

T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF HISTOLOGY AND EMBRYOLOGY



**THE EFFECT OF AGOMELATINE ON
IMMUNOMODULATORY PROPERTIES OF MICROGLIAL
CELLS IN EXPERIMENTAL NEURODEGENERATIVE
DISEASE MODEL, *IN-VITRO* STUDY**

Ph.D. Thesis

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2023

ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled “**THE EFFECT OF AGOMELATINE ON IMMUNOMODULATORY PROPERTIES OF MICROGLIAL CELLS IN EXPERIMENTAL NEURODEGENERATIVE DISEASE MODEL, IN-VITRO STUDY**” prepared by **Abubaker Yousif Abdalla ELAMİN** , and supervised by **Assoc. Prof. Dr. Mehmet Emin ÖNGER**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date 15.8.2023.

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ÖZET

DENEYSEL NÖRODEJENERATİF HASTALIK MODELİNDE, AGOMELATİNİN MİKROGLİYAL HÜCRELERİN İMMUNOMODÜLATÖR ÖZELLİKLERİNE ETKİSİ: *İN VİTRO* ÇALIŞMA

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Agomelatin, yeni bir antidepresandır. Bilişsel fonksiyonları, belleği ve uykuyu artırır. Ayrıca, nörogelişimsel bozukluklarda oksidatif stres ve iltihaplanmaya karşı koruma potansiyeline sahiptir. Ancak, agomelatinin BV2 mikrogliyal hücrelerinde LPS tarafından indüklenen hasara karşı koruyucu yetkinliği henüz araştırılmamıştır. Bu çalışmada, agomelatinin nörodejeneratif hastalıklarda BV2 hücrelerinin immünomodülatör özellikleri üzerindeki potansiyel etkisini araştırmak amaçlanmıştır.

Literatürde *in vitro* çalışmalarda kullanılan çeşitli agomelatin konsantrasyonları bildirilmiştir. Agomelatin, BV2 hücrelerinde 2 μ M'dan 200 μ M'a kadar değişen dozlar dahil olmak üzere bu değişkenlikleri tespit etmek için çeşitli konsantrasyonlarda test edildi. Benzer şekilde BV2'nin LPS'ye duyarlılığı da farklılık gösterebilir ve farklı yanıtlara ve toksik etkilere yol açabilir. Bu yüzden farklı LPS konsantrasyonları da (0,001 μ g/ml ile 2 μ g/ml arasında değişen) test edildi. Deney tasarımı, agomelatin'in BV2 hücreleri üzerindeki LPS toksisitesine karşı koruyucu özelliklerini araştırmak için MTT analizlerinin kullanımını içermektedir. Sonuç olarak; dozlar agomelatin için 25 μ M ve 50 μ M, LPS için 1 μ g/ml olarak belirlendi.

İstatistiksel analizler, agomelatin'in BV2 hücre canlılığı üzerinde doza bağlı bir etkiye sahip olduğunu ve BV2 hücrelerinin uzun süre agomelatin'e maruz bırakılmasının canlılıklarını azalttığını gösterdi. 1 μ g/ml dozunda LPS, kontrol grubuyla karşılaştırıldığında hücre canlılığını önemli ölçüde azaltırken ($P < 0.001$), hem 25 μ M hem de 50 μ M agomelatin uygulanması LPS'nin toksik etkilerini önemli ölçüde azalttı ($P < 0.001$). DMSO grupları, kontrol grubuna göre önemli ölçüde daha düşük hücre canlılığı gösterdi, bu da potansiyel bir toksik etkiyi göstermektedir.

Sonuç olarak, agomelatin, LPS tarafından indüklenen toksisiteye karşı potansiyel bir koruyucu etki göstermiştir. Bununla birlikte, bu etkilere rağmen, uzun süreli etkileri ve DMSO'nun negatif etkileri göz ardı edilmemelidir.

Anahtar Sözcükler: Agomelatin, BV2, Mikroglia, LPS, Nörodejeneratif Hastalık

ABSTRACT

THE EFFECT OF AGOMELATINE ON IMMUNOMODULATORY PROPERTIES OF MICROGLIAL CELLS IN EXPERIMENTAL NEURODEGENERATIVE DISEASE MODEL, *IN-VITRO* STUDY

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Agomelatine is a novel antidepressant. It enhances cognitive functions, memory, and sleep. It also holds potential benefits in neurodevelopmental disorders, where it protects against oxidative stress and inflammation. However, the protective capabilities of agomelatine against LPS-induced damage in BV2 microglial cells remain unexplored. This study aimed to explore the potential effect of agomelatine on immunomodulatory properties of BV2 cells in neurodegenerative diseases.

Literature reported various agomelatine concentrations used in *in vitro* studies. Agomelatine was tested across various concentrations to acknowledge these variabilities, including doses from 2 up to 200 μ M on BV2 cells. Similarly, the sensitivity of BV2 to LPS can also vary, leading to differing responses and toxic effects. Therefore; different concentrations of LPS (ranging from 0.001 to 2 μ g/ml) were also tested. The experimental design involved using MTT assay to investigate agomelatine's protective properties against LPS toxicity on BV2 cells. Finally; the doses determined to be used were 25 μ M and 50 μ M for agomelatine and 1 μ g/ml for LPS.

Statistical analysis showed that agomelatine has a dose-dependent effect on BV2 cell viability, and subjecting the BV2 cells to agomelatine for a prolonged duration decreased their viability. LPS at 1 μ g/ml significantly decreased viability compared to the control group ($P < 0.001$), while adding agomelatine at both 25 μ M and 50 μ M significantly mitigated the toxic effects of LPS ($P < 0.001$). DMSO groups showed significantly lower cell viability than the control group, indicating a potential toxic effect.

In conclusion, agomelatine has shown a potential protective effect against LPS-induced toxicity. Despite this potency, prolonged and DMSO negative effects should not be ignored.

Keywords: Agomelatine, BV2, Microglia, LPS, Neurodegenerative Disease

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CONTENTS

ACCEPTANCE AND APPROVAL OF THE THESIS.....	i
DECLARATION OF COMPLIANCE WITH SCIENTIFIC ETHIC.....	ii
DECLARATION OF THE THESIS STUDY ORIGINALITY REPORT	ii
ÖZET	iii
ABSTRACT	iv
ACKNOWLEDGEMENT.....	v
CONTENTS.....	vii
SYMBOLS AND ABBREVIATIONS	x
FIGURES LEGENDS.....	xiv
TABLES LEGENDS.....	xv
1. INTRODUCTION AND OBJECTIVES	1
1.1. Introduction	1
1.2. Objectives	4
1.2.1. General objective	4
1.2.2. Specific objectives	4
2. GENERAL INFORMATION AND REVIEW OF THE LITERATURE	5
2.1. Overview of microglial cells and their functions	5
2.2. Morphological phenotypes of the microglia	6
2.2.1. Ramified microglia	7
2.2.2. Amoeboid microglia	7
2.2.3. Hypertrophic microglia.....	7
2.2.4. Rod microglia	8
2.2.5. Dystrophic microglia	8
2.2.6. Satellite microglia	8
2.3. Ultrastructural phenotypes	9
2.3.1. Typical microglia.....	9
2.3.2. Gitter cell-like microglia.....	9
2.3.3. Dark microglia	10
2.4. Microglial cells involvement in neuroinflammation	10
2.4.1. Parkinson's disease and microglia	11
2.4.2. Amyotrophic lateral sclerosis and microglia	12
2.4.3. Alzheimer's disease and microglia.....	12
2.5. Genetic risk factors related to microglia in neuroinflammation.....	12
2.6. Oxidative stress and microglia	13
2.7. Genes related to microglia in neuroinflammation	13
2.8. BV2 cell line as an <i>in vitro</i> model of neurodegeneration.....	14
2.8.1. Overview of BV2 cell line	14
2.8.2. Characteristics of BV2 Cells	14
2.9. Toxic substance used in neurodegenerative <i>in vitro</i> studies	15
2.10. Lipopolysaccharide (LPS).....	16
2.10.1. Introduction to LPS.....	16
2.10.2. Effect of LPS	17
2.10.3. Review of neurotoxicity of LPS on BV2 microglial cells.....	19
2.11. Agomelatine and its potential as a neuroprotective agent.....	23
2.11.1. Introduction to agomelatine	23
2.11.2. The significance of agomelatine	24
2.11.3. Agomelatine application	26

2.11.4. Agomelatine effect on LPS.....	27
2.11.5. Research gaps and opportunities for further investigation	28
3. MATERIAL AND METHODS	29
3.1. Chemical reagents	29
3.2. The cell lines	29
3.3. Tools	29
3.4. Plastics and consumables	29
3.5. Working environment.....	30
3.5.1. Incubator environment.....	30
3.5.2. Laminar flow environment	30
3.5.3. Sterilization protocol	31
3.6. Substances preparations	32
3.6.1. Complete media preparation.....	32
3.6.2. Ethanol preparation.....	32
3.6.3. PBS preparation	32
3.6.4. Agomelatine preparation	32
3.6.5. MTT solution preparation.....	33
3.6.6. LPS preparation	33
3.7. Cell culture techniques	34
3.7.1. Naming the cell line.....	34
3.7.2. Check the cell morphology	34
3.7.3. Feeding and replacement of medium.....	34
3.7.4. Volume of the media	35
3.7.5. Growth curve	35
3.7.6. Cell maintenance	36
3.7.8. Freezing protocol	36
3.7.9. Thawing protocol.....	37
3.7.10. Seeding protocol	37
3.7.11. Passaging protocol	37
3.7.12. Seeding in the well-plate	37
3.8. MTT assay for measurement of the cell viability and cytotoxicity.....	38
3.8.1. MTT assay protocol.....	38
3.8.2. MTT assay data analysis for cell viability	39
3.9. Dosage determination.....	40
3.9.1. Agomelatine dose determination	41
3.9.2. LPS dose determination.....	42
3.10. Agomelatine effect on BV2 cells subjected to LPS toxicity	44
2.11. Statistical analysis	45
4. RESULTS	46
4.1. The viability of BV2 cells exposed to different doses of agomelatine.....	46
4.1.1. Statistical findings	46
4.1.2. Histological findings.....	49
4.2. The effect of duration of agomelatine on BV2 cell viability	51
4.2.1. Statistical findings	51
4.2.2. Histological findings.....	54
4.3. The viability of BV2 cells subjected to different doses of LPS	55
4.3.1. Statistical findings	55
4.3.2. Histological findings.....	57
4.4. The Agomelatine effect on BV2 cells subjected to LPS toxicity.....	59

4.4.1. Statistical findings	59
4.4.2. Histological findings.....	61
5. DISCUSSION	64
5.1. Effect of agomelatine doses on BV2 cell viability	64
5.2. Effect of agomelatine duration on BV2 cell viability	65
5.3. LPS toxicity and BV2.....	66
5.4. The protective effect of agomelatine against LPS toxicity on BV2 cells.....	67
6. CONCLUSION AND RECOMMENDATIONS.....	69
REFERENCES	71
CIRCULUM VITEA	85



SYMBOLS AND ABBREVIATIONS

5-HT2B-C	: 5-Hydroxytryptamine receptor
AD	: Alzheimer's disease
Akt	: Akt serine/threonine kinase family
ALS	: Amyotrophic lateral sclerosis
ARFGEF1	: ADP Ribosylation Factor Guanine Nucleotide Exchange Factor 1
ATG6	: autophagy gene
Bax	: Gene in Apoptosis
Bcl	: B-cell lymphoma 2
BDNF	: brain-derived neurotrophic factor
BECN1	: Beclin-1
BSC	: biological safety cabinet
BV2	: Immortalized microglial cell line.
C1q	: complement component 1q
C3	: Complement C3
C4	: Complement C4
CD11b	: A Marker of Macrophages and Microglia
Cd11b	: Anti-CD11b antibodies
CD33	: Antibody protein
CD45	: a marker of all hematopoietic cells.
ceRNA	: competing endogenous RNA
CNS	: Central nervous system
CO ₂	: Carbon Dioxide
COX-2	: cyclooxygenase-2 inhibitor
CXCL12	: motif chemokine ligand 12
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DN	: Diabetic nephropathy
DNA	: Deoxyribonucleic acid
dUTP	: Deoxyuridine Triphosphate
Dyrk2	: Dual specificity tyrosine-phosphorylation-regulated kinase 2
EDTA	: Ethylenediaminetetraacetic acid

FBS	:	Fetal bovine serum
Grm2	:	Glutamate Metabotropic Receptor 2
HO-1	:	Heme oxygenase-1
HSPA5	:	Gene - Heat Shock Protein Family A Member 5
HVA	:	Homovanillic Acid
IBA1	:	Ionized calcium binding adaptor molecule 1
IL-1	:	Interleukin-1
IL-1 β	:	Interleukin -1 β
IL-6	:	Interleukin-6
iNOS	:	inducible nitric oxide synthase
IRF8	:	the first transcription factor specific to microglia
JNK	:	c-Jun N-terminal kinase
KLF4	:	Krüppel-like factor 4
LPS	:	Lipopolysaccharide
MAFB	:	transcription factor B
MDA	:	Malondialdehyde
miR-1199-5p	:	Gene
MS	:	Multiple sclerosis
MT1	:	G-protein coupled receptors
MT2	:	G-protein coupled receptors
MTT	:	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MyD88	:	myeloid differentiation primary response gene 88
NADPH	:	nicotinamide adenine dinucleotide phosphate
NDD	:	neurodevelopmental disorders
NDDs	:	Neurodegenerative diseases
NF- κ B	:	Nuclear factor- κ B
NO	:	Nitric Oxide
Nrf2	:	nuclear factor erythroid 2-related factor 2
P2RY12	:	Purinergic Receptor P2Y12
p38MAPK	:	a marker
p62	:	protein
PAMPs	:	Pathogen-associated molecular patterns

PBS	: Phosphate-buffered saline
PD	: Parkinson's disease
PGE2	: Prostaglandin E2
PI3K	: Phosphatidylinositol 3-kinase
PI3K	: Phosphatidylinositol 3-kinase
PU1	: Transcription Factor PU 1
R47H	: Analogue for TREM2
RNA	: Ribonucleic acid
RNS	: reactive nitrogen species
ROS	: reactive oxygen species
rTMS	: Repetitive transcranial magnetic stimulation
siRNA	: Short interfering RNA
SIRT1	: Sirtuin 1
SN	: Substantia nigra
TdT	: Terminal deoxynucleotidyl transferase
TgROP18	: Toxoplasma gondii rhoptry protein 18
TLR4	: toll-like receptor 4
TNF	: Tumor necrosis factor
Trem2	: Triggering receptor expressed on myeloid cells 2
TrkB	: tyrosine protein kinase gene
TUNEL	: terminal deoxynucleotidyl transferase dUTP nick end labeling
TYROBP	: tyrosine kinase-binding protein

FIGURES LEGENDS

Figure 2.1. Microglia in normal tissue	6
Figure 2.2. Microglia in health and disease.....	6
Figure 2.3. Microglia at different states in health and disease.	8
Figure 2.4. Healthy BV2 microglial cell lines.....	15
Figure 2.5. Structures of LPS	17
Figure 2.6. LPS for cell culture	17
Figure 2.7. LPS effect when introduced to the CNS	18
Figure 2.8. LPS discrepancy in cell culture.....	19
Figure 2.9. The chemical structure of agomelatine.	24
Figure 2.10. Agomelatine effect on the CNS.	25
Figure 3.1. The chemical substances used in cell culture.	29
Figure 3.2. Plastics and consumables used in cell culture.....	30
Figure 3.3. The incubator under conventional culture conditions of 5% of CO ₂ in 95% humidified at 37°C.	30
Figure 3.4. The Laminar flow is used in the cell culture lab.....	31
Figure 3.5. Sterilization of some of equipment used in cell culture.....	31
Figure 3.6. Complete media, PBS and ethanol preparation	32
Figure 3.7. Agomelatine 1mM stock solution preparation.....	33
Figure 3.8. MTT stock solution and desired concentration preparation	33
Figure 3.9. LPS 5mg/ml stock solution preparation.....	34
Figure 3.10. Routine in the cell culture lab	35
Figure 3.11. Phases of the growth curve in cell lines.....	36
Figure 3.12. Thawing, seeding, passaging, and freezing Protocols	37
Figure 3.13. BV2 concentration at the seeding in the 96 well-plates.....	38
Figure 3.14. MTT Assay protocol for dose determination.....	39
Figure 3.15. MTT Assay protocol for examining the effect of the substances	39
Figure 3.16. MTT Assay steps for data analysis for cell viability	40
Figure 3.17. MTT Assay protocol for agomelatine dose determination.	41
Figure 3.18. MTT Assay at different stages.....	42
Figure 3.19. MTT Assay protocol for agomelatine impact of duration	42
Figure 3.20. MTT Assay protocol for LPS dose determination.....	43
Figure 3.21. MTT Assay at different stages.....	43
Figure 3.22. The experimental groups.....	44
Figure 3.23. MTT assay protocol for examining agomelatine protective effect on BV2 subjected to LPS toxicity.	44
Figure 4.1. Effect of agomelatine on BV2.	47
Figure 4.2. Inhibitory cell viability curve of agomelatine on BV2 cells.....	48
Figure 4.3. A comparative analysis of various agomelatine concentrations on BV2 cells.	48
Figure 4.4. Histological findings in BV2 cells exposed to different agomelatine concentrations.....	50
Figure 4.5. The inhibition curve and the IC ₅₀ of the impact of duration doses of agomelatine on BV2 cells.....	51
Figure 4.6. Impact of varying doses and duration of exposure of agomelatine on BV2 cells	52
Figure 4.7. A comparative analysis of BV2 cells viability subjected to various agomelatine concentrations and duration of exposure.	53

Figure 4.8. Histological findings in BV2 cells exposed to varying agomelatine concentrations for 24, 48, and 72 hrs.	55
Figure 4.9. Impact of varying doses of LPS on BV2 cells.....	56
Figure 4.10. Multiple group comparison analysis shows the dose-dependent effect of LPS.....	57
Figure 4.11. Histological findings of BV2 cells subjected to varying LPS concentrations.....	58
Figure 4.12. Cell viability of BV2 cells treated with different substances based on the experimental design.....	60
Figure 4.13. Multiple group comparison analysis of the cell viability of BV2 cells treated with different substances based on the experimental design.....	61
Figure 4.14. Histological findings of BV2 cells under various experimental conditions.	62



TABLES LEGENDS

Table 4.1. BV2 cell viability subjected to different agomelatine concentrations. .	46
Table 4.2. Impact of duration of exposure to various agomelatine concentrations on BV2 cells. The drug was administered for 24, 48, and 72 hours. .	51
Table 4.3. Cell viability of different LPS concentrations on BV2 cells.....	56
Table 4.4. Cell viability of BV2 cells treated with different substances.....	59
Table 4.5. Tukey's multiple comparisons test of the experimental groups.	60



1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

Neurodegenerative diseases are chronic and progressive disorders characterized by the degeneration and loss of neurons in the central nervous system (CNS). They involve various molecular and cellular pathologies, contributing to neuronal death (Gao & Hong, 2008; Heemels, 2016; Checkoway et al., 2011; Muddapu et al., 2020). Examples of neurodegenerative diseases include Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Huntington's disease (HD). These complex diseases involve various molecular and cellular processes (Muddapu et al., 2020).

Microglia, the resident immune cells in the CNS, have emerged as key players in the pathogenesis of neurodegenerative diseases. They play important roles in maintaining neuronal health, regulating synaptic connectivity, and clearing unwanted materials in the CNS environment (Timmerman et al., 2018; Franklin et al., 2021; Graeber and Streit, 2010). However, microglia can also contribute to disease progression. Abnormal activation and dysfunction of microglia can lead to neuroinflammation, release of inflammatory molecules, and neuronal damage (Kettenmann et al., 2011).

BV2 cells, an immortalized murine microglial cell line, have been extensively studied and serve as a valuable model for investigating microglial functions and responses (Horvath et al., 2008; Smith et al., 2012; Scheiblich et al., 2014). They exhibit properties similar to primary microglia in terms of morphology, marker expression, immune response capabilities, and phagocytic abilities (Sasaki et al., 2001; Smith et al., 2012). BV2 cells can be activated by various stimuli, producing inflammatory mediators and exhibiting immune response functions similar to primary microglia. They contribute to tissue repair, regulate immune responses, and influence neuronal survival and synaptic plasticity (Huan et al., 2016; Pocock and Kettenmann, 2007).

Moreover, studying microglial activation and the factors that inhibit it, such as certain compounds, can provide good understanding into its potential therapeutic mechanisms (Palace, 2007; Ojo et al., 2015; Fakhoury, 2015). Primary microglial cultures and BV2 cells have played significant roles in neuroinflammatory research,

allowing for studying microglial functions and responses in a controlled environment (Stansley et al., 2012).

Toxic substances are commonly used in neurodegenerative *in vitro* studies to mimic the pathological conditions observed in various neurodegenerative diseases. These substances induce specific cellular and molecular changes, provide valuable insights into the mechanisms underlying neuronal damage and cell death (Huang et al., 2018; Zhang et al., 2012). Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is frequently used as a toxic substance in these studies. LPS activates immune cells such as macrophages and microglia through toll-like receptor 4 (TLR4), leading to the production of pro-inflammatory cytokines and neuroinflammation, which are features of neurodegenerative diseases (Nam et al., 2018; Joh et al., 2012; Miyazaki et al., 2012). Reactive oxygen species (ROS) buildup also contributes to inflammatory reactions and tissue damage (Zhai et al., 2012; Wang et al., 2010).

LPS is well-characterized and standardized tool in neuroinflammatory research, allowing for comparisons across different studies and evaluation of potential therapeutic interventions (Hou et al., 2003; Lee et al., 2012; Wang-Sheng et al., 2017). It induces the release of early cytokines and activates microglia, and its effects can be modulated by anti-inflammatory compounds (Kumar et al., 2014). However, the sensitivity to LPS can vary among cell lines and experimental models, emphasizing the need for careful evaluation and standardization of protocols (Chang et al., 2005; Lu et al., 2009; Yu et al., 2012). Targeting microglia and investigating the effects of LPS toxicity on BV2 microglial cells have been areas of extensive research (Haruwaka et al., 2019).

Furthermore, LPS-induced activation of microglia and the subsequent release of pro-inflammatory cytokines have been implicated in the pathogenesis of various neurodegenerative diseases, including AD and PD. The sustained activation of microglia and the chronic release of inflammatory molecules can lead to neuronal dysfunction and contribute to the progression of neurodegeneration (Block et al., 2007; Qin et al., 2007). Therefore, understanding the underlying mechanisms of LPS-mediated neuroinflammation is crucial for developing strategies to modulate microglial activation and alleviate neuroinflammatory responses, potentially offering new avenues for therapeutic interventions in neurodegenerative disorders.

Agomelatine, a synthetic analogue of the melatonin hormone, possesses various therapeutic properties such as antidepressant, neuroprotective, and anti-inflammatory activities (Cheng et al., 2017; Saral et al., 2023). It acts as an agonist for melatonin receptors (MT1 and MT2) and as an antagonist for the serotonin 2C receptor (5-HT_{2C}) (Mahmoud et al., 2023; Papp et al., 2003; Saral et al., 2023). These receptors are widely distributed throughout the CNS, peripheral organs, and various tissues (Hafez et al., 2022; Kopustinskiene and Bernatoniene, 2021). Agomelatine presents an alternative approach to treating depression by targeting melatonin receptors and antagonizing 5-HT_{2C} receptors (Maddukuri et al., 2021).

Moreover, agomelatine is a novel melatonergic antidepressant that specifically addresses circadian rhythm disturbances associated with depression and operates through a non-monoaminergic mechanism (de Bodinat et al., 2010; Łebkowska-Wieruszewska et al., 2021). Despite its minimal affinity for most receptors, including serotonin receptors, agomelatine exhibits a well-characterized side effect profile (Łebkowska-Wieruszewska et al., 2021; Millan, 2006; Millan et al., 2003). However, its oral bioavailability is limited due to a high first-pass effect, primarily undergoing hepatic metabolism (Hafez et al., 2022). Although pharmacokinetic studies have been conducted in humans, research on canine species is lacking (Giorgi et al., 2012).

Agomelatine is used as an antidepressant that modulates endogenous melatonin secretion, regulates circadian rhythms by activating MT1 and MT2 receptors, and acts as an antagonist on the 5-HT_{2C} receptor (Castanho et al., 2014; Cherngwell et al., 2021). One of agomelatine's significance lies in its effects on cognitive functions, such as enhancing the expression of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex and hippocampus and ultimately enhances the synaptic plasticity and memory modulation (Saral et al., 2023; Martin et al., 2017; Martinotti et al., 2016).

