirkadyen ritim kontrolünü sağlayan önemli bir hormonudur. Melatoninin circadian ritim regülasyonu dışında farklı biyolojik mekanizmalar ile de ilişkisi bulunmaktadır. Bunlar; yaşlılık, antioksidan temizlenmesi ve hücresel canlılık olaylarıdır. Bu projenin arka planına bağlı olarak, bütün bu biyolojik aktiviteler içerisinde ilgi hücresel canlılığa yönlendirilmiştir. PI-3K/Akt sinyal yolağının melatoninin nöroprotektif etkilerindeki rolü, PI-3K/Akt inhibitörü olan Wortmannin in yardımıyla, hem *in vitro* model olarak primer kortikal hücre kültüründe hem de *in vivo* model olarak MCAO hayvan deneyleriyle test edilmiştir. Primer kortikal hücre kültürü yeni doğan (1. gün-3.gün) Balb/C farelerinden alınarak yapılmıştır ve devamı olarak hipoksi- OGD deneyi kültürün 7. Gününde başlatılmıştır. Melatonin ve Wortmannin tedavileri hipoksi-OGD ve reperfüzyon aşamalarında eklenmiştir. *In vivo* model olarak da yetişkin C57BL farelerine 30 dakikalık MCAO serebral iskemi deneyi uygulanmıştır. Burada, Wortmannin MCAO'dan önce ve melatonin 30 dakikalık MCAO sonrasında enjekte edilmiştir. *In vitro* hücre canlılığı analizi mikroskopta çekilen canlı hücre resimlerinin değerlendirilmesiyle yapılmıştır. *In vivo* deneyi analizleri olan Nöron canlılığı analizi ve DNA parçalarına ayırma analizi sırasıyla cresyl violet boyaması ve TUNEL boyaması sonucu hesaplanmıştır. Bu çalışmanın sonucunda; *in vitro* melatonin grubunda hipoksi ve OGD deneyi sonunda aksonal uzantılar korunmuştur ve hücresel canlılık oranı 52, 46 % olarak bulunmuştur. Benzer olarak melatonin *in vivo* deney sonucunda önemli bir ölçüde nöronal canlılığı 94.06 % oranında arttırmıştır. Bu proje sonucunda melatoninin nöronal hayatta kalma oranını arttırdığı önemli bir ölçüde *in vitro* ve *in vivo* olarak ispat edilmiştir. Bundan başka, nöronal hayatta kalma oranındaki artışın Wortmanninin aynı deney grubuna eklenmesi sonucu normal seviyelere geri dönüşü görülmüştür. Bu buluş da PI-3K/Akt yolağının melatoninin nöroprotektif etkileri üzerinde çok önemli bir rolü olduğunu kanıtlamıştır.

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ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCL-2	B-cell lymphomal leukaemia
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
CNS	Central nervous system
CO_2	Carbondioxide
DAPI	4',6-diamidino-2-phenylindole
dH_20	Distilled hydrogen dioxide
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
ECE	Endothelin Converting Enzyme
Endo G	Endonuclease G
Epo	Erythropoietin
GFAP	Glial fibrillary acidic protein
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solutioı
HIF-α	Hypoxia-inducible factor 1-alpha
ICC	Immunocytochemistry
L15	Leibovitz's L-15
MAP-K	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
mTOR	Mammalian target of rapamycin
NBA	Neurobasal-A
NeuN	Neuronal nuclei

NF-κB	Nuclear factor kappa b
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Oxide
OGD	Oxygen glucose deprivation
OH	Hydroxide
$ONOO^-$	Peroxonitrite
PDK-1	Phosphoinositide dependent protein kinase-1
PFA	Paraformaldehyde
PI-3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositide (3, 4) bisphosphate
PIP3	Phosphatidylinositide (3, 4, 5) triphosphate
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
PTP	Permeability transition pore
ROI	Region of interest
ROS	Reactive oxygen species
SPSS	Statistical Package for the Social Sciences
TGF	Transforming growth factor
TUNEL	dUTP nick end labeling

1.INTRODUCTION

1.1. THEORETICAL BACKGROUND

According to the recent topics of melatonin research, nowadays, melatonin's fame of being circadian rhythm regulator has been masked with its neuro-survival abilities. Throughout the excessive research, it has been revealed that melatonin ameliorates the neuronal loss which occurs as a response to a disease condition. Melatonin is a multitasking molecule that acts on various types of metabolic events and one of the most famous actions constitutes its effects on neuro-protective signalling pathways (1). Melatonin exerts its neuronal surviving activity via involvement of intracellular regulatory pathways. Amongst the other entire hypoxia related signalling pathways, PI-3K/Akt pathway appears to be the core basis which explains melatonin's neuro-protective action with an efficient mechanism. Here, it was found that melatonin accomplishes neuronal survival ability through the PI-3K/Akt signalling pathway. In the aspect of neuronal survival, melatonin is a well-studied molecule in the treatment fields of ischemic stroke research.

Stroke is found to be the third leading cause of death in developed countries. It is a serious health condition in which the patients that survive from stroke suffer difficult disabilities throughout their daily lives. Stroke is mainly caused by thrombosis or embolus which results in a blockage in the arteries that supply blood to brain (2, 3).Considering the results of stroke it is necessary that both clinical curing strategies and cellular level research act together. At this point, it was found that the underlying mechanisms occurred after stroke, such as apoptotic cell death, are susceptible to melatonin treatment. This fact generated a high impact for melatonin to be applied in the stroke research also with various add-on treatment combinations.

Considering the clinical importance of stroke as a neurological disease condition, although there are significant outputs reached as a result of the excessive research in this area, there are still some mechanisms remain to be unclear for stroke at cellular level including melatonin's actions.

Apart from the animal studies which have been shown to display significant results, this project also focuses to demonstrate the survival actions of melatonin through PI-3K/Akt signalling pathway based on mimicking the *in vivo* animal study model in a cellular level based research with the involvement of primary cortical neuron cell culture. In order to acknowledge the project accurately it is fundamental to gain the detail theoretical information about melatonin, stroke underlying mechanism, PI-3Ks and PI-3K/Akt signalling pathway as explained in the section below.

1.2. MELATONIN

1.2.1. General Background

Melatonin, N-acetyl-methoxytryptamine, is a highly conserved neuro-molecule observed from lower to higher organisms such as animals, plants and microbes. Melatonin is synthesised and released from the pineal gland in animals. Among all the other hormones secreted from the pineal gland, melatonin represents the most powerful hormone by having multifunctional actions throughout the body. From the discovery of melatonin there has been a variety of research carried on representing melatonin`s functions. As being a multifunctional molecule, melatonin can act on different systems in the body; central nervous system (CNS), immune system, cardiovascular system, and gastrointestinal system (1). Apart from being synthesised as a natural hormone, melatonin can also be taken externally, either by oral or intravenous medication. It was widely acknowledged that, when taken orally, melatonin could treat jet lag and cure sleep deficiency. Looking from another view, it was proven that melatonin is a powerful antioxidant and free radical scavenger. Melatonin and its derivatives can protect lipid peroxidation occurs as a result of oxidative stress and exert anti-apoptotic activity in treating neurodegenerative diseases (4).

The effects of melatonin in the CNS are generally merged with the curing activities of melatonin for neurodegenerative diseases including stroke (brain ischemia), Parkinson’s disease (PD) and Alzheimer’s disease (AD) (1). Specifically in stroke, melatonin has been shown to protect the damaged area that was exposed to oxidative stress. These protective abilities of melatonin are incorporated with its effects on mitochondria (4-6).

Melatonin concentrations are observed to be high in mitochondria, especially in the inner membrane. Mitochondria are known to be the organelle where oxidative stress is mainly arisen. The production of reactive oxygen species (ROS) is a prerequisite event for oxidative damage to initiate in mitochondria (7). It was found that under normal physiological conditions ROS are produced considerably at lower levels in comparison with disease occurring conditions. The neurological diseases that profoundly affect ROS are mainly stroke and aging (6, 8). Herein, melatonin plays a vital role in which, it prevents the diseases that are caused by oxidative stress through the help of its free radical scavenging and antioxidant properties. The presence of melatonin diminishes detrimental effects of neuronal oxidative stress and acts as a guard for mitochondrial membranes (4, 7, 9, 10).

There is growing body of evidence that melatonin can induce anti-apoptotic signals in the survival signalling pathways. Melatonin involves with the apoptotic signalling pathways by virtue of its abundant occurrence in the inner membrane of mitochondria (1, 10). Although, the signalling mechanisms that clarify melatonin's neuro-protective actions are still incompletely understood, it was found that melatonin is mainly involved with the PI3K/Akt, Bcl-2, Nuclear factor kappa-B (NF-κB), Mitogen-activated protein kinase (MAPK) pathways. Through these pathways melatonin activates signalling cascades and by this way promotes cell survival (1, 7, 11).

1.2.2. The scavenging roles of melatonin as an antioxidant

Melatonin exemplifies to be an exclusive neuro-protecting molecule by treating neuronal ischemic injury through regulating signalling pathways due to its ability as a strong antioxidant. Melatonin is a powerful antioxidant and free radical scavenger, which easily crosses Blood Brain Barrier (BBB) and by decreasing oxidative stress conditions, in mitochondria, it reduces neuronal cell death rate (12). Structurally the lipophilic and hydrophilic properties, Figure 1.1, of melatonin enable to pass through BBB, cellular membranes and fuse into cells, nucleus easily (13). In order to understand the free radical scavenging neuronal cell death protecting actions of melatonin, at first the intracellular mechanisms occur as a result of ischemic stroke should be understood carefully.

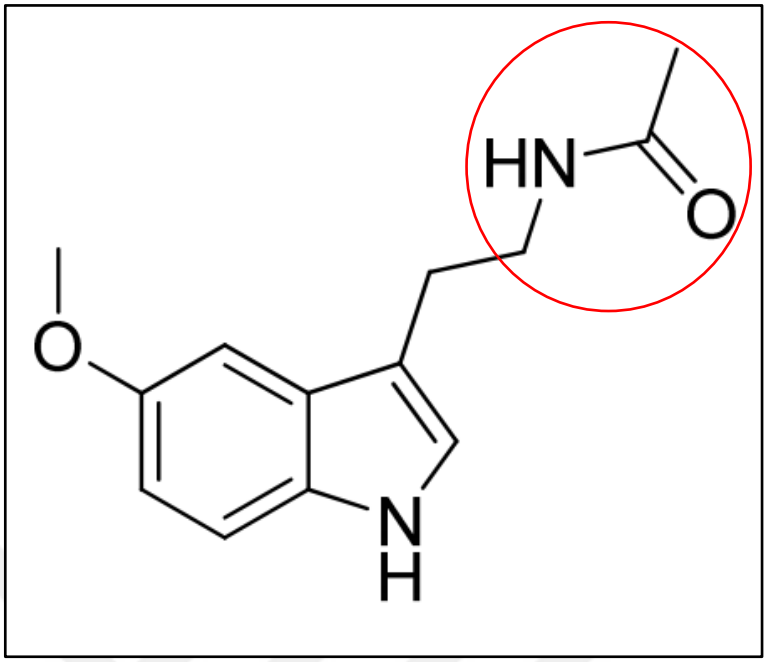

Figure 1.1. The chemical structure of Melatonin, (14).

The role of BBB in brain related research could be best explained by characterising BBB as a physiological obstacle for brain, which controls the exchange of necessary molecules between the circulatory blood and brain. In neurological disease conditions, hereof stroke, the permeability of BBB becomes increased due to the breakdown of BBB by NO, in depth by n-NOS (neuronal nitric oxide synthase) (30). Given that, BBB is a protecting obstacle for brain when BBB becomes disrupted plasma proteins freely leak into brain, which thereafter lead to the formation of vascular oedema (15, 16). NO is synthesized by the enzyme of NOS which is identified with three different isoforms; neuronal NOS (nNOS), inducible NOS (i-NOS) and endothelial NOS (e-NOS). Specifically, it was found that e-NOS reside as a powerful risk factor for stroke considering its role in the increase of vasculature after stroke. Looking at stroke from another angle, ischemic cell death could be explained by three overlapping mechanisms; apoptosis-related mechanisms and pathways excitotoxicity and oxidative stress. The role of excitotoxicity in ischemic stroke model is interconnected with oxidative stress induction and apoptotic pathways initiation; hence excitotixicity could be defined as the main toxic mechanism in ischemic stroke. Therefore it is essential to understand the underlying mechanisms that are caused by excitotoxicity. Ischemic stroke influences glutamate receptor activation followed by NMDA channel

opening influx on ischemic neurons thereafter, intracellular calcium transfer increases and accumulates in the cytoplasm and mitochondria. Consequently, these events trigger oxidative stress to be dominated in the ischemic area leading the free radicals, reactive oxygen species (ROS), which result in stimulating apoptotic pathways (5, 17).

In basal conditions, brain possesses high ATP and oxygen consumption which is produced by the electron transfer chain (ETC) from the respiratory chain reactions that reside in mitochondria (16, (18, 19). Therefore when oxidative stress is induced by stroke excitotoxicity, the lack of oxygen supply causes a dramatical drop in the normal ATP levels, which constitutes free radicals and ROS formation. Eventually, the oxygen based ROS components, H_2O_2, superoxide anion (O_2^-), hydroxyl radical (OH), peroxynitrite anion ($ONOO^-$), nitric oxide (NO) and hydrogen peroxide (H_2O_2), form to exert hazardous and abnormal events in neuronal mitochondria such as lipid peroxidation and DNA damage. Collectively following neuronal excitotoxicity and the lack of oxygen supply induced by ischemic stroke oxidative stress increases ROS formation, thereby DNA damage, which eventually results in neuronal cell death (18-22).

Brain is not well equipped with the amount of antioxidants, which would protect brain in disease conditions. Herein, vitamin C and E are known as strong antioxidants however, due to their deficiency in crossing BBB they are considered to be ineffective in brain. Recalling back to the melatonin's antioxidant and free radical scavenging roles, the bioavailability of melatonin to cross BBB easily put it one step ahead from other antioxidants. Confirming the oxidative stress underlying mechanisms explained above, hereof melatonin inserts its function by acting as an indirect antioxidant, which induces antioxidant enzymes such as NOS, to initiate free radical scavenging. Herein, the exogenous administration of melatonin in experimental ischemic stroke models indicates that melatonin acts as a free radical scavenger and reduces ROS components by the ease of crossing BBB (6, 23, 24). As considering all components of ROS, specifically NO possesses a major importance by having attacked by melatonin. Melatonin attacks NO by suppressing n-NOS synthesis which clearly then allows reduction in oxidative stress (20, 25). It was found that melatonin also involves with the detoxification of O_2^- and OH radicals but with less affinity than NO (21). Accordingly, the scavenging of NO by melatonin inhibits apoptosis followed by the lipid peroxidation and DNA damage, respectively, in ischemic neurons. On the other hand melatonin plays a crucial role by restoring the BBB integrity in the

ischemic stroke area. Conserving BBB integrity is an important step in the recovery of neuronal development and through this way melatonin protects the scattered and false vasculature by acting as a BBB protecting agent (23, 25).

1.2.3. The roles of melatonin in stroke authenticating pathways

Ischemia causes changes in several molecules such as transcription factors, growth factors and free-radical molecules. Most of these molecules stimulate MAPK pathways, as explained below.

ERK, JNK and p38-MAPK are well- studied pathways, which mediate neuro-protection in brain. Melatonin's neuro-protective effect through ERK, JNK and PI-3K/Akt signal transduction pathways were shown by researches based on the melatonin treatment.

To the best of our knowledge melatonin provokes neuro-protection and inhibits apoptosis by, most commonly, activating the PI3-K/Akt pathway and increasing Bcl-2 family proteins, Bcl-xL, expression. PI3-K/Akt pathway begins with the activation of Akt. Herein, PI3-K induces Akt phosphorylation and the activated Akt binds to the pro-apoptotic marker of Bad for blocking Bad translocation to mitochondria, hence inhibits apoptosis (26, 27). Although PI3K-Akt pathway represents the core basis of melatonin's neuro-protective action, there are also other signalling pathways that are interconnected with PI3-K/Akt.

