Neuroprotective potentials of Obestatin against ethanol-induced neurotoxicity in the SH-SY5Y and C8-D1A cell lines

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DECLARATION

I, Gizelle Govender, declare that this dissertation titled, “Neuroprotective potentials of Obestatin against ethanol-induced neurotoxicity in the SH-SY5Y and C8-D1A cell lines” is my own work that has not been submitted to any institution until now.

Signature:  Date: 09 June 2025

ABSTRACT

Ethanol negatively impacts the brain by causing structural and functional alterations leading to numerous disorders such as memory impairment, learning difficulties, cognitive decline, motor–skills delays, and psychosocial problems. As such, excessive ethanol intake is a vital risk factor for the development of many brain disorders. Moreso, excessive ethanol use is a significant health issue in South Africa with Foetal Alcohol Spectrum Disorder (FASD) being a common neurodevelopmental disorder associated with intra-uterine ethanol exposure. Ethanol exerts its damaging effects through the upregulation of oxidative stress, neuroinflammation, impairment of mitochondrial function, excitotoxicity and apoptosis. Given the effects of ethanol-induced neurotoxicity, investigations into possible ameliorative agents are needed. In this study, the neuroprotective capabilities of Obestatin against ethanol-induced neurotoxicity in the SH-SY5Y neuroblastoma cells and C8-D1A astrocytes were explored. Obestatin, a naturally occurring gastric hormone, has been shown to modulate and control cell differentiation and cellular metabolism, promote cell proliferation and survival, and suppress inflammation and apoptosis in different cells. SH-SY5Y and C8-D1A cells were treated accordingly with Obestatin to determine its impact on cell viability and neuroprotection against ethanol effects using MTT and trypan blue assays. Furthermore, the mechanism of neuroprotection of Obestatin following ethanol toxicity was determined by measuring the levels of adenosine triphosphate (ATP) activity with a cell-based assay kit. Moreover, the levels of reactive oxygen species (ROS) in the cells were determined using the fluorescent DCFH-DA staining probe following Obestatin and ethanol treatments. Autophagy was assessed using acridine orange staining by fluorescence microscopy and evaluating autophagy markers (LC3BII and p62) using Western blotting. Apoptosis was also evaluated using the nuclear stain DAPI by fluorescence microscopy, Western blotting with antibodies to Cleaved PARP and the expression of caspase-3 gene using quantitative PCR. The findings of this study demonstrated

that Obestatin protected the SH-SY5Y and C8-D1A cells by reducing the depletion of ATP and mitigated ethanol-induced oxidative stress. Moreover, Obestatin attenuated apoptosis induced by ethanol through reducing chromatin condensation, the level of Cleaved PARP and the expression of caspase-3 gene. Further, Obestatin inhibited apoptosis in the cells and regulated autophagy through a reduction in acidic vesicular organelle (AVO) formation evidenced by acridine orange as well as the protein expression levels of p62 and LC3BII. This study thus provided evidence of the neuroprotective potentials of Obestatin against ethanol-induced neurotoxicity, *in vitro*, using the SH-SY5Y and C8-D1A cell lines suggesting Obestatin could be a potential neuroprotective agent that needs to be explored further. However, additional studies are needed to substantiate its neuroprotective effects against ethanol especially in *in vivo* models.

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AD	Alzheimer's disease
ADH	alcohol dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AVO	acidic vesicular organelle
CREB	cAMP responsive element binding protein
CNS	central nervous system
CYP2E1	cytochrome P450 2E1
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DCFH-DA	2'7'-dichlorohydrofluorescin diacetate
ERK 1/2	extracellular signal-controlled kinases 1 and 2
ETC	electron transport chain
FASD	Foetal alcohol spectrum disorders
GPx	glutathione peroxidase
MAPKs	mitogen-activated protein kinases
miRNAs	microRNAs
mtDNA	mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PD	Parkinson's disease
NLR	NOD-like receptors
NF-kB	nuclear factor kappa B

NMDA	N-methyl-D-aspartate
ROS	reactive oxygen species
SOD	superoxide dismutase
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor alpha

CHAPTER 1

INTRODUCTION

1.1 Background

Ethanolic drinks (also known as alcoholic beverages) have long been consumed by people for social, cultural, and religious purposes (Brenes et al., 2021). Moreso, it is widely acknowledged that ethanol consumption is an important part of social interaction and a significant economic driver in Western society (Melia et al., 2021). In addition, ethanol acts as a psychoactive drug, serves as a nutrient, and functions as a toxin (Suter, 2004). Nevertheless, patterns of ethanol consumption, the average amount consumed, and the type of ethanolic drink appear to have a causal relationship with the morbidity and mortality associated with chronic illnesses (Shield, Parry, and Rehm, 2013). Numerous social and health issues have been linked to ethanol use (Tian et al., 2023). Ethanol misuse accounts for about three million deaths annually, representing 6% of all deaths globally. It has far-reaching negative effects, including risks to personal health, morbidity, and mortality which have serious repercussions for friends, family, and society at large (Sudhinaraset, Wigglesworth and Takeuchi, 2016). Furthermore, ethanol consumed in excess has many detrimental effects on different systemic organs or structures and could cause some health problems such as stroke, cardiomyopathy, coronary artery disease, hypertension, etc (Lucas et al., 2005).

Excessive ethanol consumption has negative health effects, and it is neurotoxic causing neuro-behavioural problems and brain dysfunctions (Guerri and Pascual, 2019). Prolonged or excessive use of ethanol causes severe to mild cognitive deficiencies in metacognitive skills, memory, and executive functions, as well as social cognition and emotional processing (Le

Berre, Fama, and Sullivan, 2017). Ethanol is thus a vital risk factor for the development of brain disorders such as dementia, Korsakoff syndrome, and Wernicke's encephalopathy (Goldstein, 1985; Fernandes et al., 2017; Planas-Ballvé et al., 2017). It is also a teratogen, as ethanol causes neuronal death in the developing central nervous system causing diverse neurodevelopmental disorders which are often seen in children with Foetal Alcohol Spectrum Disorder (FASD) (Chen et al., 2012).

Given the impacts of ethanol-induced neurotoxicity, it is pertinent to explore interventions that could reduce the neurotoxic effect of ethanol. Ethanol exerts its neurotoxic effects by disrupting oxidative stress balance, inducing neuroinflammation, impairing mitochondrial function, and promoting excitotoxicity (Virgolini and Pautassi, 2022). Consequently, the neurotoxicity brought on by ethanol may be linked to apoptotic signals that are promoted by oxidative stress and glutamate excitotoxicity. These factors ultimately damage synapses of neurons as well as causes apoptosis, leading to impairment within the nervous system and subsequently the onset of certain illnesses (Du, Shi and Zhao, 2019). It was thus necessary to investigate interventions that could reduce or prevent damages caused by ethanol. An intervention that has been shown to have neuroprotective effects is Obestatin (Anand et al., 2022).

Obestatin is a 23-amino acid peptide and a direct resultant of preproghrelin (Cowan et al., 2016) which is the gene that is responsible for the synthesis of Obestatin (Green, Irwin and Flatt, 2007). This peptide partakes in peripheral and central actions that include hormone secretion, energy homeostasis regulation, improving memory, preventing water drinking as well as sleep regulation (Shen et al., 2014). Obestatin has also been shown to reduce body weight gain, suppress the intake of food and stop jejunal contraction (Li et al., 2011). In addition to the biological functions, Obestatin also has many cellular impacts such as controlling the functions of cell differentiation and metabolism, elevating cell proliferation and survival as well as preventing inflammation and apoptosis in diverse cell types (Santos-Zas et al., 2015). These

actions are mediated by the integration of a multifaceted array of signalling pathways including the extracellular signal-controlled kinases 1 and 2 (ERK 1/2) mitogen-activated protein kinases (MAPKs) whose actions significantly impact cell proliferation (Camiña et al., 2007).

1.2 Significance of study

One of the main global risk factors for early death and the burden of disease is ethanol consumption (Sohi et al., 2021). Although ethanol misuse accounts for a small percentage of all deaths worldwide, its addictive use significantly increases the risk of neurological and memory impairments in all demographic groups (Mira et al., 2020). Heavy and prolonged ethanol consumption is linked to accelerated ageing of the brain and an increased chance of dementia, specifically Alzheimer's disease (AD) (León et al., 2022). The 2020 report from the Lancet Commission included excessive or harmful ethanol use in midlife as one of the major modifiable risk factors for dementia (Mewton et al., 2022). Consequently, the slow and progressive loss of neurons, particularly in the brain, which characterizes neurodegenerative disorders, results in incurable and devastating diseases (Baev et al., 2022). This highlights the importance of exploring potential treatments to counteract the harmful effects of ethanol.

Obestatin is a promising intervention with neuroprotective properties (Foroughi et al., 2019). With the growing evidence that Obestatin promotes cell proliferation and cell survival, it was thus necessary to further explore the capabilities of this peptide against ethanol-induced neurotoxicity on neuronal cells such as SH-SY5Y neuroblastoma cells and C8-D1A astrocytic cells. Studies on neurological disorders often involve models of glial cells and neurons (Peng et al., 2021b; Lopes et al., 2012; Mai et al., 2022) therefore, the cell lines (SH-SY5Y and C8-D1A cells) used in the present study were suitable. Subsequently, this study provided the first evidence of the neuroprotective potential of Obestatin against ethanol effects *in vitro*.

1.3 Aim

This *in vitro* study aimed to examine the neuroprotective potentials of Obestatin against ethanol-induced toxicity using the SH-SY5Y neuroblastoma cells and the C8-D1A astrocytes.

1.4 Study objectives

The specific objectives of this study are:

i. To determine the effects of Obestatin on the viability of SH-SY5Y and C8-D1A cells.
ii. To determine the concentration of ethanol that will induce neuronal cell death in the SH-SY5Y and C8-D1A cells using a cell viability assay.
iii. To determine the neuroprotective ability of Obestatin following ethanol-induced damage on the SH-SY5Y and C8-D1A cells by:
    a. measuring cell viability using the MTT and trypan blue assays.
    b. determining morphological changes in the cells using light microscopy.
    c. measuring the levels of ATP production in the cells.
    d. determining the levels of reactive oxygen species using the 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) stain.
    e. determining the presence of acidic vesicular organelles in the lysosomal compartment of the cells using an acridine orange stain.
    f. evaluating autophagy status in the cells using LC3BII and p62 biomarkers through Western blot method.
    g. determining morphological changes in the cell nuclei using a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) stain and further evaluating apoptosis with the cell death marker Cleaved PARP, using western blotting and caspase-3 gene expression utilizing quantitative real-time polymerase chain reaction (PCR).

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

The volatile and colourless liquid known as alcohol has a density of approximately 0.8 g/ml, making it less dense than water (Hendriks, 2020). Isopropyl alcohol, methyl alcohol (methanol) and ethyl alcohol (CH3CH2OH, EtOH, or ethanol) are the three distinct constituents of the alcohol family but it is illegal to consume the first two types of alcohol because they are poisonous (Guo and Ren, 2010). Ethanol (ethyl alcohol) is generated through the fermentation of yeast, which converts fruit and plant sugars into carbon dioxide and ethanol (Hendriks, 2020). It can subsequently be ingested in the form of an alcoholic beverage which mainly contains pure ethanol, water, and varying quantities of sugars, such as carbohydrates, but the levels of other nutrients like minerals, vitamins, and proteins are minimal (Eashwar, Umadevi and Gopalakrishnan, 2020; Lieber, 2003). Thus, alcoholic beverages have many calories but lack micronutrients (Lieber, 2003). Spirits (liquor) are the most widely consumed type of ethanolic drink, contributing to 44% of total ethanol consumption, while beer (34.3%) and wine (11.7%) rank second and third (Brenes et al., 2021). Consumption of these beverages has been a central aspect of many cultures for a very long time (Nwagu, Dibia and Odo, 2017). Many people, especially light-to-moderate drinkers who take one or two glasses of an alcoholic beverage per day often mistakenly assume that these beverages are part of their usual diet and that they could obtain a certain number of calories from them (Lieber, 2003). The consequence of this behaviour (i.e. long-term excessive ethanol consumption) is malnutrition with serious health problems (Butts et al., 2023).

The misuse of ethanol ranks as the fifth leading cause of early disability and death globally (Molina and Nelson, 2018). The World Health Organization reports that ethanol use contributes

to a mortality rate of 7.6% in men and 4% in women (Bete et al., 2023). Excessive ethanol use (defined as: > 4 daily beverages or 14 weekly beverages for men; > 3 daily beverages or > 7 weekly beverages for women) has been shown to have detrimental effects on an individual's health, increase the risk of traffic accidents, and change social behaviours, all of which have serious consequences for one's social, professional, and personal life (Finelli, Mottola and Agarwal, 2021).

Chronic over-indulgence in ethanol causes organs and tissues to progressively deteriorate (Erdozain et al., 2014). Many organs in the body systems (including the cardiovascular system, central and peripheral nervous systems, and gastrointestinal tract) are negatively impacted by ethanol (Argo et al., 2024). In the liver, which is responsible for metabolizing ethanol, cirrhosis/fibrosis, hepatitis, and steatosis, are the most typical liver lesions produced by excessive and persistent ethanol use (Osna, Donohue and Kharbanda, 2017). Additionally, increased cardiovascular risk is associated with chronic ethanol intake (Kawano, 2010). It is therefore not surprising that chronic ethanol users are prone to developing ischemic heart disease, cardiac arrhythmias, ischemic and haemorrhagic strokes, hypertensive disorders, and alcoholic cardiomyopathy (Rehm et al., 2016). Additionally, ethanol consumption alters the metabolism of fats, proteins and carbohydrates resulting in inadequate immune responses to infections, which hinders the host's ability to prevent haemorrhagic shock, increases corticosteroid release, and slows wound healing, all of which increase morbidity and mortality or lengthen the recovery period following trauma (Manzo-Avalos and Saavedra-Molina, 2010). The brain is also directly affected by ethanol (Oscar-Berman and Marinkovic, 2003). Overindulgence in ethanol consumption can raise the risk of ethanol-related brain damage and cognitive decline, which can lead to an increased chance of developing dementia (Nunes et al., 2019).

2.2 Structural and functional impacts of ethanol on the brain

Long-term and excessive ethanol consumption is linked to behavioural and physiological changes in the central nervous system (CNS) due to altered brain structures (Rosenbloom, Pfefferbaum and Sullivan, 1995; Bates, Bowden and Barry, 2002; Oscar-Berman and Marinkovic, 2003; Oscar-Berman and Marinković, 2007; Alfonso-Loeches and Guerri, 2011). Ethanol-induced structural changes primarily and directly affect the frontal, hippocampus, and diencephalic regions (Maharjan et al., 2022). Damage to the frontal lobe can negatively impact the orbitofrontal, ventromedial and dorsolateral frontal-subcortical circuits leading to diverse brain function deficits (Maharjan et al., 2022). Impairment to the orbitofrontal cortex may cause personality alterations with the traits of irritability and disinhibition while impairment to the dorsolateral prefrontal cortex may result in a decline in executive function (Metin et al., 2017). In addition, learning and memory formation are carried out by the hippocampus and the surrounding medial temporal lobe structures (Meda et al., 2018). These brain areas are also directly affected by ethanol which aligns with reports on animal studies that ethanol affects memory formation by interfering with hippocampal activity and neurogenesis (Klintsova et al., 2007; Taffe et al., 2010; Meda et al., 2018). These effects are possible due to the neurodegenerative impacts of ethanol on brain cells in these structures. This is evident by post-mortem analyses of the human brain and studies on animals that showed that chronic ethanol exposure results in neurodegeneration (Nixon, 2006). Likewise, ethanol affects motor skills (balance, posture, and gait) due to its damaging effects on the cerebellar cells and nuclei as well as the deterioration of the associated white matter across the brain (Fein and Greenstein, 2013). In all, the effect on the brain could be transient which often gets resolved by abstinence or it could be permanent resulting in mental or physical disabilities due to the structural damage caused by the prolonged use of ethanol (Sullivan and Pfefferbaum, 2019).

2.3 Ethanol use and prenatal exposure

The developing brain is susceptible to ethanol and exposure to ethanol during pregnancy can result in Foetal Alcohol Spectrum Disorders (FASD) (Luo, 2014). FASD is a group of neurobehavioral disorders caused by ethanol exposure during pregnancy (Mattson, Bernes and Doyle, 2019). Ethanol may also disrupt the mother's physiology, interfere with the placental physiology or directly damage Foetal brain tissues (Guerrini, Thomson and Gurling, 2007). Significant morphological abnormalities, such as enlargement of the ventricles and thinning of the corpus callosum, were noted in early case studies; however, radiological studies have shown that the effects of FASD on brain development vary widely. Reduced grey and white matter volume and increased cortical grey matter thickness and density have been consistently observed in the brain structure of FASD sufferers (Nutt et al., 2021). Consequently, FASD is linked to speech-language impairments, developmental disabilities, and behavioural and learning issues, all of which raise the need for special education (Popova et al., 2016).

Patterns of ethanol consumption (amount, frequency, length of time) and other factors are linked to the risk of FASD development (Popova et al., 2021). The prevalence of FASD seems to differ significantly between nations and regions (Popova et al., 2019). Worldwide, the prevalence of FASD between the youth and children was predicted to be 7.7 per 1000. The WHO European Region exhibited the most significant prevalence (19.8 per 1000), whereas the WHO Eastern Mediterranean Region exhibited the least (0.1 per 1000) (Lange et al., 2017). Worldwide, South Africa has the highest rate of prevalence of FASD (Louw et al., 2024). One factor contributing to South Africa's high FASD rate is the country's long-standing drinking culture, which was fuelled by the dop system, in which farmworkers were paid with ethanolic beverages. Furthermore, the absence of a thorough and multi-sectoral policy may also be contributing to the high prevalence of FASD (Adebiyi, Mukumbang and Beytell, 2019). The impairments caused by prenatal ethanol exposure impose significant social and economic costs

on society (Popova et al., 2016). FASD can lead to diminished living standards and substantial economic effects through decreasing productivity due to illness and death, as well as the rising burdens on healthcare, criminal justice system , residential care, and special education (Wynn et al., 2020).

2.4 Metabolism of ethanol in the brain

Ethanol can pass through the blood-brain barrier and subsequently be metabolized (Hernández, López-Sánchez and Rendón-Ramírez, 2016). Briefly, ethanol is metabolized by oxidizing to acetaldehyde, which is then further oxidized to acetate (Edenberg and Foroud, 2014) by different enzymes – aldehyde dehydrogenase-2, cytochrome P450 2E1 (CYP2E1), alcohol dehydrogenase (ADH), or catalase (Popova et al., 2024) (see Figure 2.1). When ethanol is converted to acetaldehyde by ADH, it also reduces NAD^+ to NADH. However, in the presence of hydrogen peroxide, catalase converts ethanol to acetaldehyde, while CYP2E1 needs oxygen and NADPH to convert ethanol to acetaldehyde, $NADP^+$ and acetate (Waddell, McKenna and Kristian, 2022). In an *in vitro* analysis, CYP2E1 was shown to be responsible for 20% of ethanol oxidation in the brain, while catalase was responsible for 60% (Zhong et al., 2012). Notably, chronic ethanol consumption causes the induction of CYP2E1, which could explain why excessive drinkers have higher elimination rates of ethanol (Hernández, López-Sánchez and Rendón-Ramírez, 2016). Nevertheless, according to recent research, the end product of ethanol metabolism, which is acetaldehyde, may contribute to ethanol neurotoxicity as it causes DNA damage and forms protein adducts, leading to cell damage and cytotoxicity (Nutt et al., 2021; Takeuchi, Suzuki and Sakai-Sakasai , 2024). It can fashion adducts with the ε-amino group of lysine residues and these covalent modifications have been proposed to impair protein function and cause cellular damage (Erdozain et al., 2014). Cortical neurons incubated with acetaldehyde protein adducts showed a dose-dependent increase in neuronal cell death (Takeuchi, Suzuki and Sakai-Sakasai, 2024). Additionally, acetaldehyde-induced protein

adduct formation was observed in large neurons of the frontal cortex and white matter in animal models of ethanol toxicity (Harper and Matsumoto, 2005). Furthermore, ethanol metabolism may lead to the formation of harmful protein adducts due to lipid peroxidation products, such as 4-hydroxy-2-nonenal (4-HNE), which can bind to tubulins and cause the loss of microtubular structures by making them more insoluble (Erdozain et al., 2014). Figure 2.1 shows a schematic of ethanol metabolism in the brain.