Agomelatine's modulation of serotonin receptors, acting as an agonist for melatonin receptors and an antagonist for 5-HT_{2B}/5-HT_{2C} receptors, contributes to its antidepressant and anxiolytic effects (Mahmoud et al., 2023; Promsan et al., 2022). Additionally, agomelatine exhibits potent anti-inflammatory and renoprotective activity against oxidative stress, particularly in diabetic nephropathy, by regulating SIRT1 and inflammation inhibition (Cherngwell et al., 2021; Mahmoud et al., 2023).

Furthermore, agomelatine shows therapeutic potential, it has anxiolytic/antidepressant effects, mitigates inflammation and oxidative stress, and

protects against obesity-related renal injury (Rebai et al., 2021; Hafez et al., 2022; Kara et al., 2008). Agomelatine has been also found to possess protective effects against LPS-induced damage (Dem andorff et al., 2022). Moreover, agomelatine has shown promising results in animal models of neurodegenerative diseases, including AD and PD, by reducing oxidative stress, inflammation, and neuronal damage (Fernández-Blázquez et al., 2021; Mahmood et al., 2022).

1.2. Objectives

The study aims to investigate the impact of agomelatine on the immunomodulatory properties of microglial cells in neurodegenerative diseases. Specifically, the study will focus on BV2 microglial cell lines and examine the introduction of agomelatine and the toxic substance LPS. The effects of agomelatine will be analyzed by evaluating cell viability, and toxicity. The objective is to determine the potential positive effects of agomelatine in reducing toxicity and preserving the cells.

1.2.1. General objective

To assess the effect of agomelatine on the immunomodulatory properties of microglial cells in an experimental neurodegenerative disease model using an *in-vitro* study.

1.2.2. Specific objectives

- To evaluate the viability of BV2 cell lines when exposed to different doses of agomelatine.
- To investigate the impact of the duration of exposure to agomelatine on BV2 cell lines subjected to different doses.
- To assess the viability of BV2 cell lines when exposed to different doses of LPS.
- To examine morphological changes under the microscope.

2. GENERAL INFORMATION AND REVIEW OF THE LITERATURE

2.1. Overview of microglial cells and their functions

Microglial cells, also known as the brain's resident immune cells, constantly survey the cerebral microenvironment, responding to pathogens and damage (van Rossum and Hanisch, 2004). They play critical roles in immune surveillance, clearance of toxic molecules, and brain homeostasis (Figure 2.1) (van Rossum and Hanisch, 2004). These cells originate from primitive macrophages in the yolk sac during early embryonic development and subsequently migrate to the CNS, where they settle in various regions (Ginhoux and Prinz, 2015). Unlike other glial cells, they have a distinctive morphology characterized by small cell bodies and highly dynamic processes that constantly survey their surrounding environment (Savage et al., 2019). Microglia are present in varying numbers throughout the CNS, representing around 10-20% of all glial cells, and their population can dynamically change in response to infection, injury, or disease (Soulet and Rivest, 2008).

Under non-pathological conditions, microglia are highly active, continually surveying their microenvironment through motile processes and ramifications (Graeber and Streit, 2010; van Rossum and Hanisch, 2004). These immune cells never truly rest but remain vigilant, constantly monitoring the brain environment and clearing toxic molecules (Graeber and Streit, 2010; Nimmerjahn et al., 2005). They possess Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (Figure 2.1), enabling them to respond to infectious microorganisms (Soulet and Rivest, 2008).

In addition to their immune surveillance role, microglia mounts the innate immune responses during acute injuries, limits the damage, and facilitates the repairment (Figure 2.2) (Hanisch and Kettenmann, 2007; Soulet and Rivest, 2008).

Microglia also clear the toxic proteins in various neurodegenerative diseases like AD, PD, and ALS which involve the accumulation of toxic proteins such as beta-amyloid, alpha-synuclein, and superoxide dismutase, respectively (Graeber and Streit, 2010; Hanisch and Kettenmann, 2007; Soulet and Rivest, 2008). While microglia are recruited in these conditions, their efficiency in phagocytosing and removing toxic proteins can be limited (Kettenmann et al., 2011).

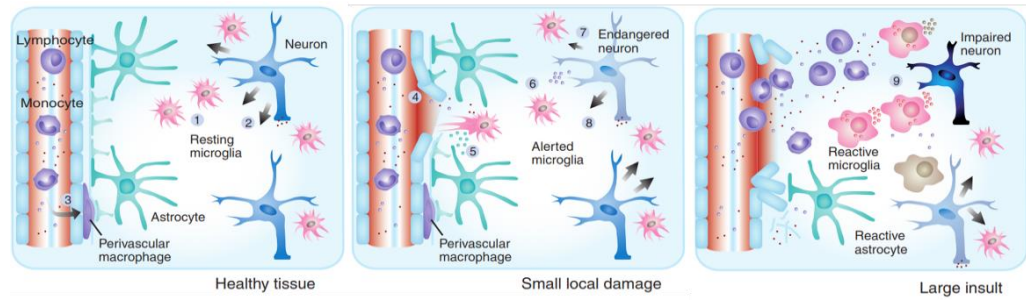


Figure 2.1. Microglia in normal tissue, they have a surveillance mode characterized by fine processes that undergo continuous rebuilding, allowing efficient scanning of their territory. In response to small damage or homeostatic disturbances, microglia undergo a directed reorganization of processes and a change in activity profile. In the case of larger insults to the CNS, microglia can acquire a reactive phenotype, potentially leading to substantial impairment of neurons and glia (Hanisch and Kettenmann, 2007).

Bone marrow-derived microglia also play in the significant role in AD. These microglia are attracted to amyloid plaques, exhibit immune responses to β -amyloid, and are efficient in eliminating amyloid deposits via phagocytosis. Thus, enhancing the recruitment of these blood-derived microglia could be a promising strategy for combating toxic senile plaques in AD (Soulet and Rivest, 2008).

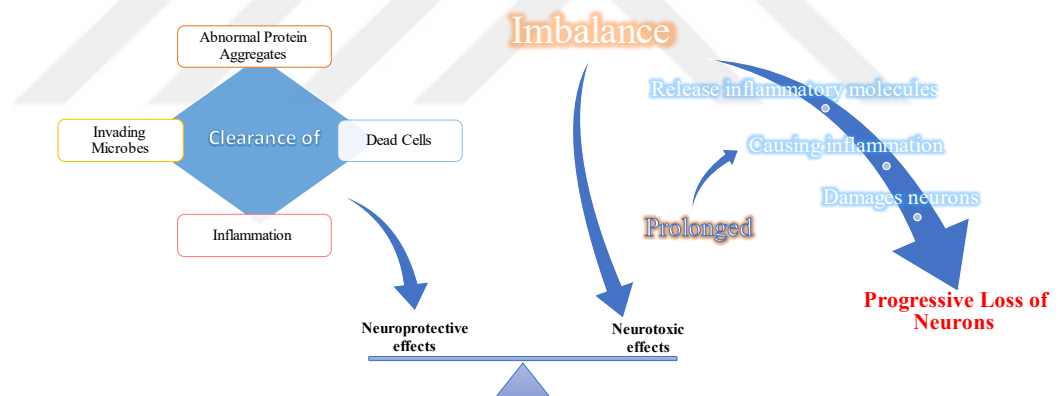


Figure 2.2. Microglia in health and disease.

2.2. Morphological phenotypes of the microglia

Microglia, resident immune cells in the CNS, exhibit diverse morphological phenotypes and have been extensively characterized using light microscopy techniques. Pió del Rí-o-Hortega's seminal work detailed microglial phenotypes in different disease conditions. At the same time, subsequent research has revealed regional heterogeneity in microglial density and morphology, particularly between grey and white matter tracts (Savage et al., 2019). Moreover, microglial morphology undergoes significant changes with age, as developing CNS microglia display more amoeboid phagocytic phenotypes than their mature counterparts. These morphological

variations of microglia play important functional roles in different developmental stages and disease contexts (Savage et al., 2019).

2.2.1. Ramified microglia

Ramified microglia are the most common phenotype in the mature CNS. These cells have small somas and extend long, highly branched processes, constantly surveying their surroundings (Figure 2.3). They make transient contact with neuronal cell bodies, blood vessels, and astrocytes. Ramified microglia are dynamically motile, interacting with various structures in the neuropil, including neurons, axons, dendrites, and synapses. They play a neuroprotective role and are involved in phagocytosis, notably clearing apoptotic newborn neurons. Their motility relies on specific channels and receptor tyrosine kinases. Ramified microglia are associated with steady-state conditions in the CNS (Nimmerjahn et al., 2005; Savage et al., 2019).

2.2.2. Amoeboid microglia

Amoeboid microglia resemble other macrophages in the body. They are round with larger cell bodies and lack processes under light microscopy, although thin filopodia may be visible under electron microscopy (Figure 2.3). Amoeboid microglia are commonly observed in embryonic and young animals and respond to acute CNS injuries. They exhibit increased overall mobility compared to ramified microglia. Amoeboid microglia express genes associated with cell cycle progression, migration, and phagocytosis. They have been found in various healthy and diseased conditions, such as the circumventricular organs, AD, ageing, and HD (Lawson et al., 1990; Nimmerjahn et al., 2005; Savage et al., 2019).

2.2.3. Hypertrophic microglia

Hypertrophic microglia, often described as "activated" or "hyper-reactive," feature enlarged somas and excessive, short, and thick processes. They appear bushy in morphology. Hypertrophic microglia are associated with regions of pathology in diseases like AD, HD, cardiovascular disease, and various mouse models of neurological conditions (Figure 2.3). These cells are functionally "primed" and respond more to proinflammatory stimuli. They upregulate certain surface markers associated with disease-associated macrophage phenotypes (Hellwig et al., 2016; Savage et al., 2019).

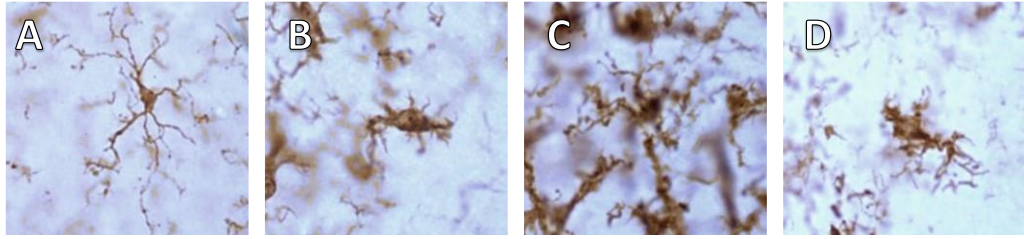


Figure 2.3. Microglia at different states in health and disease. A: Ramified microglia, B: Hypertrophic microglia, C: Rod microglia, and D: Dystrophic microglia (Savage et al., 2019).

2.2.4. Rod microglia

Rod microglia have small nuclei and exhibit few or no radial processes. They appear elongated with bipolar processes aligned in a single orientation (Figure 2.3). These cells are thought to be involved in synaptic stripping, separating presynaptic axon terminals from postsynaptic targets. Rod microglia increase with ageing and have been observed in epilepsy, encephalitis, AD, traumatic brain injury, and stroke. However, their precise role in disease is poorly understood (Y.-M. Ho et al., 2014; Savage et al., 2019).

2.2.5. Dystrophic microglia

Dystrophic microglia are characterized by beaded spherical swellings in their processes, lipofuscin deposits, and dilation of the endoplasmic reticulum. They are often observed in cognitively normal-aged individuals and are associated with neurodegenerative diseases such as AD, HD, ALS, and viral-induced encephalitis. Dystrophic microglia may represent senescent cells exhibiting reduced response to proinflammatory signalling and morphological abnormalities. They are frequently found near β amyloid plaques and display impaired lysosomal degradation and endolysosomal stress (Savage et al., 2019; Streit et al., 2014).

2.2.6. Satellite microglia

Satellite microglia were initially identified close to neurons, appearing to orbit neuronal cell bodies. They are preferentially associated with the axon initial segment in the adult cortex. Satellite microglia display unique electrical activity and make direct membrane-to-membrane contact with their associated neurons. Studies indicate spontaneous activity and membrane capacitance differences between satellite and parenchymal microglia (Hellwig et al., 2016; Savage et al., 2019).

2.3. Ultrastructural phenotypes

In addition to the morphological phenotypes visible through light microscopy, electron microscopy has been instrumental in investigating the ultrastructure of microglia and providing further insights into their complex functionality. Utilizing electron microscopy, researchers have identified distinct ultrastructural features of microglia that contribute to their diverse functions. These include the small somas of typical microglia, characterized by a distinct nuclear shape and heterochromatin pattern, and the presence of extensive endoplasmic reticulum, lysosomes, phagocytic compartments, and close association with the extracellular space. Ultrastructural studies have also revealed the intricate interactions of microglial processes within the neuropil, such as direct contact with axonal terminals, dendritic spines, perisynaptic astrocytes, and synaptic clefts. Recent advancements, such as correlative light and electron microscopy, have enabled detailed investigations of microglial interactions with synapses. In contrast, 3D scanning electron microscopy has allowed nanoscale reconstructions of microglial cell bodies and organelles (Savage et al., 2019).

2.3.1. Typical microglia

Typical microglia can be identified under an electron microscope without antibody-based staining techniques. They exhibit small somas with a clear nuclear shape and heterochromatin pattern. They possess long stretches of endoplasmic reticulum, phagocytic compartments, and lysosomes. The association with extracellular space distinguishes them from other CNS cells. Ultrastructural studies reveal that microglial processes directly contact neuropil cells, including axonal terminals, dendritic spines, perisynaptic astrocytes, and synaptic clefts. Advanced techniques such as correlative light and electron microscopy have provided detailed insights into microglial synaptic interactions. In contrast, 3D scanning electron microscopy has enabled nanoscale reconstruction of microglial cell bodies and organelles (Savage et al., 2019).

2.3.2. Gitter cell-like microglia

Gitter cell-like microglia have been identified using electron microscopy and are characterized by being filled with cellular debris resembling fat granules. In various contexts, these cells have been observed in different brain regions, such as the cerebral cortex, cerebellum, and white matter tracts. They may correspond to the cells

previously described as senescent or dystrophic microglia based on their morphology under light microscopy. Gitter cell-like microglia are associated with age-related sensory loss, accelerated-aging disorders like Werner syndrome, and other conditions. The presence of these debris-filled cells suggests their involvement in the clearance of cellular waste within the CNS (Savage et al., 2019).

2.3.3. Dark microglia

Dark microglia are a recently described cell type identified using electron microscopy. They are characterized by electron-dense cytoplasm and nucleoplasm, appearing as dark as mitochondria. Dark microglia display oxidative and metabolic stress markers, such as dilated endoplasmic reticulum, Golgi apparatus cisternae, and mitochondrial alterations. These highly active cells have thin, highly ramified processes that often encircle synaptic elements, axons, dendrites, and pockets of extracellular space. They may be involved in synaptic loss and the active digestion of their targets. Dark microglia downregulate the microglial marker IBA1 but express Cd11b and 4D4, specific markers of resident microglia. They are more prominent in aged or chronically stressed animals and in disease models such as Alzheimer's disease (AD), where they are found close to β -amyloid plaques. Dark microglia express Trem2, a marker associated with AD, and are immunonegative for the surveillant microglia marker P2RY12 (Savage et al., 2019).

2.4. Microglial cells involvement in neuroinflammation

Neuroinflammation involves activating various immune cell types that play diverse roles in the inflammatory response within the CNS. Among these cell types, microglia, the primary brain resident macrophages, are prominent players in neuroinflammation (Prinz and Priller, 2014). Microglia have important homeostatic functions and provide trophic support to neurons, clear debris, and maintain the extracellular environment in the brain. However, when activated, they can mediate neuroinflammation (Graeber and Streit, 2010). The CNS also contains other resident macrophage populations, including meningeal macrophages, choroid plexus macrophages, and perivascular macrophages, which are in specialized compartments (Jay et al., 2016; Prinz and Priller, 2014). The specific functions of these populations in health and disease are still under investigation (Jay et al., 2016).

Astrocytes, another type of brain-resident glial cells, also contribute to the inflammatory response in neuroinflammation. Both astrocytes and microglia can sense

and respond to inflammatory stimuli, migrate to injury or pathology sites, and release inflammatory cytokines and chemokines that recruit peripheral immune cells into the CNS (Jay et al., 2016; Singh, 2022). Circulating monocytes from the peripheral blood can be recruited into the CNS by chemokines released by astrocytes and microglia (Singh, 2022). Once inside the brain, these monocytes differentiate into macrophages and adopt characteristics similar to resident microglia. They contribute to releasing pro-inflammatory mediators and clearing debris (Jay et al., 2016; Prinz and Priller, 2014).

In certain disease contexts, resident and peripheral macrophages may contribute to distinct aspects of the inflammatory response within the CNS (Jay et al., 2016). T cells, a subtype of peripheral immune cells, can also be recruited from the periphery into the CNS. While their role in most neuroinflammatory contexts is poorly understood, their recruitment is particularly important and well-characterized in MS, a neurodegenerative disorder. Autoreactive T cells in MS mediate inflammation-induced damage to CNS cells (Jay et al., 2016).

Other peripheral immune cell subtypes, including neutrophils, dendritic cells, and natural killer cells, have been proposed to enter the brain in various neurodegenerative diseases (NDDs). However, their exact roles are not yet clear. It is evident that both resident and peripheral immune cells, along with the central immune system, play significant roles as mediators of neuroinflammation (Jay et al., 2016).

2.4.1. Parkinson's disease and microglia

Oxidative stress has been extensively implicated in PD, particularly in the substantia nigra (SN) where neurodegeneration occurs. When co-cultured with dopaminergic neurons or their secreted products, microglia upregulates inflammatory mediators and secrete nitric oxide, contributing to oxidative stress and neuroinflammation (M. S. Ho, 2019; Jay et al., 2016). The activation of immune cells near the SN has resulted in selective dopaminergic cell death, suggesting a crucial role for microglia in promoting neuronal stress and degeneration (Kam et al., 2020). Studies have demonstrated that reactive nitrogen species (RNS) produced by myeloid cells contribute to neuronal cell death in PD models, further emphasizing the involvement of microglia (Badanjak et al., 2021; Jay et al., 2016). Additionally, administering anti-inflammatory drugs improves dopaminergic cell survival in PD

models, highlighting the necessity of immune cell inflammatory responses in dopamine-mediated cell death (Petzinger et al., 2013).

2.4.2. Amyotrophic lateral sclerosis and microglia

ALS, primarily associated with mutations in the Sod1 gene, is strongly linked to oxidative stress ("On the Relation of Oxidative Stress to Neuroinflammation: Lessons Learned from the G93A-SOD1 Mouse Model of Amyotrophic Lateral Sclerosis," 2006). Microglial activation surrounding motor neurons undergoing degeneration is observed in ALS. Expression of mutant Sod1 in microglia leads to increased production of pro-inflammatory cytokines, contributing to oxidative stress in surrounding motor neurons (Apolloni et al., 2013). Studies utilizing mouse models have shown that myeloid cells, including microglia and peripheral macrophages, play a role in motor neuron degeneration and dysfunction (Apolloni et al., 2013). Reduced mutant Sod1 expression selectively in myeloid cells resulted in increased lifespan in mutant Sod1 mice, further supporting the involvement of microglia and peripheral macrophage dysfunction in ALS pathogenesis (Dangoumau et al., 2021; Jay et al., 2016).

2.4.3. Alzheimer's disease and microglia

In AD, microglia exhibit a dichotomous role in inflammation. While inflammation and cytokine production can modulate AD pathologies, they can also lead to neuronal damage through the generation of ROS and RNS (Singh, 2022) (9). Through cytokine production, activated microglia induce oxidative stress in neurons, contributing to neurodegeneration and cognitive decline (Jay et al., 2016). Furthermore, the respiratory burst mediated by NADPH oxidase in microglia increases ROS levels, correlating with the upregulation of NADPH oxidase observed in AD patients (Block, 2008). The inhibition of NADPH oxidase has been shown to ameliorate neurotoxicity, indicating the contribution of microglial oxidative stress to neuronal death in AD (Block, 2008; Jay et al., 2016).

2.5. Genetic risk factors related to microglia in neuroinflammation

Genetic variations in microglial genes have been identified as significant risk factors for NDDs. Numerous studies have implicated variants in genes such as TREM2, CD33, and TYROBP, primarily expressed in microglia, in the modulation of microglial function and neuroinflammation. For instance, the TREM2 R47H variant

has been associated with an increased risk of AD and other NDDs, affecting microglial phagocytosis and the clearance of pathological aggregates (Jay et al., 2016).

2.6. Oxidative stress and microglia

Oxidative stress, characterized by an imbalance between ROS production and neutralization, is closely intertwined with neuroinflammation in NDDs (COLTON et al., 2000; Peterson and Flood, 2012). Microglia, as key regulators of the neuroinflammatory response, contribute to oxidative stress by releasing ROS and RNS (Peterson and Flood, 2012; Simpson and Oliver, 2020). Activation of microglial NADPH oxidase and inducible nitric oxide synthase (iNOS) leads to the generation of ROS and RNS, which can induce DNA damage, protein modification, and ultimately neuronal cell death. The dysregulation of redox homeostasis mediated by microglia contributes to the progression of NDDs (Jay et al., 2016; Simpson and Oliver, 2020).

2.7. Genes related to microglia in neuroinflammation

The complement system, a crucial component of the innate immune response, has been implicated in neuroinflammation and synaptic pruning mediated by microglia. Genetic variants in complement-related genes, including C1q, C3, and C4, influence microglial activity and contribute to synaptic dysfunction and neuronal loss. Dysregulation of the complement cascade can lead to aberrant synaptic pruning, impairing neuronal circuitry and promoting neurodegeneration (S. Hong et al., 2016; Jay et al., 2016).

Microglial phenotype is dynamically regulated by a spectrum of genes, dictating their response to environmental cues in the CNS. Transcription factors, such as PU1, IRF8, and MAFB, play crucial roles in shaping microglial phenotypes, influencing their inflammatory or neuroprotective functions. Dysregulated expression or activation of these genes can skew microglia towards a pro-inflammatory state, exacerbating neuroinflammation and contributing to disease progression (Schwabensland et al., 2021).

Emerging evidence suggests that microglia, traditionally considered as immune cells of the innate immune system, may also interact with and influence adaptive immune responses in the CNS. The interaction between microglia and adaptive immune cells contributes to the complex immune response in NDDs (Kannarkat et al., 2013).

2.8. BV2 cell line as an *in vitro* model of neurodegeneration

2.8.1. Overview of BV2 cell line

Neuroinflammatory research commonly uses primary microglial cultures because they exhibit a phenotype similar to *in vivo* cells. However, the process of cultivating these cultures is time-consuming and yields a low number of cells. As a result, alternative methods were explored to generate larger cells in a shorter time (Stansley et al., 2012).

There has been a growing interest in studying microglia in the past few decades. Among the various cell lines used for microglia culture, the BV2 cell line is one of the most commonly used (Kučić et al., 2021). These cells are derived from mice and immortalized, allowing long-term use in experiments. BV2 cells exhibit important functional characteristics such as phagocytosis, the ability to engulf particles, and cytokine production, the release of signalling molecules involved in immune responses (Ross and Poirier, 2004; G. Zhang et al., 2012). Moreover, BV2 cells are readily accessible and cost-effective, making them popular in research on neurodegenerative disorders and related conditions (Ginwala et al., 2019).

2.8.2. Characteristics of BV2 Cells

BV2 cells are an extensively studied immortalized murine microglial cell line widely used in neuroscience research (Horvath et al., 2008; Smith et al., 2012). These cells exhibit properties similar to primary microglial cells, the resident immune cells in the CNS, making them a valuable model for investigating microglial functions and responses (Scheiblich et al., 2014).

BV2 cells' key characteristics are their resemblance to primary microglia in terms of morphology and expression of microglial markers (Figure 2.4). They possess a ramified morphology with branched processes and express markers such as CD11b, CD45, and ionized calcium-binding adapter molecule 1 (IBA1) (Sasaki et al., 2001; Smith et al., 2012).

These characteristics validate BV2 cells as a reliable model for studying microglial biology. BV2 cells exhibit immune response functions similar to primary microglia. They can be activated by various stimuli, such as LPS or pro-inflammatory cytokines, producing inflammatory mediators. Nitric oxide (NO), reactive oxygen species (ROS), cytokines, chemokines, and prostaglandins are among the immune response molecules secreted by activated BV2 cells (Huan et al., 2016; Pocock and

Kettenmann, 2007). Upon stimulation with LPS, these cells produce many molecules like IL-6, TNF and IL-1 (Kukolj et al., 2018). The BV2 cells have also been shown to up-regulate the pro-inflammatory genes, including INOS, COX-2, TNF and IL-1 β (Kukolj et al., 2018).

