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ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
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**THE POSSIBLE NEUROPROTECTIVE EFFECT OF
AGMATINE ON MICROGLIAL CELL LINE IN
NEURODEGENERATIVE DISEASE**

Ph.D. Thesis

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SAMSUN

2023

ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled “**THE POSSIBLE NEUROPROTECTIVE EFFECT OF AGMATINE ON MICROGLIAL CELL LINE IN NEURODEGENERATIVE DISEASE**” prepared by **Mohamed Suliman Osman ELHAG** , and supervised by **Assoc.Prof. Dr. Mehmet Emin ÖNGER** was found successful and unanimously accepted by committee members as Ph.D. thesis, following the examination on the date 11.8.2023 .

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ÖZET

NÖRODEJENARATİF HASTALIKLARDA AGMATİNİN MİKROGLİYAL HÜCRE HATTI ÜZERİNE MUHTEMEL NÖROPROTEKTİF ETKİSİ

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Doktora, Haziran/2023
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Sinir sistemi hastalıkları, dünya genelinde önemli bir morbidite ve mortalite nedeni olmaya devam etmektedir. Bu nedenle, nöronları koruyabilen etkili ajanların araştırılması oldukça önemlidir. Endojen bir poliamin olan Agmatin, çeşitli biyolojik özelliklere ve farmakolojik potansiyele sahiptir. Bu çalışma, Agmatin'in BV2 hücre ölümü üzerindeki nöroprotektif etkilerini araştırmayı amaçlamaktadır. Hücre canlılığının değerlendirmesi, Agmatin ve LPS için uygun dozların belirlenmesini takiben MTT testleri kullanılarak gerçekleştirilecektir.

Optimal dozun belirlenmesi için Agmatin ve LPS maddelerinin çeşitli konsantrasyonları BV2 hücreleri üzerinde test edildi. Önceki in vitro çalışmalarda literatürde bildirilen geniş konsantrasyon aralığını dikkate alınarak, deneyler 20 ila 150 μM arasındaki konsantrasyonlarla ve farklı BV2 hücre konsantrasyonları kullanılarak geniş bir doz aralığında gerçekleştirildi. Lipopolisakkaritler (LPS) için değişken duyarlılık göz önüne alındığında, farklı tepkiler ve toksik etkiler ortaya çıkabilir. Hücre canlılığı, 2 ila 0,01 $\mu\text{g/ml}$ aralığında değişen LPS konsantrasyonları kullanılarak MTT testi kullanılarak değerlendirildi. Deneysel tasarımda, yine Agmatin'in BV2 hücrelerinde LPS toksisitesine karşı koruyucu özelliklerini MTT analizleri kullanarak araştırmak amaçlandı.

Araştırma bulguları, BV2 hücrelerinin 25 μM Agmatin ile tam absorbanslı bir şekilde hayatta kalabildiğini doğruladı. Bu konsantrasyonun üzerinde, hücrelerin hayatta kalma oranı kademeli olarak azalarak sırasıyla 107,7 μM , 65 μM ve 35 μM IC50 değerlerine ulaştı. Hücre canlılığındaki bu azalma istatistiksel olarak anlamlı bulundu. Öte yandan, LPS için 0,25 $\mu\text{g/ml}$ 'ye ulaşana kadar BV2 hücrelerinin hayatta kaldığı tespit edildi. Bu noktadan sonra, hücre canlılığı azalmaya başladı ve 0,9 $\mu\text{g/ml}$ IC50 değerine ulaştı, bunu üzerindeki dozlarda hücreler öldü. LPS toksisitesine karşı Agmatin uygulaması, Agmatin'in BV2 hücrelerinde nöroprotektif potansiyelini doğruladı. 50 μM , 75 μM ve 100 μM dozlarının etkili olduğu ancak 100 μM 'yi aşan konsantrasyonların hücreler için toksik hale geldiği görüldü. Üç günlük süre boyunca optimal Agmatin dozu belirlendikten sonra IC50 değerleri birinci, ikinci ve üçüncü gün için sırasıyla 107,7 μM , 60 μM ve 35 μM olarak belirlendi.

Agmatin, sınırlı yan etkilerin avantajıyla, nöronal koruma ve tedavide yüksek etkinlik göstermektedir. Bu bulgular, Agmatinin nörolojik bozuklukların tedavisinde önemli terapötik etkisini vurgulamaktadır. Ancak Agmatinin klinik kullanıma yaygın biçimde benimsenebilmesi için daha fazla klinik araştırmaya ihtiyaç vardır.

Anahtar Sözcükler: Agmatin, nörokoruma, BV2, kronik nörodejeneratif hastalıklar, Lipopolisakkaritler, MTT

ABSTRACT

THE POSSIBLE NEUROPROTECTIVE EFFECT OF AGMATINE ON MICROGLIAL CELL LINE IN NEURODEGENERATIVE DISEASE

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Nervous system diseases remain a significant cause of morbidity and mortality worldwide. Consequently, it is crucial to search for effective agents that can protect neurons. Agmatine, an endogenous polyamine, possesses diverse biological properties and pharmacological potential. This study aims to investigate the neuroprotective effects of agmatine on BV2 cell death. The evaluation of cell viability will be conducted using MTT assay, following the determination of appropriate doses for Agmatine and LPS.

In order to determine the optimal dose, various concentrations of the agmatine and LPS substances were tested on BV2 cells. Considering the wide range of concentrations used in previous *in vitro* studies as reported in the literature, experiments were conducted across a broad range of doses, including concentrations from 20 to 150 μ M, and using different cell concentrations of BV2. Due to the variable sensitivity to lipopolysaccharides (LPS), different responses and toxic effects can occur. The cell viability was assessed using the MTT assay with varying concentrations of LPS, ranging from 2 to 0.01 μ g/ml. The experimental design aimed to investigate the protective properties of agmatine against LPS toxicity on BV2 cells, utilizing the MTT assay.

The research findings confirmed that BV2 cells can survive with full absorbance up to 25 μ M of agmatine. Beyond this concentration, the survival rate of the cells gradually declined until reaching the IC₅₀ values of 107.7 μ M, 65 μ M, and 35 μ M for the first, second, and third doses, respectively. Statistical significance was observed for this decrease in cell viability. On the other hand, for Lipopolysaccharides (LPS), BV2 cell survival was detected until reaching a dose of 0.25 μ g/ml. After this point, the viability of BV2 cells started to decline, reaching the IC₅₀ value of 0.9 μ g/ml, beyond which the cells eventually died. Combining both substances, agmatine and LPS, confirmed the neuroprotective potency of agmatine on BV2 cells. The doses of 50 μ M, 75 μ M, and 100 μ M were found to be effective, but concentrations exceeding 100 μ M became toxic to the cells. After determining the optimal dose of agmatine over a three-day period, the IC₅₀ values were determined as 107.7 μ M, 60 μ M, and 35 μ M for the first, second, and third day, respectively.

Agmatine demonstrates high efficacy in neuronal protection and treatment, with the added advantage of limited adverse effects. These findings highlight the significant therapeutic effect of agmatine in managing neurological disorders. However, further clinical trials are necessary before agmatine can be widely adopted for clinical use.

Keywords: Agmatine, neuroprotection, BV2, chronic neurodegenerative diseases, Lipopolysaccharides, MTT

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and appreciation to all those who have contributed to the completion of my thesis. First and foremost, I am immensely grateful to Ondokuz Mayıs University for providing me with the opportunity to pursue my higher education and for creating a conducive academic environment. The facilities, resources, and learning opportunities offered by the university have played a significant role in shaping my research.

I extend my heartfelt thanks to Professor Süleyman KAPLAN for providing such an opportunity and for such a wonderful experience. I am immensely grateful for all the time I spent and the knowledge I gained.

I am deeply indebted to my supervisor, Assoc. Prof. Mehmet Emin ÖNGER, for his guidance, patience, and expertise. His insightful feedback, constructive criticism, and continuous support have been instrumental throughout the program. I am grateful for his mentorship, which has not only enriched my academic knowledge but has also encouraged me to develop as a researcher.

I would also like to extend my gratitude to my colleagues Dr. Abubaker Yusof, Dr. Negwod Abdelghani, and Dr. Hiba Hassan for their welcome, help, and support throughout my academic journey. I am grateful for all the help and advice they shared, the ideas they provided, and the challenges they assisted me to overcome.

Finally, I express my deepest appreciation to my family for their unwavering support, love, and understanding. Their encouragement, sacrifices, and belief in me have been my constant source of strength. I am forever grateful for their unconditional support and for always being there for me, providing the motivation and inspiration I needed to persevere.

Thank you all from the bottom of my heart.

Mohamed Suliman Osman ELHAG

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.....	vi
SYMBOLS AND ABBREVIATIONS.....	vii
FIGURES LEGENDS.....	viii
TABLES LEGENDS.....	ix
1. INTRODUCTION.....	1
2. GENERAL INFORMATION AND REVIEW OF THE LITERATURE.....	4
2.1. Agmatine Sources	4
2.2. Function of Agmatine.....	6
2.3. Additional Cellular Activities	7
2.4. Distribution of Agmatine in Brain	8
2.5. Distribution of Agmatine Receptors	9
2.6. Agmatine Enzymatic Degradation Activity	10
2.7. Properties of Agmatine on Neuronal Damage.....	11
2.8. Mechanisms of Neuroprotection.....	14
2.9. The Neuroprotective Properties of Agmatine.....	15
2.10. Agmatine Role in Neurodegenerative Disease	17
2.10.1. Effects of Agmatine in Alzheimer's Disease.....	17
2.10.2. Effects of Agmatine On Parkinson's Disease.....	18
2.11. LPS Morphology.....	20
2.11.1. Lipopolysaccharide Functions.....	20
2.12. Effect of LPS Doses on Cells	22
2.13. Dosage Justification	22
2.13.1. Calculation of IC ₅₀	25
2.13.2. IC ₅₀ of Compounds	25
2.13.3. Calculation of IC ₅₀ Occurs in 2 Steps.....	26
2.14. Microglia.....	26
2.14.1. Source, Phenotypes and Function	26
2.14.2. Microglial Production of Cytokine and it is Measurements	28
2.14.3. Production of Inflammatory Molecules by Human Microglia	30
2.14.4 Microglia and the Histogenesis of the Nervous System	31
2.14.5. Microglia Cell Lines	32
2.14.5.1. Mouse Cell Lines.....	33
2.14.5.2. Rat Cell Lines.....	34
2.14.5.3. Human Cell Lines	35
2.15. Cell Viability Assays	36
2.15.1. Tetrazolium Reduction Assay.....	36
2.15.2. MTT Tetrazolium Assay Concept	36
3. METHODOLOGY	39
3.1. Preparation of Cells, Substance, and Reagents.....	39
3.1.1. Preparation of BV2 Cell Line Culture	39
3.1.2. Substance Preparation.....	42
3.1.3. Preparation of reagents	46
3.1.3.1. Complete media preparation protocol.....	46
3.1.4. MTT Solution.....	47
3.1.4.1. MTT Assay Protocol (Colorimetric Assay).....	48

3.1.5. MTT Calculation	48
3.2. Determination of (Agmatine) and (LPS) Proper Doses.....	49
3.2.1. Determination of Agmatine Proper Doses.....	49
3.2.2. Determination of the Proper Doses For LPS	50
3.3. Testing Agmatine Protective Effect Against LPS Toxicity on BV2 Cell.....	51
4. RESULTS	53
4.1. Statistical Analysis for Substances.....	53
4.1.1. Agmatine Statistical Analysis.....	53
4.1.1.1. First 24-hour results.....	53
4.1.1.2. Second 48 Hours Analysis Results.....	55
4.1.1.3. Third 72-hours Analysis Results.....	57
4.1.2. LPs Statistical Analysis	59
4.2. Microscopic Results Findings	62
4.2.1. First 24-hours Microscopic Findings.....	62
4.2.2. Second 48-hours Microscopic Findings	64
4.2.3. Third 72-hours Microscopic Findings	65
4.3. Agmatine treatment protect BV2 microglial cells from LPs toxicity.....	66
4.3.1. Statistical Findings.....	67
4.3.2. Microscopic Findings	69
5. DISCUSION	69
6. CONCLUSIONS	74
REFERENCES.....	76
CURRICULUM VITEA.....	82

SYMBOLS AND ABBREVIATIONS

SYMBOLS

μMol	:	Micromolar
ml	:	Milliliter
μg	:	Microgram
g/mL	:	Grams Per Milliliter
g/L	:	Grams Per Liter
mg/mL	:	Milligrams Per Milliliter
$\mu\text{g/mL}$	:	Micrograms Per Milliliter
$\mu\text{g}/\mu\text{L}$	:	Micrograms Per Microliter
ng/L	:	Nanograms Per Liter
$\text{ng}/\mu\text{L}$	:	Nanograms Per Microliter
nm	:	Nanometers
kb	:	Kilobyte
$^{\circ}\text{C}$	:	Centigrade

ABBREVIATIONS

ND	:	Neurodegenerative Disease
AD	:	Alzheimer's Disease
PD	:	Parkinson's Disease
PM	:	Primary Microglia
ROS	:	Reactive Oxygen Species
MTT	:	(Dimethylthiazol-2-Yl) Diphenyltetrazolium Bromide)
SON	:	Supraoptic Nuclei
PVN	:	Paraventricular Area
RGCs	:	Retinal Ganglion Cells
CNS	:	Central Nervous System
PAF	:	Platelet Triggering Factor
AQPs	:	Aquaporin's
BBB	:	Brain Blood Barrier
ATF3	:	Transcription Factor 3
ENOS	:	Endothelial NO Synthase
GFR	:	Glomerular Filtration Rate
GP x	:	Glutathione Peroxidase
GR	:	Glutathione Reductase
HMC-1	:	Human Mast Cell Leukemia Cells
IL	:	Interleukins
MCAO	:	Middle Cerebral Artery Obstruction
MMP-2	:	Matrix Metalloproteinase-2
IC50	:	The Half Maximal Inhibiter Concentration
MPTP	:	1-Methyl-4-Phenyl-4-1,2,3,6-Tetrahydropyridine
NMDA	:	N-Methyl-D-Aspartate
NO	:	Nitric Oxide
SOD	:	Superoxide Dismutase
TLR	:	Toll-Like Receptor
TLR4	:	Toll-Like Receptor 4
LPS	:	Lipopolysaccharide
TBI	:	Traumatic Brain Injury
TNF	:	Tumor Necrosis Factor

ND	:	Neurodegenerative Disease
i.p	:	Intraperitoneally
Bcl-2	:	B-Cell Lymphoma 2
Bclx	:	B-Cell Lymphoma-Extra Large
PCR	:	Polymerase Chain Reaction
AMPK	:	Activated Monophosphate Protein Kinase
TOR	:	Target Of Rapamycin
UV	:	Ultraviolet Light
SNP	:	Single Nucleotide Polymorphism
CNS	:	Central Nervous Stsem
dATP	:	Deoxyadenosine Triphosphate
RNA	:	Ribonucleic Acid
DNA	:	Deoxyribonucleic Acid
PS	:	Phosphatidylserine
FBS	:	Fetal Bovine Serum
im	:	Intramuscular
iv	:	Intravenous
EDTA	:	Ethylenediaminetetraacetic Acid
DMSO	:	Dimethyl Sulfoxide
DPBS	:	Dulbecco's Phosphate Buffered Saline
EtOh	:	Ethyl Alcohol
P53	:	Transformation-Related Protein 53
P13K	:	Phosphatidylinositol-3-Kinase
ATG 1	:	Autophagy
VPS34	:	phosphatidylinositol 3-kinase
LC3-I	:	Light Chain 1

FIGURES LEGENDS

- Figure 2.1. Chemical structure of agmatine modified from (Uzbay, 2012)..... 6
- Figure 2.2. Agmatine synthesis and metabolism{Barua, 2019 #498}. 11
- Figure 3.1. Laminar flow tube with UV light (A) and Microscope inverted connected to computer with a screen for visualizing and recording cell picture (B). 40
- Figure 3.2. A-Dulbecco's modified Eagle's medium (DEMM) from (gibco) for complete media B- 0.25 Trypsin-EDTA (Gibco) for cells detachment C- Antibiotic (Penicillin 10,000 units/ml +Streptomycin 10mg/ml), D- Phosphate buffer saline tabs which will be dissolved and used..... 40
- Figure 3.3. A- Incubator adjusted to 37C, 95% humidity with 5% CO₂ for cell incubation, B- 25 cm³ Flask containing cell with acidity (brown to orange colour) C- 25 cm³ flask under an inverted microscope to detect cells confluences percentage. 41
- Figure 3.4. A- Eppendorf tubes or single-use polypropylene tubes, used for mixing, centrifuging, transferring, and packing solid and liquid samples and reagents. B- DMSO is used for cryopreservation in conventional cell banking. Commonly, DMSO is added to the media for cell freezing to prevent the production of ice crystals. 42
- Figure 3.5. A- Distilled water used for dissolving substances like agmatine B- Different sizes of pipettes that are used in the calculation of fluid size in ml and µl 43
- Figure 3.6. Solutions used to emulsify LPS powder is either phosphate buffer saline A or distilled water B kept in falcon tube C in a refrigerator..... 44
- Figure 3.7. A- Fetal bovine serum B- DMEM C- Antibiotics (Penicillin 10,000 units/ml +Streptomycin 10mg/ml), usually DMEM contains glutamate for that researcher has to be careful toward duplication of glutamate dose in complete media formation. 46
- Figure 3.8. A- Falcon tube containin complete media after preparation and mixing 47
- Figure 3.9. A- Phosphate buffered saline (PBS) solutions for dilutions, cleaning culture flasks and plates, washing cell suspensions, and adding additions to cell culture media. B- MTT powder is a colorimetric test used for evaluating cell survival..... 47
- Figure 3.10. A- 96 well microplate contains BV2 in addition to agmatine + LPS in a duplicate manner, B- 96 well plate contains BV2 cells and agmatine in a duplicate manner after addition of MTT, C- 96 well plate containing agmatine+ MTT+LPS in a triplicate manner after incubation for detection absorbance. 48
- Figure 3.11. A- DNA/RNA Shield, used for the samples' expression patterns and genetic integrity protection, at room temperature. B- Digital Centrifuge C- Weighing Scale 49
- Figure 4.1. X Horizontal line represents the agmatine concentration that was added to BV2 cells while the Y vertical line represents cell viability

- percentage, and the interrupted red line represents 50% cell viability the point at which the curved line crosses the red dots line is the IC_{50} here it is $107\mu M$ 54
- Figure 4.2. Showing a 24-hours graph estimate of the similarities or dissimilarities between control group and the last three groups which contain the doses ($100\mu M$, $150\mu M$, and $200\mu M$) the first one ($100\mu M$) is significant (*) p -value < 0.05 but lower the two remaining groups ($150\mu M$ and $200\mu M$) they contain high significant (****) p -value < 0.0001 54
- Figure 4.3. Graph showing the IC_{50} in 48-hours group treated with agmatine X horizontal line axis represents agmatine doses, while Y axis represents cells viability percentage, IC_{50} is at $(65.43)\mu M$ the point in which the red interrupted line is crossed by the blue curved line 56
- Figure 4.4. Graph comparing the control with the other group contains significance and shows the level of significance in the 48-hours group, the X horizontal line axis represents agmatine doses, while the Y axis represents cell viability percentage. (**)The two stars between the brackets indicate that between these two groups, there is a significant p -value < 0.01 57
- Figure 4.5. Graph showing the IC_{50} in the second day group 72-hours treated by agmatine for three days, X horizontal line axis representing agmatine doses, while Y axis representing cells viability percentage 58
- Figure 4.6. Graph comparing the control with the other group containing significance and showing the level of significance in 72 hours. X horizontal line axis represents agmatine doses, while Y axis represents cells viability percentage, (*) the stars between the brackets indicate that between these two groups, there is a significant p -value < 0.05 , (***) three stars between the brackets indicate that between this two groups there is significant p -value < 0.001 , (****) which means the p -value < 0.0001 58
- Figure 4.7. X horizontal line represents agmatine concentration that was added to BV2 cells while Y vertical line represents cell viability percentage, the interrupted red line represents 50% cell viability the point at which the curved black line crosses the red dots line is the IC_{50} here it is $107\mu M$ in the first day and $65.43\mu M$ in the second day green line, and lastly, IC_{50} for the 3ed day was $30.25\mu M$ represented by the orange line. 59
- Figure 4.8. LPS dose groups for BV2 treatment, statistical significance was defined as a p -value of 0.05, p -values less than 0.05 are regarded as statistically significant differences in all comparisons, the symbol (*) which means the p -value < 0.05 , $150\mu M$ (****) which means the p -value < 0.0001 and $200\mu M$ (****) which means the p -value < 0.0001 61
- Figure 4.9. A- Light inverted microscope lens magnification 40X Control group of microglial BV2 cells contains no agmatine treatment 62
- Figure 4.10. Light inverted microscope lens magnification 40X of BV2 microglial cells in $25\mu M$ agmatine group after 24 hours of incubation, cells viability seems to be similar to the control group which contains no agmatine. 63

Figure 4.11. Purple formazan after the addition of DMSO	63
Figure 4.12. Light inverted microscope lens magnification40X BV2 cells after 48 hours incubation with treatment by agmatine IC ₅₀ is 65.43 µM, cells viability is declining in comparison to the same dose on the first day....	64
Figure 4.13. Light inverted microscope lens magnification40X of BV2 cells after 48 hours Microscopical findings of group 50µM agmatine, majority of BV2 cells are alive, clearly visible growing, cells morphology with their protoplasmic extension are clear as well as cells nuclei	64
Figure 4.14. Purple formazan after the addition of DMSO on the second day The MTT assay colorimetric measures the metabolic activity of cells. Under some circumstances, NAD(P)H-dependent cellular oxidoreductase enzymes represent the number of live cells present. The yellowish soluble tetrazolium dye MTT can be reduced by the enzyme to insoluble formazan, which has a purple tint.	65
Figure 4.15. Light inverted microscope lens magnification 40X of BV2 cells after 72-hours incubation with treatment by agmatine IC ₅₀ is 30.25µM, cell viability is declining to 50%.....	65
Figure 4.16. Light inverted microscope lens magnification 40X of BV2 cells treated by 1µM of LPS, few cells can be detected the majority of cells died.....	66
Figure 4.17. Purple formazan of BV2 cells treated with 1µM LPS after the addition of DMSO.....	66
Figure 4.18. The graph shows cell viability for the eight groups of the experimental BV2 cells after treatment by agmatine and LPS, The X horizontal line represents the LPS concentration that was added to BV2 cells while the Y vertical line represents the BV2 cell viability percentage.....	67
Figure 4.19. A- Light inverted microscope lens magnification 20X Control group of microglial BV2 cells contains neither LPS nor agmatine treatment, BV2 cells are alive, clearly visible, growing, cells morphology with their protoplasmic extension and cells nuclei are clear. B- Light inverted microscope 20X represents the LPS group (1µg/ml) majority of cells died.....	69
Figure 4.20. A- Light inverted microscope lens magnification 40X LPS(1µg/ml) group+Agmatine75µM + microglial BV2 cells, cells are alive, clearly visible, growing, cell morphology protoplasmic extension cell nuclei are clear. Similar to B- Light inverted microscope 40X represents agmatine group 75µM cells were, alive, cell morphology protoplasmic extension and cell nuclei are visible.....	69
Figure 4.21. A- Light inverted microscope lens magnification 40X LPS(1µg/ml) group+ agmatine 100µM + microglial BV2 cells, cells are alive, clearly visible, growing, cell morphology protoplasmic extension cell nuclei are clear. B- Light inverted microscope 40X represents the LPS group (1µg/ml) majority of cells died.	69

TABLES LEGENDS

Table 2.1. Summary of the studies showing agmatine doses and route of administration (Kotagale et al., 2019).....	24
Table 3.1. Agmatine doses preparations	43
Table 3.2. Administration of LPS in different doses to BV2 microglial cells and controls	45
Table 3.3. Administration of agmatine in different doses to BV2 cells and controls	50
Table 3.4. The different stages of the main experiments by adding the high and low doses of agmatine +LPS to BV2 cells.....	51
Table 3.5. Protocol of Cell Seeding in the well plate.....	52
Table 4.1. Mean and standard deviation of BV2 cells viability after the addition of agmatine only	53
Table 4.2. Showing comparison between control and nine doses for significance level.....	55
Table 4.3. The mean and standard deviation for the second group 48-hours	56
Table 4.4. The mean and standard deviation for the third group 72 hours for the ten groups of doses used with agmatine.....	57
Table 4.5. Mean and standard deviation of the main experiment	67

1. INTRODUCTION

Neurodegenerative disease (ND) globally is a Known and upraising reason of mortality and morbidity, predominantly in the ageing. Patients with neurodegenerative disease have deferent medical picture of presentations because they have various fundamental in medical causes and physiology, though they regularly have overlying morphology. Due to this Correct Diagnosis is serious matter on the choice of treatment and it is potency, as it permits for extra reliable prediction and frequently direct definite management (Erkkinen et al., 2018).