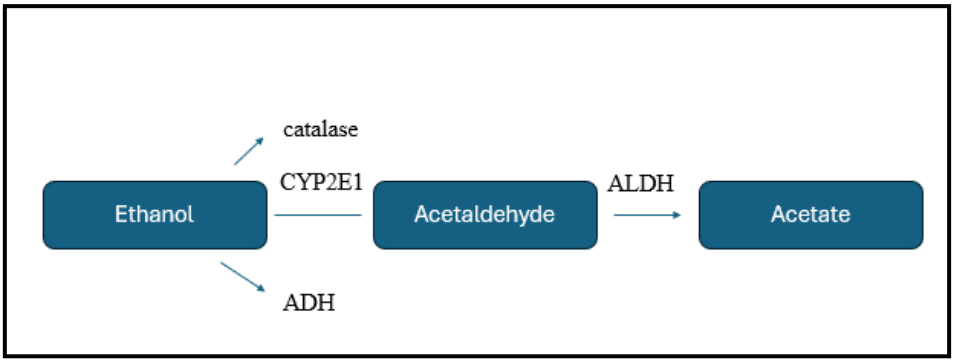

Figure 2.1: An illustration of the metabolism of ethanol in the brain adapted from Deitrich, Zimatkin and Pronko, 2006.

2.5 Mechanisms of ethanol-induced neurotoxicity

2.5.1 Generation of reactive oxygen species and oxidative stress

When ethanol is oxidized by the CYP2E1 enzyme, it results in the production of hydrogen peroxide, a type of reactive oxygen species (ROS) that acts in the form of a signalling molecule (Du, Shi and Zhao, 2019; Angelova, Esteras and Abramov, 2020). Prolonged and excessive ethanol intake may result in increased ROS generation as CYP2E1 expression in the brains of both humans and animals is notably increased by long-term ethanol intake (Du, Shi and Zhao, 2019). Excessive production of these molecules causes oxidative stress (Wilhelm et al., 2016). Oxidative stress arises when ROS production compromises antioxidant defences or surpasses the capacity of the antioxidant defence system to eradicate them (Husain et al., 2001).

Glutathione peroxidase (GPx), reduced glutathione (GSH), and superoxide dismutase (SOD) are endogenous antioxidants that are crucial for clearing ROS (Kamal et al., 2020). However, antioxidant enzyme activity is notably disrupted in the central nervous system (CNS) of animals exposed to ethanol (Hernández, López-Sánchez and Rendón-Ramírez, 2016). Ethanol has been shown to diminish the levels of GPx, SOD as well as catalase *in vivo* (Hamid et al., 2018). In addition, ethanol consumption, both acute and chronic, has been shown to diminish the levels of glutathione in the brain (Tsermpini, Plemenitaš-Ilješ and Dolžan, 2022). However, the ability of the CNS to combat oxidative stress is also reliant on exogenous antioxidants that the body consumes. The CNS's primary exogenous antioxidant is vitamin E, and after consuming ethanol, the amount of both vitamins C and E decreases while that of vitamin A rises (Hernández, López-Sánchez and Rendón-Ramírez, 2016). Consequently, the pathophysiology of neuronal degeneration is associated with the oxidative insults that emerge from the overproduction of ROS or a decline in the defence capacity of antioxidants (Jou et al., 2004). As such, Parkinson's disease (PD), Alzheimer's disease (AD), as well as other neurodegenerative disorders have all been linked to oxidative stress (Chen, Guo and Kong, 2012). In response to oxidative stress, lipids consisting of carbon-carbon double bonds in lipid bilayers of the membrane are oxidized by ROS resulting in lipid peroxidation (Mas-Bargues et al., 2021). Acrolein, 4-hydroxy-2-nonenal (HNE), and malondialdehyde (MDA) are among the aldehyde by-products that are produced because of lipid peroxidation (Singh et al., 2010). *In vivo*, it was shown that ethanol markedly elevated levels of lipid peroxide in the hippocampus and cerebral cortex (Tiwari and Chopra, 2013). Furthermore, studies conducted both *in vivo* and *in vitro* have shown that long-term ethanol administration significantly raises MDA levels (Montoliu et al., 1994; Navarro-Cruz et al., 2024). Overall, oxidative stress and lipid peroxidation byproducts like HNE or MDA have been connected in some research to reduced neuronal viability (Hernández, López-Sánchez and Rendón-Ramírez, 2016).

2.5.2 Mitochondrial dysfunction

The mitochondria serve a variety of vital purposes. Notwithstanding their many functions, the primary role of mitochondria is the synthesis of adenosine triphosphate (ATP) which is essential for cellular functions (Angelova and Abramov, 2018). ATP production occurs through oxidative phosphorylation, a process that utilizes the mitochondrial electron transport chain (ETC), where a series of electron transfer reactions take place (Nolfi-Donegan, Braganza, and Shiva, 2020). However, ethanol lowers respiratory complex activity and expression, as well as respiration in the ETC, ultimately reducing ATP content (Simon and Molina, 2022). *In vitro* studies have demonstrated that exposure to ethanol, varying in duration and concentration, is associated with decreased ATP levels (Liu et al., 2014; Mudyanselage et al., 2024).

ROS are produced as a byproduct of respiratory chain complex activity (León et al., 2022). They are mostly produced by the respiratory complexes in the mitochondria where superoxide is transformed into hydrogen peroxide by mitochondrial superoxide dismutase 2 (SOD2). Superoxide dismutase 2 (SOD2) in the mitochondria transforms superoxide, which is primarily produced by the respiratory complexes of the mitochondria, into hydrogen peroxide (Waddell, McKenna and Kristian, 2022). The rise in ROS is associated with damage to mitochondrial DNA (mtDNA) (Darbinian et al., 2023). The mtDNA encodes thirteen essential proteins involved in oxidative phosphorylation (Sylvester et al., 2004). Unlike nuclear DNA, mtDNA is more susceptible to ROS-induced damage due to its closeness to the ETC, a major site of free radical production, and its location near the inner mitochondrial membrane (Kowalczyk et al., 2021). Consequently, ethanol-induced oxidative stress leads to mtDNA depletion causing various mtDNA lesions such apyrimidinic/apurinic sites, oxidized DNA bases, and mtDNA strand breaks. Among these lesions, the most common and well-characterized mutation is the mtDNA common deletion (mtDNA-CD) (Demeilliers et al., 2017). mtDNA alterations play a key role in phenotypic ageing (Hroudová, Singh and Fišar, 2014). Furthermore, numerous

pathologies, including neurodegenerative diseases, are linked to mtDNA damage (Druzhyna, Wilson and LeDoux, 2008). Notably, AD and PD involve mtDNA mutations (Zhunina et al., 2020).

2.5.3 Induction of neuroinflammation

Following ethanol misuse, the blood-brain barrier may become compromised, which could facilitate the entry of immune cells and proinflammatory chemicals into the brain and exacerbate neuroinflammation (Rodríguez-González et al., 2023). Studies have demonstrated that ethanol excites astrocytes, microglia, and brain immune cells by causing the activation of NOD-like receptors (NLR) and Toll-like receptors (TLR) which set off signalling pathways leading to the production of chemokines and pro-inflammatory cytokines that result in neuroinflammation (Montesinos, Alfonso-Loeches and Guerri, 2016). An elevated expression of proinflammatory cytokines such as CCL2, Tumour necrosis factor alpha (TNF-α), and Interleukin-1 as well as activation of microglia are linked to ethanol-induced neuroinflammation (Lowe et al., 2020). Additionally, the glial cells have specific TLR, pattern recognition receptors, and NLR that can detect cellular damage signals which are collectively called damage-associated molecular patterns. Stimulation of TLR and NLR trigger signalling cascades linked to inflammation, primarily through the interferon response factor 3 (IRF3) and the nuclear factor kappa B (NF-κB) pathways (Anand et al., 2022). NF-κB regulates DNA transcription, cell survival, cytokine production, and neurodegeneration (Tian et al., 2021). In human astroglia cultures, ethanol elevated NF-κB activation and augmented immune response to lipopolysaccharide and cytokine expression (Nennig and Schank, 2017). Moreover, elevated TNF-α has been linked to NF-κB transcription activation. Another transcription factor that is vital for cell survival is cAMP responsive element binding protein (CREB) which protects neurons from excitotoxicity and promotes cell survival (González-Reimers, 2014). Decreased binding of DNA to CREB but increased binding of DNA to NF-κB are linked to ethanol (Wen,

Sakamoto and Miller, 2010). The treatment of brain slice cultures of the hippocampal entorhinal cortex with ethanol reduced CREB binding to DNA probes which model CREB-responsive gene promoter DNA and increased NF-κB binding to DNA probes that model gene promoter regions (Crews et al., 2015). Since CREB is involved in promoting cell proliferation and survival, a reduction in CREB binding would consequently result in a reduction of both cell survival and cell proliferation (Wen, Sakamoto and Miller, 2010). Thus, ethanol reduces trophic signals and elevates proinflammatory signals thereby inducing apoptosis (Zou and Crews, 2006; Antón et al., 2016).

2.5.4 Excitotoxicity

Chronic exposure to ethanol impacts numerous neurotransmitter receptors and their modulators in intricate and long-lasting ways (Chandrasekar, 2013). By changing the synthesis, release, and signalling of neurotransmitters such as glutamate, dopamine, and GABA, ethanol disrupts neuronal communication. Research conducted on human alcoholics has shown that chronic ethanol consumption alters dopamine signalling, which in turn alters the prefrontal cortex and impairs executive function (Tapia-Rojas et al., 2018). In addition, glycine receptors, serotonin receptors, non-N-methyl-D-aspartate (NMDA) glutamate receptors and G protein-coupled inwardly rectifying K^+ channels are among the other receptor and channel types that are impacted by ethanol (Han et al., 2005). Glutamatergic ionotropic receptors are among the ligand-gated ion channels that are impacted by long-term ethanol consumption (Chandrasekar, 2013). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, NMDA, and delta families of receptors are subgroups of glutamate receptors (Jin et al., 2014). In the CNS, ligand-gated ion channels called NMDA-type glutamate receptors mediate a significant amount of excitatory neurotransmission. They play a vital role in normal brain functions, such as synaptic plasticity and neuronal development, and are extensively distributed throughout all developmental stages (Hansen et al., 2017). Ethanol suppresses glutamate

receptor ion channel function by lowering peak-current amplitude and speeding up current desensitization, which prevents multiple types of neuronal plasticity (Jin et al., 2014). The first study showing that ethanol concentrations as low as about 0.03% directly inhibited ion flow through the NMDA receptor in rat neurons *in vitro* was published in 1989 (Gonzales and Jaworski, 1997). Research has also shown that long-term exposure to ethanol causes a marked reduction in the expression and function of AMPA receptors as well as a rearrangement of dendritic microtubules (Wirkner et al., 2000; Möykkynen, Korpi and Lovinger, 2003; Möykkynen and Korpi, 2012; Gerace et al., 2023). Notably, ethanol inhibits the Kainate receptors in some parts of the brain, which may have an impact on how neural circuits form during brain development as seen in FASD (Läck et al., 2008; Santofimia-Castaño, Salido and Gonzalez, 2011; Chandrasekar, 2013). This suggests that neuronal circuits and mechanisms may be incorrectly formed, leading to mental retardation and this may be relevant to the changes seen in newborns with FASD (Gerace et al., 2023).

2.5.5 Induction of Apoptosis

Apoptosis is a complex process that regulates cell survival and cell death. It works in response to pathogenic threats and during crucial phases like tissue turnover, embryogenesis, and metamorphosis (Mustafa et al., 2024). Nevertheless, neurodegenerative diseases, various forms of cancer, autoimmune disorders, and ischemic damage are among the human conditions that are influenced by inadequate apoptosis (Elmore, 2007). Consequently, neuronal apoptosis brought on by acute ethanol administration involves the extrinsic pathway, which is facilitated by the Fas death receptor and caspase 8 activation as well as the intrinsic pathway (Du, Shi and Zhao, 2019).

2.5.5.1 Intrinsic pathway

Oxidative stress, ischemia, and DNA damage are among the endogenous and exogenous stimuli that activate the intrinsic mitochondrial pathway (Loreto et al., 2014). Excessive exposure to ethanol may induce oxidative stress (particularly in the brain) by increasing ROS, such as hydrogen peroxide, hydroxyl and superoxide radical anions, and/or inhibiting antioxidant defences that typically inactivate ROS including superoxide dismutase, glutathione and glutathione peroxidase (Ramezani et al., 2012). Consequently, oxidative stress sets off the depolarization of the mitochondrial membrane which results in the release of cytochrome c thereby activating caspases and causing apoptosis (Luo, 2010). Cultured neurons of a Foetal rat demonstrated how mitochondrially mediated apoptosis was caused by ethanol-induced oxidative stress (Ramachandran et al., 2003). On the other hand, constituents of the Bcl-2 family, Bax and Bak, are essential modulators of the intrinsic apoptotic pathway and become activated in response to apoptotic stimuli and oligomerize at the outer membrane of the mitochondria to facilitate its permeabilization, which is thought to be a crucial stage in apoptosis (Peña-Blanco and García-Sáez, 2017). However, the administration of ethanol to mice exhibited a noteworthy increase of approximately 35% in the ratio of Bax/Bcl-2 as well as an increase of 5-fold in the ratio of cytochrome c between the cytosol and mitochondria (Karadayian, Czerniczyniec and Lores-Arnaiz, 2024). Thus, neuroapoptosis induced by ethanol seems to be an intrinsic pathway with the release of cytochrome c as well as mitochondrial membrane disruption induced by Bax as early events that set off the activation of caspase-3 (Young et al., 2003).

2.5.5.2 Extrinsic pathway

There are certain members of the TNF receptor superfamily, such as Fas (Apo-1; CD95), TRAIL receptors (DR4 and DR5), as well as Tumour necrosis factor receptor type 1 (TNFR1)

which can bind to ligands and activate the extrinsic pathway (Kalkavan and Green, 2017). Prolonged treatment of ethanol and high concentrations of hypoxemic agents have resulted in increased expression of the TNF-α cytokine, which not only triggers inflammation but also the extrinsic pathway of apoptosis (Stoica et al., 2023). In addition, other members of the TNF receptor superfamily such as Fas and DR3, communicate via kinases that trigger the transcription of NF-κB and the induction of many genes including those of the immune that are linked to neurodegenerative illnesses and cell death (Liu, Vetreno and Crews, 2020). Furthermore, if the extrinsic pathway facilitates apoptosis induced by ethanol, then the activation of caspase-8 should be seen concurrently (Young et al., 2003).

2.6 Epigenetic changes to DNA

Numerous environmental factors can influence gene expression, which is the process by which genetic information is used to produce useful products like proteins. The complex interaction between the environment and gene expression is mediated in large part by epigenetic mechanisms, including histone modification, DNA methylation, and microRNAs (Heidari et al., 2024). The part that epigenetic processes play in how ethanol affects the CNS has not received much attention until recently. However, there have been a lot more studies in the last several years that have suggested that ethanol-related molecular and behavioural changes may be related to epigenetics (Ponomarev, 2013). Even a single dose of ethanol administered to rodents during pregnancy or infancy has been found to cause profound alterations in the general epigenetic state of DNA as well as in gene expression patterns in populations of brain cells that last into later stages of development (Kim et al., 2022). Ethanol has been shown to inhibit normal DNA methylation programming of multiple genes and delay cell differentiation according to studies on neural stem cells (Zhou et al., 2011; Amiri, Davie and Rastegar, 2019). This mechanism may be crucial in the occurrence of Foetal Alcohol Syndrome (FAS) (Palmisano and Pandey, 2017). Furthermore, treatment with ethanol in P7 mice led to

decreased global DNA methylation in the hippocampus and neocortical tissues in an FASD animal model (third-trimester equivalent pregnancy) and decreased memory and learning in adult animals (Basavarajappa and Subbanna, 2016). A study on adult brains exposed to acute and chronic ethanol showed that these exposures caused opposing changes in epigenetic mechanisms. Chronic ethanol exposure leads to DNA hypermethylation, whereas acute exposure inhibits the activity of DNA methyltransferase, increases histone acetylation and reduces histone methylation (Fanfarillo et al., 2024).

On the other hand, a class of non-coding RNAs known as microRNAs (miRNAs) is crucial for controlling the expression of certain genes (O'Brien et al., 2018). Ethanol has recently been shown to change the expression of certain miRNAs in infants (Mahnke et al., 2021) and animal models (Wang et al., 2009; Mandal et al., 2018; Gerace et al., 2023). Ethanol reduced brain-specific miRNAs that are anti-inflammatory such as mir-21-5p and mir-146a-5p in female mice while simultaneously increasing the expression levels of genes that are pro inflammatory, suggesting that miRNAs may be potential biomarkers of ethanol-induced neuroinflammation (Anand et al., 2022).

2.7 Current treatment of ethanol-induced neurotoxicity

Utilizing antioxidants to reduce oxidative stress and ethanol neurotoxicity seemed logical because oxidative stress is thought to be an acknowledged mechanism behind ethanol neurotoxicity. *In vivo* and *in vitro* models have both demonstrated some success with this strategy (Chen and Luo, 2010). The neurotoxicity induced by ethanol is reduced by an antioxidant called α-tocopherol (Mitchell, Paiva and Heaton, 1999). It has been demonstrated that α-tocopherol, also known as vitamin E, acts as an antioxidant *in vivo* and protects cell membranes from hydroxyl damage of radicals as well as peroxidative damage (Mitchell, Paiva and Heaton, 1999). Nevertheless, some research indicates that ethanol neurotoxicity may not be primarily caused by oxidative stress, and antioxidants are ineffective at reducing the harm

that ethanol causes to the CNS (Chen and Luo, 2010). Although glutamatergic pathways have long been linked to ethanol-induced neurodegeneration and cognitive decline, neither calcium channel blockers nor N-methyl-D-aspartate (NMDA) receptor antagonists can effectively counteract the effects of ethanol on neuronal damage (Cippitelli et al., 2010). Another intervention in the form of curcumin has been shown to reverse the pro-inflammatory response caused by ethanol as well as improve cognitive deficits (Motaghinejad et al., 2017; Cantacorps, Montagud-Romero and Valverde, 2020; Yi et al., 2020). Curcumin has been shown to reduce ethanol-induced inflammation in rodents by inhibiting nuclear factor kappa B (NF-κB) (Cantacorps, Montagud-Romero and Valverde, 2020). However, one of its limitations is that its quick metabolism is one of the primary reasons for its low bioavailability (Peng et al., 2021a). It is thus necessary to investigate other interventions against neurotoxicity induced by ethanol. An intervention that was shown to have neuroprotective effects is Obestatin (Anand et al., 2022; Foroughi et al., 2019). More specifically, preproghrelin-derived peptide Obestatin appears to have antiapoptotic and antioxidant properties (Akki, Raghay and Errami, 2021; Erşahin et al., 2013). Furthermore, the ability of Obestatin to suppress inflammation may be one of its potential neuroprotective capabilities (Mirarab et al., 2019).

