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THE POSSIBLE EFFECT OF AGOMELATINE ON HT22 HIPPOCAMPAL CELL LINE IN EXPERIMENTAL NEURODEGENERATIVE DISEASE MODEL *IN VITRO* MODEL

Ph.D. Thesis

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ÖZET

DENEYSEL NÖRODEJENERATİF HASTALIK MODELİNDE AGOMELATİNİN HT22 HİPOKAMPAL HÜCRE HATTINA MUHTEMEL ETKİSİ *İN VİTRO* MODEL

Negwod Abdelghani Saeed ABDALLA

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Nörodejeneratif hastalıklar (NDD'ler), antioksidan sistem dengesizliğine bağlı bir ilerleyici nöronal ölüm dizisidir. L glutamik asit (Glu), sinir sisteminde nöroeksitatör bir transmitterdir ve aşırı hücre dışı üretim hücre ölümüne neden olur. Agomelatin (AGO) antidepresan bir ilaçtır. Bu çalışma, HT22 hücre hattında nöroprotektif ajan olarak kullanılan AGO ve toksik madde olarak kullanılan Glu'nun uygun konsantrasyon ve doz süresini belirlemeyi amaçlamaktadır. Ayrıca, HT22 hipokampal hücre hattı üzerinde koruyucu bir ajan olarak AGO'nun Glu kaynaklı nörotoksisiteye karşı *in vitro* modelde etkisini araştırmaktır.

HT22 hücre canlılığını değerlendirmek için MTT analizleri kullanıldı. AGO 24, 48 ve 72 saat süreyle uygulandı. Hücre canlılığı oranları, kontrol gruplarına kıyasla ortalama hayatta kalma yüzdesi olarak ifade edildi ve ardından IC_{50} değerleri hesaplandı. Optimum doz belirlendikten sonra deney grupları dokuza bölündü: Blank grup (Medium), Cont grubu (Medium+ hücre), Toksik grup (Glu 5mM), Tedavi 1 grubu (Glu 5mM + 30 µM AGO), Tedavi 2 grubu (Glu 5mM + 60 µM AGO), Proteksiyon 1 grubu (30 µM AGO), Proteksiyon 2 grubu (60 µM AGO), DMSO 1 grubu (3µL DMSO), DMSO 2 grubu (6µL DMSO).

Bulgular, AGO'nun 24 saat sonra HT22 hücre canlılığı üzerinde önemli inhibitör etki gösterdiğini ve etkinin 25 µM AGO konsantrasyonunda anlamlı olduğunu ortaya çıkardı ($p \leq 0,05$). Özellikle, daha düşük AGO konsantrasyonları, inhibitör etkilerde zamana bağlı bir artış gösterdi; veriler, 8 µM ve 2 µM dozlarının, 48 ve 72 saat sonra hücre canlılığını önemli ölçüde azalttığını gösterdi ($p \leq 0,001$). AGO için IC_{50} değerleri 24, 48 ve 72 saat sonra sırasıyla 62,85 µM, 41,86 µM ve 26,65 µM olarak bulundu. Öte yandan, Glu uygulaması, 2,465 mM dozda hücre canlılığında %50'lik bir azalmayla önemli nörotoksisite gösterirken, daha yüksek Glu dozları (3, 4 ve 5 mM) çok daha yüksek azalmaya neden oldu. AGO'nun Glu ile birlikte uygulanması, Glu'nun neden olduğu nörotoksisiteyi etkili bir şekilde bastırdı ($p \leq 0,001$). Bununla birlikte, sitotoksisite sergilediği ve kontrol grubuyla karşılaştırıldığında önemli bir etkiye sahip olduğu için yüksek DMSO konsantrasyonlarında dikkatli olunmalıdır.

Bulgular, AGO'nun HT22 hücrelerindeki nöroprotektif etkilerinin doza bağlı olduğunu ve tedavi süresinden etkilendiğini göstermektedir. Düşük AGO konsantrasyonlarının hücre canlılığı üzerinde önemli koruyucu etkiler gösterdiği gözlemlendi. Bununla birlikte, orta ya da yüksek dozların hücre canlılığı üzerinde sitotoksisite gösterdiği ortaya kondu. Son olarak; AGO uygulanması Glu nörotoksisitesini azalttı ve hücre canlılığını arttırdı.

Anahtar Sözcükler: Nörodejeneratif hastalıklar; Agomelatin; L Glutamik Asit; HT22 hücre hattı; MTT

ABSTRACT

THE POSSIBLE EFFECT OF AGOMELATINE ON HT22 HIPPOCAMPAL CELL LINE IN EXPERIMENTAL NEURODEGENERATIVE DISEASE MODEL *IN VITRO* MODEL

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Neurodegenerative diseases (NDDs) are a series of progressive neuronal deaths due to an antioxidant system imbalance. *L* glutamic acid (Glu) is a neuroexcitatory transmitter in the nervous system, and excessive extracellular generation induces cell death. Agomelatine (AGO) is an antidepressant drug. This study aims to define the appropriate concentration and dose duration of AGO used as a neuroprotective agent and Glu as a toxic substance on the HT22 cell line. Furthermore, the effect of AGO as a protective agent on HT22 hippocampal cell line against Glu-induced neurotoxicity *in vitro* model.

The MTT assay is used to assess HT22 cell viability. AGO was administered for 24, 48, and 72 hours. The survival rates were expressed as the average percent of cell survival compared to control groups then IC_{50} values were calculated. After the optimal dose was determined, the experimental groups were divided into nine as: the Blank (complete media), the Cont (complete media+ cell), the Toxic (Glu 5mM), the Treated 1 group (Glu 5mM+ 30 µM AGO), the Treated 2 group (Glu 5mM+60 µM AGO), the Protection 1 group (30 µM AGO), the Protection 2 group (60 µM AGO), the DMSO 1 group (3µL DMSO), the DMSO 2 group (6µL DMSO).

The findings revealed that AGO demonstrated significant inhibitory effects on HT22 cell viability after 24 hours and the effect was significant at 25µM AGO concentration ($p \leq 0.05$). Notably, lower AGO concentrations demonstrated a time-dependent increase in inhibitory effects, the data showed that 8 and 2µM significantly reduced the cell viability after 48 and 72 hours ($p \leq 0.001$). The IC_{50} values for AGO were 62.85 µM, 41.86 µM, and 26.65 µM after 24, 48, and 72 hours, respectively. On the other hand, Glu treatment showed significant neurotoxicity, with a 50% reduction in cell viability at 2.465 mM, while higher Glu doses (3, 4, and 5 mM) caused a very high reduction. Combining AGO with Glu treatment effectively suppressed Glu-induced neurotoxicity ($p \leq 0.001$). However, caution should be exercised with high concentrations of DMSO, as it exhibited cytotoxicity and had a significant impact compared to the control group.

The findings suggest that the neuroprotective effects of AGO in HT22 cells are dose-dependent and influenced by the duration of treatment. It has been observed that low concentrations of AGO exhibit significant protective effects on cell viability. However, moderate to high doses have demonstrated cytotoxicity on cellular viability. Finally; the administration of AGO reduced Glu neurotoxicity and increased cell viability.

Keywords: Neurodegenerative diseases; Agomelatine; *L*-Glutamic Acid; HT22 cell line; MTT

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Negwod Abdelghani Saeed ABDALLA

CONTENTS

SYMBOLS AND ABBREVIATIONS

AD	:	Alzheimer's disease
ALS	:	Amyotrophic lateral sclerosis
AMPA	:	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AGO	:	Agomelatine
BDNF	:	Brain-Derived Neurotrophic Factor
CYP 1A2	:	Cytochrome p450 family 1subfamilyAmember 2
CNS	:	Central Nervous system
DMSO	:	Dimethyl Sulfoxide
DMEM	:	Dulbecco's Modified Eagle Medium
EMEM	:	Eagle's Minimal Essential Medium
EDTA	:	Ethylene Diamine Tetra Acetic Acid
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal Bovine Serum
Glu	:	*L* glutamic acid
HD	:	Huntington's disease
MTT	:	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
NDDs	:	Neurodegenerative diseases
NCD	:	Neuronal Cell Death
NMDA	:	N-methyl-d-aspartate receptor
OS	:	Oxidative stress
PBS	:	Dulbecco's phosphate-buffered saline
PD	:	Parkinson's disease
SD	:	Standard Deviation
ROS	:	Reative Oxygen Speices

SYMBOLS

µl	:	Microliter
ml	:	Milliliter
µm	:	Micromolar
mM	:	Millimolar
µg	:	Microgram
cm^2	:	Centimeter square
°C	:	Temperature celcius
rpm	:	Rounded Per Minute
95%	:	Incubator humidity
CO_2	:	Carbon dioxide
IC_{50}	:	The half-maximal inhibitory concentration

FIGURES LEGENDS

TABLES LEGENDS

1. INTRODUCTION

The word neurodegeneration comes from the prefix neuro part, which indicates the neurons in the nervous system, while degeneration refers to any process that caused the structure or physiological aspect of neurons or any organs or tissue to disappear. Therefore, neurodegeneration is any morbidity affecting nerve cells (Dauer and Przedborski, 2003, Przedborski et al., 2003). Neurodegenerative diseases (NDDs) are irreversible and progressive disorder that potentially causes neuronal death in nervous systems and are the main factor that causes motor and cognitive impairments (Amor et al., 2014). Each NDDs has different clinical symptoms and a unique etiological pathogenesis process (Gupta, 2022). NDDs are present in Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), and amyotrophic lateral sclerosis(ALS); however, the main reasons are unidentified (Dauer and Przedborski, 2003). According to progressive neuronal death, these diseases have many signs, including loss of memory, impairment of mobility, and deficits in behavioural and cognitive emotions (Mandemakers et al., 2007).

Furthermore, the mechanisms are unclear now, but some studies reported that the pathogenesis of these diseases is related to oxidative stress (OS) and excitotoxicity (Jiang et al., 2020). On the other hand, the homeostasis unbalance of the antioxidant system plays a prominent role in producing reactive oxygen species (ROS), resulting in neurotoxicity and OS; these play a critical role in NDDs (Barnham et al., 2004). Another study noticed several intracellular molecular signs associated with these diseases, including extensive oxidative damage to genes, lipids, and proteins. The most common ROS responsible for manufacturing OS is superoxide and hydroxyl radicals agents (Kim et al., 2017).

Oxidative stress refers to a situation where there is an imbalance between the generation of ROS and antioxidant responses (Ma, 2014). These ROS are made from aerobic mitochondrial metabolism via endogenous oxygen consumption or exogenously from a foreign chemical substance (Ray et al., 2012). Additionally, ROS are typically controlled or eliminated through the antioxidant systems and possess physiologically critical roles in cellular signalling pathways. Moreover, ROS act to regulate essential cellular processes such as cell proliferation, inflammation, immune response, apoptosis, and gene expression, whereas the advanced ROS level causes subjecting the cell to excessive ROS leads to chemical alteration in the cell such as

damage in DNA, lipids, and proteins, and loss of their physiological functions (Ma, 2014, de Mello et al., 2016, Birben et al., 2012).

Moreover, calcium homeostasis, mitochondrial function, kinetics action, cell morphology, receptor distribution, cell transcytosis, and energy balance are all affected by OS (Tönnies and Trushina, 2017). The OS and an imbalance between the generation of ROS and antioxidant system defences induced cell death and generated NDDs, resulting in the morphological change features of cell apoptosis, including chromatin condensation, DNA fragmented, and leading activity of some cysteine-proteases and caspases (Radi et al., 2014). Cautionary, the OS contributes to different types of diseases such as carcinoma, atherosclerosis, blood pressure, cardiovascular, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, asthma, and ageing (Birben et al., 2012). Elimination of these hazardous substances in organisms requires complex protection substances. Exogenous antioxidants are a substance that can improve the normalization of OS in the cell and reduce the possibilities of various neurological diseases (Singh and Singh, 2017).

Recently, tissue culture has been essential for maintaining and propagating isolated cells, organs, or tissues from animals in an appropriate environment to grow *in vitro*. It is also known as a method for maintaining cell survival and growth in the optimal complete culture media. This field includes the cell culture as single cell growth outside of the body in a suitable environment and no longer organized into tissues *in vitro*. At the same time, the tissue or organ culture *in vitro* is essential to maintain or develop the tissues or part of an organ; the benefit of tissue culture promotes differentiation and maintains the structural and physiological properties of the tissue (Fedoroff, 1967). Generally, the tissue culture grows in an appropriate environment with complete media. This media contains nutrients, growth and attachment factors, serum with suitable temperature, CO_2, and pressure, and consideration of the aseptic and incubation condition (Philippeos et al., 2012, Verma et al., 2020).

The cell culture isolates the cell from the tissue of animal or plant harvesting directly through digestive enzyme treatment and subsequent collected and growth in a complete culture media (Pham, 2018). Generally, the cell culture classifies into anchorage-dependent cells, as the primary cell culture, human strain diploid cells, and cell lines. These types grow and bind to solid or substrate surfaces, whereas anchorage-

independent cells are non-adhesive cell cultures and are cultivated suspended in culture media, like the cell lines and suspension cells (Mizrahi and Lazar, 1991). Cell culture is divided into precursor, differentiated, and stem cell cultures. Cells in the precursor cell culture are committed to differentiation; on the contrary, they can no longer differentiate further in a differentiated cell culture. Whereas they can differentiate into many types in the stem cell cultures (Verma et al., 2020). Cell culture techniques are used in the fields of vaccines, proteins, and monoclonal antibodies manufacture and tissue transplantation (Pham, 2018).

The primary cell cultures are obtained from tissue or organs. After passaging/subculturing, the cells are named the cell lines. In comparison, the strain cell is generated explicitly from the primary or line cells and subculture clone. This strain of cells required genetic change to initiate the parent cell line, and the clone is the population of cells that arose from the individual cell (Handler et al., 1980).

The cell line is first a subculture or passage of the primary cell. Also, the population of cultured cells has the same phenotypic and genotypic (Sarntivijai et al., 2014). Based on the life span, the cell line is either finite or continuous. In the finite cell line, the generation is limited, and the growth takes one to four days (Masters and Stacey, 2007). On the contrary, the continuous or indefinite cell is obtained from transformed cell lines *in vitro* or cancerous cells, grows as a monolayer or suspension cell, and takes time to generate (Masters and Stacey, 2007). Several technologists in scientific fields used this cell to make vaccines, therapeutic proteins, pharmaceuticals, and anti-cancerous agents (Verma et al., 2020). They also investigated drug metabolic and cytotoxicity, manufacture of antibodies, genetic expression function, and tissue formation (Kaur and Dufour, 2012). According to several studies, these cells can help recognize the hippocampus growth, plasticity, and the action of various receptors and intracellular communication during mitotic division (Eves et al., 1995).

Immortalized cell lines are a type of transformation cell characterized by chromosomal anomalies or mutations that induce the cell to divide indefinitely, such as cancerous cells. They could also be engineered by genetic retrovirus to continuously divide and produce several generations (Carter and Shieh, 2015). These cells do not need animal extraction to live; therefore, they are used more in biological laboratories and are accessible for growth and transformation. They give new cell lines by certain continuous gene expressions and proteins. This anomalous gene expression never appears in any cell in vivo (Carter and Shieh, 2015). Immortalized oncogenes in

cultured labs could provide unlimited mitotic cells without changing gene tumor properties, such as loss of contact inhibition. Scientists and researchers utilized the immortalized cell line *in vitro* to increase numerous generations of cell culture. Additionally, this cell type applies to different neuronal differentiation processes, such as axonal selection and development (Carter and Shieh, 2015). Clinically, it assesses the effectiveness of new anti-cancerous agents and predicts their therapeutic activities (Sharma et al., 2010).

HT22 hippocampal cell line is an immortalized mouse hippocampal neuronal cell (He et al., 2013). It originated from the parent HT4 hippocampal neural cell line (Davis and Maher, 1994) and is immortalized commonly by simian virus 40 large T (TL) oncoprotein (Bryan and Redder, 1994). HT22 cells rapidly grow, inexpensive, and simple to identify their molecular and cellular mechanisms (He et al., 2013). They are commonly used for investigating the neurons of the hippocampus area *in vitro* (Alboni et al., 2014, Karri et al., 2018). Experimental studies indicated that the cell is more sensitive to oxidative cytotoxicity of Glu due to a deficiency of ionotropic Glu receptors (Sagara et al., 1998). It has physiological cholinergic properties and can be used to define the cognitive deficit mechanisms in AD (Liu et al., 2009). It explains the mechanism of OS generation, which causes neurotoxicity (Lee et al., 2019). Architecturally, it mimics hippocampal neural precursor cells (Rössler et al., 2004). Increasing the levels of Glu in the extracellular milieu is more effective in the HT22 neural line; it could block the cystine uptake by cystine-Glu antiporter and depletes glutathione (Rössler et al., 2004, Tobaben et al., 2011, Li et al., 2009). The differentiated type has exceptional properties like cholinergic protein markers. It is more affected by the activation of nerve growth factor, helps the neurite outgrowth, and increases the length during the S phase cell (Zhang et al., 2018).

Cell viability is the number of healthy cells (Adan et al., 2016). Another definition is the percentage of live cells to entire cells (Nozhat et al., 2022). In an experimental sample, cell proliferation or viability is a vital sign for understanding the mechanisms of specific genes, protein synthesis, and different pathways associated with cell survival or death after exposure to harmful chemicals or physical agents (Adan et al., 2016, Aslantürk, 2018).

Several assays were applied to assess cell viability *in vitro* to correlate cellular behavior and activities, depending on the enzyme interaction, the permeability of

plasma membrane, cell adherence, ATP energy, and nucleotide uptake activity (Kamiloglu et al., 2020, Parimelazhagan and Thangaraj, 2016). They assess the number of living cells via cytotoxicity assays. One of these assays is trypan blue, which represents an inexpensive and commonly used assay to determine vital and total accounts of the cells. In this assay, the dead cells lose their membrane capability to block the absorption from the surrounding environment; thus, they appear blue (Stoddart, 2011). Besides, the tetrazolium dye, also known as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a broadly popular tool for assessing cellular metabolic activity. It converts MTT (yellow colour) into formazan crystals (purple) in a living cell. The action of this assay depends on the mitochondrial dehydrogenase enzyme (Kamiloglu et al., 2020, Stockert et al., 2012, Wang and Jokerst, 2016).

Agomelatine (AGO) is an atypical antidepressant drug, a unique analogous melatonin, known as S20098 (Wiley et al., 1998). The most common brand name is valdoxan (Vorob'eva, 2013). It is a compound of naphthalene designated chemically as N- [2-(7-methoxy naphthalene-1-yl) ethyl] acetamide. The molecular formula is C_{15} H_{17} NO_2 (Srinivasan et al., 2012). It composes of an antagonist serotonergic 5-HT2C receptor and agonist MT1 and MT2 melatoninergic receptors (Ying et al., 1996). The activity of MT1 and MT2 controls sleep regulation. The serotonin 5-HT2C is concerned with increasing the level of norepinephrine and dopamine in depressive pathologies in the brain (Vorob'eva, 2012). The interactions of these receptors improve circadian rhythms, promote normal sleep, help mood disorders, and enhance locomotor activity (Fornaro et al., 2010, Conboy et al., 2009, De Berardis et al., 2013).

AGO enhances hippocampal neurogenesis by promoting brain-derived neurotrophic factor (BDNF) expression and diminishes depression (Racagni et al., 2011). It is demonstrated in many preclinical studies of depressive animal disorders, including chronic mild stress and regular psychosocial stress tests (Popoli, 2009). Moreover, AGO can improve cell proliferation and stimulation of the neurogenesis stages in the dentate gyrus by maintaining cell survival (Banasr et al., 2006, Dagytė et al., 2011). Also, it possesses direct neuroprotective function via anti-inflammatory besides antioxidant properties (Cankara et al., 2021). Some studies reported that AGO possesses therapeutic neuroprotective benefits against OS, which maintain the brain endothelial cell's survival after exposure to inflammation and toxicity substances (Cheng et al., 2020).

AGO can regulate sleeplessness in autism spectrum disorder and enhance thinking in adult patients (Ballester et al., 2019). It improves biological and behavioural anxiolytic abnormalities, depression, and memory in prenatal stress rates (Marrocco et al., 2014). Interestingly, AGO does not affect sexual function compared to other antidepressants (Montejo et al., 2010). It has been reported that it can increase liver enzymes (Sansone and Sansone, 2011) and improve complications associated with epilepsy disorders (Vimala et al., 2014).

The antagonism of AGO promotes an anxiolytic function (Millan et al., 2005). It has potential anticonvulsant effectiveness and antioxidant characteristics against the seizure disorder model (Aguiar et al., 2012, Aguiar et al., 2013). It features the antioxidant effect, which increases gene expressions such as cellular transcription and (BDNF). These factors are essential to avoid stress-induced hippocampal memory impairment (Gumuslu et al., 2014). Moreover, it reduces severe apathy signs in frontotemporal dementia disorder via the 5HT2C antagonistic receptor action (Callegari et al., 2016). Administration of it in preclinical experiments significantly improved neuron plasticity (Païzanis et al., 2010).

In clinical trials, AGO can improve acute complications associated with diabetic disease (Can et al., 2018). Some studies showed that AGO could modulate intracellular signalling pathways to prevent cellular apoptotic (Akpinar et al., 2014). Atanasova et., al (2021) noted that AGO has the ability to reduce neuroinflammatory and promote neurogenesis in the hippocampal dentate gyrus (Atanasova et al., 2021). Moreover, in AD, AGO prevents neuronal senescence induced by amyloid precursor protein (Wang et al., 2021).

Glutamate (Glu) is a naturally free and non-essential amino acid. It is an abundant endogenous in the brain. It is formed by different body systems such as the brain, skeletal muscle, liver, kidney, and Langerhans of the pancreas. It is an essential excitatory neurotransmitter in the central nervous system (Park et al., 2019, Fukui et al., 2009). During brain growth and development, it plays a critical role in neuronal transmission, evolution, plasticity, cell differentiation, and migration (Kritis et al., 2015). Exogenously, Glu is the salt of a sodium glutamic acid known as monosodium Glu, it is characterized by solid and white crystallized form. It is used as a flavour or enhancing taste in many foods and plays a vital role in protein metabolization (Zhou and Danbolt, 2014).

Consequently, sodium-Glu transporters on neuronal and glial cell surfaces maintain average extracellular Glu concentrations (Tan et al., 2001). The abnormality of extracellular Glu level behaves as an excitotoxic neurotransmitter in the cell, leading to irregularity production of OS, which is the primary source of most NDDs (Coyle and Puttfarcken, 1993). It may lead to several disorders, including olivopontocerebellar degeneration, epilepsy, and hypoglycemia (Greenamyre, 1986). It may also cause AD, HD, AIDS, and LSA (Lipton and Rosenberg, 1994).