The classical neurotrophins include brain- derived neurotrophic factor (BDNF) and transforming growth factor-beta1 (TGF-β1), which involve with the neuron survival mechanisms after ischemic stroke induction and other CNS injury based diseases. BDNF and TGF-β1 act on stoke through the ERK1/2 pathway. It was also found that Akt signalling in ischemic stroke models are not associated with these neurotrophins but still manage to act on stroke mechanisms. Joining this knowledge, Akt is considered to exert its action via the ERK1/2 pathway (28-30).

On the other hand it was also found that Akt leads to Bad protein phosphorylation and, by this way, inhibits mitochondrial PTP opening thus the apoptosis causative molecule cytochrome c cannot be released from mitochondria to initiate apoptosis. Along these lines,

there is a growing body of evidence that melatonin might exert its activity to inhibit mPTP through Akt associated pathway (28, 31).

Apart from ischemic stroke models, the neuro-protective roles of melatonin were also studied with the retinal axotomy model. The basic idea of this experiment is the same with the ischemic model in which the protecting roles of melatonin are examined when administered laterally to optic nerve axons damage. The roles of ERK, Akt and JNK pathways were also shown with the retinal axotomy model and optic nerve transection experiments completed by Kilic and colleagues (32). Herein, the haematopoietic growth factor erythropoietin (Epo) is applied as a neuro-protective agent, in which is more specific for retinal injury, and indicated that ERK-1/2, two similar isoforms of ERK protein kinase, and Akt signalling pathways were activated when Epo is present. On the contrary, JNK and BcL-xL were observed to be inhibited after Epo administration. This study suggests that ERK-1/2 and Akt pathways are survival pathways thus act parallel with Epo to recover the optic nerve damage, whereas JNK and BcL-xL were inhibited after Epo applied. This result indicates that the JNK and BcL-xL pathways act to be pro-apoptotic after axotomy, as laterally inhibited by Epo for neuro-protection (32). The use of retinal ganglion cells could be merged with pinealectomy to analyse melatonin's neuro-protective functions more precisely. As acting the major source for melatonin, removing the pineal gland by pinealectomy approach allowed us to examine the ability of exogenously administered melatonin in recovering the functions of endogenously circulating melatonin. It was found that the exogenous administration of melatonin succeeded to protect injured neurons against cell death in retinal ganglion cells (33). The success of analysing exogenous melatonin function by pinealectomy approach is also applied with focal cerebral ischemic model and it was found that exogenously administered as well as normal physiologically released melatonin altered neuronal protection in cerebral ischemia (34). These findings shed light to the exogenous melatonin uptake, which might be applicable for the protection of aged brain consuming the fact that in aged brain melatonin levels are lower in comparison with healthy and adolescent brain, however detailed further study is essential to understand the physiology of pineal gland and the effects of rhythmic production of melatonin (33). At the same time, the capability of endogenous melatonin improving neuronal survival and neurologic recovery should not be disregarded. The 24-hour delayed administration of exogenous melatonin approved that endogenous melatonin is able to

recover motor coordination and reduce the levels of hyperactivity and anxiety, showed by means battery of behavioural tests, in ischemic mice (12).

1.2.4. The effects of melatonin receptors in stroke mechanisms

Up till now, there have been two types of natural existing melatonin receptors identified in mammals, Mel1a (MT1) and Mel1b (MT2) (18). Although there is a variety of research carried on melatonin's protective act on neuronal injury and stroke, not much is known about the roles of melatonin receptors. The roles of melatonin receptors MT1 and MT2 in the neuro-protection and cell signalling mechanisms remain largely unknown, however some recent studies disclosed that MT1 and MT2 together exemplify as the targets for drug discovery in neuro-protection. The field of efficacy of MT1 and MT2 on melatonin's neuro-protective effects varies within themselves. Namely, MT1 was found to increase cell proliferation and neuro-protection by modulating neuronal firing in amniotic epithelial cells (AEC) after ischemic and oxidative stress injury induced *in vitro* and *in vivo* (35). On the other hand, MT2 is involved with melatonin's neuro-protective effects in ischemic stroke to a greater extent than MT1. It was found that melatonin's neuro-protecting action decreases when MT2 is absent, created by the treatment of MT2 antagonist luzindole, in ischemic-stroke mice models. Thereby melatonin might mitigate neuronal injury through MT2 receptor (36). Conversely, in a signalling factors comprehensive study, completed by Kilic and colleagues, it was shown that melatonin is able to lessen ischemic injury area without being dependent on MT1 and MT2 receptors (37). Melatonin receptors roles were investigated both in MT1 and MT2 knock out and in wild type (WT) mice through the analysis of melatonin related signalling pathways (p21, JNK1/2 and p38). As contrary evidence to other studies the results showed that MT1 and MT2 receptors involve with melatonin neuro-protection neither at signalling pathways manner nor at edema formation manner. Moreover, in MT1 and MT2 knockout mice treatment with exogenous melatonin stand for a supportive result by indicating that melatonin exerts its ischemic injury ameliorating act through decreasing p38 and p21 phosphorylation (37). Although these findings explain that MT1 and MT2 receptors have no favourable roles, further studies applying receptors' agonists and antagonists are required to fully rule out melatonin receptors' role in neuro-protection.

1.3. ISCHEMIC STROKE

1.3.1. Cerebral stroke general background

Cerebral stroke is medically described as neurological loss of an organ or tissue which occurs because of a deficiency in the blood supply. In the matter of brain, ischemic stroke or cerebral ischemia is well known and highly serious condition which deteriorates the brain functioning in order to meet metabolic requirements as a result of inadequate blood flow. There are two main types of animal models applied in stroke research; global and focal cerebral ischemia. Predictably, global ischemia is when the deficiency in blood supply is widespread, whilst in focal cerebral ischemia, the lack of blood supply occurs in a particular region. Elaborately, in animal models, focal cerebral ischemia is experimented either with or without reperfusion (9).

Brain tissue requires high amounts of glucose and oxygen for accomplishing intensive functions. It is apparent that the disruption in the oxygen and glucose consumption results in dysfunctional tissue through molecular, cellular level of deterioration and apoptotic cell death (2, 3). The molecular mechanisms underlying brain functioning consist of a compact network of signalling pathways hence the deterioration exposes a hardly irreversible situation. Mitochondria play a crucial role in the healthy functioning of the apoptosis related mechanisms and therefore stroke related signalling mechanisms (38). Mitochondria are the target for signalling mechanisms since the general feature of neurological diseases is identified to be the apoptotic cell death in CNS. Both apoptotic and anti-apoptotic survival pathways play critical roles in the formation of neuro-degeneration. From apoptotic pathways, extrinsic and intrinsic, melatonin is found to be involved with the intrinsic pathway, also known as caspase-dependent or independent mitochondrial pathways. The well-known pro-apoptotic mitochondria molecules are cytochrome c, Smac (second mitochondrion-derived activator of caspase)/Diablo and Apoptosis inducing factor (AIF). Neuronal cell death occurs as a result of series of steps starting with cytochrome c release from mitochondria, including caspase-3 activation (the key effector) and ending with NF-κB, TNF-α- induced activator protein-1 (AP-1) , p53 activation (1, 38).

1.3.2. Signalling pathways related to stroke

The signalling mechanisms that help surviving neurons progress through activating anti-apoptotic pathways (survival) or inhibiting apoptotic pathways. Since, the main topic of this project is identifying the outputs of melatonin treatment; the main stroke related signalling pathways that are merged with melatonin acting mechanisms are explained together below. Melatonin is mainly found in the inner membrane of mitochondria, hence the neuro-protective actions of melatonin through mitochondrial anti-apoptotic pathways (38).

Bcl-2 group of genes are well-known apoptosis controlling elements including both anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic (Bax, Bad and Bid) molecules. There are four mitochondrial pro-apoptotic molecules, cytochrome c, Smac (Second mitochondrion-derived activator of caspase)/Diablo, AIF and Endonuclease G (Endo-G), all of which aid to apoptosis by releasing from mitochondria. Specifically cytochrome c release from mitochondria leads to the activation of caspase-9. Once caspase-9 is activated this activation allows caspase-3 to further act on progressing DNA fragmentation and neuronal cell death. Herein, caspase-3 is known to be the "key effector" in the neuronal cell death progression (35, 39). Hence, if in a scenario caspase-3 cannot be activated therein apoptosis and neuronal cell death becomes suppressed. The anti-apoptotic molecules of Bcl-2 and Bcl-xL suppress neuronal cell death by regulating the cytochrome-c release, in parallel with (correspondingly) the prohibition of caspase-3 activation. On the other hand, the pro-apoptotic molecules of Bax, Bad and Bid accelerate cytochrome c release to progress the neuronal cell death and apoptosis (1, 27). Apart from Bad and Bid, Bax, specifically, induce apoptosis by interacting with the c-jun N-terminal kinase (JNK/Jun) pathway. It was found that when there is DNA damage and nuclear condensation, altered by the p53, AP-1 or Nuclear factor kappa-B (NF-κB) localization on DNA strands, JNK/Jun pathway becomes triggered which consequently activates the apoptotic Bax molecule to evoke apoptosis. Therefore, Bax molecule is considered as a downstream molecule for JNK/Jun pathway, in other words, Bax is essential for JNK to induce apoptosis. C-Jun N-terminal kinase (JNK) also considered as a stress-activated protein kinase (SAPK), ERK (extracellular signal regulated kinase) and p38-MAPK (p38 mitogen-

activated protein kinase) are all members MAPK family (40).

JNK, ERK and p38 are composed of MAPKKK, the un-activated form of MAPK, which phosphorylates to MAPK for further actions in nucleus. MAPK-JNK, MAPK-p38 and MAPK-ERK molecules translocate into nucleus only if the phosphorylation from MAPKKK to MAPK is occurred. All of these pathways' activation increases cell death either before or after ischemic stroke is induced. Moreover, p38/MAPK and SAPK/JNK are generally known to be the stress kinases and function as degenerating neurons by activating pro-apoptotic transcription factors for apoptosis (41). Considerately, inhibiting these kinases with specific kinase-inhibitors, such as melatonin, stand as a new therapeutic agent for stroke conditions and stroke related diseases (1, 40, 42, 43).

NF-κB is another neuro-protective agent also considered to act on apoptosis in stroke and ischemia related research. It was found that NF-κB protein factors play roles in the progression of various types of serious neurodegenerative diseases. NF-κB acts on DNA and blocks transcription therefore for its function the exact localisation of it is essential. Under physiological conditions, where there is no disease signal, NF-κB is stored in the cytoplasm and it becomes activated by canonical or non-canonical pathways to locate on DNA sequences in targeted genes for altering apoptosis. The p50 and p65 subunit localisation as heterodimers for NF-κB is an essential event in the NF-κB activation (44). NF-κB not only alters apoptosis but also plays roles in carcinogenesis and inflammation. Genes that are associated with NF-κB include κB elements which control the binding of NF-κB and via this approach NF-κB regulates the transcription of disease causing genes (over 500 genes). Since NF-κB is involved with multiple genes causing various types of serious diseases, regulating the NF-κB pathway with specific inhibitors, such as melatonin, would be a considerate approach (44). Although NF-κB induces apoptotic signalling, it was found that, it also activates survival signals in neuro-degeneration to allow neuron protection against ischemic injury (44).
Therefore melatonin can act on NF-κB through suppressing its apoptotic signalling or inducing its survival signals. Still, it was more commonly found that melatonin exert its action through suppressing the apoptotic signalling effects of NF-κB (39, 44).

Akt (v-akt murine thymoma viral oncogene homolog) also identified as protein kinase B, is

a protein kinase, which involves critical roles in regulating apoptosis. Akt includes a PH domain where PI3-K (Phosphatidylinositide 3-kinases) binds to activate it, hence the PI3-K dependent Akt activation. PI3-K/Akt complex pathway acts to inhibit apoptosis and allows survival activities in ischemic stroke administration (41). Regarding to the survival activity of PI3K/Akt, it was found that, PI-3K activates Akt by phosphorylation to allow Akt binding with Bad in which Akt acts as an obstacle for Bad thus the Bad translocation into mitochondria cannot be succeeded and apoptosis becomes suppressed (27).

The above explained neurological disease progression, specifically stroke, corresponding pathways of Bcl-2, JNK, ERK, p38-MAPK, NF-κB and PI-3K/Akt, are mainly concerned with the regulatory effects of melatonin in stroke conditions. Specifically, the PI-3K/Akt signalling pathway emerge from these pathways with its direct influence on melatonin's neuro-survival function. The neuro-protective effect of melatonin is found to be engaged with PI-3K/Akt pathway in response to the ischemic stroke conditions.

1.4. PHOSPHATIDYLINOSITIDE 3-KINASE (PI-3K)

1.4.1. Theoretical background

Phosphatidylinositide 3-Kinases (PI-3Ks) are a family of lipid kinase enzymes that are found to be involved in various types of crucial molecular processes including cellular survival. It was found that PI-3Ks act on the regulation of cell growth and survival through its act of phosphorylation on the 3′-hydroxyl group of phosphatidylinositide and phosphoinositides. Structurally, PI-3Ks are consisted of three different sub-classes; Class I, Class II and Class III (45).

The Class-I PI-3Ks are divided into two subgroups of Class IA and Class IB. Both of the subgroups are composed of regulatory (50-100 kDA) and catalytic (110 kDA) subunits by which the specific functional properties are accomplished. The Class IA subgroup is a heterodimer with specific p85 regulatory subunit and p110 catalytic subunit (46, 47). Five variants were found for p85 which are; p85α, p55α, p50α, p85β, and p55γ. On the other hand three variants for p110 were found which are; p110α, p110β and p110δ.

P85α is found to be the most famous amongst other regulatory subunits of class IA by being highly expressed and both p110α, p110β are the found to be the most famous catalytic subunit by being highly expressed. The Class IB subgroup is a heterodimer with the regulatory p101 subunit and catalytic p110γ subunit (47-49).

The Class I kinases together, are in charge of the production of PI-3-P, PI-3, 4-P2, and phosphatidylinositide (3, 4, 5)-triphosphate (PI-3, 4, 5-P3), through the phosphorylation of phosphoinositide (PI), phosphatidylinositide 3-phosphate (PI-3-P), and phosphatidylinositide (3, 4)-bisphosphate (PI-3, 4-P2), respectively (46, 48).

It was found that Class II and Class III PI-3Ks are found to be the derivatives of Class I in the matter of structure and function. The Class II kinase subgroup possesses only a catalytic subunit of p 110 which produces PI-3-P and PI-3-P and PI-3, 4-P2 through the phosphorylation of PI and PI-3-P, respectively (48).

On the other hand, class III subgroup of PI-3K are found to possess the most similar structure to class I subgroup, since both of them consist of a heterodimer structure. Class III subgroup is a heterodimer with Vps34 catalytic and Vps15/p150 regulatory subunits. It was found that class III subgroup only produces PI (3) P and PI (3, 4) P2 through the phosphorylation of PI and PIP, respectively (46, 48, 49).

1.4.2. Activation and inhibition of PI-3Ks

In order to act on signalling pathways efficiently, PI-3Ks are required to be activated. The activation of PI-3Ks is regulated through different processes which, in all, occur in the presence of extracellular stimuli. These activation regulatory processes include growth factor receptors with intrinsic protein tyrosine kinase activity, receptor substrates and with cooperative processes (48, 49). Herein, first of all, tyrosine kinase and G protein coupled receptors are required to be stimulated by growth factors of cytokines which convert PI-3Ks to the plasma membrane. On the other hand, tyrosine-phosphorylated proteins, act as receptor substrate regulators which interact with the regulatory domains of PI-3K Class I subunit and links the membrane-associated proteins with its substrates.