2.8 Obestatin

Obestatin is a peptide produced in the cerebral cortex, intestine, pancreas, spleen, and stomach (Memi, Öztürk and Palabiyik, 2023). It contains 23 amino acids obtained from the precursor, preproghrelin (Li et al., 2011). In addition, this peptide exhibits a C-terminal post-translational modification of the amide group, which was first proposed to be necessary for Obestatin binding and then shown to be necessary to stabilize the peptide into its normal conformation (Cowan et al., 2016). In addition to being transcribed from the preproghrelin gene, Obestatin requires C-terminal amidation for its biological activity (Green, Irwin, and Flatt, 2007).

2..8.1 Biological Actions of Obestatin

Obestatin has been reported to have various biological actions which are summarised in Figure 2.2 below. These actions could impact various systems including the gastro-intestinal, cardiovascular and nervous systems.

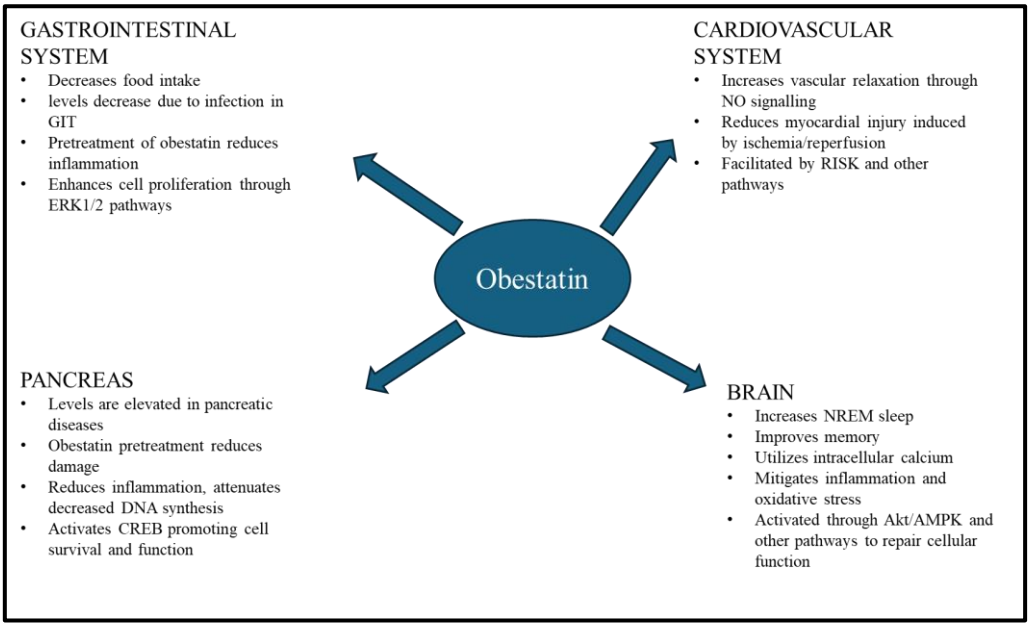

Figure 2.2: An illustration of the biological actions of Obestatin adapted from Green and Grieve, 2018.

2.8.1.1 Gastrointestinal Effects

Obestatin appears in the myenteric plexus, indicating that it plays a substantial part in gastrointestinal movement (Xing et al., 2017). When Obestatin is administered intravenously to conscious rats under a fed state, it inhibits motor activity in the duodenum and antrum, but not when it is administered to conscious rats while they are fasting (Ataka et al., 2008). Although peripheral mechanisms including expanding stomach and intestinal walls, detection of blood glucose levels as well as the breakdown products of specific compounds in the chyme, are utilized to regulate food consumption, there are also central mechanisms used to achieve regulation (Graf et al., 2020). For instance, while Obestatin contributes to gastrointestinal

movement, it does so in the duodenum via the afferent nerve of the vagus as well as the brain's receptors, corticotropin-releasing factor type 1 and 2 (CRFR1/2), which are unrelated to antrum activity (Xing et al., 2017). Therefore, some, but not all, of these impacts of Obestatin may be mediated by vagal afferent pathways (Fujimiya et al., 2008).

Apart from its suggested physiological effects, Obestatin seems to have some advantages in gastrointestinal disorders (Cowan et al., 2016). Gastrointestinal disorders, including persistent gastric ulcers, are often associated with Helicobacter pylori infection, which is closely linked to Obestatin levels (Xing et al., 2017). Thus, this infection may play a part in controlling hunger and fullness, which could impact height and body weight (Romo-González et al., 2017). Moreso, this finding implies that in models of peptic ulcers, appropriate control of Obestatin levels can hasten the process of pathology correction (Graf et al., 2020). Additionally, Obestatin has been shown to have a protective effect in the colon in one experimental study by Pamukcu et al., whereby Obestatin administration both prior to and during the onset of colitis caused by dextran sodium sulfate, lessens the intensity of this inflammation (Matuszyk et al., 2016). Furthermore, another study has demonstrated that Obestatin has a therapeutic effect on the progression of chronic stomach ulcers, the consequence of which was accelerated healing of gastric ulcers by improving mucosal blood flow, reducing the local inflammatory process, and enhancing mucosal cell proliferation (Dembinski et al., 2011). Obestatin has been shown to stimulate the proliferation of cells in the gastric cancer cell line, KATO-III. According to the results, this peptide caused the phosphorylation of extracellular signal-regulated kinases1/2 (ERK1/2) and mitogen-activated kinase kinase to proliferate cells (Pazos et al., 2007). It was further discovered that in KATO III cells, the activation of the ERK1/2 and Akt signalling pathways occurs simultaneously, contributing to the intricacy and clear cell type specificity of EGFR transactivation pathways. More specifically, Gi/o proteins control the ERK1/2 pathway,

while EGFR transactivation serves as the bridge connecting the Obestatin receptor and the Akt signalling pathway (Álvarez et al., 2009).

2.8.1.2 Pancreatic Effects

Obestatin has a noticeable impact on pancreatic diseases, particularly when it comes to early diagnosis and evaluation. The level of Obestatin in the blood is significantly higher in people with mild or severe pancreatitis (Xing et al., 2017). However, it has been shown that Obestatin pretreatment lessens the severity of cerulein-induced acute pancreatitis and subsequent pancreatic damage (Ceranowicz et al., 2009). Obestatin's protective properties appear to be associated with the suppression of the pancreatic inflammatory process, which is evidenced by the decreased release of interleukin-1β (IL-1β), the attenuation of the organ's decreased DNA synthesis as well as the suppression of leukocyte infiltration of gland tissue (Bukowczan et al., 2015b). Furthermore, by activating the cAMP response element binding (CREB) protein and upregulating the expression of pancreatic and duodenal homeobox 1 (Pdx1), which is crucial for β-cell differentiation, insulin synthesis and glucose sensing, Obestatin has been demonstrated to enhance beta cell function and survival (Villarreal et al., 2022).

2.8.1.3 Cardiovascular Effects

Obestatin may be a significant biomarker in determining the course as well as the severity of chronic inflammatory illnesses, therefore its function in inflammation and cardiovascular disease is another topic that merits more research (Villarreal et al., 2022). While ischemic heart disease and plasma Obestatin levels are unrelated, patients with chronic failure of the heart have higher plasma Obestatin levels (Bora, Prasad and Khatib, 2023). However, in the isolated heart of the rat, it has been shown that Obestatin lessens myocardial damage brought on by ischemia/reperfusion (Alloatti et al., 2010). The responses induced by Obestatin is most likely facilitated by the receptors of Obestatin existing on cardiac cells as well as through stimulation

of signalling pathways involved in the reperfusion injury salvage kinases (RISK), including extracellular signal-regulated kinases ERK 1/2, protein kinase C (PKC), and phosphoinositide 3-kinase (PI3K) (Aragno et al., 2012). Moreover, Obestatin administration led to an upregulation of Nrf2 and Mhrt in failing tissue of the myocardium and markedly improved the dysfunction of left ventricular contractility caused by doxorubicin (Li et al., 2016). Furthermore, Obestatin has been demonstrated to have vascular advantages in experimental models (Schinzari et al., 2017). Through triggering the signalling of endothelium-reliant nitric oxide, researchers have demonstrated that Obestatin impacts vascular relaxation in the isolated rat aorta and mesenteric artery (Xing et al., 2017).

2.8.1.4 Neuroprotective Actions

Obestatin, which is a result of ghrelin, was shown to suppress thirst in the brain (Samson et al., 2008) in addition to reducing anxiety, enhancing memory as well increasing non-rapid eye movement (NREM) sleep in the CNS (Stempniewicz, Ceranowicz and Warzecha, 2019). Consequently, normal behaviour and performance, both intellectually and physically, depend on NREM (de Andrés, Garzón and Reinoso-Suárez, 2011). As such, administration of Obestatin intravenously at dark onset significantly increased the duration of NREM sleep in rats during the first hour following administration (Li et al., 2011). Therefore, increased NREM sleep is associated with better hippocampus-memory consolidation, evidenced by ICV Obestatin treatment that encourages sleep onset during the dark period (Villarreal et al., 2022). Overall, the expression of Obestatin in the brain may mediate some of its suggested actions by stimulating the release of intracellular calcium and promoting calcium signalling (Cowan et al., 2016).

The endogenous and novel ligand, Obestatin, may have neuroprotective effects, according to studies (Foroughi et al., 2019). For instance, Obestatin was shown to decrease the frequency

and length of generalized seizures, resulting in a shorter latency to onset, and decreased the score of the average seizure (Koyuncuoğlu et al., 2017). In addition, by suppressing pro-inflammatory mediators and increasing endogenous antioxidants, Obestatin preserves a balance in antioxidant-oxidant status, thereby exerting strong anti-inflammatory and neuroprotective impacts against oxidative damage induced by subarachnoidal haemorrhage (Erşahin et al., 2013). Moreso, by lowering autophagic and apoptotic actions, Obestatin was able to mitigate neurotoxicity induced by methamphetamine (Foroughi et al., 2019). Furthermore, by suppressing tau hyperphosphorylation and the activity of GSK-3β, two major indicators of neuronal death in Alzheimer's disease, Obestatin also mitigated the negative effects of Aβ1-42 (Gargantini et al., 2016). Therefore, in order to fix damage and return cells to their regular functions, compensatory processes are triggered (Ronnett et al., 2009). As a result, the simultaneous activation of AMPK and Akt by Obestatin through the GPR39 receptor suggests that this system plays a part in maintaining the equilibrium between anabolic and catabolic signalling to maintain cellular viability and function (Sánchez-Temprano et al., 2021). Other processes involved in these neuroprotective actions include phosphorylation of Akt/PI3K/and ERK1/2, CREB//PKA/cAMP/Gαs signalling, and the PI3K targets mTOR and GSK-3β/β-catenin (Gargantini et al., 2016). Overall, by influencing several molecular and enzymatic processes as well as diminishing autophagy and apoptosis, Obestatin inhibits neuronal death and is accordingly neuroprotective (Foroughi et al., 2019).

CHAPTER 3

MATERIALS AND METHODS

3.1 Outline of the design of the experiment

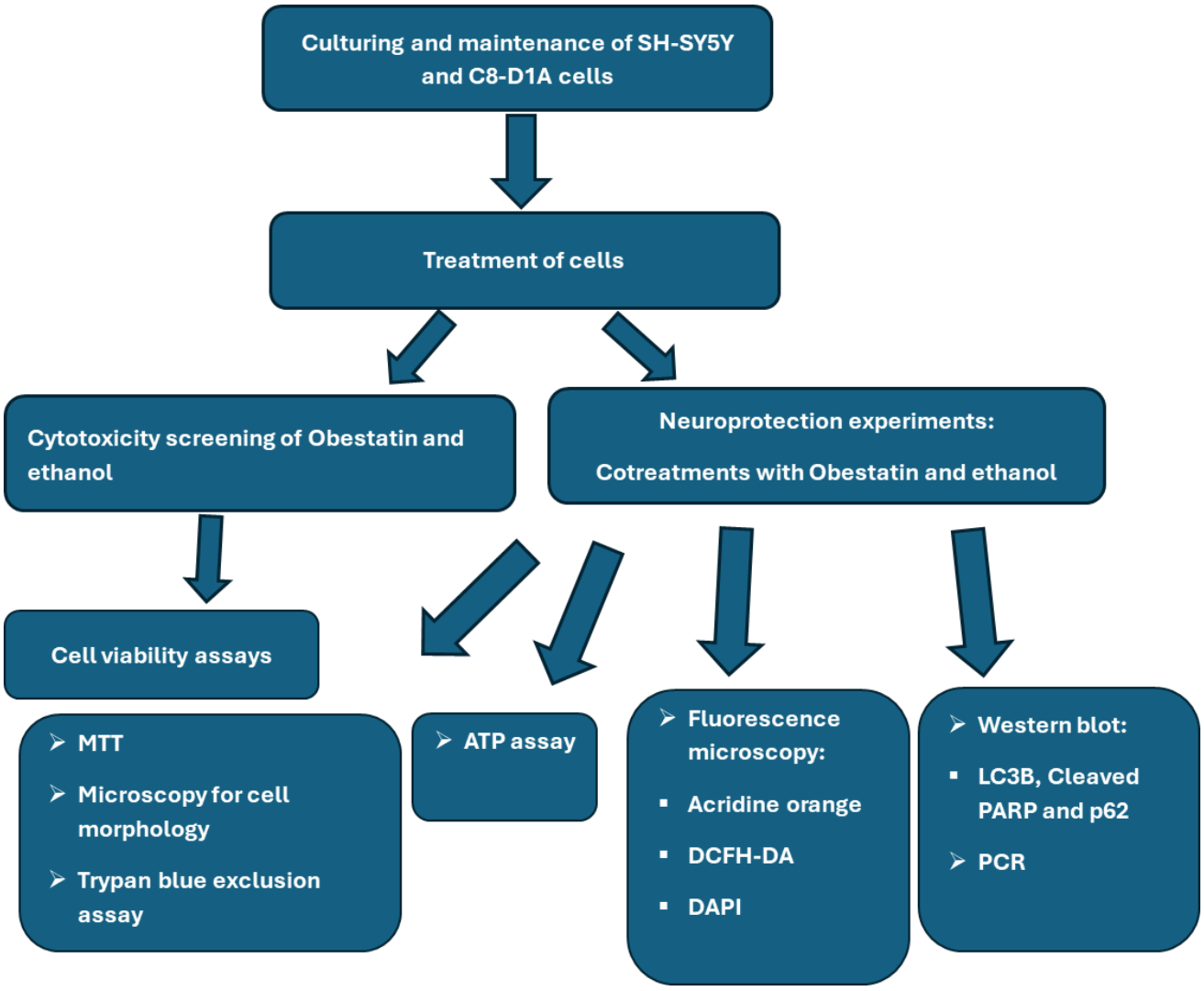

Figure 3.1 Illustration outlining the design of the experiment.

3.2 Cell culture and maintenance

Two cell lines namely, SH-SY5Y and C8-D1A, were used in the present study. The SH-SY5Y neuroblastoma cell line, which is mostly immortalized and derived from the SK-N-SH cell subline (created from a biopsy of the bone marrow of a female, 4-year-old child), is utilized in *in vitro* models of neurological disorders such as amyotrophic lateral sclerosis, Alzheimer's disease (AD), ischemia and neurotoxicity (Hoffmann et al., 2023). However, the C8-D1A cell line was created from the cerebellum of a mouse that is GFAP-positive and a type 1 astrocytic clone (Huppmann et al., 2008). The mouse C8-D1A astrocyte cell line was a kind donation by Dr. Brian Filipisi from the University of Pretoria while the human SH-SY5Y neuroblastoma cell line was a kind donation by Prof. Jonathan Blackburn from the University of Cape Town. For this study, Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Life Technologies Limited, UK) supplemented with 0.1% penicillin/streptomycin (Life Technologies Corporation, Paisley, UK) and 10% FBS (Life Technologies Corporation, Paisley, UK) was used for maintaining both cell lines.

3.2.1 Thawing cells

The SH-SY5Y and C8-D1A cell lines were kept in cryovials at -80 °C until use. For the thawing, each cell line was thawed at room temperature and subsequently suspended in 5 ml supplemented DMEM in a 15 ml centrifuge tube. The cells were then centrifuged at 2500 rpm for 5 min following which the supernatant was removed. The cells were resuspended into a 60-mm petri dish (Labocare, UK) with 5 ml supplemented DMEM and then incubated in a CO_2 water jacketed incubator with 5% CO_2 at 37 °C until the desired confluence was attained (70 - 80%). The cell growth was monitored with an inverted phase contrast microscope in order to detect abnormality in cell morphology and/or overly confluent.

3.2.2 Subculture

Once the cells reached the 70 - 80% confluence in the petri dish, the growth media was removed and then washed with 1 ml 1× Phosphate buffered saline (PBS, pH 7.4, Lonza Verviers, Belgium). After removing the PBS, 500 µl trypsin (Life Technologies Limited, UK) was added to the dish and further incubated for 2 min to enable the detachment of the cell monolayer. The cells were then observed under the microscope to check for cell detachment. The detached cells were suspended in 3 ml supplemented DMEM to neutralize the trypsin and then centrifuged at 2500 rpm for 5 min. After removing the supernatant and resuspending the pellet, the cells were cultured in a 60-mm petri dish with the medium changed every 72 hours until the start of the experiment.

3.2.3 Cryopreservation

A stock of both cell lines was made by freezing down confluent cells. This was done according to the subculture process described in 3.3.2. Only 900 µl of the cell suspension was added to a cryovial labelled with its cell line and passage number. An additional 100 µl of Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, France) was added to each cryovial before gradually freezing on ice and then stored at -80 °C for future use.

3.3 Treatments

3.3.1 Cell proliferation and cytotoxicity screening

Obestatin

A 0.2 mg/ml stock solution of Obestatin (Sigma Aldrich, St Louis, USA) was prepared in sterile water and aliquots containing 10 µl Obestatin were stored in Eppendorf tubes in the -20 °C freezer until use. A working concentration was made from which the final concentrations were obtained: 10, 20, 40, 60, 80 and 100 ng/ml. This concentration range was used in accordance

with a previous report by Granata et al. (2008), which showed that cell survival and cell proliferation were most effective at a lower concentration range between 1 to 10 nmol/l as opposed to a higher concentration range between 100 to 500 nmol/l. The SH-SY5Y cells were seeded at 10000 cells per well into 96-well plates and incubated for 24 hours to allow for cell adherence. After the 24-hour incubation, the medium was removed from all the wells and replaced with different concentrations of Obestatin described above before incubating for 24 hours. The C8-D1A cells were seeded at 5000 cells per well and treated with different concentrations of Obestatin similar to the SH-SY5Y cells.

Ethanol

For the neurotoxicity experiment, a 5000 mM stock solution of ethanol was utilized and diluted with a supplemented DMEM to obtain final concentrations ranging from 125, 250, 500, 750 and 1000 mM. Both cell lines were plated with the same seeding density as stated previously in 3.3.1 and incubated for 24 hours. After the incubation period, the medium was removed and replaced with different concentrations of ethanol. The cells were then incubated for 24 hours. The concentrations that reduced cell viability to approximately 50% were selected for the neuroprotection experiments.

3.3.2 Neuroprotection

Once the concentrations of ethanol and Obestatin were optimized, the SH-SY5Y and C8-D1A cells were plated into 96 well plates and incubated for 24 hours. Subsequently, the cells were treated with different concentrations of Obestatin (10, 20, 40, 60, 80,100 ng/ml) and treated with the obtained optimized concentration (i.e. 500 mM; see results section 4.3 A) of ethanol for the SH-SY5Y cell line or the optimized concentration (i.e. 750 mM; see results section 4.3 B) of ethanol for the C8-D1A cell line. Cells not treated with either ethanol or Obestatin (i.e.

supplemented DMEM only) served as the control cells. In addition, either cell line was also treated with their optimized ethanol concentration alone.