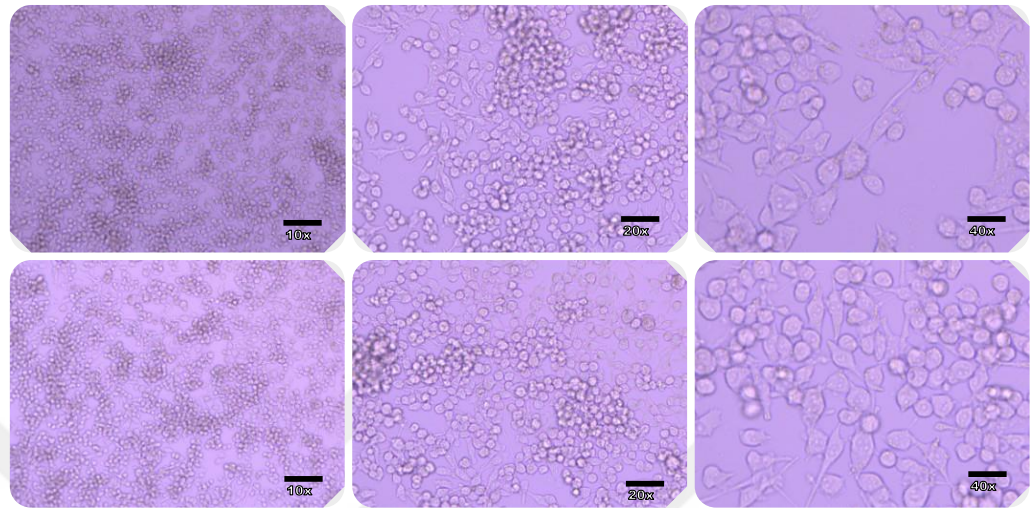


Figure 2.4. Healthy BV2 microglial cell lines.

Furthermore, BV2 cells possess phagocytic capabilities similar to primary microglia. They can engulf and clear cellular debris, pathogens, and apoptotic cells within the CNS. This phagocytic function is essential for maintaining tissue homeostasis and removing potentially harmful substances (Hanisch and Kettenmann, 2007). In the context of injury and neuroinflammation, BV2 cells play a significant role. They actively participate in the response to CNS injury and neuroinflammatory processes. BV2 cells regulate immune responses, contribute to tissue repair, and secrete factors that can influence neuronal survival and synaptic plasticity (Anna and Guy, 2001; Pocock and Kettenmann, 2007). The signalling capabilities of BV2 cells are comparable to those of primary microglia. They express many receptors, including toll-like receptors (TLRs), cytokine receptors, and purinergic receptors. These receptors enable BV2 cells to detect and respond to specific stimuli, initiating intracellular signalling pathways and influencing gene expression (Nimmerjahn et al., 2005; Pocock and Kettenmann, 2007).

2.9. Toxic substance used in neurodegenerative *in vitro* studies

In neurodegenerative *in vitro* studies, toxic substances are commonly used to mimic the pathological conditions observed in various neurodegenerative diseases. These substances play a crucial role in investigating the mechanisms underlying

neuronal damage and cell death, providing valuable insights into the pathogenesis of these disorders (M. Y. Huang et al., 2018).

Toxic substances used in neurodegenerative *in vitro* studies induce specific cellular and molecular changes that mirror the characteristics of neurodegenerative diseases. These substances can be neurotoxic agents, environmental toxins, or pathological factors associated with the studied diseases (G. Zhang et al., 2012).

LPS is a frequently used toxic substance that mimics excitotoxicity, a phenomenon observed in conditions such as AD and PD. LPS is a component of the outer membrane of gram-negative bacteria. LPS can trigger an inflammatory response in cells, causing neuroinflammation, oxidative stress, and neuronal dysfunction and death. Neuroinflammation represents a characteristic of several neurodegenerative diseases, including AD and PD (Nam et al., 2018).

These toxic substances related to the neurodegenerative diseases provide valuable tools for researchers to study the mechanisms of neuronal damage and develop potential therapeutic interventions. By replicating the toxic conditions observed in neurodegenerative diseases, *in vitro* studies contribute to the understanding of the disease progression and developing novel treatment strategies.

2.10. Lipopolysaccharide (LPS)

2.10.1. Introduction to LPS

LPS, the major portion of the outer membrane of Gram-negative bacteria such as *Escherichia coli* (Figure 2.5), is regarded as the main risk factor responsible for microglial activation (Joh et al., 2012). It is commonly used in *in vitro* studies to stimulate immune responses. It is an endotoxin recognized by pattern recognition receptors, specifically toll-like receptor 4 (TLR4), on immune cells, including macrophages and microglia (Miyazaki et al., 2012).

Upon LPS activation, the immune response can be induced, leading to the production and release of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6). Although LPS cannot pass through the blood-brain barrier, it can stimulate monocytes and macrophages, which can cross the barrier and activate the microglia cells in the local area. As a result, LPS and cytokine circulating in the bloodstream can impact microglia

activation without entering the brain (Miyazaki et al., 2012; Shigemoto-Mogami et al., 2018).

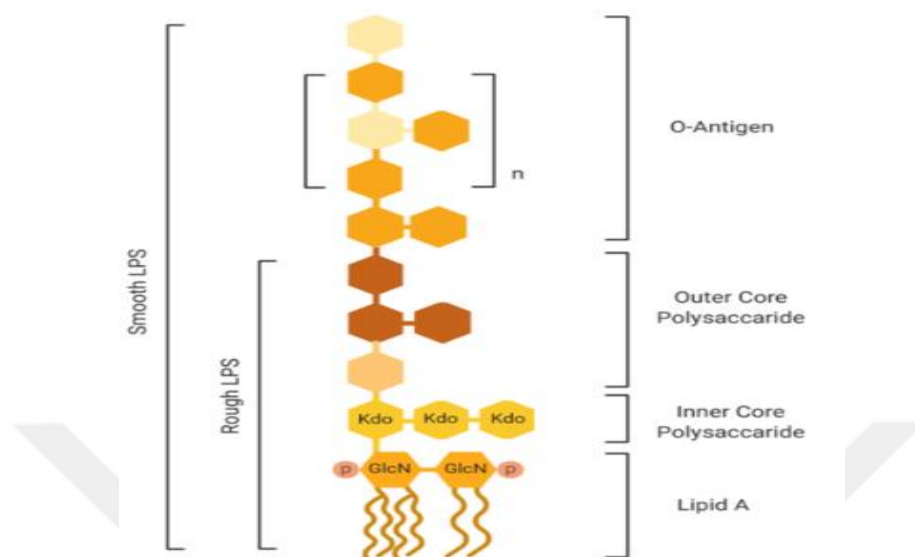


Figure 2.5. Structures of LPS (Page et al., 2022)

2.10.2. Effect of LPS

ROS buildup encourages the production of pro-inflammatory cytokines in immunocytes like macrophages, which exacerbates inflammatory reactions and damages local tissue (Zhai Z et al., 2012). As a result, the signalling pathways linked to ROS production and clearance have attracted a great deal of attention. Inhibiting ROS formation in macrophages is a general method to reduce proinflammatory signals (Figure 2.6) (Wang H et al., 2010)

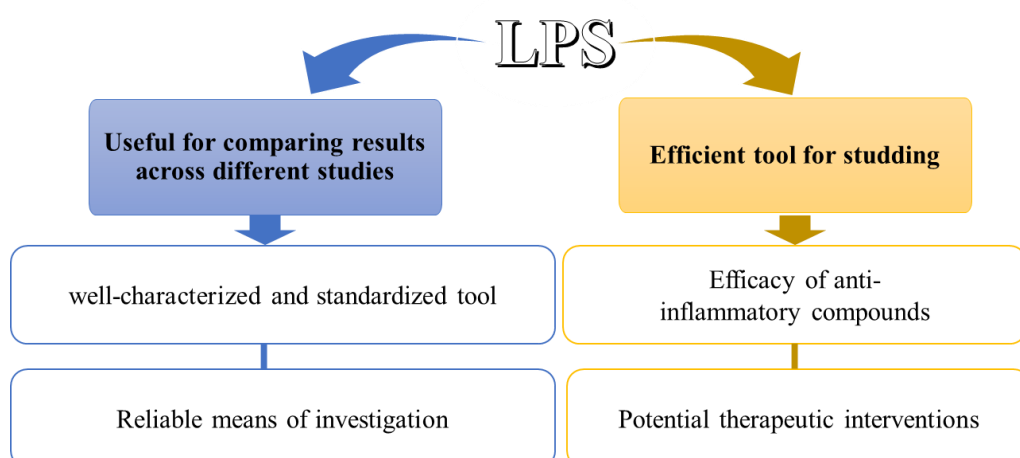


Figure 2.6. LPS for cell culture

Moreover, LPS is a well-characterized and standardized tool, making it useful for comparing results across different studies. Its consistent effects on immune cells provide a reliable means of investigating specific cellular processes, signalling pathways, and gene expression patterns associated with inflammation. Furthermore, LPS stimulation can be used to evaluate the efficacy of anti-inflammatory compounds or potential therapeutic interventions (Figure 2.6). By pre-treating cells with substances of interest and subsequently stimulating them with LPS, researchers can assess the impact of these interventions on modulating the inflammatory response (Hou et al., 2003; Lee et al., 2012; Wang-Sheng et al., 2017).

LPS was reported to provoke the release of early cytokines. Excessive production of nitric oxide (NO) through NO synthase (NOS) activity is involved in the activation of microglia in response to LPS. While NO can have protective effects in anti-inflammatory pathways, high levels can also be beneficial for defending against microbial or viral infections (Figure 2.7). This can trigger the release of early cytokines such as IL1 β , TNF α , and IL6, typically associated with an immune response (Haruwaka et al., 2019; Kumar et al., 2014).

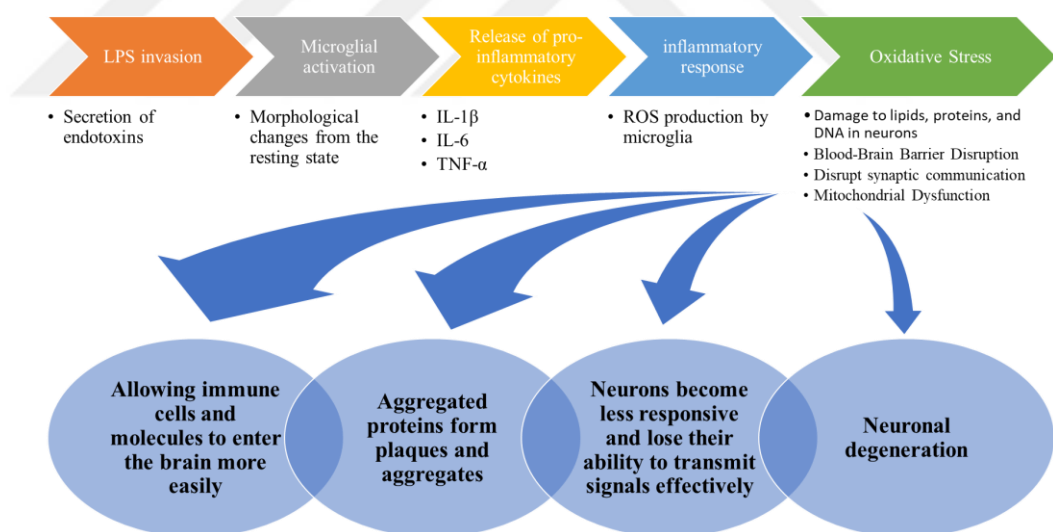


Figure 2.7. LPS effect when introduced to the CNS

The variation in LPS sensitivity among different cell lines or experimental models can lead to differing responses to LPS, resulting in varying doses that produce toxic effects. Inconsistencies in experimental conditions and protocols, such as cell culture conditions, LPS administration methods, duration of exposure, and endpoints measured, contribute to the discrepancies in reported doses. To address these conflicts, using consistent methodologies, it is important to carefully evaluate and compare

experimental details, standardize protocols, and conduct dose-response studies within individual laboratories (Figure 2.8). By doing so, we can establish a more consistent understanding of the dose-dependent effects of LPS on cellular viability and ensure accurate interpretations of experimental outcomes (Chang et al., 2005; Lu et al., 2009; Wang-Sheng et al., 2017; Yu et al., 2012).

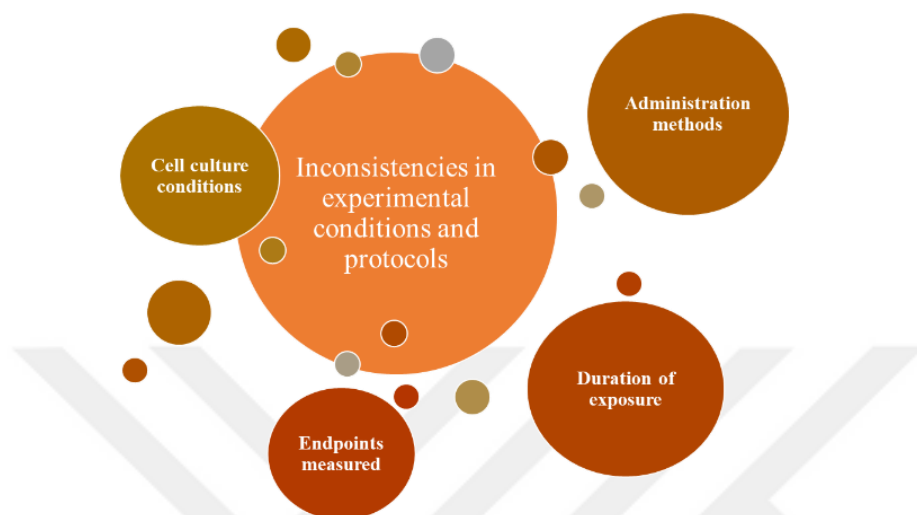


Figure 2.8. LPS discrepancy in cell culture

2.10.3. Review of neurotoxicity of LPS on BV2 microglial cells

Many studies have focused on targeting microglia as a potential means to modulate neuroinflammation and promote therapeutic or protective effects. Moreover, extensive research has investigated diverse aspects of BV2 microglial cells activated by LPS toxicity. In this section, we will investigate recent studies conducted in this field.

Bai et al. (2022) examined the role of *Toxoplasma gondii* rhoptry protein 18 (TgROP18) in regulating neuroinflammation mediated by activated microglia in BV2 cells stimulated with LPS. Their findings revealed that TgROP18 upregulated the expression of pro-inflammatory markers through the NF- κ B signalling pathway, leading to microglial polarization toward the M1 pro-inflammatory phenotype. This study highlighted the crucial role of TgROP18 in promoting neuroinflammation during *T. gondii* infection by modulating microglial activation and the NF- κ B signalling pathway.

Ban et al. (2023) investigated the anti-neuroinflammatory effects of an active fraction from *Spatholobus suberectus* Dunn (ASSD) in LPS-stimulated BV2 microglial cells. The study demonstrated that ASSD inhibited the expression of inflammatory markers and facilitated the transition of microglia from the pro-

inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. These effects were mediated by inhibiting the TLR4/MyD88/NF- κ B signalling pathway, suggesting the therapeutic potential of ASSD in neurodegenerative disorders by suppressing microglia-mediated neuroinflammatory responses.

Bui et al. (2020) examined the effects of 1,2,3,4-tetrahydroquinoline derivatives on LPS-stimulated BV2 microglial cells. Their findings revealed that these compounds attenuated the production of pro-inflammatory mediators and inhibited the nuclear translocation of NF- κ B. Among the derivatives, ELC-D-2 exhibited potent inhibition without significant cytotoxicity and showed promise as a potential therapeutic agent for modulating neuroinflammation associated with microglial activation.

Cai et al. (2018) investigated the anti-inflammatory effects of a diosgenin derivative in LPS-stimulated BV2 microglial cells. Their findings demonstrated that the diosgenin derivative inhibited the synthesis of inflammatory mediators and downregulated the expression of iNOS and COX-2. These effects were mediated by inhibiting the NF- κ B signalling pathway and suppressing JNK phosphorylation, suggesting the potential of the diosgenin derivative as a therapeutic agent for neurodegenerative disorders by targeting microglia-mediated neuroinflammation.

Dilshara et al. (2016) investigated the anti-inflammatory effects of morin, a natural flavonoid, in LPS-stimulated BV2 microglial cells. The study demonstrated that morin inhibited the expression of pro-inflammatory mediators, including NO and PGE₂, by suppressing NF- κ B activity and activating heme oxygenase-1 (HO-1). The findings suggest that morin may have therapeutic potential in preventing neuroinflammation by modulating the expression of inflammatory genes involved in the synthesis of NO and PGE₂.

Gao et al. (2021) explored the function of long non-coding RNA AK148321 in LPS-stimulated BV2 microglial cells. The study demonstrated that AK148321 was upregulated in response to LPS stimulation and acted as a competing endogenous RNA (ceRNA) for miR-1199-5p, leading to the upregulation of heat shock protein A5 (HSPA5). The AK148321/miR-1199-5p/HSPA5 axis was found to regulate neuroinflammation and microglial activation. The study suggests that targeting lncRNAs and miRNAs may provide novel therapeutic strategies for modulating neuroinflammatory responses.

In their study, L. Xu et al. (2018) investigated the role of Dyrk2 in neuroinflammation by analyzing its effect on LPS-induced microglial activation. They

found that LPS induced Dyrk2 expression in BV2 cells and interacted with Akt, p38MAPK, and NF- κ B subunit p65. Overexpression of Dyrk2 increased the expression levels of phospho-p65, phospho-Akt, and phospho-p38MAPK but decreased the release of proinflammatory cytokines TNF- α and IL-1 β . The study concluded that Dyrk2 regulates the release of proinflammatory cytokines through its phosphokinase function.

M. Xu et al. (2023) studied the effects of *Alpinia oxyphylla* and its main active components on neuroinflammation and microglial polarization. They found that AE and Chrysin, an active ingredient of AE, promoted the phenotypic transformation of microglia from M1 to M2, which was associated with the downregulation of TREM2 expression and the activation of PI3K/AKT/GSK3 β and BDNF/TrkB/TLR4 signalling pathways. The study concluded that AE improves microglial polarization and exerts anti-inflammatory effects through the TREM2-mediated signalling pathways.

S. Yang et al. (2020) investigated the anti-inflammatory effects of Isoalantolactone on LPS-activated BV2 microglia. They found that Isoalantolactone inhibited the production of proinflammatory cytokines TNF- α , IL-6, ROS, NO, and PGE2 in LPS-activated BV2 cells. The anti-inflammatory effects of Hig were attributed to its inhibition of the NF- κ B signalling pathway and activation of the Nrf2/HO-1 signalling pathway. The study concluded that Isoalantolactone exerts significant anti-inflammatory and oxidative effects by modulating these signalling pathways.

You et al. (2022) explored the effects of BIG1 (ARFGEF1) in LPS-mediated neuroinflammation and migration in BV2 cells. They found that BIG1 silencing reduced the expression of inflammatory cytokines and inhibited cell migration, while BIG1 overexpression had the opposite effect. The study demonstrated that BIG1 regulates neuroinflammation and migration through the PI3K/Akt/NF- κ B signalling pathway and is positively regulated by KLF4. The findings suggested that BIG1 activates microglia in response to LPS.

J. Zhang et al. (2019) investigated the effects of curcumin on microglial M1/M2 polarization and the underlying mechanisms. They found that curcumin inhibited LPS-induced microglial activation and switched the microglial phenotype from M1 to M2. The anti-inflammatory effects of curcumin were associated with its modulation of TLR4/NF- κ B pathways and the balance between TREM2 and TLR4 expression. The

study concluded that curcumin alleviates inflammation by regulating microglial polarization and downstream NF- κ B activation.

L. Zhang et al. (2018) studied the anti-inflammatory effects of nuciferine on LPS-stimulated BV2 microglia cells. They found that nuciferine significantly reduced the production of proinflammatory mediators, including TNF- α , IL-1 β , IL-6, NO, and PGE₂, in LPS-stimulated BV2 cells. The anti-inflammatory effects of nuciferine were attributed to its inhibition of the NF- κ B and MAPK signalling pathways. The study suggested that nuciferine has potential therapeutic value for treating neuroinflammatory diseases.

Hongxia et al. (2019) investigated the effects of zinc on LPS (LPS)-induced inflammation in BV2 microglia cells and its potential role in alleviating depression symptoms. They found that LPS treatment increased the expression of inflammatory markers and reactive oxygen species (ROS), while zinc supplementation effectively blocked ROS generation and reduced the expression of inflammatory cytokines. Additionally, zinc intervention in BV2 cells improved the viability of hippocampal HT-22 cells cultured in the medium of BV2 cells. The study suggested that zinc inhibits inflammatory responses mediated by microglia cells through the upregulation of zinc-finger A20, an anti-inflammatory factor.

Kaewmool et al. (2020) focused on the anti-inflammatory effects of protocatechuic acid (PA) on LPS-activated BV2 microglia cells and its potential modulation of SIRT1 associated with neurodegenerative diseases. Their findings indicated that PA inhibited the release of inflammatory mediators in LPS-activated BV2 microglia through the SIRT1/NF- κ B pathway, attenuating microglial activation-induced neuronal cell death. These findings suggest that SIRT1 plays a role in mediating the anti-neuroinflammatory effects of PA to protect against microglial activation-induced neuron death.

Kumar et al. (2014) investigated the role of inducible nitric oxide synthase (iNOS) in peroxynitrite-mediated protein radical formation during LPS-induced microglial activation. They observed that LPS treatment induced morphological changes, increased iNOS expression, and led to protein radical formation in BV2 cells. Inhibition of iNOS expression using siRNA or specific inhibitors attenuated protein radical formation, indicating the involvement of iNOS in peroxynitrite-mediated protein radical formation. The study established the role of iNOS in this process and highlighted its contribution to LPS-induced microglial activation.

Pan et al. (2017) focused on the anti-inflammatory effects of Shizukaol B, a compound derived from *Chloranthus henryi*, on LPS-induced activation of BV2 microglial cells. Their results showed that Shizukaol B suppressed the expression of inflammatory enzymes and the production of inflammatory mediators in LPS-stimulated BV2 microglia. The study further revealed that Shizukaol B exerted its anti-inflammatory effects by modulating the JNK-AP-1 signalling pathway, which regulates microglial activation and inflammation.

Roosen et al. (2021) investigated the effects of CXCL12, a chemokine involved in cell survival and mobilization, on microglial activation and inflammasome activity in LPS-treated BV2 cells. They found that CXCL12 significantly reduced LPS-induced inflammasome components, cytokine secretion, and caspase-1 activity in BV2 cells. These findings suggested that the neuroprotective effects of CXCL12 in spinal cord injury models may involve the modulation of microglial activation and associated inflammatory processes.

Tsou et al. (2016) explored the protective effects of olomoucine, a cyclin-dependent kinase inhibitor, on LPS-induced BV2 microglial cell death and proinflammatory responses. The study demonstrated that olomoucine reduced the generation of nitric oxide (NO) and the expression of proinflammatory cytokines in LPS-treated BV2 cells. Furthermore, olomoucine mitigated mitochondrial disruptions and cell death induced by NO.

2.11. Agomelatine and its potential as a neuroprotective agent

2.11.1. Introduction to agomelatine

Agomelatine, a synthetic analogue of the melatonin hormone (Figure 2.9), exhibits various therapeutic properties such as antidepressant, neuroprotective, and anti-inflammatory activities (Cheng et al., 2017; Saral et al., 2023). It acts as an agonist for melatonin receptors (MT1 and MT2) and as an antagonist for the serotonin 2C receptor (5-HT_{2C}) (N. M. Mahmoud et al., 2023; Papp et al., 2003; Saral et al., 2023). These receptors are widely distributed throughout the central nervous system (CNS), peripheral organs, and various tissues (Hafez et al., 2022; Kopustinskiene and Bernatoniene, 2021).

Activation of melatonin receptors, including MT1 and MT2, has shown potential in cardiovascular dysfunction, antioxidant effects, anti-inflammatory effects, and protection against pathological injuries (Dominguez-Rodriguez, 2012; Hafez et al.,

2022; Lwin et al., 2021). Agomelatine, therefore, presents an alternative approach to treating depression by targeting melatonin receptors and antagonizing 5-HT_{2C} receptors (Maddukuri et al., 2021).