Referring to the cooperative processes, in order for PI-3K class I subgroup to become

activated, the link between regulatory variant of p85 and proline-rich regions of diverse proteins and the association between p110 catalytic subunit and GTP loaded Ras are required (50, 51).

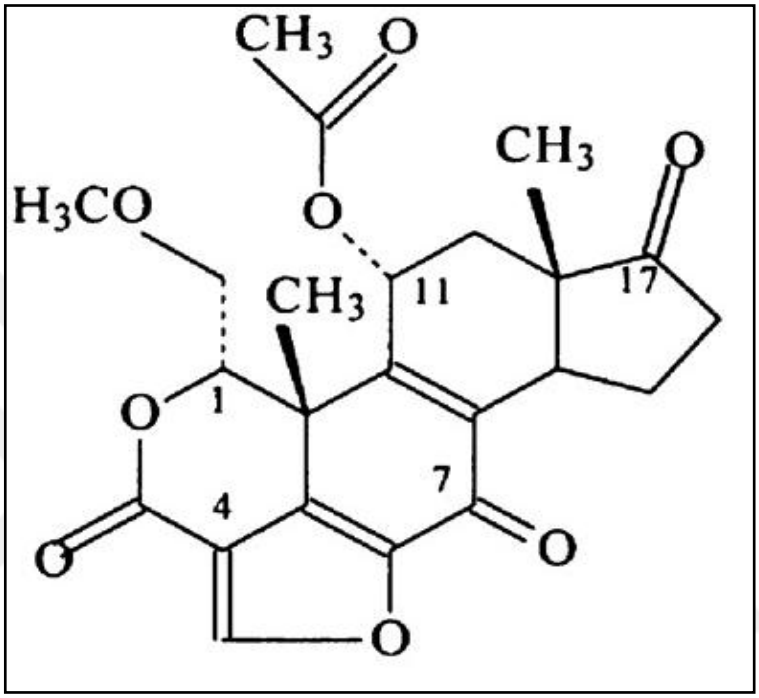

Figure 1.2. The chemical structure of Wortmannin, PI-3K inhibitor (52).

On the other hand, in order to demonstrate the roles of PI-3Ks in the survival mechanisms through PI-3K/Akt signalling pathway it is required to inhibit PI-3K from the system. This inhibition ideally illustrates the importance of PI-3K in the PI-3K/Akt pathway. Wortmannin, is a well-known non-competitive, irreversible inhibitor of PI-3K (53). Through the help of its chemical structure, as shown in Figure 1.2, Wortmannin allows a potent inhibition to PI-3K (54).

Moreover, once PI-3Ks are activated, phosphatidylinositide (3,4)-bisphosphate (PI(3,4)P2, PIP2), producing phosphatidylinositide (3,4,5)-triphosphate (PI(3,4,5)P3 and PIP3 become phosphorylated. The activation of PIP3 targets Ser/Thr kinase Akt and accelerates the mediation of Akt transition to cell membrane. Akt The activation of PIP3 activation also

co-localises phosphoinositide dependent kinase (PDK-1) and Akt becomes phosphorylated by PDK-1 on the serine 473 and threonine 308 residues. Consequently, the activated Akt inactivates the pro-apoptotic factor of BAD, in order to induce the anti-apoptotic effects (55).

1.4.3. PI-3K/Akt signalling pathway

The PI-3K/Akt pathway is an intracellular pathway which is involved with apoptosis hence neuronal survival. PI-3K/ Akt pathway progresses the signalling function via sequential sets of activations. First of all, the PI-3K/Akt signalling is initiated with PI-3K activation as explained above. Consecutively, Akt activation occurs as a result of PI-3K activation. Herein, another crucial signalling element enters the story; Mammalian target of rapamycin (mTOR).

Latterly, depending on Akt activation mTOR becomes activated. Moreover, mTOR brings with two other factors of synthase kinase (GSK3) and phosphatase and tensin homolog deleted from chromosome 10 (PTEN), which are found to be linked in this pathway and all together aim to regulate apoptosis and cellular survival (53, 56).

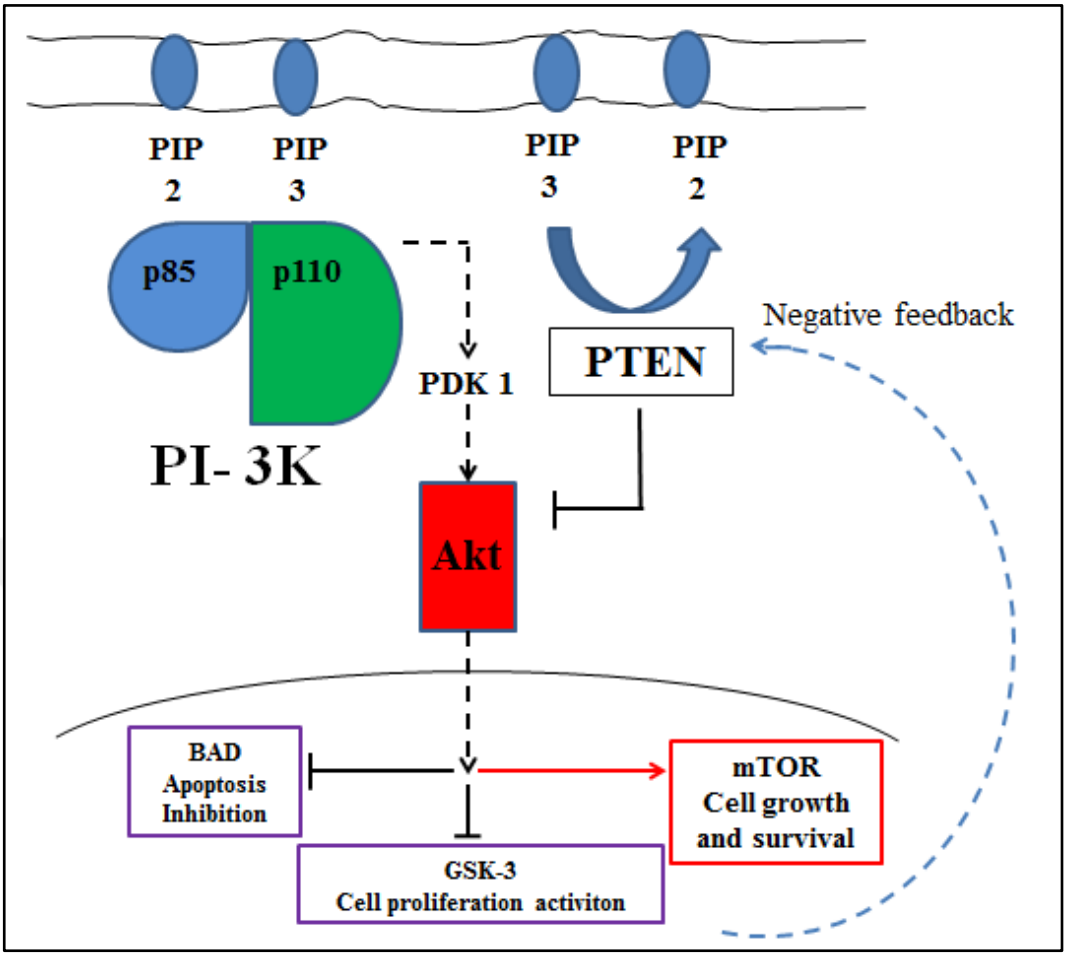

Figure 1.3. PI-3K/Akt pathway and underlying elements**.** PIP 2 and PIP3 are located on the plasma membrane. The PI-3K is consisted of the regulatory subunit of p85 and catalytic subunit of p110 which together phosphorylates the red labelled Akt protein. Phosphorylated Akt inhibits the formation of BAD and GSK3 and promotes mTOR for cellular growth. The active GSK-3 initiates negative feedback, as labelled with blue colour, allows PTEN phosphorylation and turns Akt into inactive form (57).

Figure 1.3 distinctly illustrates the PI-3K/Akt signalling pathway and its underlying elements. Focusing to thePI-3K/Akt pathway in detail, the signalling fired the Akt phosphorylation by the p85 and p110 subunits of class I PI-3K which suppresses PTEN to appear in the system and signalling process begin to initiate. Following, PDK1 accumulates to plasma membrane and Akt becomes phosphorylated. The activated Akt through phosphorylation moves into nucleus and here regulates the cellular survival through acting on several intercellular factors. Considering the trait of this project,

specifically, BAD, GSK-3 and mTOR factors are focused at this point, since they are found to be related with cell apoptosis, cell proliferation and cell growth, respectively (58, 59). Continuing, the activated Akt phosphorylates GSK3 in which the phosphorylated GSK3 suppresses GSK3's inhibitory action and allows cell proliferation. Similarly, the phosphorylated Akt also suppresses the BAD protein and inhibits the pro-apoptotic activity of BAD and through this mechanism blocks apoptosis.

From another view, the phosphorylated Akt activates mTOR and turns mTOR into an active form to freely act on producing cell growth. Considering mainly the cellular survival situation, mTOR acts as a central modulator of cellular metabolism by interrelating not only with Akt but also with intercellular elements such as, HIF-α and P70 (59, 60). The negative regulating mechanism for this PI-3K/Akt pathway is produced by the PTEN molecule. The non-phosphorylated and active GSK-3 phosphorylates PTEN on the plasma membrane. The phosphorylated and active PTEN dephosphorylates PIP3 to PIP2 by which the firing system of PI-3K pathway initiation turn into backwards and p85, p110 subunits of PI-3K becomes no longer active to phosphorylate Akt, therefore non-phosphorylation of Akt occur and Akt turns to be inactive. Overall, the cellular survival situation is regulated through the action of PI-3K/Akt signalling pathway and negative feedback mechanism (49, 55, 59).

2. AIM OF THE STUDY

There are two aims interrelated to this project. The first and the main aim is to optimise the hypoxia-OGD *in vitro* model on primary cortical neuron culture. The second aim is to investigate the role PI-3K/Akt pathway in the neuro-protective effects of melatonin through the help of both primary cortical neuron cell culture, as an *in vitro* model, and MCAO animal stroke model, as an *in vivo* model.

3. MATERIALS and METHODS

3.1. *IN VITRO* EXPERIMENTS

3.1.1. Primary cortical neuron culture

All experimental procedures were carried out with governmental approval according to the "ethical committee" guidelines for the care and use of laboratory animals. In addition, all animals were kept in an environment with 12 hours darkness and 12 hours light per day. New born (DIV from 1 to 3) BALB/C animals were dissected and cell suspension was cultured to acquire cortical neurons. The culturing method was progressed by a multistep protocol. At first the necessary mediums and equipment were prepared. Leibovitz's L-15 (L15, Sigma L5520, USA) and Neurobasal-A (NBA, Gibco,1x –L glutamine, 10888-022) mediums were supplemented with B-27® (Gibco, 17504-044), GlutaMAX-I™ (Gibco, 100X, 35050-038) and Antibiotic/Antimycotic Solution (Sigma, 100X, A5955) with the ratios of 2%, 1% and 1%, respectively.

Fluorodishes (WPI, 35mm, FD3).were coated with 750 µl, each, of 10% poly L lysine solution (Sigma, P6282, resuspended and diluted to 0.5 mg/ml with autoclaved dH_2O) and ensured that the entire glass bottom was covered with the solution. Fluorodishes were kept covered with poly L lysine solution until the final steps of culture in laminar flow (Thermo scientific, Safe 2020). Dissection procedure was continued and animals were euthanized with carbon dioxide and whole brain tissues were dissected in a dissecting dish (Nunc, Nunclon surface) including complete L15 medium. Cortical tissues were carefully dissected and separated from the brain meninx with the help of forceps (WPI) and divided into pieces of no more than six. These divided cortex tissues were incubated in L15 medium including 1% papain (Sigma, P4762) for 45 mins at + 4°C. Following the incubation, culturing process was continued with trituration steps and finally completed with two steps of centrifuging; 3ml of L15 complete medium was added to triturated tissue extract and centrifuged for 3 mins at 800 rpm + 4°C (Thermo scientific, SL16R), supernatant was aspirated and latterly 1ml of L15 complete medium was poured on the pellet and directly centrifuged, without resuspending the pellet, 3 mins at 800 rpm + 4°C.

The obtained pellet was re-suspended in 1ml of NBA complete medium. Cells were counted with a Neubauer improved haemocytometer (Isolab, 0.0025 mm^2 and 0.100 mm depth) in which one fluorodish was optimised and designed to possess 250000 cells in 1.5 ml NBA medium for all the experiments.

3.1.2. Hypoxia and oxygen glucose deprivation treatments optimisation

Hypoxia and OGD experiment's protocols for primary cortical neuron culture were optimised following various instructions as referenced in literature. Cultured primary cortical neurons were grown in a standard CO_2 incubator (Thermo scientific, forma steri cycle CO_2 incubator) by refreshing and contributing with adequate amount of culturing medium, NBA, in every other day for approximately 6 to 8 days.

In order to achieve the hypoxic conditions, fluorodish samples that included cultured cells were transferred into a specialised incubator (Thermo scientific, HeraCELL 150i) in which the O_2 level is set as 1% and CO_2 as 5%. Herein, the O_2 levels were decided to be 1% depending on the literature search (41, 61). For providing the OGD conditions NBA medium was changed with Hanks Balanced Salt Solution, HBSS (PAN Biotechnologies, w/o calcium, magnesium and phenol red, P04-341000). HBSS medium does not include glucose therefore assures the desired environment for OGD treatment by reducing the glucose uptake in cells, hence the name of OGD. On the 7^{th} day, where synaptic connections of cortical neurons were considered to be completed, both hypoxia and OGD treatments were at the same time. Hypoxia incubator's O_2 is set to 1% and mediums, HBSS and Dulbecco's Phosphate Buffered Saline (DPBS, PAN Biotechnologies, w/o calcium and magnesium, P04 36500), for OGD treatment were equilibrated and warmed within the hypoxia incubator. NBA medium was discarded and cells seeded on the fluorodishes were washed with 1ml DPBS for two times and 1.5 ml of HBSS medium was applied and fluorodishes were placed in the hypoxia incubator. Herein only the baseline protocol is explained thereby Add-on treatments. Extra drug treatment and the duration in hypoxia incubator could vary within the experiments.

3.1.3. Melatonin, Wortmannin dose determination

Melatonin and Wortmannin doses were decided after comparing and discussing the similar research completed and published in literature (41, 62). The final concentrations of Melatonin and Wortmannin doses were determined to be 5µM and 10µM, respectively. In order to reach the final concentrations, main stock solutions were prepared for each drug supplement. Both Melatonin (Sigma, M5250) and Wortmannin (Sigma, W1628) were dissolved in Dimethyl sulfoxide (DMSO, Sigma, and D1435). Both Melatonin and Wortmannin stock and working concentration solutions were prepared fresh exactly before the experiments start, kept on ice and protected from light.

3.1.4. Experimental design

There are two sets of ensuing experiments designed mainly in a similar route. At first, time versus hypoxia experiment was completed which latterly followed by the main experiment of Melatonin and Wortmannin treatments. First of all, in order to figure out the most efficient hypoxia time interval for primary cortical neuron cells, four different time depending groups were designed including 1, 2, 6 hours (1h, 2h, 6h) and a negative control group (-C). 1, 2 and 6 h (hours) groups were labelled corresponding the time that they spent in hypoxia and OGD conditions. -C did undergo neither hypoxia nor OGD conditions, therefore were kept only in normal incubator until other group's (1, 2, 6 h) samples completed the experiment. In total 4 fluorodishes were applied including one template for each groups of 1h, 2h, 6h and –C.