One of the main clinical picture of Alzheimer's Disease is the common formula of dementia global and marks up 60–80% of all dementia recorded cases, affecting predictable twenty-four million individuals globally (Doblhammer et al., 2013).

As it generally known dementia might happen in small aged peoples, but it is principally an illness of the ageing people. The occurrence and incidence of AD rises obviously with old age period, by a larger than 15-fold rise described among the aging people of 65 years and 85 years (Evans et al., 1989).

Gilad and his associates measured long-term protection of oral agmatine management by consuming everyday great dosage of agmatine orally over a period of 48-60 months. Altogether assessments continued inside ordinary values and no uneasiness was noticed throughout the follow-up time (Gilad and Gilad, 2014).

Furthermore, the neuronal defensive outcome of agmatine has been confirmed by broad publication since 1994. The small occurrence of side effects and huge therapeutic worth has gained excessive care. In 1995, Gilad et al. initially described the neuronal defensive action of agmatine(Reis & Regunathan, 2000). Henceforth, extra and additional publication exposed agmatine neuroprotection in stroke, traumatic CNS damages, neuronal degenerative disease, pathological neuronal pain, convolutions , and even in intellectual disorders (Xu et al., 2017).

To all these positive and motivating effects and outcome of using agmatine I plan to use this chemical compound agmatine in cell line field to see it is protective effect at the level of cell do it really protecting and defending for cell survival or it is effect is only when taken generally to hole body tissue, due to the link and association between microglial cells it is chosen as a cell line. Microglia, which are one of the glial cells found in the central nervous system (CNS), play a significant role as protective and engulfing cells in response to damage and disease. Del Rio Hortega, a scientist in

1927, was the first to identify microglia as a distinct type of glial cell, separate from astrocytes and oligodendrocytes. Since the 1970s, there has been growing recognition that microglia are important immune effectors in the brain that respond to various microbial conditions and contribute to the onset and progression of neurological diseases, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Hong et al., 2007).

Microglia are a type of the resident macrophages derived from monocyte located in the brain responsible about immune scrutiny in the central nervous system (Ransohoff and Perry, 2009). When these microglia cells are being stimulated, they will start secrete neurotrophic elements to make boundary around the injured tissue to minimize the extension of the damage, in order to defend susceptible neuronal tissue, and share in assistance of central nervous tissue repair progressions.

According to a study by Bodea et al. in 2014, microglia are activated by LPS, resulting in the release of inflammatory mediators and various neurotoxic substances that initiate the death of nerve cells. Most of the research on the activation of microglial TLR has been conducted in vitro, often using cell lines such as N9plus BV2 (Lee et al. 2004, Bi et al. 2005). The BV2 cell lines were originally derived from v-raf/v-myc-immortalized murine fetal microglia, and they are the most frequently used alternative to primary microglia (Duan et al., 2013)

Currently, extra laboratories have described that BV2 cell lines display numerous similarities with PM and *in vivo* models in publication of Huntington's disorders (Fujimoto, 2008). Furthermore, BV2 cell lines are used in the pharmaceutical industry (Lund et al., 2005).

Similarly, many researchers had covered BV2 microglial cells with agmatine but no researcher subjected agmatine as a neuroprotective to BV2 against LPS. All what is mentioned generate the importance of an experiments regarding protection for neuroglial cells by agmatine against LPS sharing in filling knowledge gap.

Due to high sensitivity to microglial cell to LPS contact I prefer to use it in my experiment to enhance microglial cells to initiate the defence secretion. A mechanism to assess agmatine's potency protective effect when agmatine is added to the medium, a difference between media containing agmatine and media missing agmatine will be evident in cell survival, which is clearly shown in my focal title remark regarding the

potential neuroprotective effect of agmatine on microglial cells line (Erkkinen et al., 2018).

The purpose of the current study was to acquire a better understanding of how agmatine protects against the neurodegenerative effects caused by LPS utilizing an *in vitro* model, we concentrated on the BV2 cell line.



2. GENERAL INFORMATION AND REVIEW OF THE LITERATURE

The goal of this section of our dissertation is to outline the main concepts and theories that aid in our understanding of the research issue as a whole, as well as to show you what is already known about it in a methodical manner. The review will be methodical, evaluative, and critical of the research and concepts that are pertinent to our current work.

To start solving the research question of this study, a literature review protocol is developed by using a search strategy identifying the scope and methods for the review (data sources, key search terms, and eligibility criteria). A search in an English-language literature paper between January 1984 to August 2022 in the initial phase and later extended the search from September 2022 to December 2022. The search focused on Arabic countries and Europe, because there is a critical mass of studies, enabling researchers to draw conclusions. For published journal articles, search is done through electronic databases PubMed, Scopus, and Google Scholar. Search terms used were ‘agmatine, neuroprotection, BV2, chronic neurodegenerative diseases, Lipopolysaccharides, MTT, ‘The search terms were used in combination with the Boolean operator ‘AND’. In addition, we searched the Internet to identify relevant grey literature.

Researcher then screened the list of articles independently. Titles and abstracts of the articles were read to determine their relevance to the topic. After excluding duplicate citations, the researcher excluded articles not in the English language and those published before January 1984. Researcher further excluded editorials, proposals and protocols that contained no empirical findings.

2.1. Agmatine Sources

A large and growing body of literature has investigated agmatine which is an amino acid derived from arginine that is found in the neuronal cell body and nerve ending terminals. It acts as a neuromodulator, mimicking the functional properties of other neurotransmitters (Reis and Regunathan, 2000). Agmatine is synthesized by the decarboxylation of l-arginine using the enzyme arginine decarboxylase (ADC), which involves the removal of one carbon atom from the arginine molecule. Some studies

suggest that in regions where ADC is deficient, the enzyme ornithine stimulates the decarboxylation of l-arginine instead (Atlas and Burstein, 1984).

More recent attention has focused on the provision of agmatine can be detected if checked everywhere in the brain tissue hippocampus, striatum, locus coeruleus, hypothalamus, cortex of frontal area, medulla, and along with measurable ADC activity, Conversely, these cells are gathered and concentrated in large collection number of these in the paraventricular area (PVN) and supraoptic nuclei (SON) of the hypothalamus, which likewise showed the maximum arginine decarboxylase action. Agmatine possess Numerous research teams have described various protective mechanisms for the neuronal response to neurodegenerative diseases, including blocking harmful ion channels, neutralizing harmful reactive oxygen species, promoting the formation of new neurons and blood vessels, and reducing neuronal supporting glial scarring (Barua et al., 2019; Askalany et al., 2005; Kuo et al., 2011).

In the state of acute central nervous system illnesses, mutually agmatine will be produced or synthesized inside or outside neuronal cells (via over-expression of ADC or down directive of agmatinase experiments, etc.). Also, agmatine is described to be very good in decreasing hypoxic nervous tissue destruction (Askalany et al., 2005; Binnetoglu et al., 2019; J. H. Kim et al., 2017; Kuo et al., 2011).

There is a consensus among social scientists that manufactured agmatine has also been shown to act as an anti-proliferative agent by reducing the biosynthesis and increasing the degradation of cell growth and proliferative polyamines such as putrescine, spermidine, and spermine in various cancer cell lines (M.Marx G.Trittenwein C, 1995; Song et al., 2014). Manufactured agmatine treatment also can prevent progressive glycation end product creation in diabetic renal (M.Marx G.Trittenwein C, 1995).

In the last couple of decades years, due to the importance of agmatine topic many publications have been accomplished concerning the interrelating objectives of mutually endogenous and Manufactured agmatine in the central nervous system and other parts of the body. Agmatine interacts with the various receptors as well as ion channels of the neuronal cells which are accountable for diverse neuronal degenerative illnesses (Uzbay, 2012).

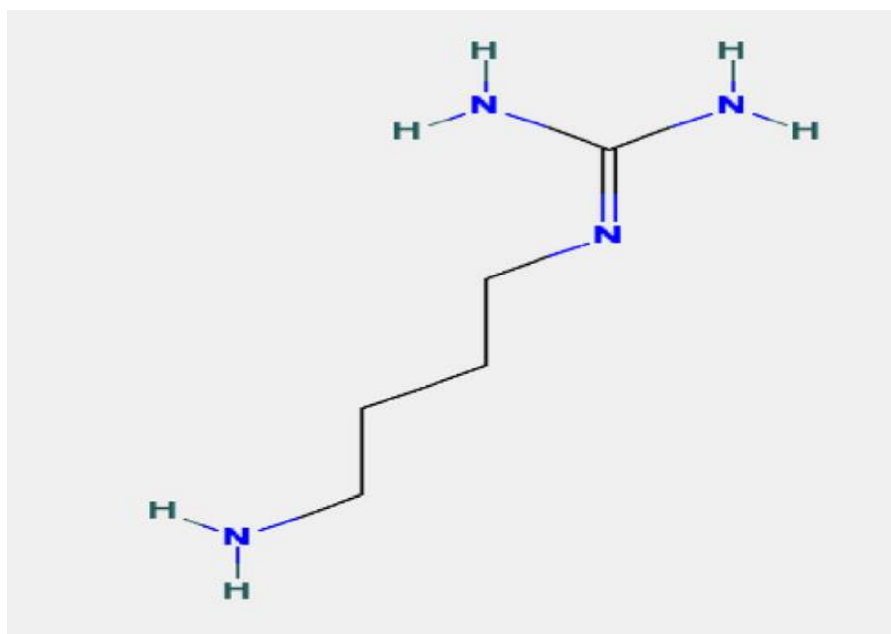


Figure 2.1. Chemical structure of agmatine modified from (Uzbay, 2012)

2.2. Function of Agmatine

Recent evidence suggests that pharmacological effect in the margin, agmatine have specific effect in insulin adaptation and sugar breakdown; it grounds dose reliant on secretion of insulin from pancreas islet of Langerhans cells stimulated by glucose (Blandini et al., 1996), has insulin-similar characteristics on insulated lipid cells (Rodriguez et al., 1998), and secretion of epinephrine and norepinephrine from suprarenal gland chromaffin cells *in vitro*. In renal, agmatine is naturistic, enhancing the elimination of Na¹ autonomously of actions on plasma flow (Sawada et al., 2010).

Additionally, agmatine inhibits the stimulated propagation of smooth muscle cells in vessels (Kuo et al., 2011). In the brain and spinal cord, agmatine secretes luteinizing hormone releasing hormone (LHRH) from the hypothalamus (Rushaidhi et al., 2013).

and as competitive for some states in blood sugar surge deprived of changing pain thresholds. Agmatine likewise concentration-dependently augments morphine insensitivity, principally when taken through lumber puncture into cerebrospinal fluid, changing the ED₅₀ of morphine by means of over 9 times (Perez Ortiz and Swerdlow, 2019).

This influence clearly to be discriminates for effective site subtype receptors. Habitually, agmatine inhibits adaptation to morphine concentration for 10 days, obstructing adaptation to the delta opioid peptide receptor ligand. Maximum

entertainingly, agmatine concentration vulnerably and considerably reduces all of the social signs of the morphine moderation syndrome that is triggered in morphine-abusing rats by naloxone (Bergin and Liu, 2010).

Agmatine has similarly been presented to be a nerve cell defendant, dropping the magnitude of segregation of oxygen from brain tissue or the loss of hippocampal neurons in rodent representations of principal or universal ischemia, respectively (Reis and Regunathan, 2000; Timmerman et al., 2018).

2.3. Additional Cellular Activities

Agmatine exhibits additional significant activities in central neurotransmission. Firstly, it can cause a voltage and dose-dependent block of the NMDA receptor channel, with the effect being selective and not affecting AMPA or kainite currents. This block is non-competitive and occurs at a site within the NMDA receptor channel pore, and is not caused by the production of polyamines putrescine or spermine (the latter being a modulator of the receptor). Hippocampal pyramidal neurons themselves contain glutamate and synapse with excitatory glutamate-containing neurons. The observation that some of the presynaptic axons contain agmatine suggests that agmatine and glutamate are co-released at the same terminal. However, the fact that agmatine inhibits the NMDA receptor while glutamate stimulates this receptor suggests that agmatine, at this receptor, acts as a co-transmitter that enhances the stimulatory actions of the primary transmitters postsynaptic. In addition, it has been proposed that agmatine may inhibit other ligand-gated cation channels (Erkkinen et al., 2018; S. U. Kim and De Vellis, 2005) comprising nicotinic acetylcholine (Prusiner, 2013) and 5-HT₃ receptors 46. It is currently unknown whether agmatine interacts with specific sites within the family of cationic channels, such as sigma-2 binding sites, and whether these sites may contain similarities with the I-binding sites. Additionally, agmatine blocks all isoforms of NOS by inhibiting their catalytic activity and decrease the new formation of the inducible NOS isoform in astrocytes (Das et al., 2016; J. H. Kim et al., 2017).

Agmatine blocks NOS in a competitive manner that is not affected by putrescine or spermine, which are potential precursors of agmatine. Regulating agmatine levels may thus help to control NO production in tissues where ADC and NOS are co-expressed or where agmatine is accumulated. The cellular properties of agmatine may explain many of its pharmacological effects. For example, other agents that inhibit

NOS or block NMDA receptors have been shown to reduce chronic pain or alleviate ischemic or excitotoxic damage. It is not surprising that agmatine, which has both of these functions, has similar effects on pain and neuronal death (Binnetoğlu D, 2019).

Agmatine's dual ability to act as a painkiller and neuroprotective agent makes it a promising candidate for therapeutic use. However, the role of naturally secreted agmatine in normal CNS function is not yet clear. An overview of the biology of agmatine at a typical synapse is presented (Reis and Regunathan, 2000).

2.4. Distribution of Agmatine in the Brain

Agmatine has been recognized chemically by high-performance liquid chromatography (HPLC) in central nervous system in addition to other body parts (Li et al., 2021; Moretti et al., 2014).

The concentration of agmatine is distributed within the entire central nervous system, which varies according to the system, represent just ten percent of which present in the stomach, the organ contains the uppermost concentration of agmatine. The percentage of presentation of agmatine in the central nervous system has been detected through the interaction between immune and chemicals by means of a particular multiclinal anti-agmatine antibody, that can by pre-absorption or enzyme connected immunosorbent assay (ELISA), does not distinguish other associated molecules as well as transporter proteins, putrescine, with L-arginine polyamines⁷. Immune response agmatine is chiefly nerve cells contains within nerve cells membrane and circulated throughout neurons and their processes. Even though glial astrocytes show ADC function they comprise light agmatine-similar defence cells reactivity, probably due to agmatine is not kept in glial cells astrocytes (Binnetoglu et al., 2019; Li et al., 2021; Sherer et al., 2003).

The supreme widespread diagramming of agmatine-containing nerve cells in the central nervous system (Binnetoğlu, 2019a) has been strongminded in rats preserved with brain ventricular colchicine, a mediator that, by obstructing neuronal end transportation, upsurges the gathering of spreaders in perikaryal then their recognition. A restriction of the colchicine method is that axonal end terminals, which in agmatine-comprising nerve cells, apparently comprise the maximum concentration of agmatine in vivo, might miscarry to gather and collect the amine and consequently would not be imagined. The central nervous system of colchicine preserved rats comprises plentiful nerve cells that precise agmatine with local heterogeneity. Nerve cells that comprise

agmatine-like defence cells reaction are usual in the brain's cerebral hemisphere cortex, where they concentrate inside laminae VI and V and, to a slighter degree, inside lamina III in the subiculum, retro splenial, cingulate, and principal body sensory cortices area. In accumulation, the hippocampus comprises agmatine (Binnetoğlu D, 2019).

Numerous agmatine-comprising neuron cell bodies are confined under cortical telencephalon with maximum quantities existing in the amygdala, septum, and bed centre of the stria terminals and in the continuum of neuron cells related to the basal forebrain. In the thalamus, categorized neurons cells are concentrated in fundamentals around brain ventricular systems and reunions of nuclei. The upper most quantities of neurons cells that show agmatine-like immune reactivity are found in the hypothalamus, behind the midbrain, around ventricular areas containing the periaqueductal grey laterodorsally nucleus, nucleus raphe dorsalis, and locus coeruleus. Inferior to brainstem, immune cell defence activation is confined choicely to visceral relay nuclei, the pontine parabrachial compound plus the nucleus tactus solitary. Thus, the distribution of agmatine-containing nerve cells is concentrated in districts of the central nervous system that serve visceral and neuroendocrine regulator, dispensation of feelings, aching awareness and perception (Sherer et al., 2003).

The subcellular spreading of agmatine has been investigated by means of electron microscopy in hippocampal pyramidal neuron cells of rat, wherever in it is limited to perikaryal, nerve terminal ending, axons and axon terminals. In perikaryal, agmatine-like immune reactivity methods condensed collections that are related to mitochondria and cylindrical vesicles, however in the axons and axon terminals, it is related with minor, synaptic vesicles that form unequal or stimulatory synapses on the spines of dendrites, numerous of which are of pyramidal cell source. The existence of agmatine inside slight axonal vesicles proposes that the amine is secreted. Furthermore, since the axons of hippocampal pyramidal neuronal cells comprise chiefly glutamate, it is probable that agmatine and L-glutamate are secreted with each other as co-transmitters (Reis and Regunathan, 2000).

2.5. Distribution of Agmatine Receptors

Corresponding to clonidine and further imidazoline medications, fixes by reasonable attraction to all subclasses of α_2 -adrenoceptors and I-binding sites 3 though, the connection between the necessary of agmatine to these locations and the organic

events that are characteristic to the interaction with α_2 -adrenoceptors² is incomprehensible. In one publication, agmatine (0.1–100 mM) did not stimulate pre connectional α_2 -adrenoceptor- controlled spreader discharge in the guinea-pig insulated second part of large intestine and rat insulated vas deferens. Extraordinary dose of agmatine 10–30 times the pK_i at α_2 -adrenoceptor binding locations, similarly unsuccessful to block α_2 -adrenoceptor stimulation by each clonidine or the choice able agonist UK14304. In rat brain cortex, agmatine failed to block for skolin-encouraged cAMP foundation (Ginhoux and Prinz, 2015).

Even though it moved binding to α_2 -adrenoceptors. By distinction, agmatine, which itself does not inspire inactive shrinkage, can quickly and rapidly upsurge the reductions of rat tail blood vessel provoked by transmural encouragement of autonomic nerves, an influence that is inhibited by suitable adversaries and is attributable to diffident barrier of prejunctional α_2 -adrenoceptor function by agmatine (Ginhoux and Prinz, 2015).

2.6. Agmatine Enzymatic Degradation Activity

In microorganisms and plants, agmatine is digested principally to putrescine and urea by hydrolysis catalyzed by agmatinase. In recent times, mammalian agmatinase has been exposed 4 in rat central nervous system and a mouse macrophage cell line, RAW 264.7 (Sastre et al., 1998).

Agmatinase, existing in rat central nervous system, contain comparable reaction characteristics to the microbial enzyme; both have a K_m for agmatine in the millimolar variety and are activated at time of need at alkaline pH. The reaction stimulated by agmatinase is alike to arginase , the enzyme that hydrolyses L-arginine to ornithine and urea. Though, the information that agmatine is a material for arginase (Stansley et al., 2012).

Though at decreased attraction, increases the inquiry of whether agmatinase and arginase are the identical enzyme. That they vary is reinforced by double results. Primary, in rat CNS, arginase is soluble and cytosolic while agmatinase is membrane-related and mitochondrial. Subsequently, arginase is stimulated, whereas agmatinase is withdrawn, by Mn^{2+} . However, more extensive properties of the construction of mammalian agmatinase are essential. However agmatine can be a substrate for amine oxidizes to akeguanidobutanoic acid in rat renal tissue (Stansley et al., 2012).

This passageway has not been verified in the brain and it seems that agmatinase is the essential enzyme that regulates the metabolic outcome of agmatine in the CNS (Uzbay, 2012).

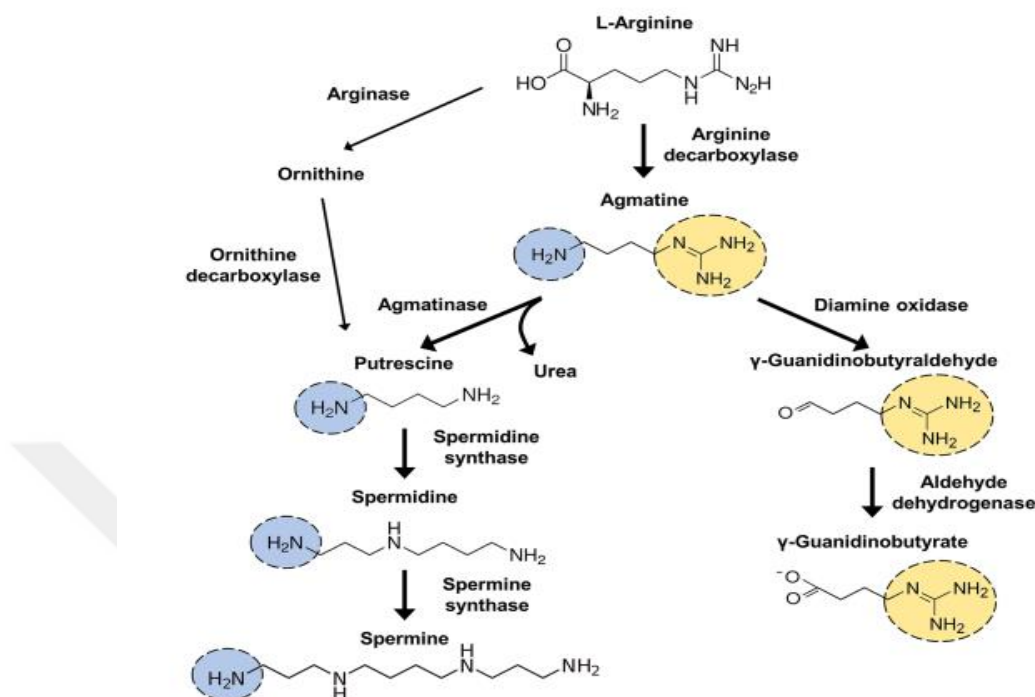


Figure 2.2. Agmatine synthesis and metabolism(Barua et al., 2019b).

2.7. Properties of Agmatine on Neuronal Damage

Gilad et al. (1995) conducted a study to investigate the neuroprotective properties of agmatine and its effects on neuronal cell toxicity using a cell line model of neuronal cells. They examined the properties of agmatine in rat models of ischemic injury.