The excitotoxicity mechanisms of Glu occur in the synaptic regions of the brain through two main methods. The first is due to the overproduction of OS, known as oxidative Glu toxicity, while the other method is an excitotoxicity-independent intermediary via N-methyl-D-aspartate (NMDA), known as ionotropic Glu receptors and other Glu receptors (Coyle and Puttfarcken, 1993, Han et al., 1997). The release of OS due to an excessive level of extracellular Glu inhibits cystine uptake and promotes glutathione reduction in the cell (Tan et al., 1998, Han et al., 1997). Altogether, these mechanisms of the Glu are assumed to be primary sources of disability of neuronal function and degeneration (Han et al., 1997, Tobaben et al., 2011). Subsequently, the Glu is the principal agent for generating several NDDs (Park et al., 2015).

The excitotoxicity pathway initiates via the activity of the NMDA Glu receptor, resulting in NDDs associated with the excessive synthesis of ROS production (Han et al., 1997, Bazzett et al., 2000). Numerous experiments have demonstrated that a high concentration of extracellular Glu level acts as a neurotoxic substance. It promotes neuronal cell death via different pathways, such as increased OS levels in various types of neural cell lines and primary cell cultures such as HT22 hippocampal cell lines (Kim et al., 2017, Fukui et al., 2009, Jeong et al., 2007), C6 glial cells (Han et al., 1997), PC12 cell (Kawakami et al., 2011, Tan et al., 2013), and HT4 cells (Tirosh et al., 2000, Kobayashi et al., 2000). The Glu toxicity causes the glutathione depletion observed in different experimental studies, for example, in the neuronal hybridoma cells (Murphy et al., 1989).

Another study concluded that Glu-induced ROS level could lead to mitochondrial membrane dysfunction, glutathione depletion, and stimulate apoptosis in glial cells (Park et al., 2019). Therefore, studies reported that Glu concentration levels above average could produce neurotoxicity and lead to neuronal damage in PC12 neuronal cells and hippocampal cell lines, whereas the lowest dose of Glu may

enhance cell viability (Bhavnani et al., 2003). Exposure to the Glu can damage primary cortical cells, such as glial and neuronal cells (Koh and Choi, 1987). Some studies demonstrated that Glu promotes PCD in the HT22 cell line. The effectiveness of Glu depends on inducing necrotic cell death in a short time, while exposure for a long time encourages the apoptosis process (Tan et al., 1998, Fukui et al., 2009).

1.1 Justification of the Study

NDDs are common. Conversely, AGO is available and frequently used in clinical therapy. Previous research recommends conducting further studies to investigate the protective effect of AGO against OS production and glutathione depletion associated with molecular signalling pathways in the cell. The study aims to identify the main properties of AGO action mechanisms that prevent cellular death, thus maintaining cellular viability. Its protective mechanism against neurotoxic substances is not well understood. There is a lack of studies regarding the neuroprotective and antioxidant effect of AGO agents against Glu-induced toxicity in the HT22 neural cell. Additionally, broad strategies around the Glu antagonistic neurotoxicity are needed to create modern treatments with the highest efficacy and the lowest adverse effects on brain function.

1.2 The Objective of the Study

1.2.1 General Objective

Assess the neuroprotective effect of AGO on HT22 hippocampal neural line cells *in vitro*-induced experimental neural disease model.

1.2.2 Specific Objective

Investigate the effectiveness of AGO on NDDs -the induced model in HT22 hippocampal neural cell line through the following:

1. Evaluate the cell counting, viability, and proliferation.
2. Distinguish and detect the cellular morphological change after exposure to different doses of Glu toxicity.
3. Determine features of the neural cell line culture treated with AGO, AGO + Glu, Glu alone, and the control groups under the inverted microscopic.

This study suggests that AGO has different characteristics, such as antioxidant and anti-apoptotic properties, which can aid in avoiding intermittent disruption of molecular signalling pathways inside the cell culture. Also, the study aims to understand the actual intracellular events associated with the morphological changes

in the cell resulting from cell toxicity. Glu can induce neurotoxicity in HT22 cell line in vitro models and is critical in cell damage and death leading to NDDs.

The novelty of my study suggests that since the AGO possesses high potential antioxidant, anti-apoptotic, and anti-inflammatory activities, it may promise us innovative neurotherapeutic medicine to improve the NDDs in the future.

2. GENERAL INFORMATION AND SUMMARY OF LITERATURE REVIEW

2.1. Introduction and Historical Background

Studying animal and human cell cultures is essential in many scientific approaches. It is not a new field; however, animal cell culture techniques were developed in the 1900s. Moreover, the cell line is sourced from a specific tissue or organ and maintained uniquely. This technique was developed in the eighteenth century (Verma, 2013). Therefore, the historical background of the cell culture technique assists us in recognizing how this cell culture started and how it applies to future cell culture technology. Subsequently, this section summarizes some successful fundamental events in cell culture development. The first attempt at animal cell culture was conducted in 1902 by Leo Loeb; *in vivo* culture, he tried to culture the tissue from the inside to the outside of the body. This procedure at that time was known as the cultivation of the tissue culture within the body. However, it was not considered a classical type of cell or tissue culture due to grafting the tissue fragments and fluid inside the living animals (Jedrzejczak-Silicka, 2017).

In vitro, the most effective technique of cell culture started in the year 1907 by Ross Harrison, using fragments of the nerve fiber from a living embryonic frog tissue and propagating in the hanging drop technique of the lymphatic sac and enclosing by glass plate on slides cover to observe the growth of neuronal processes (Harrison, 1906). In 1909, the hanging-drop technique was used again to maintain the tissue fragments of warm blood animals such as chick embryos, dogs, and cats (Ambrose, 2019). In addition, in the year 1910, Carrel and Montrose Burrows made a great effort to improve the hanging drops technique of Harrison by replacing the lymphatic sac fluid with animal blood for cell culture and using different tissue fragments from chick embryos and various mammals and also tumor tissues, and produces coagulant media for suitable mammalian cell culture (Carrel and Burrows, 1910). In 1912, Alexis Carrel manufactured the flask of tissue culture technology, which is used broadly now. He also invented the aseptic technique for the cell culture lab (Carrel, 1923). Enders and his colleagues 1949 extracted the vaccine from the poliomyelitis virus (Macdonald, 1990). Then, in 1951, the first and most widely used model of a human tumor or immortal cells, denoted as the HeLa cell, originated from a patient diseased with uterine cervical carcinoma (Korch and Capes-Davis, 2021).

The cell culture media, especially the Eagle's basal culture media, was developed in 1955 by Harry Eagle (Mizrahi and Lazar, 1991). Then, in 1961, Hayflick and Moorhead attempted to establish the first finite cell line culture known as the fibroblast human from the carcinogenic cell (Earle, 1943) (Macdonald, 1990). In 1975, Kohler and partner Milstein achieved remarkable significance in isolating the hybrid cell line and utilized it to produce monoclonal antibodies (Mizrahi and Lazar, 1991). 1976, the growth media, including growth factors and hormones, was designed (Mizrahi and Lazar, 1991). In 2012, the first new brand of therapeutic gene intervention for treating different diseases-associated with aging was described (Bernardes de Jesus et al., 2012). Entirely derivative of the embryonic stem cell lines in 1981 from mice and 1998 from humans (Solter, 2006) and the pluripotent-induced stem cells developed in 2006 (Takahashi and Yamanaka, 2006).

2.2. Definition and Application of the Cell Culture

The cell culture allows prokaryotic or eukaryotic cells to grow in a suitable artificial environment (Segeritz and Vallier). The cell culture is the most common route targeted in the tissue culture and initiates via gathering or obtaining the cell to grow out of the primary explant tissue. The cells cultured on a specific substrate surface as monolayers adherent, or the cells may float free in culture media, referred to as an independent cell suspension. These cells are collected via enzymatic treatment or specific dissociated mechanisms (Fedoroff, 1975). The cell culture has a wide range of valuable applications, from simple applications, including testing the cytotoxic effects of drugs and candidate compounds, manufacturing pharmaceutical products such as enzymes, and developing growth factors. Also, it is used to study the main biological activities of the cell, such as the cell cycle process, cell–cell communication, and cellular matrix interactions (Segeritz and Vallier, 2017). In addition, it is utilized to detect average growth and maturation of the tissue, such as gene therapy, by replacing non-functional genes with functional genes. Also, it is used to determine the main characteristics of cancer cells, viruses, and radiation in carcinoma, the production of monoclonal antibodies and pharmaceutical drugs (Verma, 2013), and in complex applications such as tissue engineering techniques.

2.3. Types of the Cell Culture

There are three types of cell culture: the undifferentiated precursor cell that can differentiate and develop. The differentiated cell culture which can never be modified to another cell. The stem cell which is undifferentiated and can be distinguished into

various cells (Verma et al., 2020). In another classification, cell culture is classified according to its origin into the primary, human strain diploid, and line cells. Also, according to the cell growth properties, they are classified as anchorage-dependent and independent cells. The anchorage-independent cells include the cell line, while the anchorage-dependent cells include the primary cell and the cell strain (Mizrahi and Lazar, Fedoroff, 1975). The strain cell generates from the subculture of a specific primary or line cell. The clonal cell refers to the population of cells that arise from the individual cell (Handler et al., 1980). However, in biological labs, the cell is three types: primary, transformed, and stem or self-renewing cells (Segeritz and Vallier, 2017). A different kind of cell culture according to rearrangement and recombination of the cell structures in simulated tissue to produce new organs, keep them functional and permit differentiation (Fedoroff, 1966). This type includes the histotypic propagation of the cell line and increases at a high density in a three-dimensional matrix. In contrast, organotypic is the interaction of more than two types of cells to generate new organ components under specific ratios and spatial relationships (Freshney, 2006, McAteer and Douglas, 1979).

2.3.1. Primary Cell Culture

2.3.1.1 Definition and Properties

It is isolated and obtained from tissues or organs by unique mechanical means and digestive enzymes. Generally, the cell proliferates in suitable equipment containing complex supplementary growth media under the appropriate condition culture (Khan and Gasser, 2016). When the cell occupies all the available substrate surface (confluence stage of the cell growth), then the cell should be subcultured or passage to the new vessels, which contain the fresh growth media. Low growth rates and heterogeneous cells are characteristic of the primary cell culture and challenging to harvest primary cells, and it is a finite lifecycle; the most significant disadvantage of these cells is easy exposure to viruses and bacteria contamination (Verma et al., 2020). Additionally, it differs from the cell line, which can grow indefinitely. The primary cell is characterized by dividing once or twice before being subjected to the senescence process or being transformed to become a continuous cell line. It requires a constant supply and re-establishes ongoing growth. Also, these cells have a finite number of cell divisions and cannot expand (Khan and Gasser, 2016). It classifies into two Anchorage-dependent adherent cell cultures and needs to grow on the substrate surface for adherent and replication (McAteer and Douglas, 1979). On the contrary,

anchorage-independent suspension cell culture is non-adhesive cells and growth floating free in a culture medium without needing a solid surface for attachment or proliferative (Philippeos et al., 2012).

2.3.2. The Stem Cells and Different Types

They are pluripotent cells generated from different parts of organisms and renew themself continuously. It resembles the immortalized cell line and is identical to the primary cell, as both originated directly from a living organism. According to the source, stem cells could be divided into embryonic, adult, and induced pluripotent stem cells. Another classification involves neural stem, Hematopoietic stem, and skin stem cells (Verma et al., 2020). Embryonic stem cells are differentiated into mesenchymal stem, brain-derived neural stem, and induced pluripotent stem cells (Takahashi and Yamanaka, 2006). The function of mesenchymal stem cells is to release protective compounds that promote immigration and facilitate the rebuilding of the malignant inflammatory tissue. These cells mainly arise from many areas, like adipose tissue, bone marrow, and partially from the placenta and blood of the umbilicus cord and jelly Wharton's (Hass et al., 2011). The induced pluripotent stem cells mainly originate from fibroblasts of the adult dermis and are modified genetically to transform into another type of stem cell (Duncan and Valenzuela, 2017).

2.3.3. The Cell Line and Immortalized Cell Line

It is a specific population of cells characterized by reserving and extending for a long time with unchanging phenotypes. The clonal cell line descends from a single progenitor cell (Li, 2011). At the same time, the immortalized cell lines are either cancerous cells that never stopped dividing or initiated from indefinite cells and can be subcultured for several generations (Phelan and May, 2017). The vital benefits of immortalized cells are homogeneous, a genetically analogue population, rapid development, and no need for living animal extraction (Phelan and May, 2017). However, the disadvantage of these cells is that they are not classified as normal cells because they can remain indefinitely in artificial environments (Jedrzejczak-Silicka, 2017). Frequently, they possess particular gene pattern expression and can be passaged to generate new altered cells from the normal cell (Phelan and May, 2017). They are essential in vitro as the study model and critical to understanding the fundamental role of neurobiological development, such as neuronal axonal selection and growth. Additionally, they can generate astrocyte, neuronal, and oligodendrocyte cells *in vitro* and *in vivo* (Whittemore and Onifer, 2000, Frederiksen et al., 1988).

2.3.4. Immortalized Cell Line

The immortal cell is a transformed cell line characterized by impairment of the checkpoint cell cycle and can be isolated from tumorous cells. Several approaches to transform or infect the cell to provide the immortal cell line, including (temperature-sensitive simian virus 40 (LT) antigen, Epstein-Barr virus, adenovirus E1A, papillomaviruses, saimiri herpesvirus, T-cell leukaemia human virus, telomerase reverse transcriptase) (Soice and Johnston, 2021). Generally, the most common vectors utilized to generate cell transformation are SV40 and Human papillomavirus (McKay et al., 1988). This oncogene causes the inactivation of regulatory factor genes like p53 and retinoblastoma, which control cell cycle genes. It leads to the proliferation of the cells for a limited number of populations after the normal cells are subject to senescence (Bryan and Redder, 1994, Freed et al., 2005). In contrast, telomerase gene expression causes genome expression instability, generating cell mutation that transforms into immortal cells (Freshney, 2015). Immortality is potentially significant in determining several life processes, including cellular proliferation and differentiation (Kohno et al., 2011). Also, it is used to clarify malignant cancerous intercellular signalling pathways. The benefits promote tissue regeneration in medicine (Irfan Maqsood et al., 2013). It is a valuable tool for developing regeneration therapy for patients with periodontitis and for studying cementogenesis (Saito et al., 2005).

2.4. Normal Morphological Appearance of the Cell Culture

The cell cultures' morphological features contain epithelial-like, fibroblast-like, and lymphoblastoid-like, broadly describing the cell morphological characteristics focused on natural observations. Many elements may be essential to determine if the cell type is a fibroblast, epithelial, or lymphoblast.

2.4.1. Fibroblast-like

It is bipolar or multipolar, stellate-shaped, and grows on the substrate aspect (Schaeffer, 1979, Nims and Reid, 2017).

2.4.2. Epithelial-like

The epithelioidal cells are flatted or cuboidal in shape, cobble, and appear as polygonal shape (Schaeffer, 1979, Nims and Reid, 2017).

2.4.3. Lymphoblastoid-like

The suspension cell usually appears to have rounded or spherical growth as single or cluster suspension cells without being attached to the substrate surface.

Besides these types, there are other types of cells regarding the microscopical morphological changes that develop due to infection by certain viruses or the progress of PCD, like apoptosis or cytotoxicity (Nims and Reid, 2017).

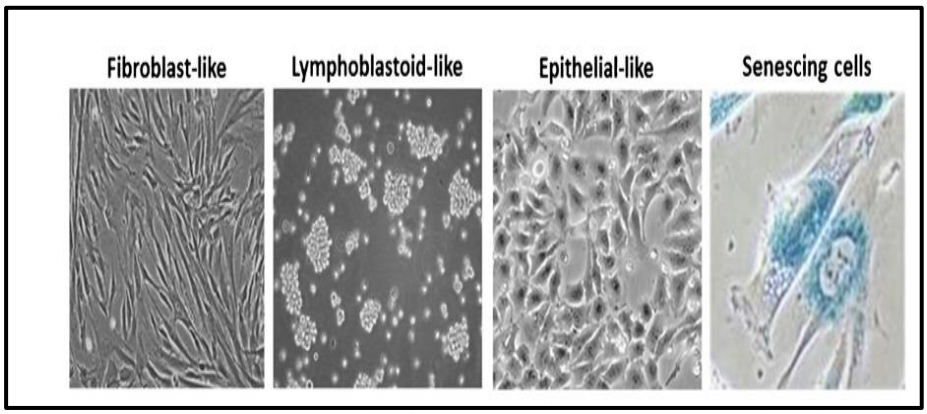

Figure 2.1. Showed some normal morphological appearance of the cell culture under an inverted microscope (20x, 20x, 40x, and 100x magnifications, respectively, from left to right) (Nims and Reid, 2017).

2.5. The Cell Cycle Phases for Growth

All different types of cell culture pass through four distinctive culture phases, including the lag, log, plateau, and decline phases, respectively (Ravi et al., 2016).

2.5.1 The Lag Phase

The initial stage is when cells adapt to new environmental conditions without dividing. This phase depends entirely on the cell type, seeding, cell density, medium culture components, and cell treatment (McAteer and Douglas, 1979).

2.5.2. The Log or Exponential Phase

It is characterized by increasing the cellular number exponentially to cover the whole growth substrate (reach confluency). The cells are subculture or passage before they become entirely confluent. Also, the maximum metabolic activity is indicated in this stage (McAteer and Douglas, 1979).

2.5.3. The Plateau Phase

In this phase, the cell growth rates break down, and some events occur, like discontinuous cell proliferation and the stationary phase.

2.5.4. The Stationary Phase

It is known as the plateau phase, where cell growth reduces from 0 to 10. Several alterations in molecular and physical markers of the cell, especially protein synthesis, are observed (Verma et al., 2020).

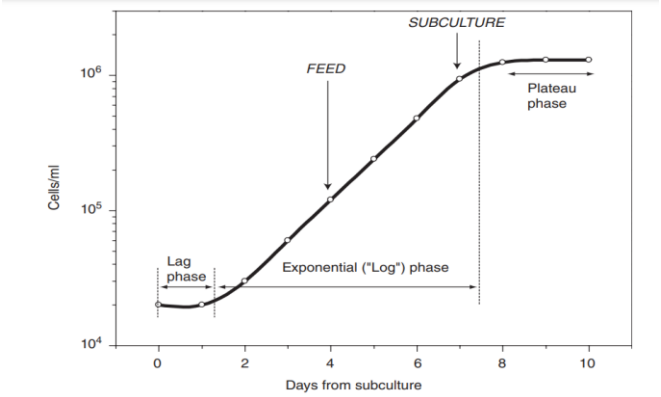

Figure 2.2. The growth curve and maintenance of cell concentration versus time from subculture displayed the lag phase, exponential phase (confluence), plateau, and identifying the best periods for subculture and feeding (Freshney, 2005).

2.6. The Environmental Conditions of Culture Cell

The environment differs from one cell to another. Usually, a suitable environment to maintain the cell is created by complete fresh culture media with additional elements, serum, hormone, the factor of growth, amino acids, carbohydrates, vitamins, minerals, nitrogen, mineral salts, nucleotide precursors, antibiotics, oxygen consumption, the pH balance, and buffering product (Mizrahi and Lazar, 1991). The cell culture incubates at 37 ºC, 5% CO_2, and humidity 95%. The oxygen level is essential to keep and regulate pH (Freshney, 2015).

2.7. Aseptic Condition

It is essential to prevent the cell from contamination by bacteria, fungi, and viruses. Because the contamination alters the physiology functions of the cells and may cause cell death. An aseptic technique is necessary for all people working in cell culture, and daily sterile of the instrument is essential. The biosafety cabinet hood is the appropriate environment for animal cell culture (Freshney, 2015).

2.8. The Hippocampus

2.8.1. General Structure and Functions of Hippocampus

In humans, the hippocampus is neural, an elongated structure located deep within the temporal lobe. It is an integral part of the limbic system and is highly associated with learning and memory functions. It participates in the formation of the hippocampal formation. It is more C-shaped and prominent in the inferior horn of the cerebrum lateral ventricle. Anatomically, the hippocampus is formed mainly by four areas: dentate gyrus, entorhinal cortex, Cornu ammonis, and subiculum

complex(Schultz and Engelhardt, 2014). The proper hippocampal area is known as Cornu Ammonis and is usually formed by four main anatomical areas, including (CA1, CA2, CA3, and CA4) (Sweatt, 2004). At the same time, the dentate gyrus and Cornu Ammonis structures are the hippocampal formations (Lothman et al., 1991).

Another region is the para-hippocampal, which divides into the rostral region (head), which curves dorso-caudal to form the uncus and lies on the medial part of the temporal lobe. In comparison, the caudal portion (tail) divides into the fimbria that runs ventrodorsally to the corpus callosum and the gyrus fasciolaris that becomes the fornix and indusium griseum (Destrieux et al., 2013). The One-way direction of the sensory pathway interconnects all the hippocampal structures. So initially, the cortical sensory input enters the hippocampal via the dentate gyrus. The information comes mainly from the entorhinal cortex via the perforant path input (Knierim, 2015). Subsequently, the dentate granular cell sends the fibers of mossy into the CA3 region of the hippocampus. The neuronal pyramid in the CA3 area gives some axonal fibers to end in this CA3 as a collateral association, and remnants of axonal fibers input terminate in the CA1 hippocampus. This CA1 pyramidal neuron is the central region of the hippocampal neuronal output, containing glutamic neurotransmitter axons. It acts to distribute the information from the hippocampal proper by the Schaeffer securities fibers to contralateral and ipsilateral of entorhinal, subiculum, and subcortical structures (Knowles, 1992, Amaral and Witter, 1989). However, the output from CA1 neurons also projects into the contralateral hippocampus via the fornix and distributes to different areas such as anterior stria and mammillary bodies (Sweatt, 2004). CA1 neuronal returns to project into the entorhinal cortex again, forming a complete hippocampal circuit known as the trisynaptic loop (Knierim, 2015). In the adult brain, the dentate gyrus plays a vital role in hippocampal cell proliferation and neurogenesis, and the cell can differentiate into new neuronal and glial cells (Nyberg et al., 2005).