3.3.3 Measuring viability of cells (MTT)

The colorimetric method that measures the metabolic activity of cells is known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Bahuguna et al., 2017). NADPH dependant cellular oxidoreductase enzymes mainly found in the mitochondria of viable cells reduce the tetrazolium dye, MTT, into an insoluble formazan derivative that can be solubilized and then the colour intensity is measured as an indicator of cell viability (Winter et al., 2017). Figure 3.2 shows a schematic of the procedure of MTT.

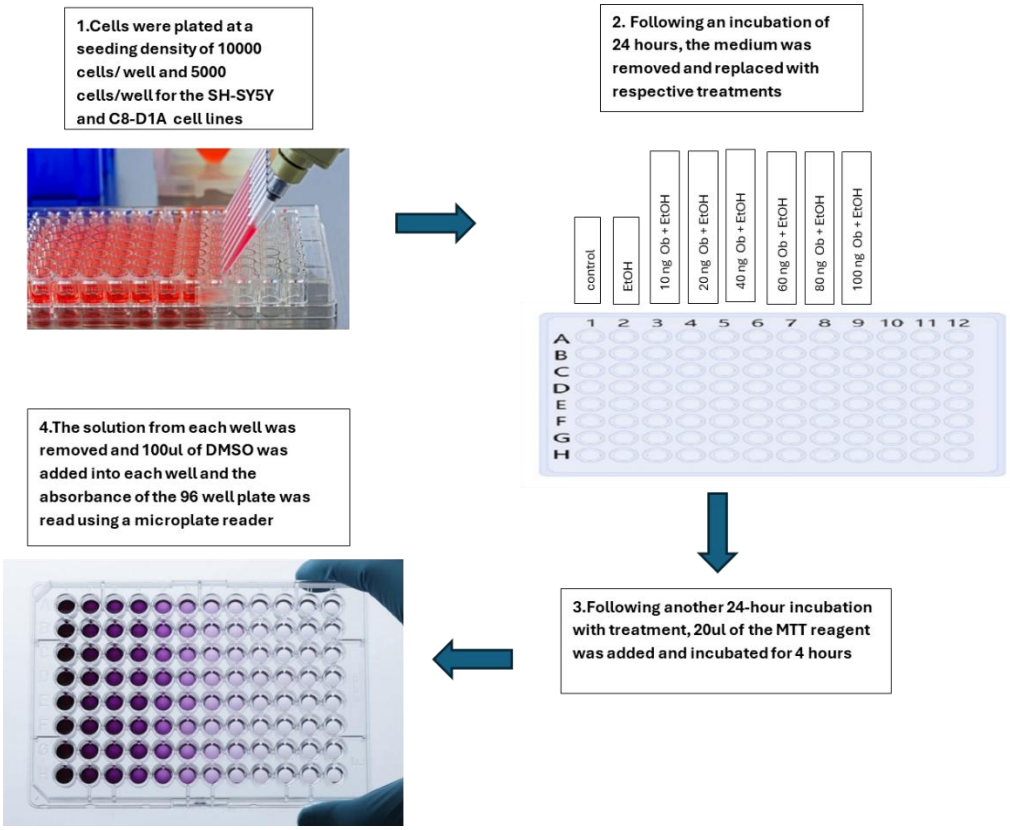

Figure 3.2 Illustration demonstrating the procedure of measuring cell viability of the neuroprotective assay utilizing the MTT reagent.

In this study, a working solution of MTT (Sigma Aldrich, St Louis, USA) was made (5 mg/ml in PBS). Subsequently, 10 µl of the working solution of MTT was added to each well containing a volume of 100 µl supplemented DMEM that was treated with either Obestatin or ethanol for the cytotoxicity testing. However, 20 µl of MTT working solution was added to the neuroprotection treated wells that contained a volume of 200 µl of supplemented DMEM with ethanol and or Obestatin. Following the administration of the MTT, the plates were incubated for 4 hours in a water-jacketed incubator. After the incubation period ended, the growth media was removed and replaced with 100 µl of DMSO to solubilize the formed formazan crystals. The intensity of the dissolved crystals was quantified using the microplate reader (Anthos 2010, Austria) at a wavelength of 570 nm. The cell viability was quantified as a percentage and calculated using the absorbance of the treatment relative to the absorbance of the control and multiplied by 100. Based on the results of the neuroprotection assay, the 10 and 100 ng Obestatin were chosen for subsequent assays.

3.3.4 Trypan blue exclusion assay

The number of viable cells in a cell suspension can be ascertained using the dye exclusion test. This test is predicated on the principle that dead cells, lacking intact cell membranes, cannot exclude certain dyes like trypan blue, propidium or eosin, whereas living cells maintain intact membranes that prevent dye penetration (Strober, 2015). Consequently, for the trypan blue assay, cells were seeded into four petri dishes (35-mm) at a density of 2.70×10^5 cells per dish for the SH-SY5Y cell line and 1.38×10^5 cells per dish for the C8-D1A cell line. After a 24-hour incubation with the cells reaching a 70% confluence, cells were treated with optimized concentrations of Obestatin and/or ethanol. Each dish represented a treatment: control, optimized concentration of ethanol (500 or 750 mM), 10 ng Obestatin with 500 or 750 mM ethanol, and 100 ng Obestatin with 500 or 750 mM ethanol. Post-treatment, the growth media

was aspirated, and the cells were washed with PBS before being harvested and suspended in DMEM. Subsequently, 10 µl of 0.4% trypan blue (Life Technologies Corporation, Paisley, UK) was added to an Eppendorf tube containing 10 µl of suspended cells. This mixture was then added to a countess cartridge and the cells were counted automatically using a cell counter (Countess). The number of live cells was recorded.

3.3.5 Changes in cell morphology

To visualize the morphology of the cells after respective treatments, the cells were plated onto coverslips in 4 petri dishes (35-mm) at a density of 2.70 x 10^5 cells per dish for the SH-SY5Y cell line and 1.38 x 10^5 cells per dish for the C8-D1A cell line. After a 24-hour incubation with the cells reaching a 70% confluence, cells were treated with the optimized concentrations of Obestatin and/or ethanol. Following the treatment incubation period, the cells were fixed with 4% paraformaldehyde for 15 min, subsequently washed twice with PBS and then viewed with the confocal microscope (MICA) using the brightfield function at the 10× objective.

3.3.6 Adenosine triphosphate (ATP) assay

Both acute and long-term ethanol exposure have been linked to mitochondrial energy metabolism dysfunction, which not only increases ROS production but lowers ATP synthesis, making cells more vulnerable to oxidative-induced apoptosis and/or death (Liu et al., 2014). A mitochondrial Tox Glo ATP assay kit (Promega, Madison, USA) was used to determine the changes in ATP level according to the manufacturer's instructions. Cells were plated in a white 96-well plate at a seeding density of 10000 cells per well for the SH-SY5Y cells and 5000 cells per well for the C8-D1A cells. Subsequently, each cell line was treated after 24 hours using the optimized concentrations of ethanol and/or 10 or 100 ng/ml Obestatin. Each well contained a volume of 200 µl growth medium. After the treatment duration, 150 µl of growth media was substituted with 50 µl of ATP detection reagent comprising luciferin as a substrate, detergent

to lyse the cells, ATPase inhibitors to stabilize the ATP discharged from the lysed cells, and the stable form of luciferase to propel the reaction that produces light photons (Riss, 2013). The intensity of the luminescence was measured utilizing the Promega GloMax® explorer multimode microplate reader at a wavelength of 520 nm.

3.3.7 Measurement of intracellular reactive oxygen species (ROS) production

The brain experiences oxidative stress because of ROS produced by exposure to ethanol (Chen et al., 2012). As such, a 2'7'-dichlorohydrofluorescin diacetate (DCFH-DA) cell-permeant dye was used to detect the immunofluorescence of the reactive oxygen species. DCFH-DA is absorbed by cells, where acetyl groups are broken off by cellular esterase to produce DCFH. The molecule is changed from DCFH to DCF by ROS oxidation, and DCF emits green fluorescence at 530 nm emission and 485 nm excitation wavelengths (Kim and Xue, 2020). Figure 3.3 shows a schematic of the principle of DCFH-DA.

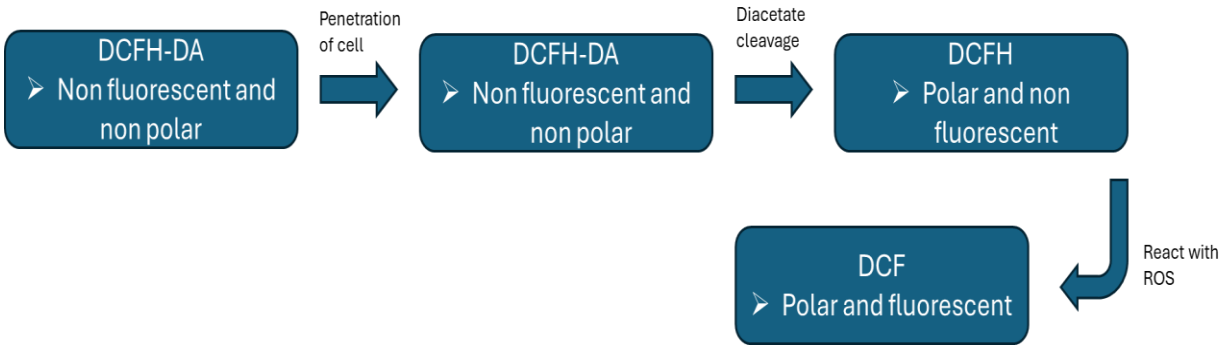

Figure 3.3 A flowchart showing the principle of measuring ROS levels using DCFH-DA adapted from Ezequiel et al., 2022.

A 20 µM stock solution of DCFH-DA was made in DMSO and 10 µl aliquots were made into Eppendorf tubes and stored in the freezer until ready to use. Cells were seeded onto coverslips in 35-mm petri dishes at a density of 2.70 x 10^5 cells per dish for the SH-SY5Y cell line and 1.38 x 10^5 cells per dish for the C8-D1A cell line. After a 24-hour incubation with the cells

reaching a 70% confluence, cells were treated with the optimized concentrations of Obestatin and/or ethanol. Post-treatment, the growth media was aspirated, and cells were washed with PBS twice and stained with 500 µl of DCFH-DA working solution for 35 min in the incubator. After this staining period ended, the staining dye was aspirated, and cells were washed twice with PBS. The cells were then fixed with 4% paraformaldehyde for 15 min. Post fixation, the cells were washed twice for 5 min per wash. Once the PBS was removed, the cells were imaged using a fluorescent microscope (M7000, evos) at the 20× objective utilizing the appropriate filter for DCFH-DA. Quantification of fluorescence intensity was done using image J software.

3.3.8 Determining the presence of acidic vesicular organelles in the lysosomal compartment of cells with acridine orange

A morphological feature indicative of autophagy, which is the formation of acidic vesicular organelles (AVOs), is identified using acridine orange staining (Shin, Kim and Park, 2012). Cells were seeded onto coverslips in 35-mm petri dishes at a density of 2.70 x 10^5 cells per dish for the SH-SY5Y cell line and 1.38 x 10^5 cells per dish for the C8-D1A cell line. After a 24-hour incubation with the cells reaching a 70% confluence, cells were treated with optimized concentrations of Obestatin and/or ethanol. Post-treatment, the growth media was aspirated, and cells were washed with PBS. The cells were stained with 1 µg/ml acridine orange for 15 min and then washed with PBS twice. After that, the cells were fixed with 4% paraformaldehyde for 15 min, subsequently washed twice with PBS and then viewed with the confocal microscope (MICA) at the 20× objective utilizing the appropriate filters for acridine orange. The emission and excitation of the red filter were as follows: excitation was 460nm and emission was 650 nm. The emission and excitation of the green filter were as follows: excitation was 502 nm and emission was 525 nm.

3.3.9 Evaluating the protective effects of Obestatin against ethanol-induced nuclear morphological changes in the SH-SY5Y and C8-D1A cells

Cells were seeded onto coverslips into 35-mm petri dishes at a density of 2.70 x 10^5 cells per dish for the SH-SY5Y cell line and 1.38 x 10^5 cells per dish for the C8-D1A cell line. After a 24-hour incubation with the cells reaching a 70% confluence, cells were treated with optimized concentrations of Obestatin and/or ethanol. Post-treatment, the growth media was aspirated, and cells were washed with PBS twice. The cells were then fixed with 4% paraformaldehyde for 15 min and subsequently washed twice with PBS for 5 min per wash. After the washes, the cells were stained with a 1 in 10000 solution of DAPI (5 mg/ml) for 10 min. The cells were then washed twice with PBS for 5 min per wash and then viewed with the fluorescent microscope (M7000, evos) at the 20× objective utilizing the appropriate filter for DAPI. Quantification of fluorescence intensity was done using image J software.

3.3.10 RNA extraction, cDNA synthesis, and quantitative real-time PCR

The SH-SY5Y cells were plated into the 60-mm petri dishes at a density of 6.72 x 10^5 cells per dish and the C8-D1A cells were plated into 60-mm dishes at a density of 3.36 x 10^5 cells per dish. After 24 hours, both cell lines were treated with the optimized concentrations of ethanol and/or Obestatin. Post-treatment, the cells were harvested and centrifuged. After discarding the supernatants, the pellets were washed with 1 ml of PBS each to remove the excess medium. RNA was then extracted from the cells using an RNeasy mini kit: The PBS was aspirated and 350 µl of Buffer RLT was added to the cells for a duration of 5 mins. Thereafter, 350 µl of 70% ethanol was added to the cells and 700 µl of the contents were transferred to an RNeasy spin column which was then placed into an Eppendorf tube and centrifuged for 15 sec at 14.5 rpm. The flow-through from the Eppendorf tube was discarded and replaced with 700 µl of RW buffer to each RNeasy spin column and centrifuged for another 15 sec at the same rpm.

The flow-through was discarded and 500 µl RPE buffer was added to the spin column and centrifuged for 15 sec. Consequently, the flow through was discarded and another 500 µl RPE buffer was added to the spin column and centrifuged for 2 mins. Thereafter 40 µl RNase free water was added directly to the membrane of the spin column and centrifuged for 1 min. This was done to elute the RNA. The extracted RNA concentration was measured using a spectrophotometer. The SH-SY5Y RNA concentrations were recorded for the control, 500 mM ethanol, 10 ng Obestatin with ethanol and 100 ng Obestatin with ethanol were as follows: 560, 293, 644 and 926 ng/µl. The C8-D1A RNA concentrations were recorded for the control, 750 mM ethanol, 10 ng Obestatin with ethanol and 100 ng Obestatin with ethanol were as follows:1239, 320, 811 and 845 ng/µl. Subsequently, RNA was diluted to a concentration of 50 ng/µl and stored at -80 °C. The extracted RNA was then reverse transcribed to complementary DNA (cDNA). Subsequent cDNA dilutions were made and stored at -20 °C.

The stock solution of the primers (Table 3.1) was made first at a concentration of 100 µM. Following this, further 10 µM solutions were made. The 100 µM concentrations of primers were made and stored at -80 °C and 10 µM concentrations were stored at -20 °C. The synthesized cDNA of each treatment with the addition of the above-mentioned primers underwent a quantitative PCR. The qPCR technique used in this study measured the expression of genes using the Syber green method.

Table 3.1 Primer design sequences used for the quantitative polymerase chain reaction

Mammal	Gene	Forward primer sequence	Reverse primer sequence
Homo sapien	GAPDH	AAGACGGGCGGAGAGAAACC	CCGACCTTCACCTTCCCCAT
	Caspase-3	TGGGTGCTATTGTGAGGCGG	CCAGAGTCCATTGATTCGCTTCC

3.3.11 Western blotting

The SH-SY5Y cells were plated into the 60-mm petri dishes at a density of 6.72 x 10^5 cells per dish and the C8-D1A cells were plated into 60-mm dishes at a density of 3.36 x 10^5 cells per dish. After 24 hours, both cell lines were treated with the optimized concentrations of ethanol and/or Obestatin. Post-treatment, the cells were trypsinized and centrifuged. After discarding the supernatants, the pellets were washed with 1 ml of PBS each to remove the excess medium. The cells were lysed using boiling blue lysis solution (Appendix I) and the protein concentration was stored at -20 °C before use. The exact quantity of protein concentration cannot be determined using the boiling blue method. However, the protein of interest was normalised to control, and this method of protein extraction has been previously reported in another study (Omoruyi et al., 2020). The gels were made according to the molecular weight of the antibodies: LC3B required a 15% resolving gel (Appendix I). Whereas Cleaved PARP and GAPDH required an 8% resolving gel (Appendix I). Equivalent quantities of protein were resolved by SDS-PAGE. However, the 15% resolving gel required 2 hours whereas the 8% resolving gel required 1 hour for the bands to separate completely and evenly. The running buffer used for the separation of gels required 100 ml running buffer (10×) and 900 ml distilled water. After the proteins were resolved, they were transferred to a nitrocellulose membrane with the utilization of 1× transfer buffer (Appendix I). The bands were transferred at 100V for the first 30 min and were increased to 120V for the remaining 1 hour. Thereafter, membranes were blocked with either TBS-Tween (for LC3B, p62 and Cleaved PARP) or PBS-Tween (for GAPDH) in 5% non-fat milk for 1 hour and then incubated with a primary antibody (Table 3.2) overnight at 4 °C.

Table 3.2 List of antibodies used in Western blotting

Antibody	Dilution	Manufacturer	CAT number	Buffer
LC3B (1°)	1:1000	Cell Signalling Technology	2775S	TBS/Tween
P62 (1°)	1:1000	Cell Signalling Technology	88588S	TBS/Tween
Cleaved PARP (1°)	1:2000	Cell Signalling Technology	5625S	TBS/Tween
GAPDH (1°)	1:1000	Cell Signalling Technology	97166S	PBS/Tween
HRP (2°)	1:4000	Cell Signalling Technology	7074P2	PBS/Tween
HRP (2°)	1:10000	Abcam	Ab205718	TBS/Tween

The membranes were washed the next day with either PBS-Tween or TBS-Tween and were incubated with a secondary antibody (HRP) for 1 hour. However, LC3B required a different secondary antibody. Thereafter the membranes were washed with PBS-Tween or TBS-Tween and protein expression was visualized using the Bio-Rad machine. Densitometry was then done using Image Lab Software (Bio-Rad; version 4.0).

3.4 Statistical analysis

The experiments performed in this study were done at least in triplicates except otherwise stated. The experimental data sets were exported from an Excel spreadsheet into GraphPad Prism 8.01 where the One-Way Analysis of Variance (ANOVA) was used. To determine the significance of the difference between the control and treated cells, Dunnett's multiple comparisons test was done. Finally, the data generated were then illustrated as means ± standard error of the mean (SEM).

CHAPTER 4

RESULTS

4.1 Cytotoxic impacts of Obestatin on the viability of SH-SY5Y and C8-D1A cells

The effects of Obestatin on the SH-SY5Y and C8-D1A cells were evaluated after a treatment duration of 24 hours using the MTT assay. The optimum concentration of Obestatin that would have minimal effects on the viability of SH-SY5Y cells was determined as the cells were treated with different Obestatin concentrations ranging from 10 to 100 ng/ml. In Figure 4.1 A, the results showed no significant decrease in the cell viability of the SH-SY5Y cells. Indeed, the percentage cell viability stood at 96%, 91%, 85%, 85%, 84%, and 80% for the 10, 20, 40, 60, 80 and 100 ng/ml concentrations respectively relative to the control cells. It was also observed that the lower concentrations of Obestatin had a higher percentage of cell viability as opposed to the higher concentrations. This was evidenced by the 10 ng/ml concentration which had a 96% cell survival rate while the 100 ng/ml concentration was 80%. However, Obestatin was unable to increase cell viability above 100% in the SH-SY5Y cells. Figure 4.1 B showed Obestatin markedly increased the proliferation of C8-D1A cells with percentage viability of 120%, 140%, 118%, 105%, 106%, and 90% for the same concentrations compared to the control. The impact of Obestatin on the C8-D1A cells demonstrated its ability to increase cell viability above 100%. More specifically, the 20 ng/ml Obestatin resulted in a higher proliferation rate of 140% as opposed to the 100 ng/ml concentration which showed a lower cell proliferation rate of 90%. Consequently, Obestatin did not significantly reduce the cell viability of the C8-D1A cells compared to the SH-SY5Y cells. Together, these results show that Obestatin has minimal impact on the cell viability of the cells.