In their experiment, Gilad et al. tested 72-hour-old cerebellar nerve cells from newborn rats. These cells were exposed to 1 mM NMDA for three hours and then treated with different concentrations of agmatine (ranging from 1 to 2000 μ M). They observed that the cells remained viable after one day, as confirmed by phase-contrast microscopy and the trypan blue segregation test. Agmatine supported the survival of nerve cells at concentrations between 10 and 100 μ M. However, when the dose was increased beyond that range, it became lethal (with an obvious toxic concentration TD50 = 700 μ M), possibly due to its negative impact on cell propagation. The study by Gilad et al. demonstrated the neuroprotective properties of agmatine against NMDA-induced damage in cerebellar neuronal cells (G. M. Gilad and Gilad, 1995).

The findings of Gilad et al. regarding the neuroprotective properties of agmatine in cerebellar neuronal cells challenged by NMDA were further supported by Olmos et al. in their research. Olmos et al. evaluated the effects of agmatine and several imidazoline medications on glutamate-induced necrosis and apoptosis in primary cultures of newborn rat cerebellar granule cells (M. Gilad et al., 1995).

Considering the relatively low affinity of agmatine for the NMDA receptor ($K_i = 219 \text{ mM}$) and the normal concentrations of agmatine in rat neuronal cells ($1.5\text{-}8.5 \text{ }\mu\text{M}$), it raises the question of whether internally produced agmatine could be responsible for the observed neuroprotective effects detected *in vivo* (Abe et al., 2000).

It is possible that the effective dose of agmatine can reach the NMDA receptor site due to uneven distribution in the brain and its secretion if it occurs. Glutamate at concentrations of $1\text{-}100 \text{ }\mu\text{M}$ caused a reduction in the breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a reduced formazan product in brain cells. This suggests the possibility of mitochondrial damage due to excessive NMDA receptor stimulation, leading to increased calcium concentration and disruption of osmotic balance, ultimately resulting in tissue necrosis. The neuronal toxicity induced by glutamate was equally inhibited by treatment with agmatine ($100 \text{ or } 500 \text{ }\mu\text{M}$) and the NMDA receptor antagonist dizocilpine (MK-801), as well as other imidazoline treatments such as antazoline, cirazoline, and idazoxan. These findings indicate a direct interaction of agmatine with the NMDA receptor and its neuroprotective effects (Gad M. Gilad and Gilad, 2000).

Furthermore, it was observed that agmatine and structurally similar compounds did not provide protection against decreased potassium-induced apoptotic cell death, in which NMDA receptors are not involved. Additionally, the ability of agmatine to directly inhibit glutamate-induced currents was demonstrated in HEK-293 cells transfected to express the NR1-1a/NR2C subunits of the NMDA receptor in concentration- and voltage-dependent manners. Due to the challenges associated with agmatine's delivery across the cell membrane, Gilad & Gilad patented novel derivatives of agmatine, guanidine, hydrazine, polyamines, and related combinations referred to as "polyamino guanidine derivatives" for the treatment of neural injury and neurodegenerative diseases (Gad M. Gilad and Gilad, 2000).

The guanidine moiety of agmatine has been identified as the active component responsible for inhibiting the NMDA receptor channel by interacting with a site

located within the NMDA channel opening in rat hippocampal neurons. When agmatine was applied externally to cultured hippocampal neurons, it exhibited voltage- and dose-dependent inhibition of NMDA currents. Agmatine, unlike other internally synthesized biogenic amines, selectively acts as an antagonist at non-glycine β sites of NMDA receptors. Stimulation of the NMDA receptor leads to rapid mitochondrial calcium uptake, and the I2-BS imidazoline binding sites are predominantly located on the mitochondrial membrane. Agmatine may mediate its neuroprotective effects by activating I2-BS, thereby increasing mitochondrial calcium influx and reducing excessive cytosolic calcium accumulation (Abe et al., 2003).

However, no association was found between the effectiveness of agmatine on the central nervous system I2-BS and its protective effects against glutamate toxicity. This suggests that the activation of I2-BS is not involved in the neuroprotective effects of agmatine against glutamate-induced neuronal toxicity. In contrast to the findings of Olmos et al., agmatine was found to be neurotoxic in primary cultures of newborn rat brain cerebellar hemisphere cells under high potassium conditions (27.5 mM) but not under reduced extracellular potassium concentration (10 mM) (Abe et al., 2003).

In another experiment, agmatine treatment (200-800 μ M) of primary cultures of cerebellar granule nerve cells from one-week-old rats resulted in a significant reduction in cell survival, with cell death observed as early as 6-12 hours after treatment initiation. Cell death was attributed to a substantial increase in extracellular L-glutamate concentration due to exocytosis triggered by high potassium concentrations. Pretreatment with botulinum toxin C, which inhibits exocytosis, prevented agmatine-induced glutamate release and cell death. This glutamate toxicity was reduced by NMDA receptor antagonists and enzymatic breakdown of L-glutamate by glutamic pyruvic transaminase. Wang et al. (2006) reported potassium concentrations below 6 mM in their experiments with cultured rat hippocampal nerve cells, where agmatine showed neuroprotective effects against NMDA and glutamate toxicity (Wang et al., 2006).

In summary, the neuroprotective or neurotoxic effects of agmatine may depend on the experimental conditions, including the concentrations of potassium and agmatine used. Nonetheless, the inhibition of the NMDA receptor is the most frequently observed mechanism underlying the neuroprotective effects of agmatine. Zhu et al. attributed the neuroprotective effects of agmatine to the inhibition of

NMDAR channels in nerve cells, as well as PC12 cells derived from the rat adrenal medulla (Olmos et al., 1999).

2.8. Mechanisms of Neuroprotection

Primary brain damage occurs when cells are damaged directly at the time of trauma, such as contusion, blood vessel injury, axonal shearing, injury to the blood-brain barrier (BBB), and meningeal injury. The secondary effects of primary damage manifest as additional damage in neurons that were initially unaffected. Secondary injury can result from conditions like ischemia, cerebral hypoxia, hypotension, cerebral edema, alterations in blood flow and supply to the central nervous system (CNS), increased intracranial pressure, reduced CO₂ levels, acidosis, inflammation of the meninges, and brain abscess (Kotagale et al., 2019).

The primary event is the lack of oxygen, which impairs the normal ATP production in neurons. The cellular adaptations to generate energy without oxygen lead to the production of lactic acid. As ATP-dependent ion transport processes cease, the cell becomes depolarized, allowing ions like Ca²⁺ to enter the cell. In the absence of active ion pumps, the intracellular levels of Ca²⁺ become elevated. The presence of Ca²⁺ triggers the release of the excitatory amino acid neurotransmitter glutamate into the extracellular space, leading to its toxic levels (Kotagale et al., 2019).

If the cell undergoes necrosis, it releases glutamate and toxic substances into the surrounding cells, affecting them as well. Glutamate stimulates AMPA receptors and Ca²⁺-permeable NMDA receptors, allowing more Ca²⁺ to enter the cells. The high levels of Ca²⁺ activate enzymes that disrupt structural and regulatory proteins, cleave lipid sheaths, and increase the production of harmful chemicals like free radicals and reactive oxygen species (a process known as excitotoxicity). Ca²⁺-dependent enzymes, such as phospholipases, modify membrane permeability and the function of receptors, ion channels, and other proteins. Disruption of mitochondria leads to the release of toxins and apoptotic factors into the cellular matrix (Kotagale et al., 2019).

The caspase-dependent apoptosis pathway is activated, and during reperfusion, several factors contribute to reperfusion damage. Structural damage during reperfusion is believed to result from excessive generation of oxygen free radicals. Peroxynitrite, formed by the reaction of oxygen with NO (produced by neuronal constitutive or inducible nitric oxide synthase), is one of the main initiators of lipid peroxidation

during reperfusion, along with the inflammatory response following reperfusion damage (Kotagale et al., 2019).

2.9. The Neuroprotective Properties of Agmatine

To demonstrate the potential of agmatine as a promising target for future pharmacotherapies, the following sections aim to evaluate the findings reported in medical and veterinary studies, providing a comprehensive understanding of agmatine's role in neuronal degenerative disorders. Over the years, several independent research clusters have examined the neuroprotective properties of agmatine using various animal models (Gad M. Gilad and Gilad, 2000) were among the first to provide evidence of the beneficial effects of agmatine in a medullar ischemic model. Agmatine administration improved motor function recovery and prevented damage to medullar motor neurons following ischemic injury in rodents. Similarly, in a temporary ischemic cat model, agmatine administration reduced apoptotic cells detected through TUNEL staining and diminished ischemic neuronal damage observed with hematoxylin and eosin staining (D. J. Kim et al., 2006).

Furthermore, studies have shown that agmatine injection exhibited positive effects against temporary cerebral ischemia in rodents by reducing edema formation and neuronal toxicity. In a recent study, the properties of agmatine and piperazine-1-carboxamidine (an agmatinase blocker) were assessed in hippocampal slices subjected to oxygen deprivation. Consistent with previous findings, the treatments demonstrated beneficial effects compared to the control group treated with saline after reoxygenation (John E. Piletz et al., 2013).

Several cellular pathways have been associated with the protective properties of agmatine in ischemic models. In cell culture, agmatine demonstrated neuronal protective properties against hypoxia by reducing lactate dehydrogenase levels, phosphorylated c-Jun N-terminal kinase (JNK), and nuclear kappa B (NF- κ B) proteins, which are linked to cellular death (Hong et al., 2007).

Additional studies evaluated the effects of agmatine on oxygen-glucose-deprived primary-cultured neuron-supporting astrocyte cells. Agmatine treatment improved cell survival and promoted NF- κ B translocation to the nucleus, indicating that this polyamine can prevent astrocytic cell death (Lee et al., 2009) . It was also found that agmatine treatment reduced aquaporin levels, resulting in improved motor

functions and reduced edema formation and blood-brain barrier damage in rodents (J. H. Kim et al., 2010).

These findings suggest that agmatine can mitigate ischemic effects by reducing aquaporin expression and central nervous system fluid volume. Agmatine has also demonstrated beneficial effects in experimental models of spinal cord injury. Mice chronically treated with agmatine for one month showed enhanced recovery of medullar tissue integrity (indicated by reduced collagen markers, which impede axon regeneration), increased expression of the neuroprotective marker bone morphogenetic protein 7 (BMP-7), and decreased expression of transforming growth factor-beta 2 (TGF- β 2), a marker of collagen deposition (J. H. Kim et al., 2011).

Another recent publication reported that chronic agmatine treatment (35 days) reduced damage caused by spinal cord compression. The protective effects of agmatine included improved motor and urinary bladder function, reduced demyelination, and decreased neuronal damage and glial cell accumulation at the lesion site (Park et al., 2013).

A study examining the effects of agmatine on cellular models of glutamatergic neuronal toxicity demonstrated that agmatine exerted neuronal protective effects in cerebellar granule cells in the presence of glutamate. Moreover, studies conducted on hippocampal neuron cultures (Wang et al., 2006), and PC12 cells, derived from rodent adrenal medulla pheochromocytoma, showed that pharmacological treatment with agmatine had a protective role in a glutamatergic model of epilepsy (Zhu et al., 2003).

Overall, these findings suggest that the antagonism of NMDA receptors plays a significant role in the neuroprotective mechanism of agmatine. Another crucial factor that may contribute to agmatine's neurodefensive effects is its antioxidant properties. Previous studies have demonstrated that agmatine exhibits neuroprotective effects against hydrogen peroxide-induced impairment of retinal culture cells (Yoko et al., 2008).

Mitochondrial function is vital for optimal neuronal function. In this regard, agmatine's involvement in the mitochondrial scenario has been investigated. It has been shown that agmatine administration preserved mitochondrial function and reduced apoptosis, necrosis, DNA damage, and chromatin condensation in cells. Agmatine also demonstrated antioxidant properties by reducing oxidative stress, the expression of B-cell lymphoma 2 (Bcl2), caspase-3, and apoptosis induced by

compounds such as 5-fluoracil. Taken together, these findings strongly suggest that agmatine's mitochondrial defense may contribute to its neuroprotective effects (Arndt et al., 2009).

2.10. Agmatine Role in Neurodegenerative Disease

2.10.1. Effects of Agmatine in Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder, characterized by the accumulation of amyloid-beta peptide ($A\beta$) outside the cells and the presence of neurofibrillary tangles composed of the tau protein. These pathological hallmarks initiate a series of events leading to synaptic dysfunction, neuronal damage, and ultimately the loss of cognitive abilities and memory seen in AD. The global prevalence of AD varies due to differences in nutritional, environmental, and social factors. Age is the primary risk factor, with the incidence increasing with advancing age, ranging from 0.5% in individuals aged 65-69 years to 6% in those over 85 years (McDowell, 2000; Perez Ortiz and Swerdlow, 2019).

Various risk factors have been associated with an increased likelihood of developing AD, including the presence of the ApoE e4 allele, cerebrovascular disease, hyperlipidemia, smoking, diabetes, obesity, and traumatic brain injury. Conversely, protective factors include a higher cognitive reserve, adherence to a Mediterranean diet, and regular exercise (Mayeux and Stern, 2012).

While the exact pathophysiology of AD remains unclear, studies have implicated mitochondrial dysfunction, oxidative stress, neuroinflammation, synaptic deficits, and apoptotic cell death in the progression of the disease. Agmatine, a compound with potential therapeutic properties, has been investigated in animal models of AD.

Administration of agmatine before and after $A\beta$ peptide infusion was found to mitigate memory impairment in mice tested in various cognitive tasks (Bergin and Liu, 2010). Moreover, alterations in arginine metabolism and reduced levels of NOS and agmatine in the hippocampus and prefrontal cortex have been observed in AD, suggesting the involvement of the agmatinerbic system in the cognitive deficits associated with $A\beta$ (Liu et al., 2011).

Studies examining polyamine metabolism in AD have demonstrated abnormal activity of ornithine decarboxylase and altered polyamine levels in the brains of AD patients. The upregulation of polyamine metabolism by $A\beta$ peptides has been observed

in cell cultures, suggesting that polyamine supplementation may hold therapeutic potential in AD (Perez Ortiz et al., 2019).

Although limited research exists on the effects of agmatine in AD models, both in animals and humans, further studies are warranted to fully evaluate the benefits of agmatine for the prevention and treatment of AD (Moretti et al., 2014).

2.10.2. Effects of Agmatine in Parkinson's Disease

Recently, Parkinson's disease (PD) can be diagnosed with a consistent level of accuracy, and there is a reasonable clinical correlation with pathological findings. The characteristic symptoms of resting tremor, bradykinesia, postural instability, and autonomic dysfunction, which typically manifest in older age and progress slowly, align well with the underlying pathology observed in post-mortem examinations of PD patients. However, non-motor symptoms such as cognitive and emotional fluctuations are more variable, although it is increasingly recognized that a significant proportion of patients experience cognitive difficulties, particularly in the later stages of the disease. The exact causes of PD remain unknown, but substantial evidence suggests a multifactorial etiology involving genetic and environmental factors (Ross and Smith, 2007).

While the primary pathological feature of PD is the degeneration of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNc), several studies have indicated that neurotransmitters, particularly glutamate, play a significant role in the pathophysiology of the disease. Dopamine deficiency leads to increased activity of the subthalamic nucleus (STN), which consists of neurons that utilize glutamate as a neurotransmitter. These STN neurons project to different regions such as the external and internal segments of the Globus pallidus (GPe and GPi), the substantia nigra pars reticulata (SNr), and the pedunculopontine nucleus (PPN) (Di Monte, 2003).

Additionally, STN neurons provide excitatory innervation to dopaminergic neurons in the substantia nigra pars compacta (SNc), which also possess glutamate receptors. Stimulation of the STN leads to increased firing of SNc dopaminergic neurons. This suggests that the excessive activity of STN neurons resulting from dopamine depletion may induce toxic damage in vulnerable brain regions, including the SNc. Moreover, the decreased activity of complex I, an enzyme involved in energy production, observed in the nigra of PD patients, may contribute to mitochondrial dysfunction and make SNc dopaminergic neurons more susceptible to even normal

levels of glutamate. It is hypothesized that the dopamine loss in PD leads to increased STN activity, which, in turn, exacerbates the damage to vulnerable dopaminergic neurons, creating a vicious cycle of neuronal loss in the SNc. Additionally, increased STN activity may affect the GPi, SNr, and PPN, potentially explaining the development of parkinsonian symptoms that are unresponsive to levodopa treatment in advanced stages of the disease (Barua et al., 2019a).

Based on this theory, pharmacological or surgical interventions aimed at reducing STN neuronal overactivity or inhibiting glutamate receptors in the SNc and other target structures may have neuroprotective effects and potentially slow or halt the progression of neurodegeneration in PD. Studies investigating glutamate receptor signaling in the basal ganglia have demonstrated increased glutamatergic transmission, which can be detrimental to remaining dopaminergic neurons, leading to further deterioration of dopaminergic transmission and disease progression (Kotagale et al., 2019).

Supporting the involvement of the glutamatergic system in PD, clinical and preclinical evidence has shown the efficacy of memantine and amantadine, both NMDA receptor antagonists, in the management of PD. Considering that drugs modulating the glutamatergic system may have beneficial properties in PD treatment, some studies have explored whether agmatine, when administered, could provide neuroprotection in experimental models of PD. In a rat model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), agmatine administration after MPTP treatment provided partial protection (31%) against dopaminergic toxicity in the SNc. However, the same study found that concurrent administration of agmatine and MPTP exacerbated the dopaminergic toxicity induced by MPTP (Barua et al., 2019a).

Another study investigated the effects of agmatine on motor and cognitive impairments in aged rats following intranasal administration of MPTP, a newly established model of PD. Agmatine, administered for five consecutive days before MPTP treatment, was able to prevent the development of motor, cognitive, and emotional deficits caused by MPTP. Furthermore, agmatine protected the hippocampus from glutamate-induced damage and preserved tyrosine hydroxylase levels in the SNc. Notably, agmatine reduced the MPTP-induced neuronal death by approximately 40% (Berman and Bayati, 2018).

The neuroprotective effects of agmatine were also observed in SHSY5Y cell cultures exposed to rotenone, another experimental model of PD. Agmatine administration dose-dependently reduced cellular damage caused by rotenone by inhibiting oxidative stress. Agmatine also prevented rotenone-induced activation of apoptotic pathways (caspase-3, Bax, cytochrome c). Moreover, Fourier transform infrared spectroscopy analysis revealed that agmatine significantly attenuated rotenone-induced redox changes and cellular damage at the molecular level, further supporting the potential therapeutic role of agmatine in PD management (Rushaidhi et al., 2013).

2.11. Lipopolysaccharide Morphology

The core oligosaccharide, and the O antigen are the three structural domains that make up the massive glycolipid known as LPS, the acylated glucosamine disaccharide that makes up Lipid A, the hydrophobic part of the molecule, forms the OM's outer leaflet, the glucosamines are phosphorylated at the 1 and 4' locations and acylated at the 2, 3, and 2' and 3' positions in *Salmonella* and *E.coli*, the distal glucosamine generally also contains two extra secondary acyl chains, making mature lipid A mostly hexa-acylated, the nonrepeating oligosaccharide that makes up the heart of the lipid A is linked to its glucosamines, heptoses, other hexoses, and 3-deoxy-Dmanno-oct-2-ulosonic acid (Kudo) residues are typically found in the core structure which can be altered by adding phosphates and other substituents, like phosphoethanolamine, the core oligosaccharide of the O antigen is joined to an extended polysaccharide. It consists of an oligosaccharide that repeats and contains two to eight sugars (Raetz & Whitfield, 2008).

LPS general structure is conserved, although there are numerous species- and strain-specific changes that can happen. In a similar way the fundamental oligosaccharides differ between organisms and even among certain strains of one species, but the O antigen is the most variable part of LPS. The lipid A structure is conserved at the species level, but as will be discussed below, it can undergo controlled variations as a reaction to environmental circumstances (Bertani & Ruiz, 2018).

2.11.1. Lipopolysaccharide Functions

LPS may have different structures in different bacteria, but in all cases, this glycolipid covers a large portion of the cell surface and creates a permeability barrier

to stop the entry of harmful chemicals like antibiotics and bile salts. LPS frequently plays a significant role in bacterial pathogenicity since it is the main bacterial component that the host immune system encounters. The strong amphipathic character of LPS contributes to its barrier function. Like other bi layer lipids, lipid A's acyl component has hydrophobic properties that prevent molecules that are hydrophilic from passing through the OM. The OM is highly resistant to hydrophobic substances as well because, unlike other bilayers, the core oligosaccharide and O antigen both give LPS substantial hydrophilic property (Carpenter et al., 2016).

LPS (1 g/kg to 250 g/kg) significantly decreased body weight, raised corticotrophin hormone (ACTH) (doses ranging from 15 g/kg to 250 g/kg), and decreased serum corticosterone levels (doses ranging from 5 g/kg to 250 g/kg) after 24 hours post-intraperitoneal injection (Bison et al., 2009). The serum levels of LPS also increased those of TNF- and IL-1 β , with dosages ranging from 1 to 250 ng/kg and 5 to 250 ng/kg, respectively (Ramírez et al., 2019).

LPS is a PAMP, or pathogen-associated molecular pattern, since it is widely distributed on the surface of bacterial pathogens and because the host immune system has evolved to react strongly to its presence (20, 23, 24). This response may even be so severe as to be harmful to the host. Consequently, LPS has historically been referred to as an "endotoxin" in relation to the cell-associated (endo) toxicity seen in numerous Gram-negative species. Since the lipid A structure is the most conserved component of LPS and the host toll-like receptor 4 (TLR4) is its major receptor, it makes sense that the immune system has evolved to respond to it first (Bertani & Ruiz, 2018).

LPS is a bacterial endotoxin that causes neuroinflammation and is used as an inflammagen in numerous animal model studies to examine the relationship between inflammation, brain function, and memory. Several lines of research have shown that LPS and other toxic agents such as ethanol and d-galactose activate numerous ROS-mediated neuroinflammatory and apoptotic signaling pathways that in turn cause neurodegeneration and memory deficits. It is generally known that LPS therapy increases the production of cytokines and ROS, which ultimately causes neuronal cell death and memory problems (Khan et al., 2019).

The most well-known of the various mechanisms for LPS-induced neuronal damage is the increased creation of ROS, which consequently increases oxidative stress and neuronal damage. Additionally, an increased level of ROS can activate other

mediators such as phosphorylated-nuclear factor kappa B (p-NF-B), tumor necrosis factor (TNF), and interleukin-1 (IL-1), which can impair memory by affecting the function of neuronal cells in the hippocampus through neuronal apoptosis. Additionally, recent investigations have shown that intraperitoneal administration of LPS can activate astrocytes and microglial cells, resulting in the processes of microgliosis and astrogliosis, respectively. By inducing proinflammatory mediators, astrocyte and microglial activation causes neurotoxicity, neuroinflammation, and the generation of ROS (Khan et al., 2019).

2.12. Effect of LPS Doses on Cells

Different cell lines have wildly varying levels of sensitivity to the toxicity of LPS. The levels of circulating tumor necrosis factor alpha (TNF- α), which form in response to LPS, seem to be connected with host susceptibility to LPS, despite the fact that the processes underlying this variability are not well known. As an illustration, human cells are incredibly sensitive to LPS, with a fatal dose of just 1 to 2 g. There are various previous studies done on human and animals (rats and rabbits) (Redl et al., 1993) shows in rats 100 μ g LPS/kg BW alone resulted in TNF production at 2 hours post-endotoxin that was comparable to animals that received 15.0 mg LPS/kg BW. These findings show a correlation between TNF synthesis and LPS concentrations up to 100 μ g/kg BW, at which point a plateau is attained. Injection of 100 μ g/kg BW of LPS alone did not have any deadly consequences in normal rats, whereas sensitized rats experienced an 82% mortality rate after 48 hours. For normal rats, a dose of 15 mg/kg would be necessary for such a mortality rate. On the other hand, deficient literature for cell line to detect the exact toxic dose of LPS (Redl et al., 1993).