2.8.2. The Hippocampal Function

It denotes a unique and vital functional part of the brain due to the neurogenesis process, which is generated continuously in the adult brain. Also, It plays a fundamental role in long-term memory, learning, and spatial navigation because it connects with the neocortex (Stella et al., 2012). In addition, it has been associated with olfactory function for a long time since it receives direct input from the secondary olfactory blub (Soudry et al., 2011). Moreover, it plays an essential role in motor behaviour because it is the central part of the ventral striatal loop. It also can regulate

emotional behaviour. Therefore, the hippocampus fibers projected into the hypothalamus and influenced the function of the hypothalamus by affecting the secretion of adrenocorticotropic hormones. The hippocampus has bilateral tasks as part of the limbic system. Hence, it plays a fundamental role in learning and memory functions (Tsukahara et al., 2006, Milner and Klein, 2016). The hippocampus and other cortical structures assist with predictions of future events while making decisions, planning, and envisioning a novel scenario. However, certain studies on amnesic individuals indicate that this function is impaired during hippocampal injury (Buckner, 2010).

2.8.3. Hippocampal Neurogenesis

It occurs during the embryonic period and is completed after birth (Kriegstein, 2005). It is a continuous process that follows throughout postnatal life and can generate new neurons for memory operational process circuits in the adult brain of the mammalian (Zhao et al., 2008, Valero et al., 2017). In addition, neurogenesis remains exclusively in the hippocampus in the dentate gyri and the bulb of olfaction of the adult brain. The hippocampal has a unique heterogeneous neuron that can reorganize its synapses and dendrites. The structural alteration associated with neuronal growth continues from birth to early adulthood and into middle age and senescence (Leuner and Gould, 2010). The hippocampal neurogenesis is affected by many NDDs connected with cognitive dysfunction, such as depression, epilepsy, cerebral ischemia, AD, and PD (Zhao et al., 2008).

The assessment of the hippocampus growth and regulation of trophic interactions are essential in supporting and maintaining the growth of synaptic inputs because the synaptic inputs are necessary for controlling the cognitive function that is relatively associated with many neural diseases (Eves et al., 1995). It is used as a model to study malignant diseases (Capes-Davis et al., 2010).

2.8.4. Stages of the Adult Hippocampal Neurogenesis

Generation of new neurons in adult hippocampal passes through precursor cell early survival, postmitotic maturation, and late survival stages (Kozareva et al., 2019).

2.8.4.1. Precursor Stage

New neurons originate from stem cells like glia or precursor cells. This precursor is frequently divided into progenitor cells and can differentiate into immature neuroblasts or glioblasts (Kempermann et al., 2015, Encinas et al., 2011). that subsequently differentiate into mature dentate granule neuron(Gonçalves et al., 2016)

2.8.4.2. Early Survival Phase

This stage indicates that by exiting from the cell cycle, the most significant number of the newborn cells after birth are eliminated or die by apoptosis or maybe survive to continue their functional and morphological maturation (Valero et al., 2017a, Chancey et al., 2013).

2.8.4.3. Post Mitotic Maturation Phase

The newborn, lineage-committed cells exit the cell cycle and enter a maturation stage. Through this stage, dendrites grow and extend into the molecular layer, while axons grow to reach CA3, as this stage is characterized by synaptogenesis.

2.8.4.4. Late Survival Phase

The adult-born dentate granule cell matures into highly excitable neurons, which integrate with the existing circulatory of the dentate gyrus and increase synaptic plasticity (Valero et al., 2017, Kozareva et al., 2019).

2.9. HT22 Hippocampal Neural Cell Line

These cells are generated by retrovirus infection of the primary hippocampal cell culture and established from the embryonic animal at 18 days old. It subcloned HT4 immortalized cell lines (Frederiksen et al., 1988, Lendahl and McKay, 1990). The HT22 cell line resembles neuronal cells and lacks ionotropic Glu receptors. It uses laboratory experience as an appropriate neural cell line to study the disorders induced by Glu toxicity (Kim et al., 2017, Rössler et al., 2004). Also, it is a cholinergic neuronal cell used as a model to study the cognitive deficits of AD (Liu et al., 2009) and to investigate the neuronal diseases associated with OS (Rao et al., 2013).

2.10. Neurodegenerative Diseases

These diseases serve as a severe problem that is threatening human health in the world (Gitler et al., 2017). The pathophysiological signs are still not fully understood. The components that may contribute to the pathophysiology include PD, AD, and ALS. These disorders are complex in many cellular milieus, such as neurons, oligodendrocytes, Schwann's cells, and muscle cells, which gradually deteriorate until degenerating in a wide range of pathological symptoms affecting the nervous and the muscular systems (Radi et al., 2014). Additionally, some study explains that the leading causes of these diseases are associated with the deposition of protein screening in the cell cytoplasm, followed by toxicity and damage of the cell, which leads to impairing memory, dysfunction of synapses, and cognitive defect (Pathak et al., 2022). The result is in many NDDS developments (Gupta et al., 2021). The most common

causes are excessive production of ROS and OS levels more than the antioxidant defence system (Niedzielska et al., 2016). Mitochondrial malfunction, protein misfolding, heavy metal toxicity, and disruption of energy supply (Gupta, 2022). Other risk factors for this disease include environmental chemicals and genetic abnormalities in numerous genes (Mandemakers et al., 2007). Progressive OS and DNA mitochondrial alteration are dangerous factors and contribute to aging, which is the leading cause of neurological disorders (Lin and Beal, 2006).

Further insufficiency in ATP synthesis accompanies inappropriate ROS production, buffering calcium ions, and uncontrolled apoptosis of cells. All these irregular processes lead to CNS degeneration (Mandemakers et al., 2007). The essential proteins associated with these diseases involve B- amyloid, prion protein, tau, a-synuclein, and TDP-43 (Kovacs et al., 2013).

2.11. Oxidative Stress

They are associated with high quantities of ROS, such as oxygen, free hydroxyl radicals, and deficiency of the oxidant or antioxidant system balance. DNA fragmentation, lipids, and protein can initiate excessive ROS production, which eventually induces apoptotic and necrosis processes and causes the tissue to die (Singh et al., 2019, Prasad et al., 2014). OS potentially causes cellular malfunction or damage (Birben et al., 2012). Generally, ROS compounds are natural products in all different organisms and act as defence mechanisms in the body (Kim et al., 2017).

2.12. Antioxidant

The word antioxidant refers to a substance inhibiting oxygen consumption (Ma, 2014). It is necessary for the survival of plants and animals, also known as chemical molecules that prevent the risk of unpredictable molecules such as free radicals, by reducing or inhibiting the oxidation of substances that produce these free radicals (Hamid et al., 2010). The human antioxidant system prevents tissue from being damaged by toxic substances and comprises exogenous and endogenous antioxidants. These exogenous and endogenous antioxidants are biologically enzymatic and non-enzymatic (Pisoschi and Pop, 2015). It also contains minor antioxidants, such as antioxidant proteins and ROS-metabolizing enzymes. In addition, this antioxidant can regulate proteins that influence oxidant stress reactions (Ma, 2014). Functionally, the system has resistance ability against unregulated production of free radicals and stimulated oxygen species or prevents their interactions with biological cellular structures (Chaudière and Ferrari-Iliou, 1999). The essential antioxidant enzyme is

superoxide dismutase, mainly transformed to H_2O_2, and protects cells from damage (Prasad et al., 2014). Subsequently, inhibition of this enzyme's activity can increase the superoxide that induces the cellular apoptosis process (Prasad et al., 2014). Another function of this system is protecting the neuronal cells from OS and maintaining the brain tissue from damage by encouraging antioxidant defence to inhibit ROS production (Lee et al., 2020). It also reduces cell damage by elevating the activity of antioxidant enzymes or interacting with free radicals (Lü et al., 2010).

2.13. Agomelatine

It is an innovative and new anti-depressive drug that recently acquired authorization from the European Medicines Agency to treat severe episodes of depression in adults (Tardito et al., 2012). The mechanism results from the interaction of melatonergic receptors and 5-HT2C serotonin receptors. The AGO promotes neurogenesis and cell viability (Tardito et al., 2012). AGO treatment boosted neurogenesis outgrowth, increased the number of granular cells, and expedited the maturity and lifespan of new cell granules (Soumier et al., 2009). AGO improves the expression of pre and post-synaptic neurons and microtubular proteins of the cytoskeleton and BDNF (Ladurelle et al., 2012). The prior experimental study suggested that AGO therapy can prevent cardiovascular system disorder by suppressing the action of proinflammatory cytokines and chemokines. That can inhibit the activity of angiotensin II, facilitating the adhesion of endothelial and monocytic cells and leading to endothelial disorder (Hong et al., 2021). Another study uses AGO to inhibit proinflammatory cytokine activities in murine macrophage cell lines (Hyeon et al., 2017). Several types of research have highlighted that AGO treats depression and anxiety mainly through the melatoniergic receptors and 5-HT2C antagonistic receptors and regulates circadian rhythm (Rainer et al., 2012). Some findings indicate that agomelatine therapy has antioxidant ability alone or combined with other antidepressants. That leads to suppression of intracellular signalling molecules that cause the cell to be apoptotic, such as oxidative stress production caspase-3, caspase-9, mitochondrial membrane depolarization, reduces glutathione, and lipid peroxidation, as well as blocking voltage-gated of Ca^{2+} Channel in neuronal PC12 Cells (Akpinar et al., 2014). Another recent study has shown that AGO therapy inhibits macrophage cell migration and prevents brain endothelial cell damage by improving the strength of the tight junctions of the cell and preventing permeability of the blood-brain barrier (Cao et al., 2021). In addition, it plays a pivotal role in reducing

the growth and migration of breast cancer cells more than melatonin (de Carnevale Galeti et al., 2021). A previous study showed that the antidepressant prevents the negative effect of stress on hippocampal memories and induces the systemic storage of memory in responses to experimental learning (Conboy et al., 2009). It also demonstrated the sub-chronic treatment of it to promote brain energy metabolism and reduce OS markers (de Mello et al., 2016). Indeed, clinical therapy reveals that AGO more effectively affects depression, enhances the normal circadian rhythms and delayed sleep phase syndrome, and improves the disorganization of sleep-wake beats (Popoli, 2009). In addition, it has a vital role in preventing peripheral neuropathy (Yucetas et al., 2019). Many articles report that AGO is effective in the chronic mild stress study by protecting the neurogenesis process and neonatal cell life in hippocampal rat models. Also, It acts in the dentate gyrus to regulate doublecortin protein synthesis (Dagytė et al., 2011). However, it can prevent amyloid beta to induce tau phosphorylation and oxidative damage in PC12 cells that may affect AD progress (Yao et al., 2019). The AGO significantly minimizes neuropathic pain hypersensitivity via melatonergic and 5-HT_{2C} receptors in several experimental animals (Chenaf et al., 2017).

2.13.1. Chemical Composition of Agomelatine

AGO is a nonionizable amide compound that is poorly soluble and acts as a melatonergic antidepressant drug (Skořepová et al., 2017). It has a structural formula resemblances to melatonin. It is a naphthalene compound. It is a agonist MT_1 and MT_2 melatonin receptor and antagonistic 5-HT2c serotonin receptor (Srinivasan et al., 2012). Its properties are weakly soluble in water, poor bioavailability, and pleiomorphisms (Liao et al., 2016)**.** In humans, the half-life of AGO is greater than melatonin, about 2 hours (Srinivasan et al., 2012). Additionally, it is more differentiated from melatonin because it has higher affinities with 5-HT2C and 5-HT2B receptors than 5-HT2A (Millan et al., 2003).

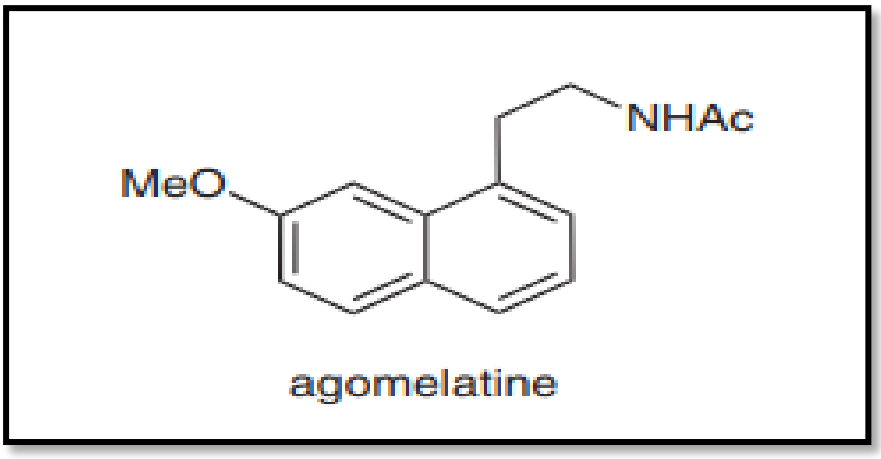

Figure 2.3. Chemical structure of agomelatine (Markl and Zlotos, 2011)

2.13.2. Discovery of Agomelatine

It is a new and novel non-SSRI for treating major depressive disorders. It was discovered and developed by the European Pharmaceutical Company Servicer Laboratories Limited in 1992 (Su et al., 2023). In 2005, it was refused by the Agency of European Medicines, submitted again in 2007, and approved in 2009 (Liu et al., 2016). To treat the depressive disease. This year, it was also approved in different countries, for example, Canada, South America, and Australia (Skořepová et al., 2017).

2.13.3. Absorption and Distribution of Agomelatine

Eighty percent of AGO is absorbed rapidly after oral administration in the gastrointestinal tract (Sansone and Sansone, 2011). It is mainly absorbed in parts of the small intestine, such as the duodenum and jejunum, through passive diffusion (Liao et al., 2016). It has a short life span in blood plasma, just between one to two hours. *In vitro*, the ratio of AGO to plasma in blood concentration is 5:1. Less than 95% of AGO bounds directly with plasma proteins such as albumin and α-1 acid glycoprotein and contributed 35% and 36%, respectively (Sansone and Sansone, 2011). It has moderate distribution throughout the body with a volume of about 35L. It has greater bioavailability in women than in other genders, and food intake does not affect the absorption or bioavailability of AGO (Sansone and Sansone, 2011).

2.13.4. Agomelatine Metabolism Process

The metabolism process initiates in the liver via hepatic CYP 1A2 of the cytochrome P450 isoenzyme system and is expelled through the urine. More than 80%

of the AGO is hydroxylated metabolically through this enzyme to form (S-21540) and 3,4-dihydrodiol (S-22380) (Hong et al., 2021). The primary metabolic process of AGO is known as hydroxylation and demethylation (Green, 2011). The remanent of metabolism occurs via 7-O-demethylation such as CYP2C 9 or CYP2C19 to produce (S-21517) (Zupancic and Guilleminault, 2006, Saiz-Rodríguez et al., 2019). About eighty percent of this substance is expelled through urinary excretion, While small parts of the metabolites are eliminated through fecal (Green, 2011).

2.13.5. Melatonergic Receptors of Agomelatine

They are classified as G protein-coupled melatonergic receptors in seven specific trans-membrane domains (Tardito et al., 2012). There are two mammalian melatonin receptors, MT1 and MT2 melatonin subtypes (von Gall et al., 2002). The MT1 receptor prevalence is significant in the brain tissue that is associated with the dopamine signalling pathways such as the suprachiasmatic nucleus, hippocampus, retina, basal ganglia, the nucleus of accumbens, substantia nigra, and ventral tegmental region (De Berardis et al., 2013). Also, they are scattered in some parts: the paraventricular, periventricular, supraoptic, and tuberomammillary nucleus, Broca's diagonal band, the nucleus of Meynert's, sexually dimorphic nucleus, and mammillary body (Wu et al., 2006). However, MT2 receptors frequently are distributed in the hippocampal region, the suprachiasmatic nucleus, and the retina. MT1 receptors may appear in another body tissue, while the MT2 receptors distributed are more constrained. Also, these receptors are present in the thalamus, cerebellum neurons, and glial cortex (De Berardis et al., 2013, Tardito et al., 2012, von Gall et al., 2002). These agonists receptors act as neurogenic and anti-depressant receptors through the inhibiting of the acid sphingomyelinase, that leads to reduced ceramide and pro-inflammatory cytokines or elevated the level of monoamine oxidase in the hippocampal region (Boiko et al., 2022).

2.13.6. Serotonergic 5-HT2C Agomelatine

Also known as serotonin 2C receptors, it belongs to the 5-HT family receptor. It is expressed through serotonergic postsynaptic terminals on GABAergic, glutamatergic, and dopaminergic neurons. The anatomical distribution is prevalent in broad brain areas, including hypothalamic nuclei, hippocampus, ventral midbrain, striatum, nucleus accumbens, and amygdala (Jensen et al., 2010). They are enhancement the norepinephrinergic activity in some parts of the brain at the locus coeruleus (De Berardis et al., 2015).

Table 2.1. AGO Different Concentration Doses Effected in Different Types of The Cell Culture Previous Studies

Agent	Dose	Duration	Assay	Cell types	Effect	Reference
AGO	0.1, 0.5, 1, 5, 10, 50, and 100 μM But (5, 10 μM) Are optimized dose	24 h	MTT for 4 h	Brain Endothelial Cells		(Cheng et al., 2020)
AGO	(1–100 μM) for various times (1–24 h). The optimal dose is 20 μM after 24 h	1- 24 h		Neuronal PC12 cell		(Akpinar et al., 2014)
AGO	2 μM, 20 μM,200 Mm	24 hours		PC12 cells		(Yao et al., 2019)
AGO	(1 μM, 2 μM, 4 μM, 8 μM and 16 μM	48-hours	MTT (1 mg/ml) 4 h	HT-22 mouse hippocampal neuronal cell line		(Cankara et al., 2021)
AGO	(0, 100, 200, 400 and 600 μM)	24 h	MTT for 2h	RAW264.7 murine macrophage cell line,		(Hyeon et al., 2017)
AGO	concentrations of 1.5, 3, 15, 30, 150, 300, and 500 μM the optimal dose is 15 and 30 μM	24 h		Human SH-SY5Y neuronal cells		(Wang et al., 2021)
AGO	10 and 20 μM	24 h		Human umbilical vascular endothelial cells& human monocytic Leukemia cell line THP-1 cells		(Hong et al., 2021)

2.14. Glutamate

Glu is an amino acid that is thought to be the primary excitatory signal negotiator and is noticeable in the majority of functions of the brain, such as memory, learning, and understanding (Danbolt, 2001). The metabolism of Glu is introduced in protein synthesis, ATP generation, and ammonia and acts as a neurotransmitter in synaptic neurons. It reaches the synaptic vesicle through a Glu transporter and is expelled by exocytosis transport (Danbolt, 2001). The abnormality of Glu is known as the excitotoxicity process, which causes toxicity to neuronal cells. This excitotoxicity occurs in the Long-term action of ionotropic Glu and over-stimulus of NMDA receptors, which promote Ca^{2+} influx thought to be the main cause of neuronal death (Jazvinšćak Jembrek et al., 2018). The overproduction of Glu level suppresses OS and promote neuronal death in both types of immature primary cortical neuron and HT22 cell line (Park et al., 2020, Stanciu et al., 2000, Ma et al., 2021). Also, another study observed that Glu-induced ROS elevation and glutathione depletion in C6 glial cells

caused apoptosis cell death (Han et al., 1997). In neuronal pc12 and hippocampal line, cells had reported the neurotoxic effect of Glu in a typical dose-related manner. The result found that the lowest dose of Glu can promote cell viability (Bhavnani et al., 2003). The Glu significantly induced neuronal cell death or OS in HT22 due to preventing the uptake of cystine into the cells via the cystine-Glu transporter system, thus leading to the depletion of glutathione (Rössler et al., 2004). A recent study showed that Glu caused neurotoxicity by producing ROS, accumulating around 9 hours after HT22 cell culture Glu exposure (Fu and Koo, 2006).

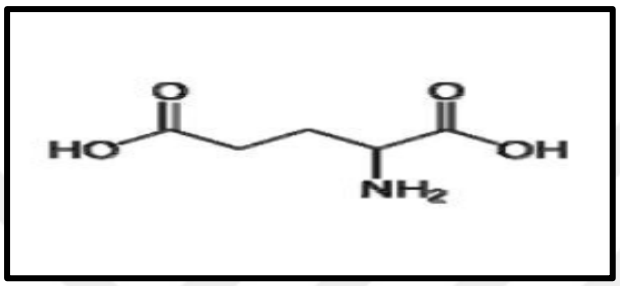

Figure 2.4. Chemical structure of L glutamic acid

2.14.1. Glutamate Neurotoxicity Process

Glu neurotoxicity passes through three sequential induction, amplification, and expression stages. Each one may be deceptive to interference therapy. Excessive Glu receptor stimulation induces the Ca^{2+} and Na^{+} inside cells (Choi, 1990). The most common approaches to prevent the induction of Glu by blocking antagonizing postsynaptic Glu receptors, the extenuating release of Glu from presynaptic terminals, and accelerating Glu removal from synaptic clefts, as well as obstructed or stopped Ca^{2+} influx and stores. Abnormal intracellular free Ca^{2+} concentrations may cause damaging cascades and neuronal degeneration (Choi, 1990).

Figure 2.5. *L* glutamic acid

2.14.2. Glutamate Receptor

The brain tissue's Glu action mediates by activating ionotropic and metabotropic receptors (Kew and Kemp, 2005). These receptors are abundant in the CNS on both neuron and glial cells, facilitating speedy excitatory Glu transmission. These receptors control many processes in the brain, spinal cord, peripheral nervous system, and retina (Traynelis et al., 2010). Specific receptors found on the surface of neurons selectively bind with Glu. It is generated through other neurons, triggering the events that lead to the activation of the postsynaptic neuron (Dingledine and Conn, 2000).

Moreover, these Glu receptors have been identified, as the metabotropic are associated with second messenger enzymatic through G proteins and routinely act to regulate a variety of cellular operations involving genetic transcriptional and phosphorylation of ligands and channels of voltage-ion gated. In contrast, ionotropics are responsible for the passaging of sodium and calcium ions through a pore. The ionotropic Glu receptors are three agonists kinetic acid, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA (Dingledine and Conn, 2000, Asztély and Gustafsson, 1996).

These receptors, when activated, permit the positively charged ions to move into the cell. Not all these variant subtypes of Glu receptors contributed to excitotoxicity. Exclusively, most neuronal cell death briefly accompanies exposure to Glu NMDA receptor activation (Choi, 1992). The NMDA receptor plays an essential role in regulating the brain's adaptation to environmental conditions and is critical for the growth of new synapses. Also, they are linked to various neurological illnesses, including retinal dystrophy, cerebellar ataxia, autism spectrum disorder, intellectual disability, paraparesis, and schizophrenia (Burada et al., 2020). The effective

functioning and expression of many Glu receptors and transporters are necessary for competent Glu transmission (Iovino et al., 2020). The NMDA receptor is vital in early brain growth, while AMPA and kinetic receptors associate with the mid-gestation of the fetus. Malfunction of Glu receptors is associated with developing brain disorders or may later extend the brain in adults (Egbenya et al., 2021).