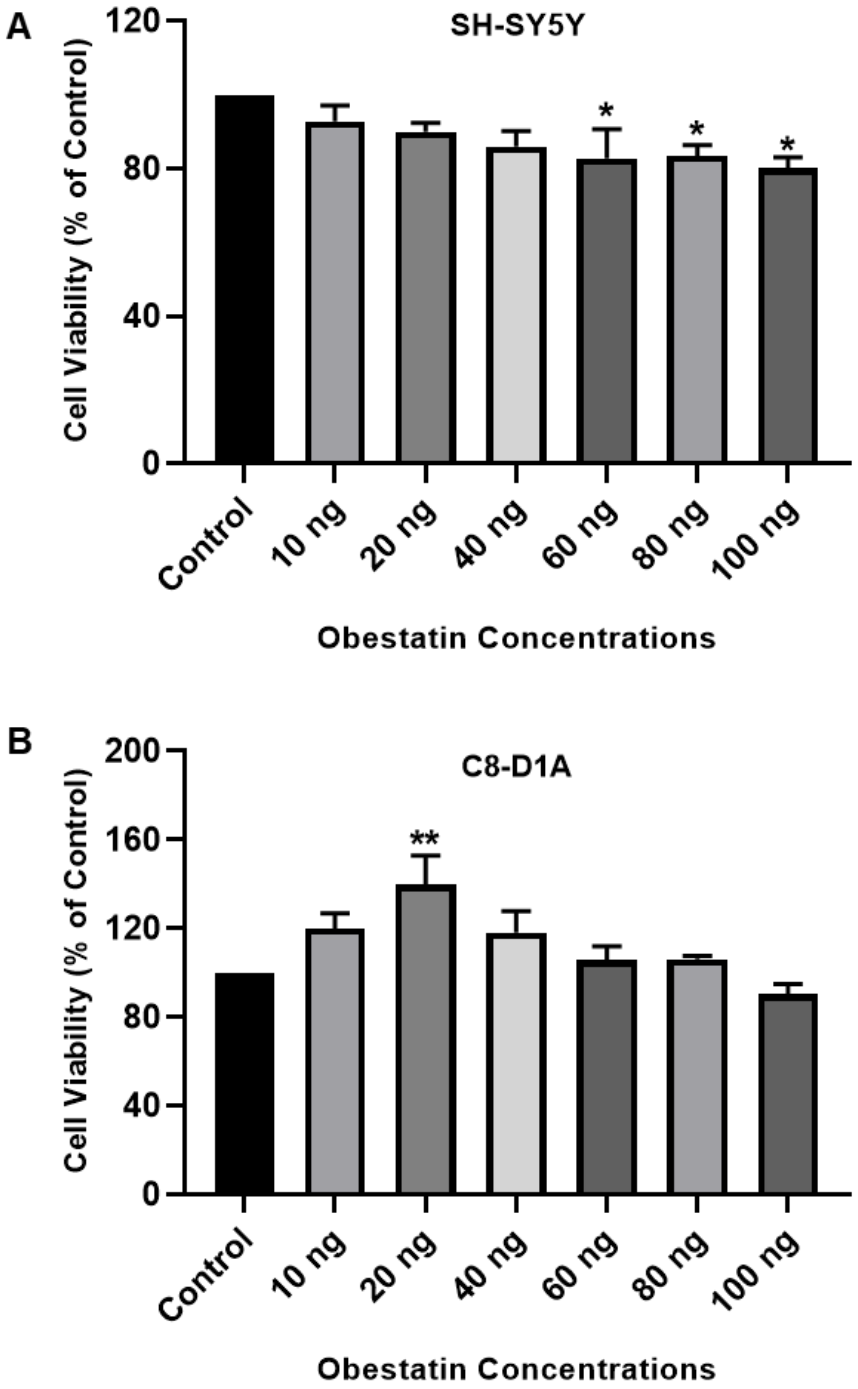

Figure 4.1 Assessment of the impact of Obestatin on the viability of SH-SY5Y (A) and C8-D1A (B) cells. Graphs A and B show the effects of varying concentrations of Obestatin on the SH-SY5Y and C8-D1A cells after a 24-hour exposure duration. The untreated cells served as the control and cell viability was assessed utilizing the MTT assay. Graphs were formulated as an average of experiments done in triplicate (n=3). Statistically significant changes are represented by asterisks (*$p<0.05$, **$p<0.01$).

4.2 Cytotoxicity of ethanol on the viability of SH-SY5Y and C8-D1A cells

The cytotoxic effects of ethanol on the viability of the SH-SY5Y and C8-D1A cells were assessed following a treatment duration of 24 hours utilizing an MTT assay. Varying concentrations of ethanol were used, including 125, 250, 500, 750, and 1000 mM. In Figure 4.2, the results showed the impact of different ethanol concentrations with a consecutive decrease in cell viability. Figure 4.2 A showed a consecutive reduction in the percentage cell viability of the SH-SY5Y cells (82%, 76%, 68%, 56% and 39%) for the 125, 250, 500, 750 and 1000 mM concentrations compared to the control. It was observed that the lowest concentration of ethanol, 125 mM, had the least toxic impact on the viability of the SH-SY5Y cells demonstrating a cell viability of 82%. However, the highest concentration of ethanol,1000 mM, had the most toxic effect reducing cell viability to 39%. Figure 4.2 B also showed a consecutive reduction in the viability of the C8-D1A cells (90%, 88%, 68%, 48% and 30%) for the same ethanol concentrations compared to the control. The same trend was observed in Figure 4.2 B with the lowest concentration having the least impact on viability (90%) for the C8-D1A cells whereas the highest concentration had the most toxic impact markedly reducing cell viability to 30%. The concentration that reduced cell viability to approximately 50% was chosen. Hence, the optimum concentrations selected were the 500 mM concentration for the SH-SY5Y cells and the 750 mM concentration for the C8-D1A cells. These concentrations were subsequently used in the present study.

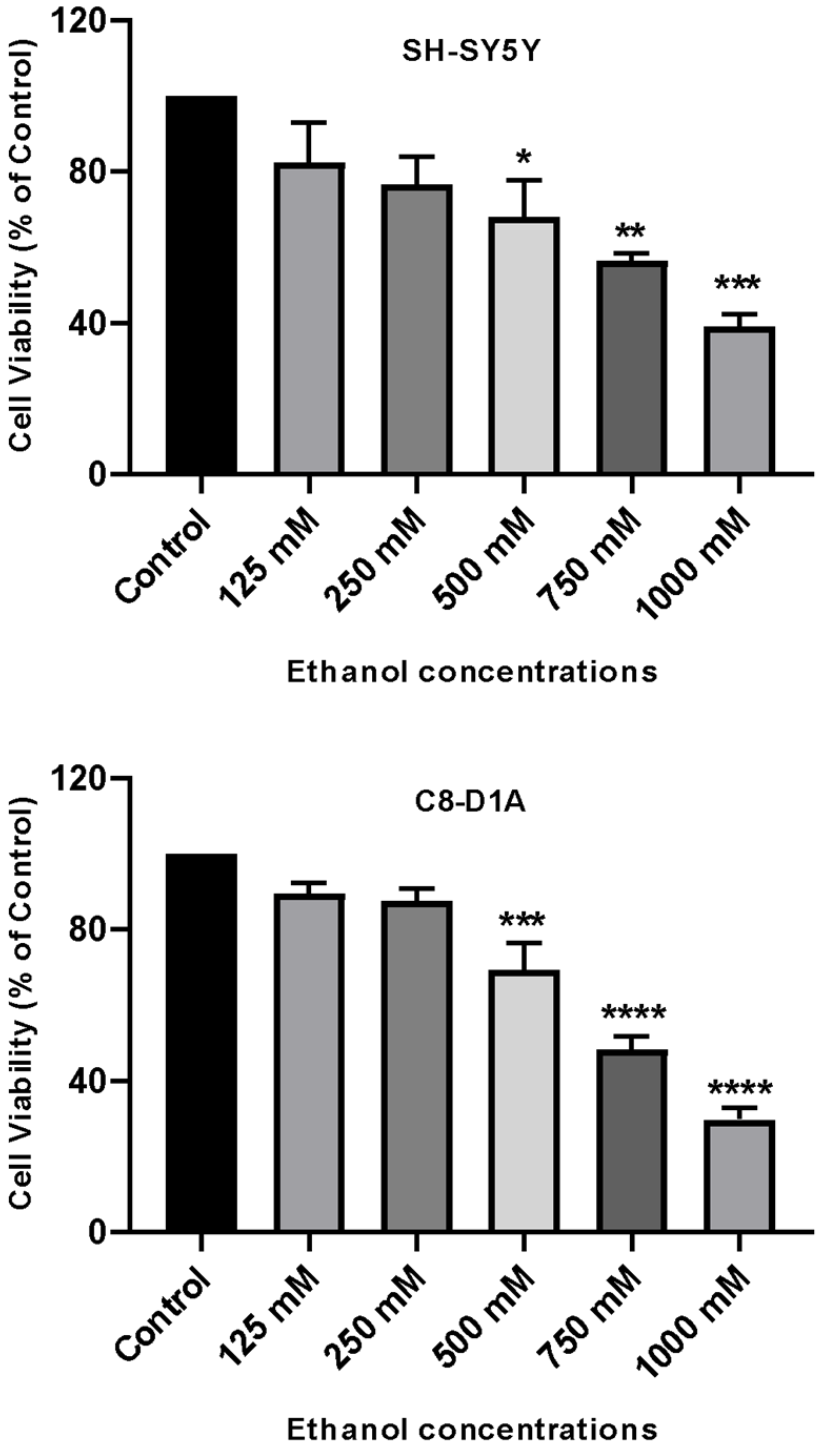

Figure 4.2 Cytotoxic impact of ethanol on the SH-SY5Y (A) and C8-D1A (B) cells. Graphs A and B show the effects of ethanol after 24-hour exposure duration. The untreated cells served as the control and cell viability was assessed utilizing the MTT assay. Graphs were formulated as an average of experiments done in triplicate (n=3). Statistically significant changes of the ethanol-treated cells against the control cells are represented by asterisks (* $p<0.05$, **$p<0.01$, *** $p< 0.001$, ****$p< 0.0001$).

4.3 Evaluation of the neuroprotective capabilities of Obestatin against ethanol-induced SH-SY5Y and C8-D1A cytotoxicity

Once the optimum concentration of ethanol was determined, the SH-SY5Y and C8-D1A cells were then treated with 10, 20, 40, 60, 80 and 100 ng/ml concentrations of Obestatin with 500 mM ethanol for the SH-SY5Y cells or 750 mM ethanol for the C8-D1A cells. Figure 4.3 showed that the cells treated with ethanol alone diminished cell viability to approximately 50% when compared to the control. However, in the cells treated with Obestatin and ethanol, Obestatin reduced the toxicity induced by ethanol when compared to the ethanol-treated cells. In Figure 4.3 A, SH-SY5Y cells treated with different Obestatin concentrations with 500 mM ethanol showed significant increases in viability at the lower concentrations (approximately 81%, 74%, 66%, 61%, 63% and 62% for the 10, 20, 40, 60, 80 and 100 ng/ml Obestatin concentrations respectively) when compared to the ethanol-treated cells. Similarly, in Figure 4.3 B, C8-D1A cells treated with the same concentrations of Obestatin but with 750 mM ethanol also demonstrated significant increases in viability at the lower concentrations (approximately 105%, 91%, 85%, 77%, 80% and 78%) when compared to the ethanol-treated cells. Based on these observations, the lowest concentration of Obestatin (10 ng/ml) offered the most neuroprotection against ethanol effect with cell viability of 81% for the SH-SY5Y cells and 105% for the C8-D1A cells. However, the highest concentration of Obestatin (100 ng/ml) produced the least cell viability. Collectively, these results demonstrated the neuroprotective capabilities of Obestatin in both the SH-SY5Y and C8-D1A cell lines against ethanol toxicity and therefore 10 ng/ml and 100 ng/ml concentrations of Obestatin were selected for further studies.

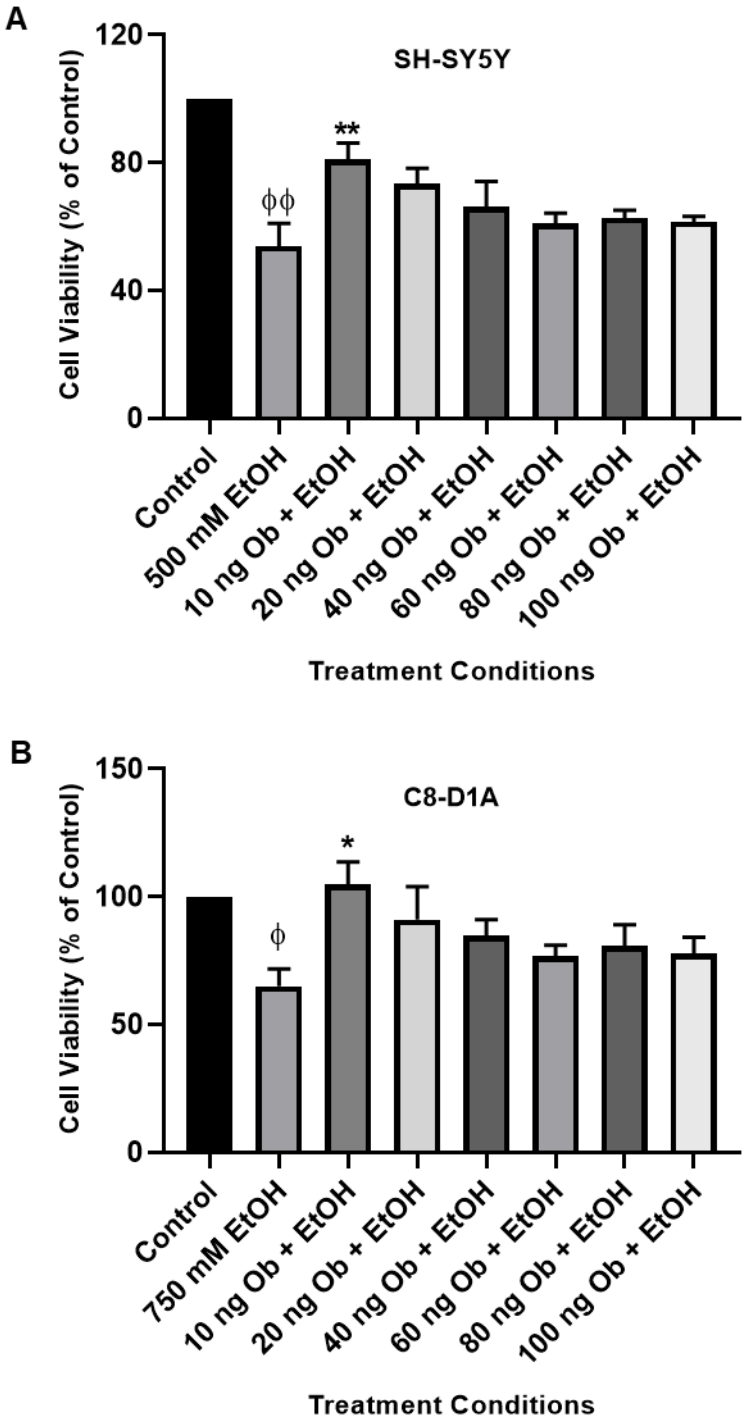

Figure 4.3. Neuroprotective capabilities of Obestatin on the viability of SH-SY5Y (A) and C8-D1A (B) cells after the administration of ethanol. Graphs A and B show the neuroprotective capabilities of Obestatin against exposure to ethanol for 24 hours. The untreated cells served as the control and cell viability was assessed utilizing the MTT assay. Graphs were formulated as an average of experiments done in triplicate (n=3). Statistically significant changes are represented by asterisks (*$p<0.05$, **$p<0.01$). The ethanol-treated cells is represented by ϕ.

To further determine the neuroprotective effects of Obestatin, the number of live cells were quantified following treatment with Obestatin and ethanol using a Trypan blue assay. Figure 4.4 showed that the cells treated with ethanol alone significantly diminished the number of live cells when compared to the control. However, in the cells treated with Obestatin and ethanol, Obestatin significantly reduced the toxicity induced by ethanol when compared to the ethanol treatment alone. In Figure 4.4 A, SH-SY5Y cells treated with (10 or 100 ng/ml) Obestatin and 500 mM ethanol significantly increased the number of live cells than in the ethanol-treated cells. In Figure 4.4 B, C8-D1A cells treated with (10 or 100 ng/ml) Obestatin and 750 mM ethanol also significantly increased the number of live cells than in the ethanol-treated cells. Collectively, these results demonstrated the neuroprotective capabilities of Obestatin in both the SH-SY5Y and C8-D1A cell lines against the toxicity induced by ethanol.

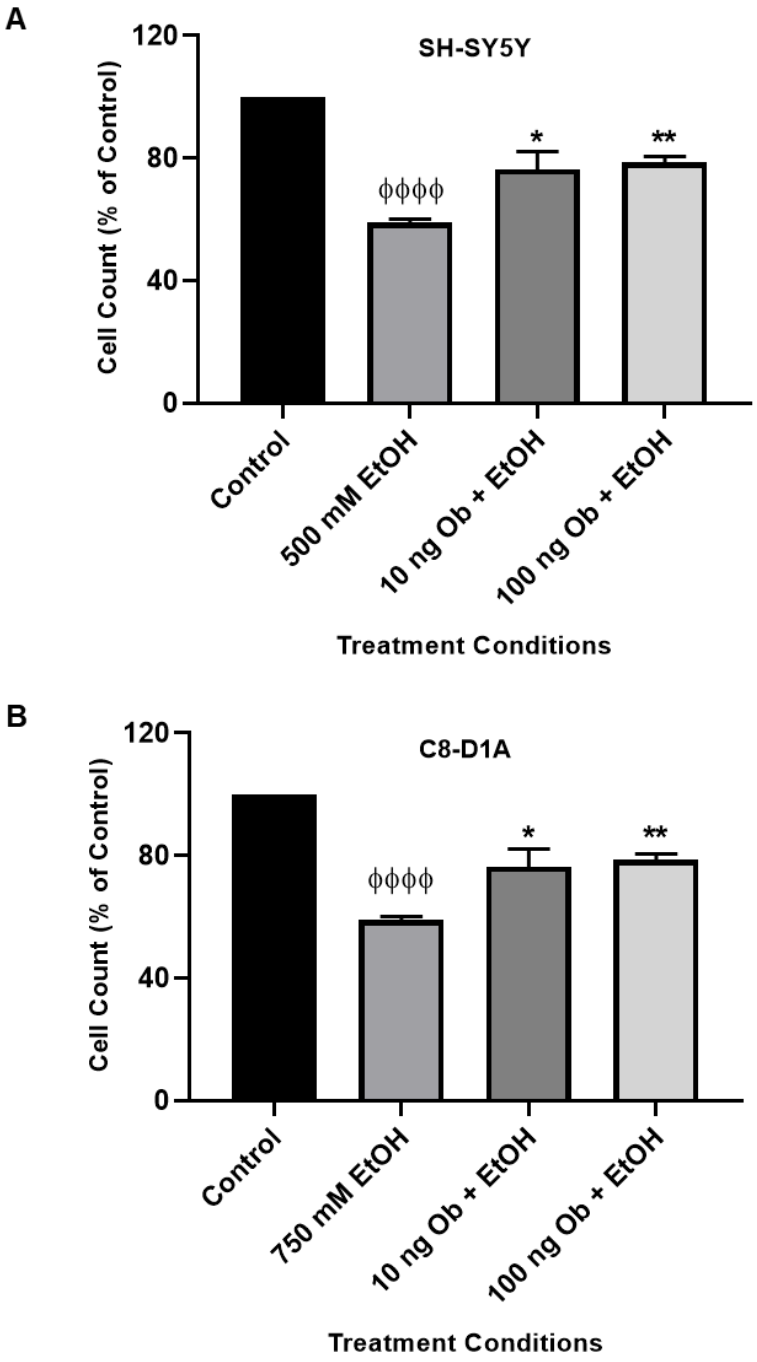

Figure 4.4. Neuroprotective capabilities of Obestatin on the viability of SH-SY5Y (A) and C8-D1A (B) cells after the administration of ethanol. Graphs A and B show the neuroprotective capabilities of Obestatin against ethanol exposure for 24 hours. The untreated cells served as the control and cell viability was assessed utilizing the Trypan blue assay. Graphs were formulated as an average of experiments done in triplicate (n=3). Statistically significant changes are represented by asterisks (*$p<0.05$, **$p<0.01$, ****$p<0.0001$). The ethanol-treated cells is represented by ϕ.

