EVALUATION OF NEUROPROTECTIVE ACTIVITY OF DNA POLYMERASE INHIBITOR AGAINST SCOPOLAMINE INDUCED AMNESIA MODEL IN RATS

A Thesis Submitted to

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in Partial Fulfilment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

BY

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Under the guidance of

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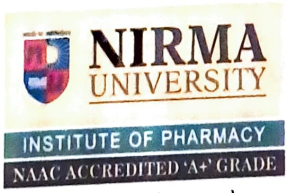

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CERTIFICATE

This is to certify that the dissertation work entitled "EVALUATION OF NEUROPROTECTIVE ACTIVITY OF DNA POLYMERASE INHIBITOR AGAINST SCOPOLAMINE INDUCED AMNESIA MODEL IN RATS" submitted by Prateeksha Sharma (22MP211) in partial fulfilment for the award of Master of Pharmacy in Pharmacology is a bonafide research work carried out by the candidate at the Department of Pharmacology, Institute of Pharmacy, Nirma University under my guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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CERTIFICATE OF ORIGINALITY OF WORK

This is to undertake that the dissertation work entitled "EVALUATION OF NEUROPROTECTIVE ACTIVITY OF DNA POLYMERASE INHIBITOR AGAINST SCOPOLAMINE INDUCED AMNESIA MODEL IN RATS" submitted by Prateeksha Sharma (22MP211) *in partial fulfillment for the award of Master of Pharmacy in Pharmacology is a bonafide research work carried out by me at the Institute of Pharmacy, Nirma University under the guidance of Dr. Jigna Shah. I am aware about the rules and regulations of Plagiarism policy of Nirma University, Ahmedabad. According to that, this work is original and not reported anywhere as per best of my knowledge.*

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DECLARATION

*I hereby declare that the dissertation entitled "**EVALUATION OF NEUROPROTECTIVE ACTIVITY OF DNA POLYMERASE INHIBITOR AGAINST SCOPOLAMINE INDUCED AMNESIA MODEL IN RATS**", is based on the original work carried out by me under the guidance of Dr Jigna Shah, Professor and Head, Department of Pharmacology, under the Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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Regards,

Prateeksha Sharma

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LIST OF ABBREVATIONS

Aβ: AMYLOID BETA

ACH: ACETYLCHOLINE

ACHE: ACETYLCHOLINESTERASE

AD: ALZHEIMER'S DISEASE

DPI-1: LOW ORAL DOSE

DPI-2: MIDDLE ORAL DOSE

DPI-3: HIGH ORAL DOSE

APP: AMYLOID PRECURSOR PROTEIN

BACE: B-SITE AMYLOID PRECURSOR PROTEIN CLEAVING ENZYME

BBB: BLOOD BRAIN BARRIER

CAT: CATALASE

CDK: CYCLIN-DEPENDENT KINASE

CHAT: CHOLINE ACETYLTRANSFERASE

FDG-PET: FLUORODEOXYGLUCOSE POSITRON EMISSION

GFAP: GLIAL FIBRILLARY ACID PROTEIN

GSH: GLUTATHIONE

GSK: GLYCOGEN SYNTHASE KINASE

H&E: HEMATOXYLIN AND EOSIN

HSV: HERPES SIMPLEX VIRUS

MACHRS: MUSCARINIC ACETYLCHOLINE RECEPTORS

MAP: MICROTUBULE ASSOCIATED PROTEIN

MDA: MALONDIALDEHYDE

MRI: MAGNETIC RESONANCE IMAGING

NFT: NEUROFIBRILLARY TANGLES

NMDA: N-METHYL D-ASPARTATE

PBS: PHOSPHATE BUFFER SOLUTION

PHF: PAIRED HELICAL FILAMENT

PSEN: PRESENILIN

RNS: REACTIVE NITROGEN SPECIES

SOD: SUPEROXIDE DISMUTASE

STZ: STREPTOZOTOCIN

ROS: REACTIVE OXYGEN SPECIES

VC: VEHICLE CONTROL

DC: DISEASE CONTROL

DPZ: DONEPEZIL

VD: VOLUME OF DISTRIBUTION

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ABSTRACT

AIM: To evaluate neuroprotective activity of DNA Polymerase Inhibitor against scopolamine induced amnesia model in rats.

INTRODUCTION: Alzheimer's disease stands as the primary contributor to dementia cases, which is characterized by a progressive loss of cognitive ability. The pathogenic hallmarks of this condition include the development of amyloid beta plaques outside of brain cells, accumulation of tau proteins forming neurofibrillary tangles within cells, reduced acetylcholine levels in the brain, oxidative stress, and neuroinflammation. The signs of AD include anterograde amnesia, aphagia, agnosia and anomia. Scopolamine is an anti-muscarinic agent which causes short-term memory loss. The investigational drug, a DNA Polymerase Inhibitor (DPI) used in many viral infections, according to the literature, can reduce cognitive impairment by inhibiting the AChE enzyme.