2.14.3. Glutamate Uptake

The uptake mechanism is essential to reducing extracellular concentrations of Glu. The Glu proteins transporter achieves uptake. The neuronal cell releases Glu into the cleft of the synapse and is attractive to both receptors to transmit an excitatory message. Astroglia cell uptake of Glu released from neural synapses occurs through sodium-dependent and independent transport pathways and is crucial for preventing toxic extracellular accumulations of Glu (Danbolt, 2001).

2.14.4. Metabolism of Glutamate

Extracellularly, enzymes never metabolize Glu, but it is taken up through the glial and neuronal cells from the extracellular fluid around its receptors (Trotti et al., 1998). After being taken up by cells, the Glu can be recycled as a transmitter or used for physiological activities like protein synthesis, carbohydrate metabolism, and ammonia fixation (Danbolt, 2001). Glu is released by cellular exocytosis from an axon terminal and connected with transporters in pre and post or extra-synaptic astroglia cells. Astroglia cell is responsible for detoxifying Glu to convert it into glutamine by synthetase enzyme of glutamine (Iovino et al., 2020). This glutamine is released by the glial cell and uptake by neurons. This neuronal cell can transform glutamine into Glu (Danbolt, 2001). Another study suggested that the tricarboxylic acid cycle metabolizes a small amount of the Glu, and the remnant is stored as glutamine (McKenna et al., 1996). The Glu is terminated and released as ammonia in the liver, then detoxifies into urea. In pancreatic Beta-cells, the Glu should be oxidated as an amino acid to stimulate insulin release. Glu is a neuroexcitatory transmitter in the CNS (Kelly and Stanley, 2001).

2.15. The Cell Death

It commonly plays a significant role in average growth in living animals (Clarke, 1990). There are three main types of cell death. The first one is hetero phagocytosis; in it, the cell dies and degenerates without any detectable role being destroyed by its lysosomes, but the lysosomes of other cells kill its fragments. The second type is autophagocytosis; the cell is destroyed and degraded with its lysosomes. And the third

type is the cell destroyed without lysosomes (Clarke, 1990). The cell death may be apoptosis, also known as PCD or necrosis and autophagy cell death, or associated with different proteases, caspases, and immunology disorders (Galluzzi et al., 2007, Duprez et al., 2009). Cell death is also connected to protein aggregates, damage to axon transport, and synaptic dysfunction (Andreone et al., 2020).

3. MATERIAL AND METHOD

3.1. The Daily Routine of Experimental

All procedures of the cell culture are carried out by using an aseptic technique to prevent biohazard chemical substances, guaranteeing the environmental working area of the cell line culture is safe, including a laminar flow cell culture hood, and an incubator, with clothing, personal protective equipment, (PPE) such as labs coats and nitrile gloves. The decontamination of the surface room culture must be sterilized by 70 % (v/v) ethanol. Any contaminated cell, changed media, or potential pathogenesis-containing solutions must be drained into the trash. Additionally, all disposable vessels, flasks, and pipettes dispose of them after use. Wipe down bench surfaces with 70 % ethanol alcohol.

Table 3.1. The equipment required in the laboratory cell culture.

Materials for the experiment consumables:		
Latex gloves & wipes	Pipettes and Pipette tips (10 µl/ 200 µl/ 1000 µl) and stereological tip (5,10,25ml) gun pipette	Sterile cell culture 96, 24,12,6 well-plates
Sterile conical tube 50&15ml	Cell culture Flasks (T25, 75, 175cm^2)	Waste container
2. Electronic equipment		
Laminar flow / microbiological safety cabinet Model MN120, serial NO, 02-0461, class II EN 12469:2000, HEPA % 99.999 POWER 760 W AC MAINS 230 v.50Hz		
Co_2 Incubator: (5% of atmospheric CO_2 in 95% humidified at 37◦C) with UV system installed. It is made in Japan. Sanyo Electric Co. Ltd serial no: 11100425 model: MCO 18ac. Internal volume:170 l. rated voltage: ac220-240 v, frequency 50 Hz, amperage max: 1.4, power consumption max: 310 w, net weight: 92 kg. Aspiration pump (peristaltic or vacuum) - centrifuge		
Refrigerator -20°C		
Elisa absorbance reader		
Inverted ELISA microscope		
3. Software: Excel, graph pad prism version 9.5.1(733)		
4. Reagents:		
Dulbecco's modified Eagle's medium (DMEM) high glucose4.5g/ID-glucose was purchased from (Biological Industries Israel Beit Haemek Ltd.)	Trypsin-EDTA was purchased from (Biological Industries Israel Beit Haemek Ltd.)	Dulbecco's phosphate-buffered saline (PBS)
Fetal bovine serum.	10,000 U/ml penicillin 10mg/ml streptomycin (Biological Industries Israel Beit Haemek Ltd)	L-glutamine 200 mM (29.2mg/ml) (Biological Industries Israel Beit Haemek Ltd)
DMSO	MTT assay	

Figure 3.1. The cabinet flow hood

Figure 3.2. CO_2 Incubator 5% of atmospheric carbon dioxide in 95% humidified at 37◦C

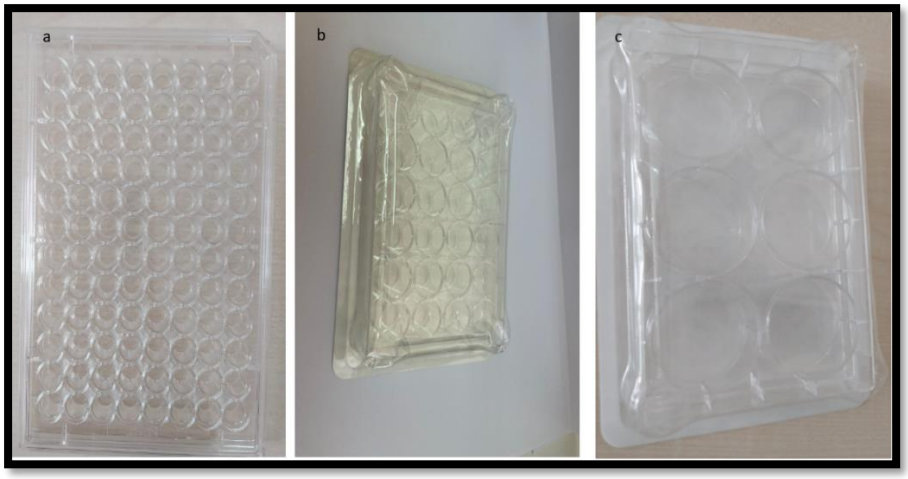

Figure 3.3. Different types of well-plate a= 96. b=24. c=6 well-plate for cell culture seeding

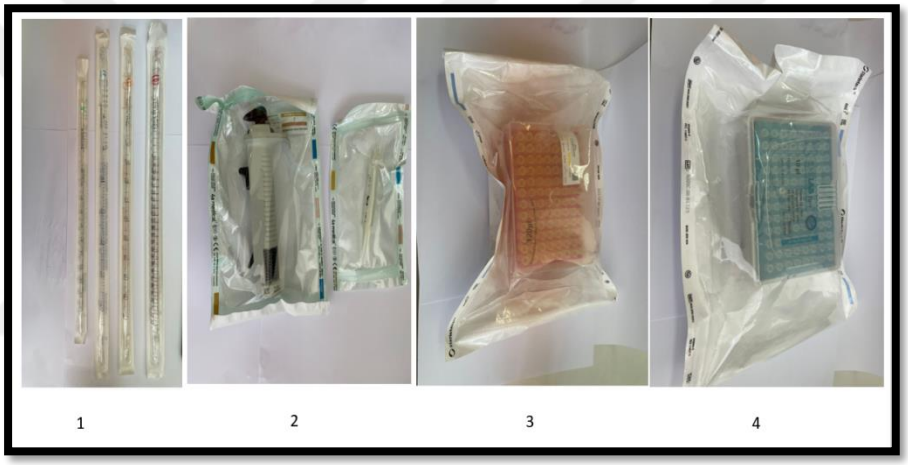

Figure 3.4. Showed different tools are used in laboratory culture 1- serological pipette tip 2- 3- pipette tip 200µl 4- pipette tip 20µl.

Figure 3.5. Microbiological safety cabinet and centrifuge

3..2. The cell culture

This study was conducted at the Ondokuz Mayis University at the Department of Histology and Embryology for Cell Culture Laboratory (Samsun, Turkey). All experiments were performed in the HT22 hippocampal cell line (Figure 3.6) in a laminar flow hood cabinet under aseptic conditions. The cells were kindly gifted by the Histology and Embryology Department - Ondokuz Mayis University-Samsun - Turkey.

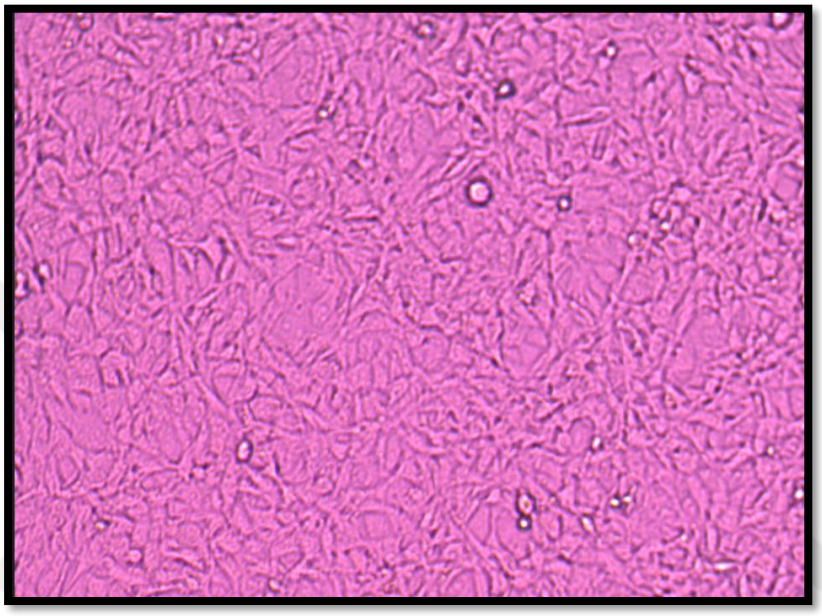

Figure 3.6. Showed the HT22 hippocampal cell line in the culture cell flask at the confluence stage.The image was captured by an inverted microscope Evos XI core (AMG, WA, U.S.A) at 40um x magnification.

3.3. Freezing HT22 Hippocampal Neural Cell Line for Long Storage

After the HT22 hippocampal cell line culture reached the 70 to 80% confluency stage, it dissociated using trypsin EDTA, as described in the steps below. Centrifuged the cell at 3500 ×g for 5 minutes and removed all the supernatant. We resuspended the pellet in freezing media, formed by (900 µl complete fresh media +100 µl DMSO). The counting number of cells for freezing in each vial is according to the number of cells in the flask. If the cells seeding in the flask 25cm^2, the cell number about 200.000 cells, then the vial contained the same number in this flask. Then we distributed the re-suspended cell in many cryovials if needed and started the steps of cooling slowly to vials by transferring them into ice, then to −20 °C, then to −86 °C, and lastly, the next day, storing them in a liquid nitrogen container if possible.

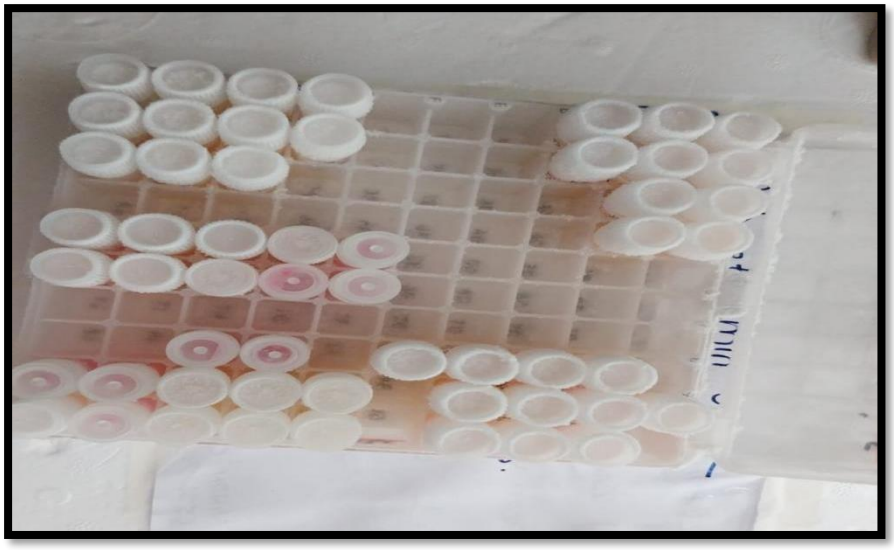

Figure 3.7. HT22 hippocampal cell line in a cryovial and freezing at −86 °C.

3.4. Thawing of the HT 22 Hippocampal Cell Culture

Cells were thawed immediately after being removed from the liquid N2, shaken slightly at room temperature, and we waited until all contents of the vial were thawed wholly; the suspended cells were put in a 15 ml falcon tube and added 5 ml of complete fresh DMEM. The cells were centrifugated at 3500 rpm for 5 minutes, then removed the supernatant and resuspended the pellet in 5ml of growth media. Then, cells are ready for seeding in a flask containing suitable growth media. After 24 hours, we removed all the complete media and replaced them with another to eliminate any debris and remnants from DMSO.

3.5. Preparation of Complete Media

The fresh complete media formed by adding some supplementary reagents to 80% of Dulbecco's Modified Eagle Medium (4 ml of DMEM), 20% fetal bovine serum (1ml of FBS), 50 microliter L glutamine, 50 microliters penicillin-streptomycin antibiotics solution at room temperature (Phelan and May, 2015).

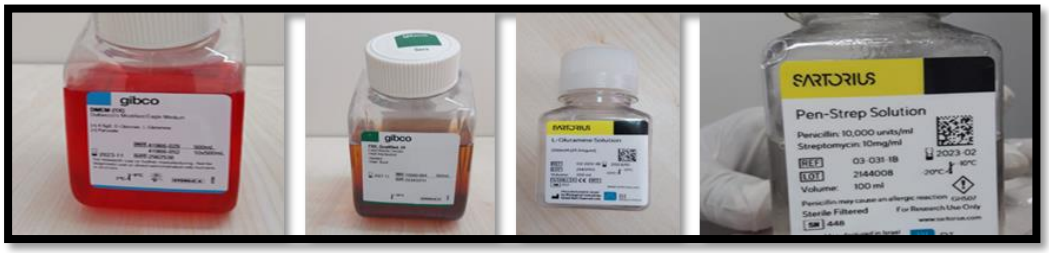

Figure 3.8. Dulbecco's Modified Eagle Medium (DMEM) with supplementary (FBS, L glutamine, penicillin-streptomycin solution).

3.6. HT22 Hippocampal Cell Line Maintenance

Before beginning the experimental treatment with AGO or Glu. The HT22 hippocampal cell line maintained in 4 ml 80% of complete Dulbecco's Modified Eagle Medium (DMEM) (Biological Industries Israel Beit Haemek Ltd)) media containing 50 microliter L-glutamine (Biological Industries Israel Beit Haemek Ltd) and also supplemented with 1ml 20% fetal bovine serum (Gibco Company), and 50 microliter penicillin/ streptomycin (Biological Industries Israel Beit Haemek Ltd), the cells were placed horizontally in an incubator under conventional cell culture conditions 5% of atmospheric carbon dioxide in 95% humidified at 37◦C to allow the cells to settle and attach to the surface of the culture flask. All my experiments were performed on the cell cultures when the cells reached the 70 – 80 % confluent or exponential stage of the cell cycle, which was detected under the inverted microscope. Then, the cells were dislodged from the flask or substrate surface by 1 to 2ml trypsin-EDTA 25% for 3 minutes (Biological Industries Israel Beit Haemek Ltd).

3.7. Trypsinization and Passaging of the Cell Culture

The HT22 hippocampal cell line is used in all investigations of this methodology chapter in passaging between cells 5 and 13. The cell passage in the biology laboratory refers to the number of times the cell divides to grow. For instance, passage 9 means the cell can split into a ninth cell splitting. Before the cell passage, it disassociated from the flask or substrate surface, emptied the flask from all growth culture media, and washed twice with phosphate-buffered saline (PBS). Then, the cells were exposed to 1 ml of 0.25% (v/v) trypsin-Ethylene Diamine Tetra Acetic Acid (EDTA) solution (Biological Industries Israel Beit Haemek Ltd). Put 5 ml of new growth media or FBS to deactivate trypsin.

When all the cells were detached, the Pipetting sterile was used to transfer the cell culture from the flask and put it inside the falcon tube. Removed the media slightly and put 2ml of media inside the falcon tube, then used the Pipette sterile again, put the cell inside the new flask, added complete growth media to cover the flask, and finally returned the flask to the incubator. After cell counting, cell suspensions were either split in a ratio of 1 to 10 into a cell culture flask or seeded into well- plates to be used for subsequent experiments. The cell was incubated at 37°C, 5% CO_2, and 95% humidified.

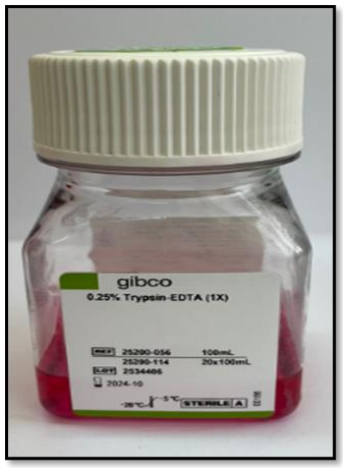

Figure 3.9. 0.25% Trypsin EDTA used to disassociate the cell culture from substrate surface of the flask culture

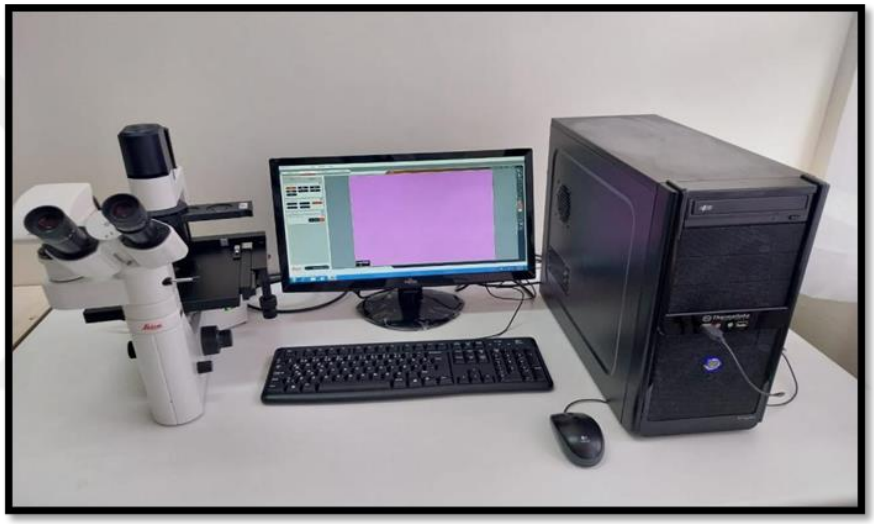

Figure 3.10. Inverted microscope Elisa system for cell culture investigation

3.8. The Determination of the Optimized AGO & Glu Concentration Dosage

To determine the optimal and more neuroprotective effective concentration dose of AGO and Glu as a toxic substance, an MTT assay was to assess the cell viability of HT22 hippocampal cell line and calculated IC_{50} of each agent after being the cells treated with AGO or Glu at different concentrations and different duration times.

3.8.1. AGO Stock Solution Preparation

AGO is an antidepressant agent purchased as a powder from the pharmacy in Samsun -Turkey; different concentration therapy doses in range (2, 4, 8, 16, 25, 50,75,100, 150, and 200 µM) were administrated to HT22 hippocampal cell line culture treatment, in each well- plate group for a different duration. We prepared a 200 µM stock solution of AGO by adding 1.2mg powder of AGO to dissolve in a 5 ml

volume of DMSO according to the molecular weight of the AGO, which is equal to 243. 30g, then taken 1 ml from the stock of AGO solution and diluted in 9 ml of fresh complete media. The dose concentration is represented in (Table 3.2.).

Table 3.2. AGO concentration dosages preparation for experiments

AGO concentration doses preparation stock (1 µM)	The dose taken from the stock added to the well plate (µL)	Diluted by Complete Media (µL)	Complete solution (µL)	(Dilution)% (µM)
200	200	0.00	200	200
150	150	50	200	150
100	100	100	200	100
75	75	125	200	75
50	50	150	200	50
25	25	175	200	25
16	16	184	200	16
8	8	192	200	8
4	4	196	200	4
2	2	198	200	2

3.8.2. Stock Solution Preparation for Determination Toxicity Dose of Glu

Glu is a neurotransmitter purchased from (Biological Industries Israel Beit HaEmek Ltd) as *L* glutamic acid. The cell was administered with different concentration doses (from 1 to 5 mM). I first dissolved this reagent as 11mg of Glu in 15 mL of distilled water. At a stock concentration or molarity, 5 mM and stored at −20°C. Each concentration dose was diluted with the corresponding cell culture media. The molecular weight of the Glu is 146.12g. These dose concentrations are shown in (Table 3.3.).

Table 3.3. The preparation of *L* glutamic acid concentration doses

Glu concentration (50 mM) Stock)	Complete media (µl)	The dose is taken from the complete solution and added to the well plate (µl)	(Dilution)% (mM)
5	0.00	250	5
4	50	200	4
3	150	150	3
2	150	100	2
1	200	50	1

3.8.3. Protocol of the Cell Seeding

Before starting the MTT assay, the cells were seeded in a 96 well-plate. The HT22 hippocampal cell line was first expanded in 25cm^2 culture flasks in a complete growth media until it reached 70-80 % confluency. After completing the growth, the flask 25cm^2 contained about 200.000 cells, and these cells were distributed equally in number for seeding in each well plate, which included maximumly from 5000 to 6000 cells and growth in 200 µl of complete media. Then, the cell was incubated overnight or for 24 hours at 37°C under an atmosphere of 5% CO_2 and 95% humidity. Before treated with substances.