4.5 Evaluation of Obestatin against the morphological changes induced by ethanol on the SH-SY5Y AND C8-D1A cells

The morphology of the SH-SY5Y and C8-D1A cells was observed after cells were treated for 24 hours using a confocal microscope. Figure 4.5 demonstrated the morphological alterations induced by ethanol compared to t he control, but treatment with Obestatin showed cell morphology similar to that of the control. Figure 4.5 A and B illustrated that the ethanol treated cells induced marked changes to the cells, with both the SH-SY5Y and C8-D1A cells displaying more rounded-shaped cells relative to the control. The rounding of the cells is indicative of the cells probably under stress and encountering a type of programmed death. In addition to the alteration of cell morphology, the ethanol-treated cells displayed a significant reduction in cell density. However, in the cells treated with Obestatin and ethanol, cells appeared to have a normal cell morphology similar to the cells in the control which seems to indicate that Obestatin prevented the damaging effect of ethanol on cell morphology and cell density. Therefore, Obestatin was shown to mitigate morphological changes induced by ethanol as well as a reduction in cell density in both SH-SY5Y and C8-D1A cells.

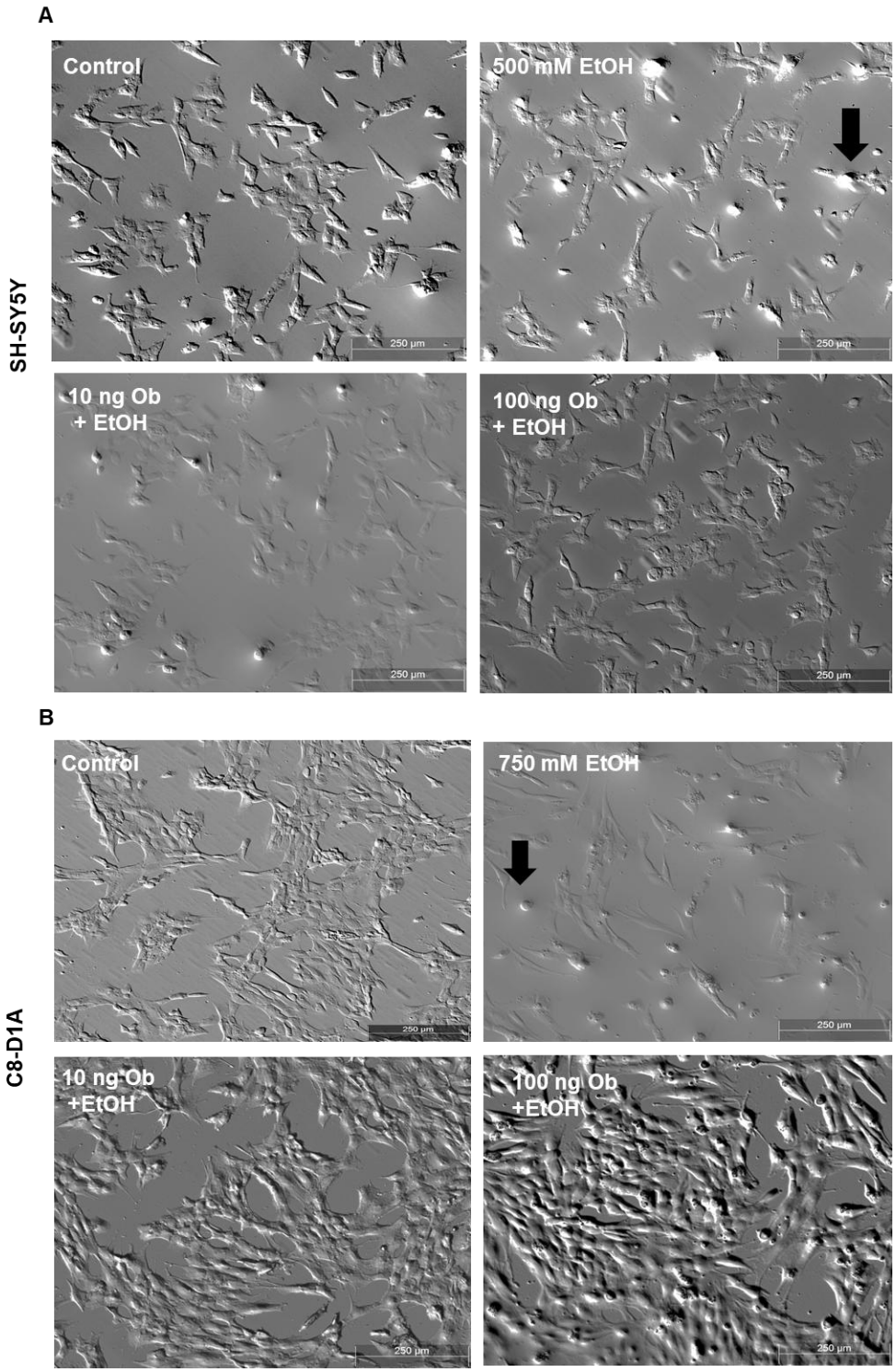

Figure 4.5 Morphological evaluation of the neuroprotective capabilities of Obestatin on the SH-SY5Y (A) and C8-D1A (B) cells after the administration of ethanol. SH-SY5Y (A) and C8-D1A (B) show the neuroprotective capabilities of Obestatin against morphologically induced changes to the cells exposed to ethanol for 24 hours. The black arrow illustrates round-shaped cells which is an indication of cells probably undergoing a programmed cell death. Representative images were taken under a 10× magnification. Scale bar = 250 µm.

4.6 Neuroprotective capabilities of Obestatin against ethanol-induced ATP depletion

The neuroprotective effects of Obestatin were evaluated against the diminishing effects of ethanol on ATP production. Figure 4.6 indicated that ATP production was diminished in the cells treated with ethanol compared to the control. It was also observed that both Obestatin concentrations inhibited ATP depletion by ethanol and these concentrations displayed ATP production levels similar to the control. However, it was noted that the lower Obestatin concentration (10ng/ml) with ethanol had a higher level of ATP compared to the 100 ng/ml. More specifically, the SH-SY5Y cells had an ATP level of 97% for the 10 ng/ml Obestatin with ethanol while the 100 ng/ml Obestatin with ethanol-treated cells expressed an 89% ATP production level (Figure 4.6 A). The C8-D1A cells followed a similar trend as the SH-SY5Y cells with an ATP production level of 100% for the 10 ng/ml Obestatin with ethanol which is also similar to the control while the 100 ng/ml Obestatin with ethanol expressed a 99% production of ATP (Figure 4.6 B). Collectively, these results confirmed that Obestatin mitigates ethanol-induced ATP depletion in the SH-SY5Y and C8-D1A cells.

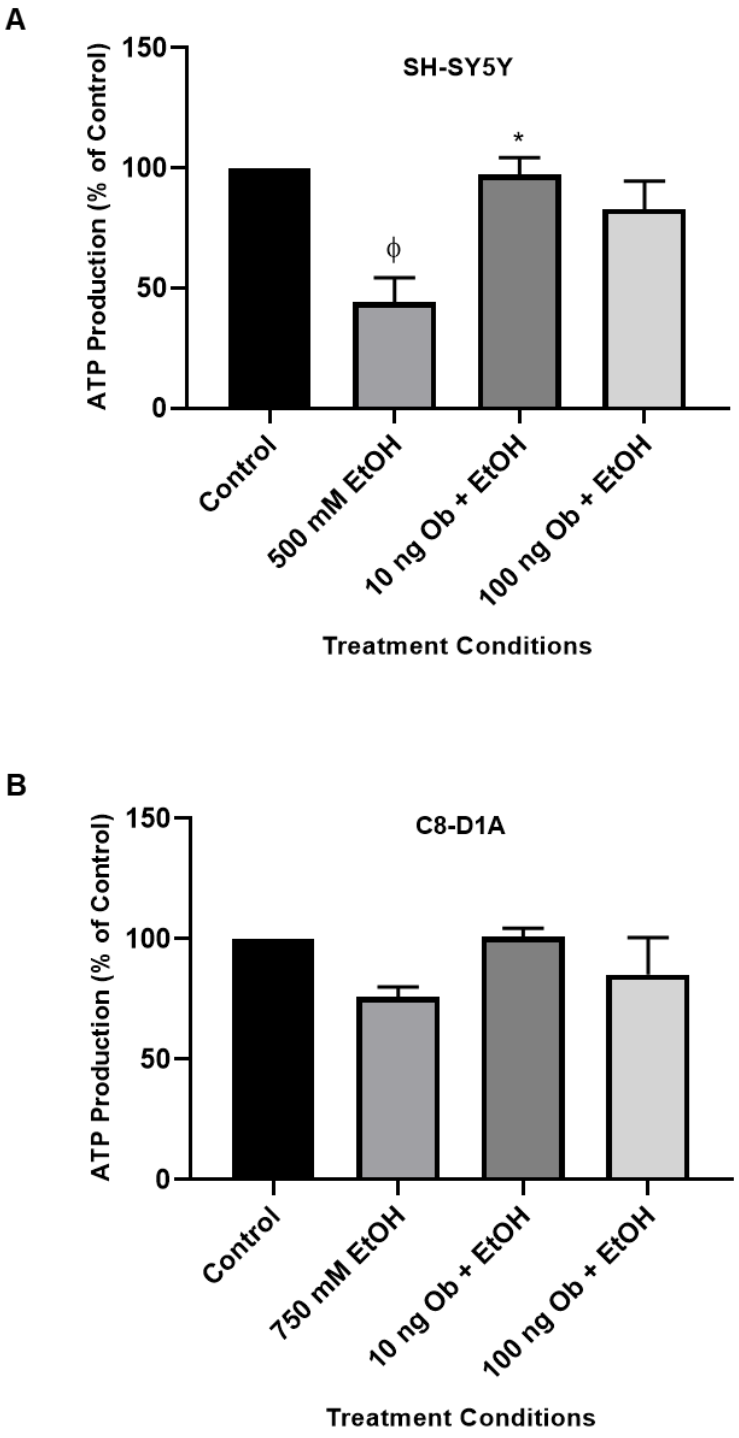

Figure 4.6. Neuroprotective capabilities of Obestatin against ethanol-induced ATP depletion on SH-SY5Y (A) and C8-D1A (B) cells. Graphs A and B show the neuroprotective effects of Obestatin diminishing ATP depletion induced by ethanol for 24 hours The untreated cells served as the control and cell ATP production was assessed utilizing the ATP bioluminescent kit. Graphs were formulated as an average of experiments done in duplicate(n=2). Statistically significant changes are represented by asterisks (*$p<0.05$). The ethanol-treated cells is represented by ϕ.

4.7 Neuroprotective capabilities of Obestatin against ethanol-induced increased production of ROS

Superoxide anions, hydroxyl and peroxyl radicals, as well as other non-radicals that can produce free radicals, are all constituents of the ROS family of free radicals. Oxidative damage to proteins, lipids, and DNA has been linked to an excess of ROS (Bhatti, Bhatti and Reddy, 2017). Consequently, the neuroprotective capabilities of Obestatin against ethanol-induced excessive formation of ROS were evaluated by using the fluorescent DCFH-DA assay. The intensity of fluorescence correlates with the level of ROS production with a bright emission of green fluorescence indicating a high level of ROS whereas a low emission of green fluorescence indicates a low level of ROS. Figure 4.7 illustrated that ethanol had a significantly higher level of ROS production in both cell lines compared to the control evidenced by the exhibited bright green fluorescence. However, Obestatin reduced ethanol-induced ROS production and was subsequently significantly different in the 10 ng/ml concentration.

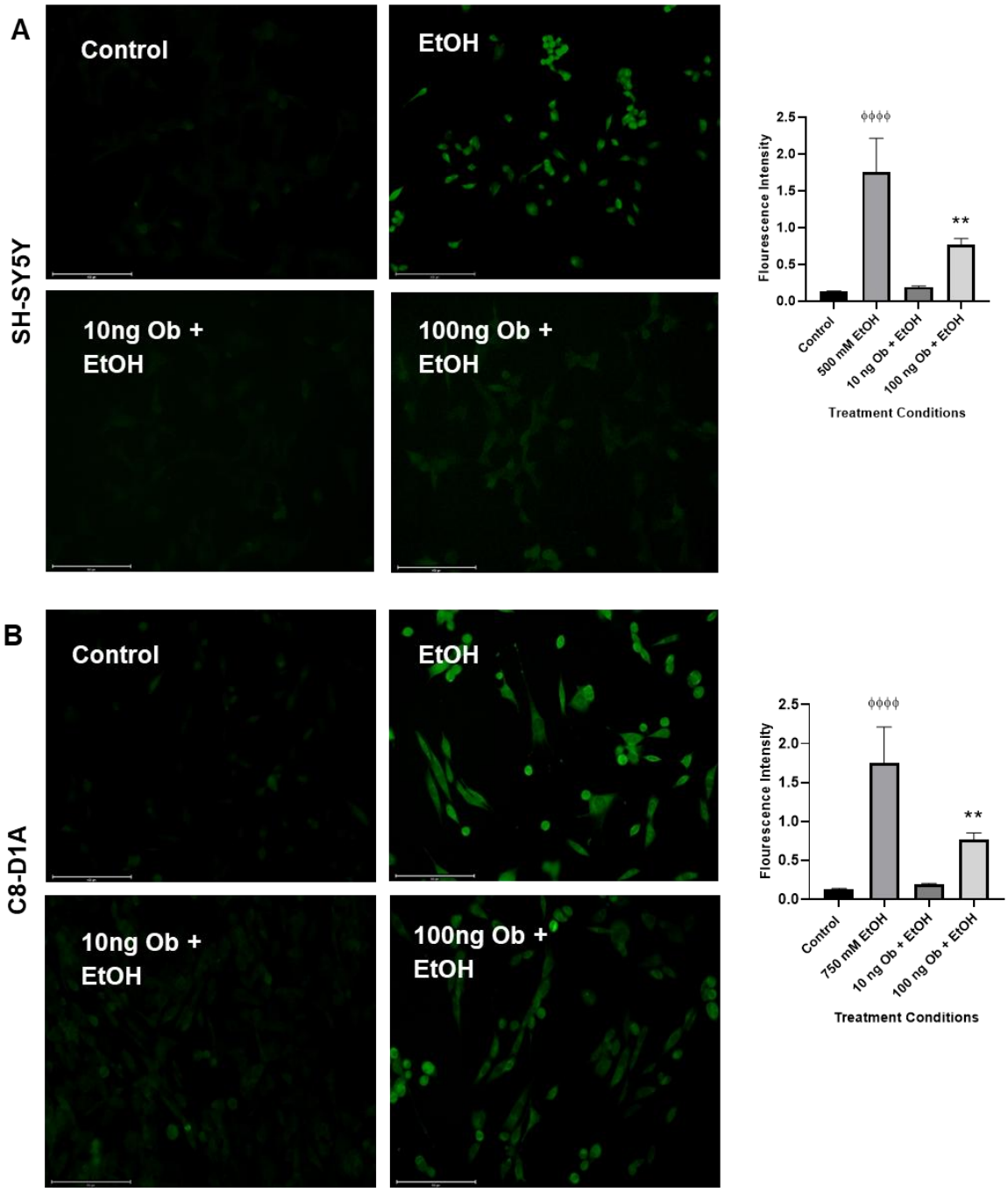

Figure 4.7 Impact of Obestatin on ethanol-induced increase in ROS production in SH-SY5Y (A) and C8-D1A (B) cells. Total levels of intracellular ROS were measured using the DCFH-DA assay. Cells were exposed to treatment for 24 hours and then incubated with 10 µM DCFH-DA for 30 min. Cells were analyzed using a fluorescent microscope. Quantification of fluorescence intensity was reported as an average of 8 different fields of view (n = 8). Representative images were taken under 20× magnification. Scale bar = 100 µm. Statistically significant changes are represented by asterisks (**p<0.01, ****p<0.0001). The ethanol-treated cells is represented by ϕ.

4.8 Neuroprotective capabilities of Obestatin against ethanol-induced acidic vesicular organelle (AVO) formation and autophagy

A morphological feature indicative of autophagy is the formation of AVOs, which is identified using acridine orange staining (Shin, Kim and Park, 2012). It is commonly stated that acridine orange emits red fluorescence in acidic lysosomal compartments, which supports its value as a probe to measure autophagy (Thomé et al., 2016). Figure 4.8 illustrates the presence of autophagy through the emission of bright red fluorescence within the ethanol-treated cells compared to the control which illustrated little to no red fluorescence. It was also observed that both the neuroprotective treated cells expressed little to no emission of red fluorescence in both cell lines, indicating a significantly lower level of AVO formation compared to the ethanol-treated cells. Furthermore, 100 ng/ml Obestatin expressed a slightly more increased emission of red fluorescence compared to the 10 ng/ml Obestatin. Nonetheless, both concentrations of Obestatin illustrated a marked reduction in AVO formation despite exposure to ethanol.