OBJECTIVES: To evaluate the neuroprotective action of DNA Polymerase Inhibitor and to investigate the mechanism of action of drug against scopolamine induced amnesia model in rats.

METHODOLOGY: Healthy Sprague Dawley female rats were utilized in the experimental study. Rats were divided into 6 groups: Normal Control; Disease Control (Scopolamine 2mg/kg I.P); Standard Treatment group (Disease treated with Donepezil 5mg/kg PO); DPI-1 treatment: Disease treated with DNA Polymerase Inhibitor at the dose of 87mg/kg PO; DPI-2 treatment: Disease treated with DNA Polymerase Inhibitor at the dose of 130mg/kg PO; DPI-3 treatment: Disease treated with DNA Polymerase Inhibitor at the dose of 173mg/kg PO. The rats were initially (Day -7 to -1) trained for neurobehavioral paradigms i.e., Morris Water Maze, and Novel Object Recognition. The disease was induced from Day 0 to Day 15 by administering scopolamine 2 mg/kg intraperitoneally (I.P) which was followed by 28 days treatment with standard treatment (donepezil) and test drug (DNA polymerase inhibitor) orally along with scopolamine IP. At the end of the protocol, the neurobehavioral paradigms were evaluated and finally, the rats were sacrificed and brain samples were isolated for biochemical estimations, histopathology, and immunohistochemistry.

RESULTS: In neurobehavioral paradigms, animals in Morris water maze test showed a decrease in escape latency in DNA Polymerase Inhibitor groups, but no significant changes were seen in the discrimination index of novel object recognition test. In biochemical assays, acetylcholinesterase level was reduced in treatment groups, but the difference was not significant. A significant increase in the levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were observed in the treatment groups in comparison to the disease control group. Decreased malondialdehyde (MDA) level was observed in the treatment groups in comparison to the disease control group. Histopathological studies revealed that the treatment ameliorated neuronal degeneration and increased neuronal density in the hippocampus. The immunohistochemistry studies showed that scopolamine administration increased glial fibrillary acidic protein (GFAP) immunoreactivity and decreased synaptophysin immunoreactivity in the CA1 region of the hippocampus which was reversed by the DPI treatments.

CONCLUSION: The results of the present study showed that the DNA polymerase inhibitor drug inhibited acetylcholinesterase enzyme and thus in-turn increased the available stores of acetylcholine and thus improved memory and cognitive functions. It decreased oxidative stress by increasing GSH, SOD and CAT levels and decreasing the MDA levels. Further it reduced neuroinflammation in the brain as observed from GFAP immunohistochemistry and enhanced synaptic plasticity as shown by increased immunoreactivity to synaptophysin. The results showed that DNA Polymerase Inhibitor drug conferred neuroprotection by decreasing neuroinflammation and oxidative stress and improved synaptic plasticity. Further studies to elucidate the mechanistic pathway in advanced animal models are warranted.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Dementia, serving as an overarching term, refers to a wide range of progressive neurological disorders that impact brain functions. It is one of the most commonly occurring neurodegenerative disorders is distinguished by cognitive decline in brain, abnormalities in motor and sensory functions, memory impairment, and neuronal alterations [1,2]. It majorly impacts on the performance of daily activities, which is gradually seen in geriatric patients. One of the main causes of dementia is Alzheimer's disease (AD), which is marked by the degeneration of the nerve cells and resulting in slowing of function[3]. According to WHO estimates, 10 million new cases of dementia are diagnosed year, out of an estimated 50 million people worldwide. By 2050, 19.1% of Indians are expected to be 60 years of age or older, or 316 million people – nearly the entire population of the United States at the moment[6]. The most common pathologies are neuroinflammation where overactive immune cells such as microglia causes brain dysfunction and neuronal damage, oxidative stress, Aβ peptides are produced when β- and γ- secretases gradually cleave the amyloid precursor protein (APP) therefore abnormal Aβ accumulation in the brain results in the hyperphosphorylation of tau protein and the formation of neurofibrillary tangles, which impair synaptic and neuronal function causing neuroinflammation or neuronal death and ultimately cognitive impairment. Due to the loss of cortical and subcortical neurons, all this cause cerebral atrophy, which causes AD and memory loss[3]. Anterograde amnesia, aphasia, agnosia, and apraxia are among the symptoms of AD, a progressive neurogenerative condition [4,5]. AD can be classified into two categories: genetic and sporadic where more than 95% of the cases are of the sporadic form, which often appears in the individuals between the ages of 80 and 90, and the familial early- onset form[1]. The three dominant autosomes, which are encoded by the mitochondrial proteins those are Amyloid Precursor Protein (APP), Presenilin 1 (PSEN1), and Presenilin 2 (PSEN2), respectively, are analogous to the genetic form. The other variety resembles apolipoprotein. The majority of symptoms that have been identified clinically and molecularly include tau hyperphosphorylation in the brain, neuroinflammation, amyloid beta deposition, insulin desensitization or resistance status, and oxidative stress[7]. Beta amyloid deposition is a well acknowledged pathogenesis, despite the complicated nature of its origin. Preclinical application of research on non-human subjects has showed little promise because AD is