Jenny Resiliac et al., (Resiliac et al., 2020) With relatively low dosages of LPS (0.1–0.03 mg), LPS-mediated survival from a deadly paramyxoviral infections is highest. Interestingly, these are the doses that have been linked to the onset of atopic disease.

2.13. Dosage Justification

Determining the appropriate dosage of agmatine is a crucial step that requires careful consideration based on previous studies. Concentrations of agmatine sulfate should be adjusted, taking into account absorption values assuming high solubility and low permeability reabsorption. Additionally, considering the permeation of agmatine

through cell walls via selective carriers, which may interact with other substances such as polyamines, is important. It is essential to evaluate the effectiveness of agmatine at similar or different dosing regimens and consider cellular enzymatic events that might impact its dosage (Gad M. Gilad and Gilad, 2013).

To investigate the effects of agmatine on solute and water elimination, studies were conducted in Sprague-Dawley rats. Agmatine was administered either through intracerebroventricular injection (0, 10, 100, 300, or 1000 nmol in 5 microliters) or direct intra-renal distillation (0, 3, 10, 30, or 100 nmol/kg/min at 3.4 microliters/min) in pentobarbital-sedated mice. Only intra-renal administration of agmatine led to an increase in creatinine clearance, with the lowest dose (3 nmol/kg/min) and highest dose (100 nmol/kg/min) showing this effect (Penner and Smyth, 1996).

A second group of researchers (J. E. Piletz et al., 2003) employed two models of analysis. In the first, rats received arginine (600 mg/kg) or agmatine (10, 50, or 300 mg/kg) intraperitoneally (i.p). After 1 or 3 hours, the rats were gas anesthetized, their CNS was perfused with frozen saline, and whole-CNS agmatine was determined using HPLC (High-Performance Liquid Chromatography). A rhesus monkey was given either agmatine (0.3 mg/kg i.c.v.) or arginine (150 mg/kg) intravenously (i.v) or intracerebroventricularly (i.c.v.) under gas anesthesia in parallel studies (J. E. Piletz et al., 2003).

Table 2.1. Summary of the studies showing agmatine doses and route of administration (Kotagale et al., 2019)

Investigational model	Subject	Agmatine dose/ route	Detected Outcome	Reference
Spinal cord ischemia	Rat	100 mg/kg (i.p.)	Quicker recovery of motor task. Avoidance of damage of motor neurons in the spinal cord.	(Gilad and Gilad, 2000)
Ischemia/ reperfusion injury	Cat	100 mg/kg (i.v.)	Decrease of TUNEL-positive cells. Reduced neuronal injury.	(Kim <i>et al.</i> , 2006)
Middle cerebral artery occlusion	Rat	100 mg/kg (i.p.)	Hasten the recovery of motor and proprioception shortages. Inhibition of CAN infarction, gliosis, oedema, apoptosis plus neurotoxicity.	(Wang <i>et al.</i> , 2010)
Occlusion of the middle cerebral artery	Mouse	100 mg/kg (i.p.)	Decrease of CNS edema, infarct volume, liquid content and reduction in the altitudes of aquaporin	(Kim <i>et al.</i> , 2010)
Spinal Cord Injury	Mouse	100 mg/kg (i.p.)	Enhanced surface righting reflex. Decrease of collagen scar	(Kim <i>et al.</i> , 2011)
Spinal Cord Injury	Mouse	100 mg/kg (i.p.)	Blocking of demyelination actions. Reduced neuronal loss and glial wound near by the lesion location.	(Park <i>et al.</i> , 2013)
Oxygen-glucose deprivation	Rat hippocampal slices	100 mg/kg (i.p.)	Reduced deterioration of neurons.	(Piletz <i>et al.</i> , 2013)
Hypoxic culture	Retinal ganglion cells	100 μ M and 500 μ M	Reduced quantities of LDH, phosphorylated JNK plus NF-KB.	(Hong <i>et al.</i> , 2007)
Oxygen-glucose deprivation	Primary-cultured astrocytes	100 μ M	Amplified the cell feasibility.	(Lee <i>et al.</i> , 2009)
Glutamate-induced neurotoxicity	Hippocampal neurons culture	100 μ M	Protection of glutamatergic neurotoxicity. Reduced neuronal expiry encouraged by glutamate.	(Olmos <i>et al.</i> , 1999)
Oxidative stress injury	Retinal ganglion cells	upto 100 μ M	Amplified cell feasibility. Reduction of DNA destruction.	(Arndt <i>et al.</i> , 2009)

2.13.1. Calculation of IC₅₀

The medication concentration needed to provide a 50% inhibition is known as the IC₅₀. The assay circumstances have an impact on the operational term "IC₅₀." If total inhibition is desired, IC₉₀ or IC₉₉ may be utilized. The calculation of Fractional occupancy demonstrates that, under the assumption of one site binding at equilibrium and a Hill coefficient of 1, the IC₉₀ concentration is around ten times greater than the IC₅₀ concentration. The IC₅₀ concentration is around 100 times higher than the IC₉₉ value. The percent bound, also known as fractional occupancy, results from the equation $\text{percent bound} = \frac{[I]}{[I] + K_I}$, where [I] is the concentration of the inhibitor and K_I is the equilibrium dissociation constant for I (Swinney, 2011b).

How does the half maximal inhibitory concentration (IC₅₀) relate to binding kinetics? As was already said, k_{on}, k_{off}, and K_I are fundamental to a bimolecular interaction, whereas IC₅₀ is an operational term based on the test circumstances. For instance, even when the K_I, k_{on}, and k_{off} for a competitive inhibitor remain unchanged, the IC₅₀ will rise with rising substrate concentrations. The Cheng Prusoff relationship, where [S] is the substrate concentration and K_m is the Michaelis constant, uses math to describe how the IC₅₀ changes as a result of competition. Inhibition that is not competitive has an IC₅₀ that is equal to K_I (Swinney, 2011a).

2.13.2. IC₅₀ of Compounds

Calculating the percent inhibition as a function of each drug concentration, a four-parameter logistic model was used to fit the data. The IC₅₀ value, which was calculated as the mean of at least two independent experiments, was defined as the concentration of a substance at which the percent inhibition is equal to 50. Since the concentration of VPS34 was 5 μM, the minimal IC₅₀ should be 2.5 μM, according to Eq. The smallest value that we could really quantify was 1 μM, which falls in line with the hypothesis that some protein was lost owing to nonspecific binding to surfaces. Our best inhibitors were thought to have sub nanomolar affinities. Tight binding formulae can be used to determine a K_i below the enzyme concentration; however, this wasn't originally planned due to practical issues. By using surface plasmon resonance, we evaluated the affinities of our top inhibitors (Fassy et al., 2017a).

2.13.3. Calculation of IC₅₀ Occurs in 2 Steps

Perform the following calculation: % inhibition at each inhibitor concentration. % inhibition is equal to 100 times $[1 - (\text{ABS} - \text{MIN}) / (\text{MAX} - \text{MIN})]$, where ABS is the absorbance after treatment with the inhibitor, MAX is the absorbance at a concentration of 0, and MIN is the absorbance when a control inhibitor completely inhibits cell growth. Use nonlinear regression to fit the data on the percentage of inhibition versus inhibitor concentration to an appropriate IC₅₀ equation, such as the logistic or Hill equation. Among them is: Where n is the Hill coefficient, percentage inhibition is calculated as $(100 \times [I]^n) / (\text{IC}_{50} + [I]^n)$ (Fassy et al., 2017b).

2.14. Microglia

2.14.1. Source, Phenotypes and Function

The initial description of microglia was provided by Río-Hortega in the early twentieth century, who identified them as non-neuronal elements originating from oligodendroglia and astroglia. Despite extensive and repeated research, the origin of microglia has remained a topic of debate. Researchers have proposed that microglia can derive from mesodermal cells, which belong to the middle layer of the three embryonic layers, as well as from pial rudiments, pericytes, and neuroectodermal macroglia (Ginhoux and Prinz, 2015).

However it remained by now projected that microglia originate from yolk-sac macrophages Alliot et al. (1999), confident proof was just providing a ten years later when it was presented that microglia originate after yolk-sac primitive myeloid antecedent cells. The apparent phenotypic resemblances among microglia and further mononuclear cells groups guide to organized recognition of the conception of their myeloid source, though the correct uniqueness of microglial precursors remains debatable up to now. Preliminary studies designated the existence of microglial cells throughout initial growth, signifying that microglia ascend since embryonic progenitors. These progenitors remained initial suggested by del Río-Hortega to exist meningeal macrophages penetrating the CNS through premature embryonic development. However, the mainstream of the scientific society at that period, comprising del Río-Hortega himself, assumed that microglia might similarly be derivative from plasma monocytes (Ajami et al., 2007).

Monocytes are certainly engaged to the newborn and mature brain, in the concluding situation, maximum frequently under inflammatory situations, anywhere they can differentiate into microglia-like cells. These clarifications long supported the fundamental perspective that circulating monocytes exemplified microglial progenitors, substituting those scattering in the CNS throughout embryonic growth. In fact, pending up to now, the maximum consensual suggestion was that fetal and perinatal hematopoietic waves of microglial enrolment and discrepancy happened in the CNS (Ginhoux & Prinz, 2015). In rats, movement and settlement of yolk-sac-derived macrophages to and into the central nervous system begins among E8 and E10. During fetal development and throughout adulthood, microglia are sustained through self-renewal without replacement from blood-derived stem cells, establishing them as distinct residents separate from hematopoietic macrophages and monocytes (Ginhoux and Prinz, 2015).

Under homeostatic conditions, microglia constantly survey the brain environment using their highly mobile processes, and they are best described as "neutral" or "M0" rather than "resting" cells. M0 microglia receive inhibitory signals from the brain environment, including the transforming growth factor beta (TGF- β). Interactions between microglia and neurons, facilitated by CD200R-CD200 interaction, provide further inhibitory signals to microglia. Disruption of these blocking signals leads to alterations in microglia phenotype (Butovsky et al., 2014).

One characteristic of activated microglia is the increased expression of activation markers such as CD11b and CD45. Additionally, microglia undergo a loss of their ramified morphology and can form clusters, which are not typically observed in healthy brain tissue (Hoek et al., 2000).

It is important to note that the M0 phenotype itself may vary with age and also shows regional differences within the central nervous system (Olah et al., 2011).

Microglia have the ability to change their phenotype in response to specific growth factors or cytokines. *In vitro* exposure to interferon gamma (IFN γ) and/or lipopolysaccharide (LPS) has been associated with morphological changes from ramified to amoeboid and the induction of an activated "M1" phenotype, which is linked to neuroinflammation. Conversely, *in vitro* exposure of microglia to anti-inflammatory cytokines such as interleukin-4 (IL-4) can induce an alternative "M2" phenotype. Recent studies have demonstrated that macrophages and microglia can

exhibit a wide range of intermediary phenotypes both *in vitro* and *in vivo* (Nimmerjahn et al., 2005).

The current understanding of the ApoE pathway as a driver of microglial phenotype modulation in Alzheimer's disease has raised further doubts about the usefulness of the traditional dichotomous M1-M2 phenotype model. Microglia, similar to other macrophages, are phagocytic cells capable of releasing a wide range of chemokines and pro- and anti-inflammatory cytokines. While microglial inflammatory responses have been extensively studied, there is growing recognition of their contribution to brain development and homeostasis. Microglia release neuronal growth factors that regulate the proliferation of oligodendrocytes, astrocytes, and neural stem cells, and they contribute to the formation of neural circuits (Ransohoff, 2016).

During brain development and the maturation of neurons, microglia participate in the elimination of excess neurons. Additionally, microglia are involved in synapse pruning or synaptic remodeling, which is crucial for establishing efficient neuronal connections. Inappropriate synaptic contacts are labeled by complement components C1q and C3, and microglia expressing the complement C3 receptor can recognize and eliminate these synapses (Schafer et al., 2012).

2.14.2. Microglial Production of Cytokine and It's Measurements

Microglia, which are fixed macrophages of the central nervous system (CNS), play a crucial role in the innate immune response of the CNS (Cytology and cellular pathology of the nervous system. In Penfield W (ed) Microglia. P. B. Hoebaer, New York, NY, pp 483–534). Microglial activation is typically characterized by the release of cytokines and chemokines in response to microbial invasion, such as LPS (Kaushik et al., 2010).

Microglia share functional similarities with macrophages and exhibit functional plasticity during activation. While they are relatively quiescent in the resting or "ramified" state, they perform surveillance functions (Hoek et al., 2000).

In addition to the resting state, microglia can adopt two functionally distinct activated states, M1 and M2. The former is typically induced by stimuli like LPS or IFN- γ and leads to neuronal damage through the release of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and reactive oxygen/nitrogen species (ROS/NOS). In contrast, the amoeboid M2 state acts as an anti-inflammatory state by suppressing the release of pro-inflammatory cytokines, phagocytosing debris, promoting tissue

repair, and releasing neurotrophic factors . *In vitro* culture methods can be used to manipulate microglial activation states to study various homeostatic or pathological conditions (Henkel et al., 2009).

Cytokines and chemokines are low-molecular-weight proteins that mediate cell-to-cell communication and play crucial regulatory roles in various cell types (Borish and Steinke, 2003) . Their quantification can be achieved through enzyme-linked immunosorbent assay (ELISA) or immunoblot procedures, which are time-consuming and labor-intensive (D. J. Kim et al., 2006).

Flow cytometry, including cytokine bead array (CBA), offers a more efficient and rapid method for quantifying and analyzing cytokines. CBA is based on antigen-antibody reactions similar to ELISA, where polymer beads are coated with specific antibodies for different cytokines. Detection reagents bind to the already-bound cytokines on the beads under various conditions, allowing highly specific measurement of cytokines. The beads are then analyzed using a cytometer, and the levels of different cytokines are determined based on their fluorescence intensities. CBA allows for the simultaneous analysis of multiple cytokines in a single experiment without additional hassle . Various companies offer ready-to-use kits and software for the quantification and analysis of cytokines (Neurochemistry, 2008).

Cytokine analysis using a mouse inflammation kit from BD (Becton Dickinson) was performed using a BD™ FACS Caliber instrument. The principles and ethics of the procedure are generally similar among different providers. Cytokines from the CNS can be measured from whole CNS homogenates, although it is important to note that other cell types, such as astrocytes, can also secrete cytokines. However, microglia, as the predominant immune cells in the brain and spinal cord, are responsible for releasing cytokines and executing innate immune responses during CNS pathologies (Helmut et al., 2011).

Assessing cytokines from CNS homogenates provides information about the inflammatory state of the tissue. The protein concentration of the homogenate needs to be standardized, and sensitive assays can detect as little as 10 ng/L of cytokines (M. Moretti et al., 2014). Typically, 10 µg of protein in a 50 µl sample volume is used for cytokine analysis. The cytokine beads are precoated with antibodies specific to different cytokines, and each bead corresponds to a particular cytokine. The beads may have different sizes, allowing their identification based on size. The beads are gated

and analyzed individually using forward scatter (FSC) and side scatter (SSC) plots. The BD Biosciences CBA kit includes six different beads corresponding to different cytokines, and they have similar fluorescence intensities, enabling the identification of various bead populations (Neumann et al., 2009).

There are different bead populations that can be distinguished depending on the cytokines being analyzed. The number of cytokines that can be detected depends on the assay configuration, as some systems allow for the analysis of multiple cytokines simultaneously. For example, using the mouse infection CBA kit from BD on a FACS Calibur instrument, beads corresponding to different cytokines are distributed on FL3 tubes, from brightest to dimmest, to estimate the levels of various cytokines (Neurochemistry, 2008).

Various models of microglia and microglia-like cell lines are used to study neuroinflammatory events. These models include primary microglial cultures and cultures of immortalized microglial cells, which can be retrovirus or non-retrovirus converted. While these models share similarities, they also have important differences that need to be considered when selecting an appropriate model for neurodegenerative research (Stansley et al., 2012).

2.14.3. Production of Inflammatory Molecules by Human Microglia

Microglia has been revealed to generate numerous inflammatory particles like interleukin-1 and -6 and α -tumor necrotic factor (α TNF). Human embryological microglia discharge plentiful quantity of α -TNF upon encouragement with LPS. The release is controlled by extra cytokines like IL-6, IL-10 or transmuting growth-factor. Moreover, α -TNF upregulated humanoid microglial cell creation of IL-10 *in vitro*. Cytokine generation by mature humanoid microglial cells is encouraged by humanoid T cell lymphotropic virus type I Tax protein. Between the various inflammatory particles, the question of *in vitro* invention of platelet triggering factor (PAF) is raised, a lipid particle introduced in inflammatory procedures and in cell-cell interaction (Bito et al., 1992).

PAF is secreted from stimulated inflammatory cells like white blood cells, platelets and endothelial cells, monocytes, additionally by renal, cardiac, hepatic and spleen tissues. In the brain and spinal cord PAF they are producing an extensive variety of effects. There is increasing proof that PAF is an intermediary of neuronal-damage produced by blow and shock. Furthermore, PAF produces cell toxicity effects on nerve

cells, resulting in cerebral blood vessel narrowing, is a facilitator of microbial meninges inflammation, and can play a part in prolonged CNS illnesses (Tansey et al., 2007).

However, CNS cells are definitely targets of PAF Bito et al. (1992), the cellular origin of PAF in the brain is still unclear. Nerve cells are known to generate PAF *in vitro* either naturally or after particular encouragement, although filtered astrocytic cultures remained without the ability to produce PAF. Mutually TNF α plus LPS encouraged PAF creation in a dose- and time-dependent PAF was not secreted by microglia, as previously revealed in other systems, Together TNF α and LPS encouraged PAF creation in a dose - and time-dependent means. Therefore, human microglia are introduced in a diversity of measures associated with brain illnesses, together through the collaboration with the cells of the immune system and via the creation of particles of the inflammatory reaction (Bito et al., 1992).

2.14.4. Microglia and the Histogenesis of the Nervous System

Microglia proposed to participate in the histogenesis of the nervous system via the phagocytic elimination of the dead cells happening through the monotonous cell expiry linked with growth. And for selective removal of assured axonal projections. Furthermore, they have been discharging neurotoxic elements. Adversely microglia can collect nerve growing factors, thus exercising a differing outcome, bFGF can motivate neurite development and exert trophic functions on neurons *in vitro* and *in vivo* (Tansey et al., 2007).

It also induces cell propagation in astroglia cells and in peripheral cells oligodendrocytes. Therefore, the proposition can be advanced that bFGF created by cells of the growing brain and spinal cord plays an important role in regular histogenesis of the human CNS. It was of importance to detect whether or not microglia could be a source as well as a target of bFGF, though, triggered microglia might similarly increase the production of , cytokines, chemokines, prostaglandins, and reactive O²⁻ and nitrogen kinds which might take a damaging influence on neuronal existence by stimulating oxidative trauma plus triggering cell damaging pathways, elevation of the interesting probability that long-lasting microglia stimulation might participate to the initiation and/or development of neuronal degenerative disorder (Tansey et al., 2007).

In vitro, studies of microglia phenotype plus their effect or tasks are predicted to considerably increase the capability to produce theories that are easy to be tested regarding the *in vivo* way of method of microglia in normal physiological ordinary and pathological abnormal condition states in the central nervous system. Yet conservative methods followed to attain standardized populations of microglia necessitate preservation of primary neuroglia cultures for prolonged phases of time (from one to two weeks), raising the probability of losing its external feature and microglial functional characteristics (Das et al., 2016).

Microglia shows a comprehensive kind of pattern recognition receptors in the toll-like receptor (TLR) series to distinguish bacterial and viral invaders and CNS mutilation. Between these, microbial cell walls internal toxin lipopolysaccharide (LPS), the ligand for toll-like receptor 4 (TLR4), is one of the maximum strong motivations that encourages microglial stimulation (Tansey et al., 2007).

2.14.5. Microglial Cell Lines

Microglia are immune cells that play a predominant role in the central nervous system and contribute to maintaining homeostasis and responding to changes in their microenvironment. While there have been numerous studies investigating microglial activity in CNS diseases, there is still a lack of knowledge regarding the fundamental molecular mechanisms that regulate different activation states and phenotypes of microglia. Most microglial research has been conducted using rodent cells and models, but it is increasingly evident that there are significant differences between rodent microglia and their human counterparts (Dragunow, 2008).

Although studies on microglial function often utilize neonatal and rat microglia, further research is needed to determine which microglial processes and characteristics are conserved in the mature human CNS and where they diverge from rat microglia. There is also evidence that immune responsiveness changes with age. Additionally, microglia may respond differently to peripheral immune cells due to their specific CNS environment. Therefore, the isolation and culture of mature human microglia provide a valuable tool to complement other approaches in microglial research. Primary mature human microglia offer a suitable means to study human CNS diseases due to their tissue origin and characteristics (Gibbons et al., 2007).

Cultured primary human microglia can be easily employed for various studies, including functional assays, drug screening and development, and gene profiling.

Utilizing mature human CNS cells as a research tool can play a crucial role in conjunction with *in vivo* proof-of-concept studies and contribute significantly to the translation of microglia-based treatments to the clinic. Microglia can be cultured within a mixed glial system that includes astrocytes and oligodendrocyte precursor cells, providing a high yield of microglia for high-throughput investigations when combined with 96-well microplates and advanced imaging techniques (Hanisch and Kettenmann, 2007).

Various microglial cell lines derived from mice, rats, macaques, and humans are available. Many of these lines are derived from primary microglial cultures of the CNS, which were infected with oncogenes (e.g., v-myc, v-raf, v-mil, SV40T antigen) through viral transduction. Non-transformed microglial cell lines originating from primary microglial stem cell cultures have also been described. The advantages of cell lines include ease of maintenance and their abundant availability due to their proliferative capacity. However, a major limitation is their susceptibility to dedifferentiation. Additionally, viral transformation or immortalization can alter the microglial phenotype. Recent studies have highlighted the inherent and functional differences between microglial cell lines and primary microglia or ex vivo microglia (Timmerman et al., 2018).

Furthermore, microglial cell lines derived from new-born or embryonic brain sources may not accurately reflect the phenotype of mature or aging microglia. Despite these limitations, microglial cell lines are suitable for biological, chemical, and molecular studies, as well as high-throughput screening assays that require a large cell population (Timmerman et al., 2018).

2.12.5.1. Mouse Cell Lines

Over the years, a wide range of mouse microglial cell lines has been developed. In Table 2.2, we provide a non-exhaustive list of these cell lines and will briefly discuss the two most commonly used ones, BV2 and N9. The BV2 cell line is generated by transducing fetal primary microglia with the J2 retrovirus carrying the v-raf/v-myc gene. Non-transformed cells die off during culture, while the converted cells survive for more than four weeks (Blasi et al., 1990).

BV2 cells exhibit macrophage markers such as MAC1 and MAC2, but they test negative for astrocyte marker glial fibrillary acidic protein and oligodendrocyte marker galactocerebroside. The expression of MAC2, also known as galectin-3, is particularly

important when considering murine microglial cell lines for studying neuroinflammatory processes (Burguillos et al., 2015).

For several years, BV2 cells have been used to investigate neuronal inflammation and neurodegenerative diseases, including Alzheimer's and Parkinson's disease. BV2 cells are responsive to lipopolysaccharides, possess phagocytic abilities, and increase their expression levels of reactive oxygen/nitrogen species (ROS/RNS) and pro-inflammatory genes upon exposure to A β fibrils or α -synuclein (Stansley et al., 2012).

N9 is one of a series of clonally derived microglia cell lines obtained from fetal primary microglial cultures transduced with v-myc or v-mil oncogenes of the avian MH2 retrovirus. Among these cell lines, N9 is the most widely used and cited in publications (Lynch et al., 2010).

It has been confirmed that N9 cells express immunoglobulin G receptors, glycoprotein F4/80, and MAC1 while testing negative for GFAP and GalC. Similar to BV2 cells, N9 cells produce and release pro-inflammatory cytokines in response to LPS stimulation and are capable of phagocytosing A β fibrils (Stansley et al., 2012).