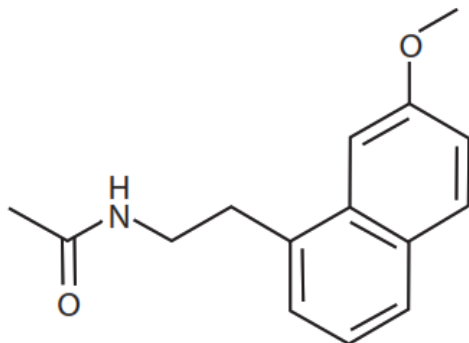


Figure 2.9. The chemical structure of agomelatine. (Promsan et al., 2022)

Moreover, agomelatine is a novel melatonergic antidepressant that specifically addresses circadian rhythm disturbances associated with depression and operates through a non-monoaminergic mechanism (de Bodinat et al., 2010; Łebkowska-Wieruszewska et al., 2021). Its affinity for most receptors, including serotonin receptors, is minimal, and it is associated with a well-characterized side effect profile (Łebkowska-Wieruszewska et al., 2021; Mark J. Millan, 2006; M. J. Millan et al., 2003). However, its oral bioavailability is limited due to a high first-pass effect, and it primarily undergoes hepatic metabolism (Hafez et al., 2022). Although pharmacokinetic studies have been conducted in humans, research on canine species is lacking (Giorgi et al., 2012).

Clinically, agomelatine is used as an antidepressant that modulates endogenous melatonin secretion, regulates circadian rhythms by activating MT₁ and MT₂ receptors, and acts as an antagonist on the 5-HT_{2C} receptor (Castanho et al., 2014; Cherngwell et al., 2021).

2.11.2. The significance of agomelatine

Agomelatine has significantly affected cognitive functions by modulating limbic regions, including the hippocampus (Figure 2.10). Studies indicate that agomelatine enhances the expression of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex and hippocampus (Saral et al., 2023). BDNF, a crucial neurotrophic factor in the hippocampus, acts through the tropomyosin receptor kinase B (TrkB) receptor present in hippocampal cells' nerve terminals, axons, and dendritic spines (Colucci-

D'Amato et al., 2020). BDNF is involved in synaptic plasticity, neuroprotection, and memory modulation (Kowiański et al., 2018), and agomelatine has been shown to regulate BDNF signalling, exerting beneficial effects on cognitive functions affected by stress (Martin et al., 2017; Martinotti et al., 2016; Saral et al., 2023).

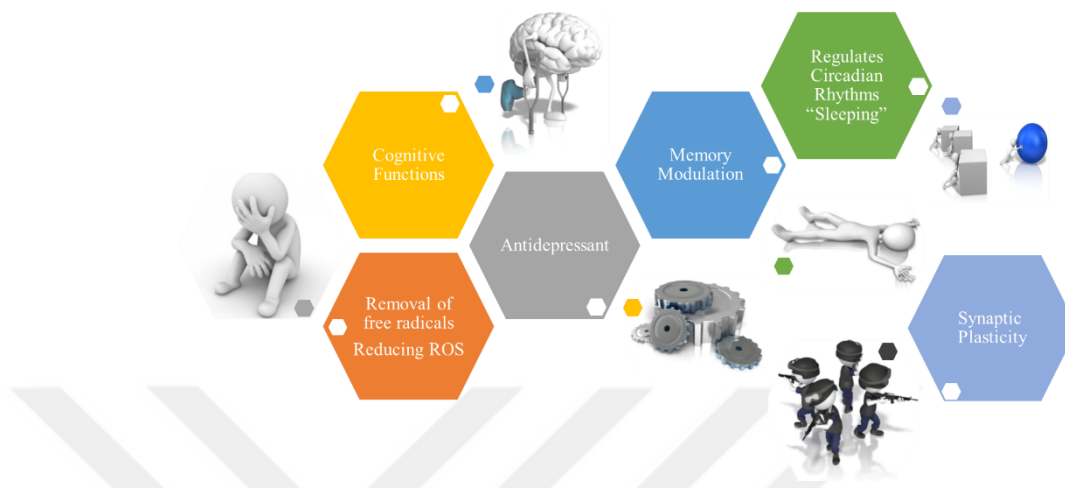


Figure 2.10. Agomelatine effect on the CNS.

Regarding serotonin receptor activity, 5-HT_{2C} receptor agonists can enhance stimulant or opioid-reinforced behaviours at low doses, whereas high doses can attenuate drug-taking (De and Grasing, 2023; Johns et al., 2021). Agomelatine, acting as an analogue of melatonin, functions as a melatonin receptor agonist (MT₁/MT₂) and a serotonin receptor antagonist (5-HT_{2B}/5-HT_{2C}), exhibiting antidepressant and anxiolytic effects (N. M. Mahmoud et al., 2023; Promsan et al., 2022). It has also demonstrated protective effects against oxidative stress and inflammation in various organs (Demirdaş et al., 2016).

Furthermore, agomelatine has been found to possess potent anti-inflammatory activity and provide protection against oxidative stress in multiple organs (Demirdaş et al., 2016; N. M. Mahmoud et al., 2023). It exerts renoprotective activity in diabetic nephropathy (DN) by regulating SIRT1, a histone deacetylase involved in cell survival and inflammation inhibition (Cherngwell et al., 2021; N. M. Mahmoud et al., 2023).

Melatonin and its analogues have beneficial effects in managing various conditions, including depression, insomnia, epilepsy, Alzheimer's disease, diabetes, obesity, cancer, and immune and cardiac disorders (Cherngwell et al., 2021).

Additionally, agomelatine exhibits anxiolytic/antidepressant effects and mitigates inflammation and oxidative stress (Rebai et al., 2021). It scavenges reactive oxygen species (ROS), regulates oxidant and antioxidant enzymes, and improves renal

oxidative stress and endoplasmic reticulum stress (Hafez et al., 2022; Kara et al., 2008). Agomelatine may also protect against obesity-related renal injury by modulating oxidative stress, inflammation, and endoplasmic reticulum stress (Cherngwell et al., 2021).

2.11.3. Agomelatine application

Agomelatine, a melatonergic antidepressant, has been extensively studied for its therapeutic effects in various conditions. It acts as a non-selective agonist of melatonin receptors (MT1/MT2) and an antagonist of serotonin receptor 5-HT_{2C}, exhibiting unique pharmacological properties (Atanasova et al., 2021; Tchekalarova et al., 2020).

One area of investigation has focused on the potential of agomelatine in preventing kidney injury in an obese rat model. Studies have shown that agomelatine exerts antioxidant, anti-inflammatory, and anti-apoptotic effects, thereby ameliorating alterations associated with a high-fat diet (Cherngwell et al., 2021; N. M. Mahmoud et al., 2023). Agomelatine's protective effects extend beyond kidney injury and have been observed in the cardiovascular system and testes in various models (Khalaf et al., 2020; et al., 2020; Mahmoud et al., 2021) (Aslankoc et al., 2020; Hafez et al., 2022; Khalaf et al., 2020; A. M. Mahmoud et al., 2021).

In the field of depression and sleep quality, agomelatine has demonstrated promise as a novel antidepressant that acts on both melatonin and serotonin receptors simultaneously (Pu et al., 2023). Combination studies with repetitive transcranial magnetic stimulation (rTMS) have explored its effects on sleep quality and biomarkers in patients with mild to moderate depression (N. M. Mahmoud et al., 2023).

Agomelatine has also been investigated for its potential therapeutic effects in neurodevelopmental disorders (NDD). By targeting limbic regions, including the hippocampus, and modulating cognitive functions, agomelatine shows promise in regulating brain-derived neurotrophic factor (BDNF) expression and influencing neuronal signalling pathways (Papp et al., 2003; Saral et al., 2023).

Furthermore, agomelatine's effects on oxidative stress and inflammation have been explored in various contexts. Studies have indicated its protective effects against oxidative stress-induced injuries and inflammation (Demirdaş et al., 2016). In rat models of sepsis-related acute kidney injury and kidney injury under obese conditions, agomelatine demonstrated beneficial effects by reducing markers of oxidative stress, inflammation, and apoptosis (Cherngwell et al., 2021).

Agomelatine's potential therapeutic applications extend to obesity and metabolic syndrome. Studies have suggested that agomelatine, through its modulation of the intestinal melatonin system, could potentially improve metabolic syndrome by influencing gut microbiota and restoring the Firmicutes/Bacteroidetes ratio (Diez-Echave et al., 2022).

2.11.4. Agomelatine effect on LPS

Many studies have demonstrated the effects of agomelatine on LPS and its potential as a protective agent. This review aims to provide insights into its role in mitigating LPS-induced damage:

Savran et al. (2020) evaluated the effects of agomelatine on LPS-induced brain damage. They found that LPS administration increased inflammation, oxidative stress, and apoptosis. Agomelatine treatment reversed these effects, enhanced SIRT-1 levels, and ameliorated inflammation, oxidative stress, and apoptosis.

Molteni et al. (2013) studied agomelatine's ability to modulate the rat brain's immune response after LPS-induced inflammation. They found that agomelatine reduced the up-regulation of pro-inflammatory cytokines (IL-1 β and IL-6) in the rat brain and peripheral tissues. Agomelatine also inhibited NF- κ B translocation and affected the expression of enzymes related to the kynurenine pathway, which is associated with inflammation-related depression.

Asci et al. (2022) investigated the effects of agomelatine on LPS-induced endothelial and cardiac damage. Their study showed that LPS administration increased inflammation, oxidative stress, and apoptosis in cardiac tissues. Agomelatine treatment reversed these effects and exhibited antioxidant, anti-inflammatory, and antiapoptotic activities.

Inanir et al. (2015) explored the effects of agomelatine on a psychosis-relevant behaviour model induced by LPS. They observed that agomelatine reduced TNF- α , HVA, and MDA levels and the stereotype score in the model of psychosis. These effects were associated with its anti-inflammatory properties and influence on dopamine metabolism.

Lan et al. (2022) studied the molecular mechanisms underlying the antidepressant effects of agomelatine in an LPS-induced rat model of depression. They found that agomelatine reduced neuroinflammation, abnormal autophagy, and neuronal apoptosis while promoting neurogenesis in the hippocampus. Agomelatine

also affected the 5-HT_{2C} receptor-Gai-2 protein interaction and modulated the ASK1 pathway, which is involved in pathological processes.

Rossetti et al. (2018) used a genome-wide strategy to investigate agomelatine's immune-regulatory activity in response to LPS challenge. They found that agomelatine modulated pathways associated with immune/inflammatory systems and prevented the activation of genes induced by LPS, including IL-1 β and the metabotropic glutamatergic transporter Grm2.

2.11.5. Research gaps and opportunities for further investigation

Although much literature has explored the effects of agomelatine on LPS, no study has delved into its potential protective capabilities against LPS toxicity in the BV2 cell line. This unexplored territory presents a unique opportunity to uncover novel insights into the innovative use of agomelatine and its potential as a safeguard against LPS-induced damage in BV2 cells.

3. MATERIAL AND METHODS

3.1. Chemical reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), 10,000 U/ml penicillin 10mg/ml streptomycin, 0.25% (v/v) Trypsin-Ethylene Diamine Tetra Acetic Acid (EDTA) and Dulbecco's Phosphate-Buffered Saline (PBS) solutions, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, 70% Ethanol, DW, Agomelatine, LPS were obtained from recognized companies.



Figure 3.1. The chemical substances used in cell culture.

3.2. The cell lines

BV2 cell lines were kindly gifted by the Histology and Embryology department, Ondokuz Mayıs University, Samsun, Turkey.

3.3. Tools

Laminar flow/biological safety cabinet (BSC), CO₂ incubator, microscope, centrifuge, pipettes, water bath, autoclave, mixer, and shaker.

3.4. Plastics and consumables

Cell culture flasks, pipette tips, falcon tubes, serological pipettes, cryovials, reagent reservoirs, filters, cover foils, and eppendorf tubes.

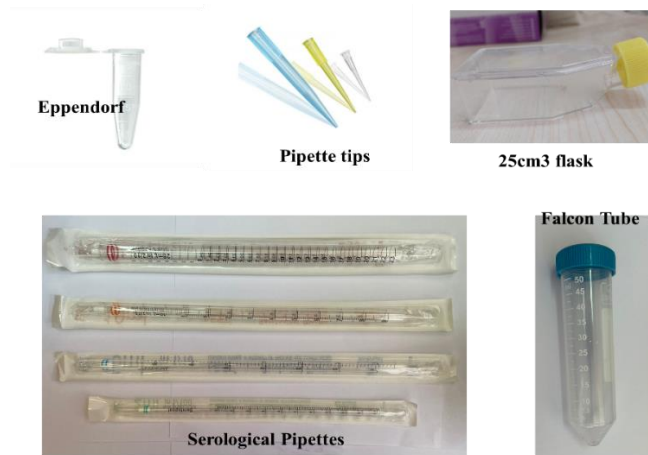


Figure 3.2. Plastics and consumables used in cell culture.

3.5. Working environment

3.5.1. Incubator environment

The incubator is under conventional culture conditions of 5% carbon dioxide in 95% humidified at 37°C to allow the cell to attach to the surface of the 25 cm² flask.



Figure 3.3. The incubator under conventional culture conditions of 5% of CO₂ in 95% humidified at 37°C.

3.5.2. Laminar flow environment

Laminar flow is an essential component in the cell culture environment. It involves using specialized laminar flow cabinets to provide a continuous supply of clean, filtered air to the workspace. The laminar flow creates a unidirectional airflow that helps to minimize the introduction of contaminants and maintain a sterile working area. The air passes through high-efficiency particulate air (HEPA) filters, removing airborne particles, including bacteria and viruses. It creates a sterile environment for

cell culture procedures, reducing the risk of contamination and ensuring the integrity of cell cultures. Laminar flow cabinets are designed to provide a clean, controlled environment for the aseptic handling of cells, media, and other materials, promoting successful cell growth and minimizing the risk of contamination-related issues (Phelan and May, 2015).



Figure 3.4. The Laminar flow is used in the cell culture lab.

3.5.3. Sterilization protocol

Sterilization protocols are the way to maintain aseptic conditions and prevent microbial contamination in laboratory settings. These protocols involve the use of various methods to eliminate the presence of microorganisms on equipment, surfaces, or media. Common sterilization techniques used include autoclaving, which uses high temperature and pressure to achieve sterilization, and filtration, which removes microorganisms through a physical barrier. Other methods, such as chemical sterilization using ethanol 70% disinfectants, were used depending on the specific requirements (Phelan and May, 2015).

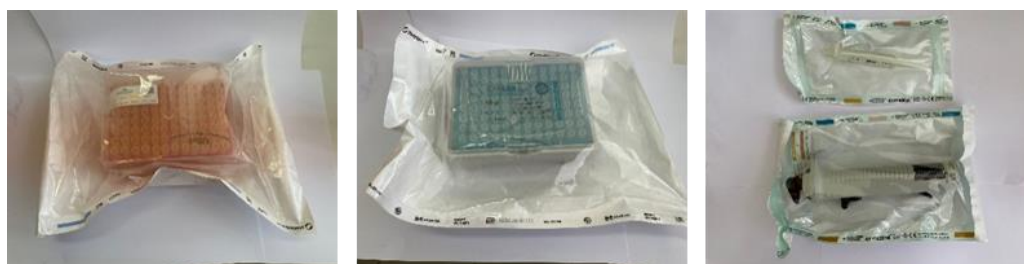


Figure 3.5. Sterilization of some of equipment used in cell culture.

3.6. Substances preparations

3.6.1. Complete media preparation

The complete media was prepared as follows: 80% DMED, 20% FBS, 1 % L- Glutamine, 1% penicillin 10,000 U/ml streptomycin 10mg/ml, and when needed 1% amphotrcin B was used (Masters and Stacey, 2007).

3.6.2. Ethanol preparation

Ethanol was used at a concentration of 70%. To prepare 750 mL of ethanol at 70% concentration, 525 mL of ethanol 96% was measured and added to a clean container. Then, 225 mL of a DW was added, and the solution was thoroughly mixed. The container was tightly sealed for lab sterilization (Phelan and May, 2015).

3.6.3. PBS preparation

To prepare PBS (phosphate-buffered saline), each tablet of PBS was dissolved in 100 mL of distilled water. A total of 5 tablets was added to 500 mL of distilled water in a single large bottle and dissolved properly before usage.

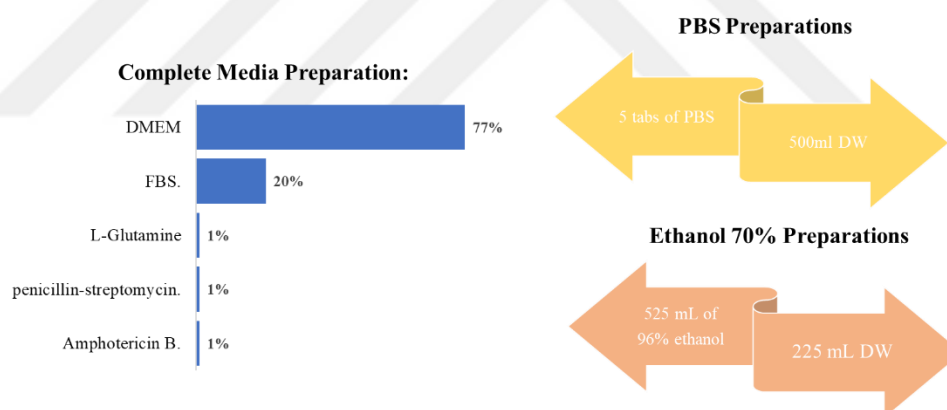


Figure 3.6. Complete media, PBS and ethanol preparation

3.6.4. Agomelatine preparation

To prepare a 10 mL solution of agomelatine at a concentration of 1 mM, 2.4 mg of agomelatine powder was measured and added to a sterile falcon tube. Then, 1 mL of dimethyl sulfoxide (DMSO) was measured and added to the falcon tube. Then, the mixture was gently mixed until the agomelatine powder was completely dissolved in the DMSO, ensuring a homogenous solution. Finally, 9 media was added to reach the final concentration of 1mM. The agomelatine solution was properly labelled with the contents, concentration, and date of preparation. The solution was stored following the recommended storage conditions for agomelatine and DMSO.

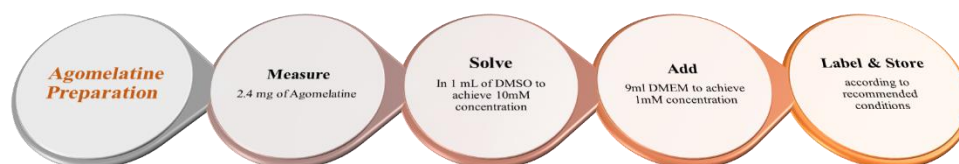


Figure 3.7. Agomelatine 1mM stock solution preparation

3.6.5. MTT solution preparation

To prepare a stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in a sterile and clean Falcon tube. First, 50 mg of MTT powder was measured and added to the 15 ml Falcon tube. Next, 10 mL of PBS (phosphate-buffered saline) was measured using the electric pipette and added to the Falcon tube containing the MTT powder. It was then gently mixed to dissolve the MTT powder with the PBS thoroughly.

Once the MTT powder was completely dissolved in the PBS, the stock solution of MTT was successfully prepared. The Falcon tube was appropriately labelled, indicating the concentration and date of preparation. It was then stored in the refrigerator, adhering to the recommended storage conditions for MTT.

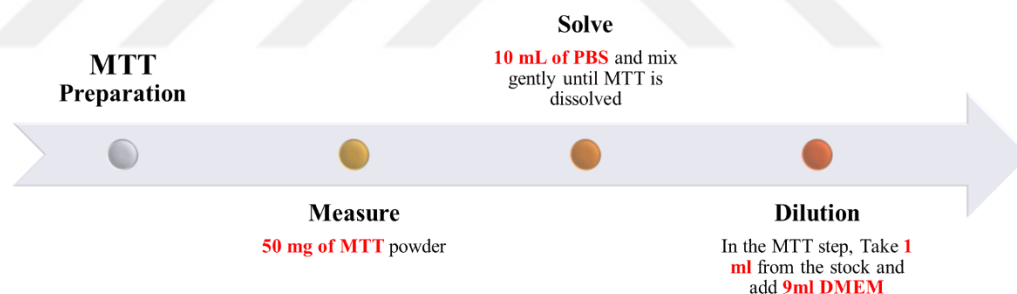


Figure 3.8. MTT stock solution and desired concentration preparation

3.6.6. LPS preparation

The following procedure was followed to prepare a stock solution of LPS at the 5 mg/mL concentration. A clean and sterile Eppendorf container was obtained and ensured to be free from contaminants. Then, 5mg of LPS powder was accurately measured. Next, an appropriate solvent (sterile water/PBS) was obtained, and 1 mL was measured using a 1ml pipette. With caution, the entire 5mg of LPS powder was slowly added into the container while gently mixed to facilitate dissolution and ensure the powder was completely dissolved in the solvent.

Once the LPS powder was fully dissolved, a stock solution of LPS at a 5 mg/mL concentration was successfully prepared. The container was appropriately labelled, indicating the concentration and date of preparation. It was stored in refrigeration/freezing according to the recommended storage conditions. Throughout the preparation process, proper safety measures and sterile techniques will be adhered to, ensuring the integrity and quality of the stock solution.

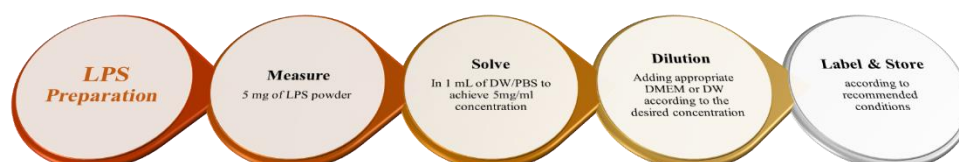


Figure 3.9. LPS 5mg/ml stock solution preparation

3.7. Cell culture techniques

3.7.1. Naming the cell line

New cell lines should be given a **code name**. For BV2, the cell line will be given a **number for the passage**. An additional number was added for several cell lines that were derived from the same source (BV2 P1) and given a **clone number** for cloned (BV2 P1-1, BV2 P1-2) (Phelan and May, 2015).

3.7.2. Check the cell morphology

A thorough examination of the culture at every step of the procedure is crucial to ensure its integrity and verify the absence of contamination or any signs of deterioration. These signs can indicate the need for a medium change or suggest more significant issues such as inadequate or toxic medium or serum, microbial contamination, or the ageing of the cell line. Inadequate medium components can even trigger apoptosis. Being familiar with the cell's morphology also enables the early detection of potential cross-contamination or misidentification, which is vital for maintaining accurate and reliable results (Phelan and May, 2015).

3.7.3. Feeding and replacement of medium

For medium replacement, several factors should be considered. These include a drop in pH, colour change of the medium, rate of pH decline, cell concentration, and cell type. Regular examination and familiarity with the cell line are essential to identify

morphological deterioration. If not addressed in time, irreversible damage may occur as cells enter apoptosis (Phelan and May, 2015).

When a culture has a low density and slow growth, a half-feed will be performed by removing only half of the medium and replacing it with the same volume (Phelan and May, 2015).

3.7.4. Volume of the media

The medium volume to surface area ratio is typically 0.2 to 0.5 mL/cm². The optimal ratio depends on the oxygen requirement of the cells. Cells with high oxygen need to thrive in shallow medium, while those with lower requirements may do better in deeper medium (Phelan and May, 2015). For BV2, I will add 4-5 ml Complete media to 25cm³.

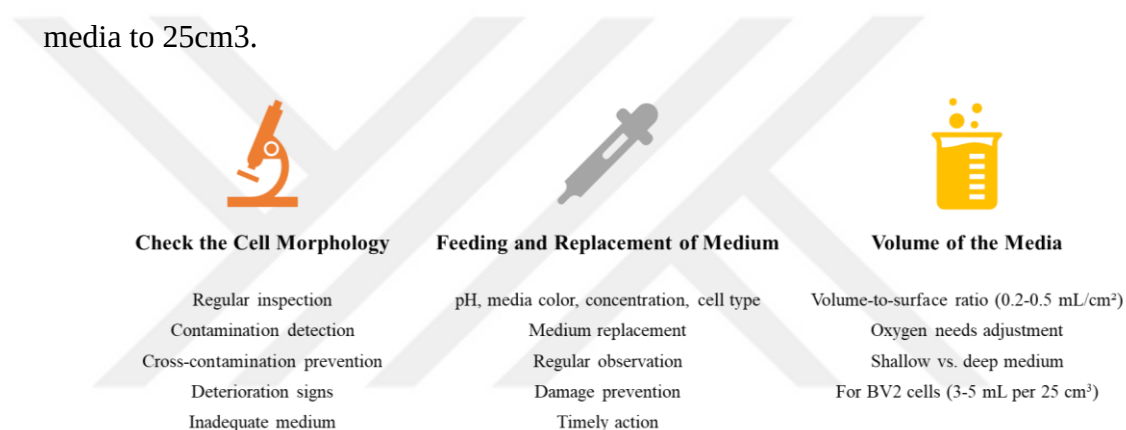


Figure 3.10. Routine in the cell culture lab

3.7.5. Growth curve

The growth curve consists of different phases. The lag phase occurs after seeding, followed by the log phase characterized by exponential growth. The medium must be changed or the culture divided when the cell density reaches a certain limit. The plateau phase, also known as the stationary phase, is where cell growth slows down due to a growth-limiting factor. The death phase is marked by a predominance of cell death and reduced viable cells (Phelan and May, 2015).