On the 7^{th} day of the culture, pictures from eight separate areas were taken by Hoffmann microscopy (Zeiss, Axio observer A1) before the hypoxia-OGD experiment was started. The hypoxia and OGD equipment and medium preparations were arranged as explained in the second paragraph of the 3.1.2 section. Once the media (HBSS, DPBS) were equilibrated and hypoxia incubator was set to 1% O_2, HBSS medium was applied and Hypoxia-OGD experiment was started as explained in the section 3.1.2. Herein, groups were placed in the hypoxia-OGD incubator at the same time and incubated for different time intervals (1h, 2h, and 6h). As soon as their duration was finished in hypoxia-OGD

incubator, mediums were changed back into NBA culturing medium and samples placed in normal CO_2 incubator for 24 hours of reperfusion.

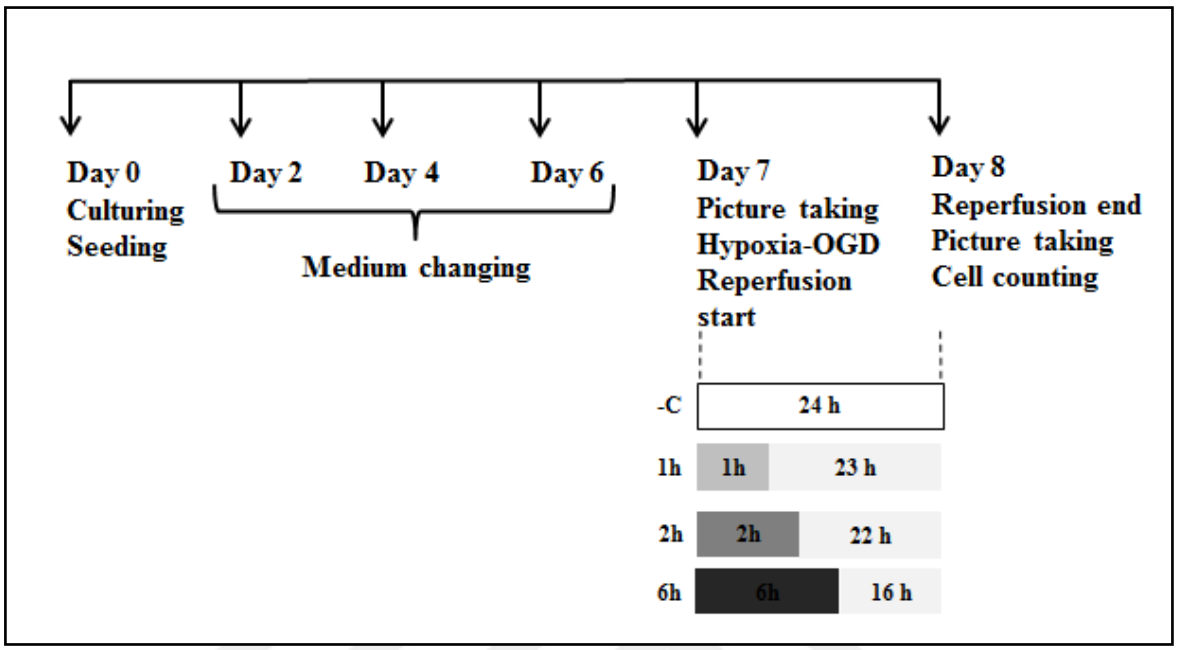

Figure 3.1. Time versus Hypoxia-OGD experiment design.1h, 2h, 6h and –C refer to the experimental groups. refer to the time spent in hypoxia and grey bars refer to the time spent in reperfusion conditions for the groups 1h, 2h and 6h. 24 h bar refers to the time spent in normoxia.

Hereof, the experiment was designed in a way to allow all groups ending the reperfusion at the same time. In order to meet this aim, as it is indicated in Figure 3.1, the time spending in hypoxia-OGD was subtracted from the 24 hours reperfusion and groups were stayed in reperfusion only to make the total time 24 hours after their time spent in hypoxia-OGD. After reperfusion, pictures were taken with the same method as they were taken before hypoxia-OGD and cells were counted for further cell survival percentage analysis. Within this content, the cell counting and cell survival percentage analysis processes were applied exactly the same way with Melatonin- Wortmannin treatment experiment below.

Secondly, for the Melatonin and Wortmannin treatment experiment, in order to induce cells with Melatonin and Wortmannin chemicals, the correct working solutions were introduced in OGD, HBSS, and reperfusion, NBA, mediums. In total five groups were applied for this experiment. Three of them cover the chemical treatment groups; Melatonin

(mel), Wortmannin (wort), Melatonin + Wortmannin (mel+wort) which included the sample treatments with Melatonin, Wortmannin and the combination of Melatonin and Wortmannin respectively.

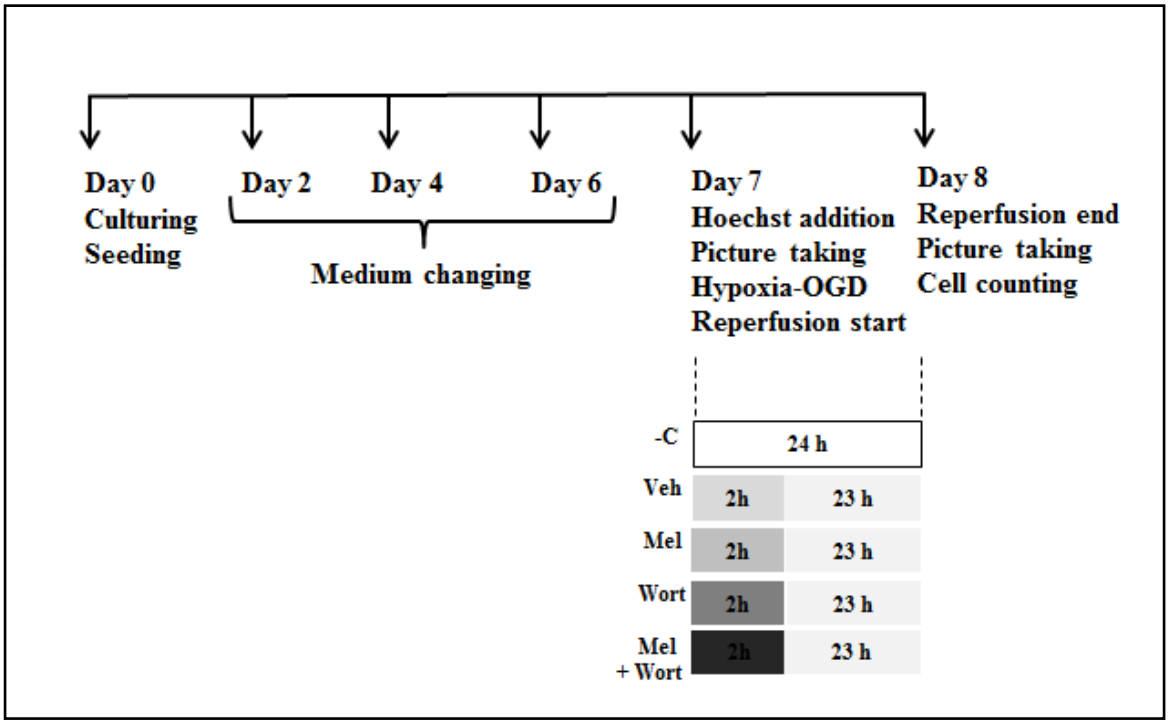

Figure 3.2. Melatonin-Wortmannin treatments with Hypoxia-OGD experiment design**.** Mel, Wort, Mel+Wort, Vehicle and –C refer to the experimental groups. Grey to black bars refers to the time spent in hypoxia and grey bars refer to the time spent in reperfusion conditions for the groups of Mel, Wort, Mel+Wort and Vehicle. 24 h bar refers to the time spent in normoxia.

Other two groups include the vehicle, which the samples were only treated with (baseline) hypoxia conditions and without any chemical treatments and -C, same group as it is applied in time versus experiment. In total there were nine fluorodishes applied, covering two templates for each of the mel, wort, mel+wort, vehicle groups and a template for the –C group. Primary cortical neuron culture and seeding processes were completed referring to the section of 3.1. The haemocytometer counting step calculations were adapted for 9 dishes each possessing 250000 cells. Once seeding process was finished and samples were healthily brought up to the day 7, hypoxia- OGD experiment was started. At first 20 µl of

Hoechst (1mg/ml) dye (bis benzimide trihydrochloride, Sigma,H33342) was added into these nine fluorodishes and incubated in CO_2 incubator (37 °C) for approximately 1 hour. The decisions of 20 µl and 1 hour incubation were made depending on various literature research and several trials. After 1 hour incubation, pictures from eight separate areas per fluorodish were taken by the use of confocal microscope (Zeiss, LSM 780) under 20X objective and DAPI (blue) filter.

Accordingly, once the Melatonin and Wortmannin chemical stock and final concentration solutions were prepared, as explained under the section of 3.1.3, calculations were made in order to cover two templates for each group. Once the stock solutions were prepared, as explained in section 3.1.2, final concentrations were calculated for two fluorodishes in each group except –C. As one dish supposed to include 1.5 ml of medium with cells, 3 ml of medium was needed for two fluorodishes and calculations were completed using this knowledge.

In order to find how much of stock solution is needed to prepare 5 µM of melatonin final concentration and 10 µM of Wortmannin final concentration, 2 mM stock solution and 1 mM, respectively, was diluted. Calculations were completed by the use of the formula explained below.

Melatonin	Wortmannin
$M_1V_1=M_2V_2$	$M_1V_1=M_2V_2$
2000 µM. V_1 = 5 µM. 3 ml	1000 µM.V_1= 10 µM. 3 ml
V_1: 7.5 µl	V_1: 30 µl

The Melatonin+Wortmannin group contained both melatonin and Wortmannin final concentration amounts of 7.5 µl and 30 µl, respectively, which was complemented with medium to reach 3 ml solution to cover two fluorodishes. Therefore Melatonin + Wortmannin group included 37, 5 µl of Melatonin and Wortmannin mixture (suspended) in

DMSO. This group determines the DMSO set value, 37, 5 µl for all other groups. All groups were designed to include 37, 5 DMSO in 3 ml, hence 18, 75 in 1.5 ml for –C group. Following the information explained above, the table 1 below shows the correct amounts of supplements for each group. In order to standardise the experiment all the groups -were treated with exactly same amount of DMSO and these values were decided by taken into consideration that DMSO toxicity is not more than 1-1.5%, in which herein toxicity is 1.25% (37.5 µl in 3000 µl).

Table 1: Experimental groups design on the intake of necessary supplement**.** Table shows the amount of supplements designed to cover all samples for each group. "_ "refers to the absence of the supplement for that specific group. The DMSO amount is optimised to be the same for all groups' samples in which one fluorodish includes 18.75 µl of DMSO.

	Supplements			
Groups	Medium	DMSO	Melatonin	Wortmannin
Melatonin	2.963 µl	30 µl	7.5 µl	_
Wortmannin	2.963 µl	7.5 µl	_	30 µl
Melatonin+Wortmannin	2.963 µl	_	7.5 µl	30 µl
Vehicle	2.963 µl	37.5 µl	_	_
-C	1.481 µl	18.75 µl	_	_

After incubating in the hypoxia incubator, mediums and final concentrations for each groups were made ready, the cultured nine fluorodishes NBA was discarded and washed with DPBS as explained in the section 3.1.2.

Following, the five different group solutions were applied into fluorodishes with correct labelling. 8 fluorodishes corresponding to the groups of mel, wort, mel+wort and vehicle were transferred into hypoxia incubator and left for 2 hours. On the other hand –C group was located back into the CO_2 incubator. After 2 hours in hypoxia incubator samples were taken out from the incubator and HBSS mediums changed with the NBA mediums including Melatonin, Wortmannin, Melatonin Wortmannin, vehicle group's supplements and located in CO_2 incubator for twenty four hours of reperfusion under normal incubator conditions. Herein, -C groups medium and place were not changed and kept the same until other groups completed with reperfusion. On the day 8, after reperfusion, Pictures were taken after twenty four hours reperfusion exactly the same procedure applied for picture taking before hypoxia. Fluorodishes were carefully located back into the CO_2 incubator and kept with the same mediums until day 9. On the day 9 samples were taken out from the CO_2 incubator and experiment was ended by fixation with 4% PFA for further ICC staining.

3.1.5. Immunocytochemistry staining

The amounts of all solutions below are explained for one sample/ fluorodish. Therefore for nine fluorodishes in this experiment all of the values were multiplied with nine. For all washing processes and other step, the same PBS and PBS-T solutions were applied. The orbital shaker steps were carried out the same and set for the same rpm (approximately 150). Herein, for this experiment double staining was administered in which primary and secondary antibodies were designed to mark and visualise glia and cortical neurons. In order to detect cortical neurons NeuN (MAB377C3 Anti NeuN, clone A60, cy3 conjugate, mouse, Millipore) and to detect glia GFAP (sc-9065, rabbit, Santa Cruz) primary antibodies were applied. Moreover, to be able to visualise nucleus 4', 6-Diamidino-2-phenylindole (DAPI, Sigma D9542) was applied. For the secondary antibody, Alexa Fluor 633 conjugated Goat anti Rabbit (Invitrogen, A21070) was applied in order to match with GFAP Rabbit primary antibody. For the NeuN primary no secondary antibody was needed as a result of primary antibody being cy3 conjugated.

Samples mediums were discarded and washed with 1ml DPBS twice. Samples were fixed with 750 µl 4% paraformaldehyde (PFA, Sigma 158127-5009) for 10 minutes at room temperature. PFA is securely discarded and samples were washed with 1ml PBS for 5 minutes and 3 times on shaker, approximately 150 rpm (Heidoplh, Rotamax 120). 1 ml of 0.2% Triton X (Sigma, X-100) was applied for permeabilisation for 10 minutes on shaker.

Samples were washed with 1ml PBS-T solution (0.2% Tween 20, Sigma P1379 in PBS), 500 µl of blocking solution including 10% Normal Goat Serum (NGS, Sigma, G9023) in PBS-T was added to the glass covered bottom end of fluorodish and incubated for 1 hour on shaker at room temperature.

Double staining primary antibody solution was prepared on ice with light sensitive conditions by mixing 1:100 dilutions of NeuN, GFAP with 3% NGS in PBS-T. For one fluorodish it was optimised to apply 100µl of this double staining primary antibody solution. Therefore for one fluorodish, 1µl of GFAP and NeuN antibody, 3µl of NGS was mixed in 95 µl of PBS-T. In order to prepare the solution for nine fluorodishes for this experiment these values were multiplied with nine and 100 µl was applied for each sample. Fluorodish lids were covered with parafilm (PM-996) which was located in a box including wetted dH_2O cotton in order to reduce the ability of samples becoming dried. Samples were located in +4°C on shaker for overnight incubation. On the following day, protocol was carried out with 1ml PBS-T washing step 3 times for 10 minutes on shaker. Secondary antibody solution was prepared on ice with light sensitive conditions. Secondary antibody solution was prepared similar to primary antibody solution in which 1:1000 dilution of Goat anti Rabbit 633 antibody was mixed with 3% NGS in PBS-T. For one sample 0.1 µl Goat anti Rabbit 633 and 3 µl of NGS were mixed in 96.9 µl of PBS-T to make up the solution 100 µl in total. Secondary antibody was added to all samples and incubated for 1 hour on shaker at room temperature. 1 ml 0.1% DAPI was prepared in PBS-T and applied on fluorodish to incubate 2 minutes on shaker at room temperature. Hereof, the actual DAPI incubation was optimised to be 3 minutes but because the samples for this experiment already included Hoechst duration was reduced to 2 minutes. Samples were washed with PBS for 5 minutes at room temperature. PBS discarded and one drop of fluoromount (Sigma, F4680) was added on each fluorodish that were instantly covered with special rounded cover slips (Deckglöser, 100 st, pcs, 15 mm) and left on bench with light sensitive conditions for drying.

Once coverslips were dried and adhered to the fluorodishes, the borders of glass bottom were nail polished in order to reduce further cover slip movement. After sealing cover slips with nail polish step samples were left on bench with light sensitive conditions over night and analysed on the next day with confocal microscopy. All confocal microscopy images were taken with 20X objective. The images taken for cell counting analyses were pictured by using bright field and DAPI light options. Only in ICC extra cy3 light was selected in order to visualise the NeuN marker.