most likely caused by a combination of environmental and genetic factors that combine to produce variety of neurodegenerative diseases [8].

Different chemical inducing AD models are streptozotocin, scopolamine, okadaic acid, colchicine, ethanol and other dysregulated heavy metals.

Scopolamine, which is frequently used in pilot studies, causes hippocampal cell loss and learning impairment in rodents by inhibiting Ach muscarinic receptors in the cerebral cortex, hence inducing memory issues. Its effects on M1 and M5 receptors lead to potential adverse effects including agitation and hallucinations, as well as dose-dependent deficiencies in memory and learning. Additionally, scopolamine affects proteins linked synaptic plasticity such as DNA methyl transferase-1 and histone deacetylase-2, as well as NMDA receptor pathways in memory loss. When injected into particular brain regions, it interferes with memory and learning functions, including spatial encoding[9].

Given that scopolamine can cause memory and cognitive impairments, it is an often-utilized model in the research of dementing- related conditions. Working memory-related muscarinic acetylcholine (ACh) receptors were traditionally stimulated by this compound. Numerous neurobehavioral studies have demonstrated that scopolamine can negatively affect both human and rodent function, especially short-term memory and learning acquisition. A few traditional techniques for assessing these cognitive functions in rodents are radial arm maze, water maze, and passive avoidance tests. Numerous studies have employed scopolamine to identify and explain potential AD treatment targets. The application of scopolamine as a pharmacological model to investigate the cellular and molecular alterations associated with AD is yet unknown, despite the fact that scopolamine-induced amnesia provides a great behavioural model for studying dementia-related disorders like AD[10].

AD is incurable while only being able to manage the disease to keep at a pause currently. Till date, only four drugs have been approved for the treatment of Alzheimer's disease which can only treat symptoms of the disease but not the disease itself. Three of which belongs to the cholinesterase inhibitors; Donepezil, Rivastigmine and Galantamine. The other class of drug belongs to the NMDA receptor blocker i.e., Mementine[11]. Due to problems with drug permeability across the BBB, which necessitates higher dosages and raises the risk of side effects, drug trails for AD often end in failure. Drug delivery to CNS

is challenged by BBB, prompting the development of various strategies like aducanumab and lacanemab antibodies. Preclinical studies may not always take into account age-related changes in neuronal membranes and receptors, which could potentially impair medication efficacy. Treating AD in its late stages may reduce the efficacy of the medications as well. Lack of curative treatments and challenges in early diagnosis highlight the preventive and neuroprotective measures in order to slow down the neurodegeneration and lower the risk of AD[12].

Pathogens can enter the CNS in a various way, depending on the organism that is causing the infection which could accelerate the development of AD. A frequent pathway involves a breached blood brain barrier, where the involvement of cerebral spinal fluid occurs in transporting the pathogens. This is especially dangerous for the elderly and people with compromised immune systems since some viruses, like herpesvirus, can lie dormant after an initial infection and then revive later in life, causing problems that are delayed. Bacteria and viruses can still access the brain through a number of different channels even when the BBB is intact. From a recent investigation in 2020, revealed that immunocompromised patient's olfactory cortex and hippocampal regions could produce neurofibrillary tangles and amyloid beta plaques when exposed to *C. pneumoniae*. Studies conducted *in vivo* have demonstrated a link between and viral infections and the accumulation of amyloid beta peptides. The brain of mice infected with HSV-1, *P.gingivalis, C. pneumoniae*, and pseudorabies virus showed noticeably greater amount of Aβ1-42. Rats that were subjected to bacterial infections also displayed elevated Aβ expression. Interestingly, it was shown that HSV-1 infects the hippocampal region more frequently, which is also the location where AD- related Aβ plaque deposition is highest. Cells co-cultured with HSV-1, HSV-2, *P. gingivalis,* or *B. burgdoferi* showed increased intracellular levels of Aβ, according to *in vitro* tests. HSV-1 has also been connected to elevated beta-secretase expression and suppression of the non-amyloidogenic route of APP metabolism[14].