Split ratio or dilution establishes an appropriate subculture interval and prevents the cells from entering a plateau phase before the next subculture. (Phelan and May, 2015). It will ensure that the cells are not diluted below a concentration that allows them to re-enter the growth cycle within a reasonable timeframe of 24 hours or less (Phelan and May, 2015). The ideal method of determining the correct seeding density

was performed at different seeding concentrations to determine the ideal concentration.

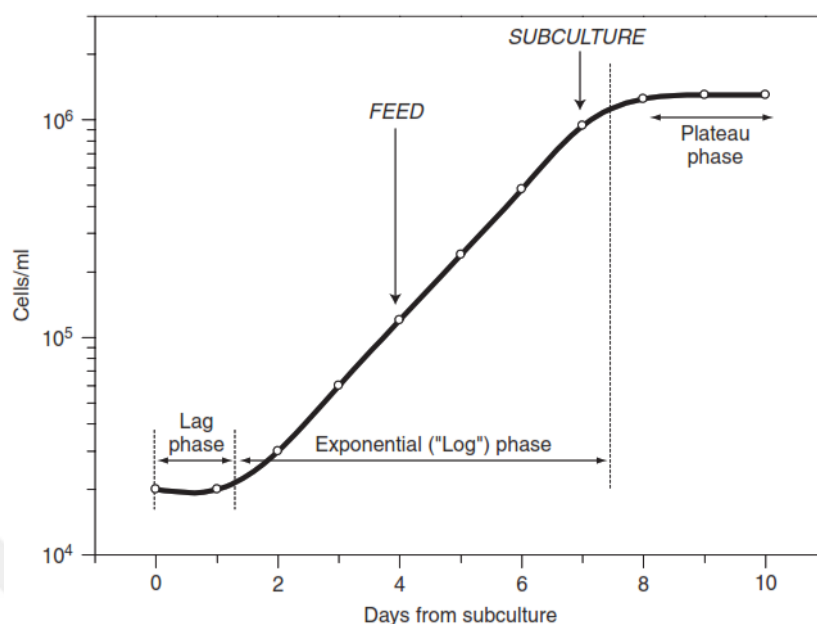


Figure 3.11. Phases of the growth curve in cell lines. (Phelan and May, 2015).

3.7.6. Cell maintenance

All experiments were performed under the laminar flow in a sterile condition. The flasks were continuously checked under the inverted microscope for any sign of contamination or feeding process (Phelan and May, 2015).

3.7.8. Freezing protocol

Cells have been observed to exhibit higher survival rates when frozen at higher cell concentrations. This phenomenon is attributed to reduced viability during thawing, necessitating a higher seeding concentration. Additionally, cells that have experienced cryogenic damage tend to be leaky. Freezing cells at a higher concentration also allow for sufficient dilution of the cryoprotectant upon reseeded after thawing, eliminating the need for centrifugation in most cases. The number of cells to be frozen should permit a 1:10 or 1:20 dilution upon thawing, effectively diluting out the cryoprotectant while maintaining a higher cell concentration than normal passage (Phelan and May, 2015).

(DMSO is considered more effective as it penetrates the cells efficiently, although it may occasionally exhibit toxicity or induce cell differentiation after thawing. With 7.5% or 10% DMSO Concentrations being more commonly used. For

my protocol, 10% DMSO solution was added to 90% complete media to prepare the cell suspension for freezing.

Thawing & Seeding		Passaging		Freezing	
Cell Retrieval	Retrieve frozen cells from -80 fridge.	Media removal	Aspire all the culture media and rinse with PBS.	Media removal	Remove culture media and rinse with PBS.
Thawing	warm the vial rapidly to minimize intracellular ice crystal growth during warming	Trypsinization	Detach cells at 80% confluence using Trypsin-EDTA for 3 minutes, then adding complete culture media to inhibit trypsin.	Trypsinization	Detach cells at 80% confluence using Trypsin-EDTA for 3 minutes, then adding complete culture media to inhibit trypsin.
Complete Media preparation	Prepare and pre-warm complete media	Centrifugation	Centrifuge cells at 2500 rpm for 5 minutes, remove supernatant, then add pre-warmed complete media to the cells.	Centrifugation	Centrifuge cells at 2500 rpm for 5 minutes, remove supernatant, then add pre-warmed complete media to the cells.
Centrifugation	Centrifuge cells rpm for 5 minutes, remove the supernatant, then add pre-warmed complete media to the pellet.	Splitting and Seeding	Resuspend, split, and seed the cells into 25 cm ² culture flasks.	Freezing	Add 900 ul complete media, transfer the cells into Cryovial, then Add 100 ul DMSO
Seeding	Pipette and seed the cells into 25 cm ² culture flasks at a high concentration for optimal recovery				

Figure 3.12. Thawing, seeding, passaging, and freezing Protocols

3.7.9. Thawing protocol

Frozen cells were brought from -80°C, thawed and reseeded into 25 cm³. The seeding density was performed at a relatively high concentration to optimize recovery. The vial was thawed rapidly to minimize the growth of intracellular ice crystals during the warming process (Phelan and May, 2015).

3.7.10. Seeding protocol

Vials of frozen BV2 cells were obtained and pre-warmed in a water bath. Complete media was pre-prepared and prewarmed. The cells were centrifuged, and the supernatant was removed. Then, complete media was added, and the cells were seeded into 25cm³ flasks (Masters and Stacey, 2007).

3.7.11. Passaging protocol

Upon reaching 80% confluence, cells were detached using Trypsin/EDTA for 3 minutes after being eliminated from culture media in the flask and rinsed with PBS. The trypsin was inhibited by adding 3 ml of complete culture media. Followed by centrifuging at 2500 rpm for 5 min and removing the supernatant (Masters and Stacey, 2007).

3.7.12. Seeding in the well-plate

The cells were seeded in 96 well-plates at a density of 200,000 cells per well-plate, each well contained in a 200 µl complete culture media with 5000 to 6000 cells and incubated for 24 hours before administration of the experimental treatment (Masters and Stacey, 2007).

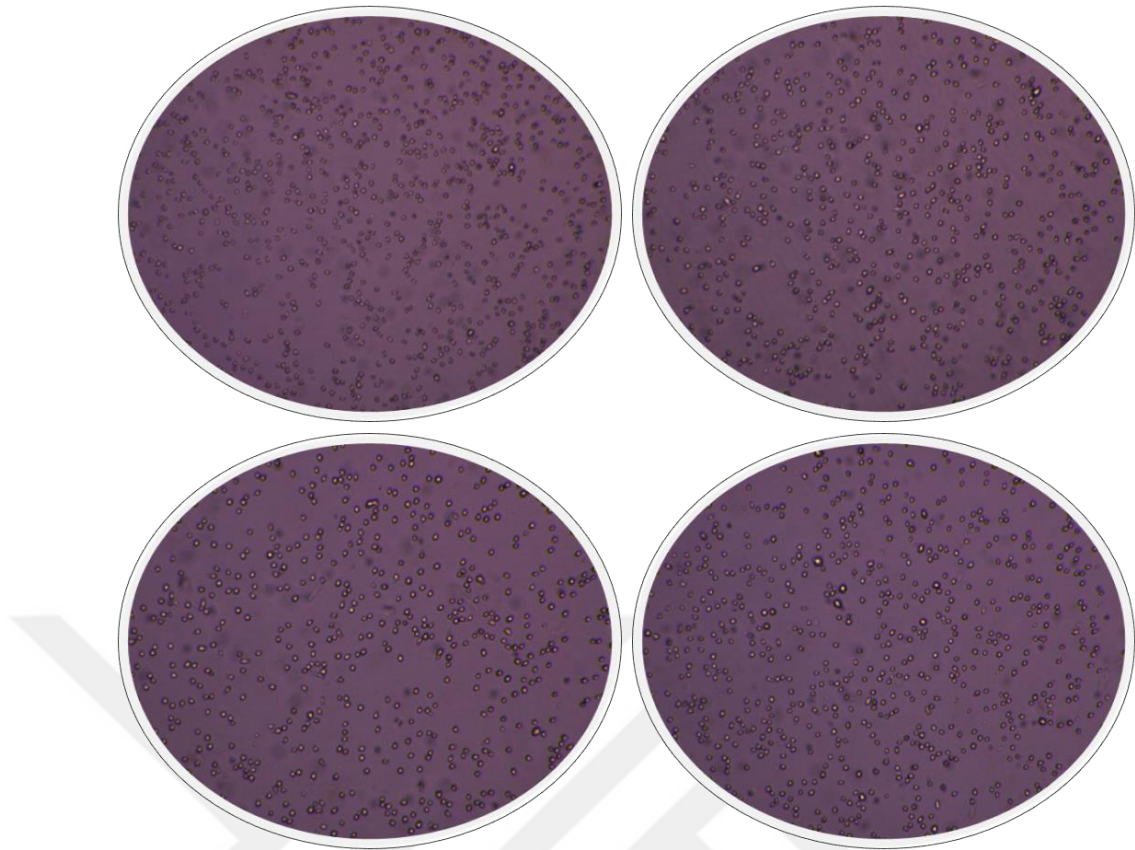


Figure 3.13. BV2 concentration at the seeding in the 96 well-plates.

3.8. MTT assay for measurement of the cell viability and cytotoxicity

To evaluate the cell viability and determine the IC_{50} , I used the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium). It is used for multiple applications such as cell viability, cytotoxicity, cell proliferation, and cell activity. The reaction of this assay depends on the ability of the mitochondria metabolic activity of viable cells to transform the water-soluble yellow tetrazolium salt into a water-insoluble formazan (purple). This action does not occur in dead cells (Uğuz and Nazıroğlu, 2012).

3.8.1. MTT assay protocol

BV2 cells were seeded in different concentrations in a 96-well plate (with three replicates). After 24 hours, the complete media was aspirated, and cells were washed with PBS. The dosage determined from the dose determination experiment was applied, and the 96-well plate was incubated for another 24 hours.

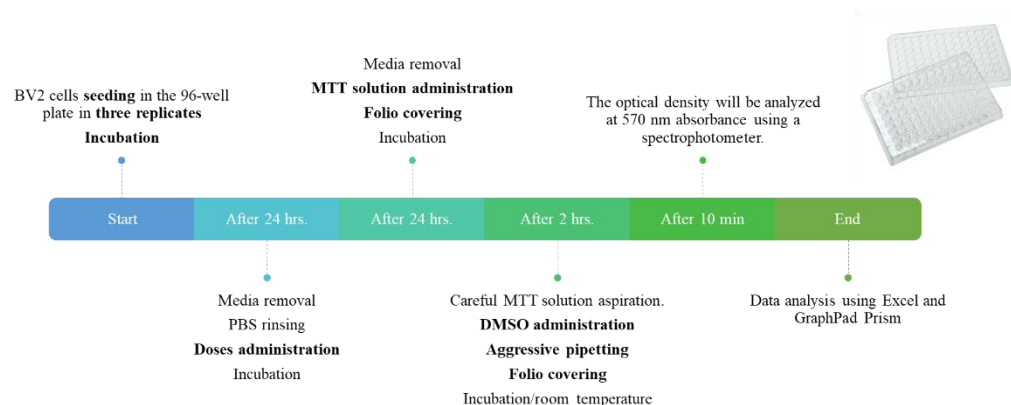


Figure 3.14. MTT Assay protocol for dose determination

MTT solution was added, and the well-plates was incubated for 2 hours, followed by careful aspiration. DMSO was added, and aggressive pipetting was performed. The plate was covered with folio and incubated or left at room temperature for 10 minutes. The results was analyzed at 570 nm absorbance using a spectrophotometer (Uğuz and Nazıroğlu, 2012).

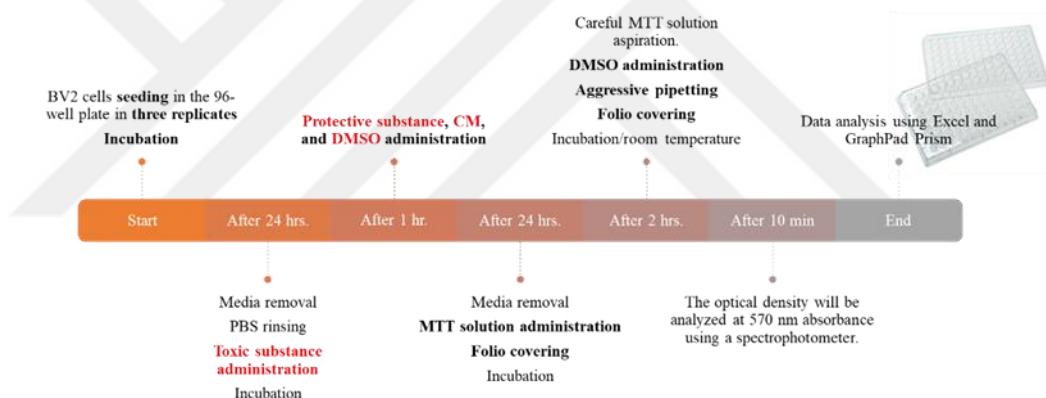


Figure 3.15. MTT Assay protocol for examining the effect of the substances

3.8.2. MTT assay data analysis for cell viability

The following steps will be used for calculating the cell viability of the cells:

1. Obtain the absorbance values

Retrieve the absorbance values obtained from the microplate reader for each well of the MTT assay.

2. Background correction

Subtract the absorbance value of the blank wells (containing only the complete media) from the absorbance values of the sample wells. This correction accounts for non-specific absorbance from the solubilization buffer or other factors.

3. Normalize the data

The corrected absorbance values of the treated samples were normalized to the absorbance of the control group to account for any variations in cell number or experimental conditions. The corrected absorbance values of the treated samples were then divided by the control group's absorbance.

4. Calculate cell viability

Cell viability can be expressed as a percentage relative to the control. The normalized absorbance values were multiplied by 100 to obtain the cell viability percentage.

The equation for calculating the cell viability:

$$\text{Viability (\%)} = (\text{Sample absorbance} / \text{Control absorbance}) \times 100$$

5. Statistical analysis:

Descriptive statistics were calculated as mean and standard deviation of the cell viability values. One-way ANOVA / non-parametric statistical tests followed by Tukeys' multiple comparison test were conducted to determine the significance of any observed differences between experimental groups.

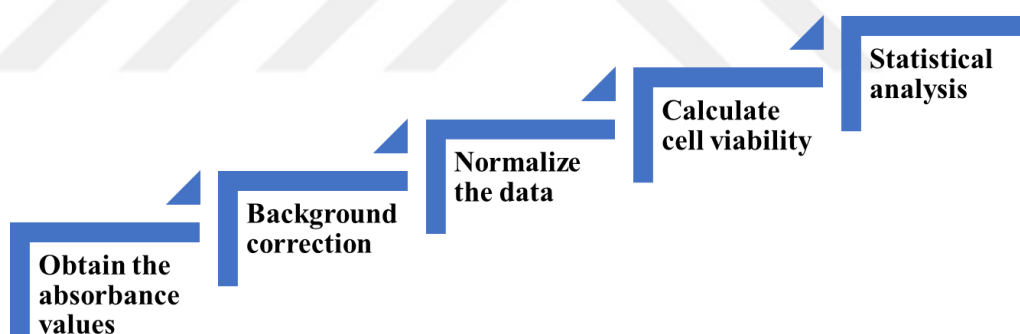


Figure 3.16. MTT Assay steps for data analysis for cell viability

3.9. Dosage determination

The ideal cell concentration was determined through an experimental study in the study protocol. It was done by testing different concentrations of the cells to a different dose of the protective and toxic substances. BV2 microglia cells were seeded into 96-well plates and then exposed to various concentrations of agomelatine to assess their ideal neuroprotective and LPS for its toxic effects.

To determine the effects of these substances, MTT assays were performed with different doses and durations. The MTT assay is commonly used to evaluate cell viability. The primary objective was to examine the IC₅₀ value for each substance,

which represents the concentration at which 50% of the cells' viability is affected. It guided us to the ideal dosage and duration of drug administration.

3.9.1. Agomelatine dose determination

3.9.1.1. Experiment description

The study aimed to assess the effects of different concentrations of agomelatine on BV2 cells in order to determine the optimal dose for our research project. According to the literature, agomelatine doses used in *in vitro* studies showed a range of concentrations. For instance, some studies reported using 2 and 4 μM doses, while others utilized 2 to 16 μM doses. Some studies used higher doses, such as 100, 200, and 300 μM . Considering this variability, thus, experiments were conducted to evaluate the effects of agomelatine across a range of concentrations, including doses up to 200 μM , on different cell concentrations of BV2.

3.9.1.2. Study design

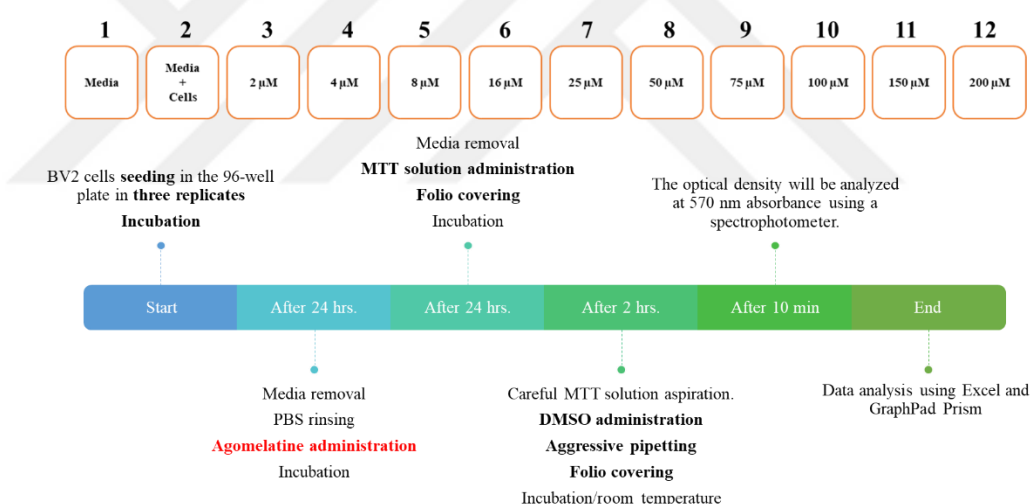


Figure 3.17. MTT Assay protocol for agomelatine dose determination.

BV2 cells were seeded in a 96 well-plate using appropriate seeding methods. Three repetition designs were used for the reliability of the results. The 96 well-plate was then incubated for 24 hours. After incubation, the complete media was aspirated, and the cells were washed with PBS. Agomelatine was then administered at different concentrations (ranging from 1 to 200 μM), and the well-plate was later incubated for 24 hrs.

After 24 hours, agomelatine-containing media was aspirated, and the cells were washed again with PBS. Then 110 μl MTT was added, and the well-plate was

incubated for 2 hours. The MTT was then carefully aspirated without disturbing the formazan crystals. Then, 100µl DMSO was added, pipetted and left for 10 minutes. The results were analyzed using a spectrophotometer at 570 absorbance.



Figure 3.18. MTT Assay at different stages. A: after agomelatine administration, B after MTT administration, and C after DMSO administration.

3.9.1.2. Effect of duration

The same procedure was conducted in the second well plate after 48 and for the third 96 well-plate after 72hrs. The results were also analyzed for both at 570 nm absorbance using a spectrophotometer. The experiment aims to test the effect of these doses of agomelatine on BV2, determine the IC₅₀ after 24 hrs and the effect of duration after 48hrs and 72 hrs., and compare all three results using one-way ANOVA.

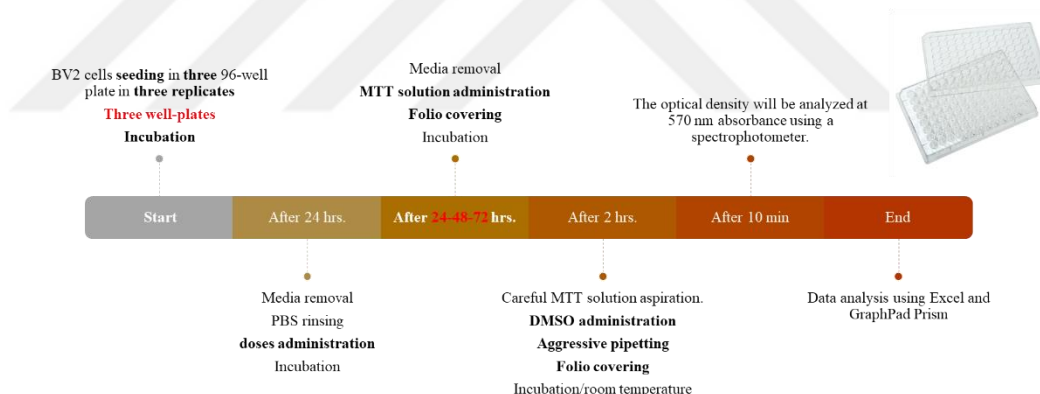


Figure 3.19. MTT Assay protocol for agomelatine impact of duration

3.9.2. LPS dose determination

3.9.2.1. Experiment description

The variation in LPS sensitivity among different cell lines or experimental models can lead to differing responses to LPS, resulting in varying doses that produce toxic effects. Inconsistencies in experimental conditions and protocols, such as cell culture conditions, LPS administration methods, duration of exposure, and endpoints measured, contribute to the discrepancies in reported doses. To address these conflicts,

using consistent methodologies, it is important to carefully evaluate and compare experimental details, standardize protocols, and conduct dose-response studies within individual laboratories. By doing so, we can establish a more consistent understanding of the dose-dependent effects of LPS on cellular viability and ensure accurate interpretations of experimental outcomes.

3.9.2.2. Study design

BV2 cells were seeded in different concentrations in a 96-well plate (with three replicates per concentration). After 24 hours, the complete media was aspirated, and cells were washed with PBS. Different concentrations of LPS (2, 1, 0.5, 0.25, 0.125, 0.01 $\mu\text{g/ml}$) were administered. The 96 well-plate was incubated for 24 hours.

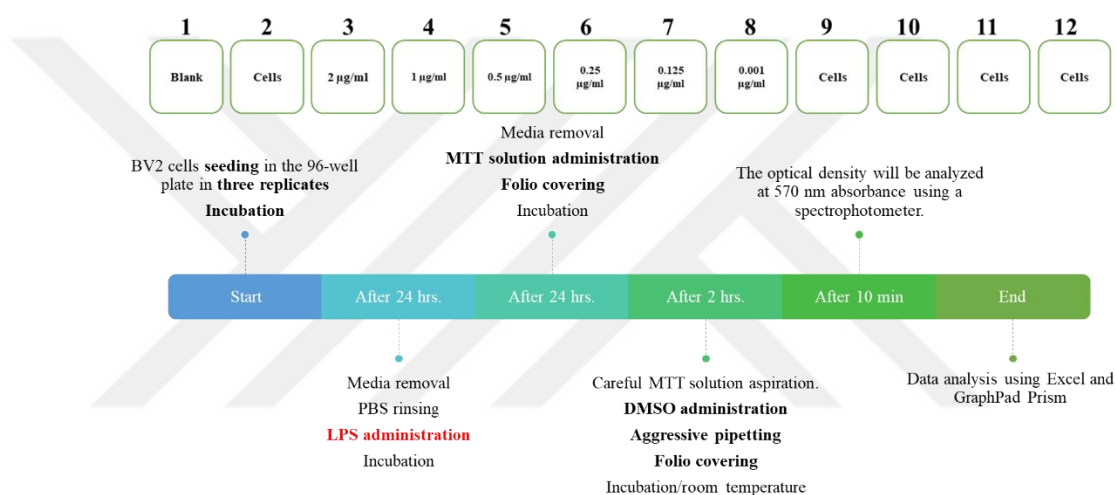


Figure 3.20. MTT Assay protocol for LPS dose determination.

MTT solution was added and incubated for 2 hours, followed by careful aspiration. 100 μl DMSO was added, and aggressive pipetting was performed. The plate was covered with folio and incubated for 10 minutes at room temperature. The results were analyzed at 570 nm absorbance using a spectrophotometer.