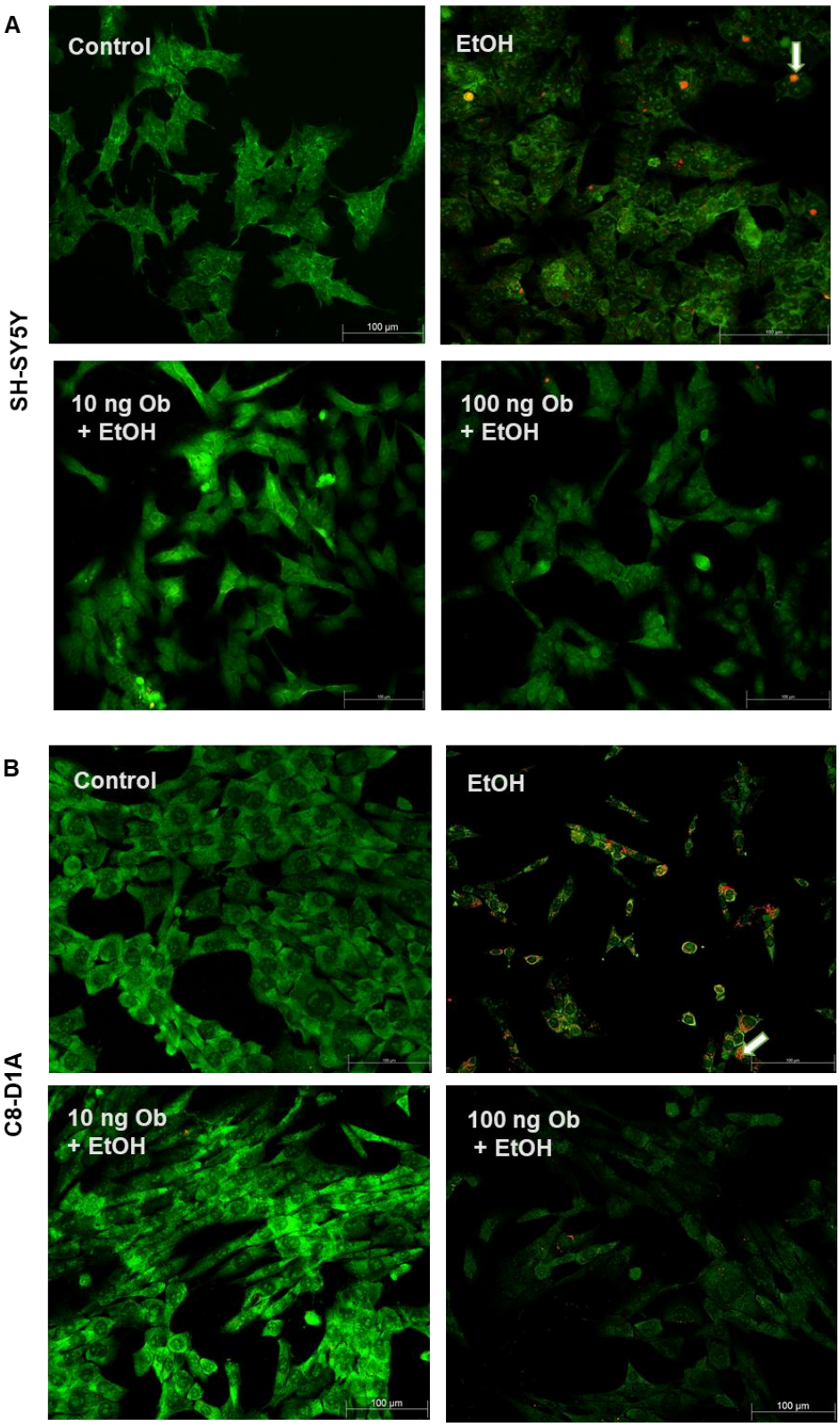

Figure 4.8 Evaluation of the presence of acidic vesicular organelles. SH-SY5Y (A) and C8-D1A (B) underwent a treatment duration of 24 hours and were then stained with acridine orange to determine the presence of AVO formation that is indicative of autophagy. The white arrow illustrates AVO formation. Representative images were taken under a 20× magnification. Scale bar = 100 µm.

To further confirm autophagy at the molecular level, Western blotting with antibodies to key autophagy markers like LC3B and p62 was conducted. In Figure 4.9, it was observed that autophagy status in the SH-SY5Y and C8-D1A cells differed. While ethanol treatment reduced the expression levels of LC3BII in the SH-SY5Y cells (Figure 4.9 A), an opposing result was evident in the C8-D1A cells treated with ethanol (Figure 4.9 B). Consequently, the SH-SY5Y cells treated with both Obestatin, and ethanol showed an increase in LC3BII expression compared to the ethanol-treated cells alone. This might indicate that the inhibition of autophagy is involved in the neuroprotection of Obestatin in ethanol-treated cells. Conversely, in the C8-D1A cells, it was observed that while ethanol increased LC3BII expression levels, treatment with Obestatin at all concentrations decreased the levels of LC3BII in the cells. Thus, suggesting that inhibiting ethanol-induced autophagy in the C8-D1A cells might be involved in the neuroprotection of Obestatin. This was substantiated by densitometric analysis whereby LC3BII was normalized against GAPDH. In Figure 4.9 A, it was observed that the ethanol-treated cells showed a very low level of LC3BII compared to the control, but the neuroprotective treatments showed higher levels of LC3BII similar to that of the control. It was also noted that the lower concentration of Obestatin (10 ng/ml) expressed a higher level of LC3BII compared to the higher concentration of Obestatin (100 ng/ml) as indicated by the densitometric analysis. The differentials in autophagy status in both cell lines can be attributed to differences in the cell lines as the SH-SY5Y cells are of human origin and the C8-D1A cells are of mouse origin. Furthermore, there was a significant increase in the level of p62 in the ethanol-treated cells compared to the control (Figure 4.9 A). However, the Obestatin and ethanol treated cells showed a marked decrease in the level of p62. Based on these results it was observed that ethanol treatment caused defects in autophagic degradation, but Obestatin was able to mitigate these defects. Importantly, it was stated that p62 was undetectable for the C8-D1A cells due to the reactivity of the antibodies used.

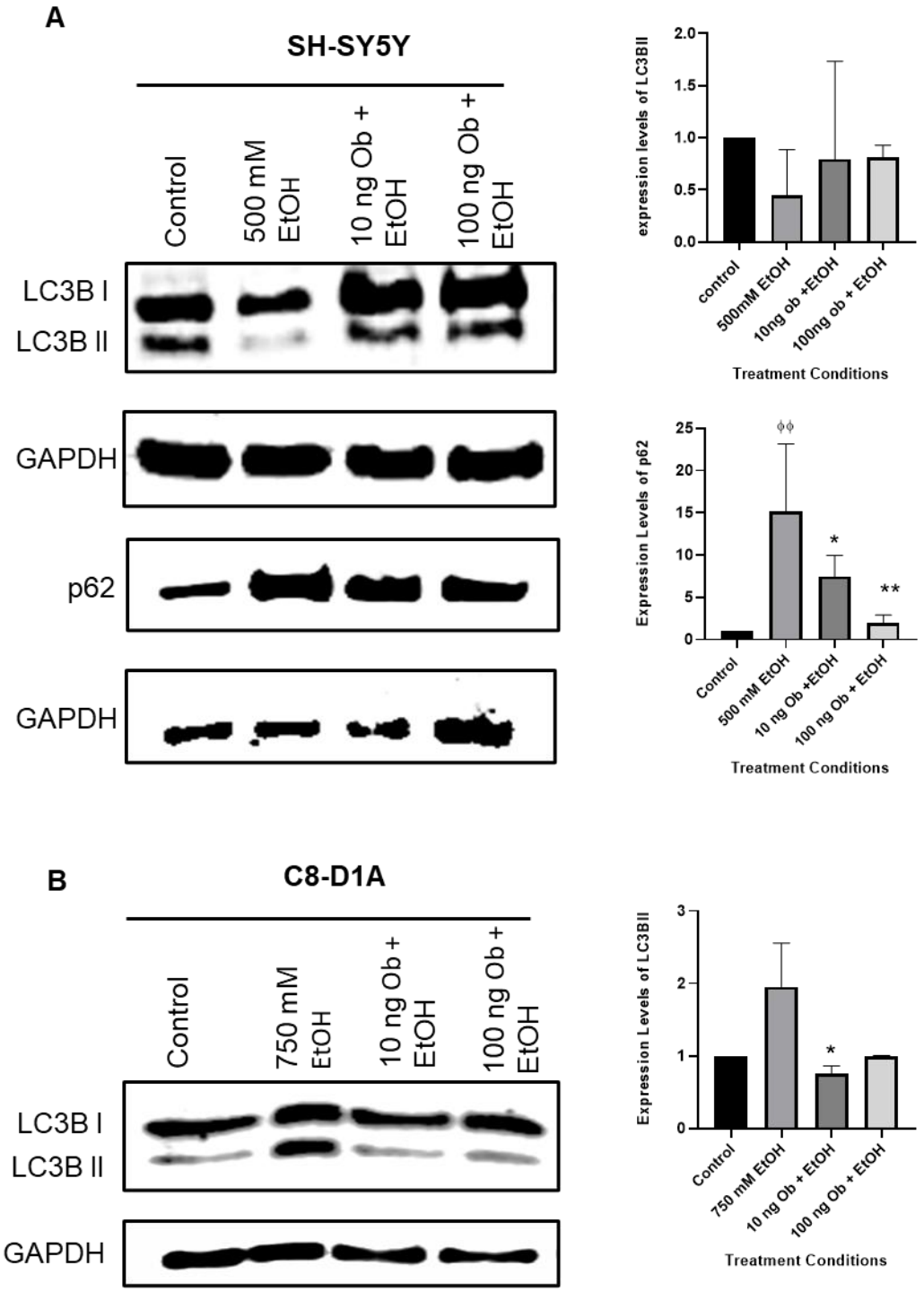

Figure 4.9 Evaluation of autophagy in SH-SY5Y (A) and C8-D1A (B). Protein samples of the treatment groups were analyzed by western blotting with GAPDH (loading control) and LC3BII and p62 antibodies. Densitometric values were quantified using Bio-Rad image lab 4.0 software. Graphs were formulated as an average of experiments done in duplicate (n=2). Statistically significant changes are represented by asterisks (*$p<0.05$, **$p<0.01$). The ethanol-treated cells is represented by ϕ.

4.10 Evaluation of Obestatin against ethanol-induced apoptosis and nuclei morphological changes to the SH-SY5Y and C8-D1A cells

Fluorescence microscopy generally provides excellent images of apoptotic bodies and chromatin condensation in comparison to non-apoptotic cells. It is thus a commonly used method to detect apoptosis (Mandelkow et al., 2017). Rounding up of the cell, retraction of pseudopods, chromatin condensation, cellular volume reduction (pyknosis), nucleus fragmentation (karyorrhexis), minimal or no ultrastructural alteration of cytoplasmic organelles, blebbing of the plasma membrane, and preservation of an intact plasma membrane until the end of the process are all signs of apoptosis (Miyoshi et al., 2008). Subsequently, the nuclear stain, DAPI, was used to visualize nuclear morphology and assess apoptosis of the respective treatments in SH-SY5Y and C8-D1A cells. Figure 4.10 illustrates the nuclear morphology of the SH-SY5Y and C8-D1A cells for the different treated cells. Furthermore, it was observed that the ethanol treatment caused a significant condensation of the nucleus compared to the control as observed when the fluorescent intensities of the Obestatin and ethanol treated cells were compared with those treated with ethanol alone. However, the ethanol and Obestatin treated cells (10 and 100 ng/ml) showed reduced condensation and fragmentation of the nucleus.

To further assess apoptosis at the molecular level, western blotting with antibodies to Cleaved PARP and PCR for mRNA levels of caspase-3 was performed. The 116-kDa polypeptide PARP is cleaved by caspase-3 to its distinctive 89-kDa fragment, which stops pointless DNA repair cycles and is regarded as a clear indicator of apoptosis (Joshi et al., 2003). It was observed that the ethanol-treated SH-SY5Y cells had a higher level of apoptosis compared to the control as indicated by the formation of a second band at 89 kDa. However, the neuroprotective treatments illustrated a significant reduction in apoptosis as both second bands were barely visible. This was further substantiated by the densitometric analysis (Figure 4.11).

Again, due to antibody reactivity and specificity, Cleaved PARP was undetectable for the C8-D1A cells.

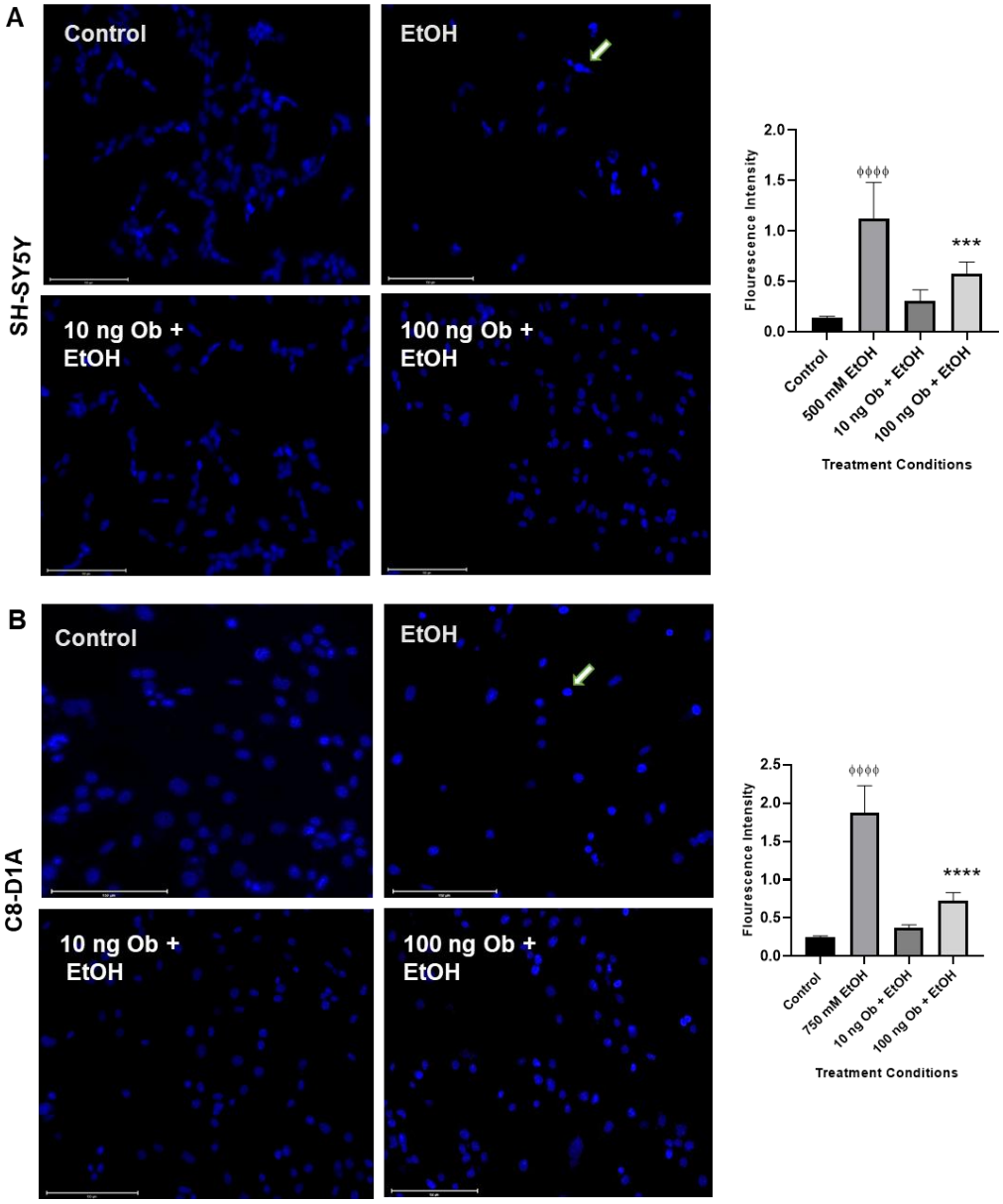

Figure 4.10 Morphological assessment of the SH-SY5Y and C8-D1A nuclei. SH-SY5Y (A) and C8-D1A (B) underwent a treatment duration of 24 hours and were then stained with DAPI to assess changes to the nuclear morphology. Cells were analyzed using a fluorescent microscope. The white arrow illustrates condensation of the nucleus which is indicative of apoptosis. Quantification of fluorescence intensity was reported as an average of 8 different fields of view (n = 8). Representative images were taken under 20× magnification. Scale bar = 100 µm. Statistically significant changes are represented by asterisks (***p<0.001, ****p<0.0001). The ethanol-treated cells is represented by ϕ.

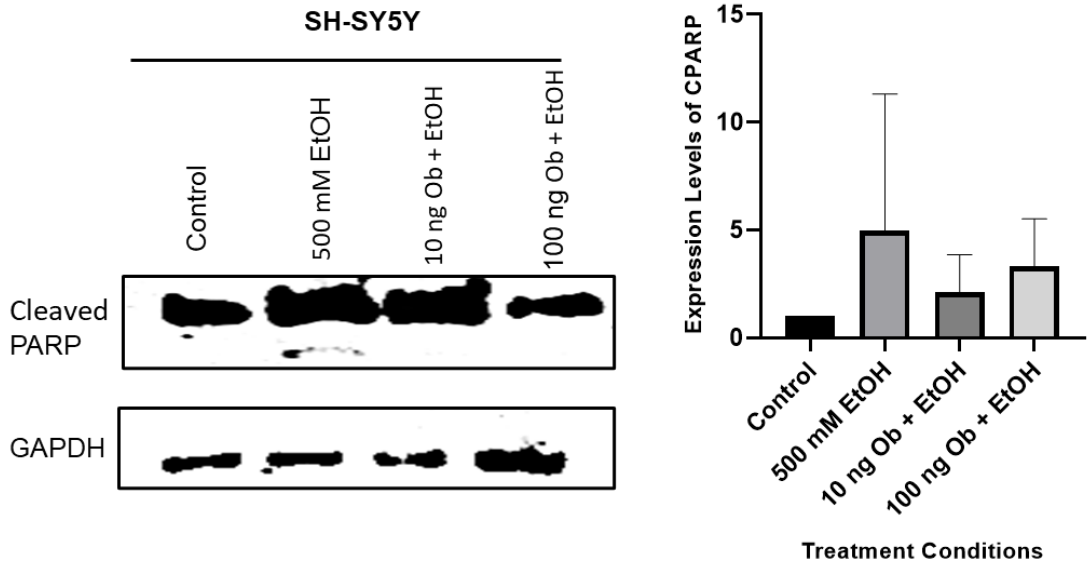

Figure 4.11 Evaluation of apoptosis in SH-SY5Y cells. Protein samples of the treatment groups were analyzed by western blotting with GAPDH (loading control) and Cleaved PARP antibody. Densitometric values were quantified using Bio-Rad image lab 4.0 software. The graph was formulated as an average of experiments done in duplicate (n=2).

In addition, the execution of apoptosis is significantly influenced by the activation of cysteine proteases known as caspases (Saraste, 2000). The most activated cell death protease among them is caspase-3 which catalyses the precise cleavage of numerous important cellular proteins (Porter and Jänicke, 1999). Figure 4.12 presents a trend in the gene expression levels of caspase-3 across the groups. In SH-SY5Y cells, caspase-3 expression was significantly higher in the ethanol-treated cells compared to the control. However, the neuroprotective treatments of Obestatin in combination with the optimized ethanol concentration showed no significant difference in caspase-3 expression relative to the ethanol-treated cells.

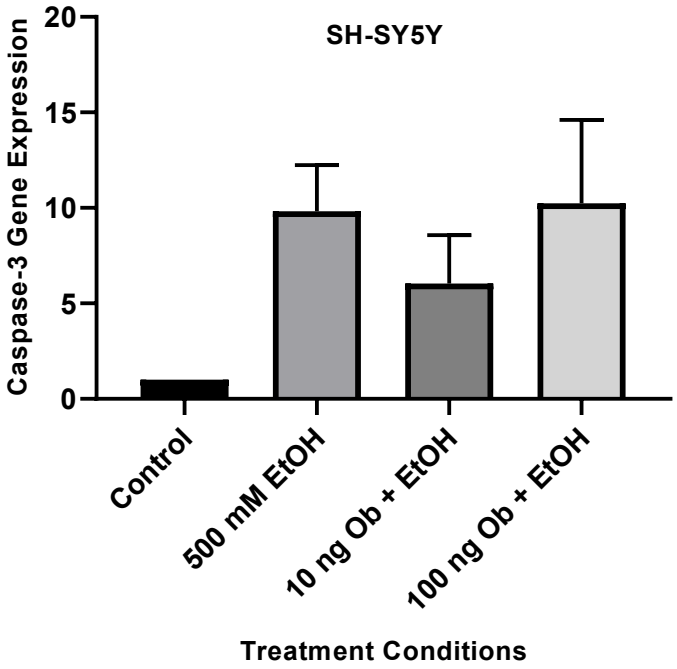

Figure 4.12 Neuroprotective capabilities of Obestatin against ethanol-induced upregulation of caspase-3. Gene expression levels of caspase-3 were assessed by quantitative PCR in SH-SY5Y cells. The graph was formulated as an average of experiments done in duplicate (n=2).