As per the established theory of the aetiology of AD, viruses present in the brain specifically herpes simplex virus (HSV), HSV1 causes oral herpes and HSV2 that causes genital herpes, could contribute to the pathology of AD. According to experimental research in phosphorylation of tau protein, a rise in intracellular amyloid beta-protein, and a decrease in APP. Amyloid plaques in AD frequently contains HSV1 DNA, amyloid plaques and

neurofibrillary tangles have elevated levels of HSV1 binding proteins, ranging from 11 to 15 times higher. In autopsy research on AD, HSV1 DNA was found in 90% of amyloid plaques in 72% of cases. On the other hand, just 24% of HSV1 DNA was linked to plaques in older, normal brains, and there were fewer plaques overall [13].

The DNA Polymerase Inhibitor, which is used to treat HSV1 infections, has shown good safety and tolerability characteristics. This antiviral medication efficiently reduces the HSV1 viral load by stopping viral replication as it is a DNA polymerase inhibitor. DNA Polymerase Inhibitor treatment has also shown to have a protective effect against HSV1-induced neuronal death. Reduced levels of beta-secretase and a gamma-secretase component, which are involved in the metabolism of APP into Aβ, were found to be associated with this condition. Moreover, it has been demonstrated that DNA Polymerase Inhibitor administration can stop neuronal damage caused by HSV-1. To combat the cognitive decline associated with AD, a study has been done to explore the effects of DNA Polymerase Inhibitor on Aβ oligomer-induced spatial cognitive deficits, which revealed that administering DPI along with dexamethasone helped to lessen the deficits in spatial cognition.[14].

An appealing theory explains that the aberrant DNA replication ultimately leads to neuronal death through a noncanonical enzymatic machinery, possibly triggered by the pathological conditions associated with AD. Different enzymes and pathway than those involved in the canonical DNA replication machinery maybe involved in this noncanonical machinery. In this case, DNA polymerase β, an enzyme normally involved in DNA repair, becomes quite essential. Base excision repair (BER), a mechanism that fixes small base lesions in DNA, involves DNA Pol- β. Nonetheless, it seems that DNA Pol-β maybe dysregulated or repurposed in case of AD, engaging in aberrant DNA replication processes as opposed to regular repair mechanisms[71].

Several downstream consequences are seen when DNA Pol- β is inhibited or blocked, including:

Reduced S phase: DNA synthesis takes place during the S phase of the cell cycle. Reduction in DNA Pol- β causes a disturbance in the cell's progression through S phase, which lowers the amount of DNA replication.

Apoptotic death: the process of programmed cell death, or apoptosis, is strictly regulated and essential for maintaining the tissue homeostasis. On the other hand, dysregulated apoptosis in diseases such as AD can result in excessive neuronal death. Inhibition of DNA Pol- β in this process decelerates apoptotic death of neurons[71]. The early involvement of DNA Pol- β in the onset of AD maybe reduced by the administration of DNA Polymerase Inhibitor in treatment.

This study was undertaken to evaluate the neuroprotective activity of DNA Polymerase Inhibitor against scopolamine induced amnesia model in rats. The purpose of this work is to determine if administration of DNA Polymerase Inhibitor can prevent loss of cognition and to elucidate the mechanism through which it confers neuroprotection in scopolamine induced AD.

CHAPTER 2
REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Background

The most prevalent type of dementia is Alzheimer's which is an intriguing demonstration of the link between higher-order cognitive deficits and neurophysiological abnormalities. The pathophysiology and aetiology of AD have been studied since the disease was first identified in 1906. These studies have revealed an incredibly complex set of genetic and molecular mechanisms for the disease's progression, which go far beyond the neuropathological hallmarks of beta- amyloid plaques and neurofibrillary tangles[11].

An early sign of this disease is episodic memory loss, or the inability to recall recent events[15]. The clearest example of this is the inability to learn new knowledge or even remember past conversations. The next stage is a gradual deterioration in other cognitive functions, which may be followed by modifications in how one manages their emotions, their behaviour, and their ability to connect with others. Patients will find it harder and harder to eat, dress themselves, and use the washroom on their own as the disease worsens[16].

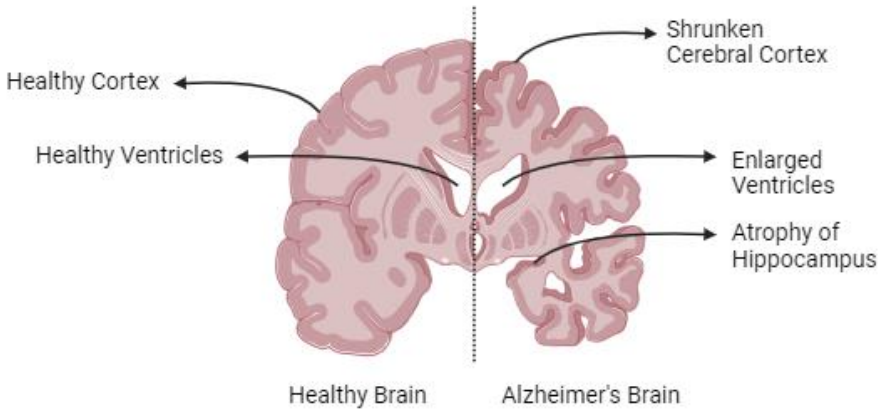


Figure 1. Comparison between a healthy and Alzheimer's brain[66].