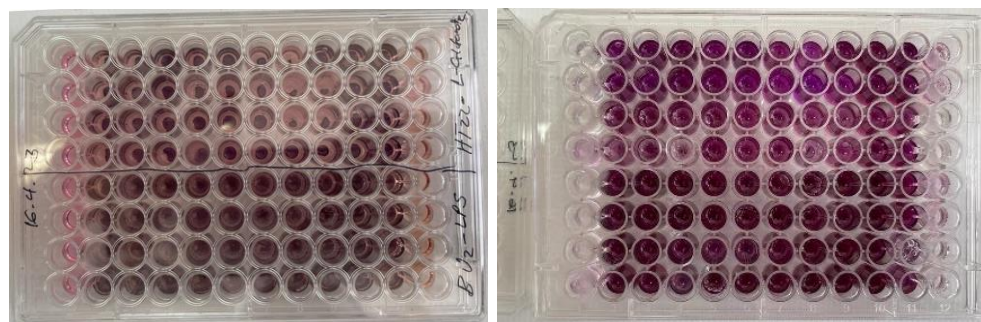


Figure 3.21. MTT Assay at different stages. A: after MTT administration, B after DMSO administration

3.10. Agomelatine effect on BV2 cells subjected to LPS toxicity

The study organized experimental groups (Figure 3.22) to investigate the effects of LPS and agomelatine on BV2 cells. The groups included "Blank" group with fresh media, "Control" group with untreated BV2 cells, and "Toxic" group with BV2 cells exposed to 1 μ g/ml LPS. Additionally, "Treated 1" and "Treated 2" groups had BV2 cells treated with agomelatine post LPS exposure, while "Protection 1" and "Protection 2" groups were controls treated with agomelatine without LPS exposure. "DMSO 1" and "DMSO 2" groups involved BV2 cells treated with different DMSO concentrations. This structure facilitated a systematic study of potential therapeutic strategies.

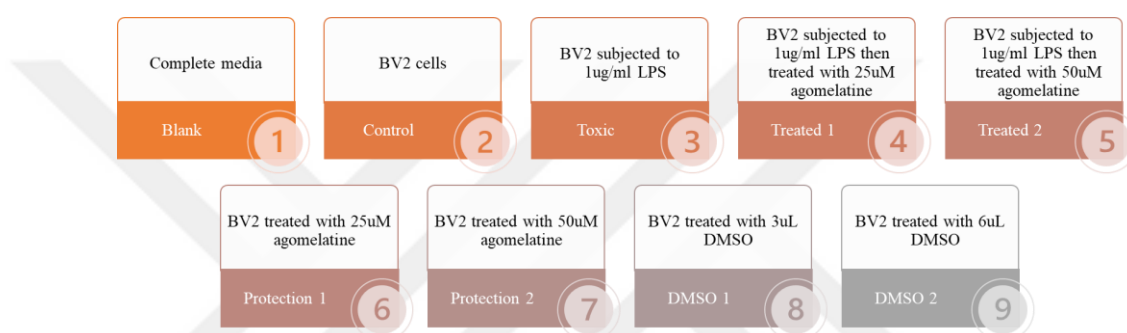


Figure 3.22. The experimental groups.

In this experimental study (Figure 3.22), the following steps were carried out. Initially, 100 μ l of complete media was added to the Blank group of a 96 well-plate. Subsequently, BV2 cells were seeded in 100 μ l of complete media in the second through ninth columns and incubated for 24 hours to allow cell attachment and growth.

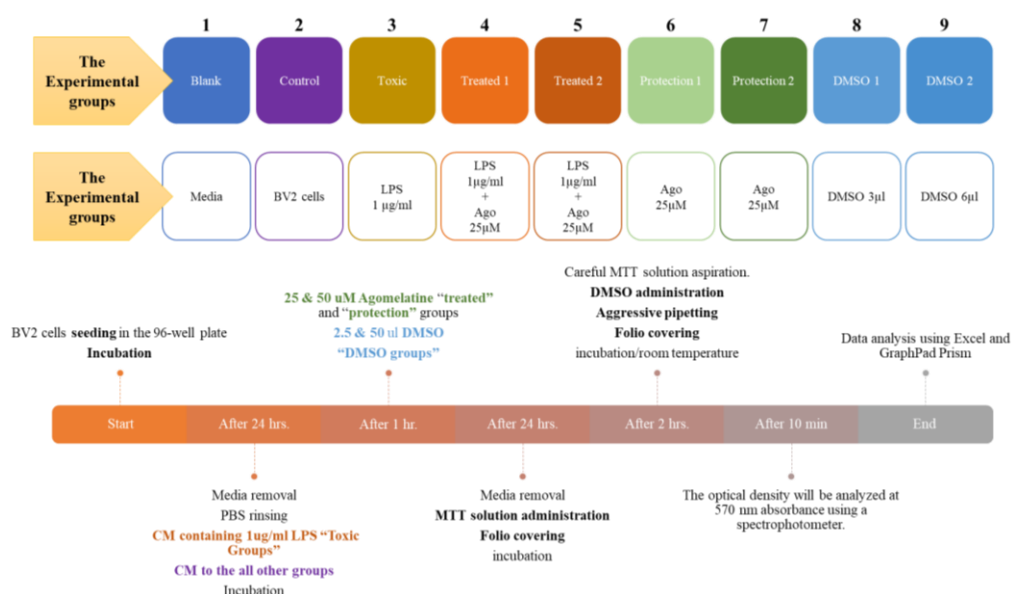


Figure 3.23. MTT assay protocol for examining agomelatine protective effect on BV2 subjected to LPS toxicity.

After the 24-hour incubation period, the media in all groups was aspirated, and an additional 80 μ l of fresh media was added. In the third (Toxic), fourth (Treated 1), and fifth (Treated 2) groups, 20 μ l of LPS at a concentration of 5 μ g/ml was added as a toxic substance to induce cellular stress.

In the first (Blank), second (Control), sixth (Protection 1), seventh (Protection 2), eighth (DMSO 1), and ninth (DMSO 2) groups, 20 μ l of complete media was added, serving as control groups. The 96 well-plate was then incubated for one hour to allow the substances to interact with the cells.

Following the one-hour incubation agomelatine was added. In the fourth (Treated 1) group, 3 μ l of agomelatine 1mM was added as a protective agent to achieve a concentration of 25 μ Mol. In the sixth (Treated 2) group, 6 μ l of agomelatine 1mM was added as a protective agent to achieve a concentration of 50 μ Mol. Similarly, the same doses of agomelatine were added in the (Protection 1) and seventh (Protection 2) groups. In the eighth (DMSO 1) group, 3 μ l of DMSO was added to match the volume of DMSO used to prepare 25 μ M of agomelatine. Similarly, in the ninth (DMSO 2) group, 6 μ l of DMSO was added to match the volume of DMSO used to prepare 25 μ M agomelatine.

The 96 well-plate was then incubated for 24 hours to allow the protective substances to exert their effects. Following this incubation period, the MTT assay assessed cell viability. The results were subsequently analyzed to evaluate the impact of the experimental conditions on the cells.

2.11. Statistical analysis

The data was collected and analyzed using SPSS 26, Excel 365, and GraphPad Prism 9. The means and standard deviation were obtained. A normality test was conducted before data analysis to ensure appropriate statistical analysis. A one-way ANOVA test with a post-hoc Tukey Test was applied, considering a p-value of less than 0.05 as statistically significant. In cases where the data violated normality assumptions, the nonparametric Kruskal-Wallis Test was utilized to explore significant differences among the groups.

4. RESULTS

The study aimed to determine the protective effects of agomelatine in reducing the toxicity of the LPS and preserving the microglial cells. This experiment investigated the immunomodulatory effect of agomelatine BV2 microglial cells. BV2 microglial cell lines were obtained and treated with different doses of agomelatine and LPS to determine the ideal doses for the experiment and construct the ideal design. The viability of the cells was investigated using the MTT assay. Additionally, the expression of genes associated with apoptosis and autophagy was investigated. The mean and standard deviation (SD) were obtained. One-way ANOVA test with a post-hoc Tukey Test was applied to investigate the significance among the groups. The experiment aimed to determine the potential positive effects of agomelatine in reducing the toxicity of LPS and preserving the microglial cells.

4.1. The viability of BV2 cells exposed to different doses of agomelatine

4.1.1. Statistical findings

The experiment's findings demonstrated the effect of different doses on the BV2. With a dose of 0, the control group exhibited a mean value of 100, with a standard deviation equal to 0, serving as the baseline reference for comparison.

Table 4.1. BV2 cell viability subjected to different agomelatine concentrations.

Concentration (μM)	Mean% (SD)
2	108.1% (SD ± 8.18)
4	77.19% (SD ± 1.727)
8	67.36% (SD ± 0.2899)
16	67.59% (SD ± 7.743)
25	65.74% (SD ± 2.114)
50	55.09% (SD ± 2.231)
75	43.59% (SD ± 3.426)
100	25.5% (SD ± 0.8208)
150	5.248% (SD ± 0.5809)
200	2.359% (SD ± 0.7242)

The findings demonstrate a dose-dependent effect on the BV2. Initially, as the dose increased to 2 μM , the mean value exhibited an upward trend. However, beyond a dose of 4 μM , the mean value progressively decreased, indicating an inhibitory effect with higher doses (Figure 4.1).

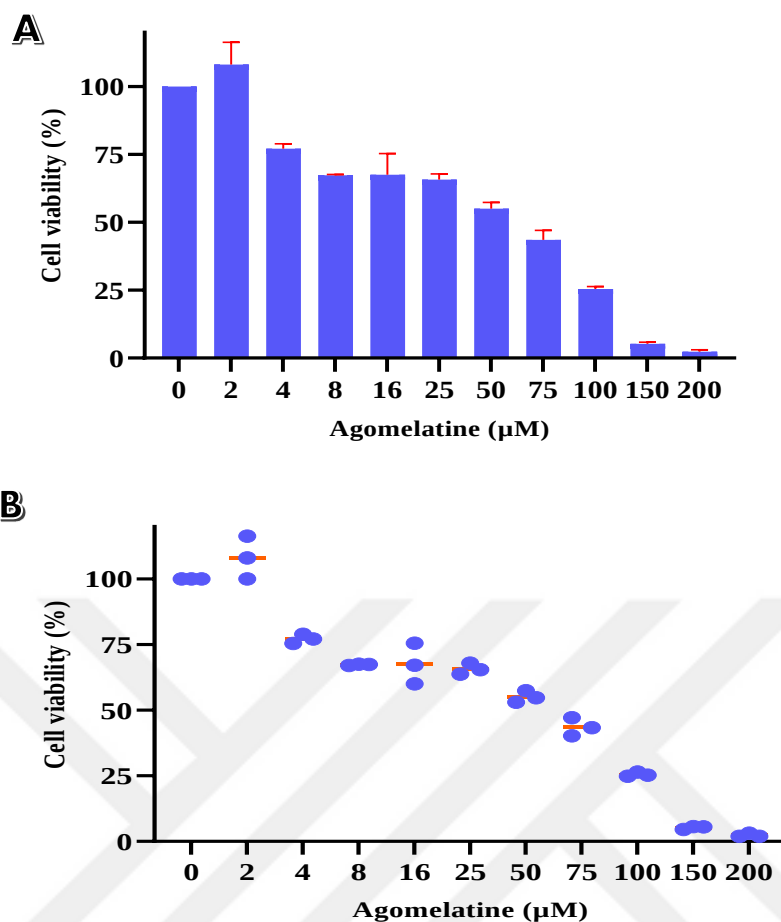


Figure 4.1. Effect of agomelatine on BV2. Cells were treated with different concentrations of agomelatine for 12 hrs. (A) The values presented as mean and SD of BV2 cells viability of three replicate experiments. (B) The values indicate the individual outcome of all three replicates.

The findings revealed that with the administration of the agomelatine at 2μM, the viability increased by 108.1% (SD ±8.18). However, with the increase of agomelatine dose to 4μM, the mean value decreased by 77.19 % (SD ±1.727). This negative trend slightly continued down at doses (8, 16 and 25μM), but the death rate has increased significantly beyond that, with cell viability reaching 55.09% (SD ±2.231) at 50μM (Table 4.1).

The non-linear analysis of the agomelatine effect was conducted, and the inhibition curve showed a significant impact on BV2 cells beyond 25μM. With the increase in agomelatine concentration beyond 100μM, there was an obvious decline in cell viability, while 150μM concentration led to a nearly complete loss to the cells. The results also showed that the agomelatine IC₅₀ was determined to be 36.24 μM (Figure 4.3).

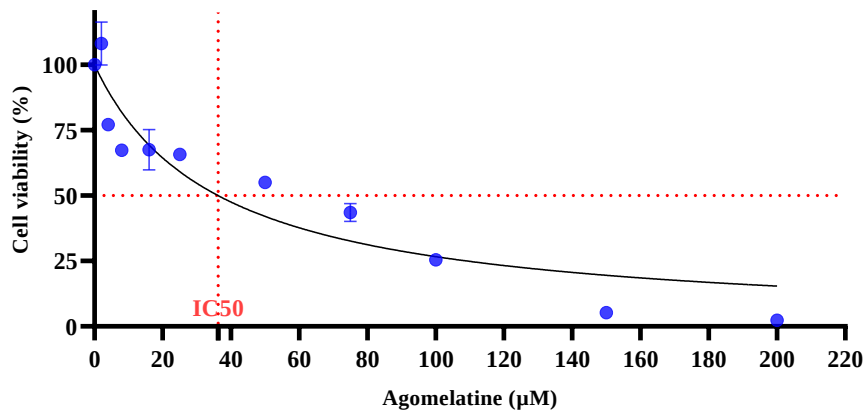


Figure 4.2. Inhibitory cell viability curve of agomelatine on BV2 cells. The cells were treated with agomelatine for a duration of 24 hrs. The curve indicates the gradual decrease of cell viability with increased agomelatine concentration. The IC_{50} was calculated, revealing that 50% of the cells could be lost at agomelatine concentration of 36.24 μ M.

One-way ANOVA was conducted to test the differences between agomelatine concentrations compared to the control group. Our findings revealed that the difference in the cell viability started to show a significant difference in cell viability at 4 μ M. The difference was highly significant in all subsequent groups. When comparing cell viability at 4 μ M and onward doses, the data showed no significant difference until 25 μ M (Figure 4-3).

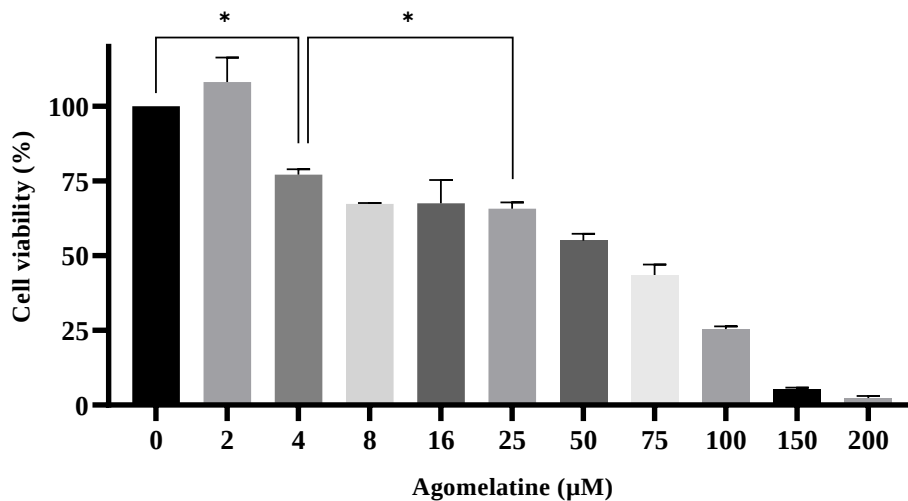


Figure 4.3. A comparative analysis of various agomelatine concentrations on BV2 cells. The agomelatine started to demonstrate an inhibitory effect at 4 μ M concentration where BV2 cells viability significantly decreased ($P < 0.0001$). However, no significant differences were observed when comparing the subsequent doses to the 4 μ M concentration. Notably, a significant effect was observed at a concentration of 25 μ M compared to the 4 μ M concentration ($P = 0.0335$).

4.1.2. Histological findings

Regarding the histological findings, Control Group (no agomelatine): In the control group, histological examination of BV2 cells reveals healthy, normal cellular morphology characterized by intact cell membranes and cytoplasm. The cells exhibit abundant mitochondrial activity, leading to the formation of formazan crystals in the MTT assay. These findings indicate baseline cell viability and metabolic activity, serving as the reference point for assessing the impact of agomelatine (Figure 4.4).

At a low concentration of agomelatine, BV2 cells exhibit mild morphological changes, such as slightly enlarged or elongated cell structures. There is evidence of formazan crystals, although their density is lower than the control group. These histological observations suggest that agomelatine decreased cell viability even at this low concentration (Figure 4.4).

With a moderate concentration of agomelatine, BV2 cells show further changes in morphology, including increased cell size and metabolic activity. The formation of formazan crystals becomes less pronounced, and the density observed is way below the control group. These histological findings indicate a significant decrease in cell viability, suggesting that with the concentration increase, there is a detrimental decrease in cell viability (Figure 4.4).

At a high agomelatine concentration, BV2 cells exhibit pronounced cellular changes, such as loss of cell membrane and cytoplasm integrity. Cells also have lost their attachment, and cell bubbling and fragmentation indicate cells entering apoptosis. The MTT assay reveals very few formazan crystals, indicating low metabolic activity within the cells. Importantly, there is a significant decrease in cell viability compared to the control group, suggesting that high concentrations of agomelatine significantly inhibit BV2 cell viability (Figure 4.4).

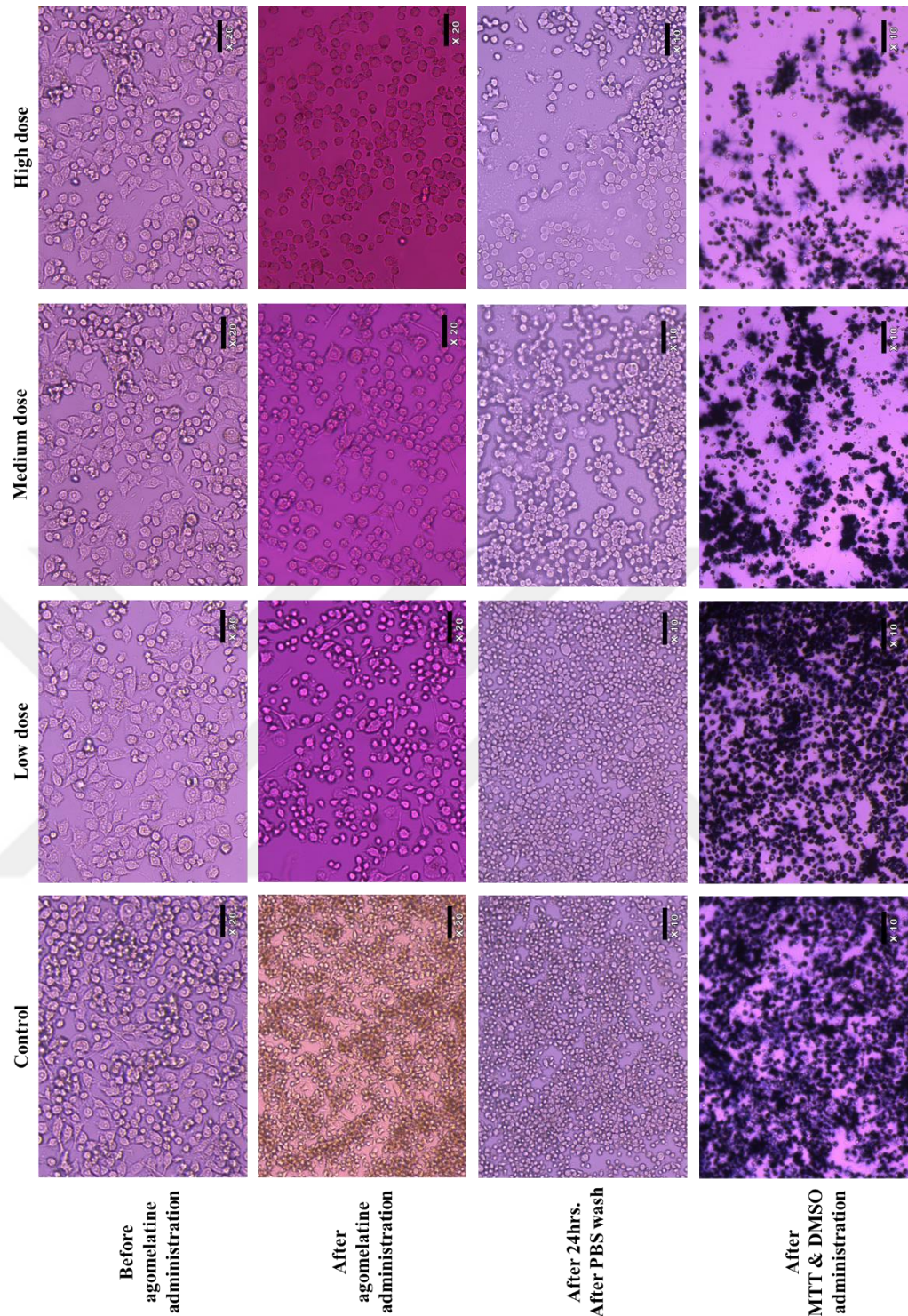


Figure 4.4. Histological findings in BV2 cells exposed to different agomelatine concentrations. In the Control Group, normal cellular morphology with intact membranes and abundant mitochondrial activity, indicating baseline cell viability. At low agomelatine concentration, there were slight morphological changes, decreased formazan crystal density, and a modest decrease in cell viability. Moderate agomelatine concentration increased cell size, reduced formazan crystal formation, and decreased cell viability significantly. In contrast, high agomelatine concentration caused pronounced cellular damage, loss of cell integrity, signs of apoptosis, minimal formazan crystals, and a substantial decrease in cell viability.

4.2. The effect of duration of agomelatine on BV2 cell viability

4.2.1. Statistical findings

The study also investigated the effect of different concentrations of agomelatine on BV2 cell viability at various time points. Three replicates of BV2 cells were seeded in 96-well plates and treated with different concentrations of agomelatine (ranging from 2 μ M to 200 μ M). Cell viability was assessed after 24, 48, and 72 hours of treatment using the MTT assay, and the mean of the results was calculated (Table 4.2).

Table 4.2. Impact of duration of exposure to various agomelatine concentrations on BV2 cells. The drug was administered for 24, 48, and 72 hours.

Concentration	24 hrs. Mean% (SD)	48 hrs. Mean% (SD)	72 hrs. Mean% (SD)
2 μ M	109% (SD $\pm$ 5.67)	90.14% (SD $\pm$ 3.42)	78.12% (SD $\pm$ 1.54)
4 μ M	77.19% (SD $\pm$ 1.41)	73.61% (SD $\pm$ 4.75)	66.41% (SD $\pm$ 3.85)
8 μ M	67.36% (SD $\pm$ 0.24)	71.57% (SD $\pm$ 3.98)	52.16% (SD $\pm$ 3.43)
16 μ M	67.59% (SD $\pm$ 6.32)	62.3% (SD $\pm$ 5.18)	43.65% (SD $\pm$ 2.54)
25 μ M	65.74% (SD $\pm$ 1.73)	53.98% (SD $\pm$ 3.91)	24.94% (SD $\pm$ 9.53)
50 μ M	55.09% (SD $\pm$ 1.82)	43.32% (SD $\pm$ 2.57)	25.26% (SD $\pm$ 2.77)
75 μ M	43.59% (SD $\pm$ 2.8)	27.23% (SD $\pm$ 1.45)	23.53% (SD $\pm$ 1.64)
100 μ M	25.5% (SD $\pm$ 0.67)	14.73% (SD $\pm$ 1.86)	16.14% (SD $\pm$ 2.63)
150 μ M	5.25% (SD $\pm$ 0.47)	4.81% (SD $\pm$ 2.09)	5.58% (SD $\pm$ 1.25)
200 μ M	2.36% (SD $\pm$ 0.59)	1.06% (SD $\pm$ 0.03)	1.72% (SD $\pm$ 0.5)

The results showed that agomelatine expressed a dose-dependent effect on BV2 cell viability across all three durations. At 24 hours, cell viability was slightly increased at the lowest concentration (2 μ M) compared to the control group, which was normalized to 100%. However, at higher concentrations ($\geq$ 4 μ M), cell viability showed a gradual decline in cell viability (Figure 4-5).