Table 3.4.Protocol of cell Seeding in the well-plate

No.	Steps
1.	We 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 complete media to deactivate the trypsin reaction
5.	Transferred the cell using a pipette, put it in a falcon tube, and centrifuged at 3500 rpm for 5 minutes.
6.	We removed all the supernatant, added 3.5 or 4 ml of new complete media, pipetted the cell in the falcon tube, and then distributed it to the 96 well -plate.
7.	We then incubated it for 24 hours to attach.
8.	Serial dilutions of different AGO or Glu concentrations were prepared within complete culture media or distilled water. After incubation, the culture media in a 96-well plate was removed gently and washed with PBS or 200 µl fresh media with various drug concentrations; this step was triplicated.
9.	We returned the well plate to the incubator for 24 hours or more according to the experimental duration needed. All experiments were conducted within 5-13 passages of original thawed cell culture stocks.
10.	We added an MTT kit, but it must be prepared before use.

3.8.4. Formation of the Experimental HT22 Cell Line Groups

The cell samples were subdivided randomly into nine well plate groups for treatment in the present study. In all experiments, cells were seeded in well-plate groups triplicated and remained for 24 hrs. to grow at 37 °C, 5 % CO_2, and 95% humidity before the cell treatment when the cells received the treatment also incubated again (37 °C and 5 % CO_2). We used the MTT assay analysis to determine the cell viability.

Plate (1) group: This plate is blank, or the background contains complete media only.

Plate (2) group: This plate includes cell + cell media.

Plate (3) group: This plate was treated with the Glu

plate (4) group: This plate was treated with Glu and a low dose of AGO.

Plate (5) group: This plate was treated with Glu and a high AGO dose.

Plate (6) group: This plate was treated with AGO low dose

Plate (7) group: This plate was treated with an AGO high dose.

Plate (8) group: This plate was treated with a DMSO low concentration.

Plate (9) group: This plate was treated with a DMSO high concentration.

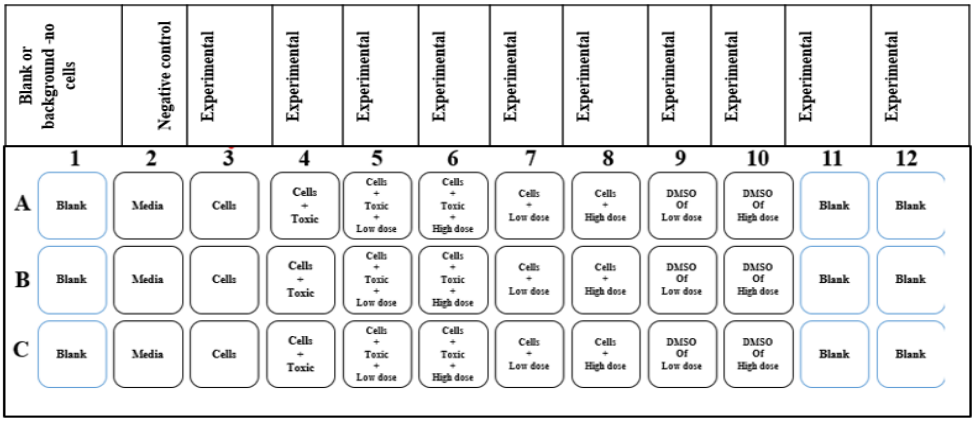

Figure 3.11. The picture represented the 96 well-plate of the AGO neuroprotective effect against Glu neurotoxicity on the HT22 cell line.

Table 3.5.The experimental design well-plates groups of the HT22 neural cell line.

NO.	Group	Details of each group
1.	Blank	This background contained 50 µl complete fresh media without cells.
2.	Control	This plate group was positive and did not treat contained cell+ complete fresh media.
3.	Toxic	This plate group contained cells in 50 µl complete media and was treated with Glu (5 mM concentration of Glu as a toxic substance for 1 hr.) before adding the protection substance.
4.	Treated 1	This plate group contained cells in 50 µl complete media and was treated with Glu (for 1hr) and added.
5.	Treated 2	This plate group contained cells in 50 µl complete media and was treated with Glu (for 1hr) and added with a high dose of AGO (Glu 5 mM + AGO 60 µM) for 24 hrs.
6.	Protection 1	This plate group contained cells in 50 µl complete media and was treated with AGO (AGO 30 µM as a low dose) for 24 hours of incubation.
7.	Protection 2	This plate group contained cells in 50 µl complete media and treated with AGO (AGO 60 µM as a high dose) for 24 hrs.
8.	DMSO 1	This plate group contained cells in 50 µl complete media and treated with DMSO (3 µl low concentration) for 24 hours.
9.	DMSO 2	This plate group contained cells in 50 µl complete media and treated with DMSO (6 µl high concentration) for 24hrs

3.8.5. MTT Assay for Dosage Determination

In this study, we subjected the HT22 neural cell line to various concentration dosages of AGO and Glu agents and commonly used the MTT assay (Biological Industries Israel Beit Haemek Ltd) to evaluate the cell viability Known as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium). The MTT is used for multiple applications such as cell viability, cytotoxicity, cell proliferation, and cell activation. The mechanism depended on the ability of the mitochondria of viable cells or the cellular metabolic activity to convert water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan. These results did not occur in dead cells (Uğuz and Nazıroğlu, 2012). We quantified the number of viable cells using an Elisa microplate reader at 570 nm. In addition, The primary objective of this study is to calculate the IC_{50} value for different duration dosages of AGO and Glu, representing the concentration at which 50% of the cell's viability is affected.

3.8.5.1 MTT Assay Preparation

100 µl (MTT+ DMSO) during triplicated the experimental needed = 36 well × 100 ml =3.600 ml = 4 ml for all well- plate.

20% of stock = 2ml MTT+ 8 ml complete media = 10 ml.

1ml MTT+4 ml complete media = 5 ml complete media. If the MTT is powder, I dissolved 5mg MTT in 1 ml PBS and then diluted with 9 ml complete fresh media.

Figure 3.12. MTT kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium)

In this current study, we used the cells that did not administrate with any treatment dose as the control group (untreated group), whereas the blank group, also known as background, contains only the complete DMEM media or DMSO. Then, we used MTT 110 µl to treat each group of cells and the control and incubated for a proper result of approximately 2 to 4 hours.

Table 3.6. MTT assay Protocol

	Steps of MTT assay
1	500 ml MTT +500 ml PBS = 1 ml MTT (each cell in the well-plate received 110 µl of MTT).
2	We removed all the media from the well- plate, washed the cell with PBS, and Added an MTT concentration of about 110 µl after preparing the cell in the well- plate and placed it in the incubator for 2 to 3 hours.
3	Removed all solution, carefully added 110 µl of DMSO, and pipetted for 10 minutes.
4	We read the cell viability absorbance in the 570nm ELISA reader.

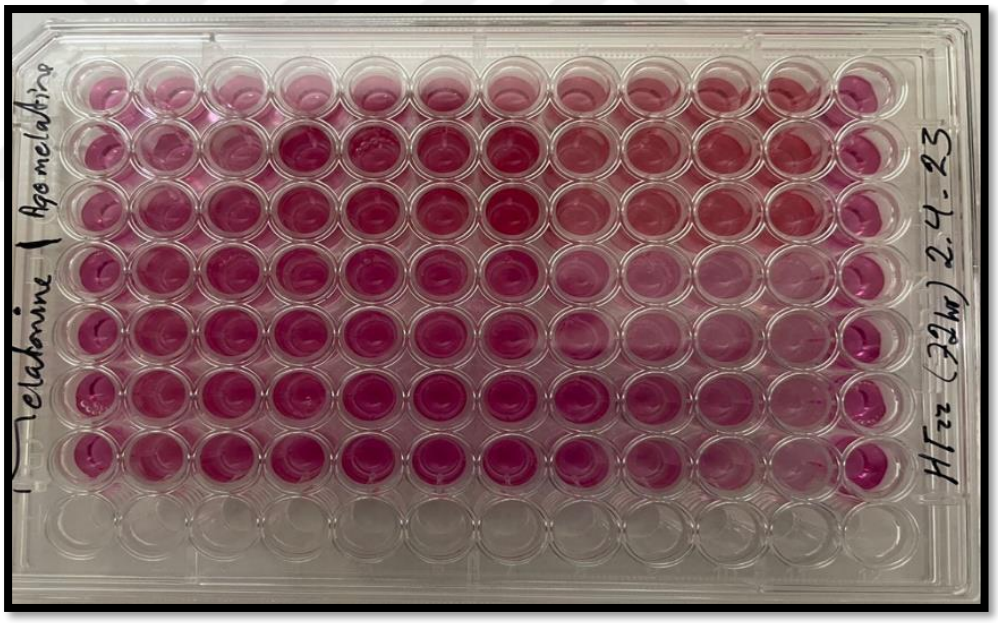

Figure 3.13. The HT22 neural cell seeded in the 96 well-plate administrated with different substance dosages of AGO and incubated for 24 hours to assess the effect of AGO concentration doses by MTT assay.

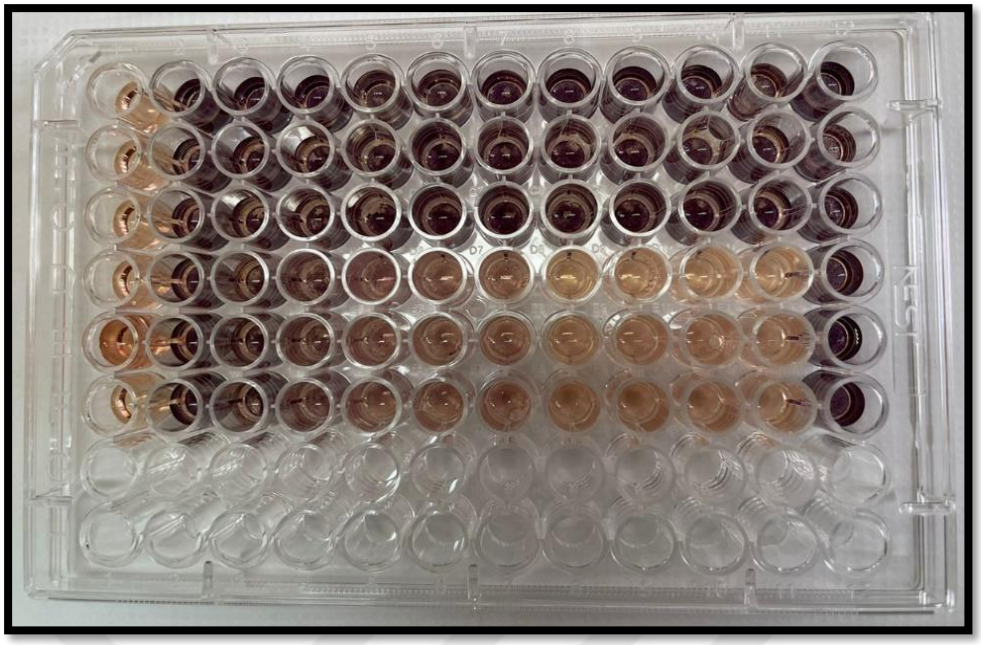

Figure 3.14. HT22 neural cell administrated with 110µl MTT and incubated 2hrs, after being treated with the dose concentrations for 24hrs

3.8.5.2. Dimethyl Sulfoxide Solution (DMSO)

We removed the purple formazan crystallization that formed after adding the MTT solution. We dissolved them in a solubilization solution such as DMSO by adding 110 µl in seeded cells for 10 minutes and pipetted carefully after the purple crystal precipitated, clearly visible under the contrast inverted microscope.

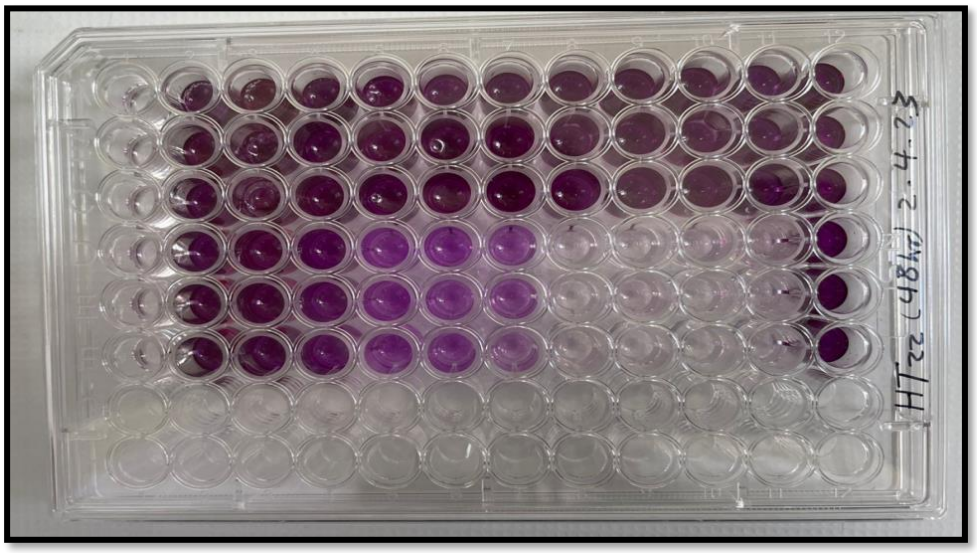

Figure 3.15. The HT22 neural cell was Administered with DMSO 110 µl for 10 minutes and incubated for formazan crystals dissolved.

3.8.5.3. Elisa Absorbance Reader

We measured the cell viability samples absorbance (calorimetric measurement of MTT reduction) and recorded by Elisa microplate absorbance reader 570 nm. All experimental treatments of HT22 neural cells were triplicated and seeded. The mean value of the cell viability was calculated from these three triplication.

Figure 3.16. Elisa absorbance reader 570 nm to determine the number viable cells and give the data in Excel sheet for analysis.

The cell viability (%) was calculated using this formula:

The viability (%) = (sample absorbance/ control absorbance) ×100

3.9. Statistical Analysis

Each calculation of the cell viability of triplicated well-plate analytical results was expressed as average ± standard deviation values. In the current study, the data was analyzed by the SPSS 26.0 program, Microsoft Excel 365, GraphPad Prism version 9.5.1, one-way ANOVA application, and Tukey's multiple comparisons test used in the present study. The significance of the *p*-value was compared with the results of the samples less than 0.05.

4. RESULTS

The study aimed to investigate AGO therapeutic and neuroprotective effects on NDDs in HT22 hippocampal cell line model. It aimed to investigate AGO cell protective effect against Glu-induced excitotoxicity. This study assessed the impact of AGO and Glu effect on the HT22 cells. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Assay was utilized to determine the viability of the cells, Elisa absorbance reader was used to calculate the optical density of the cells. The findings of the study were represented as mean, standard deviation and standard errpt of means in tables and graphs. One way anova was used to compare between the experimental groups.

4.1. The Finding of MTT Assay for HT22 Cell Viability

4.1.1. Determination of the Optimal Neuroprotective Doses of AGO and the IC_{50} Value for Different Duration

The cells were seeded in 96-well plates at a density of 5000-6000 cells per well, with even distribution. After 24 hours, the cells were exposed to various concentrations of AGO (2, 4, 8, 16, 25, 50, 75, 100, 150, and 200 μM) for durations of 24, 48, and 72 hours. Cell viability was assessed using MTT assay and the IC_{50} for AGO was calculated.

4.1.2. The Effect of various Concentrations of AGO on HT22 Cell Viability and the IC_{50} Value for 24hrs

The mean and standard deviation of the cell viability of HT22 subjected to different doses of AGO after 24 hrs. (Table 4.1. ; Figure 4.1.).

Table 4.1. Shows the mean value and the standard deviation (SD) of cell viability in different concentration dosages of AGO for 24h.

Groups/AGO μM	Mean value of cell viability % ± SD
Cont	100 ±0.000
2	92.7 ±4.038
4	106.6 ±12.06
8	88.49 ±15.1
16	85.39 ±5.291
25	79.91 ±3.629
50	66.1 ±2.105
75	46.24 ±3.711
100	31.7 ±4.399
150	8.223 ±2.149
200	3.979 ±1.163

Table 4.2. Shows the significant degrees of different concentrations doses of AGO after the HT22 neural cell was treated with AGO for 24 hours.

AGO μM	Significance
0 vs. 2	0.9457
0 vs. 4	0.9710
0 vs. 8	0.5601
0 vs. 16	0.2509
0 vs. 25	0.0347
0 vs. 50	0.0001***
0 vs. 75	<0.0001***
0 vs. 100	<0.0001***
0 vs. 150	<0.0001***
0 vs. 200	<0.0001***

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

The statistical analysis results for the cell viability of the HT22 cells exposed to different concentration doses of AGO for 24 hrs showed no significant impact ($p > 0.5$) for the doses between 2 and 16 μM. Whereas the viability started to decrease clearly at the dose 25μM where it showed significant impact ($p \leq 0.05$). Whereas the cytotoxicity effect of AGO on the cell viability was highly significant from the dose 50μM up to 200μM when compared with control groups ($p \leq 0.001$). The IC_{50} was calculated for AGO after 24hrs. and the findings showed that 50% of the cells were viable at 62.85μM (Figure 4.2.).

Table 4.3. Shows the mean number of the cell viability, the standard deviation (SD), and the Error of Mean of AGO as neuroprotective in different concentrations for 24 hrs.

AGO μM	Mean	Std. Deviation	Std. Error of Mean
0	100	0.000	0.000
2	92.7	4.038	2.332
4	106.6	12.06	6.963
8	88.49	15.1	8.721
16	85.39	5.291	3.054
25	79.91	3.629	2.095
50	66.1	2.105	1.215
75	46.24	3.711	2.143
100	31.7	4.399	2.54
150	8.223	2.149	1.241
200	3.979	1.163	0.6712

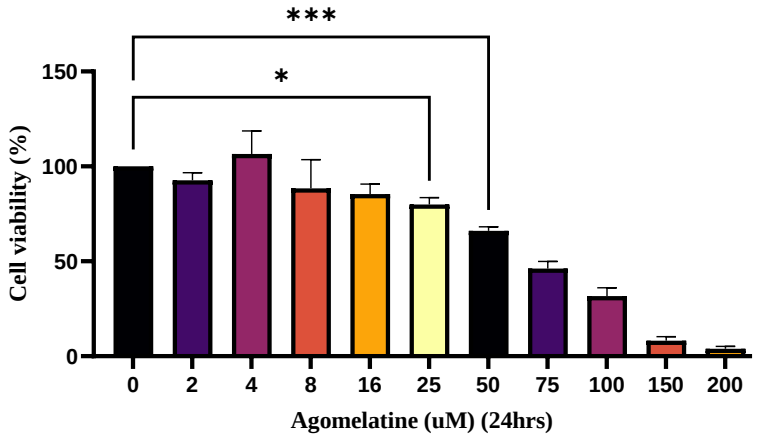

Figure 4.1. Neuroprotective effects of AGO on the HT22 neural cell line after exposure to different concentration dosages for 24 hrs. The values are given as the mean ± standard deviation. The significant difference at $p \leq 0.05$ was indicated by *, whereas the highly significant at $p \leq 0.01$ was indicated by **, and the very high significance at $p \leq 0.001$ indicated ***.

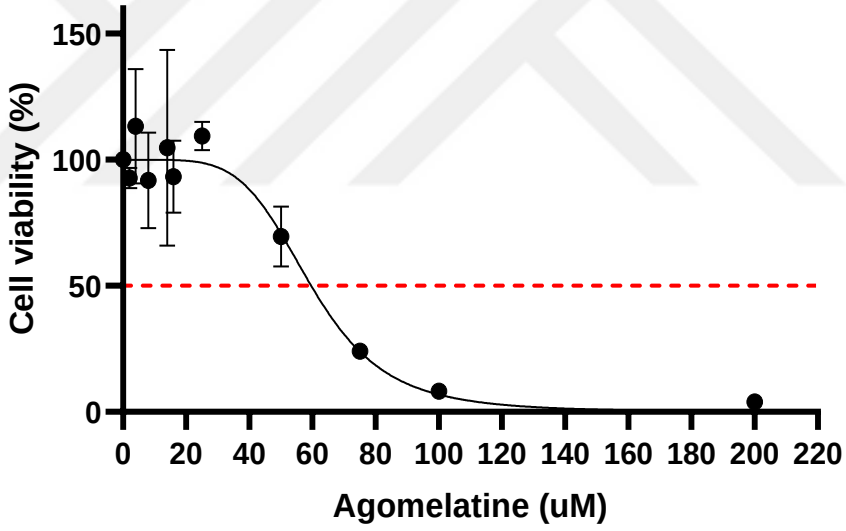

Figure 4.2. Shows the IC_{50} value of HT22 after the AGO was administrated for 24 hrs.

Table 4.4. Illustrate the degree of significance between different concentration doses of AGO on HT22 cell viability after 24 hrs.

AGO (μM)	Significance	AGO (μM)	Significance	AGO (μM)	Significance
0 vs. 2	0.9457	4 vs. 8	0.0748	16 vs. 150	<0.0001***
0 vs. 4	0.971	4 vs. 16	0.022*	16 vs. 200	<0.0001***
0 vs. 8	0.5601	4 vs. 25	0.0022**	25 vs. 50	0.3185
0 vs. 16	0.2509	4 vs. 50	<0.0001***	25 vs. 75	0.0001***
0 vs. 25	0.0347*	4 vs. 75	<0.0001***	25 vs. 100	<0.0001***
0 vs. 50	0.0001***	4 vs. 100	<0.0001***	25 vs. 150	<0.0001***
0 vs. 75	<0.0001***	4 vs. 150	<0.0001***	25 vs. 200	<0.0001***
0 vs. 100	<0.0001***	4 vs. 200	<0.0001***	50 vs. 75	0.038*
0 vs. 150	<0.0001***	8 vs. 16	>0.9999	50 vs. 100	<0.0001***
0 vs. 200	<0.0001***	8 vs. 25	0.8661	50 vs. 150	<0.0001***
2 vs. 4	0.3086	8 vs. 50	0.0136*	50 vs. 200	<0.0001***
2 vs. 8	0.9991	8 vs. 75	<0.0001***	75 vs. 100	0.2564
2 vs. 16	0.9453	8 vs. 100	<0.0001***	75 vs. 150	<0.0001***
2 vs. 25	0.4172	8 vs. 150	<0.0001***	75 vs. 200	<0.0001***
2 vs. 50	0.0023**	8 vs. 200	<0.0001***	100 vs. 150	0.0086**
2 vs. 75	<0.0001***	16 vs. 25	0.9923	100 vs. 200	0.0014**
2 vs. 100	<0.0001***	16 vs. 50	0.0478*	150 vs. 200	0.999
2 vs. 150	<0.0001***	16 vs. 75	<0.0001***		
2 vs. 200	<0.0001***	16 vs. 100	<0.0001***		

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

4.1.3. The Effect of various Concentrations of AGO on HT22 Cell Viability and the IC_{50} Value for 48 hrs.

The cells were seeded in 96-well plates at a density of 5000-6000 cells per well, with even distribution. After 24 hours, the cells were exposed to various concentrations of AGO (2, 4, 8, 16, 25, 50, 75, 100, 150, and 200 μM) for durations 48 hours. Cell viability was assessed using MTT assay and the IC_{50} for AGO was calculated.