2.2 Prevalence

Alzheimer's disease is an age-related disorder primarily associated with elderly populations. The World Alzheimer's Report 2021 states that although the prevalence of

dementia is expected to increase globally from 50 million in 2010 to 113 million in 2050, dementia was currently the sixth largest cause of death globally[17]. Today, an estimated 6.7 million Americans with 65 years of age and older suffer from AD, if medical advancements are not made to prevent, slow or cure AD, this figure may rise to 13.8 million by 2060. In 2019 United States, 121499 fatalities from this disorder were documented in official death certificates. Deaths from heart disease, stroke, and HIV declined between 2000 and 2019, which recorded deaths from AD rose by more than 145%. This increased mortality rate from AD was probably made worse in 2020 and 2021 by the COVID-19 pandemic[11].

2.3 Risk Factors

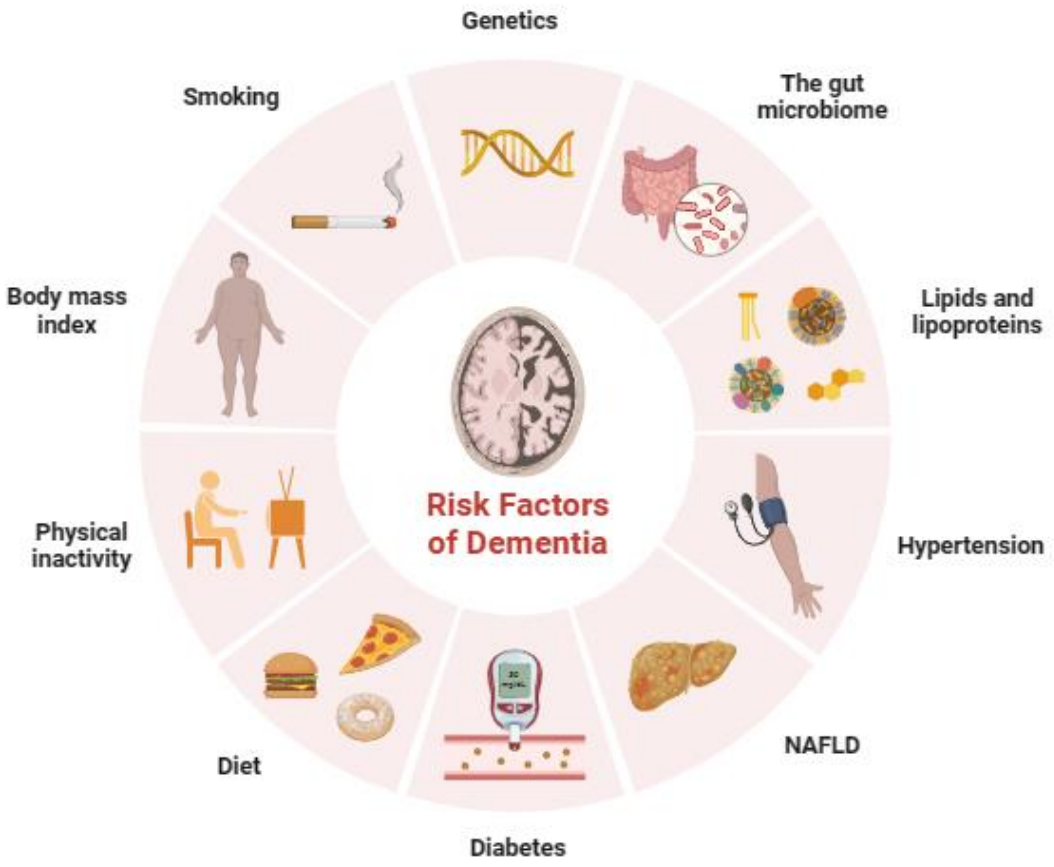


Figure 2: Risk factors for AD[62]

2.3.1 Age

One of the primary demographic factors linked to AD and dementia is age. The risk of getting AD rises steadily with age, emphasizing the disease's progressive character. As people age, the proportion of those suffering from the AD rises sharply. This factor plays a

crucial role for the cause of AD. It affects 5% of adults with age 65-74, 13.1% of people 75 to 84 and 33.3% of people with age 85 years or older [18,19]. According to age-specific incidence rates, the risk of AD doubles for every six years of extra life, suggesting an exponential increase in risk as people age.