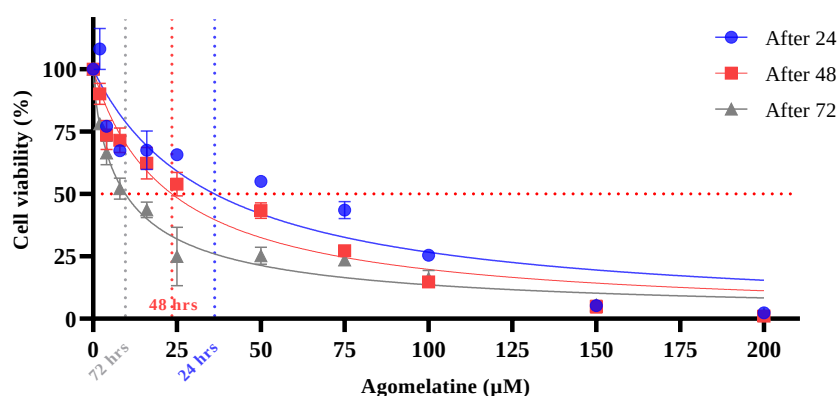


Figure 4.5. The inhibition curve and the IC₅₀ of the impact of duration doses of agomelatine on BV2 cells. The drug was administered for 24, 48, and 72 hours.

When examining the effect of duration, it was observed that the cytotoxic effect of agomelatine became more pronounced over time. At 48 and 72 hours, the decrease in cell viability was more evident, particularly at higher concentrations. Significant differences in cell viability were observed between the control and agomelatine-treated groups at all concentrations and time points ($p < 0.0001$) (Figure 4-6).

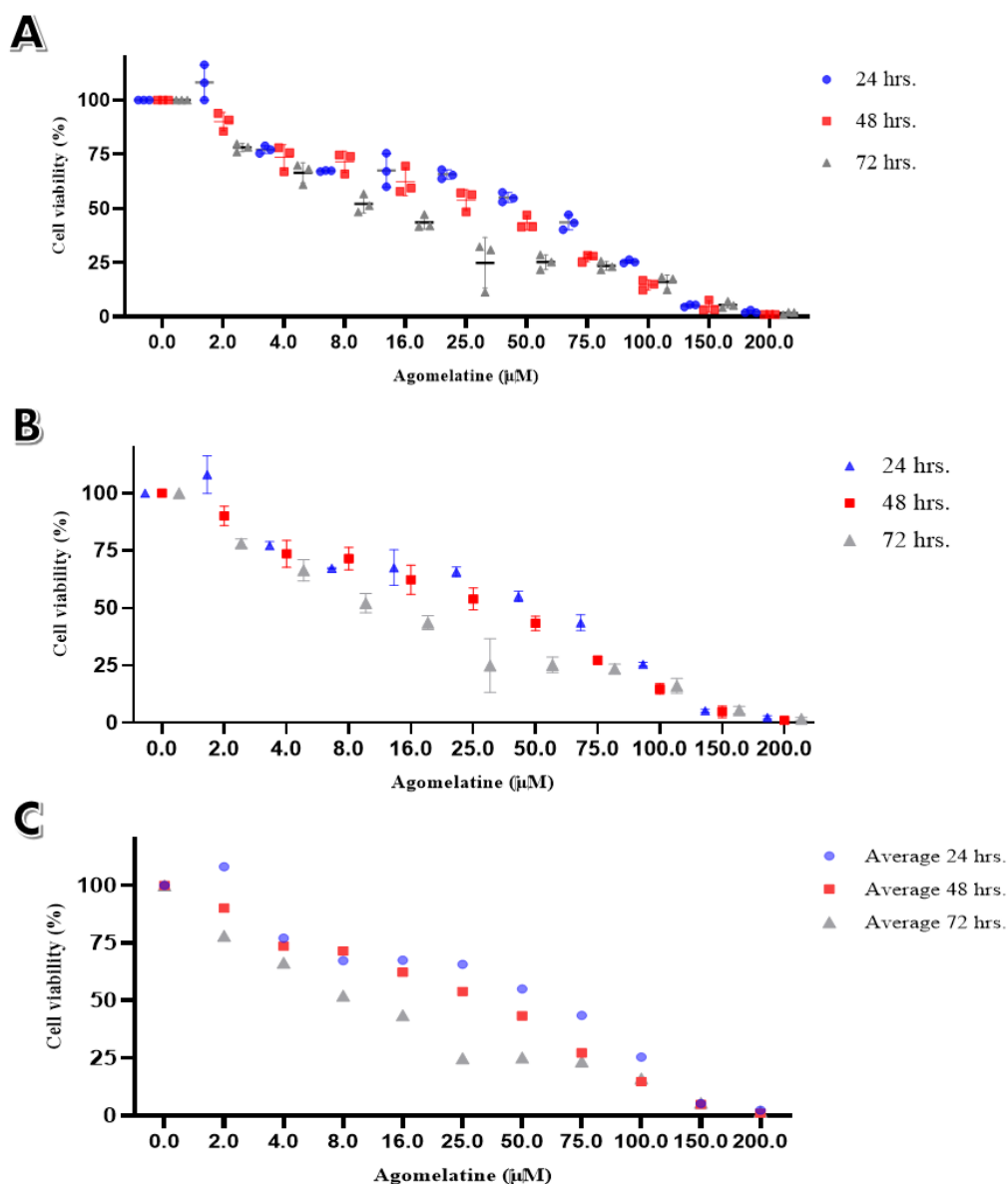


Figure 4.6. Impact of varying doses and duration of exposure of agomelatine on BV2 cells, (A) the detailed values, means and SD per dose and duration, (B) the means and SD of different doses and duration of agomelatine, (C) the effect of duration of exposure of agomelatine per dose.

Furthermore, agomelatine's IC_{50} (the concentration at which 50% of cell viability was inhibited) after 24 hrs. was estimated to be approximately 40μM. While after 48 and 72 hrs., it was approximately around 24 and 8μM, respectively. This value

indicates the potency of agomelatine in reducing BV2 cell viability within the tested timeframe (Figure 4-5).

One-way ANOVA and Tukey's multiple comparison tests revealed significant differences between the control group and all agomelatine concentrations at all three-time points ($p < 0.001$). It indicates that agomelatine significantly impacts BV2 cell viability compared to the control group (Figure 4-8).

Comparing the mean cell viabilities across all three durations for each agomelatine concentration using Tukey's multiple comparisons test, significant differences were observed between the control and all agomelatine doses ($p < 0.05$). These results suggest that agomelatine has a dose-dependent effect on BV2 cell viability regardless of the duration of exposure (Figure 4-8).

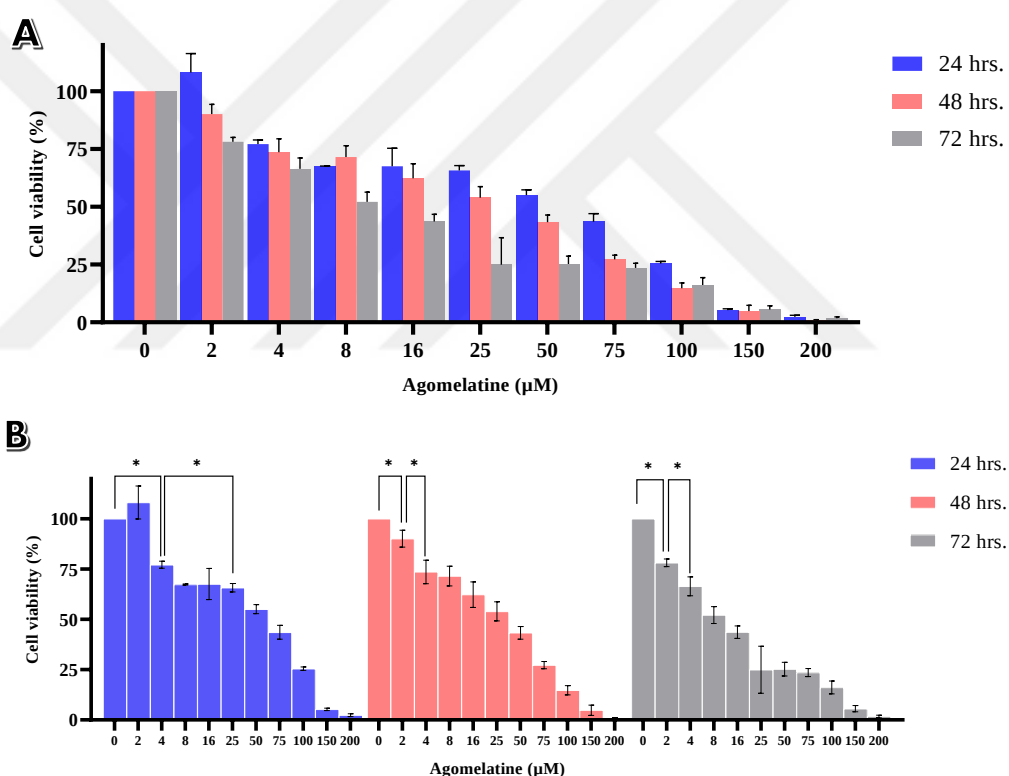


Figure 4.7. A comparative analysis of BV2 cells viability subjected to various agomelatine concentrations and duration of exposure. (A) demonstrate the mean and SD of all concentrations across all durations. (B) demonstrate the impact of the duration where a concentration of 4 μM led to a significant decrease in cell viability. However, the significance was observed at 2 μM after 48 and 72 hours of exposure.

These findings suggest that agomelatine has a dose-dependent cytotoxic effect on BV2 cells, with increasing concentrations leading to a significant decrease in cell viability. Moreover, the duration of exposure to agomelatine accentuates its

cytotoxicity. These results highlight the importance of considering the dose and duration of agomelatine treatment when assessing its impact on BV2 cells.

4.2.2. Histological findings

Regarding the histological findings, Histological examination of BV2 cells subjected to a range of agomelatine concentrations after 24 hrs. and of agomelatine administration revealed distinct effects. The control group showed normal cellular morphology with intact membranes, high mitochondrial activity, and abundant formazan crystals, indicating baseline cell viability. At low agomelatine concentrations, mild morphological changes and reduced formazan crystal density hinted at decreased cell viability. Moderate concentrations increased cell size and decreased formazan crystal formation, thus decreasing cell viability. High concentrations resulted in severe cellular damage, loss of integrity, signs of apoptosis, minimal formazan crystals, and a substantial decrease in cell viability (Figure 4-8).

Over 48 hours of agomelatine exposure, BV2 cells displayed an aggravation of the observed effects. The control group maintained normal cellular morphology, although a slight decrease in mitochondrial activity and formazan crystal density was noted. At low agomelatine concentrations, the previously observed mild morphological changes intensified, accompanied by a further reduction in formazan crystals, indicating a continued decrease in cell viability. Moderate concentrations led to more pronounced changes in cell size and metabolic activity, with significantly fewer formazan crystals, further corroborating the decrease in cell viability. At high concentrations, cellular damage worsened, characterized by increasing membrane and cytoplasm integrity loss, detachment, and evident signs of apoptosis. Very few formazan crystals were observed, underscoring the substantial decrease in cell viability (Figure 4-8).

After 72 hours of agomelatine treatment, the effects on BV2 cells continued to intensify. Cellular morphology remained relatively normal in the control group, but a noticeable decrease in mitochondrial activity and formazan crystal density indicated a prolonged exposure effect. Low agomelatine concentrations further exacerbated the morphological changes, with significantly diminished formazan crystals, emphasizing the persistent decrease in cell viability. Moderate concentrations resulted in a more substantial increase in cell size, reduced formazan crystal formation, and a pronounced decrease in cell viability. High concentrations induced severe cellular damage, with

extensive membrane and cytoplasm integrity loss, detachment, and pronounced signs of apoptosis. Minimal formazan crystals and a marked decrease in cell viability were evident (Figure 4-8).

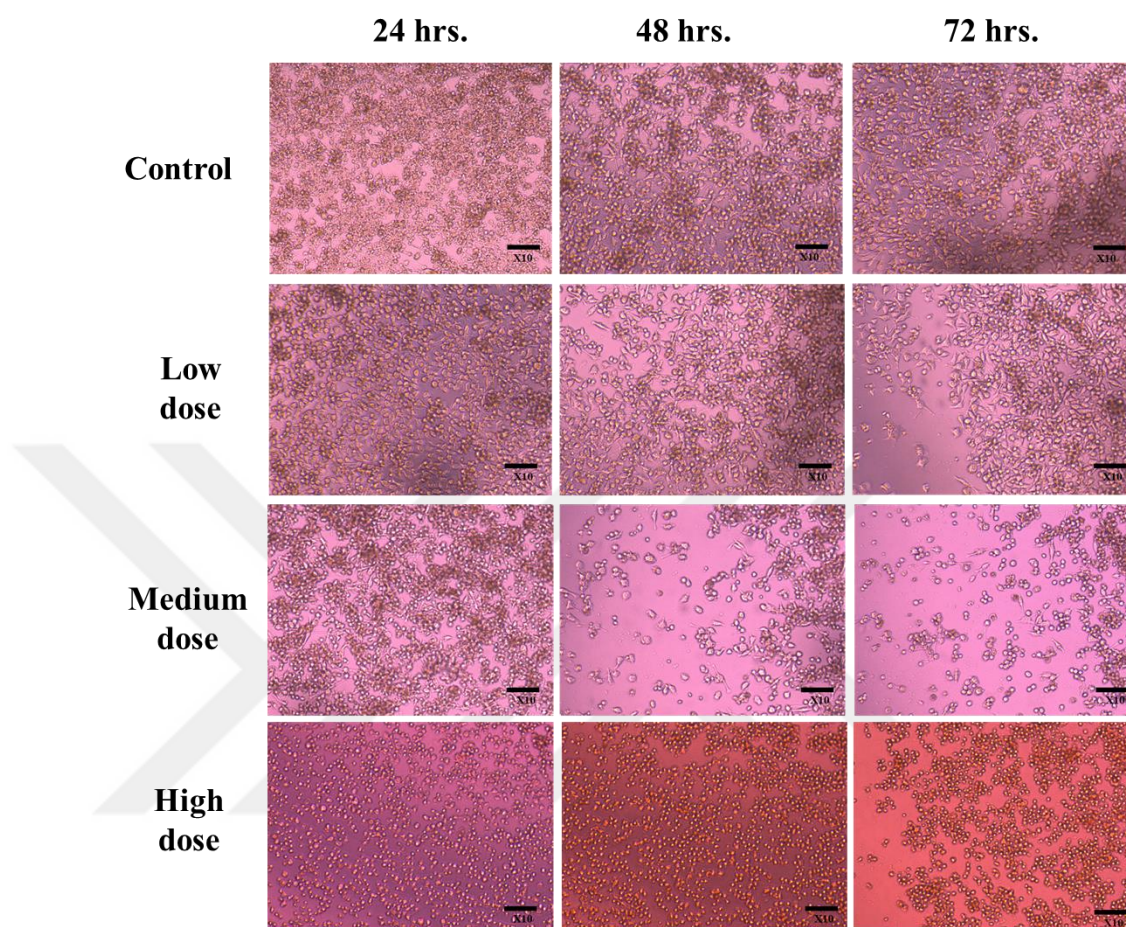


Figure 4.8. Histological findings in BV2 cells exposed to varying agomelatine concentrations for 24, 48, and 72 hrs. Increasing agomelatine concentration led to a dose-dependent decrease in cell viability. Moreover, with prolonged exposure, cell viability continued to decline, indicating the time-dependent detrimental effects of agomelatine on BV2 cells.

4.3. The viability of BV2 cells subjected to different doses of LPS

4.3.1. Statistical findings

BV2 cells were exposed to different concentrations of LPS (ranging from 0.001 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$). Cell viability was assessed using the MTT assay. One-way ANOVA and Tukey's multiple comparisons test were performed to test the groups' differences. The aim was to investigate the toxic effect of LPS on BV2 cells and to determine the appropriate toxic dose for the main experiment.

The results revealed a clear dose-dependent toxic effect of LPS on BV2 cells. At the lowest tested concentration of 0.001 $\mu\text{g/ml}$, the cell viability was 95.14% (SD $\pm$

2.245), which did not significantly differ from the control group ($p > 0.05$). Similarly, at 0.125 $\mu\text{g/ml}$, the cell viability was 91.09% (SD $\pm$ 2.271), indicating no significant difference compared to the control ($p > 0.05$) (Table 4-3).

Table 4.3. Cell viability of different LPS concentrations on BV2 cells.

Drug concentration	Mean% (SD)
0.001 $\mu\text{g/ml}$	95.14% (SD $\pm$ 2.245)
0.125 $\mu\text{g/ml}$	91.09% (SD $\pm$ 2.271)
0.25 $\mu\text{g/ml}$	79.59% (SD $\pm$ 7.362)
0.5 $\mu\text{g/ml}$	73.86% (SD $\pm$ 4.825)
1 $\mu\text{g/ml}$	49.18% (SD $\pm$ 14.01)
2 $\mu\text{g/ml}$	2.69% (SD $\pm$ 0.46)

However, as the LPS concentration increased, a notable decrease in cell viability was observed. At 0.25 $\mu\text{g/ml}$, the cell viability decreased to 79.59% (SD $\pm$ 7.362), demonstrating a moderate reduction compared to the control group ($p < 0.05$). Increasing the LPS concentration further to 0.5 $\mu\text{g/ml}$ resulted in a more significant reduction in cell viability, with a mean of 73.86% (SD $\pm$ 4.825) (Table 4-3), showing a highly significant difference from the control ($p < 0.001$) (Figure 4-9 & 10).

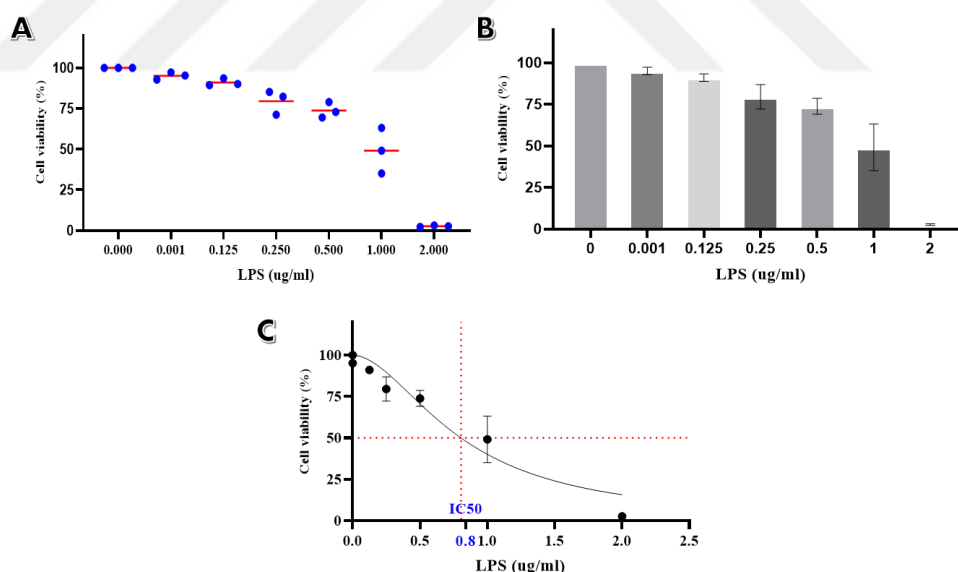


Figure 4.9. Impact of varying doses of LPS on BV2 cells.(A) demonstrates the values per concentration indicate the individual outcome and the mean of all three replicates (B) demonstrates the mean and SD of different doses of LPS administered to BV2 cells (C) shows the inhibition curve of LPS on BV2 cells after 24 hrs. administration. The results indicate that the IC_{50} was determined to be 0.801 $\mu\text{g/ml}$.

Significant cytotoxicity was observed at higher LPS concentrations. At 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$, the cell viabilities were 49.18% (SD $\pm$ 14.01) and 2.69% (SD $\pm$ 0.46),

respectively. These concentrations exhibited highly significant differences compared to the control ($p < 0.001$). These findings indicate that LPS concentrations above 0.5 $\mu\text{g/ml}$ induce a potent toxic effect on BV2 cells (Figure 4-10).

Nonlinear fit analysis of the data revealed that the IC_{50} value of LPS was determined to be 0.801 $\mu\text{g/ml}$, which provides insights into the sensitivity of BV2 cells to LPS-induced toxicity (Figure 4-10).

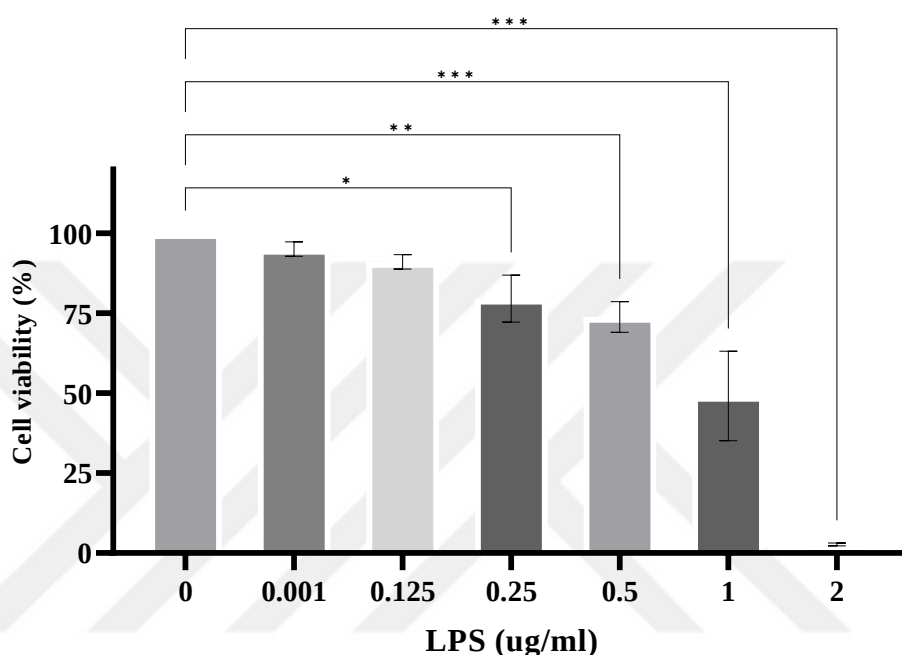


Figure 4.10. Multiple group comparison analysis shows the dose-dependent effect of LPS. LPS was applied at different doses, and the viability of the doses was compared to the control group. It showed very high significant effect at 0.5 $\mu\text{g/ml}$ ($P < .001$) and the subsequent groups.

4.3.2. Histological findings

Regarding the histological findings, in the control group, histological examination of BV2 cells reveals healthy, normal cellular morphology characterized by intact cell membranes and cytoplasm. The cells exhibit abundant mitochondrial activity, leading to the formation of formazan crystals in the MTT assay. These findings indicate baseline cell viability and metabolic activity, serving as the reference point for assessing the impact of LPS.

At a very low concentration of LPS (0.001 and 0.125 $\mu\text{g/ml}$), BV2 cells showed mild changes. However, the cells also showed high mitochondrial activity, as indicated by formazan crystals' abundant formation. With increased LPS doses (0.25 $\mu\text{g/ml}$), BV2 cells exhibit more morphological changes. Nevertheless, there was evidence of

formazan crystals, although their density was lower than the control group. These histological observations suggest that even at this low LPS concentration, there are initial signs of decreased cell viability.

At the concentration of 1 ug/ml LPS, BV2 cells show further changes in morphology, including increased cell size, changes to the cell membrane, and nucleus integrity. The formation of formazan crystals becomes less pronounced, and the density observed is significantly below the control group, indicating a significant decrease in cell viability.

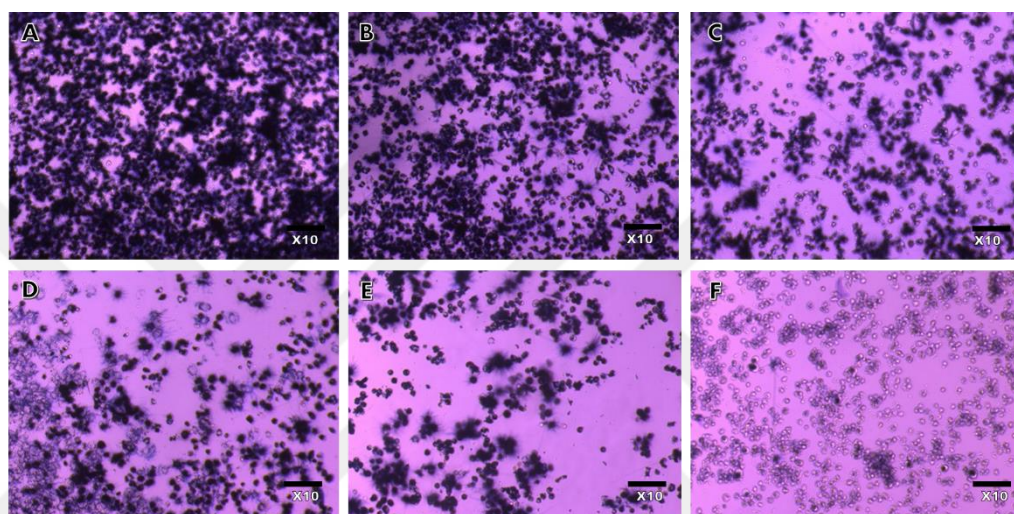


Figure 4.11. Histological findings of BV2 cells subjected to varying LPS concentrations. At very low LPS levels (B & C), mild morphological changes were observed alongside high mitochondrial activity indicated by abundant formazan crystal formation. With the increase in dose concentration (D), additional morphological changes were evident, though formazan crystal density remained reduced compared to the control group (A). Notably, at 1 ug/ml LPS, BV2 cells exhibited altered cell size, cell membrane and nucleus integrity, accompanied by significantly reduced formazan crystal formation, indicating a substantial decrease in cell viability. At the highest LPS concentration (2 ug/ml), BV2 cells displayed high cellular alterations, including membrane and cytoplasm integrity loss, detachment, bubbling, and fragmentation, suggesting apoptosis. The MTT assay indicated minimal formazan crystal formation and a marked decrease in cell viability compared to the control group.