Table 4.5. Showed the mean value and the standard deviation (SD) of cell viability in different concentration dosages of AGO for 48h.

AGO (μM)	Mean value of cell viability % ± SD
Cont untreated	100 ±0.000
2	88.99 ±3.877
4	95.95 ±10.85
8	75.22 ±12.84
16	72.58 ±4.497
25	67.92 ±3.085
50	56.19 ±1.789
75	39.3 ±3.155
100	26.94 ±3.739
150	6.99 ±1.827
200	3.383 ±0.988

Table 4.6. Showed the mean number of the cell viability, the standard deviation (SD), and the Error of Mean of AGO as neuroprotective in different concentrations for 48hrs.

AGO (µM)	Mean	Std. Deviation	Std. Error of Mean
0	100	0	0
2	88.99	3.877	2.238
4	95.95	10.85	6.266
8	75.22	12.84	7.413
16	72.58	4.497	2.596
25	67.92	3.085	1.781
50	56.19	1.789	1.033
75	39.3	3.155	1.821
100	26.94	3.739	2.159
150	6.99	1.827	1.055
200	3.383	0.9882	0.5705

Table 4.7. Showed the degree of significance between different concentration doses of AGO and control groups of protection doses in HT22 cell viability after 48 hrs.

AGO (µM)	*P* Value
0 vs. 2	0.431
0 vs. 4	0.998
0 vs. 8	<.001***
0 vs. 16	<.001***
0 vs. 25	<.001***
0 vs. 50	<.001***
0 vs. 75	<.001***
0 vs. 100	<.001***
0 vs. 150	<.001***
0 vs. 200	<.001***

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

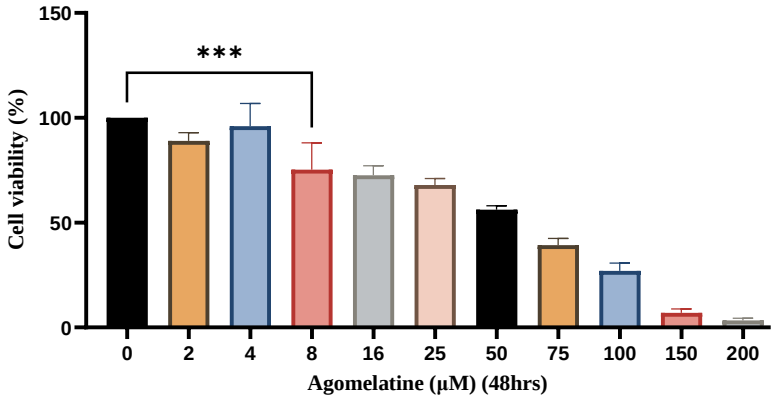

Figure 4.3. Neuroprotective effects of AGO on HT22 cells viability for 48 hrs. No significant impact was observed at doses 2 and 4, but the significance was observed at AGO concentrations of 8 µM. The significant difference at $p \leq 0.05$ was indicated by *, whereas the highly significant at $p \leq 0.01$ was indicated by **, and the very high significance at $p \leq 0.001$ indicated ***.

Regarding statistical analysis of the cell viability for 48 hrs., no significant impact was detected at concentrations 2μM and 4μM when compared with the control group. However, the viability of the cell decreased, the death clearly observed from dose 8 μM and the higher concentrations with very high significant impact ($p \leq 0.001$). The IC_{50} calculation of AGO showed that 50% of the cells were lost by the dose of 41.86 μM (Table 4.5.; Figure 4.7.).

4.1.5. The Effect of various Concentrations of AGO on HT 22 Cell Viability and IC_{50} Value for 72 hrs.

The HT 22 cells were treated with using different doses of AGO (2, 4, 8, 12,16, 25, 50, 75, 100 and 200 μM) and incubated for 72 hrs, the mean and standard deviation of HT2 cell viability, and IC_{50} of AGO were calculated.

Table 4.8. Showed the mean value and the standard deviation (SD) of cell viability in different concentration dosages of AGO for 72h.

Groups/ μM AGO	Mean value of cell viability % ± SD
Cont untreated	100 ±0.000
2	85.43 ±3.722
4	86.36 ±9.768
8	63.93 ±10.91
16	61.69 ±3.822
25	57.73 ±2.622
50	47.76 ±1.521
75	33.41 ±2.681
100	22.9 ±3.178
150	5.941 ±1.553
200	2.875 ±0.8399

The statistical findings of MTT assay for cell viability after 72hrs showed significant difference at 2 μM ($p \leq 0.05$) and highly significant difference from 8 μM and higher doses (25 μM, 50 μM, 75 μM, 100 μM,150 μM, and 200 μM ($p \leq 0.001$) (Table 4.10.).

Table 4.9. Showed the degree of significance between different concentration dosages of AGO and control groups in HT22 cell viability after 72hrs

AGO (µM)	*P* Value
0 vs. 2	0.04*
0 vs. 4	.078
0 vs. 8	<.001***
0 vs. 16	<.001***
0 vs. 25	<.001***
0 vs. 50	<.001***
0 vs. 75	<.001***
0 vs. 100	<.001***
0 vs. 150	<.001***
0 vs. 200	<.001***

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

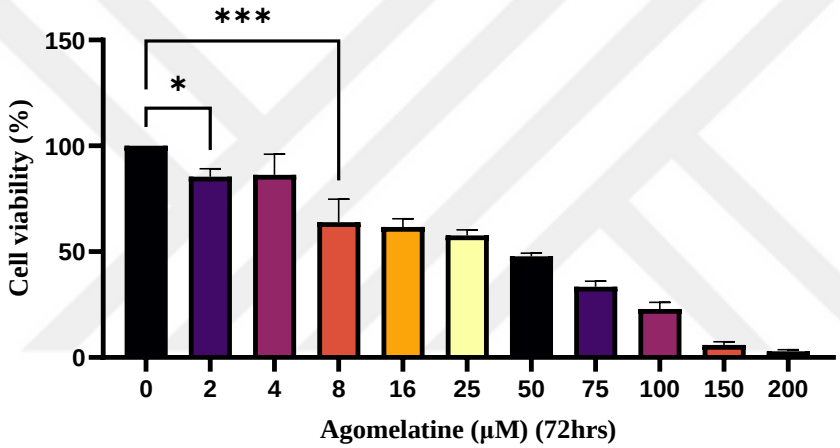

Figure 4.4. Neuroprotective effects of AGO on HT22 cells viability for 72 hrs. The values were given as the mean ± standard deviation. Significance was observed at AGO concentrations 2 doses, and very high significance started from 8 µM compared with the control group. Very high significance showed from 8 µM at ($p \leq 0.001$) and determined by (***).

4.1.6. The IC_{50} Values for Different concentrations and Durations of AGO on HT22 Cells

The IC_{50} of AGO was calculated for 24, 48, and 72 hours drug administration. It was determined to be 62.85, 41.86, 26.65µM for 24, 48, and 72 hours respectively (Table 4.11.).

Table 4.10. Showed the result of inhibitor concentration doses of AGO for different times.

IC_{50}	After 24 hrs.	After 48 hrs.	After 72 hrs.
[Inhibitor] vs. normalized response – Variable slope	62.85	41.86	26. 65

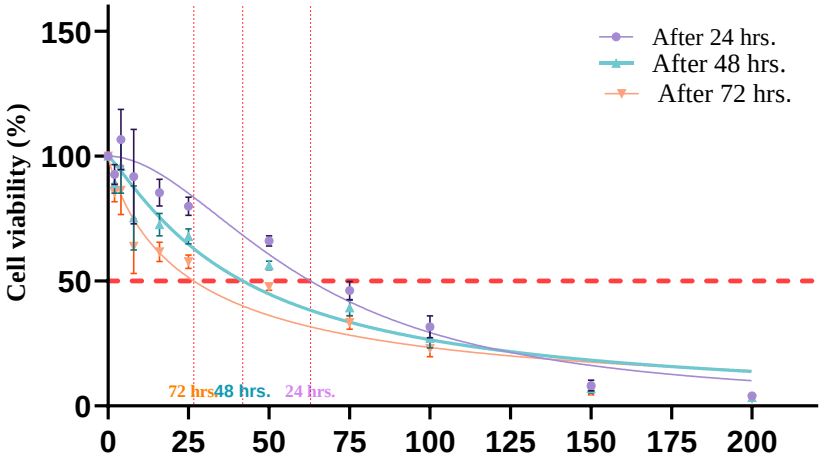

Figure 4.5. Showed the comparison of the inhibitory concentration value of AGO after different durations of 24, 48, and 72 hrs. IC_{50} = 62.85µM, 41.86µM, 26.65µM respectively, these concentration doses dropped the cell viability or growth into 50 percent, it was determined by non-Lin fit from the absorbance line of the cell and crossed with the concentration curve line.

4.1.7. Determination of Toxic Dose of Glu on HT22 Cell Viability and calculate the IC_{50}

In this study, the toxic effect of Glu on HT22 cell viability was assessed, different concentrations of of Glu (1, 2, 3, 4, and 5mM) were applied for 24 hours, MTT assay was used to evaluate the cell viability, and the IC_{50} value was calcuated. The the cell viability of of Glu different concentration doses were presented as mean and the standard deviation (SD) (Table 4.12.).

Table 4.11. Showed the mean value and the standard deviation (SD) of cell viability in different concentration dosages of Glu.

Glu (mM)	Mean Value of cell viability % ± SD
0	100 ± 0.000
1	88.63 ±18.04
2	66.72±20.14
3	40.16 ±19.64
4	9.681±1.546
5	10.71 ±4.043

One way Anova and Post-hoc Tukey were conducted, no significant difference was observed at low doses; however, it was found that the cell viability reduced by more than half the total number at concentration 3mM with very high significance ($p \leq 0.001$). While the doses 4 and 5mM killed over 50% of the cells compared to the

control group ($p \leq 0.001$) (Figure 4.6.). On ther other hand, Glu IC_{50} value was calculated and determined to be 2.465 mM (Figure 4.7).

Table 4.12. Showed the significant degrees of cell viability in different concentrations of Glu for 24 hrs. of cell treatment.

Glu (mM)	*P* value
0 vs. 1	.846
0 vs. 2	.031*
0 vs. 3	<.001***
0 vs. 4	<.001***
0 vs. 5	<.001***
1 vs. 2	.263
1 vs. 3	.001**
1 vs. 4	<.001***
1 vs. 5	<.001***
2 vs. 3	.117
2 vs. 4	<.001***
2 vs. 5	<.001***
3 vs. 4	.055
3 vs. 5	.067

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

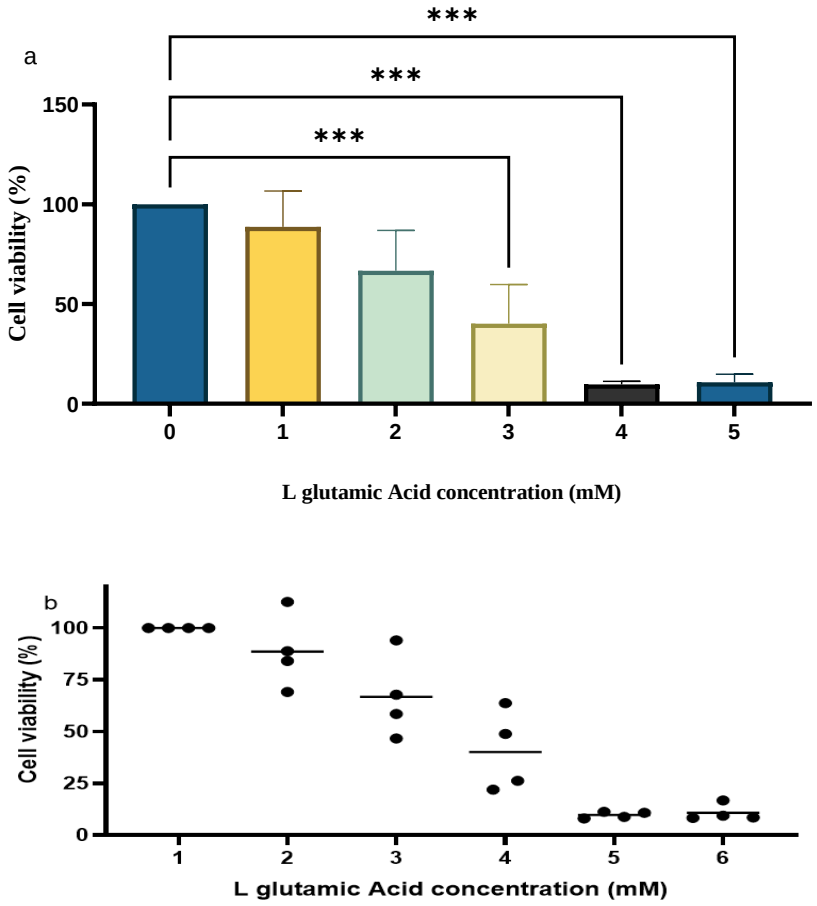

Figure 4.6. The concentration-dependent effect of Glu on HT22 cells viabilityin a 24h, a: multiple group comparison and b: individual concentration values and means.

4.1.8. The Toxic Effect of Glu on HT22 cells after 24 hrs Administeration

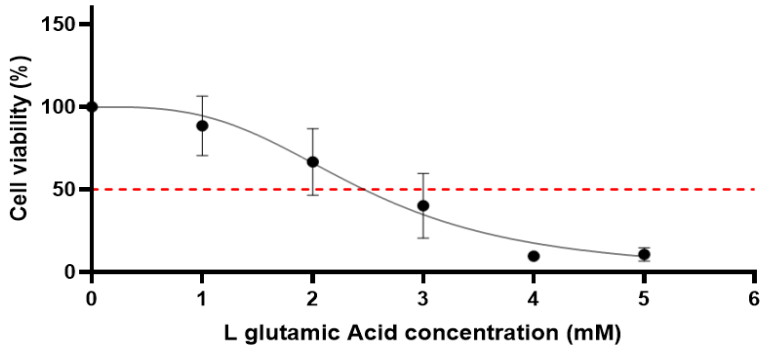

Figure 4.7.The IC_{50} values of Glu at (2.465mM) concentration dose after 24h exposure

4.1.9 The protective effect of AGO against Glu toxicity on HT22 Cell Viability

In this study, to ensure HT22 toxicity, variuos doses of Glu induced cell death was exmined, the results showed that the IC_{50} value of Glu was (2.465mM). To ensure mimicing the neurodegenerative model even higher dose was considered. Thus, the cells were administered with 5 mM Glu.

HT22 cells were seeded in 96 well-plates in nine groups and the cells were seeded equally in each group in 100µl complete media. In the blank (first) group, it involved only media to calculate and deduct the background effect. In the Cont (second) group, the cells were not treated with any substances; In the toxic (third) group, the cells were administrated with 5mM of Glu. In the treated 1 (toxic + low AGO / fourth) group, the cells were administrated with 5mM of Glu for one hour, and then 30 µM of AGO for 24 hours. In the treated 2 (toxic + high AGO / fifth) group, the cells were administrated with 5mM of Glu for one hour, and then 30 µM of AGO for 24 hours. In the protection 1 (low AGO / sixth) group, the cells were administrated with 60 µM of AGO for 24 hours. In the protection 2 (high AGO / seventh) group, the cells were administrated with 60 µM of AGO for 24 hours. In the DMSO groups 1 and 2 (eighth and nineth) groups cells were administrated 3 and 6 µl of DMSO respectively. These doses of DMSO were applied based on the volume used to dissolve AGO.

4.2. The Finding of MTT Assay of the Experimental Groups

The mean number of the cell viability of diverse groups and the degree of significance are visible below in the following table and figure (Table 4.15. ; Figure 4.8.).

Table 4.13. Represented the mean value and the standard deviation (SD) of cell viability

Group	Doses	Mean cell viability % ± SD
Cont	only cells	100 ±0.000
Toxic	Glu 5mM	8.705 ±0.5698
Treated 1	Glu 5mM +AGO 30µM	46.04 ±5.431
Treated 2	Glu 5mM AGO 60µM	39.59 ±0.5426
Protection 1	AGO 30 µM	73.01 ±1.626
Protection 2	AGO 60µM	56.17 ±1.928
DMSO 1	DMSO 3µL	51.15 ±6.034
DMSO 2	DMSO 6µL	43.98 ±0.6029

Table 4.14. Illustrates the degree of significance between various groups of HT22 cell viability.

Group	P value
Cont vs. Toxic	<.001***
Cont vs. Treated 1	<.001***
Cont vs. Treated 2	<.001***
Cont vs. Protection 1	<.001***
Cont vs. Protection 2	<.001***
Cont vs. DMSO 1	<.001***
Cont vs. DMSO 2	<.001***

Toxic group: Glu 5mM, Treated Group 1: Glu + AGO 30µM, Treated Group 1: Glu + AGO 60µM, Protection Group 1: AGO 30µM, Protection Group 2: AGO 60µM, DMSO Group 1: DMSO 3µL, DMSO Group 2: DMSO 6µL.
* indicates significance at 0.05, ** indicates significance at 0.01, *** indicates significance at 0.001.

Table 4.15. Showed the significant degrees of cell viability in different treated groups.

Group	Sig.	Group	Sig.
Cont vs. Toxic	<.001	Treated 1 vs. Treated 1	<.001
Cont vs. Treated 1	<.001	Treated 1 vs. Treated 2	0.015
Cont vs. Treated 2	<.001	Treated 1 vs. DMSO 1	0.471
Cont vs. Protection 1	<.001	Treated 1 vs. DMSO 2	0.988
Cont vs. Protection 2	<.001	Treated 2 vs. Protection 1	<.001
Cont vs. DMSO 1	<.001	Treated 2 vs. Protection 1	<.001
Cont vs. DMSO 2	<.001	Treated 2 vs. Protection 1	0.005
Toxic vs. Treated 1	<.001	Treated 2 vs. Protection 1	0.64
Toxic vs. Treated 2	<.001	Protection 1 vs. Protection 2	<.001
Toxic vs. Protection 1	<.001	Protection 1 vs. DMSO 1	<.001
Toxic vs. Protection 2	<.001	Protection 1vs. DMSO 2	<.001
Toxic vs. DMSO 1	<.001	Protection 2 vs. DMSO 1	0.493
Toxic vs. DMSO 2	<.001	Protection 2 vs. DMSO 2	0.003
Treated 1 vs. Treated 2	0.221	DMSO 1 vs. DMSO 2	0.137

Toxic group: Glu 5mM, Treated Group 1: Glu + AGO 30µM, Treated Group 1: Glu + AGO 60µM, Protection Group 1: AGO 30µM, Protection Group 2: AGO 60µM, DMSO Group 1: DMSO 3µL, DMSO Group 2: DMSO 6µL.
* indicates significance at 0.05, ** indicates significance at 0.01, *** indicates significance at 0.001.

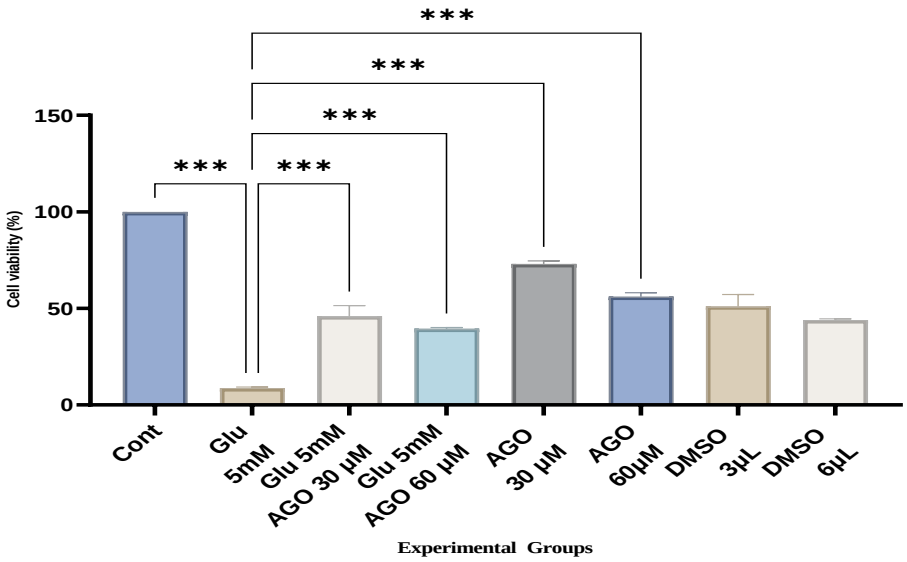

Figure 4.8. Showed the difference in the cell viability of HT22 cell line for the experimental groups.The significant difference at $p \leq 0.05$ is indicated by *, whereas the highly significant at $p \leq 0.01$ is indicated by ** and the very high significance at $p \leq 0.001$ indicates ***. The means values and bars represent standard deviation.

One-way ANOVA accompanied by Tukey's multiple comparisons test were used to compare between the experimental groups. The results revealed very high significant neurotoxic effect of Glu on HT22 ($p \leq 0.001$). Similarly, there were highly significant differences between the low and high AGO in compared with Glu toxic group ($p \leq 0.001$) indicate AGO protective effect. Additionally, there were highly significant differences between the pretection groups 1 and 2 in compared with Glu toxic group ($p \leq 0.001$). On the controary, DMSO at both doses (3 and 6ul produced high toxicity, when compared to the control group, the statistical analysis showed very high significant difference ($P \leq 0.001$).

4.2.1. AGO as Neuroprotective Agent Against HT22 Cell Cytotoxicity Induced by Glu

The results were presented as the mean and SD of HT22 cell viability (Table 4.15.; Figure 4.8.).

4.2.2. Effect of Glu Toxicity Induced Death in HT22 Neural Cells Line

The treatment of the HT22 cells with 5 mM Glu for 1 hr. ultimately reduced the cell viability to less than 10% . It caused a significant change in the cell morphology, and the neurites shortened or disappeared **(**Figure.4.12.).

4.2.3. Effect of Low Dose of AGO on HT22 Cell Viability Against Glu-Induced toxicity

The cells were treated with 5 mM Glu for 1hr, and then with 30 μM AGO as low neuroprotective doses. The findingd showed that the low dosage of AGO inhibited the Glu-induced death of HT22 cells, prevented cellular morphological appearance and decreased cell death rate **(**Figure 4.12.).