2.3.2 Genetics

According to a study, genetic risk factors for AD, such as gene mutations linked to the disease, account for 70% of cases. Under normal circumstances, the soluble isoform Amyloid-β40 is formed; however, if a mutation occurs, the amount of the insoluble isoform, Amyloid-β42, increases and plaques are formed. Because plaques are insoluble, they eventually cause AD. 15% of early-onset AD cases are caused by mutations in the APP gene, of which 85% are caused by Presenilin 1 and 2, which account for 80% and 5% of cases, respectively. Late-onset AD is associated with polymorphisms in the APOE-e4 gene, while early-onset AD is caused by mutations in the Amyloid Precursor Protein gene, Presinilin 1 and 2 genes. Each parent gives birth to one of the three APOE gene types (alleles), which are e2, e3, and e4. This results in the six potential APOE pairs: e2/e2, e2/e3, e2/e4, e3/e3, e3/e4, and e4/e4[20].

2.3.3 Cardiovascular health problems

The significant increase in dementia risk that the stroke, a cerebrovascular event caused by the blocked or burst blood vessel, causes is one of the best examples. The other two variables that raise the risk of dementia are diabetes and hypertension. Patients with excessive cholesterol, and heart illness have increased risks of getting AD. These illnesses harm the blood vessels in the brain, resulting in decreased blood flow and early death of brain cells[21].

2.3.4 Smoking, physical activity and diet

Adding to the evidence supporting the link between heart and brain health, scientists have discovered that heart-healthy practices may also have an impact on brain function and, consequently, dementia risk. One habit that raises the risk of dementia is smoking and on other hand, exercises with physical activities seems to lower the risk. There is growing evidence that eating a heart-healthy diet can also decrease the incidence of dementia. Patients with AD often experience deficits in protein and other nutrients, which can lead to

the production of fibrils due to a lack of calcium and magnesium. Significant vitamin deficits (A, E, D, and K) were also discovered in the plasma of AD patients, indicating that frequent supplementation may be beneficial in reducing beta-amyloid production and memory loss [22,19].

2.3.5 Hypertension

Hypertension is capable of causing changes in the vascular walls which can lead to cerebral hypoxia, ischemia and hypoperfusion contributing to trigger the development of AD. Research studies have provided evidence that cerebral ischemia has the potential to result in the build-up of amyloid precursor protein (APP) and amyloid-beta (Aβ). Furthermore, it has been observed that cerebral ischemia can also induce the expression of presenilin, a protein implicated in the manufacture of Aβ [21].

2.3.5 Obesity

The association between obesity and the development of Alzheimer's disease (AD) remains unclear, as several research have shown inconsistent findings. Based on a meta-analysis conducted, there is a strong and independent association between obesity and the chance of acquiring AD. Conversely, a meta-analysis has demonstrated that obesity throughout middle age is associated with an increased risk of developing dementia. However, in the later stages of life, there appears to be an inverse relationship between obesity and the risk of dementia. The aforementioned writers have also documented a correlation between being below the appropriate weight and an elevated susceptibility to dementia. Weight loss in older individuals is commonly observed alongside various medical conditions and is frequently associated with compromised health. In fact, it has been suggested that weight loss in this population may serve as an early indicator of deteriorating cognitive function, perhaps preceding the onset of dementia within a decade. A separate study undertaken has demonstrated that there is a correlation between both underweight and overweight, as well as obesity, throughout middle age and an increased likelihood of getting Alzheimer's disease in later stages of life[21].

2.4 Types of Alzheimer's Disease

1) Mild AD - At this stage, the temporal, lateral, and parietal lobes have been affected by the sickness. The issue gets worse as the neurodegenerative disease progresses. Before things become worse, it is expected that this condition will endure for two years. The patient's cognitive impairment gets worse as reading, remembering things, and figuring out directions get harder.
2) Moderate AD - In this stage of AD, the prefrontal cortex begins to show signs of neurodegeneration. Similar to the previous phase, this is expected to endure for about two years. The signs of progressive neurodegeneration include the inability to pass judgment, the capability to act and make plans, and the inability to focus on outside stimuli.
3) Severe AD - When neurodegeneration reaches the occipital lobe in the final stage of AD. This causes the patient to experience visual impairments, delusions, and hallucinations in addition to the problems from the previous stages. It is expected to happen in three years. The terrifying patient death occurred.