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Ethanol misuse has increased significantly in recent years, negatively impacting public health and leading to a rise in ethanol-related healthcare expenses (Carrino et al., 2021). Ethanol use has wide-ranging and diverse effects on the central nervous system and its neurotoxic effects are caused by intricate and mostly unidentified molecular mechanisms (Guerri and Pascual, 2019). Although many theories have been put forth to explain ethanol neurotoxicity, oxidative stress is widely acknowledged to be a key one (Ramezani et al., 2011). Acetaldehyde and oxidants produced during ethanol metabolism may harm DNA, proteins, and lipids (Dudek et al., 2020). In addition to the toxic byproducts of ethanol metabolism and the oxidative microenvironment generated by oxidative/nitrosative stress, other well-acknowledged mechanisms of ethanol-induced neuronal dysfunction include excitotoxicity, an imbalance in excitatory/inhibitory currents, inflammation and apoptosis (Sangaunchom and Dharmasaroja, 2020; Virgolini and Pautassi, 2022). To reduce the neurotoxicity linked to cytotoxic agents, neuroprotective agents are required to shield healthy tissues without sacrificing their ability to fight abnormal cell growth (Beijers, Jongen and Vreugdenhil, 2012). Furthermore, by minimizing or halting the loss of neurons, neuroprotective agents help preserve the structure and function of neurons, potentially preventing or partially reversing damage (Pekdemir et al., 2024). Therefore, this study evaluated the neuroprotective potential of Obestatin against ethanol-induced neurotoxicity in SH-SY5Y and C8-D1A cells.

The current study evaluated the neuroprotective impacts of Obestatin by investigating the cytotoxicity of Obestatin at varying concentrations (10 - 100 ng/ml) to determine its optimum concentration. The lowest concentration of Obestatin produced the most cell proliferation and

cell survival in both cell lines. This is also consistent with Granata et al. (2008) who reported that lower concentrations of Obestatin (1 and 10 nM) produced a significant increase in the survival and proliferation of cells of the pancreas. Another study by Gurriarán-Rodríguez et al. (2015) also showed that 5 nM Obestatin produced the most proliferation in C2C12 myoblasts. These findings show a U-shaped dose-response curve with higher protection at lower doses and lower protection at higher levels, as seen with many other peptides (Koç et al., 2014). It has also been shown that Obestatin activates PI3K/Akt and ERK1/2 thereby promoting the survival and proliferation of various cell types (Gargantini et al., 2016). Interestingly, Gurriarán-Rodríguez et al. (2011) demonstrated the stimulation of Obestatin signalling pathway, Akt, in 3T3-L1 adipocytes in a dose-dependent manner. As such the Akt phosphorylation at the C-terminal hydrophobic motif (Ser 473) was detected at the 10 nM concentration, whereas at 100 - 200 nM showed a plateau in the amount of pAkt (Ser 473). Considering the reported activities of Obestatin, in the present study, it was evident that Obestatin prevented ethanol-induced neurotoxicity in the SH-SY5Y and C8-D1A cell lines. The neuroprotective role of Obestatin was demonstrated by the retention of normal cell morphology as revealed by the light microscopy in the cells that were treated with Obestatin and ethanol. Other *in vitro* studies have found that Obestatin exerts neuroprotective effects on other neurotoxins such as methamphetamine on PC12 cells (Foroughi et al., 2019) and 1-methyl-4- phenylpyridinium (MPP^+) on MES23.5 dopaminergic cells (Shen et al., 2014). The neuroprotective effects of Obestatin appear to be dependent on mechanisms such as the enhancement of the antioxidant system and/or the inhibition of astrogliosis and apoptosis (Mirarab et al., 2019).

5.1.1 Amelioration of oxidative stress

Following ethanol exposure, ROS overproduction leads to oxidative stress in the brain (Chen et al., 2012). More specifically, the excessive generation of free radicals brought on by a

deficiency in the opposing antioxidant response system results in oxidative stress (Salim, 2017). In the present study, a high level of ROS was observed in the ethanol-treated cells and this finding is consistent with Haorah (2005) who reported that brain microvascular endothelial cells exposed to ethanol markedly increased ROS production. Wang et al. (2012) also reported that ethanol exposure promoted ROS production in SH-SY5Y cells indicated by the intensity of DCF fluorescence. Nonetheless, the intervention with Obestatin provided significantly reduced ROS levels based on the quantification of fluorescence as well as fluorescence intensity. Consequently, it confirms that Obestatin is effective in reducing oxidative stress induced by ethanol. This finding aligns with Koyuncuoğlu et al. (2017) who showed that Obestatin administered peripherally had antioxidant impacts which mitigated the oxidative stress caused by seizures induced by pentylenetetrazolein in an *in vivo* study using albino Wistar rats. A study by Mirarab et al. (2019) also showed that Obestatin raised the levels of antioxidant enzymes, GSH and SOD following brain ischemia. Overall, these findings as well as the results of the present findings are indications that Obestatin has antioxidant properties and can inhibit ROS production in the cells. It is thus suggestive of one of the mechanisms through which Obestatin may exert its neuroprotective capabilities.

5.1.2 Amelioration of ATP depletion

The role of mitochondria in maintaining the bioenergetics of the cell is well known; they are referred to as the "powerhouses" of the cell and, in addition to their involvement in important metabolic processes like the metabolism of fats, carbohydrates, and amino acids, they are the primary location for ATP production via the electron transport chain (ETC) and oxidative phosphorylation coupling (Peggion, Calì and Brini, 2024). However, ethanol lowers respiratory complex activity and expression, which ultimately results in a reduction in ATP content (Simon and Molina, 2022). Consequently, the effects of Obestatin against the damaging effect of ethanol on the levels of ATP were assessed. The present findings revealed that ATP production

was significantly diminished by ethanol in both cell lines. More specifically, 500 mM ethanol reduced ATP levels in the SH-SY5Y cell line by about 60% when compared to the control. This finding is consistent with Liu et al. (2014) who utilized SH-SY5Y cells to demonstrate inhibition in the generation of intracellular ATP by ethanol. The impact of ethanol on the bioenergetic capacity of cells was also determined by Mudyanselage et al. (2024) via ATP level quantification which showed a reduction in ATP levels for both undifferentiated and differentiated SH-SY5Y cells. However, the findings of this study indicate that Obestatin significantly mitigates ethanol-induced ATP depletion. Moreover, there are no prior reports of Obestatin directly influencing ATP depletion in the brain, as such this is showed for the first time in this study. Thus, to further substantiate this finding, it is imperative to investigate the mitochondrial complexes impacted by Obestatin treatment.

5.1.3 Apoptotic cell death reduction

Consequent upon ROS overproduction and ATP depletion, cells undergo programmed cell death including apoptosis. Physiological apoptosis eliminates damaged or mutated cells and regulates cell numbers, tissue and organ patterning and morphology. However, diminished or increased cell death due to dysregulated apoptosis frequently results in cancer, neurodegenerative diseases, and other hyperproliferative conditions (Chaitanya, Alexander and Babu, 2010). To further investigate the impact of ethanol on apoptosis in this study, the nuclear morphology was visualized, and the levels of Cleaved PARP were assessed as well as the activation of caspase-3 using mRNA levels. The qPCR analyses showed an upregulation in caspase-3 gene expression following ethanol treatment, which is consistent with Qin and Crews, (2012) which also showed that activated caspase-3 immunoreactivity was increased in mice treated with ethanol for 24 hours. In addition, Han et al. (2005), indicated a robust caspase-3 activation was caused by ethanol exposure in an FASD model using Sprague Dawley rats. Interestingly, it has been demonstrated that in various experimental models of

neurodegeneration, caspase inhibition postpones cell death (Li and Yuan, 2008). As such, neuroprotective treatments with Obestatin showed a concentration-dependent effect on caspase-3 gene expression in this study. The lowest concentration (10 ng/ml) resulted in reduced caspase-3 activation, while the highest concentration (100 ng/ml) led to increased caspase-3 activation. Similarly, a study by Toosi et al. (2019) showed that Obestatin also reduced activation of caspase-3 in a FASD model of ethanol-induced neurotoxicity in Wistar rat male pups. Obestatin was also shown to diminish caspase-3 activity induced by subarachnoidal haemorrhage in a study by Erşahin et al. (2013).

Furthermore, in caspase-dependent apoptosis, activated caspases 3 and 7 bind to PARP1 and PARP1 becomes inactive and cleaved, which inhibits DNA repair (Mashimo et al., 2021). More specifically, PARP (116 kDa) is broken down into fragments with relative molecular sizes of 85 and 27 kDa during apoptosis (Oliver et al., 1998). Subsequently, the 89 kDa fragment's appearance has been identified as an indicator of apoptosis (Castri et al., 2014). The analysis of Cleaved PARP revealed increased apoptosis in the ethanol-treated cells compared to the control. However, neuroprotective treatments significantly reduced apoptosis levels. In addition, ethanol appears to activate several apoptotic pathways in neurons, including the well-established intrinsic elements linked to DNA damage. The latter is demonstrated by end-point DNA fragmentation in apoptotic neurons (Cherian, Schenker and Henderson, 2008). More specifically, chromatin condensation and genomic DNA fragmentation are indicators of the final phases of apoptosis (Widlak et al., 2002). As such, the nuclear morphology of the ethanol-treated cells showed that the nuclei of both SH-SY5Y and C8-D1A cells had more chromatin condensation compared to the control. However neuroprotective treatments with Obestatin showed reduced condensation of nuclei. Therefore, the neuroprotective effects of Obestatin mitigated apoptosis.

5.1.4 Autophagy regulation

The lysosome-dependent cellular catabolic pathway known as autophagy breaks down organelles, protein aggregates, and cytoplasmic proteins. Damaged organelles and toxic protein aggregates are among the cytoplasmic components that are sequestered by double-membrane vesicles called autophagosomes during autophagy (Lipinski et al., 2015). Beclin1, the conjugate marker ATG16L, ATG5-ATG12, and the LC3BI/LC3BII ratio are significant indicators of autophagy (Parry et al., 2021). Subsequently, LC3BII, p62 and acridine orange were utilized to assess autophagy in this study. During autophagy, cytoplasmic constituents are confined within vesicles that have two membranes which are known as autophagosomes. These autophagosomes unite with lysosomes (vacuoles in plants and yeasts) to break down their contents (Anding and Baehrecke, 2017). The fusion of autophagosomes and lysosomes results in the formation of AVOs (Kassis, Grondin and Averill-Bates, 2021). A key morphological indicator of autophagy, AVO formation, can be detected using acridine orange staining (Shin, Kim and Park, 2012). Through acridine orange staining, autophagy induction was shown in this study due to the presence of acidification in the lysosomal compartments of both the SH-SY5Y and C8-D1A cells in the ethanol-treated cells.

The expansion of phagophores and the development of autophagosomes depend on the transition from LC3BI (an unconjugated form) to LC3BII (a conjugated form) (Hwang and Kim, 2022). The transition of MAP1LC3 (LC3) from the soluble form (LC3I) to the autophagosome-associated form (LC3II) indicates that there is an increase in autophagosomes within the cells (Chen et al., 2012). This was evident in this study in the C8-D1A cells that expressed a high level of LC3BII in the ethanol-treated cells. On the other hand, ethanol seemed to have the opposite effect on autophagy in the SH-SY5Y cells whereby LC3BII levels were significantly decreased by ethanol which is an indication of suppression of autophagy. These opposing findings of this study are consistent with Pla et al. (2016) which showed that *in vivo*

mouse astrocytes exhibited an increased LC3BII level induced by ethanol and subsequently decreased p62 levels. However, in this study, protein expression of p62 was only detectable in the SH-SY5Y cell line but not in the C8-D1A cell line. This difference may be attributed to the antibody used which, unfortunately, was not reactive for the cell line derived from the mouse. Pla et al. (2016) also observed that the SH-SY5Y cells showed a decreased level of LC3BII and a subsequent increase in p62 levels. It has been demonstrated that among the various elements implicated in the strict modulation of autophagy, mTORC1—but not mTORC2—is essential for coordinating the corresponding catabolic and anabolic processes as a response to physiological and environmental stressors (Paquette, El-Houjeiri and Pause, 2018). Chronic ethanol exposure increases mTOR phosphorylation, which inhibits the brain's autophagic pathways *in vivo* (Luo, 2014). Akt and Erk1/2 are the two main signalling pathways that are involved in the activation of mTORC1 (Cid-Díaz et al., 2017). Consequently, neuroprotective impacts of Obestatin modulating the AKT-driven anabolic program have been demonstrated in PC12 cells (Sánchez-Temprano et al., 2021). Therefore, it can be suggested that Obestatin may mediate the regulation of autophagy through any of these pathways but will have to be substantiated through further experiments.

5.2 Conclusion

The present findings highlight the neuroprotective potential of Obestatin, demonstrating its effectiveness against ethanol-induced damage on the SH-SY5Y and C8-D1A cell lines. This study shows that Obestatin has antioxidant properties by significantly reducing ROS production induced by ethanol, thereby suggesting its role in limiting oxidative stress in brain cells. Obestatin also protects the cellular bioenergetics of the mitochondria by mitigating ethanol-induced depletion of ATP, which further provides evidences of its neuroprotective capabilities against ethanol-induced mitochondrial dysfunction. In addition, Obestatin also appears to control apoptosis in ethanol-treated cells. This was evidenced by decreased caspase-

3 activation, reduced chromatin condensation, and lower levels of Cleaved PARP in the Obestatin and ethanol co-treated cells. Furthermore, Obestatin could influence autophagic pathways to protect neurons and cells from ethanol-induced damage. Collectively, these results align with previous studies on neurotoxicity that have demonstrated the neuroprotective properties of Obestatin and consequently underline the multifaceted neuroprotective capabilities of Obestatin, including antioxidant effects, regulation of apoptotic pathways, and regulation of autophagy. Therefore, Obestatin's potential as a therapeutic agent for counteracting ethanol-induced neurotoxicity is promising, providing valuable insights into its possible clinical applications in preventing or treating ethanol-related neurodegenerative conditions. Future studies are thus necessary to explore its long-term effects and the underlying signalling pathways involved in its neuroprotective actions.

5.2.1 Study limitations and future recommendations

This study provided valuable insight into the neuroprotective properties of Obestatin however there are some limitations. Only reactive oxygen species were evaluated in this study, however exploring reactive nitrogen species could be evaluated in the future by measuring nitric oxide through the Griess assay. Furthermore, the autophagic marker, p62 and the biomarkers for cell death, Cleaved PARP, were not expressed for the C8-D1A cell line as the antibodies was unreactive for the cell line. Future recommendations would be to utilize different mouse antibodies. Moreso the gene expression of caspase-3 was unable to be evaluated for the C8-D1A cell line therefore in the future, a different designed primer should be used. Future recommendations would be to explore other biomarkers of cell death, such as BCL2, using Western blotting. It will also be beneficial if gene expression of inflammatory markers in response to the neuroprotective capabilities of Obestatin against the negative impact of ethanol could be investigated on these cell lines. Likewise, the expression of proinflammatory markers such as interleukin1B, interleukin 6, CREB, TNF alpha and TGF beta in response to

Obestatin's effect against ethanol would also be beneficial. Due to the antioxidant effects of Obestatin, it will also be necessary to further validate the neuroprotective properties of Obestatin using an antioxidant enzyme kit to further show that the levels of SOD and glutathione are mitigated by Obestatin. Using a wider array of antibodies (such as antibodies to ERK1/2 and Akt) could elucidate the mechanism of action of Obestatin. Lastly, *in vitro* models using the SH-SY5Y and C8-D1A cell lines may not fully illustrate the complexity of human neurotoxicity, it would therefore be beneficial for *in vivo* models of ethanol to further investigate the beneficial effects of Obestatin.

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APPENDIX I

Boiling blue ingredients (10 ml):

Quantity	**Reagent**
832 µl	1 M Tris- HCl (pH 6.8)
4 ml	10% SDS (Sodium Dodecyl Sulfate) solution
1 ml	β-mercaptoethanol
2 ml	100% glycerol
2.168 ml	Distilled water
0.1 g	Bromophenol blue

Transfer buffer (1000 ml):

Quantity	**Reagents**
100 ml	10x Transfer buffer
700 ml	Distilled water
200 ml	Methanol

Resolving gel reagents:

Reagents used	**8% (10ml)**	**15% (10ml)**
Distilled water	5.3 ml	3.5 ml
40% acrylamide	2 ml	3.8 ml
1.5M Tris (pH 8.8)	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl
10% APS	100 µl	100 µl

TEMED	10 µl	10 µl

APPENDIX II: ETHICS WAIVER

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Innovation)

19/05/2023

Ref: W-CBP-230519-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Ms GB Govender
Student No. (if appropriate): 2553781
Staff No. (if appropriate):

Supervisor: Dr S Omoruyi

School: Anatomical Sciences
Department:

Medical School
University

Project title: *Neuroprotective potentials of Obestatin against ethanol-induced neurotoxicity in SHSY5Y neuroblastoma cells*

Reason: In vitro laboratory study
No human participants will be involved in the study

Dr CB Pelmy
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Third Floor, Philip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
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Tel Nos: +27 (0)11 717 1234/1252/2656/2700
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https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Innovation)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
ETHICS WAIVER

10/02/2025

Ref: W25/02/01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Dr S Omoruyi
Student No. (if appropriate):
Staff No. (if appropriate): A0076030

Supervisor: N/A

School: Anatomical Sciences
Department:
Division:
Faculty of Health Sciences

Project title: *Protective role of Obestatin in alcohol-induced oxidative stress in brain cells*

Degree: N/A

Reason: In vitro laboratory study
No human participants will be involved in the study

Professor P Ruff
Chairperson: Human Research Ethics Committee (Medical)

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APPENDIX III: AI STATEMENT

Wits University Faculty of Science post-graduate student AI declaration

I understand that the use of generative AI tools (such as ChatGPT or similar) without explicitly declaring such use constitutes a form of plagiarism and is classified by Wits University as academic misconduct.

I declare that in the course of conducting the research towards my degree or in the preparation of this thesis/dissertation/research report (select one by marking with an X):

I **did not** make use of generative AI tools	
I **did** make use of generative AI tools for the following (tick all that apply):	
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2. Sourcing Related Work (summarising, identifying sources)	
3. Methods and Experiment Design (experiment setup, model tuning)	
4. Data Analysis (presentation, coding, interpretation)	
5. Theoretical Development (theorem proving, conceptual analysis)	
6. Code Development (generating algorithms, writing scripts)	
7. Presentation (rendering graphics, formatting)	
8. Editing (grammar, readability)	
9. Writing (text generation, document structuring)	
10. Citation Formatting (structuring, organising)	X

If other uses were involved, please specify below:

Generative AI tool used (list all)	**Used for?**
Scribbr paraphrasing tool	Editing (grammar, readability)
Grammarly	Editing (grammar, readability)
NotebookLM	Sourcing related work (summarizing sources)

If generative AI tools were used as an integral part of the experimental design or in the direct execution of my research, I confirm that details of this use are clearly outlined in the relevant experimental/methodology chapters of my thesis/dissertation/research report.

Student number: 2553781

Candidate signature:

APPENDIX IV: TURNITIN REPORT

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