4.4. The Agomelatine effect on BV2 cells subjected to LPS toxicity

4.4.1. Statistical findings

This study aimed to assess the effect of agomelatine on the immunomodulatory properties of the BV2 cells subjected to LPS toxicity. The viability of BV2 cells was assessed using the MTT assay, and the obtained results were analyzed using one-way ANOVA and Tukey's multiple comparisons test. The results were presented as mean viability percentage and SD of all experimental groups.

Table 4.4. Cell viability of BV2 cells treated with different substances.

Group	Substances	Mean% (SD)
Control	Control	100% (SD $\pm$ 0)
Toxic	LPS 1 ug/ml	25.84% (SD $\pm$ 3.051)
Treated 1	LPS 1 ug/ml + Ago 25 μ M	44.35% (SD $\pm$ 4.875)
Treated 2	LPS 1 ug/ml + Ago 50 μ M	43.07% (SD $\pm$ 6.607)
Protection 1	Ago 25 μ M	65.74% (SD $\pm$ 2.114)
Protection 2	Ago 50 μ M	55.09% (SD $\pm$ 2.231)
DMSO 1	DMSO 2.5 μ L	38.89% (SD $\pm$ 3.534)
DMSO 2	DMSO 5 μ L	33.6% (SD $\pm$ 4.785)

The experiment results demonstrated that the presence of LPS -which represented the toxic group- at a concentration of 1 ug/ml significantly decreased the viability of BV2 cells compared to the control group. Moreover, adding agomelatine at both 25 μ M and 50 μ M has effectively mitigated the toxic effects of LPS, as indicated by significantly higher cell viability than the LPS-treated (toxic) group. The protective effect of agomelatine was dose-dependent, with no significant difference observed between the two agomelatine concentrations.

Furthermore, the groups treated with DMSO alone showed lower viabilities than the agomelatine (protective) groups, suggesting a potential toxic effect of DMSO. However, it is important to note that DMSO might impact cell viability.

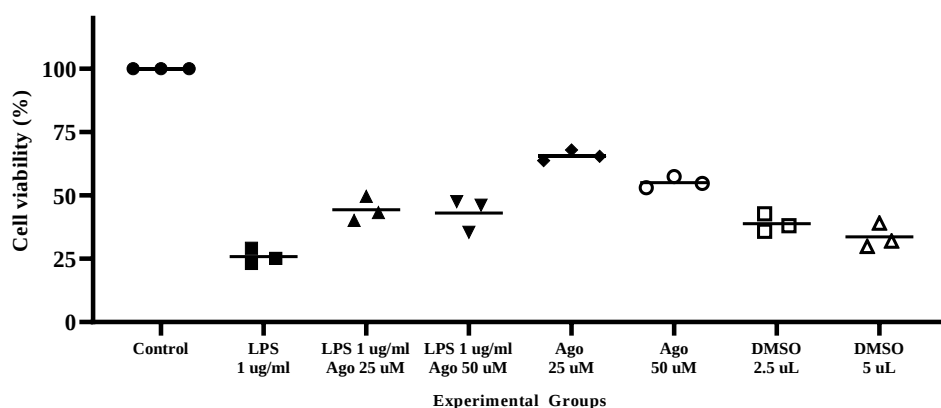


Figure 4.12. Cell viability of BV2 cells treated with different substances based on the experimental design.

One-way ANOVA analysis revealed a significant difference in BV2 cell viability among the experimental groups ($p < 0.05$). Tukey's multiple comparisons test was then conducted to examine the pairwise differences between all groups.

Table 4.5. Tukey's multiple comparisons test of the experimental groups.

Experimental Groups	Significance
Control vs. Toxic	< 0.001***
Control vs. Protection 1	< 0.001***
Control vs. Protection 2	< 0.001***
Toxic vs. Treated 1	< 0.001***
Toxic vs. Treated 2	0.001**
Treated 1 vs. Treated 2	> 0.999
Protection 1 vs. Protection 2	0.062
Protection 1 vs. DMSO 1	< 0.001***
Protection 2 vs. DMSO 2	< 0.001***
DMSO 1 vs. DMSO 2	0.711

* indicates significance at 0.05, ** indicates significance at 0.01, *** indicates significance at 0.001.

The multiple comparisons of the experimental doses on BV2 cells revealed significant differences in cell viability among the various groups. Compared to the control group, the LPS (Toxic) group showed a significantly lower viability ($p < 0.001$). Thus, the presence of LPS at a concentration of 1 ug/ml had a detrimental effect on BV2 cell viability and affirmed the desired effect.

However, the addition of agomelatine at both concentrations 25µM and 50 µM (low-dose and high-dose treated groups) against LPS toxicity resulted in significantly

higher cell viability compared to the toxic group ($p < 0.001$). It suggests that agomelatine has effectively mitigated the toxic effects of LPS on BV2 cells at both concentrations. The protective effect of agomelatine was dose-dependent, with no significant difference observed between the two agomelatine concentrations ($p > 0.05$).

Although agomelatine at both protective groups (25 μ M and 50 μ M) also exhibited significantly higher cell viability than the LPS (toxic) group ($p < 0.001$), it also held a possible toxic effect on the viability of BV2 cells when compared to the control group.

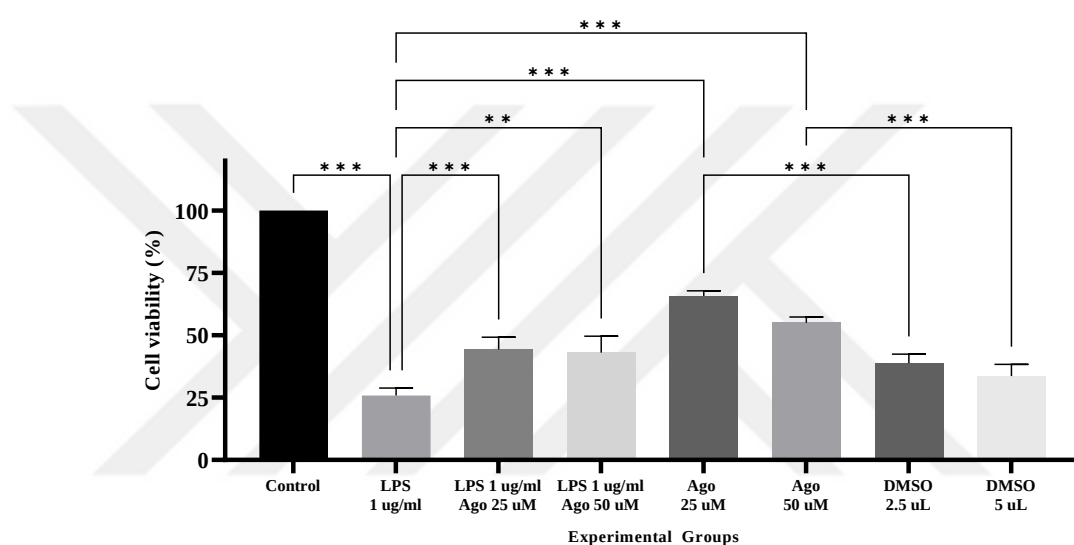


Figure 4.13. Multiple group comparison analysis of the cell viability of BV2 cells treated with different substances based on the experimental design. *: indicate $P < 0.05$, **: indicate $P < 0.01$, ***: indicate $P < 0.001$.

Finally, BV2 cell viability in both DMSO groups (2.5 μ L and 5 μ L) groups showed significantly lower cell viability than in the control group ($p < 0.001$), as well as the protective groups of the same doses, indicating its potential toxic effect. It is important to note that although DMSO showed a toxic effect, the literature has shown that it is recommended not to use it beyond 1%. So, it may have produced this impact on cell viability.

4.4.2. Histological findings

Regarding the histological findings, in the control group, histological examination of BV2 cells reveals a healthy, normal cellular morphology characterized by intact cell membranes and cytoplasm. The cells exhibit abundant mitochondrial activity, leading to the formation of formazan crystals in the MTT assay. These

findings indicate baseline cell viability and metabolic activity, serving as the reference point for assessing the impact of various treatments.

In the toxic group exposed to LPS at a concentration of 1 ug/ml for 24 hours, BV2 cells displayed significant cellular alterations. These changes included decreased cell size, compromised cell membrane integrity, and nucleus abnormalities. Formazan crystal formation in this group became notably less pronounced, with a density significantly below that of the control group, indicating a substantial decrease in cell viability.

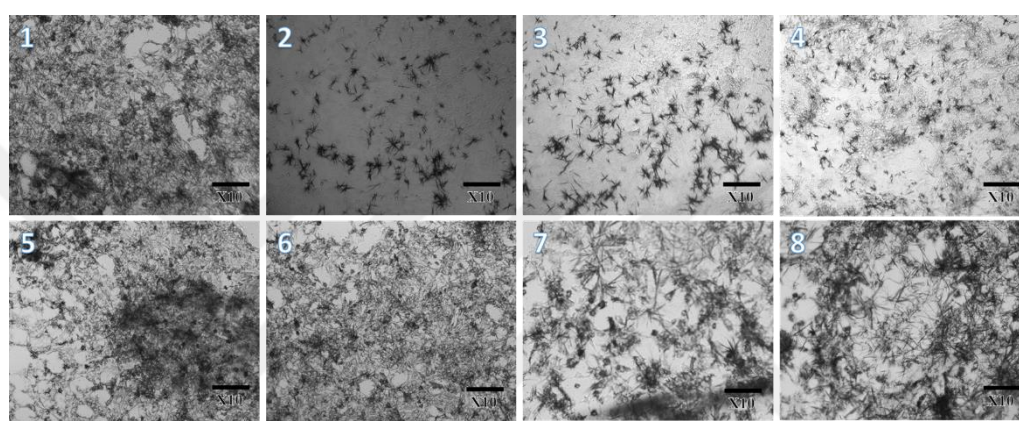


Figure 4.14. Histological findings of BV2 cells under various experimental conditions. It shows the difference in formazan crystal formation among all experimental groups. The control group (1) exhibited very high formazan crystal formation, while the toxic group subjected to LPS toxicity (2) showed significant cellular alterations and very low formazan crystal formation. Treated 1 and 2 groups (3) and (4), exposed to LPS and treated with agomelatine, displayed partial recovery of cell viability indicated by higher formazan crystal formation than the toxic group. Protection 1 and 2 groups (5) and (6) treated with agomelatine alone exhibited lower formazan crystal formation than the control group indicating deteriorated cell morphology. DMSO groups one (7) and two (8), exposed to DMSO, demonstrated lower cell viability indicated by lower formazan crystal formation than the protection groups.

In treated 1 group, BV2 cells were initially exposed to LPS at 1 ug/ml for 1 hour, followed by treatment with agomelatine at a concentration of 25 μ M for 24 hours. Histological examination revealed cellular improvements compared to the toxic group, including increased cell size, a more intact cell membrane, and less severe nucleus abnormalities. While reduced compared to the control group, Formazan crystal formation was significantly higher than in the toxic group, indicating a partial recovery of cell viability.

Treated 2 group followed a similar protocol as treated 1 group, with BV2 cells exposed to LPS for 1 hour before receiving agomelatine treatment at a higher concentration of 50 μ M for 24 hours. Histological findings in this group demonstrated cellular improvements similar to treated 1 group, including increased cell size, improved cell membrane integrity, and fewer nucleus abnormalities. Though reduced compared to the control group, Formazan crystal formation was significantly higher than in the toxic group, indicating a partial recovery of cell viability.

In protective 1 group, BV2 cells were treated with agomelatine alone at a concentration of 25 μ M for 24 hours. Histological examination revealed cellular improvements compared to the toxic group, including increased cell size, improved cell membrane integrity, and fewer nucleus abnormalities. While reduced compared to the control group, Formazan crystal formation was notably higher than in the toxic group, indicating a protective effect of agomelatine.

Protection 2 group underwent a similar treatment regimen as protection 1 group but with a higher agomelatine concentration of 50 μ M for 24 hours. Histological findings in this group showed more cellular changes and detachment than protection 1 group, indicating a higher inhibitory effect of agomelatine, albeit with a higher concentration.

In DMSO group one, BV2 cells were exposed to 3 μ L of DMSO used in preparing the agomelatine 25 μ M concentration for 24 hours. Histological examination revealed lower cell viability than in the agomelatine protective groups, suggesting a potential toxic effect of DMSO on cell viability.

DMSO group two followed a protocol similar to DMSO group one, but BV2 cells were exposed to a higher dose of DMSO 6 μ L used to prepare the agomelatine 50 μ M concentration for 24 hours. Histological findings indicated significantly lower cell viability than in the control and agomelatine protection groups, further suggesting a potential toxic effect of DMSO on cell viability.

5. DISCUSSION

In the context of injury and neuroinflammation, BV2 cells, besides their similarity to primary microglia, play a significant role in CNS injury and neuroinflammatory processes. They actively regulate immune responses, contribute to tissue repair, and secrete factors that can influence neuronal survival and synaptic plasticity (Anna and Guy, 2001; Pocock and Kettenmann, 2007). So, they represent a good model and line of treatment in neurodegenerative diseases.

However, there are discrepancies in the literature regarding the optimal dose of agomelatine used in *in vitro* studies. Hong et al. (2021) used two doses 10 and 20 μ M, for their cell viability experiment. Similarly (Hyeon et al., 2017) and (Sharma and Singh, 2022) used doses ranging between (100 to 600 μ M) and (1 to 200 nM) for their experiment, respectively. Jia et al. (2019) examined the effects of agomelatine duration of exposure for doses ranging between 10 to 500 μ M. These variations highlight the need for experimental studies to determine the ideal doses that accurately represent the study model.

5.1. Effect of agomelatine doses on BV2 cell viability

Agomelatine, as a melatonergic antidepressant, has shown promising effects on various cellular processes. Many studies in the literature were conducted on BV2 cells, highlighting the variations of agomelatine doses and recommending the need for experimental studies to determine the ideal doses that accurately represent the study model.

My findings revealed a dose-dependent relationship, where lower doses (up to 2 μ M) of agomelatine exhibited increased cell viability. This finding suggests a potential positive effect of agomelatine in preserving the viability of BV2 cells. However, as the dose exceeded 4 μ M, the cell viability started to decline, indicating an inhibitory effect of agomelatine.

Several previous studies have observed the dose-dependent effects of agomelatine on cell viability. Researchers have reported varying outcomes depending on the dose used. For instance, Cankara et al. (2021) investigated the effects of agomelatine at different doses ranging from 1 to 16 μ M. They found that the 4 μ M dose and below showed a moderate increase in cell viability, while 8 μ M and 16 μ M exhibited a more pronounced improvement (Cankara et al., 2021). On the contrary, our finding revealed that this dose produced a mild toxic effect on the cells.

Similarly, de Carnevale Galetti et al. (2021) conducted a study using agomelatine in various doses. They observed a dose-dependent response, with the IC₅₀ at 0.3mM dose demonstrating the most significant increase in cell viability compared to the higher doses (de Carnevale Galetti et al., 2021). These findings did not align with our findings, which indicated that doses higher than 150µM produced significant cell death.

In another study conducted by (Moreno-SanJuan et al., 2023), who investigated a range of doses, the study found that 0.1 mM dose showed significance in cell viability while the higher doses decreased the rate of cell viability enhancement.

Furthermore, the impact of agomelatine duration on BV2 cell viability was also investigated. The experimental findings showed that cell viability decreased over time for most doses, suggesting a potential time-dependent effect of agomelatine. This observation is consistent with a study by Jia et al. (2019), which reported a significant effect of agomelatine on cell viability after 48 hours of exposure. However, the decrease in viability varied across different doses and durations, indicating complex interactions between agomelatine, treatment duration, and cell viability.

Moreno-SanJuan et al. (2023) also investigated the effect of the duration of agomelatine exposure and observed a significant decrease in cell viability over time (Moreno-SanJuan et al., 2023). These findings support my results in which the dose and duration significantly affect cell viability. However, agomelatine at 100µM revealed a toxic effect of its own.

These doses and duration-dependent effects of agomelatine on BV2 cell viability emphasize the need for careful consideration when designing experiments and interpreting results. Optimizing experimental conditions, including dose and duration, is necessary to ensure the findings' relevance and reliability. Therefore, it is important to determine the optimal dose and duration of agomelatine treatment to achieve the desired therapeutic effects while minimizing any potential adverse effects on cell viability.

5.2. Effect of agomelatine duration on BV2 cell viability

Understanding the impact of agomelatine duration of administration on BV2 cell viability is vital for clarifying the dynamics of its effects. The study aimed to explore the relationship between agomelatine duration of exposure and BV2 cell viability and shed light on this drug's time-dependent effects.

The study examined the effect of agomelatine on BV2 cells at different durations of exposure and how treatment duration influenced cell viability. The results indicated that cell viability decreased over time for most agomelatine doses. This observation suggests a potential time-dependent effect, where prolonged exposure to agomelatine may harm BV2 cell viability.

These results align with the findings of Jia et al. (2019), who reported a significant effect of agomelatine on cell viability after 48 hours of exposure. The time-dependent effects of agomelatine on cell viability may be attributed to various factors, such as cumulative drug toxicity, alterations in cellular homeostasis, or the activation of specific signalling pathways. Further research is needed to unravel the underlying mechanisms driving these temporal dynamics.

It is important to note that cell viability decreased across different agomelatine doses and treatment durations. It suggests that the relationship between agomelatine duration of exposure and BV2 cell viability is complex and influenced by multiple factors.

5.3. LPS toxicity and BV2

The use of LPS as a toxic substance in cell cultures has been extensively studied concerning their impact on cell viability and various cellular activities. However, there are discrepancies in the literature regarding the optimal concentrations of LPS used for experimental purposes. Studies have examined a wide range of LPS concentrations, demonstrating the need for dose determination to accurately evaluate their effects and establish optimal experimental or clinical conditions.

Several studies have explored the effects of LPS at various concentrations and durations. For instance, Hyeon et al. (2017) investigated LPS effects on cell viability using a 10 µg/ml concentration. Kitchens et al. (1992) observed significant impacts on cellular functions within the 1 to 10 ng/ml concentration range. Cario et al. (2000) examined LPS effects at 5 µg/ml and 10 µg/ml concentrations, revealing significant effects on cell viability. Similarly, Y. Yang et al. (2007) conducted a comprehensive study investigating the effects of LPS at various concentrations (10 and 100 ng/ml, as well as 1 and 10 µg/ml) and durations (15, 30, 60, and 120 minutes, as well as 24 hours), demonstrating its significant effects on cell viability and the need for exploring them.

Böcker et al. (2003) investigated the LPS effect using concentrations ranging from 0.001 to 10 µg/ml and various durations. They observed significant effects on cell viability under these conditions. Katsuyama et al. (1998) examined the effects of LPS at concentrations ranging from 1 to 1000 ng/ml and found a significant effect at a concentration of 1 µg/ml. McKinney et al. (1998) also studied LPS at concentrations ranging from 1 to 1000 ng/ml and reported a significant effect at a 1 µg/ml concentration. Furthermore, Wittwer et al. (1997) observed a significant increase in lysosomal activity when using a concentration of 1 mg/ml of LPS for various durations. Kawamata et al. (2000) investigated the effects of LPS at a concentration of 1 µg/ml for different durations and reported a significant increase in nitrate production during the study period.

In light of these discrepancies in the literature, I conducted a dosage determination experiment to identify the appropriate dose of LPS for generating a neurodegenerative model using BV2 cells. Based on my findings, the selected dose for LPS in the main experiment was 1 µg/ml.

My results and the literature findings highlight the need to determine optimal LPS concentrations to better understand their effects on cell viability and various cellular activities. The wide range of concentrations used in previous studies demonstrates the necessity of evaluating the impact of different LPS doses on cell viability and other cellular functions. Therefore, determining the appropriate LPS doses is critical for accurately assessing its effects and establishing optimal conditions for experimental or clinical applications.

5.4. The protective effect of agomelatine against LPS toxicity on BV2 cells

This experimental study aimed to investigate the impact of different substances on the viability of BV2 cells. The study's results revealed several important findings that shed light on the effects of these substances on BV2 cell viability. Firstly, the presence of LPS at a concentration of 1 µg/ml significantly decreased the viability of BV2 cells compared to the control group. This observation indicates that LPS harms BV2 cell viability, confirming its known pro-inflammatory and cytotoxic properties. The decrease in cell viability suggests that LPS-induced inflammation and cellular stress pathways contribute to cytotoxicity.

To explore potential protective effects, agomelatine was added to the LPS-treated groups at 25 µM and 50 µM concentrations. The results demonstrated that

agomelatine effectively mitigated the toxic effects of LPS on BV2 cells. Agomelatine concentrations significantly increased cell viability compared to the LPS group, indicating the ability of agomelatine to attenuate LPS-induced cytotoxicity. Importantly, there was no significant difference in the protective effect between the two concentrations of agomelatine. However, there is still a dose-dependent effect of agomelatine within the tested range.

It has been previously reported that agomelatine possesses anti-inflammatory and neuroprotective properties. The current study's findings provide further evidence for the potential therapeutic value of agomelatine in protecting BV2 cells against LPS-induced cytotoxicity. Agomelatine's mechanism of action in this context may involve modulating intracellular signalling pathways and reducing oxidative stress, ultimately promoting cell survival.

Furthermore, the groups treated with DMSO alone, at concentrations of 3 μ L and 6 μ L, exhibited significantly higher cell viability compared to the LPS-treated group. However, the decrease in cell viability was also significant compared to the control group. It suggests a potential toxic effect of DMSO against BV2 cells.

It is important to acknowledge that the DMSO may impact cell viability, as it possesses cytotoxic properties depending on the concentration and experimental conditions. Therefore, caution should be exercised when interpreting the results of DMSO-treated groups and considering its potential confounding effects.

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, the experimental study investigated the effects of agomelatine and LPS on BV2 cell viability. The findings revealed dose-dependent effects of agomelatine, where lower doses (up to 2 μ M) increased cell viability, while higher doses (above 50 μ M) decreased cell viability. Agomelatine treatment duration was also impacted, as prolonged exposure decreased cell viability for most doses. These dose and duration-dependent effects highlight the importance of optimizing experimental conditions to achieve desired therapeutic effects while minimizing adverse effects.

Regarding LPS toxicity, it was found that LPS at a concentration of 1 μ g/ml significantly decreased BV2 cell viability, confirming its pro-inflammatory and cytotoxic properties. However, the study also demonstrated the potential protective effect of agomelatine against LPS-induced cytotoxicity. Both concentrations of agomelatine tested (25 μ M and 50 μ M) significantly increased cell viability compared to the LPS-treated group. It suggests that agomelatine can attenuate the toxic effects of LPS on BV2 cells.

Additionally, DMSO treatment alone showed potential toxicity, as the DMSO-treated groups exhibited lower cell viability compared to the control group. However, the impact of DMSO itself on cell viability should be considered, as it can be very cytotoxic depending on the concentration and experimental conditions.

Overall, the study provides evidence for the potential therapeutic value of agomelatine in protecting BV2 cells against LPS-induced cytotoxicity. Further research is needed to reveal the underlying mechanisms and optimize the conditions for agomelatine treatment to maximize its beneficial effects and minimize potential adverse effects on cell viability.

Several recommendations can be made based on the study's findings. Firstly, conducting additional studies to determine the optimal dosage of agomelatine for achieving the desired outcomes is advisable. Secondly, investigating the time-dependent dynamics of the substances by examining different treatment durations before proceeding with the experiment is crucial. Additionally, it is recommended to compare the efficacy of agomelatine with other neuroprotective agents or antidepressants to assess its potential advantages. Furthermore, assessing the long-term effects of agomelatine and LPS exposure on cell viability and inflammatory

markers is essential. Lastly, in future experimental designs, it is important to consider the effect of potential confounding factors that may influence cell viability.



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