2.5 Signs and Symptoms

Patients with dementia suffers from behavioural and mental changes. Main signs with Alzheimer’s are the symptoms of depression, delusion, irritability, agitation, changes in sleep pattern, hallucinations, and difficulty with the auditory and visual performances of the patients. Alzheimer’s consists of 7 different stages having different symptoms. Stage 1, where there is no impairment and in stage 2, very earliest signs of forgetfulness may be mistaken for age-related change. No memory loss, but symptoms may be noticeable to close family and friends. Stage 3, has mild cognitive decline but no memory loss to this point. Stage 4, shows moderate cognitive decline (Mild or early-stage Alzheimer’s disease) forgetfulness of recent events, impaired ability to perform challenging work such as complex tasks. Stage 5, moderately severe cognitive decline (Moderate or mid-stage Alzheimer’s disease) whereas in stage 6 and 7 are kind of similar having severe cognitive decline like reduced impairment performance in difficult work settings, complexity at new locations, impaired ability to perform tasks (complex tasks), requires caretaker in clothing etc.

2.6 Pathogenesis

As it is well known, Alzheimer's disease is a neurodegenerative condition with tau protein and amyloid-β serving as its hallmarks. The pathophysiology of AD is a conglomeration of different theories and mechanisms rather than being limited to one.

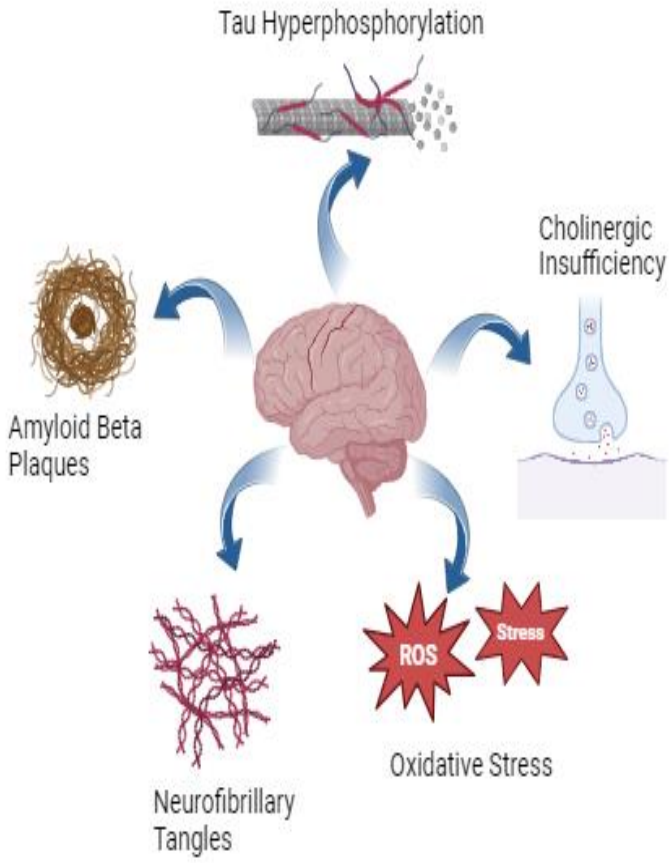


Figure 3: Pathogenesis involved in the progression of Alzheimer's Disease

2.6.1 Amyloid beta hypothesis

Amyloid pathogenesis starts when the integral protein of the plasma membrane, APP is abnormally cleaved by secretases and the β-site amyloid precursor protein cleaving enzyme (BACE1). This results in the formation of insoluble Aβ fibrils. Subsequently, A oligomerizes, diffuses into clefts in synapses, and interferes with synaptic transmission. Consequently, when it polymerizes into insoluble amyloid fibrils, plaques are formed. Neurofibrillary tangles (NFTs) are insoluble because of the hyperphosphorylation of the microtubule-associated protein brought on by this polymerization. The microglia that

surround the plaques become active once plaques and tangles have formed. By encouraging the activation of microglia and a local inflammatory response, this adds to neurotoxicity[23]. The cause of dementia, vascular damage, NFTs, and cell loss is the Aβ protein. There are 39–42 amino acids in Aβ. The precursor of Aβ produces insoluble extracellular deposits. In the void created by the nerve cells, amyloid builds up. APP pass through the membrane of the neuron. β- and α-secretases split the APP into Aβ fragments that are harmful. Many clumps grow in dementia, interfering with neurons' ability to function. It impacts different areas of the cerebral cortex in addition to the hippocampal region. According to Xiong et al. (2020), APP is a type 1 transmembrane glycoprotein that regulates the secretory or endosomal-lysosomal pathway. Different glycosylation's secreted by APP increase due to complicated proteolysis, phosphorylation, glycosylation, and splicing changes[24]. Gene expression function is translocated by APP mutation. In a sick state, it cleaved differently and released Aβ via BACE-1. There are two types of Aβ that cause neurotoxicity and plaque development. Aβ40 is more prevalent and less harmful. Aβ42 is extremely neurotoxic, less common, and insoluble. Aβ degeneration impairs neuronal activity and results in cell death[23].