3.1.6. Cell counting and cell percentage analysis formula

All confocal microscopy images were taken with 20x objective. The images taken for cell counting analysis were pictured by using bright field and DAPI light options and only in ICC extra cy3 light were selected in order to visualise NeuN marker. Differently, the images of non-stained time-versus hypoxia experiment were taken by Hoffman microscopy still under 20 X objectives but with no light application.

Once the images were taken at the before hypoxia-OGD and after reperfusion stages, blue labelled cells, referring to the Hoechst dyed nucleus thus alive cells, were counted from the eight separate areas for each experimental groups. The raw data was processed by averaging eight separate areas and templates of each group. The cell survival percentages were calculated in EXCEL by proportion calculation, in which, for example if the before hypoxia-OGD count average of a group is X_1 and the after reperfusion count average is X_2, the percentage of cell survival would be calculated by multiplying X_2 with 100 and dividing this to X_1. Through this way cell survival percentages were found for all groups and illustrated with bar chart graphics.

3.1.7. Statistics

In order to support the data statistically, a standard 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.

3.2. *IN VIVO* EXPERIMENTS

3.2.1. Animals

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 (n=7/ group).

3.2.2. Cerebral ischemia induction and reperfusion

Animals were anesthetised 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 an intraluminal filament technique. 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. 30 min after MCA occlusion, reperfusion was initiated by withdrawal of the monofilament. The wound was closed with sutures carefully and anaesthesia was discontinued and animals were placed back into their cages.72 hours (for 30 min occlusion) later, animals were deeply re-anesthetized and decapitated. Brains were removed and cut on a cryostat in 18 μm coronal sections.

The experimental groups of the *in vivo* arm of this project were designed by generally mimicking the *in vitro* experimental groups. There were four groups designed to represent the Melatonin and Wortmannin groups;

Control (DMSO + Vehicle): DMSO was applied 30 minutes before the MCAO with 2µl volume via i.c.v. After the 30 minutes, 50 µl vehicle (5% ethanol) was intraperitonally (i.p.) (administered.

Melatonin (DMSO + Melatonin): DMSO was applied 30 minutes before the MCAO with 2µl volume via i.c.v. After the 30 minutes, 50 µl 4mg/kg melatonin was i.p. administered.

Wortmannin (Wortmannin + Vehicle): Wortmannin was applied 30 minutes before the MCAO with 2µl volume and 0.1 mM/ 2µl i.c.v. After the 30 minutes, 50 µl vehicle (5% ethanol) was i.p. administered.

Wortmannin + Melatonin (Wortmannin + Melatonin): Wortmannin was applied 30 minutes before the MCAO with 2µl volume and 0.1 mM/ 2µl i.c.v. After the 30 minutes, 50 µl 4mg/kg melatonin was i.p. administered.

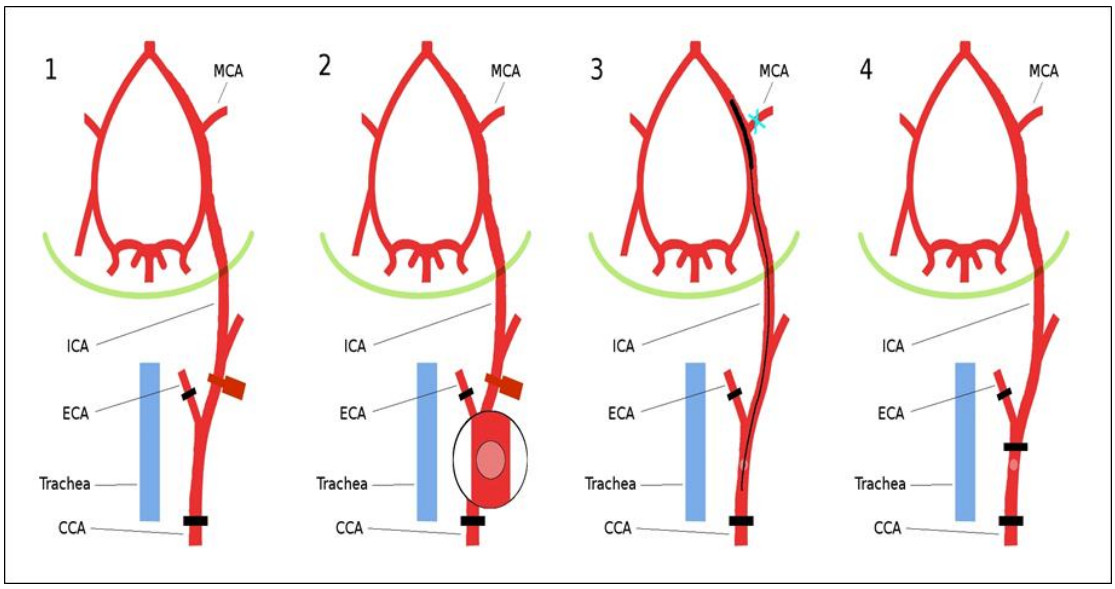

Figure 3.3. Graphical representation of the surgical operation of cerebral ischemia.

Herein being distinctive from the *in vitro* experiments, Melatonin was introduced to animals at the end of 30 mins MCAO. The same cerebral ischemia induced with MCAO procedure was applied to all groups and further treatments were applied as explained separately for each group above.

3.2.3. Cresyl violet staining

72 h after 30 min MCAO animals were re-anesthetised 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 bregma level, 2 mm apart, were stained with cresyl violet stain according to standard histological staining procedures. The cresyl violet protocol was progressed through the steps of were 4% Paraformaldehyde (PFA) fixation, Phosphate Buffered Saline (PBS) washing, cresyl violet staining, alcohol series administration and finally mounting the slides with Entellan (Merck, 1.07961).

3.2.4. Surviving neuron analysis

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.

3.2.5. TUNEL

In order to detect and quantificate the number of apoptotic cells, DNA strand breaks on the coronal brain sections were labelled via terminal transferase biotinylated-dUTP nick end labelling (TUNEL) technology (In Situ Cell Death Detection Kit; Roche, Switzerland). Cryostat sections from 30 min MCA occlusion 72 h reperfusion were fixed with 4% PFA/0.1 M PBS.

Sections were stained with TUNEL and counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Sections were analysed by quantifying DNA-fragmented cells in nine adjacent regions of interest in the striatum each measuring 62,500 μm^2 under a fluorescence microscope

3.2.6. DNA fragmentation analysis

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.

3.2.7. Statistics

In order to support the data statistically, a standard 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. TIME VERSUS HYPOXIA VISUAL ANALYSIS, *IN VITRO*

The primary cortical neuron culturing optimisation for OGD experiments, including culturing techniques, cell counts for each fluorodishes and solution's effect on cells, was performed (see section 3.1.1). The first aim of this study was the establishment of OGD method for primary cortical neuron cell culture. For this aim, we have studied the most suitable time interval for OGD.

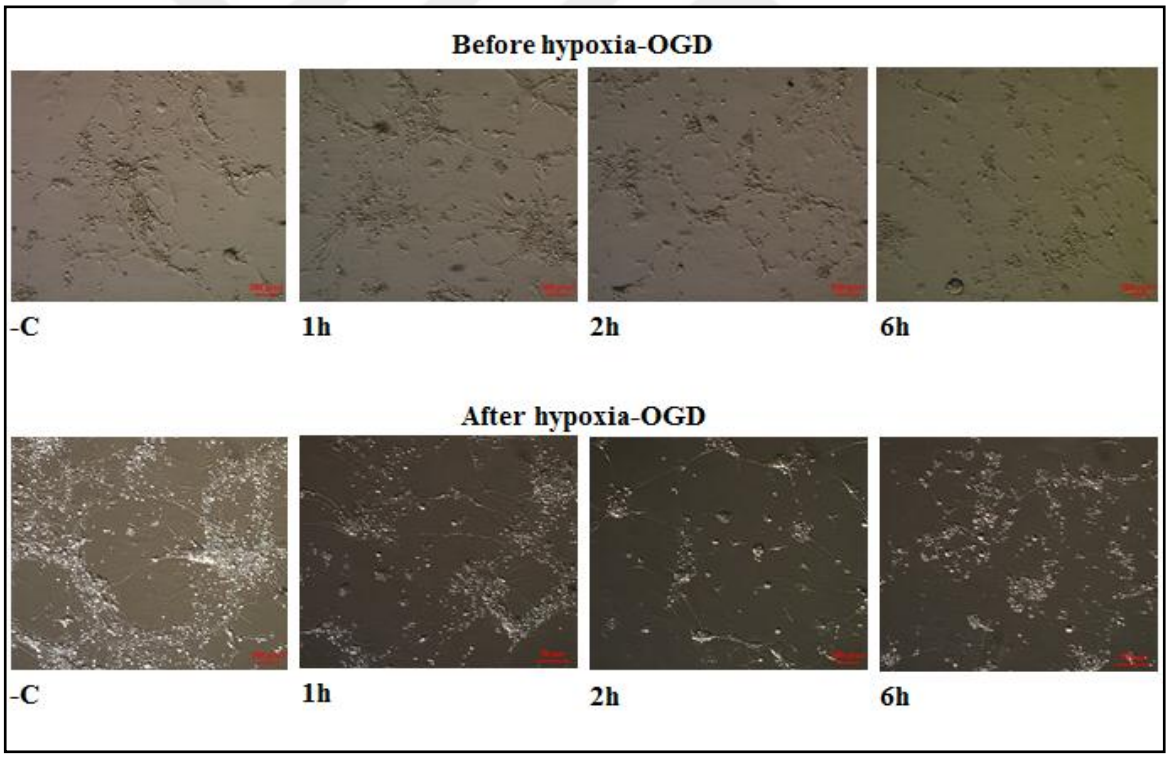

Figure 4.1. Time versus hypoxia-OGD experiment before and after images. These images were chosen from the 8 separate pictures that were taken for each group of –C, 1h, 2h and 6h. Number of the cells was optimised as 25 x10^4 per 35 mm culture dish for all groups. –C represents the negative control which was not subjected to hypoxia-OGD conditions.

Four groups were designed including three different time intervals with a negative control group. These four groups were treated equally same in considering the culturing methods until the 7th day when the OGD was induced. Herein, in order to address the most significant time interval, groups were imposed to OGD conditions at the same time but with different time durations; 1 hour, 2 hours and 6 hours. The microscopic images were taken before the hypoxia-OGD treatments were applied from the eight separate areas under the 20X objective for one culturing fluorodish (for further information see section 3.1.2 and 3.1.4).

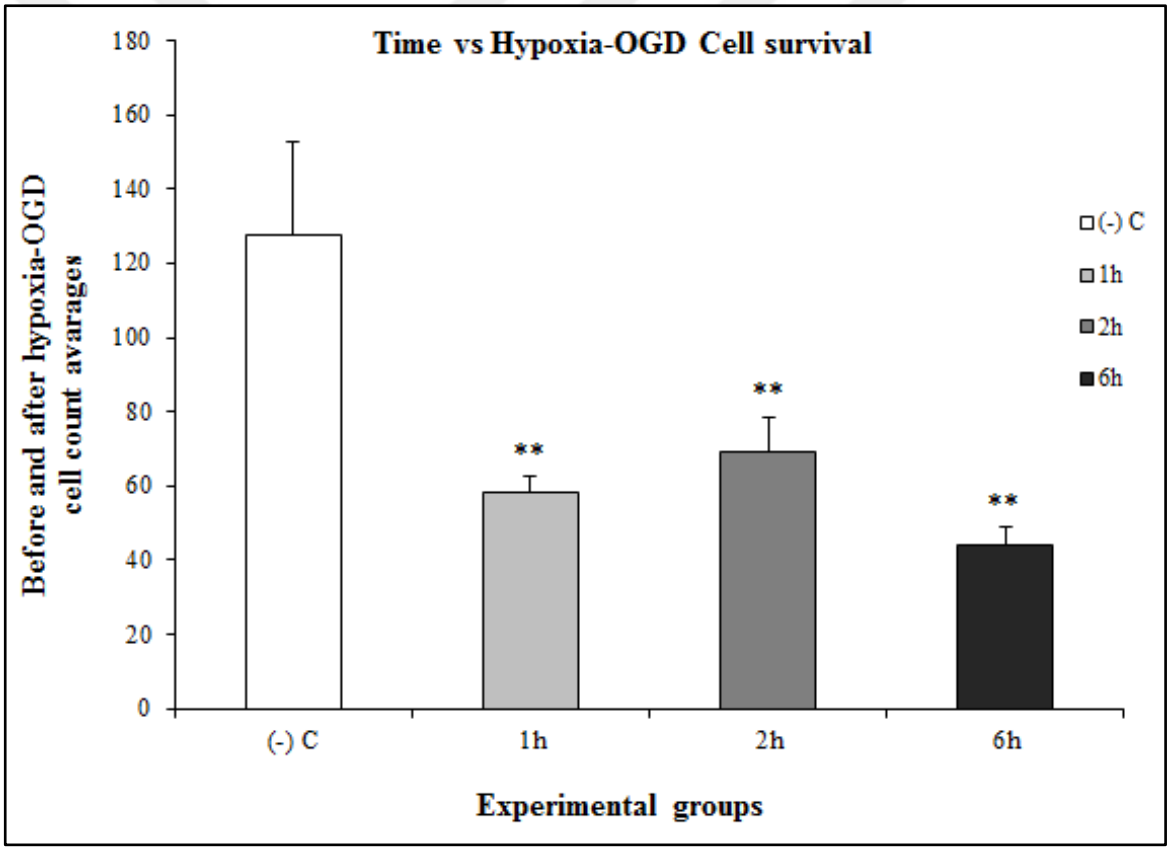

Figure 4.2. Time versus hypoxia-OGD experiment cell survival percentages. This graphic illustrates the cell survival percentages that were calculated by the averages of the cell counts counted from 8 separate areas for each group. The –C, 1h, 2h and 6h groups represent percentages of 127.67 %, 58.23%, 69.26%, and 43.93 %, respectively. 1h ** p= 0.01, 2h** p=0.06, 6h** p=0.000

The samples were prepared for the OGD treatment with appropriate OGD mediums. Samples were placed in the hypoxia incubator (1% O_2) at the same time and incubated for the indicated time durations. The –C group sample was placed in CO_2 until other groups finished their time in reperfusion. The different group samples were separately taken out from the hypoxia incubator, mediums changed back into culturing medium of NBA and placed in CO_2 incubator for further reperfusion process. The reperfusion durations were designed in a matter to meet the 24 hour time set in total including both the time spent in hypoxia and CO_2 incubator. To be able to address this, the reperfusion durations varied for each group depending on the time that they spent in hypoxia incubator (for further info see section of experimental design; 3.1.4). Once all groups completed their reperfusion durations the images of all groups, including –C, were taken for each group with exactly the same device and procedure as applied before the hypoxia treatment, Figure 4.1. In order to obtain the cell survival rate for hypoxia-OGD treatments on primary cortical neuron culture, the cells with the correct morphology as cortical neurons were counted from each group's eight separate pictures of before and after hypoxia-OGD treatments. Figure 4.1 demonstrates one area images of four groups before and after hypoxia-OGD treatment pictures. The eight separate area cell counts for before and after hypoxia-OGD treatments of each groups were averaged and analysed with a proportion calculation as explained in section 3.1.6.

Figure 4.2 illustrates the cell survival percentage data of four different groups after being exposed to hypoxia-OGD treatments. This experiment was designed to find the most significant time interval for hypoxic and OGD conditions, in terms of achieving the highest and the most meaningful drop in the cellular survival rate. The negative control group stained positive for DAPI appears to have the highest survival rate as it was expected since –C was not subjected to hypoxia-OGD and kept at CO_2 incubator in normal culturing conditions. Figure 4.2 indicates that 6 hours group possessed the highest cell survival percentage after hypoxia-OGD treatments. Although 6 hours group appeared to cause the highest drop, most meaningful group was decided to be the 2 hours group. This was simply because 6 hours duration in hypoxic and OGD conditions caused deteriorations in cortical cells morphology, Figure 4.1. The most significant and meaningful time interval for primary cortical neuron culture was therefore decided to be 2 hours for further experiments.