2.12.5.2. Rat Cell Lines

The greatly violently multiplying immortalized (HAPI) cell line is derivative from new-born primary cultures enriched for microglia and was the 1st cell line known to be the outcome of impulsive immortalization. The inherited mutation that is accountable for immortalization is unidentified yet. HAPI cells show the microglial indicators isolectin B4, OX-42 and fructose carrier GLUT5, and are not positive for GFAP and the oligodendrocyte indicator A2B5. Contact with LPS encourages discharge of tumour necrosis element α . The invention of ROS/RNS, and HAPI cells can phagocytosis such as verified by the acceptance of fluorospheres that were mixed to the culture medium (Cheepsunthorn et al., 2001).

Cell lines derivative from rats are regularly being used in microglial functions experimental research. As current publications have exposed significant varieties amongst primary microglia and rodent microglial cell lines, and similarly among human beings and rodent microglia together in terms of elderly plus task, outcomes achieved by rodent microglial cell lines must continuously be generalized with precaution (Smith and Dragunow, 2014).

2.12.5.3. Human Cell Lines

The HMO6 cell line is a human microglial cell line created by transducing fetal primary microglia from telencephalon nerve cells using a v-myc-containing PASK 1.2 retroviral vector. Among the transduced cells, 99% were positive for the macrophage/microglia markers Ricinus communis agglutinin1 lectin and CD11b, and negative for the nerve cell marker neurofilament-medium, the astrocyte marker GFAP, and the oligodendrocyte marker myelin basic protein (Nagai et al., 2001).

The ATP accessibility and phagocytic capability of HMO6 cells were found to be similar to those of human fetal primary microglia. However, a comprehensive analysis of the induced protein profile following exposure to LPS or A β 25-35 showed that HMO6 cells secreted a less diverse range of soluble mediators compared to primary microglia (Nagai et al., 2001).

While mRNA expression analysis revealed increased levels of several soluble inflammatory mediators in HMO6 cells compared to primary microglia after 6 hours of exposure to LPS or A β 25-35, contact with LPS did not induce the expression of mRNA encoding macrophage inflammatory protein (MIP)-1 α or tumour necrosis factor alpha (TNF- α) in HMO6 cells, unlike primary microglia. Furthermore, primary human microglia exposed to LPS released IL-1 β , IL-6, IL-8, TNF- α , and MIP-1 α proteins, while exposure to A β 25-35 stimulated the release of IL-1 β , IL-8, TNF- α , and MIP-1 α proteins. In contrast, HMO6 cells exposed to LPS or A β 25-35 only triggered the release of IL-8 and TNF- α proteins (Nagai et al., 2001).

The reason for this reduced responsiveness in HMO6 cells is unclear and may be related to the cell line's derivation process. However, the limited availability of human microglial cell lines has hindered their broader use. Other immortalized human microglial cell lines, such as HMC3 (S. Martin et al., 2003), have been reported, but it is worth noting that a recent study indicated that the CHME-5 cell line, from which HMC3 originates, is of rodent origin (Garcia-Mesa et al., 2017).

Until further clarification is provided, caution should be exercised when using these cell lines. Interestingly, the group that revealed the origin of CHME-5 has developed a method to immortalize mature primary microglia obtained from mice, macaques, and humans by transducing the cells with either SV40 large T antigen alone or in combination with human telomerase reverse transcriptase (hTERT) (Garcia-Mesa et al., 2017).

2.15. Cell Viability Assays

2.15.1. Tetrazolium Reduction Assay

Now a day a diversity of tetrazolium combinations has been utilized to identify living cells. The greatest generally utilized materials comprise MTT, XTT, MTS, plus WST-1. These combinations are classified into double elementary groups:

- 1) MTT which is very charged and actively enters living nucleated cells
- 2) XTT, MTS, plus WST-1 which remain-very charged without the capability to pierce cells. The former group (MTS, XTT, WST-1) remains characteristically utilized through a transitional electron acceptor that might move electrons from the cell matrix or cell membrane to simplify the decrease of the tetrazolium into the stained formazan outcome (Riss et al., 2004).

2.15.2. MTT Tetrazolium Assay Concept

The MTT tetrazolium reduction assay is a widely used cell viability assay, especially in high throughput screening (HTS) applications. The assay involves treating cells with MTT substrate in a physiologically balanced liquid, typically at a final concentration of 0.2-0.5 mg/ml, and incubating them for 1 to 4 hours. The resulting formazan product, which is proportional to the number of viable cells, is quantified by measuring absorbance at 570 nm using a plate reader. A reference wavelength of 630 nm may be used in some cases, but it is not essential for most assay conditions (Mitra et al., 2016).

Living cells with active metabolism convert MTT into purple formazan, which can be detected by its absorbance at 570 nm. If cells die, they lose the ability to convert MTT to formazan, making the staining a reliable indicator of cell viability. The exact cellular mechanism of MTT reduction into formazan is not well understood but likely involves reactions with NADH or similar reducing agents that transfer electrons to MTT. Some studies have suggested a link between MTT reduction and mitochondrial activity based on observations with specific mitochondrial enzymes. The formazan product accumulates as insoluble precipitates within the cells' cytoplasm, near the cell walls, and in the culture medium. Before measuring absorbance, the formazan needs to be solubilized. Various solubilization methods have been used to dissolve the formazan product, fix the staining, prevent cell lysis, and reduce interference from phenol red and other medium components (Kamiloglu et al., 2020).

Common solubilization methods include acidified isopropanol, DMSO, dimethyl formamide, SDS, and combinations of detergent and organic solvents. Acidifying the solubilization solution has the advantage of changing the colour of phenol from red to yellow, which can minimize interference with absorbance measurements. Adjusting the pH of the solubilization solution can also optimize absorbance readings if sensitivity is a concern (Mitrus et al., 2018).

However, it is important to note that other assay techniques offer much greater sensitivity than the MTT assay. The amount of formazan produced depends on various factors, including the MTT concentration, incubation time, number of viable cells, and their metabolic activity. All these parameters need to be considered when optimizing assay conditions to generate a detectable signal above background. Prolonged incubation can lead to increased colour development and sensitivity up to a certain point, but the incubation period is limited due to the cytotoxic nature of the assay reagents, which deplete cellular energy sources like NADH (Kamiloglu et al., 2020).

In logarithmic growth phase cell populations, the amount of formazan product is generally proportional to the number of metabolically active viable cells, as indicated by a linear response. However, culture conditions that alter cellular metabolism can disrupt the conversion of MTT to formazan. For example, when adherent cells reach confluence and growth becomes contact-inhibited, metabolism may decrease, resulting in lower MTT reduction per cell. This can lead to a loss of linearity between absorbance and cell number. Other culture conditions, such as changes in pH or depletion of essential nutrients like glucose, can also affect cells' ability to reduce MTT. The MTT assay was developed as a non-radioactive alternative to tritiated thymidine incorporation for assessing cell proliferation (Pintor et al., 2020).

In many experimental settings, the MTT assay can directly replace the tritiated thymidine incorporation assay. However, it is important to note that MTT reduction is a measure of viable cell metabolism, not strictly cell proliferation. Tetrazolium reduction assays are often mistakenly referred to as cell proliferation assays without proper controls to confirm effects on metabolism (Huyck et al., 2012).

Immediately after the addition of MTT, the external appearance of certain cell types may undergo noticeable changes, suggesting altered physiology. The toxicity of the MTT reagent is likely dose-dependent, and increasing the dose can result in lower toxicity. Given the cytotoxic nature of MTT, the assay should be considered an

endpoint assay. A recent report suggested that formazan crystals could contribute to cell damage by membrane penetration through exocytosis (Stockert et al., 2012).

The presence of formazan crystals outside the cell, often several times larger than the cells themselves, makes it unlikely that exocytosis of these large structures is involved. The growth of crystals may indicate the accumulation of peripherally located liquid formazan where seed crystals began to form. Reducing agents are known to interfere with tetrazolium reduction assays. Substances such as ascorbic acid or sulfhydryl-containing compounds like reduced glutathione, coenzyme A, and dithiothreitol can non-enzymatically reduce tetrazolium salts and lead to increased absorbance readings in assay wells (Lü et al., 2012).

A culture medium with an elevated pH or prolonged exposure of reagents to light can also result in increased spontaneous reduction of tetrazolium salts, leading to higher background absorbance readings. Suspected chemical interference of assay compounds can be assessed by measuring absorbance readings from control wells without cells incubated with a culture medium containing MTT and various concentrations of the test compound (Momeni et al., 2020).

3. METHODOLOGY

3.1. Preparation of Cells, Substance and Reagents

3.1.1. Preparation of BV2 Cell Line Culture

After preparing the working environment and all necessary equipment are on hand. Cell culture work is done under a laminar flow hood (model mn120, power 760W AC mains 230 v.50Hz) through infection control by using sterile procedures, and all cell culture equipment and fluids are sterilized (sterilization is done using Ethanol 70% for cleaning and UV).

BV2 cells are a type of microglial cells derived from C57/BL6 murine, BV2 immortalized murine microglial cell line displaying reactive microglial cells' morphological and functional characteristics of reactive microglial cell, is maintained in 80% Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin 50µl penicillin streptomycin 50µl L-Glutamine. Material used in this study including Dulbecco's phosphate-buffered saline, and 25% trypsin-EDTA, were purchased (Sigma and Biological Industries Israel Beit HaEmek Ltd). BV2 Cell was a generous gift from my supervisor Assoc. Prof. Dr. Mehmet Emin ÖNGER (Histology and Embryology Department – Ondokuz Mayıs University Samsun -Turkey).

BV2 cells for this study were brought frozen in cryovials and kept in the refrigerator they were firstly being warmed in the water path to 37 °C, after that cells are examined under an inverted microscope to confirm their fitness, for this researcher should be familiar with spot changes in morphology and indicators of healthy cells feature under an inverted light microscope, light refracts in a ring around the cells, largely attached to the bottom of the flask and expanding at normal growth rate (typically, media will be turned from brilliant red to bright orange as a result of acidity). Cell culture is monitored for contamination and colour changes in the culture medium.

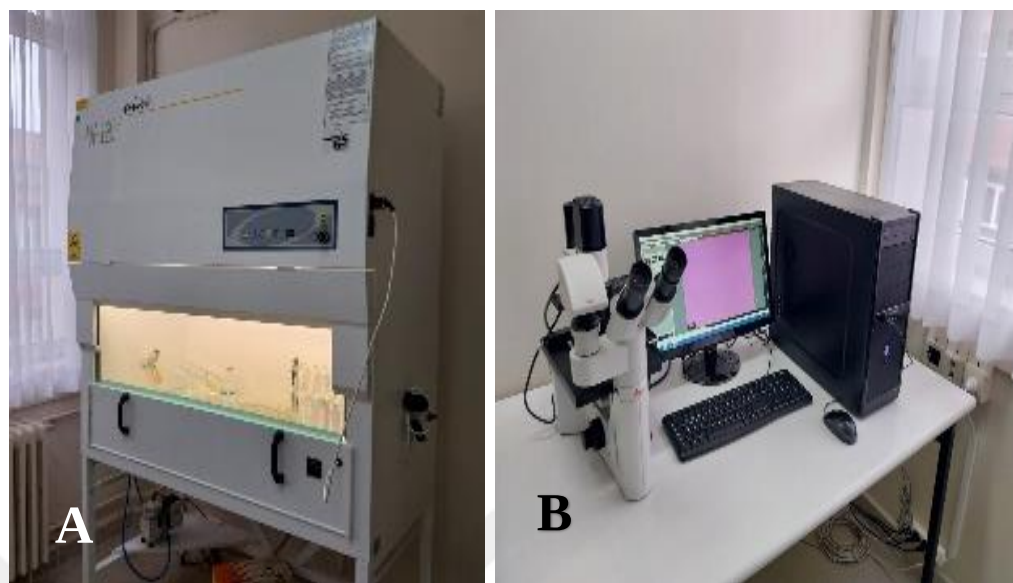


Figure 3.1. Laminar flow tube with UV light (A) and Microscope inverted connected to computer with a screen for visualizing and recording cell picture (B).



Figure 3.2. **A**-Dulbecco's modified Eagle's medium (DEMM) from (gibco) for complete media **B**- 0.25 Trypsin-EDTA (Gibco) for cells detachment **C**- Antibiotic (Penicillin 10,000 units/ml +Streptomycin 10mg/ml), **D**- Phosphate buffer saline tabs which will be dissolved and used

Subculturing, many microglial cell lines are sensitive to being over-confluent and should be subcultured when reaching around 80-90 % confluence, sub culturing is performed every 2-3 days, depending on the rate of cell line growth and the consumption of the full media, sub cultured used to be in 25 cm³ flasks, cells are

frequently split at 1:4-1:10 ratio, firstly used medium is discarded from the cell flask, and cleaned with 5ml of PBS at room temperature, flask is gently tipped to clean out any remaining PBS from the flask. 2-3ml of trypsin-EDTA will be added for cell detachment from the flask based with help of gently shaking to flask, trypsinized flask is kept for 3-5 minutes to allow the cells to detach. Cells are being checked frequently during trypsinization to avoid lengthy durations in trypsin. If the cells are taking too long to detach, tapping the flask helps to get them to lift off.

As soon as the cells have detached, 5-10ml of complete media is added (2-3 times the amount of trypsin) to inhibit trypsin action with pipetting after that transfer the cell suspension to a falcon tube for spinning all in a centrifuge at 3500g for 5 minutes. After centrifugation supernatant is removed, BV2 cells pellet is either:

Redistributed seeded in triplicated manner in 96 well plate each single well contains 5000 to 6000 cells which grow in 200 μ l complete media and incubated for 24, 48 and 72, in 95% humidity, 5% of CO₂, and 37°C, for the experiment.

Seeded in a 25 cm³ flask to repeat subculturing with writing the passage number on the culture flask before returning it to the incubator. The number of passages was kept modest in order to reduce selection pressure and divergence from the mother cell line, or frozen in cryovials with entire media and DMSO added. DMSO is added to cell freezing media as a cryoprotective agent to prevent the production of ice crystals during the freezing process, which would otherwise damage and kill the cells.

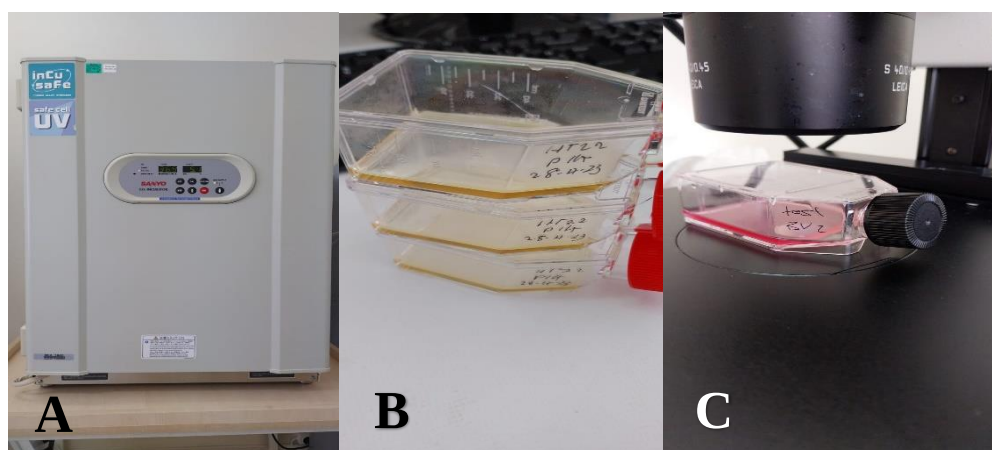


Figure 3.3. **A-** Incubator adjusted to 37°C, 95% humidity with 5% CO₂ for cell incubation, **B-** 25 cm³ Flask containing cell with acidity (brown to orange colour) **C-** 25 cm³ flask under an inverted microscope to detect cells confluences percentage.

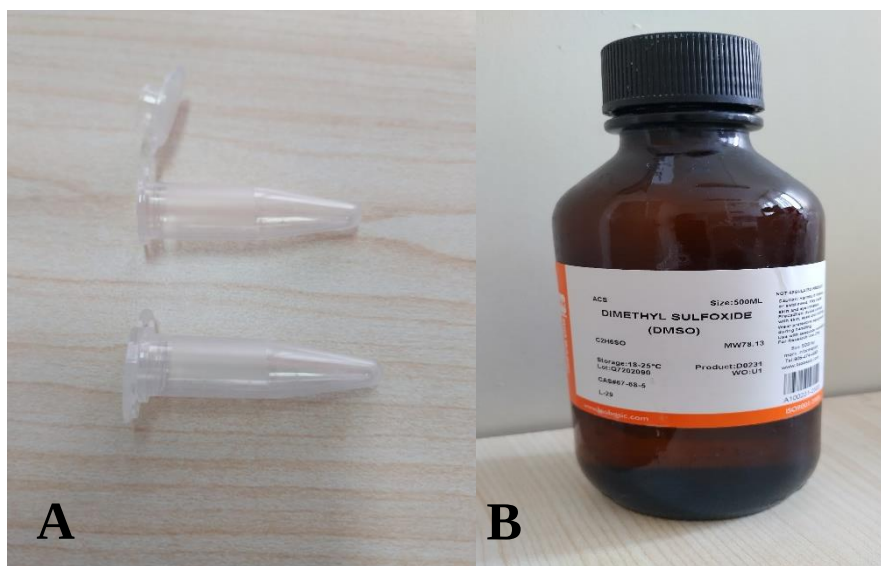


Figure 3.4. **A-** Eppendorf tubes or single-use polypropylene tubes, used for mixing, centrifuging, transferring, and packing solid and liquid samples and reagents. **B-** DMSO is used for cryopreservation in conventional cell banking. Commonly, DMSO is added to the media for cell freezing to prevent the production of ice crystals.

3.1.2. Substance Preparation

Regarding agmatine preparation, it is brought in a form of powder for the sake of this experiment. Its doses were calculated according to the equation in Table 3.1 below to make the proper concentration molarity. Agmatine will be dissolved in distilled water.

Table 3.1. Agmatine doses preparations

Dose	Concentration	Unit	V From stock	Unit	From C.M	Unit
1	5	μmol	0.050	mL	1.950	mL
2	10	μmol	0.100	mL	1.900	mL
3	15	μmol	0.150	mL	1.850	mL
4	25	μmol	0.250	mL	1.750	mL
5	50	μmol	0.500	mL	1.500	mL
6	75	μmol	0.750	mL	1.250	mL
7	100	μmol	1.000	mL	1.000	mL
8	125	μmol	1.250	mL	0.750	mL
9	150	μmol	1.500	mL	0.500	mL
10	175	μmol	1.750	mL	0.250	mL
11	0	μmol	0.000	μL	2.000	mL
12	0	μmol	0.000	μL	2.000	mL
13	0	μmol	0.000	μL	2.000	mL

The idea of this table is to prepare the needed substance in advance to keep the total amount fixed in all wells that will not affect the molarity of agmatine, and convert the mol into ml which will make easier the addition of such a small amount for instance: in the first well in which the required dose is 5 ml, only 0.05 ml from agmatine stock to the first well plate in triplicate manner in addition to 1.950 ml of complete media total amount per well is 2ml per each well. This will be repeated for the all remaining 12 doses.



Figure 3.5. **A-** Distilled water used for dissolving substances like agmatine **B-** Different sizes of pipettes that are used in the calculation of fluid size in ml and μl

Regarding the preparation of LPS, the toxic substance is brought in a form of powder, but in order to be added to the well plates it should be emulsified either in

distilled water or in phosphate buffer saline, in my experiment I prefer to use distilled water after that stock is made in order to be used in the experiment of proper dose and IC_{50} for LPS, the following table showing the calculations of doses percentage between LPS and complete media. LPS is an abbreviation for lipopolysaccharides which is extracted from bacterial cell wall to use the toxic characteristic in experimental practice, such as which is running in this research.

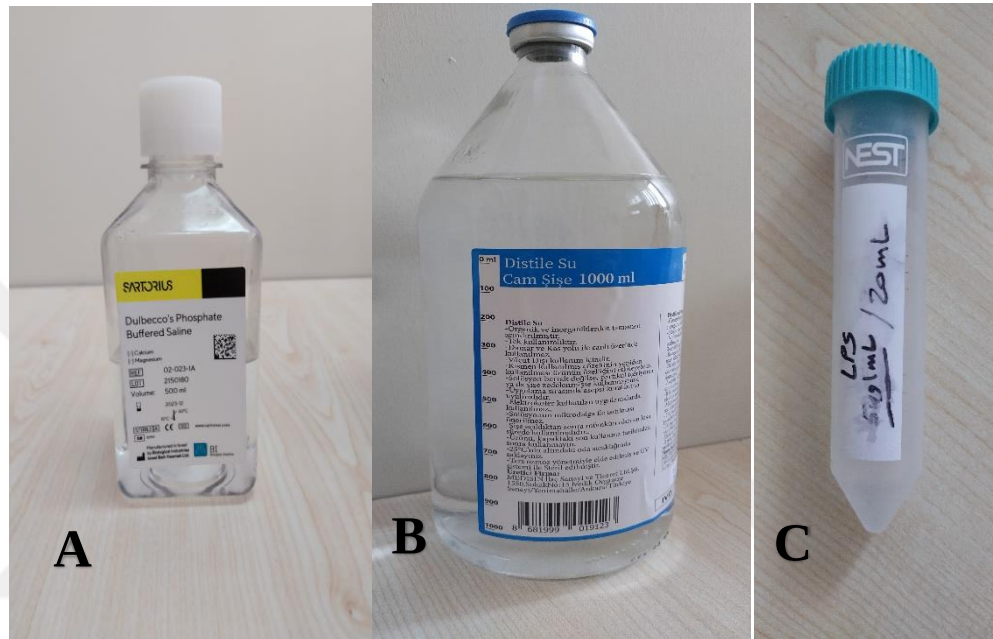


Figure 3.6. Solutions used to emulsify LPS powder is either phosphate buffer saline **A** or distilled water **B** kept in falcon tube **C** in a refrigerator

Table 3.2. Administration of LPS in different doses to BV2 microglial cells and controls

			LPS drug administration to BV2						
	1	2	3	4	5	6	7	8	9
A	Media	Media +Cells	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
			1 µg/ml	0.5 µg/ml	0.25 µg/ml	0.2 µg/ml	0.1 µg/ml	0.05 µg/ml	1 µg/ml
B	Media	Media +Cells	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
			1 µg/ml	0.5 µg/ml	0.25 µg/ml	0.2 µg/ml	0.1 µg/ml	0.05 µg/ml	1 µg/ml
C	Media	Media +Cells	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
			1 µg/ml	0.5 µg/ml	0.25 µg/ml	0.2 µg/ml	0.1 µg/ml	0.05 µg/ml	1 µg/ml

3.1.3. Preparation of reagents

3.1.3.1. Complete media preparation protocol

Materials: FBS, Pen-Strep, DMEM, a sterile filter, sterile container or bottle, 1.5 mL Eppendorf tube, 70% EtOH, serological pipettes, 10 μ L pipette tips, pipettor, and 10 μ L micropipette. The FBS should be heated for 30 minutes after it has completely thawed. The hood blower should be run for at least 15 minutes before starting.

When working in the hood, gloves and a lab coat are mandatory the following items are placed within the hood, spray them with 70% Ethanol, and then wipe them down with paper towels.