4.2.4. Effect of High Dose of AGO on HT22 Cell Viability Against Glu-Induced toxicity

The cells were treated with 5 mM Glu for 1hr. and then with 60 μM AGO as high neuroprotective doses. The result showed enhanced cells morphology and higher cell viability rate compared to the Glu group. The results showed that this dose significantly protected the cells and blocked Glu-induced morphological alterations in HT22 neural cells **(**Figure 4.12.).

4.2.5. Effect of Low Dose of AGO on HT22 Cell Viability

The low dosage of AGO produced significant effect on cell viability compared to the cont group **(**Figure 4.12.).

4.2.6. Effect of High Dose of AGO on HT22 Cell Viability Against

The high dosage of AGO also produced significant effect on cell viability compared to the cont group (Figure 4.12.).

4.2.7. Effect of Low DMSO Dose on HT22 Cell Viability

These doses of DMSO (3μl) caused a significant decrease in cell viability compared to the cont group, however, no significant effect was observed when compared with low AGO group (Figure 4.12.)

4.2.8. Effect Of High DMSO Dose On HT22 Cell Viability

These doses of DMSO (6μl) caused a significant decrease in cell viability compared to the cont group, however, no significant effect was observed when compared with High AGO group **(**Figure 4.12.).

4.3. The histological Finding Different Phase Under the Inverted Microscope

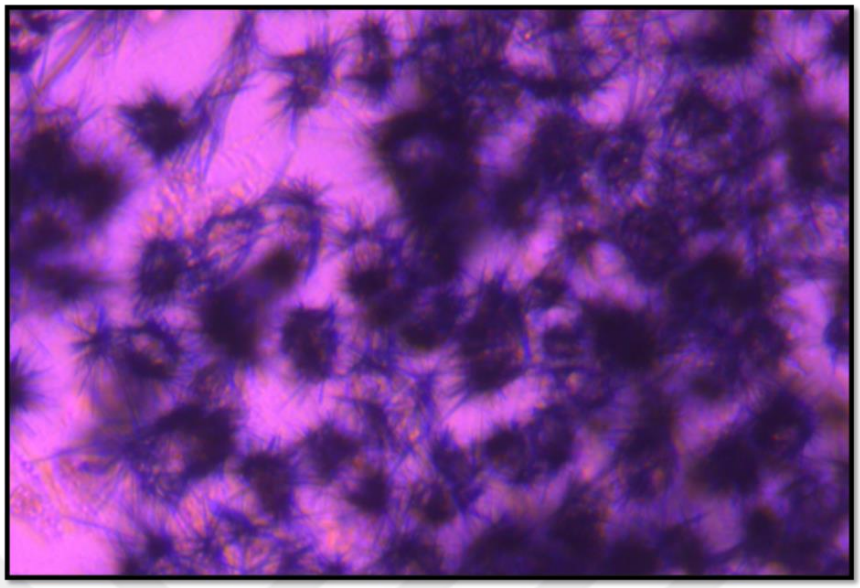

Figure 4.9.Showed dissolved formazan crystallization by DMSO after exposure of the cell with MTTassay.

4.3.1. The Finding of Different Concentration Doses of AGO

Regarding the morphological analysis of the HT22 neural cell after administration with different concentrations of AGO (2, 4, 8, 16, 25, 50. 75, 100, 150, and 200 μM). In the control group, healthy and viable cell was observed. When the cells treated with 2μM of AGO, the cells were slightly detached from the surface but mostly were healthy and with no change in morphology. With dose increase (4, 8 and 16 μM) AGO, more cells detached from the surface with few morphological changes. At higher AGO (25,50,75,100 μM) doses, there was changes in the cells' morphlogy with many cells losing their attachment indicating higher death rate in compare to the lower doses, the morphological changes become more obvious in the high doses. The cell viability decreased even more at 150 and 200 μM, with cellular membrane changes, and reduced sizes. Moreover, the dendrites of cells became short and thin and the cell number dropped **(**Figure 4.10.).

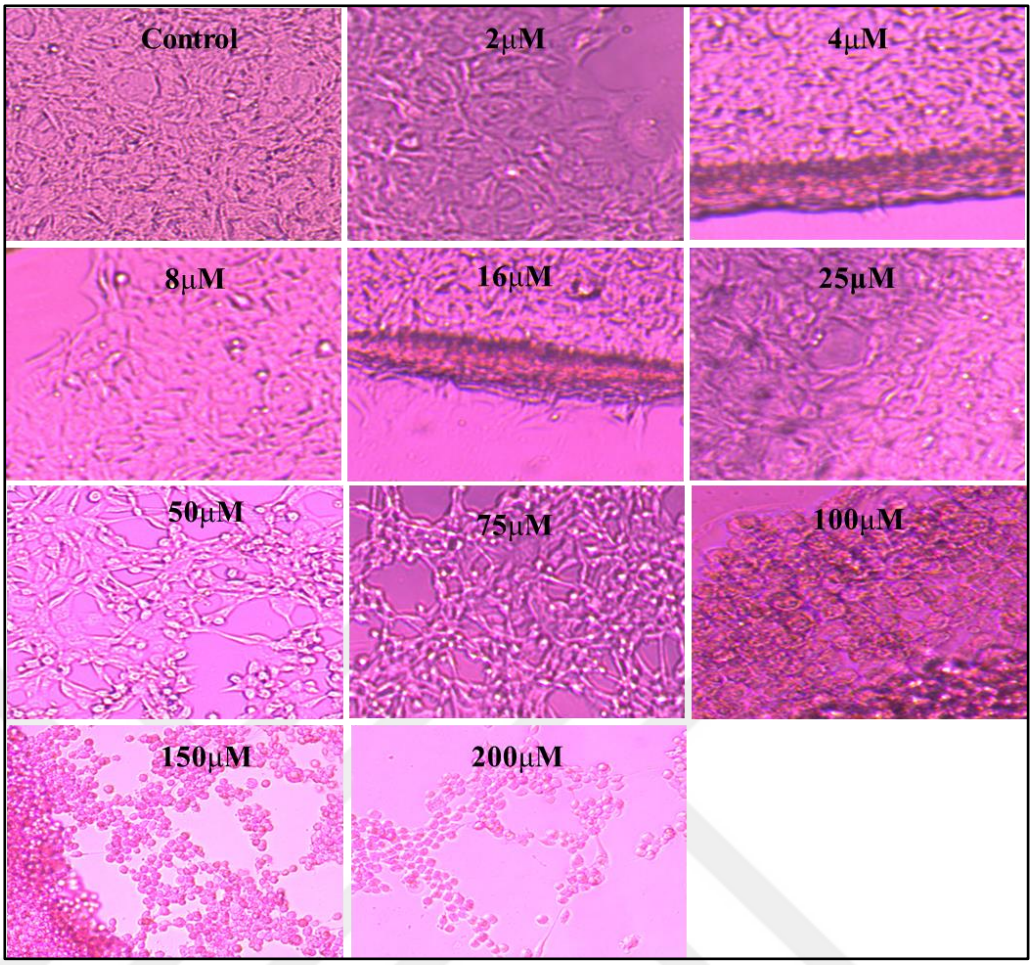

Figure 4.10. A Photomicrograph was taken from the HT22 neural cell line after exposure to different concentrations of AGO as neuroprotective doses for 24 hrs. The cell viability started to decrease in number from dose 16μM and change in morphological appearance compared clearly with the untreated group. Additionally, large spaces represented detachment and death of the cells, also observed to reduce the cell size and loss of their neurites.

4.3.2. The Finding of Different Concentrations of Glu on HT22

As microscopical analysis of the HT22 neural cell after administration with different concentrations of Glu as the toxic (1, 2, 3, 4, and 5 mM), we observed that the cells in all different doses appeared to have a morphological change in shape and size. The shape changed from a typical spindle to oval or rounded and became very little or dropped in viability; it also lost most organelles, such as the nucleus and cell membrane. Furthermore, the cell's dendrites became short and thin; all this change showed under the contrast phase of an inverted microscope **(**Figure 4.11.).

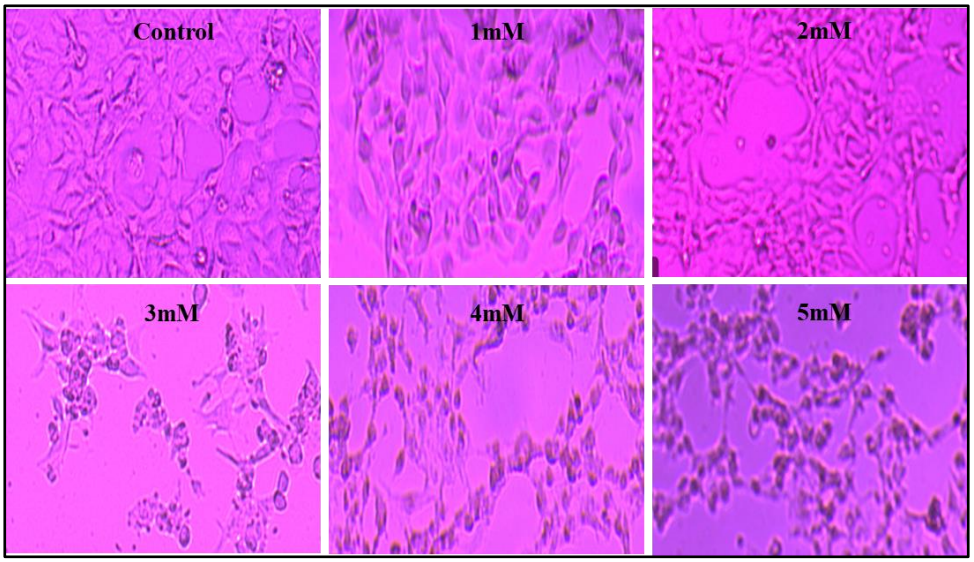

Figure 4.11. Showed the HT22 neural cell exposure to different concentration doses of Glu-induced cell toxicity and morphological cell change and death control group (0mM) the cell is expected, the cell treated with 1mM, and 2 mM were slightly reduced viability, cells administrated with 3mM, 4mM, and 5mM led to reduce cell viability and change in morphological appearance compared with untreated group.

4.3.3. Microscopic Findings of Cell Viability for the Experimental Groups

In the morphological analysis of the Cont group, we observed the cell had a normal morphological appearance and spindle in shape and grew rapidly to reach the confluence stage. The nuclear membrane surrounded the cell membrane and the nucleus, and a considerable number of neurites were synapsis. Whereas the Glu+ a low AGO and Glu a high AGO groups revealed that the cells had defined morphological appearance of these groups was clear, and the structure of neurons is commonly well preserved, the numerical density of the cell viability in these groups was higher than in the Glu group. Furthermore, the Glu dose led to the degeneration and death of some cells in Glu+ low AGO and Glu+ High AGO, but the effect was fewer than in the Glu group. On the other hand, many cell death and large spaces that suggested the detachment and death of the cell decreased the cell number, appeared in black, lost all organelles, and terminated by lysed were observed in this group treated with Glu. The cells reduced in size and number and lost most of their neurites in each DMSO concentration, which was lower than that of the Cont and protective groups. The regular cells became small and lost the neurites determined by (white arrow), and degenerated cells are depicted by (blue arrow) changed in shape, and reduced viability that was observed in the low AGO and High AGO groups compared to the Glu group (Figure 4.12.).

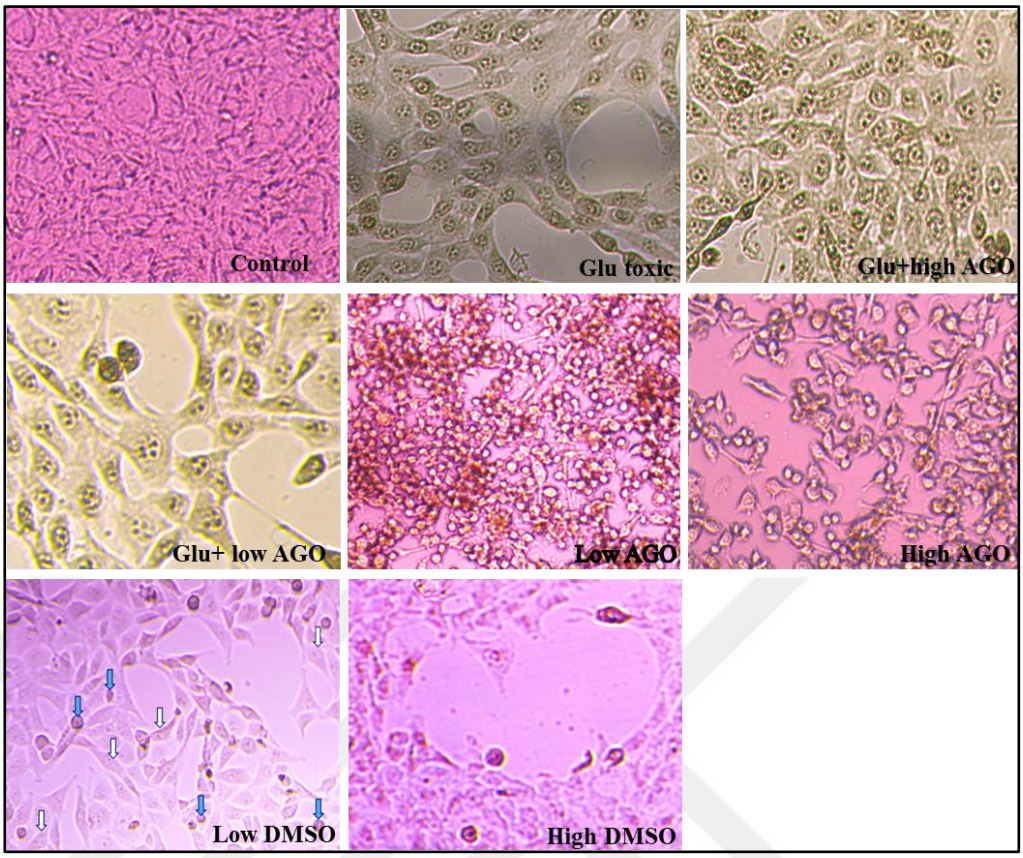

Figure 4.12. Photomicrograph was taken from the HT22 neural cell line after exposure to Glu toxicity and AGO as neuroprotective doses.The large spaces indicated detachment and cell death, while the black spots indicated the degeneration of the cell and reduced viability, and some cells in the toxic group were losing their neurites. The contrast phase of the inverted microscope assessed the image.

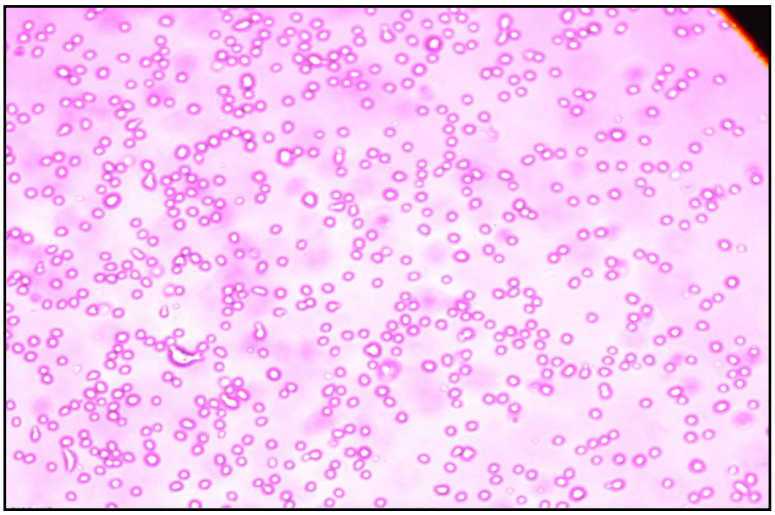

Figure 4.13. Showed the HT22 neural cell line was subculture in a new flask with fresh complete media after being detached by trypsin and centrifugated under an inverted microscope (4 x magnification) and put at 37°C and CO_2 5% and humidity 95%in incubator.

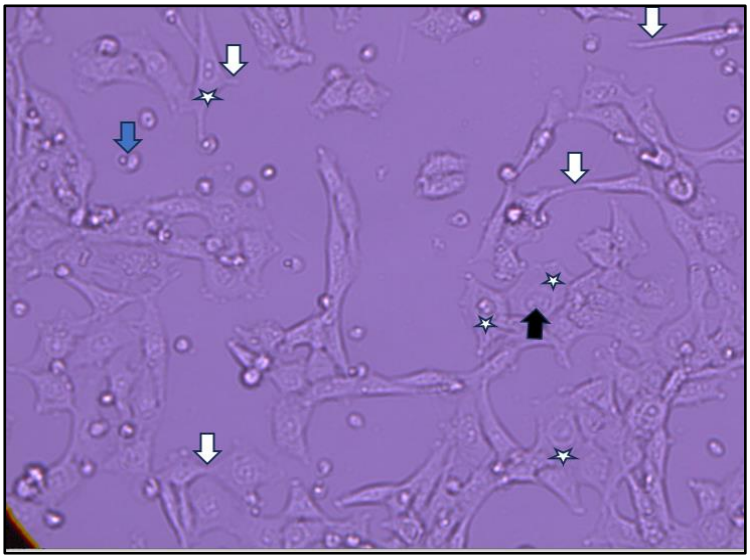

Figure 4.14. Photomicrograph taken from the routine and healthy attached HT22 neural cell line,after seeded in flask 25cm^2 the cell in the level of growth and proliferative noted the development of the many neurites determined by (white arrow), and cell membrane (star) and nucleus of the cells surrounded by nuclear membrane are depicted by (black arrow). The contrast phase of the inverted microscope assessed the picture.

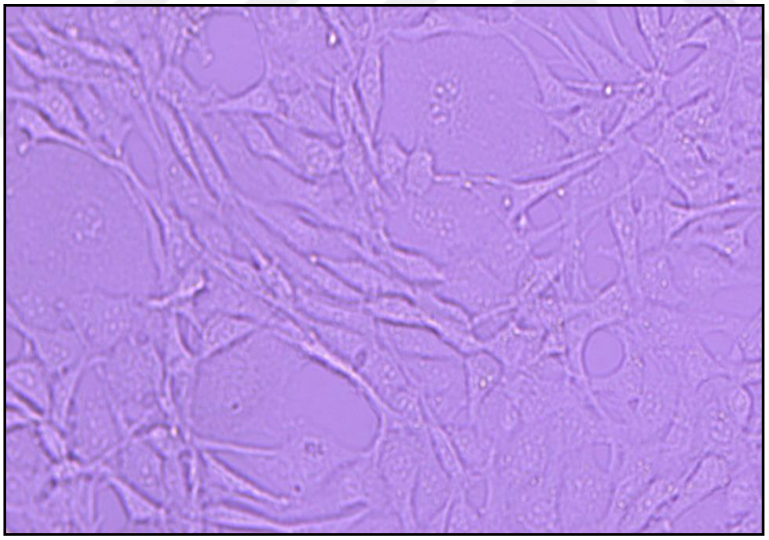

Figure 4.15. HT22 at the confluence stage under the inverted microscope.

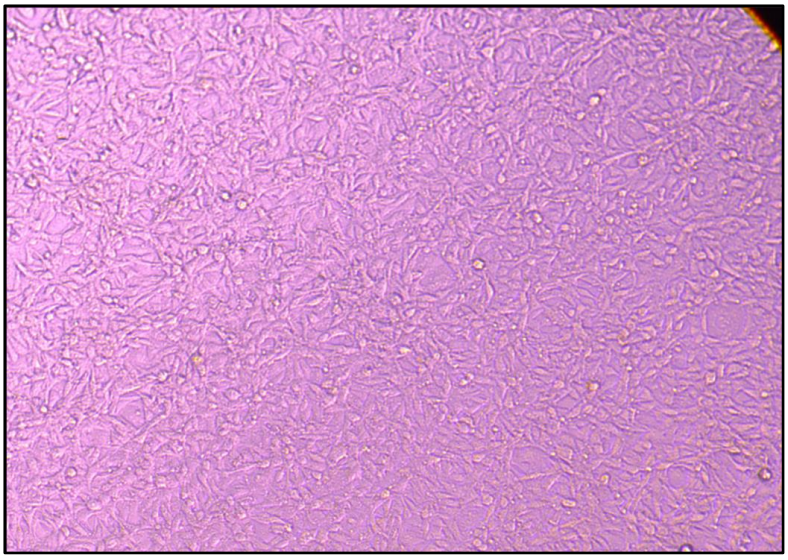

Figure 4.16. This image shows that the HT22 neural cell line reached 100% of the confluence cell cycle stage under the contrast inverted microscope with 10x magnification, ready for treatment or splitting again.

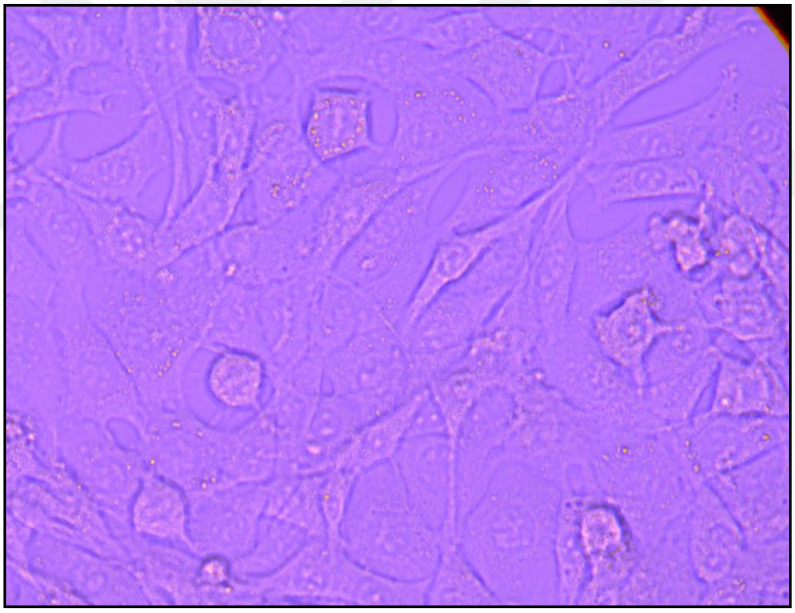

Figure 4.17. The HT22 neural cell line reached 90% of the confluence cell cycle stage under the contrast inverted microscope with 40x magnification, the synapse between cells and cytoplasm and cell nuclei very clear.