4.2. MELATONIN AND WORTMANNIN TREATMENTS VERSUS HYPOXIA, *IN VITRO*

4.2.1. Melatonin- wortmannin treatment responses to hypoxia-OGD conditions

After determining the optimum hypoxia-OGD time interval to be used for the primary cortical neurons, project was carried out with introducing drug treatments Melatonin and Wortmannin. The same cortical neuron culturing method with time versus hypoxia-OGD experiment was applied and the hypoxia-OGD experiment was started on the day 7.

Melatonin and Wortmannin treatments were introduced into samples within the OGD medium (HBSS) before hypoxia and within the NBA medium before the reperfusion stages. Melatonin and Wortmannin doses were decided after a literature search as explained in the section 3.1.3. Distinctively Hoecsht dye was added 1 hour before the experiment (see section 3.1.4) to all of nine samples for more effective cell counting process since, Hoechst dyes the nuclei of cells with blue marker. The hypoxia incubator was set to 1% $O_{2.}$ Fresh stock solutions were prepared in DMSO (see section 3.1.3) for both Melatonin and Wortmannin therefore vehicle was designed to carry DMSO as a control group. The Melatonin + Wortmannin (mel + wort) group included both Melatonin and Wortmannin final concentration solutions in DMSO. DMSO amount was standardised to include equal amounts of DMSO with the mel+wort group, since the combined group contained the highest DMSO volume. Therefore the groups that possess lower DMSO than the mel+wort group were received extra DMSO to equilibrate the DMSO amount with mel+wort group. All final concentration solutions, HBSS and NBA mediums were prepared as explained in sections 3.1.2 and 3.1.4.

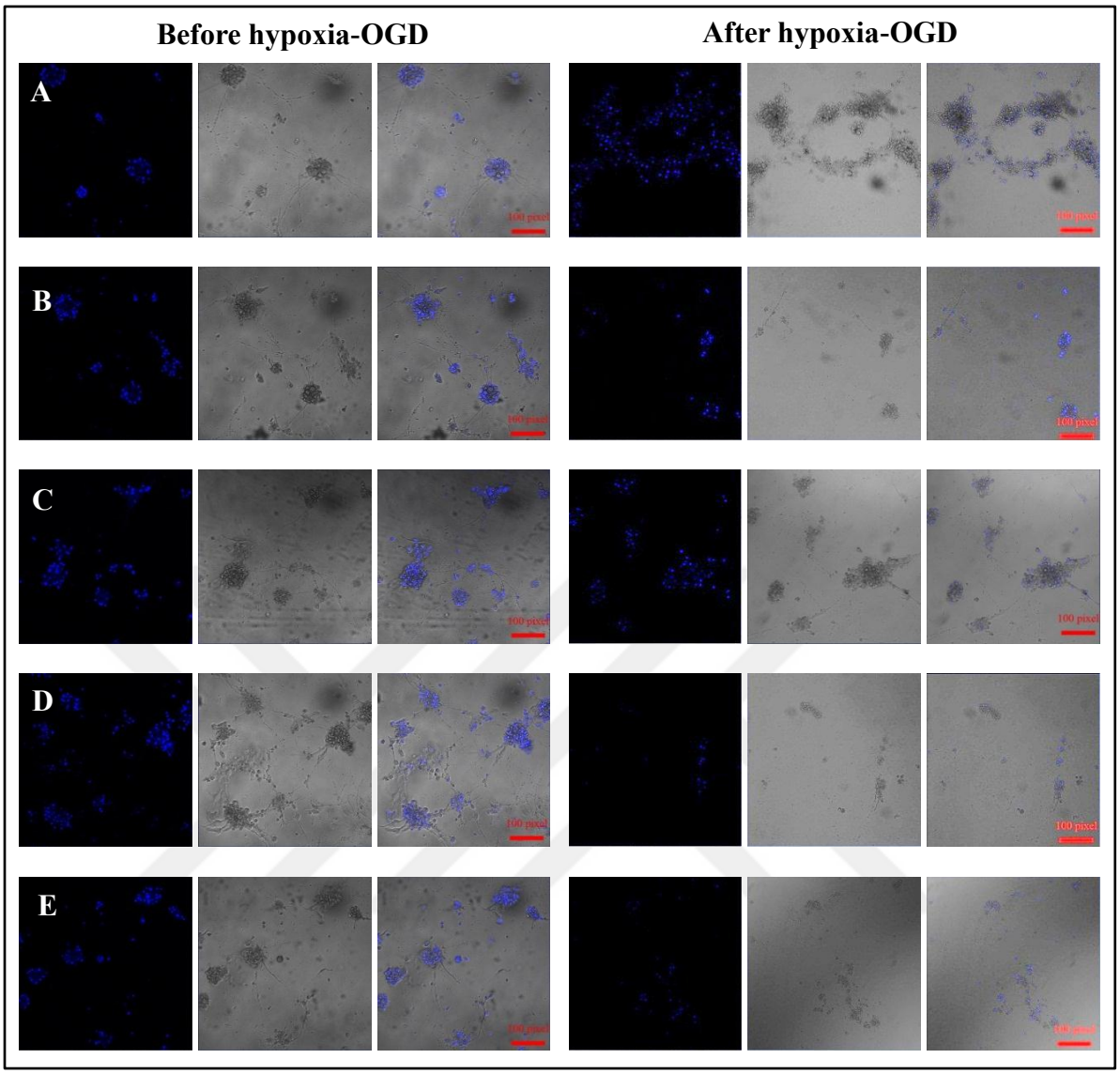

Figure 4. 3. Melatonin and wortmannin treatments versus hypoxia-OGD experiment cell survival percentages. Blue stains (Hoechst) corresponds to nucleus of cortical cells. These images were chosen from the 8 separate pictures that were taken for each group of -C, Veh, Mel, Wort and Mel+Wort. A, B, C, D and E refer to the –C (negative), mel (Melatonin), veh (Vehicle), mel (Melatonin), wort (Wortmannin), mel + wort (Melatonin and Wortmannin) groups, respectively. Number of the cells per well was optimised as 25 x10^4. The sequentially aligned 3 sets of images, before and after hypoxia-OGD section, represent the DAPI (blue), bright field and merged versions, respectively.

The before hypoxia-OGD images from 8 areas per dish were taken with the same protocol as explained in the "time versus Hypoxia-OGD" results for the final comparison analysis after the experiment.

The equilibrated and prepared final concentration solutions in HBSS were applied on 8 samples excluding –C and located in hypoxia incubator. Only the standardised amount of DMSO was applied to –C group in NBA and placed in the CO_2 incubator. After 2 hours of incubation, 8 samples including 2 fluorodishes each of Melatonin, Wortmannin, mel+wort and vehicle group were taken out (better word) from the hypoxia incubator, final concentrations of the groups prepared in NBA then applied on all of 8 samples and placed into the CO_2 incubator for reperfusion. All samples were incubated for 22 hours in the CO_2 incubator to fulfil the 24 hours duration from the beginning of the hypoxia-OGD process using the same logic as "time versus hypoxia-OGD" experiment. Following reperfusion the after hypoxia-OGD images from 8 separate areas of each sample were taken following the same protocol as the before hypoxia-OGD pictures. Cell counting analysis was applied for the before and after hypoxia-OGD images, and the calculated cell survival percentages were transferred into a bar chart graph (Figure 4.4) with the same method applied on "time versus hypoxia" experiment. Herein, because the cell nuclei were stained using Hoechst dye they were visualised clearly for the counting.

Figure 4.4 indicates that the melatonin group appeared to have the highest cell survival percentage amongst all the other groups excluding –C. The –C group possessed the highest cell survival rate with 75.04% as it was expected since –C stands for the control group which only included the standardised amount of DMSO and did not receive any hypoxic treatment.

The melatonin group showed the second highest cell survival rate with 52.94% indicating that melatonin treatment modulates the neuron survival as expected. On the other hand the Wortmannin group's cell survival rate was found to be lower than the vehicle with the percentages of 31.20 and 38.61, respectively. This result demonstrated that the Wortmannin treatment did not stimulate neuronal survival in the same extent with melatonin, which was observed as expected to be since Wortmannin stands for the well-known PI-3K pathway inhibitor. PI3-K is a known pathway which together with Akt phosphorylation and through this way regulates the cell survival.

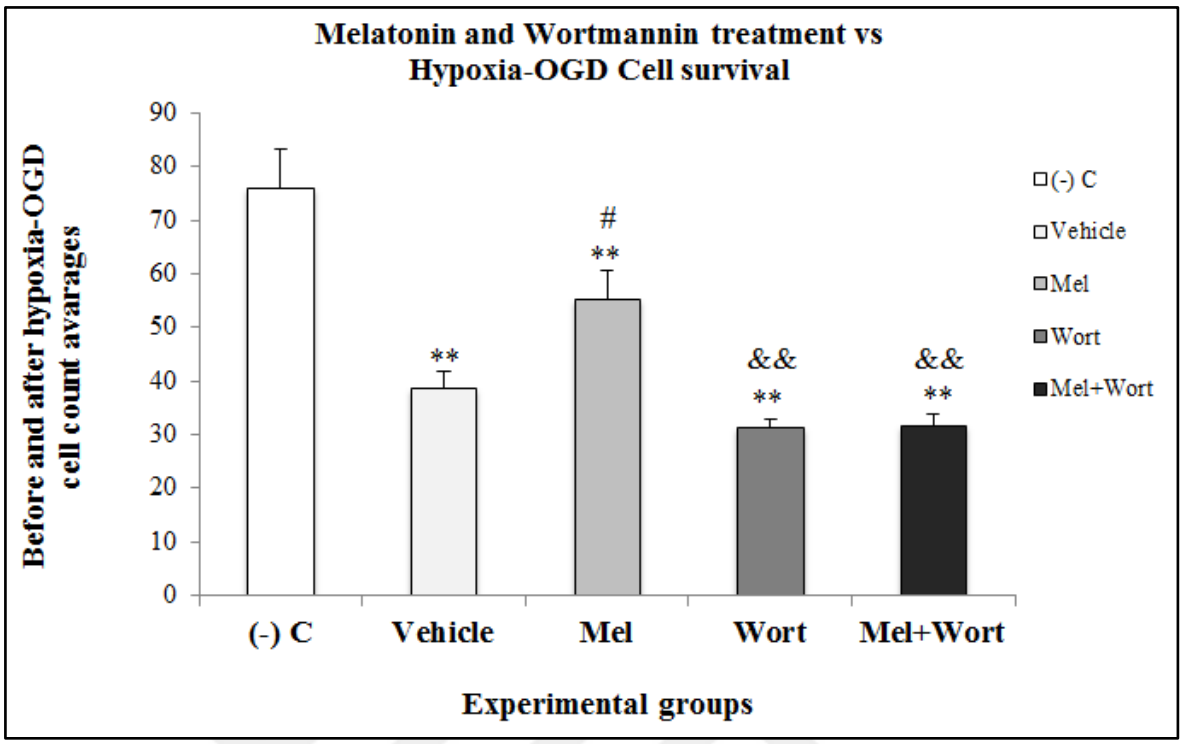

Figure 4.4. Melatonin and Wortmannin treatment versus hypoxia-OGD experiment cell survival percentages**.** This graphic illustrates the cell survival percentages that were calculated by the averages of the cell counts counted from 8 separate areas for each group. The –C, Veh, Mel, Wort and Mel+Wort groups represent percentages of 75.94%, 38.61%, 52.46%, 31.20% and 31.55%, respectively. . Veh **p= 0.00, Mel **p= 0.008, # p= 0.031, Wort ** p= 0.00 && p= 0.03, Mel+Wort** p= 0.00 && p= 0.03.

Herein, when Wortmannin was induced in the system this regulating pathway appeared to be blocked therefore cell survival decreased. This is why in Figure 4 even though both of them were being exposed to hypoxia-OGD conditions; the cell survival rate of wortmannin is lower than vehicle, compared to the control group that was related with DMSO.

The combined treatment group data of mel+wort was the third highest cell survival ranking and fell in between the vehicle and Wortmannin group with the percentage of 31.55. This result was expected since the combined group included Melatonin as well as Wortmannin. Although the mel+wort group possessing melatonin, the melatonin appearance was yet to be powerful to transcend the Wortmannin's inhibitory effect. Considering all cell survival percentages summarised in Figure 4.4, data authenticated that melatonin promotes

neuronal cell survival via PI-3K dependent pathway. These results were supported with the images specifically designed to focus on axonal extension analysis. Similar to the Figure 4.1 and 4.3, on Figure 4.5 before and after hypoxia-OGD pictures were taken with regard to axonal network, for each group of –C, Vehicle, Melatonin, Wortmannin and Melatonin +Wortmannin. The axonal extensions before hypoxia-OGD were measured for all groups whereas; the axonal extensions after hypoxia-OGD could only be measured for the –C and Mel group. Figure 5 represents the axonal extension differences before and after hypoxia-OGD conditions for the –C, Mel and Mel+Wort group only. The reason of emphasising on these groups relied on the output of after hypoxia-OGD images. Presumable to the cell survival percentages, the vehicle and Wortmannin group's images after hypoxia-OGD did not occur to possess any significant axonal extensions. Figure 4.5C and 4.5B significantly shows that axonal extensions tend to be recovered mostly in the Melatonin, whereas in the Melatonin+Wortmannin group the axonal extensions were begun to degenerate as directed with black arrows on Figure 5F. The negative control group which possessed the most recovered axonal extensions was represented in Figure 4.5A and 4.5B in order to make a clear comparison with the Melatonin group. On the other hand, Melatonin+Wortmannin, combined group, Figure 4.5E and 4.5F, were shown in order to investigate the role of PI-3K/Akt pathway in the presence of Wortmannin.

The Melatonin+ Wortmannin treatments versus hypoxia-OGD experiment was supported with a side- experiment of immunocytochemistry (ICC), where the 9 fluorodish samples were stained for specific neuronal markers. Consequent to the after hypoxia-OGD pictures were taken for the Melatonin+Wortmannin treatment experiment, samples staying in the reperfusion stage mediums were discarded and the ICC staining protocol was performed with DPBS washing and fixing (see section 3.1.5).