Sterile filter, sterile bottle or container, MEM, FBS, and pen-strep. Pipette and a 1.5 mL eppendorf tube, a 10 mL micropipette, and 10 mL pipette tips, and spray 70% ethanol on the entire hood surface and all items, then wipe everything dry. The following information should be labelled on the container:

Media MEM a + 10% FBS + 1% PS + 6.5 L/L of 2-ME Name / Date

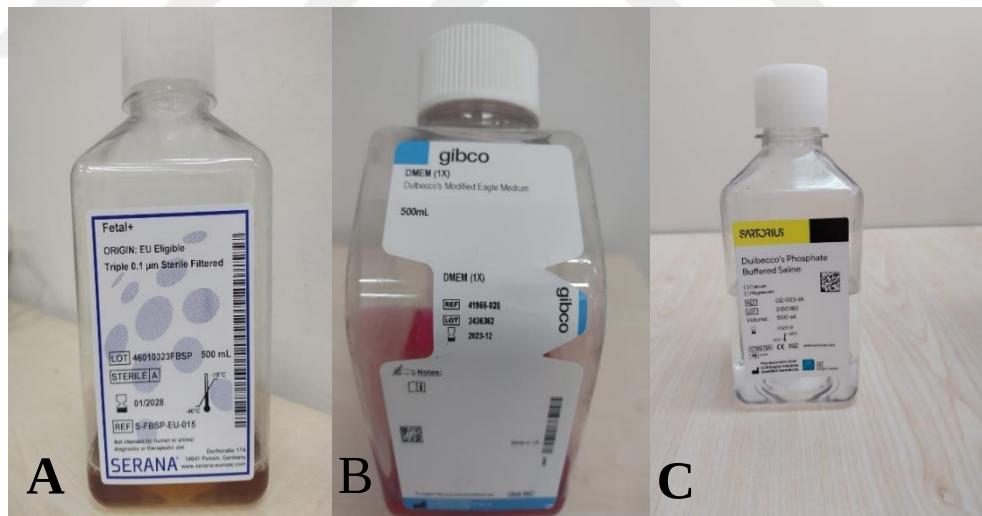


Figure 3.7. **A-** Fetal bovine serum **B-** DMEM **C-** Antibiotics (Penicillin 10,000 units/ml + Streptomycin 10mg/ml), usually DMEM contains glutamate for that researcher has to be careful toward duplication of glutamate dose in complete media formation.

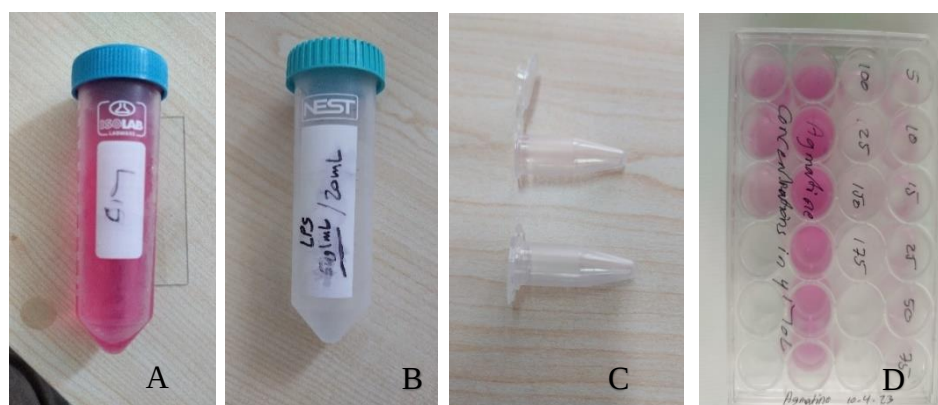


Figure 3.8. **A-** Falcon tube containing complete media after preparation and mixing **B-** LPS Solution **C-** Cryovials **D-** Stock volume of agmatine

3.1.4. MTT Solution

MTT powder is dissolved in Dulbecco's phosphate buffered saline, pH=7.4 (DPBS) to 5mg/ml, was purchased from (biological industries Israel Beit HaEmek Ltd and Gibco BRL (Grand Island, NY, USA), stored, and -20°C for prolonged storage, 4°C for regular use, and secured against light. The outcome of this test depends on the mitochondrial-dependant NADPH oxidoreductase to convert the water-soluble yellow tetrazolium salt into the water-insoluble formazan (purple), a process that does not take place in cells that have died.



Figure 3.9. **A-** Phosphate buffered saline (PBS) solutions for dilutions, cleaning culture flasks and plates, washing cell suspensions, and adding additions to cell culture media. **B-** MTT powder is a colorimetric test used for evaluating cell survival.

3.1.4.1 MTT Assay Protocol (Colorimetric Assay)

To select the effective doses in which the BV2 cell line will be 100% a life with full absorbance, and to select IC₅₀ in which 50% of cells die determined by using 3-(4,5-Dimethyl-2-thiazolyl) -2,5-diphenyl tetrazolium bromide (MTT) assay. After the supernatant was discarded from every single well of the 96 well, two hours' incubation purple formazan is created preceded by adding 10 µL of a 5 mg/mL MTT solution per well that contained (100 µL/well). The purple formazan was then dissolved in each well by using 100 µL of DMSO solution per well mixed by pipetting to confirm comprehensive solubilization, the container should be protected from light to avoid DMSO damage, the outcomes were assessed using a microplate reader (Biotek, Winooski, VT, USA) at an absorbance of 570 nm. The MTT viability growth curve provided the IC₅₀ values.

3.1.5. MTT Calculation

The following equation is the standard used for the cell's viability percentage calculation:

$$\text{Viability (\%)} = (\text{Mean OD sample} / \text{Mean OD blank}) * 100$$

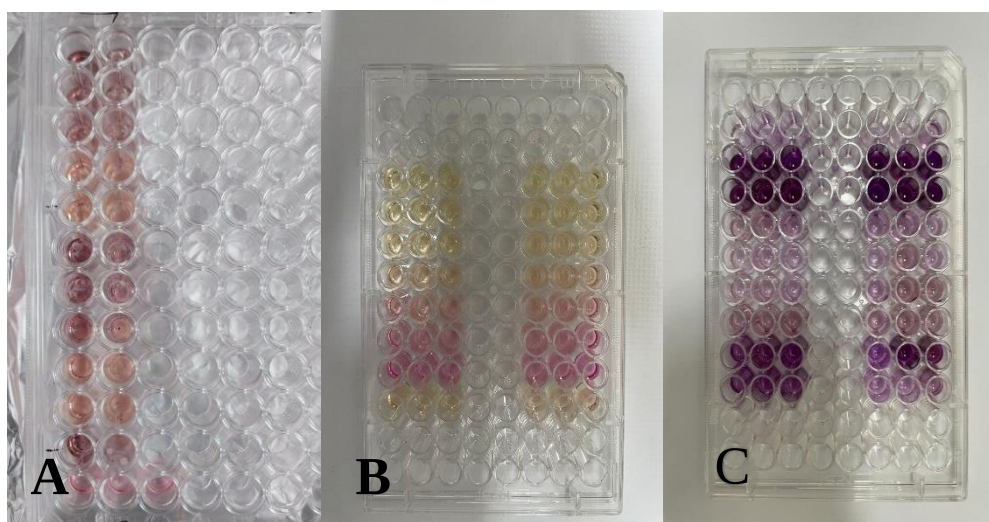


Figure 3.10. **A**- 96 well microplate contains BV2 in addition to agmatine + LPS in a duplicate manner, **B**- 96 well plate contains BV2 cells and agmatine in a duplicate manner after addition of MTT, **C**- 96 well plate containing agmatine+ MTT+LPS in a triplicate manner after incubation for detection absorbance.

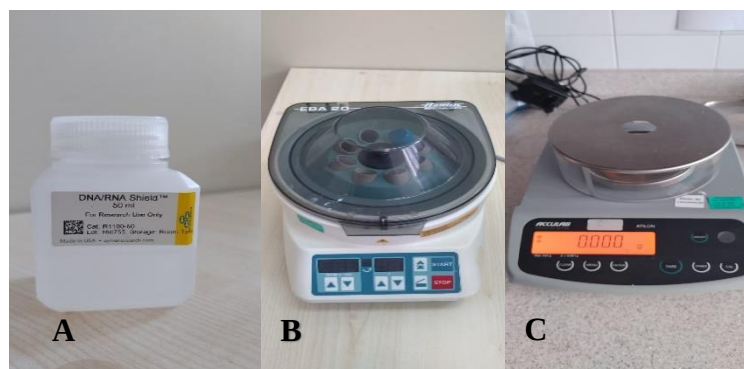


Figure 3.11. **A-** DNA/RNA Shield, used for the samples' expression patterns and genetic integrity protection, at room temperature. **B-** Digital Centrifuge **C-** Weighing Scale

3.2. Determination of Agmatine and LPS Proper Doses

3.2.1. Determination of Agmatine Proper Doses

Previous studies are using the doses agmatine (10, 50, or 300 mg/kg) (J. E. Piletz et al., 2003), and (10, 100, 300, or 1,000 μmol in 5 microliters) (Penner & Smyth, 1996) researcher aim to choose dose which is close to IC_{50} to be used in this study as high doses. To select this IC_{50} (high dose) several doses are being examined according to previous studies, and to select IC_{50} dose to be used in this experiment as high dose.

It is crucial to ensure that all equipment is in excellent functioning order prior to beginning any cell culture operation. Moreover, frequent inspections and ongoing equipment maintenance are necessary if cell culture is to become a common technique used in laboratories. Following is a partial list of items to think about: Verify that the incubator's temperature and CO_2 levels are where they should be; Verify that the water bath in the incubator contains copper sulphate to prevent bacterial development and is filled with clean water to prevent contamination; Verify that the water bath is at the appropriate temperature and has enough clean water in it; Verify that the centrifuge has been cleaned and is free of contamination. Verify that the biological safety cabinet being utilized is certified and functioning properly.

BV2 microglial cell line in full sterilized condition, in 96 well plate BV2 cell line was seeded at a density of 5000 cells per well after reaching a confluence of 70–80% cells were exposed to agmatine in deferent doses (2,5,10,15,25,50,75,100,125,150 and 175) μM agmatine in the triplicated manner

and incubated for 24,48 and 72-hours consecutively. Agmatine is dissolved in distilled water.

Table 3.3. Administration of agmatine in different doses to BV2 cells and controls

BV2 agmatine drug administration													
	1	2	3	4	5	6	7	8	9	10	11	12	13
A	Media	Cell	2	5	10	15	25	50	75	100	125	150	175
B	Media	Cell	2	5	10	15	25	50	75	100	125	150	175
C	Media	Cell	2	5	10	15	25	50	75	100	125	150	175

96 well plates will be used in a triplicate manner, the first column contains 110 μ L complete media only, the second column contains complete media plus BV2 cells were seeded, from the third up to the 13th column contains complete media plus BV2 cells, and agmatine dose (2,5,10,15,25,50,75,100,125,150 and 175) μ M respectively. Groups were cultured in a 95% humidified incubator with 5% CO₂ for 24, 48, and 72 hours respectively. 10 μ L of 5 mg/mL MTT solution was added to form the water-insoluble purple formazan which reflects cell viability. 100 μ L of crystal dissolving solution (DMSO) was added to each well in order to dissolve the generated purple formazan crystals. Results were determined at an absorbance of 570 nm using a microplate reader (Biotech, Winooski, VT, USA). The MTT viability growth curve was used to get the IC₅₀ values.

3.2.2. Determination of the Proper Doses for LPS

In a 96 well plate, BV2 cells were seeded at several concentrations to determine the optimal cell concentration for *in vitro* studies, with two replicates per concentration. The entire medium was aspirated after 24-hours, and all cells were rinsed with PBS. The LPS was then given in various concentrations (2, 1, 0.5, 0.25, 0.125, 0.01) μ g/ml. After that, the 96 well plate was incubated for 24 hours. Following incubation, the media containing the LPS was aspirated. Solution (5 mg/ml), 10 μ L MTT was then added to each well and incubated for 2 hours in 37 C° 95% humidity and 5% CO₂ before being aspirated adequately. Forceful pipetting was used to add 100 μ L of crystal-dissolving solution (DMSO) to each well in order to dissolve the generated formazan crystals. The sample was kept at room temperature

and covered with a folio for ten minutes. Finally, an ELISA plate reader was used to measure the absorbance with a spectrophotometer at 570 nm. The MTT viability growth curve provided the IC₅₀ values. The following equation was then used to determine the cell viability: (A treated/A control) * 100 (A: Absorbance).

3.3. Testing Agmatine Protective Effect Against LPS Toxicity on BV2 Cell

After the preparation of agmatine and LPS solutions and doses in addition to the preparation of the complete media stock volume needed for the experiments, BV2 cells were seeded and treated by the prepared agmatine and LPS stocks when the cell density reached 80-90%, in triplicated manner, according to the Table 3.4. which represents a real well plate.

Table 3.4. The different stages of the main experiments by adding the high and low doses of agmatine +LPS to BV2 cells.

No	1	2	3	4	5	6	7	8	9
Components	Media	Control	Cells + Toxic	Cells+ Toxic +Low dose	Cells+ Toxic + High dose	Cells +Low dose	Cells+ High dose	Cells + D water Low dose	Cells + D water High dose

Seeding: The first column of a well-plate was initially filled with 100 µl of complete media, from the second through ninth columns, BV2 cells were seeded in 100 µl of complete media, and incubation was carried out for 24 hours to allow cell attachment and growth.

Incubation: 96 well plate with their contents will be incubated for 24 hours in 95% humidity, and 5% of CO₂. After the 24 hours incubation period, media in all groups was aspirated by gentle pipetting, and an additional 80 µl of fresh media was added.

Addition of toxic substance: Before agmatine addition in the third, fourth, and fifth column 20µl LPS was added to BV2 at a concentration of 5µg/ml and introduced as a toxic substance to induce cellular stress. In the first, second, sixth, seventh, eighth, and ninth columns, 20µl complete media was added then, the well-plate was

incubated for an hour to allow the substances to interact with the cells, before adding the protecting substance agmatine to them.

Following one-hour incubation: Different columns received the addition of specific protective substances: In the fourth column, 7.5 μ l agmatine was added to the BV2 cell (1mM) to achieve a concentration of 75 μ M (low dose) added as a protective agent.

In the fifth column, (which already contains the toxic substance LPS), 10 μ l of agmatine (1mM) to achieve the concentration of 100 μ M (high dose) is added as a protective agent. Similarly, in the sixth and seventh columns received the addition of the same doses of agmatine as protective substances and 10 μ l agmatine is added as a low dose of the protective substance.

The eighth column was introduced with 7.5 μ l of DW to match the volume used for preparing agmatine at a concentration of 75 μ M, similarly, the ninth column was introduced with 10 μ l of DW to match the volume used for preparing agmatine at a concentration of 100 μ M.

Then, the well-plate was incubated for 24 hours, after that the MTT assay was conducted and the results were analysed.

Table 3.5. Protocol of Cell Seeding in the well plate

No	Steps
1	Removed all the complete media.
2	Rinsed the cell with 3ml of PBS without Ca ²⁺ /Mg ²⁺
3	Added trypsin EDTA 1 to 3ml (according to flask volume) for 3 minutes
4	Added 4 to 5 ml fresh media to deactivate the trypsin reaction
5	Transferred the cell by using a pipette and put it in a falcon tube and centrifuged at 3500 rpm for 5 minutes.
6	Removed all the supernatant and added 3.5 ml of new complete media, pipetted the cell in the falcon tube, and then distributed into the 96 well plates.
7	Then incubated for 24 hours to attach.
8	Serial dilutions of different doses of LPS or agmatine concentrations were prepared within complete culture media. After incubation the culture media in a 96 well plate was removed gently and washed with PBS and added 200 μ l fresh medium with various drug concentrations, this step is triplicated.
9	Added MTT kit, must be prepared for it.

4. RESULTS

4.1. Statistical Analysis for Substances

All findings have been presented as means and, standard deviation. GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA, US) was used to analyse our data, which are shown as the mean and standard deviation for the six separate dosages, One-way analysis of variance (ANOVA) was used to assess the data, and multiple comparisons, in addition to descriptive analysis. Statistical significance was defined as a *p*-value less than 0.05, *p*-values less than 0.05 are regarded as statistically significant differences in all comparisons, the symbol (*) which means the *p*-value < 0.05, 150 μ M (****) which means the *p*-value < 0.0001 and 200 μ M (****) which means the *p*-value < 0.0001.

4.1.1. Agmatine Statistical Analysis

4.1.1.1. First 24-hour results:

From the three samples treated and incubated before 24 hours the first sample was analysed it showed the following different analytical results below which are analysed by the analytical program Prism 9.

Table 4.1. Mean and standard deviation of BV2 cells viability after the addition of agmatine only

Dose/ μ M	Cell viability(%) mean	Std. Deviation
2	105.48	12.74
5	99.59	22.94
10	103.88	19.24
15	102.67	15.76
25	99.47	14
50	88.61	11.99
75	70.56	9.432
100	60.36	9.96
150	31.45	6.926
200	3.93	1.157

This table shows different doses of agmatine in the first column that is added to BV2 cells, the second column the mean and the third column shows the Standard deviation. 100 μ M is only toxic to 40% of cells.

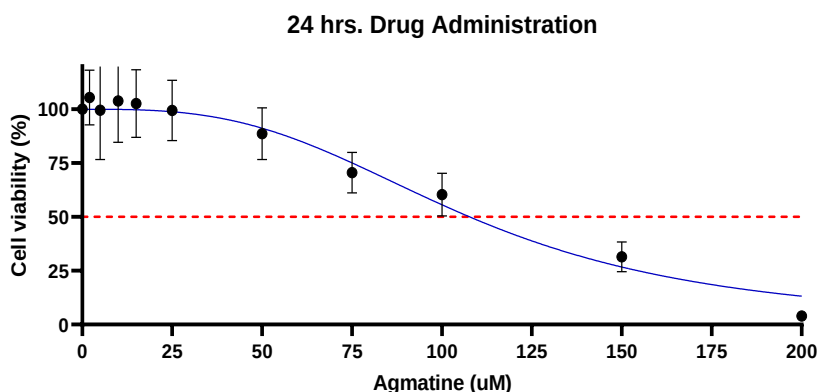


Figure 4.1. X Horizontal line represents the agmatine concentration that was added to BV2 cells while the Y vertical line represents cell viability percentage, and the interrupted red line represents 50% cell viability the point at which the curved line crosses the red dots line is the IC₅₀ here it is 107 μ M

The absorbance of cells up to 25 μ M was 100% after this concentration cells' viability started to decrease dramatically until it reached the dose 107.7 μ M at which 50% of cells were only able to remain alive while 50% of cells lose their ability to survive this point is known scientifically by IC₅₀. After IC₅₀ by increasing the dose cells continue to die in alignment to increase in dose, until they reach zero viability.

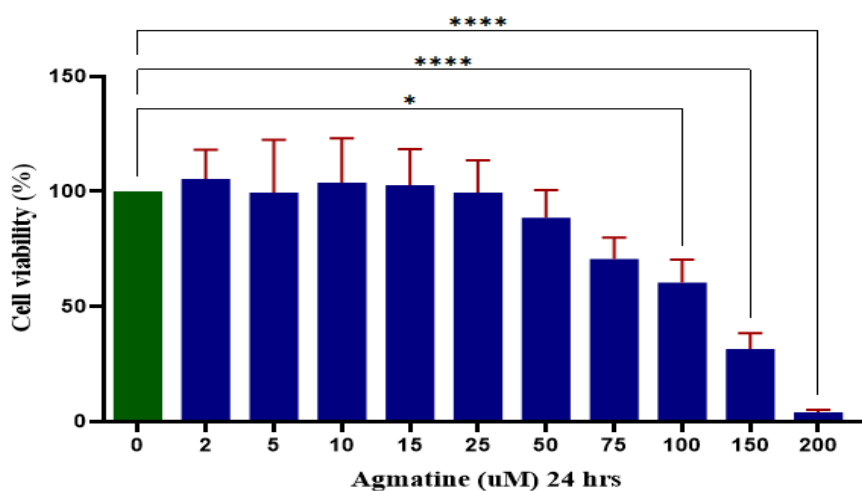


Figure 4.2. Showing a 24-hours graph estimate of the similarities or dissimilarities between the control group and the last three groups which contain the doses (100 μ M, 150 μ M, and 200 μ M) the first one (100 μ M) is significant (*) p -value < 0.05 but lower the two remaining groups (150 μ M and 200 μ M) they contain high significant (****) p -value < 0.0001

Table 4.2. Showing comparison between control and nine doses for significance level.

Comparison between	significance	significance level	Significance level calculator
0 vs. 2 μ M	No	ns	>0.9999
0 vs. 5 μ M	No	ns	>0.9999
0 vs. 10 μ M	No	ns	>0.9999
0 vs. 15 μ M	No	ns	>0.9999
0 vs. 25 μ M	No	ns	>0.9999
0 vs. 50 μ M	No	ns	0.9895
0 vs. 75 μ M	No	ns	0.2384
0vs.100 μ M	Yes	*	0.0376
0vs.15 μ M	Yes	****	<0.0001
0vs.200 μ M	Yes	****	<0.0001

Table 4.2. In estimating and comparing the control group with the other remaining ten concentration groups to assess the presence of significance, the outcome was the presence of significance in group 100 μ M (*) which means the p -value < 0.05, 150 μ M (****) which means the p -value < 0.0001 and 200 μ M (****) which means the p -value < 0.0001

4.1.1.2. Second 48 Hours Analysis Results

After two days the second sample 48-hours Group analysis it shows the following table and diagram :

Table 4.3. The mean and standard deviation for the second group 48-hours

Dose in μM	Cell viability(%) Mean	Std. Deviation
0	100	0
2	83.54	24.77
5	112.4	41.47
10	83.09	20.89
15	87.11	32.17
25	82.72	45.6
50	67.43	27.44
75	60	18.36
100	12.13	5.522
150	9.666	1.849
200	7.358	1.312

The mean is inversely proportional to the increase of dose, likewise, standard deviation decreases with the increase in dose, and 100 μM becomes toxic to more than 50% of cells.

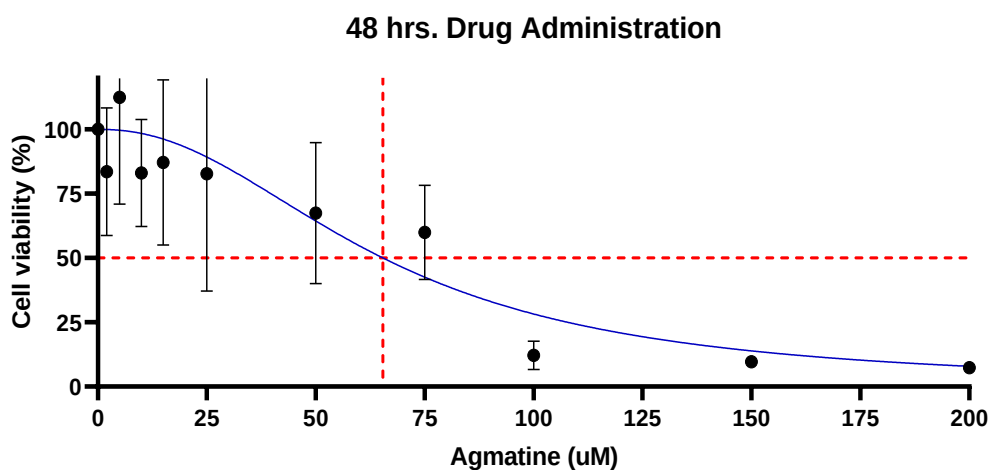


Figure 4.3. Graph showing the IC_{50} in 48-hours group treated with agmatine X horizontal line axis represents agmatine doses, while Y axis represents cells viability percentage, IC_{50} is at (65.43) μM the point in which the red interrupted line is crossed by the blue curved line

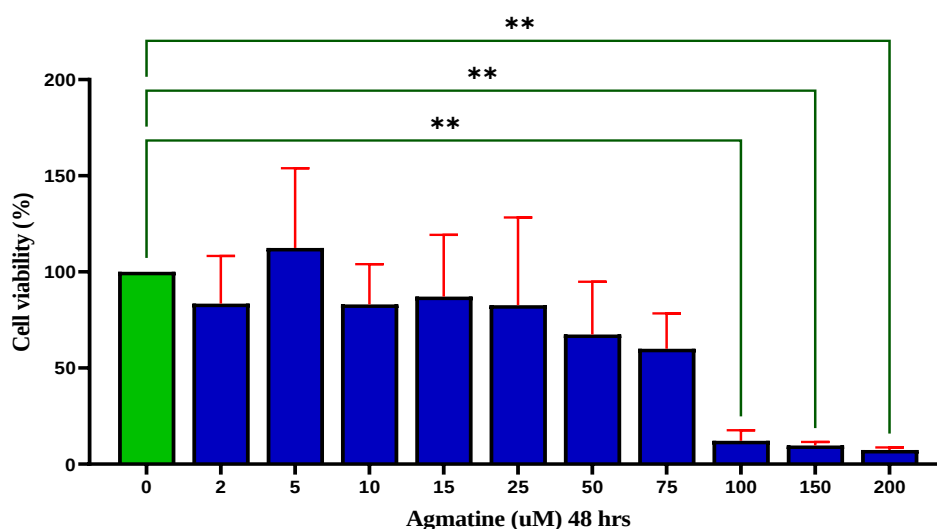


Figure 4.4. Graph comparing the control with the other group contains significance and shows the level of significance in the 48-hours group, the X horizontal line axis represents agmatine doses, while the Y axis represents cell viability percentage. (**)The two stars between the brackets indicate that between these two groups, there is a significant p -value < 0.01

4.1.1.3. Third 72-hours Analysis Results

The last group to be analysed is the 72-hours group, analysis for this group shows in the table and diagram below.