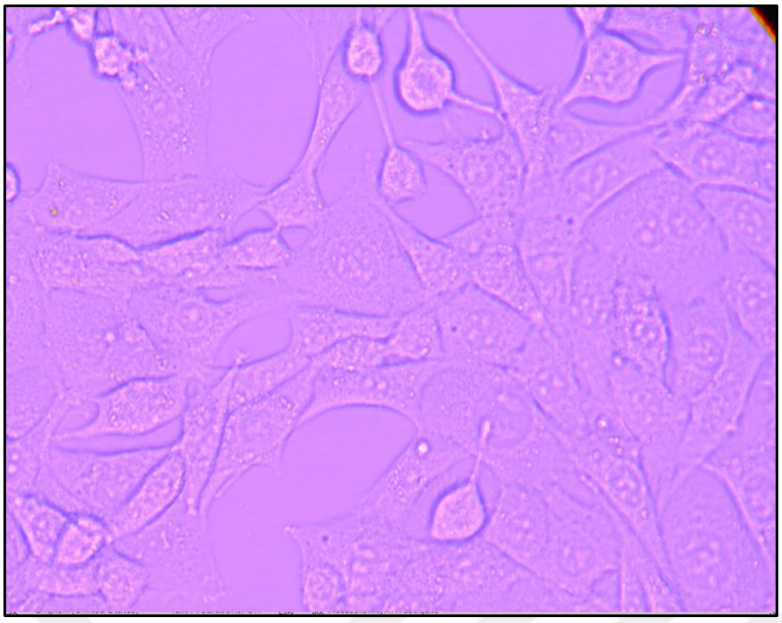

Figure 4.18. Showed that the HT22 neural cell line reached 90% of the confluence cell cycle stage under the contrast inverted microscope 40 magnification

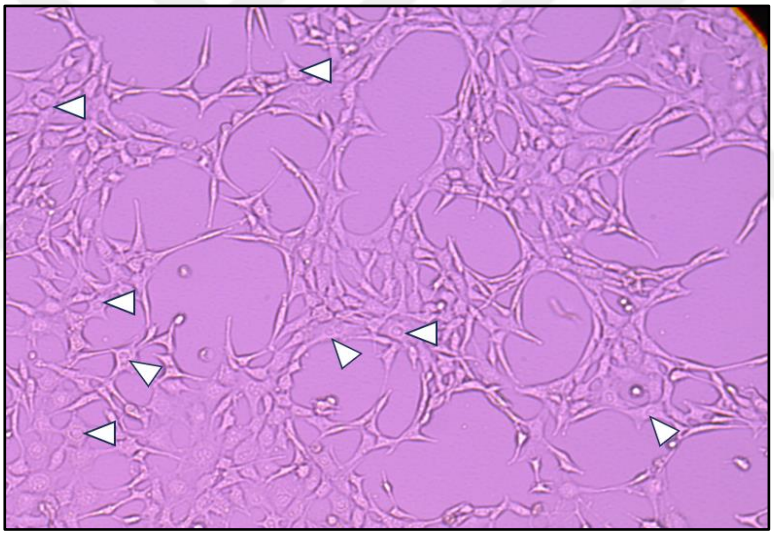

Figure 4.19. Photomicrograph was taken from the HT22 neural cell line after seeded in flask 25cm^2 complete culture media and showed under an inverted phase contrast microscope, incubated in environmental condition at a temperature 37° C, 95% humidity, 5% of CO_2. The cell is obvious and healthy with organelles like cell membrane and nucleus indicated by (head arrow)

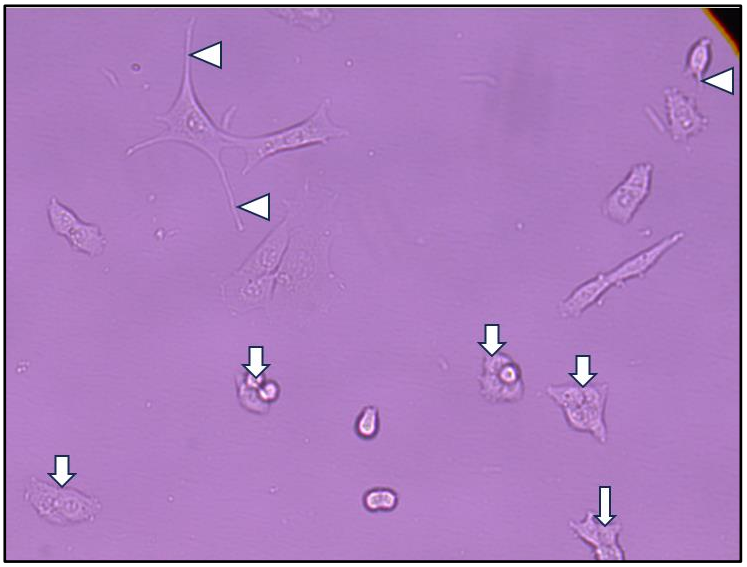

Figure 4.20. A Photomicrograph taken from the usual and healthy attached HT22 neural cell line after seeded in flask 25cm^2 cell culture, the cell at the beginning of mitotic division and growth determined by (white arrow). Developed the neurites, and some appeared as long as determined by (head arrow); this is assessed of the image by the contrast phase of an inverted microscope.

5. DISCUSSION

NDDs are a variety of irreversible and more progressive disorders that potentially cause neuronal death of the central or peripheral nervous systems and are recognized as the main factor that causes motor and cognitive impairments (Amor et al., 2014). More than thirty million people are affected by these diseases, which result in death and disability (Radi et al., 2014). The most common NDDs involve PD, AD, HD, and ALS, but the causes are unclear (Dauer and Przedborski, 2003).

Common signs of these disorders include impaired mobility, loss of memory, and behavioural and cognitive deficiencies due to progressive neuronal death (Mandemakers et al., 2007). The leading causes are excessive OS levels and an imbalance between the free radicals release and antioxidant defence system, which are associated with cell death and neurological diseases (Radi et al., 2014). Each NDDs condition has a different etiology, pathophysiology, and specific clinical signs (Gupta, 2022). Generally, in these diseases, severe oxidative disturbance can cause cell death by several mechanisms, either by inhibiting essential cellular activities or stimulating harmful substances (Barnham et al., 2004).

In vitro studies, toxic substances simulate the NDDs observed in these diseases and attempt to investigate the mechanism triggered by neuronal damage and cell death (Huang et al., 2018). Glu is commonly used as a model system to stimulate cell excitotoxicity that generates the NDDs according to the overproduction of (OS) and excessive glutamic receptor action that causes neuronal damage(Coyle and Puttfarcken, 1993). Besides this harmful toxicity influence, in this current study, we attempted to intervene with AGO as the neuroprotective substance to minimize Glu neurotoxicity in the cell line by combining this protection substance with Glu toxicity. This combination approach may be promising with beneficial therapeutic effects on NDDs. Hence, this study was designed to explain the possible neuroprotective effect of AGO on HT22 neural cell line *in vitro* to protect against the Glu neurotoxicity that induced the depletion of the antioxidant system and signalling molecules pathways associated with neuronal cell death.

To assess the effectiveness of AGO and Glu with different concentration doses in the HT22 cell line, we selected some of the methods in this study to determine the viability and metabolic activity of the cell after administration with different concentration doses either from AGO or Glu, these methods are including MTT assay

which is colourimetric analysis a commonly used to determine the appropriate affected concentration doses of substance on the cell viability through the calculation the IC_{50} value which is a measure used in pharmacology to determine the concentration of a drug that inhibits 50% of cell viability.

Besides, this method used the Elisa absorbance reader at 570nm to measure the number of cell survival and obtained the data as an Excel sheet.

The viability is the ratio of living cells to total cells (Nozhat et al., 2022). This viability of cell culture is frequently evaluated through the metabolic mitochondrial activity of cells (Vega-Avila and Pugsley, 2011). The MTT assay is a sensible, accurate, and credible test. It acts based on the capacity of the mitochondria dehydrogenase enzyme in viable cells to reduce the yellow water-soluble MTT bromide substrate into a purple or dark-crystalized formazan that is insoluble in water (Vega-Avila and Pugsley, 2011). DMSO was then added to dissolve the crystallization.

Statistical analysis is used to collect quantitative data to provide reliable results. This present study was conducted in HT22 neuronal cell lines among various investigation tools, which is the primary and most used *in vitro* model for relevant mechanistic and pharmaceutical studies (Davis and Maher, 1994, Karri et al., 2018, Alboni et al., 2014). Experimental studies indicated that the HT22 hippocampal cell line is more sensitive to oxidative Glu neurotoxicity due to a deficiency of ionotropic Glu receptors (Sagara et al., 1998).

The first part of this current experimental study is to determine the appropriate effect of the concentration dose of both substances AGO as neuroprotective and Glu as neurotoxicity on the HT22 neural cell line. Different concentrations of Glu were administered against the viability of HT22 for different durations, and an MTT assay and ELISA reader were used to assess the cell viability and calculate the IC_{50}.

In this study, we attempted to measure the effect of AGO in various dosages depending on time and concentration on cell viability and determined IC_{50} on 24h, 48h, and 72hrs at a concentration (2, 4, 8, 16, 25, 50, 75, 100, 150, 200 μM).

The current study found that IC_{50} of AGO value increased from 62.85μM at 24 hours to 41.86 μM at 48 hours and then decreased to 26.65μM at 72 hours. These results suggested that the AGO potency may vary over time, potentially impacting its therapeutic efficacy. These findings indicate that the effectiveness of AGO may vary depending on the duration of exposure.

These results are consistent with a study conducted by Yang, J et al. (2016), who explored the time-dependent efficacy profile of AGO *in vitro* hypothalamic slice preparation (Yang et al., 2016). It was perceived that AGO has a dose-dependent therapeutic outcome on depression and anxiety caused by Fluoride intoxication in mice (KARADEMİR, 2023). Limited research has been conducted to investigate the impact of AGO on HT22 cell viability *in vitro*.

In this study, we focus on understanding how various concentrations and durations of AGO agents influence cell viability. The objective is to underscore the significance of studying the effects of AGO drugs on cellular health.

Our study investigated the effect of AGO administration on HT22 cells at various concentrations. Interestingly, we did not observe any significant differences in the cell viability between doses ranging from 2 μM to 16 μM ($p > 0.05$) compared to the control group for 24hrs, whereas at 48 hrs., the significance began from 8μM.

These findings indicated that the range of 2 μM to 16 μM doses is safe and possesses neuroprotective properties for 24 hrs. compared with the control group, This finding suggests that the range of 2 μM to16 μM doses are safe and include neuroprotective properties. This study aligns with several studies demonstrating that AGO could improve cell proliferation, stimulate neurogenesis in the dentate gyrus, and maintain cell survival (Banasr et al., 2006). The neuroprotective effect of AGO to minimize cytotoxicity and improve hippocampal cell integration is mediated through its anti-inflammatory and antioxidant (Cankara et al., 2021). Another study demonstrated that AGO can increase cellular transcription and BDNF to enhance neurogenesis in the brain's hippocampus region (Gumuslu et al., 2014). Additionally, investigations showed that AGO therapy has an antioxidant impact and the capacity to change the brain's metabolism ATP energy (de Mello et al., 2016). Another research analysis showed that the administration of AGO encourages cytokine production and stabilizes the glutathione level and glutathione peroxidase activity (Demirdaş et al., 2016).

However, when the dose was increased and extended from 25 to 200 μM, there was a noticeable decrease in cell viability compared with the untreated control group ($p \leq 0.001$) after 24 and 48 hours.

Regarding the effective dose of AGO in HT 22 cell viability for 72 hrs., only low concentrations dose 4μM AGO administration showed safety and neuroprotection compared to the control group ($p > 0.05$). Moderate doses of AGO (2 μM) displayed a

noticeable decrease in cell viability compared to the control group with a *p*-value < 0.05. The doses, such as those at levels of 8μM up to 200 μM, significantly impacted cellular viability compared to untreated control ($p \leq 0.001$). These findings strongly suggest that cytotoxicity or adverse effects are dose-dependent and associated with increased concentrations of AGO.

Glu is a major CNS excitatory neurotransmitter and vital in many physiological processes. Excessive Glu production might be implicated in several acute and chronic neurological disorders, including cerebral ischemia, AD, PA, and HD. Glu low concentration dose between 10 to 100 μM exerts its cytotoxic effects through receptor-mediated excitotoxicity and non-receptor-mediated oxidative toxicity (Tan et al., 1998). OS caused by glutathione depletion after prolonged exposure to high extracellular concentration among 10 to 100 mM of Glu inhibited cystine transport and led to a form of NCD that exhibits apoptosis and necrosis (van Leyen et al., 2005).

In this current study, the statistical analysis measured the IC_{50} value of Glu at 2.645 mM and showed that it led to the destruction of 50% of the cell. At the same time, other little concentrations of Glu did not exhibit significant cytotoxicity compared with the untreated control for 24h. However, for one day of treatment, the cell viability significantly decreased at high doses 3mM, 4 mM, and 5mM of Glu. These findings strongly suggested that cytotoxicity or adverse effects are dose-dependent and associated with increased concentrations of Glu and affect the cell appearance and intracellular structure by reducing the cell size according to findings present under the inverted microscope. We showed a higher significant level began from low concentrations dose 1 mM, which significantly increased excessively when the concentration was elevated, affecting the cell appearance and reducing the cell size under the microscope. Dose 4 and 5 mM, the viability dropped significantly compared with the control group ($p \leq 0.001$).

These results align with those previously published the Glu toxicity in C6 cells, induced cell death via inhibit cystine transport (Han et al., 1997). Another study was supportive of the hypothesis that the treatment effect of the high concentration level of extracellular Glu acted as a neurotoxic substance and promoted neuronal death by increasing OS levels, causing a deficit in the antioxidant system in various types of neural cell lines and primary cell cultures (Kim et al., 2017, Fukui et al., 2009, Jeong et al., 2007).

Another comparative study supported and reinforced that Glu toxicity stimulated neuronal apoptosis cells (Han et al., 1997, Murphy et al., 1989). Furthermore, the subsequent study reported that Glu concentration levels were more than average and able to activate neurotoxicity and led to neuronal damage also in PC12 neuronal cells and hippocampus neural cell line, whereas the lowest dose of Glu does not appear to affect the cell clearly (Bhavnani et al., 2003). Exposure to Glu can damage the primary cortical cells, such as glial and neuronal cells (Koh and Choi, 1987). Another study showed that Glu caused neurotoxicity by producing ROS in the cell line culture (Fu and Koo, 2006). An early decrease in intracellular glutathione precedes Glu-induced cell death.

Furthermore, this resulted in an agreement with previous reports, indicating that Glu can induce cell death and damage (Schelman et al., 2004). Other findings suggest Glu toxicity in cell death due to an apoptotic mechanism (Nishi et al., 1999). Excessive extracellular stimulation induced death in HT 22 by inhibiting or activating some proteins related to cell death (Lee et al., 2021). Glu-induced mitochondrial dysfunction in HT22 cells and reduced membrane potential releases cytochrome c and causes cell death (Fukui et al., 2009). In addition, the excitotoxicity of Glu can increase autophagic genetic proteins that are associated with neuronal death (Kulbe et al., 2014). Some studies support our results of the Glu-induced excessive production of OS and mitochondrial dysfunction (Jeong et al., 2022). Much of the data from the above research appears to be consistent with the present study's findings, which indicate that Glu has a neurotoxic influence on cell viability and causes cell damage and death.

Based on the beneficial outcome of AGO on neuronal cell viability and its acts as the substance that supports the endogenous antioxidant defence system and after determining the IC_{50} of AGO substances in this current study, we attempted to examine the possible effect of AGO to mitigate the negative impacts of the Glu induced neurotoxicity on the HT22 neural cell line. In addition, we used two concentration doses of AGO neuroprotective effect to determine the IC_{50} value is 60 μM as a high neuroprotective dose, and half of 60 μM dose as a low neuroprotective concentration dose 30 μM

In comparison, to reach the high impact of Glu neurotoxicity that induced the cell death, we used a high concentration dose, such as 5mM of Glu toxicity, to give an elevated effect to decrease the cell viability instead of the IC_{50} value 2.465mM dose.

As MTT assay results showed, significant statistical differences among the cell groups treated with the different doses of AGO (30 µM and 60µM) could protect the cell viability and survival compared with the group treated with Glu toxicity in the mean of the total number of absorbance cell viability. These results are at the same level as other studies that reported that AGO therapy could increase neuronal survival and proliferation (Banasr et al., 2004).

Furthermore, AGO promotes neuronal maturation ratio, and enhanced outgrowth of hippocampal granular cells' neurite might accelerate the growth in different phases of neurogenesis (Soumier et al., 2009, Tardito et al., 2012). According to some research, AGO regulates cell survival by collaborating with the action of MT1/MT2 and 5-HT2C receptors, whereas cell proliferative control through the activity of the 5-HT2C is antagonistic (AlAhmed and Herbert, 2010). There is widespread agreement that AGO can potentially treat AD since it reduces OS and the inflammatory response and prevents cellular senescence via regulating SIRT 6 (Wang et al., 2021). Some studies published by Soumier et al. reported that AGO therapy enhanced the neuronal cell's proliferation, maturation, and survival (Soumier et al., 2009). In this case, we suggested that AGO resembles melatonin, which can act by the exact mechanism of melatonin to produce antioxidant and anti-inflammatory properties. Another study supported our findings that AGO administration can promote neurogenesis and prevent cell death in stress hippocampal rats (Yucel et al., 2016). In addition, the results showed a significant decrease in cell viability was observed in the Glu group compared with the control and neuroprotective groups and the significant presence at ($p \leq 0.001$). This cell viability may be inhibited because Glu acted as a neurotoxic substance and promoted neuronal cell death by increasing oxidative stress levels (Kim et al., 2017, Fukui et al., 2009, Jeong et al., 2007).

According to several previous studies, our study suggested the positive result of AGO as neuroprotective typically had many benefits and positive outcomes to improve the proliferative and enhanced neurogenesis and maintain cell viability. There remains a substantial degree of significance When comparing the low and high levels doses of AGO groups with the Glu toxicity group ($p \leq 0.001$).

These findings underscore the considerable impact of AGO treatment on cellular viability as neuroprotective. These findings support and align with some research by Akpinar et al. (2014) that AGO treatment reduced OS generation and prevented the death of PC12 cells. Additionally, this study omitted previous research on AGO,

which has been shown to have an anti-inflammatory response triggered by the suppression of pro-inflammatory cytokines. (Hong et al., 2021a, Hyeon et al., 2017).

Further assessment confirms and strengthens my findings that AGO had effects resembling melatonin to reduce Ca^{2+} influx from the endoplasmic reticulum mitigated Glu excitotoxicity (Chanmanee et al., 2022).

In line with previous studies, AGO promotes neuronal regeneration, prevents the nuclear morphological change in the cell, and has an anti-inflammatory effect in the hippocampal area. Recently reported studies showed that it would have therapeutic neuroprotective benefits against OS to maintain the brain endothelial cells' survival after exposure to inflammation and toxicity (Cheng et al., 2020).

Additionally, these findings disagree with some studies investigating AGO's potential hepatotoxic effects on immortalized human hepatic cell line L02. Results show significant suppression of cell survival in both concentration-dependent and time-dependent manners (Jia et al., 2019).

Another study was conducted that AGO effectively inhibited colorectal adenocarcinoma cell proliferation and reduced G1 phase cell number in HCT-116 and HCT-116 p53 null cells, its potency compared to melatonin *in vitro* in dosages and time-dependent manner (Moreno-SanJuan et al., 2023). In addition, AGO led to elevated protein oxidation levels and reduced the number of neurons in the rats' brain tissue striatum (Günaydın et al., 2019).

The DMSO is an amphipathic molecule consisting of two nonpolar methyl groups and a polar domain sulfinyl group, and it may switch hydrophobic barriers and lead to solubilizing both polar and nonpolar molecules (de Abreu Costa et al., 2017). It is frequently used to solubilize medical medicines. Besides, these groups also treated cells with different doses of DMSO; one group was given 3µl, and the other group of the cells administrated 6 µl This dose depends on dilution of AGO in the DMSO solution to detect the toxicity impact in this current study.

The statistical analysis showed that the cell death induced by Glu toxicity was significantly higher than that caused by DMSO toxicity, as evidenced by a reduction in cell numbers. When comparing these concentration doses of DMSO with the control group, the significant degree was at ($p \leq 0.001$). Some study reinforces, supports, and are in the same line with my findings. At the same time, some previous studies showed that DMSO caused retinal apoptosis when used at low concentrations (5 µl), and the toxicity demonstrated clearly in retinal neuronal cells (Galvao et al., 2014). On the

other hand, some studies showed that a 10% concentration of DMSO did not affect the viability of human peripheral blood mononuclear cell culture (De Abreu Costa et al., 2017). More than 10 percent of DMSO suggested unaffected the apical membrane's permeability or the tight junction of the cell (Da Violante et al., 2002).

6. CONCLUSION

Our findings concluded that the neuroprotective effects of AGO in HT22 cells are dosage and duration dependent. The study indicates that lower concentrations of AGO have shown notable benefits in maintaining cell viability. However, higher doses appear to adversely affect cellular viability as they exhibit cytotoxicity. These results emphasize the importance of carefully considering the concentration and length of treatment with AGO when assessing its potential neuroprotective properties. Further research is needed to determine the optimal concentration and duration for achieving the desired outcome without causing harm to cellular viability.

Based on MTT assay and statistical analysis, Glu treatment significantly reduced the viability of cultured HT22 cells. Also, treatment with either low or high AGO concentrations in the neural cell significantly exhibited neurotoxicity and increased cell viability compared to the Glu-treated group ($p \leq 0.001$). Also, we found that dimethyl sulfoxide at high concentrations dosage is slightly cytotoxic compared with a Glu group. Our findings suggest that the AGO administration may inhibit Glu neurotoxicity and increase cell viability compared with a toxic group.

Recommendation

- Administration of AGO may heal the neurodegeneration caused by Glu toxicity.
- Further investigations, additional data on the determination of reactive oxygen species release, the level of glutathione associated with Glu cytotoxicity, and oxidative stress activity will be beneficial to support our result for the AGO protection on cell viability.

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8. CURRICULUM VITA

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Publications:

1. Negwod, A, S., Önger, M. E.,and Kaplan, S. (2023). *Evaluation, The Effective Doses Of Agomelatine In Ht22hippocampal Neural Cell Line Viability, Using MTT Assay* abstract presented at the 9thınternational Congress on innovative scientific approaches Samsun-Türkiye.
2. Negwod, A, S., Önger, M. E., and Kaplan, S. (2023). *In Vitro* Assessment the toxicity doses of glutamic acid In HT 22hippocampal neural cell line viability using MTT Assay abstract presented at the 9thınternational congress on innovative scientific approaches - Samsun, Türkiye.