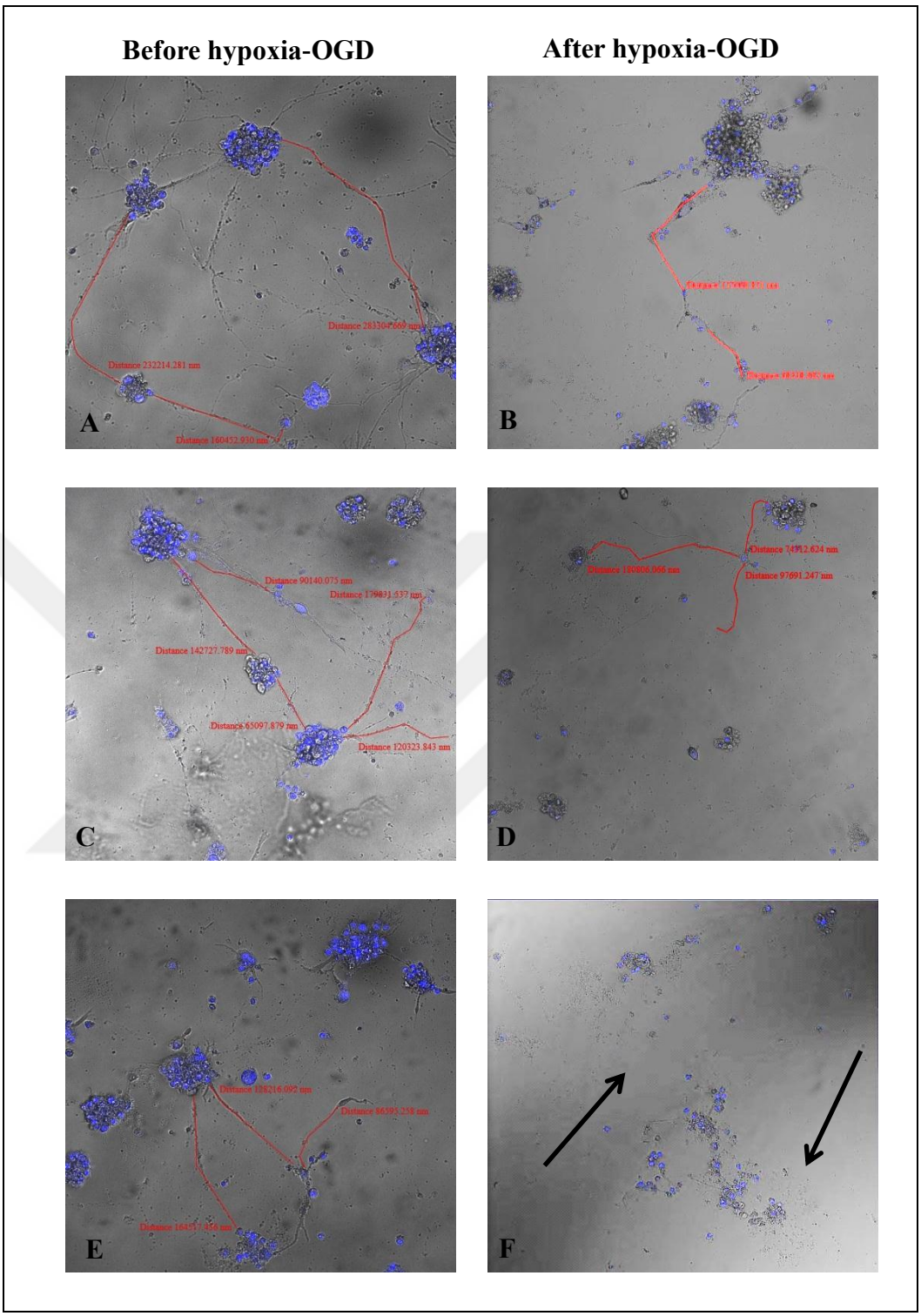

Figure 4. 5. The axonal extension images of melatonin and wortmannin treatment experiment. Blue stain (Hoeschst) corresponds to nuclei of cortical neurons. A and B: -C group, C and D: Melatonin group, E and F: Melatonin+Wortmannin group. The black arrows on the F directs to the areas where loss of axonal extension degeneration.

4.2.2 Melatonin-wortmannin treatment experiment immunocytochemistry results

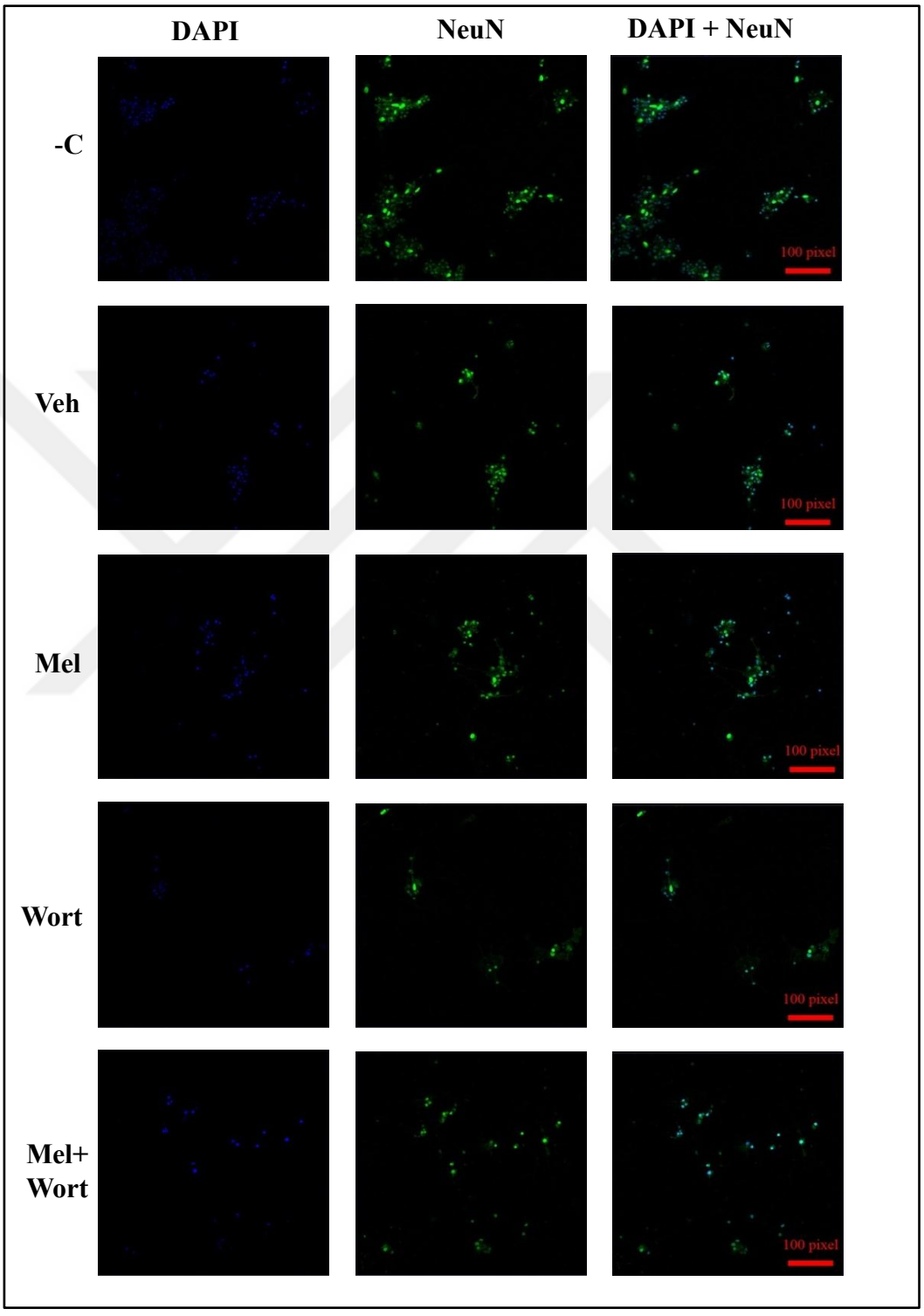

Figure 4.6. Immunocytochemistry (ICC) staining**.** -C, Veh, Mel, Wort and Mel+Wort correspond to the groups of negative control, vehicle, Melatonin, Wortmannin and Melatatonin+Wortmannin, respectively. DAPI stains with blue stains refer to nucleus of cortical cells and NeuN stains with green refer to neuronal nuclei.

Double staining was applied with GFAP and Anti-NeuN cy3 markers in order to mark glial cells and neuronal nuclei of consisting neurons, respectively. The GFAP marker was managed to mark glial cells however, did not exhibit qualified signals due to microscopy and antibody related technical issues. The ICC images thereupon were taken by applying cy3 and DAPI in a confocal microscopy, 20X objectives.

Figure 4.6 demonstrates the ICC images of Melatonin+Wortmannin treatment versus hypoxia-OGD experiment for all groups of –C, vehicle, Melatonin, Wortmannin, mel+wort. The blue stains refer to the appearance of DAPI, hence nuclei, whereas the green stain confirms the appearance of NeuN (Neuronal nuclei) hence neuronal nuclei. Depending on the fact that both ICC staining and image taking steps were applied after reperfusion, the number of cells dropped steadily between the groups of –C and Wortmannin by following the group order of –C, melatonin, vehicle, mel+wort, Wortmannin. The melatonin image evidently shows that cell survival and axonal extensions were protected, to an extent, in comparison with the other groups of vehicle, Wortmannin and mel+wort. The –C group appeared to have the highest cell survival as expected since it received neither hypoxia-OGD nor drug treatments. Mel+wort group happened to visualise better survival and axonal protection in comparison with the Wortmannin group, supporting the Figure 4.4 bar chart graphic result. Overall, the ICC staining side experiment results appear to be in the same line with the previous experiment, as expected, and openly support the Melatonin+Wortmannin treatment versus hypoxia-OGD experiment results.

4.3. *IN VIVO* RESULTS

30 minutes on going cerebral ischemia induced with MCA occlusion was applied as explained in the section 3.2.2 followed with reperfusion.

Experimental groups of Control (c), Melatonin (mel), Wortmannin (wort) and Wortmannin + Melatonin (w + m) were introduced (better word) as explained in the section 3.2.1. Next, 72 hours after 30 min MCAO animals were re-anesthetised and decapitated. The whole brains of these animals were cross sectioned onto charged cover slides through the aid of a crytostat and tissues adhered on cover slides were frozen down at -80°C.

Following, by the use of these frozen slides TUNEL and Cresyl violet staining were applied in order to attain DNA fragmentation and Neuronal Survival results, respectively. TUNEL and Cresyl violet staining were carried out as explained in the sections between 3.2.3-3.2.6.

4.3.1. DNA fragmentation

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was applied in order to detect DNA Fragmentation. After TUNEL assay protocol progressed successfully, the images were analysed by calculating nine random Region of Interest (ROI) and the mean values for each sample in the same group. The obtained results were transferred into a bar chart graphic to clarify the overall results for all groups. Figure 4.7 clarifies the mean values of calculated ROI for each group of c, mel, wort, w + m and these mean values of TUNEL positive cells/square were revealed to be 8.10 ± 3.75 , 3.23 ± 2.33 , 12.72 ± 4.65, 7.49 ± 3.14, respectively.

Figure 4.7 illustrates that the mel group possess the lowest mean value amongst other groups. This result proves that melatonin treatment significantly decreased the TUNEL positive cell number ($p < 0.05$) which supports the fact of melatonin promoting neuronal survival. On the other hand the highest mean value appeared to be in the wort group which means that Wortmannin treatment significantly increased the TUNEL positive cell number ($p < 0.05$). This result hints that the i.c.v injection of Wortmannin causes DNA fragmentation since Wortmannin is a PI-3K inhibitor, as expected. Although the i.c.v injection of Wortmannin on its own increases TUNEL positive cell number, when melatonin was administered latterly, it was found that Wortmannin significantly dropped the favourable effect of melatonin as shown on Figure 4.3.1 group w+m ($p < 0.05$).

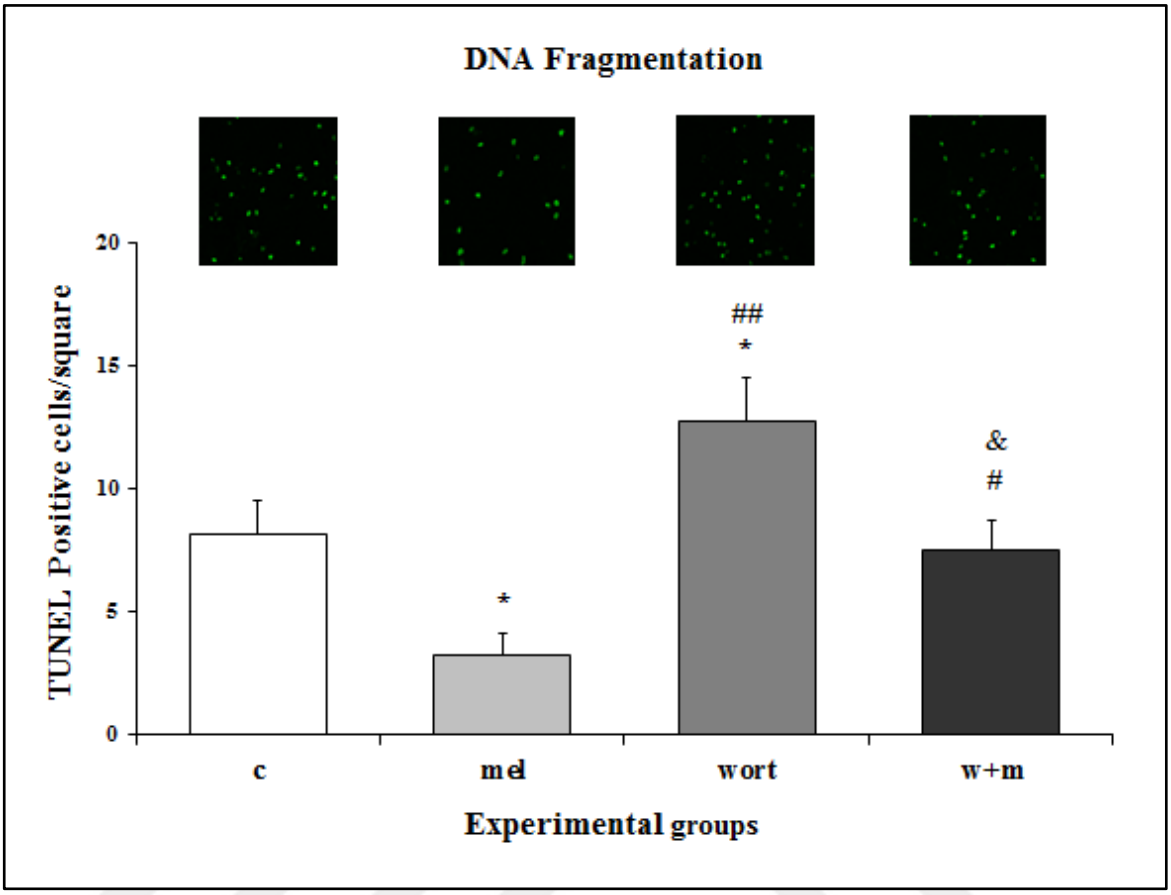

Figure 4.7. DNA Fragmentation analysis with TUNEL staining. The c, mel, wort and w+m corresponds to the control, melatonin, wortmannin and wortmannin+melatonin groups, respectively. The c, mel, wort and w+m represent percentages of 8.10%, 3.23%, 12.72% and 7.49%, respectively. The figures above the bars correspond to the TUNEL staining images. The green stains represent the TUNEL positive cells.

This data indicates that when PI-3K/Akt pathway is inhibited DNA fragmentation increases, hence the loss of cell survival. Moreover, because melatonin administration was not enough to regain the cell survival, here this data supports the *in vitro* results in a matter that melatonin promotes cell survival through the PI-3K/Akt pathway.

4.3.2 Neuronal survival

The DNA Fragmentation results were accompanied with the Neuronal Survival analysis after Cresyl violet staining, in order to show the cell survival rate diversity amongst all groups.