Table 4.4. The mean and standard deviation for the third group 72 hours for the ten groups of doses used with agmatine

Dose μ M	Cell viability(%) Mean	Std. Deviation
0	100	0
5	84.35	10.31
10	77.68	8.113
15	64.21	10.54
25	50.69	7.275
50	64.15	6.005
75	26.06	8.104
100	8.46	3.626
150	7.855	0.2976
200	6.79	1.653

Mean is inversely proportional to the increase in dose, likewise, standard deviation decreases with the increase in dose, and 75 μ M becomes toxic to more than 50% of cells.

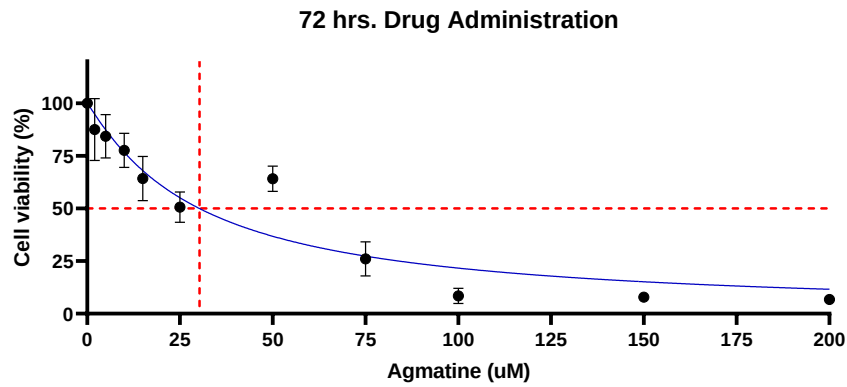


Figure 4.5. Graph showing the IC_{50} in the second day group 72-hours treated by agmatine for three days, X horizontal line axis representing agmatine doses, while Y axis representing cells viability percentage

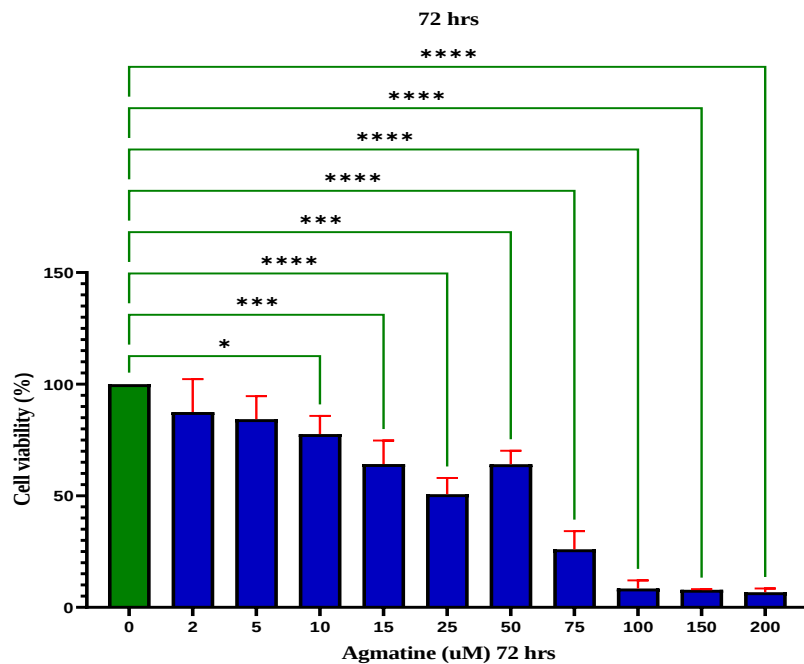


Figure 4.6. Graph comparing the control with the other group containing significance and showing the level of significance in 72 hours. X horizontal line axis represents agmatine doses, while Y axis represents cells viability percentage, (*) the stars between the brackets indicate that between these two groups, there is a significant p -value < 0.05 , (***) three stars between the brackets indicate that between this two groups there is significant p -value < 0.001 , (****) which means the p -value < 0.0001

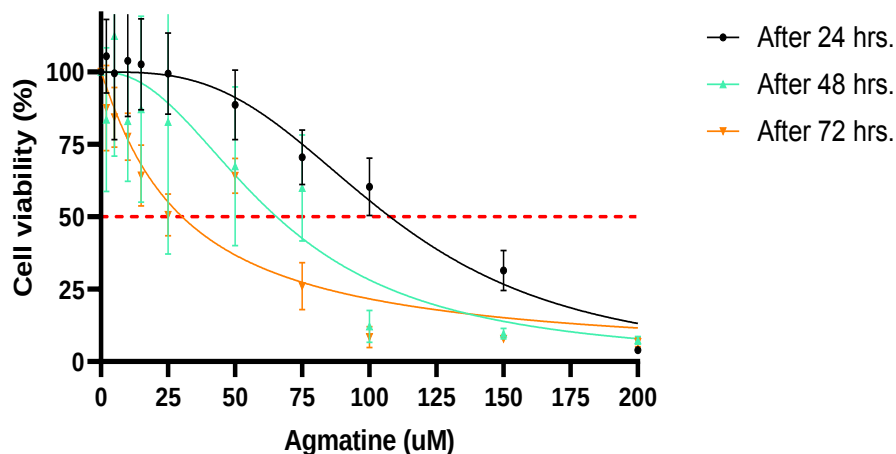


Figure 4.7. X horizontal line represents the agmatine concentration that was added to BV2 cells while Y vertical line represents cell viability percentage, and the interrupted red line represents 50% cell viability the point at which the curved black line crosses the red dots line is the IC_{50} here it is $107\mu M$ in the first day and $65.43\mu M$ in the second day green line, and lastly, IC_{50} for the 3ed day was $30.25\mu M$ represented by the orange line.

4.1.2. LPS Statistical Analysis

The LPS was particularly hazardous at $2\mu g/ml$, according to observations, there was also a significant decrease in cell viability at $1\mu g/ml$ drug concentration, and the cell died to a large extent at 0.5 concentrations as well. LPS did not give a suitable model for a neurodegenerative model below these concentrations. Conclusion: the best dose of LPS on BV2 for creating a neurodegenerative model was established to be $1\mu g/ml$ for future use as a toxic agent causing neurodegenerative illnesses.

Table 4.5. Mean and standard deviation of LPS

LPS Doses / $\mu\text{g/ml}$	Cell viability(%) Mean	Std. Deviation
0	100	0
0.1	94.5	12.42
0.125	94.59	0.61
0.25	85.9	0.031
0.5	74.7	0.03
0.9	58.47	3.2
1	49.61	11.99
2	2.56	0.332

This table shows the mean and standard deviation with alignment to ten dose groups of LPS added to BV2 cells to estimate the proper doses to be used as toxic doses in the experiment with BV2 cells, the mean is more than 50% for the first six doses up to the dose 0.9 $\mu\text{g/ml}$ IC_{50} , after that the mean started to decrease reflecting the toxicity of LPS doses.

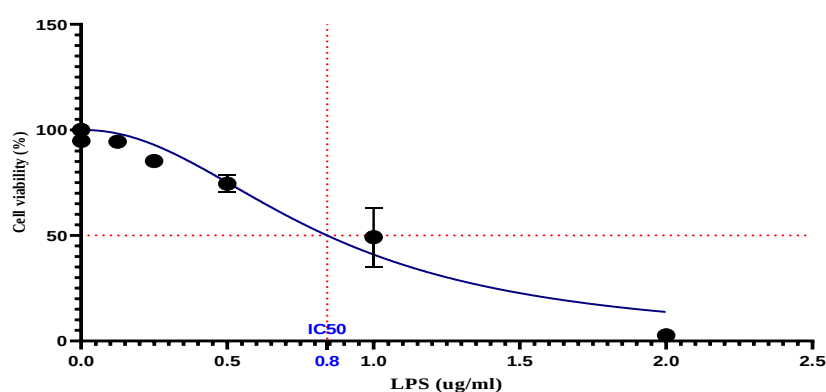


Figure 4.8. X horizontal line represents the LPS concentration that was added to BV2 cells while the Y vertical line represents the BV2 cell viability percentage, the interrupted red line represents 50% cell viability aligned with all LPS concentrations, the point at which the curved black line crosses the red dots line is the IC_{50} here it is approximately 1 $\mu\text{g/ml}$.

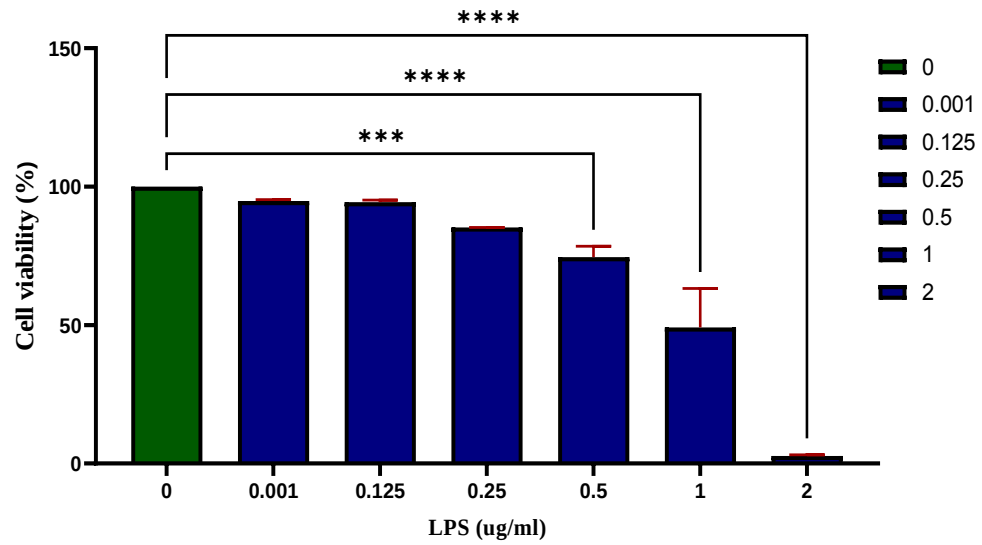


Figure 4.8. LPS dose groups for BV2 treatment, statistical significance was defined as a p -value of 0.05, p -values less than 0.05 are regarded as statistically significant differences in all comparisons, the symbol (*) which means the p -value < 0.05, 150 μ M (****) which means the p -value < 0.0001 and 200 μ M (****) which means the p -value < 0.0001.

4.2. Microscopic Results Findings

During and after the processing of all experiment's cells were checked under a microscope for confluency, attachment, detachments, and morphological changes, information will be recorded for comparisons with other groups, following are the microscopic findings for the three consecutive days of dose determination of agmatine

4.2.1. First 24-hours Microscopic Findings

In the first day after the addition of agmatine to BV2 cells in the control group in which there is only complete media for cell nutrition without agmatine and LPS cells are incubated for 24 in 95% humidity, 5% of CO² and 37°C, after that the following microscopical analysis were done under confocal microscope lens magnification 10X, cells were visible growing, cells morphology with their protoplasmic extension are clear in addition to cell nuclei.

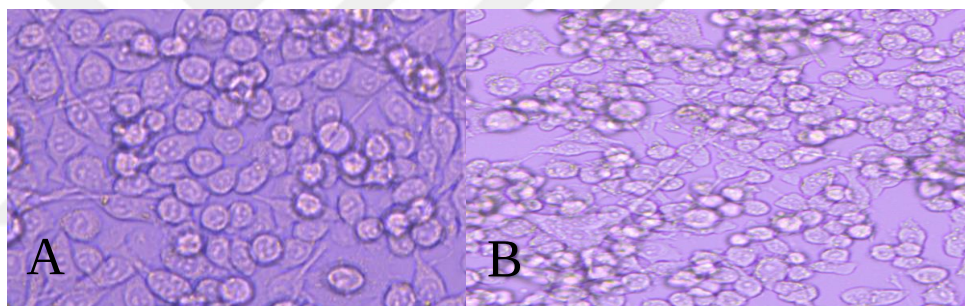


Figure 4.9. **A-** Light inverted microscope lens magnification 40X Control group of microglial BV2 cells contains no agmatine treatment

after 24 hours of incubation in 95% humidity, 5% of CO² and 37°C, 100% of the BV2 cells are alive, which were visible growing, cell morphology with their protoplasmic extension are clear in addition to cell nuclei. **B-** Light inverted microscope 40X represents agmatine group (107.7 μM) IC₅₀ group after 24 hours to BV2 cells treated by agmatine, 50% of the cells only were viable, dead cells.

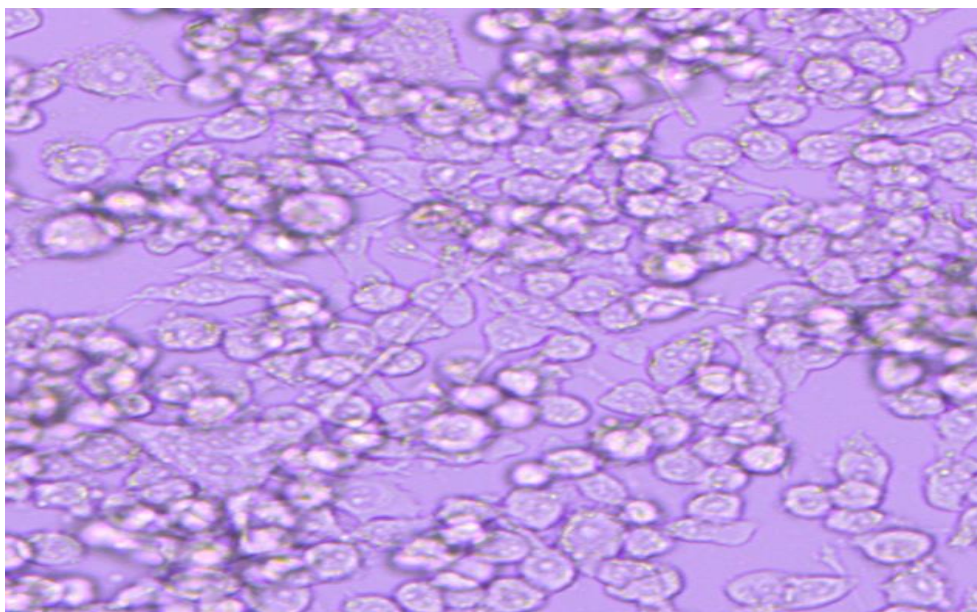


Figure 4.10. Light inverted microscope lens magnification 40X of BV2 microglial cells in 25µM agmatine group after 24 hours of incubation, cells viability seems to be similar to the control group which contains no agmatine.

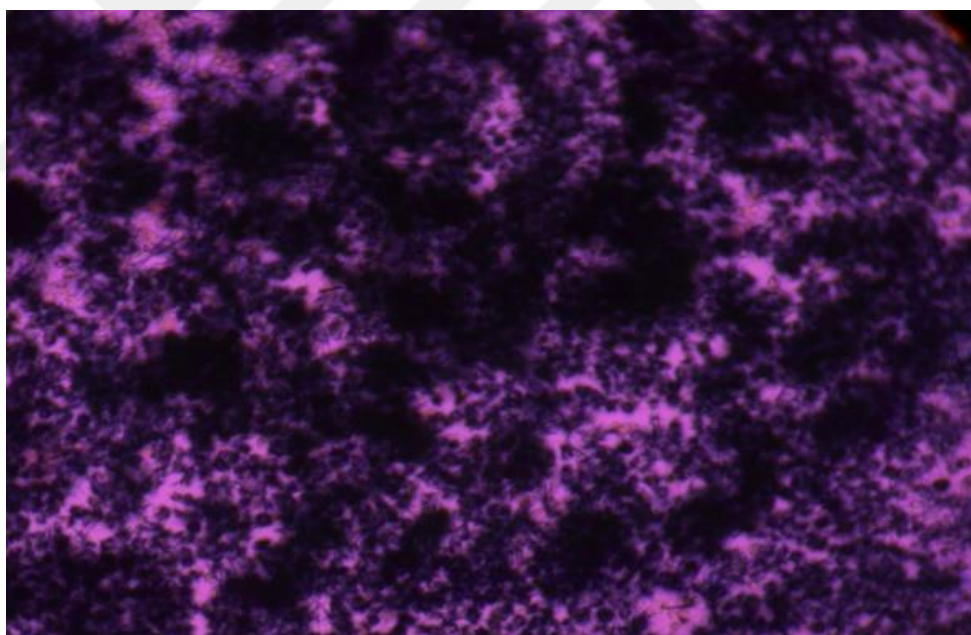


Figure 4.11. Purple formazan after the addition of DMSO

The MTT assay colorimetric measures the metabolic activity of cells. Under some circumstances, NADPH-dependent cellular oxidoreductase enzymes represent the number of living cells present. The yellowish soluble tetrazolium dye MTT can be reduced by the enzyme to its insoluble formazan, which has a purple tint.

4.2.2. Second 48-hours Microscopic Findings

After 48 hours of incubation at 37C in 5 % carbon dioxide in which IC_{50} is 65.43 μ M the following microscopical findings are detected

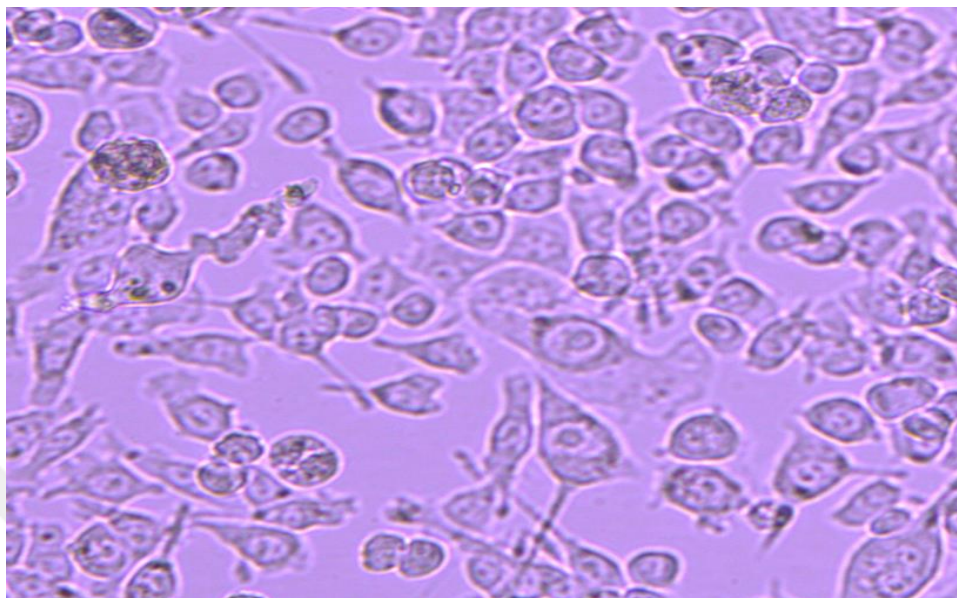


Figure 4.12. Light inverted microscope lens magnification40X BV2 cells after 48 hours incubation with treatment by agmatine IC_{50} is 65.43 μ M, cells viability is declining in comparison to the same dose on the first day

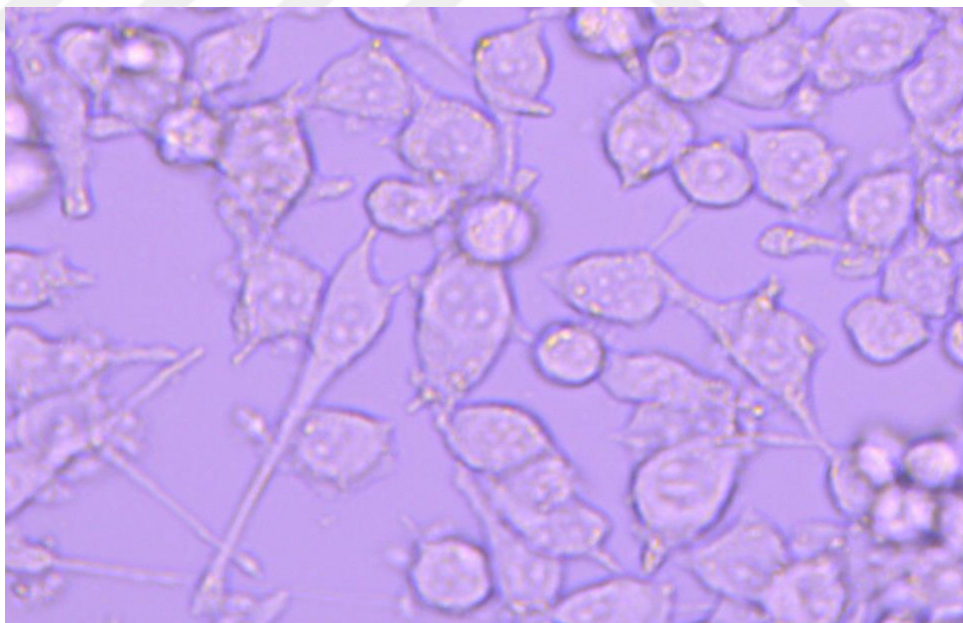


Figure 4.13. Light inverted microscope lens magnification40X of BV2 cells after 48 hours Microscopical findings of group 50 μ M agmatine, majority of BV2 cells are alive, clearly visible growing, cells morphology with their protoplasmic extension are clear as well as cells nuclei

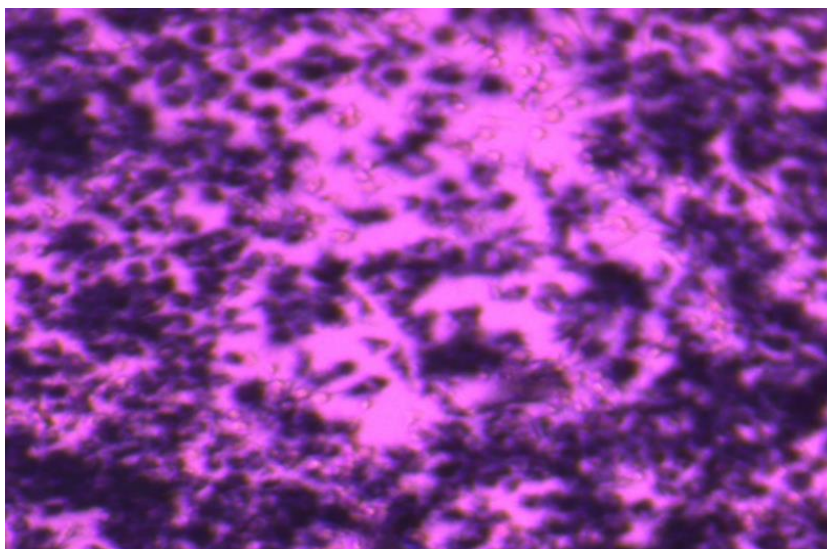


Figure 4.14. Purple formazan after the addition of DMSO on the second day The MTT assay colorimetric measures the metabolic activity of cells. Under some circumstances, NAD(P)H-dependent cellular oxidoreductase enzymes represent the number of live cells present. The yellowish soluble tetrazolium dye MTT can be reduced by the enzyme to insoluble formazan, which has a purple tint.