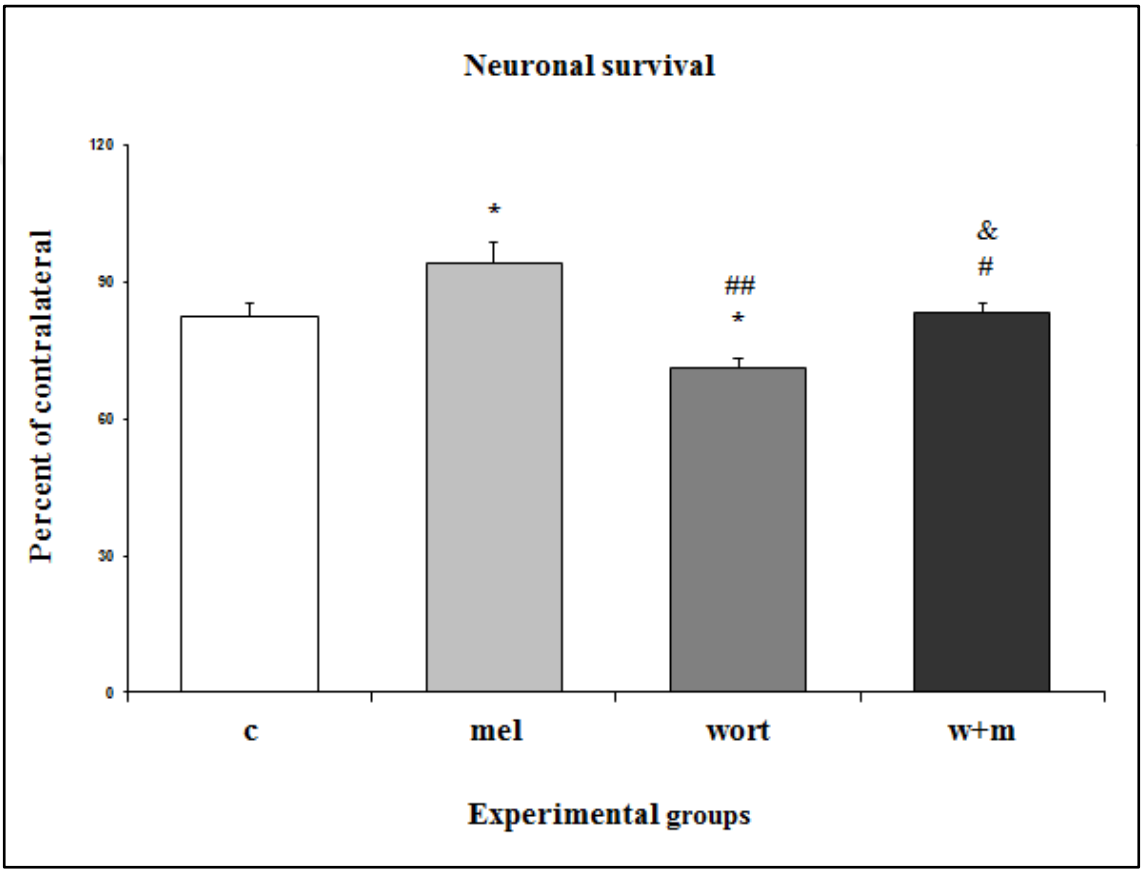

Figure 4.8. Neuronal survival analysis. The c, mel, wort and w+m corresponds to the control, melatonin, wortmannin and wortmannin+melatonin groups, respectively. The c, mel, wort and w+m represent percentages of 82.66%, 94.06%, 71.16%, 83.31% with the SD of 6.54, 12.82, 5.93 and 5.87, respectively.

The frozen brain tissues were cross sectioned. Following, the same protocol of cell counting and analysing were applied as explained in the DNA Fragmentation result. The sections from defined rostrocaudal levels, specifically from the bregma striatum level 2mm apart, of frozen brain cross sectioned slides were picked and stained with cresyl violet according to standard histological staining procedures (see section 3.2.3).

Neuronal survival analysis was applied after the specific cross sectioned brain slides were successfully stained with Cresyl violet staining protocol and the images were analysed by calculating nine random ROI and the mean values for each sample in the group. Throughout this mean value data the percentage of surviving neurons was determined for each group of c, mel, wort and w + m.

Figure 4.8 shows the mean values of calculated ROI for each group of c, mel, wort, w + m and these mean values of Neuronal survival percentages were revealed to be 82.66 ± 6.54, 94.06 ± 12.82, 71.16 ± 5.93, 83.31 ± 5.87, respectively. Opposite to the Figure 4.7 results, herein mel group possess the highest mean value amongst other groups, corresponding to the neuronal survival to be highest on the melatonin group, as expected. This result proves that melatonin treatment significantly increased neuronal survival percentage ($p < 0.05$) which again supports the fact of melatonin promoting neuronal survival. On the other hand the lowest mean value appeared in the wort group illustrates that the i.c.v injection of Wortmannin significantly decreased neuronal survival percentage ($p < 0.05$). Although the treatment of melatonin increases neuronal survival percentage, when melatonin was administered after i.c.v injection of Wortmannin it was found that Wortmannin significantly reduced the favourable effect of melatonin as shown on Figure 4.8 group w+m ($p < 0.05$). This result indicated that the success of melatonin in surviving neurons could be masked when combined with Wortmannin, hence the crucial role of PI-3K/Akt pathway in Melatonin promoted neuronal survival.

5. DISCUSSION

The unceasing attention on stroke research relies on the fact of stroke, as a disease, being described to be the third most common death cause in the developed countries, including cancer and cardiovascular diseases. Focusing on the stroke research, it was found that almost 80% of different stroke cases occur as a result of brain vessel blockage by MCAO (63). Considering the clinical reputation of stroke, it is inevitable for research to head towards into the field of treatment by mimicking the MCAO in animal studies.

Animal MCAO model is found to be the most practised method for ischemic stroke research since it allows strong resemblance with the clinical stroke disease caused by MCAO. At this stage, melatonin enters the story by its compelling feature of providing a significant therapy to stroke. Melatonin possess a colossal impact at ischemic stroke curing aimed research including both animal and cell culture studies (41, 61, 64). Melatonin treats ischemic stroke through its act on obtaining neuronal survival and this specificity occurs through signalling mechanisms (28). For this reason, our group focused on melatonin for more than 15 years.

PI-3K family consists of enzymes involved in various cellular functions including survival. PI-3K function in cellular survival is better explained with a signalling pathway which is known to be merged with the Akt pathway. Amongst other cellular survival roles in cancer research, PI-3K/Akt pathway plays a critical role by mediating neuronal survival in brain, hence the importance in the matter of stroke (29, 32, 58). Herein combining the knowledge with the appearance of melatonin in stroke research, it was found that melatonin inserts its neuro-survival effects through the PI-3K pathway.

The neuro-survival role of melatonin through PI-3K/Akt pathway is a well-studied and understood topic in ischemic stroke related animal studies, whereas remains unfocused and yet to be supported with a cell culture based study.

This project was designed to address two interrelated aims designed as; (i) to understand once again the effect of melatonin on animal based study, since the neuro- survival act of melatonin has been well proved with animal study by several researchers, and (ii) to

elucidate the remedial action of melatonin on a cell culture based study with highlighting the inclusion of PI-3K/Akt pathway.

In order to prove the survival effects through animal study ischemic stroke was introduced via MCAO animal model. The MCAO introduced ischemic stroke model was combined with specific treatments including melatonin and Wortmannin. Herein, Wortmannin was applied to show the beneficiary effects of melatonin by testing the relationship of Melatonin with neuronal survival signalling pathways. Wortmannin is a well-known irreversible PI-3K inhibitor which blocks PI-3K pathway and therefore cellular survival (52, 53). This *in vivo* part of the project was carried out with four distinct groups of c, mel, wort and w+m. The effects of these treatments were addressed with two separate analysis; DNA Fragmentation and Neuronal Survival. DNA fragmentation analysis was measured through examining TUNEL staining results and Neuronal Survival analysis was measured through examining Cresyl violet staining results. TUNEL positive results indicated that the least DNA fragmentation occurred in the melatonin group and oppositely the highest DNA fragmentation occurred in the Wortmannin group. This result suggests that melatonin promotes neuronal survival after stroke conditions by having the least DNA fragmentation rate, hence death cell rate.

On the other hand, Cresyl violet staining supported this result from the opposite view that where melatonin group possessed the highest Neuronal survival percentage and Wortmannin group possessed the lowest Neuronal survival percentage. This result illustrates once again that melatonin significantly decreases the number of damaged cells while promoting survival after stroke conditions. Melatonin+Wortmannin group's cell survival percentage was significantly higher than the Wortmannin group's cell survival percentage. In both analyses the combined group of Melatonin + Wortmannin cell survival percentages were appeared to be in between the Melatonin group and the Wortmannin group cell survival percentages. This result highlights that the beneficiary effect of melatonin becomes less effective when Wortmannin is introduced, hence the role of PI-3K/Akt pathway in the neuro-protective action of Melatonin.

The cell survival percentage difference between Wortmannin and Melatonin+Wortmannin group suggests that Melatonin inserts the neuro-protective effects not only through PI-3K/Akt pathway but also with the involvement of other molecular pathways, which could possibly be MAPK, NF-κB, Bcl-2, ERK and JNK pathways. These results are in line with

the findings published by similar projects. For example, in the related study, we, Kilic's group, demonstrated the involvement of ERK1/2, JNK and Akt pathways in acute and prophylactic (chronic) application of melatonin after focal cerebral ischemic stroke is induced in mice. The results of this study revealed that the PI-3K/Akt pathway activation was observed more in acute melatonin treatment in comparison with prophylactic melatonin treatment. However, the ERK1/2 and JNK pathways' activation were only observed in prophylactic melatonin treatment, shown via western blotting experiments (28, 42). Therefore it can be understood from this research that PI-3K/Akt pathway responds to melatonin for quick and short neuro-protection function whereas ERK1/2 and JNK pathways act through the long-term effects of melatonin. These results stand for evidence of which ERK1/2 and JNK-MAPK phosphorylation might maintain the melatonin function, since they are only observed in prophylactic melatonin treatment. The prophylactic use of melatonin in protecting the focal ischemic stroke is also shown through the inhibition of ECE-1, endothelin converting enzyme-1, which is induced in the ischemic model to detect the vascular effects of melatonin (65). The prophylactic delivery of melatonin inhibits ECE-1 and decrease vascular dysfunctioning after ischemia (65). Hereof, besides mediating neuronal protection, melatonin could also reduce the levels of blood fluid extravasation, which is increased in ischemic brain due to the damaged capillaries. Melatonin administration managed to diminish the degree of oedema to a significant extent, MCA occlusion in rats (66). Therefore we could confirm that apart from its neuro-survival actions, melatonin exposes some vascular effects such as reducing arterial blood pressure by increasing cerebral blood flow, CBF, after focal ischemia (12, 34).

The *in vitro* part experiments of this project were started with optimisation of the hypoxia-OGD procedure for primary cortical neuron culture. Hypoxia-OGD method was carried out in order to mimic the MCAO ischemic stroke model on primary cortical neurons. The blockage of oxygen transfer, hypoxia, was achieved with reducing the oxygen levels of the culture existing environment to 1% from normal condition of 21%. Moreover, the oxygen-glucose deprivation, OGD, was achieved by switching the normal culturing medium into the OGD medium of HBSS in the presence of 1% O2. This main part of the project was addressed by following two consecutive experiments; at first the time versus hypoxia-OGD was applied for time optimisation and later the aim was reached with Melatonin-

Wortmannin treatments versus hypoxia-OGD. Once the hypoxia-OGD method was optimised significantly, the convenient time duration for applying hypoxia-OGD on primary cortical neurons was determined through the time versus hypoxia-OGD experiment. It was obtained that 2 hours of hypoxia-OGD revealed the most efficient time for primary cortical neurons. Here, the *in vitro* model for ischemic stroke was therefore developed for further applications. Thereafter, a Melatonin and Wortmannin treatment versus hypoxia-OGD experiment was completed with containing the groups of –C, Mel, Wort, Vehicle and Mel+Wort. The OGD protocol was successfully optimised with 1% O_2 on mice primary cortical culture in comparison with a similar study applied on primary cortical cultures of rat cortex (61). Referring back to the literature, the *in vitro* protocol optimisation with 5 μM Melatonin and 10μM Wortmannin concentration was significant enough to show the effects of both treatments in comparison with a similar study applied in primary astrocytes (41).

According to the cell survival percentages calculated for Melatonin and Wortmanin treatments versus hypoxia-OGD experiment the general overview of results appear to have a similar trait with the *in vivo* neuronal survival graphics. The Melatonin occurred to be the second highest percentage and the –C group expectedly possesses the highest cell survival rate amongst other groups, since –C group was exposed to neither hypoxia nor OGD conditions. The lowest cell survival percentage belongs to the Wortmannin group which is predictable since Wortmannin blocks the PI-3K/Akt pathway and similar to the *in vivo* neuronal survival analysis results. The vehicle group refers to the group which received hypoxia-OGD conditions without any treatments and appears to possess the second highest cell survival rate after Melatonin group's result. In comparison with the lower survival rate of the vehicle group, this result illustrates the beneficiary effects of melatonin that when applied at hypoxia-OGD and reperfusion phase Melatonin ameliorates neuronal survival. Although the general overview of graphical results follows the similar trait as *in vivo* neuronal survival analysis results, there were some variable results between *in vivo* and *in vitro* results. For example *in vivo* results show that control group's neuronal survival rate is lower than melatonin group's whereas, *in vitro* results show that –C appear to have the highest survival percentage. This difference was considered to occur depending on the reason of the *in vivo* control group being exposed to MCAO ischemic stroke conditions

whereas *in vitro* –C group being treated only in the normal culturing conditions and not receiving any hypoxia-OGD conditions.

On the other hand Melatonin + Wortmannin combined results occur in between vehicle and Wortmannin group. This result of Melatonin + Wortmannin group being not higher enough to reach the melatonin group's survival rate and not lower enough to fall under the Wortmannin's survival rate illustrates that Wortmannin suppresses the neuro-protective effect of melatonin. Although Wortmannin suppresses melatonin's survival effects, the presence of melatonin in the Melatonin Wortmannin group, forced neuronal survival percentage to be higher than the Wortmannin group. Together, these results prove that Melatonin significantly ameliorates the number of damaged neurons after hypoxia-OGD conditions and noticeably completes this role through the PI-3K /Akt signalling pathway.

Moreover, *in vitro* results also revealed that melatonin not only modulates neuronal survival but also protects the axonal extensions of cortical neurons. The visual results of Melatonin + Wortmannin treatment versus hypoxia-OGD experiment proves that axonal extensions were only protected in the Melatonin group with efficient proportions.

6. CONCLUSION AND FUTURE RECOMMENDATIONS

6.1. CONCLUSION

In this study, the neuro-protective roles of melatonin through PI-3K/Akt pathway was investigated by both primary cortical neuron culture *in vitro* model and MCAO animal *in vivo* model. The *in vitro* model was created with hypoxia-OGD experiment and further supported with Melatonin and Wortmannin treatments. The cell survival rates were calculated analysing before and after hypoxia-OGD images. It was found that melatonin treatment significantly increased the cell survival percentage after hypoxia-OGD conditions. This finding was supported with the *in vivo* animal experiments, where 30 min MCAO was applied, that Melatonin significantly decreased infarct volume, brain swelling and DNA fragmentation when compared to the vehicle-treated control groups. Both *in vivo* and *in vitro* results followed the same trait. The effects of PI-3K/Akt on Melatonin treatment were addressed with the Wortmannin treatment and results revealed that the presence of Wortmannin masked melatonin's protective effects to a significant level, in the Melatonin+ Wortmannin combined group. This combined groups' results from both *in vivo* and *in vitro* experiments proved the crucial role of PI-3K/Akt pathway in the neuro-protective effects of Melatonin.

To conclude, both aims of this study were reached without any major deviations. At first the hypoxia-OGD *in vitro* model was optimised on primary cortical neuron culture which then further supported with illustrating the role PI-3K/Akt pathway in the neuro-protective effects of melatonin through the help of both primary cortical neuron culture, as an *in vitro* model, and MCAO animal stroke model, as an *in vivo* model. Overall, the sets of experiments demonstrated in this project stand as evidence that PI-3 Kinase/Akt pathway has a crucial role in the neuro-protective effect of Melatonin.

6.2. FUTURE RECOMMENDATIONS

As future projects, there are some further experiments recommended to improve the findings of this project. Focusing on in vitro experiments, the roles of -PI-3K/Akt pathway on melatonin should be targeted with the addition of treatments that could possibly block melatonin's action, as looking to the overall picture from another view, for example by administering luzindole as a melatonin receptor inhibitor. On the other hand, for both *in vitro* and *in vivo* arms of the project more quantitative experiments should be applied including western blot analysis to show the involvement of Akt protein. Moreover, focusing on the PI-3K/Akt pathway, apart from the inhibition of PI-3K by Wortmannin, the pathway should be targeted at different levels in order to show the importance of PI-3K/Akt in melatonin's protective actions with more confidence.

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