4.2.3. Third 72-hours Microscopic Findings

On the third day of incubation between the BV2 microglial cells and toxic substance LPS in addition to the protective substance agmatine the following microscopic findings are detected, IC_{50} for 72-hours is $35.45\mu M$ which is the lowest between the three days' groups for this reason majority of cells died and cannot able to survive in concentrations more than $75\mu M$ and seems to be losing their viability.

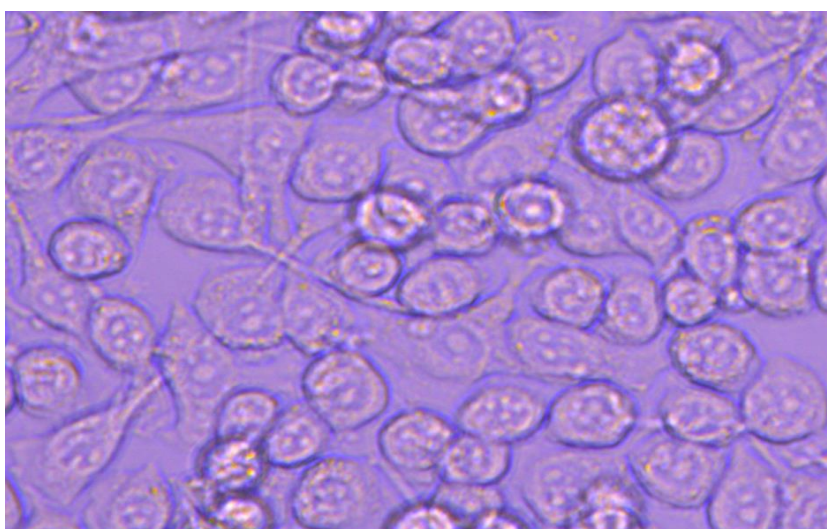


Figure 4.15. Light inverted microscope lens magnification 40X of BV2 cells after 72-hours incubation with treatment by agmatine IC_{50} is $30.25\mu M$, cell viability is declining to 50%.

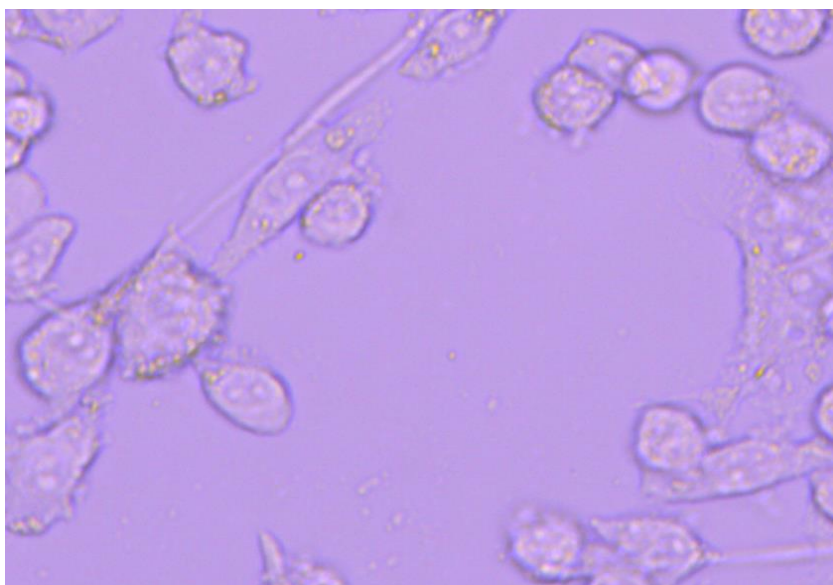


Figure 4.16. Light inverted microscope lens magnification 40X of BV2 cells treated by 1 μ M of LPS, few cells can be detected the majority of cells died

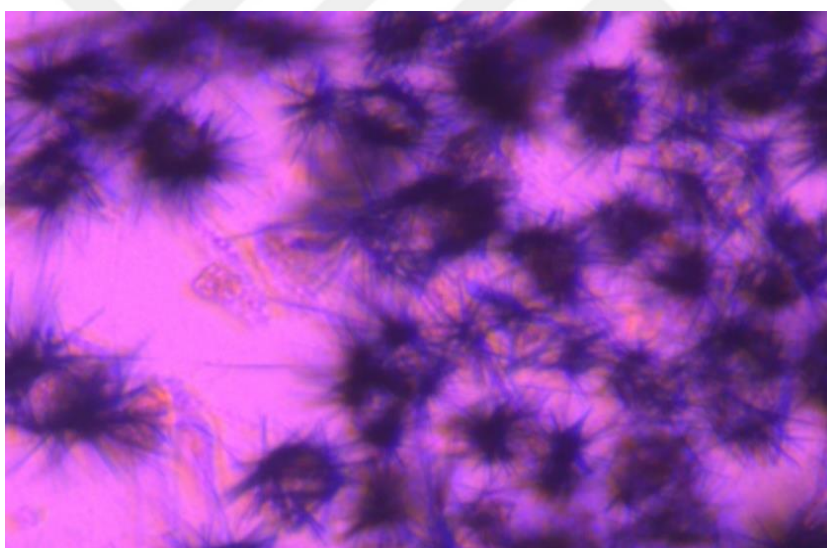


Figure 4.17. Purple formazan of BV2 cells treated with 1 μ M LPS after the addition of DMSO.

4.3. Agmatine Treatment Protects BV2 Microglial Cells from LPS Toxicity

BV2 cells were alive in well number (2,4,5,6,7,8,9) the joining factor between these wells is the absence of LPS except for cells in well number (3) which contain only LPS that reflects the toxicity of LPS and the protective effect of agmatine to BV2 cells against LPS.

4.3.1. Statistical Findings

Table 4.6. Mean and standard deviation of the main experiment

Column1	Mean% SD
Control	100 SD $\pm$ 0
LPS 1 ug/ml	12.54 SD $\pm$ 2.64
LPS 1 ug/ml - Agma 75 μ M	73.13 SD $\pm$ 2.351
LPS 1 ug/ml - Agma 100 μ M	45.09 SD $\pm$ 5.329
Agma 75 μ M	75.98 SD $\pm$ 5.035
Agma 100 μ M	64.13 SD $\pm$ 1.851
DW 75 μ L	51.15 SD $\pm$ 6.034
DW 100 μ L	47.32 SD $\pm$ 6.186

Mean of cell viability % with LPS only is very low, while agmatine 75 is the higher than that of agmatine 100 which reflect higher doses of agmatine can be toxic.

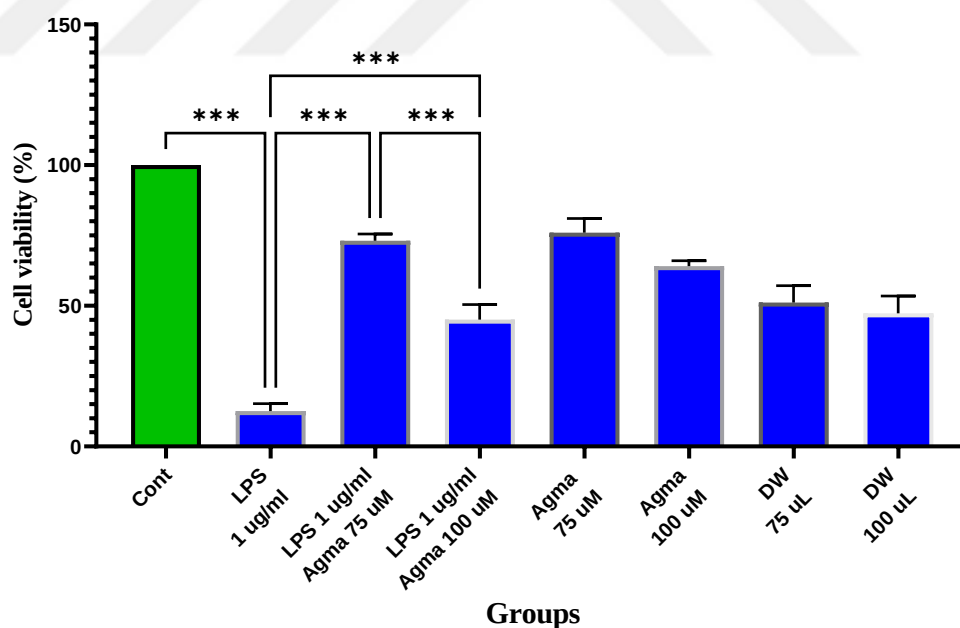


Figure 4.18. The graph shows cell viability for the eight groups of the experimental BV2 cells after treatment by agmatine and LPS, The X horizontal line represents the LPS concentration that was added to BV2 cells while the Y vertical line represents the BV2 cell viability percentage.

The X line represents the experimental eight groups for 8 well plate, while the Y vertical line represents the percentage of cell viability, results shows 100% cell viability in control in the second well which contains LPS 1µg/ml without agmatine cells viability around 80% while in the 3ed well which contains LPS 1µg/ml in addition to agmatine 100µM cell viability dramatically decreased to around 15 % similar to group number four which contain LPS 1µg/ml in addition to 100µM agmatine cell viability is around 20%. In wells number 5 and 6 which contain 75µM and 100µM only agmatine respectively cell viability was 13% and 20% respectively.

In the last two consecutive well, which contained distilled water in addition to 75µM and 100µM Agmatine respectively cell viability was around 50%.



4.3.2. Microscopic Findings

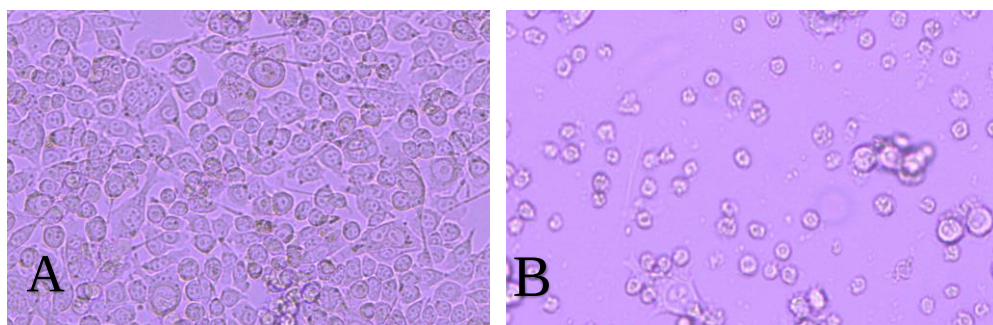


Figure 4.19. **A-** Light inverted microscope lens magnification 20X Control group of microglial BV2 cells contains neither LPS nor agmatine treatment, BV2 cells are alive, clearly visible, growing, cells morphology with their protoplasmic extension and cells nuclei are clear. **B-** Light inverted microscope 20X represents the LPS group (1µg/ml) majority of cells died.

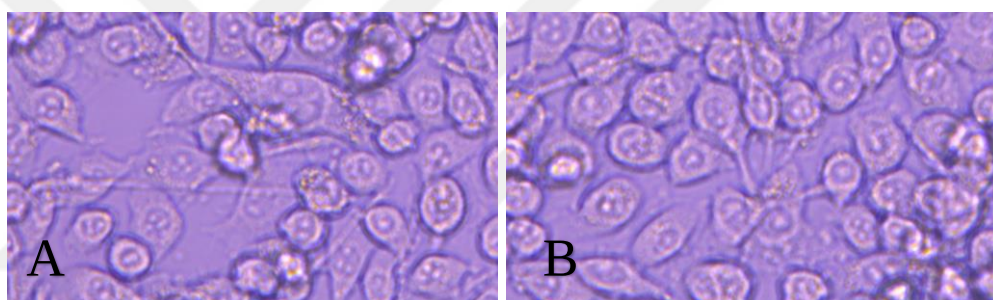


Figure 4.20. **A-** Light inverted microscope lens magnification 40X LPS(1µg/ml) group+Agmatine75µM + microglial BV2 cells, cells are alive, clearly visible, growing, cell morphology protoplasmic extension cell nuclei are clear. Similar to **B-** Light inverted microscope 40X represents agmatine group 75µM cells were, alive, cell morphology protoplasmic extension and cell nuclei are visible.

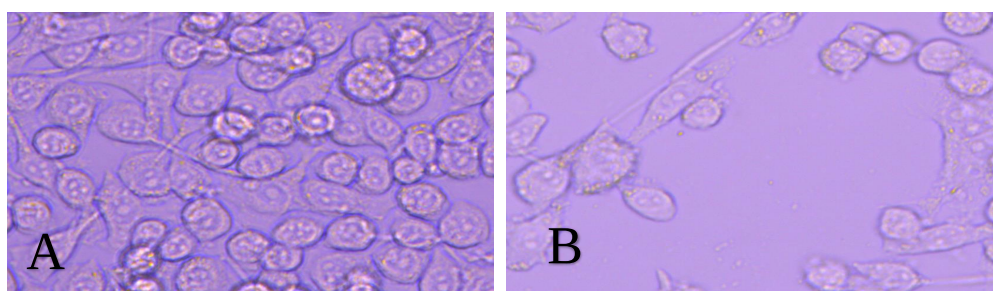


Figure 4.21. **A-** Light inverted microscope lens magnification 40X LPS(1µg/ml) group+ agmatine 100µM + microglial BV2 cells, cells are alive, clearly visible, growing, cell morphology protoplasmic extension cell nuclei are clear. **B-** Light inverted microscope 40X represents the LPS group (1µg/ml) majority of cells died.

5. DISCUSSION

Previous surveys such as that conducted by Binnetoğlu and his coworkers (Binnetoglu et al., 2019b) demonstrated that agmatine has the highest neuroprotective impact in low dosages, while glutamate decreased cell viability in larger concentrations. The effectiveness of agmatine was greatly enhanced by increased total oxidant capacity brought on by toxicity. Again, the administration of agmatine improved the antioxidant capacity that had diminished in the toxicity group. In addition, agmatine treatment significantly reduced the elevated caspase 3 and caspase 9 mRNA expressions in the toxic condition (Binnetoğlu D, 2019).

Agmatine, a strong NMDA receptor antagonist, reduced many chemical-induced neurotoxicity in light of all these findings. Agmatine has mostly been investigated as a neuroprotective drug for central nervous system neuronal ischemia damage.

Recently, in another *in vitro* study, researchers had used lactate dehydrogenase (LDH) assay, to determine that LPS caused cytotoxicity in BV2 microglia. Approximately 20% of LDH was released at baseline, and more than 80% of LDH was released 24 hours following LPS exposure. Using 100µM agmatine, this LPS-induced cytotoxicity was reduced to less than 60% in the treated cells. Nitrite generation was evaluated in LPS-damaged BV2 microglial cells to look into the potential neuroprotective mechanism of agmatine. The amount of nitrite in the culture media grew after 24-hour exposure to LPS, while agmatine treatment dramatically reduced it (Ahn et al., 2012).

In this study similar dose of agmatine was used in alignment with what was used by Ahn et al., 100µM as a high dose to effectively demonstrate is neuroprotective properties of agmatine against LPS-induced toxicity in BV2 cells using this theory in a microglia model however this study looked into the neuroprotective effects of agmatine on BV2 cell, after the cells were subjected to harm. The current findings encourage to consideration of agmatine as a potential protective drug because it can prevent BV2 cells from dying as a result of toxicity. However, more research is needed to fully understand the neuroprotective mechanisms of agmatine.

Previous research findings of Ahn and his peers (Ahn et al., 2012) were treating BV2 cells with 1 g/mL of LPS in serum-free DMEM for 24 hours in order to examine

the cytotoxic damage caused by LPS. Other studies have considered the relationship between the protective effect of 100 μ M agmatine we decided to utilize 100 μ M agmatine as well in future studies since cell mortality was markedly decreased at doses of agmatine of 100 μ M and higher. A lactate dehydrogenase assay was used to confirm LPS-induced cytotoxicity in BV2 microglia. After being exposed to LPS for 24 hours, LDH release increased to over 80% from a baseline of roughly 20%. Using 100 μ M agmatine, this LPS-induced cytotoxicity was reduced to less than 60% in the treated cells (Ahn et al., 2012),

Likewise in this research 1 μ g/ml of LPS is confirmed among several other LPS doses to be effective to be harmful to BV2 cells, similarly Ahn and his colleagues found 100 μ M agmatine was protective which is a coincidence with this study that confirmed 100 μ M of agmatine is protective to BV2 against LPS induced cytotoxicity. Differently, they used a lactate dehydrogenase assay in contrast to the MTT assay that was used in this study.

Chai et al. (2016), prove the effect of agmatine in reducing the Rise in nitric oxide (NO) and iNOS levels caused by LPS in LPS-stimulated macrophages, NO, a product of the nitric oxide synthase NOS family, functions as a biological mediator and is crucial. A colorimetric assay was used to assess the NO production in LPS-treated cells, and a western blot was used to assess the expression of iNOS. The findings demonstrate that in RAW 264.7 cells, LPS greatly increased NO production and improved iNOS protein levels. Treatment with agmatine dramatically reduced the LPS-induced overproduction of NO and overexpression of iNOS (Chai et al., 2016).

In contrast to this study result and other researchers' results Abe and his colleague showed that when agmatine is added alone even in high or low concentrations 1–300 μ M did not affect the viability of cultured microglial cells Abe et al. (2000), otherwise in this study agmatine is harmful after exceeding 25 μ M to BV2 cells start dying, but Abe agreed with other when they add agmatine they found that agmatine is significantly inhibited the LPS-induced nitrite gathering. The suppressive effect of agmatine on nitrite accumulation is dose-dependent in the variety of 1–300 μ , (Abe et al., 2000).

This is similar to this study's result on the suppressive effect of agmatine on LPS when added to the BV2 cells. Similarly, our result demonstrates that agmatine

is toxic to LPS and protective of BV2 cells when they are mixed together. Agmatine has been shown in numerous researches to have antioxidant and anti-inflammatory effects in various animal models. This study documents the neuroprotective properties of agmatine against the toxicity of the LPS-induced microglial BV2 cell line. Agmatine at doses of 75 μ M and 100 μ M greatly reduced the damage to the microglial BV2 cell line caused by LPS in a dose-dependent manner. Agmatine also reduced the action of LPS against BV2 microglial cells. Therefore, the focus of our research is on the molecular impacts of agmatine's antioxidant and anti-inflammatory properties.

To investigate the protective effects of agmatine on hypoxia-induced apoptotic death in RGC-5 cells, additional studies were conducted (Hong et al., 2007).

After 12 hours of hypoxia exposure, the proportion of apoptotic cells did not change compared to the normoxic culture, but a significant increase was observed after 24 hours. However, the addition of 100 μ M agmatine and 10 ng/mL BDNF resulted in a reduction in the percentage of apoptotic cells. Caspase-3 activity, determined using the Ac-DEVD-pNA substrate, showed a significant increase after 24 hours of hypoxia-induced damage, which was inhibited by 100 μ M agmatine (Hong et al., 2007).

Agmatine has shown potential protective properties in other different studies El-Sherbeeney et al. (2016) demonstrated that agmatine could protect the rat tissue from nicotine-induced damage by inhibiting the production of the proapoptotic protein. Arndt et al., (Arndt et al., 2009) found that agmatine displayed protective effects *in vitro* by reducing death in Ha-Ras-transformed murine NIH-3T3 cell line. Furthermore, agmatine was shown to modulate programmed cell death in rats, inhibit the proliferation of human mast cell leukemia cells (HMC-1) and HL-60 cells (Haenisch et al., 2011).

Numerous studies have demonstrated protective properties of agmatine in various neurological diseases. For instance, M. Kim et al. (2017), found that agmatine reduced cellular death in rats with traumatic brain injury by decreasing MAPK phosphorylation and enhancing nuclear translocation of NF κ B Zarifkar et al. (2010), reported that agmatine prevented LPS-induced spatial memory impairment and hippocampal cell death by regulating caspase-3 expression. In a rat model of Alzheimer's disease, Sheng et al. (2018) showed that agmatine increased the levels

of Bcl2, PI3K, Nrf2, and gamma-glutamyl cysteine, thereby reducing cellular death by suppressing caspase-3 and Bax production.

Agmatine was found to protect the human-derived dopaminergic neuroblastoma cell line (SH-SY5Y) in several *in vitro* investigations. By increasing the quantity of phosphorylated Akt/Akt, limiting GSK-3 activity, and reducing the expression of apoptotic markers such as caspase 3, Bax, and cytochrome c, agmatines exercised their anti-apoptotic actions. Agmatine's neuroprotective properties were demonstrated by Kim et al, both *in vivo* and *in vitro* by lowering the generation of nitric oxide (NO) by competitively inhibiting nNOS and iNOS. Agmatine can also activate the endothelial cell enzyme eNOS, which increases the generation of nitric oxide (NO), a vasodilator that improves blood flow in ischemic regions (Huang et al., 1994).

6. CONCLUSIONS

The present study was designed to estimate the protective ability of agmatine in the BV2 cell line against LPS *in vitro*, after determining the effect of duration and the proper dose of agmatine that can be used with the BV2 cell line exactly IC_{50} , in addition to determining the proper dose of LPS that can assure 50% of the cells died. It is concluded that the proper dose of agmatine and LPS is $100\mu M$ and $1\mu g/ml$ respectively.

Experiments were run in fully sterilized conditions in a laminar hood flow, 96 well plates were used for cell culture after seeding in a 25 cm^3 flask, after 70-90 confluence of cells, cells will be distributed in 96 well plates with complete media, incubated for one day after that it will be treated with substances and incubated for one hour to be treated by MTT to detect cell viability.

The main goal of the current study was to determine and to share in fulfilling the gaps and share in agmatine and lipopolysaccharides research results further, research findings can be beneficial in a variety of experimental practice and clinical practices due to the wide spreading of neurodegenerative disease in the need of avoidance and treatment of such cases.

The findings of the MTT test revealed that agmatine had a strong proliferative effect at the lowest 4 doses, whereas continuing at high doses reduced cell viability. However, given that this decrease was statistically significant, agmatine was probably hazardous because of this. Agmatine proliferation was studied over a long period of time to ascertain how long it would last. As a result of our findings, only the lowest dose of the amino acid provided throughout a 24-hour period was statistically significant for its proliferating compared to other groups and periods. Agmatine has also been demonstrated to significantly alter the minimum two concentrations when applied for 24 hours straight compared to other occasions.

One of the more significant findings to emerge from this study is that, although we found satisfactory results, there is a need for other investigations in the same field to fulfill the limitations that faced during this experiment. It is recommended to use more confirmatory methods to prove the most obvious finding that emerged from this study is that the IC_{50} proper doses for agmatine with the BV2 cells in the 24, 48, 72 hours are $107\mu M$ in the first day, $65.43\mu M$ in the second-day, and $30.25\mu M$ for the third day.

Several questions remain unanswered at present, it is recommended that to use more confirmatory methods to prove that the IC₅₀ proper dose for LPS with the BV2 is 0.9 µg/ml. It is recommended to use more assay methods to prove the protective effect of agmatine on BV2 against LPS.



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