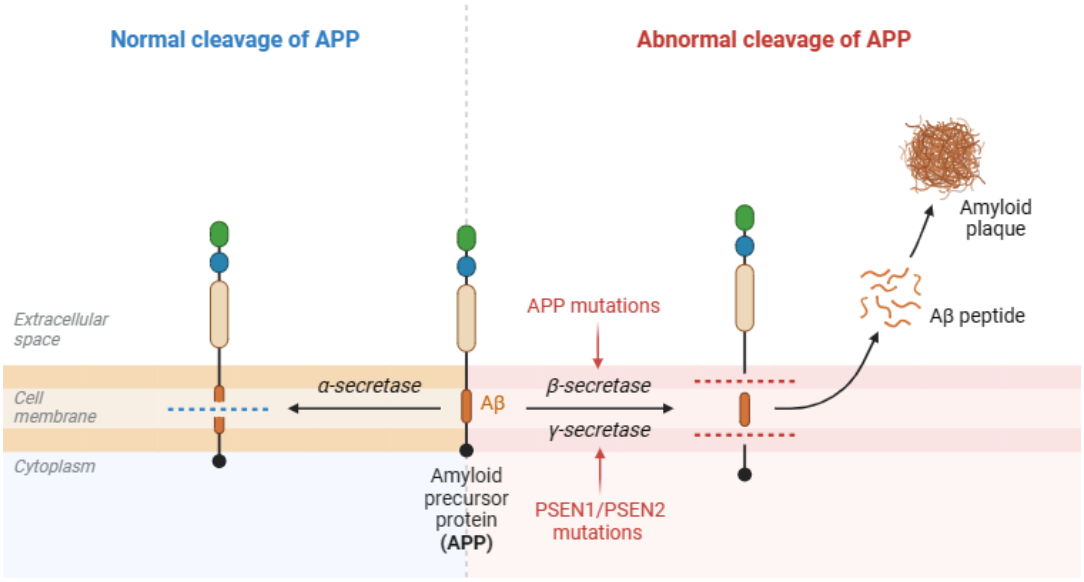


Figure 4: Amyloid beta plaques accumulation[61]

2.6.2 Tau Hypothesis

The Tau Hypothesis describes how Paired Helical Filament (PHF)-Tau forms and how this results in neurofibrillary. The increased phosphorylation of Tau protein is the cause of tangles. Tau protein is a soluble form of microtubule associated protein (MAP), and both phosphorylated tau and other isoforms of Tau are helpful in maintaining microtubule structure.

There are six different types of Tau, when a mutant version of Tau experiences hyperphosphorylation, it causes microtubules to break apart and transforms ubiquitin, MAP-1, MAP-2, and normal Tau into insoluble formations (PHF), which eventually result in neurofibrillary tangles. Because these tangles are intractable, they affect axon-related and cytoplasmic activities, which eventually results in neuronal death[25]

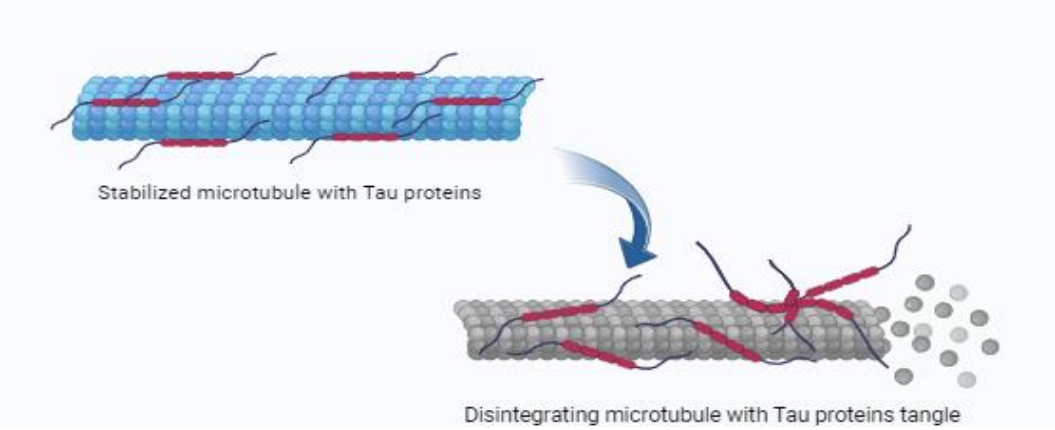


Figure 5: Tau Pathogenesis[63]

2.6.3 Cholinergic Hypothesis

Acetylcholine is the neurotransmitter in a normal person that is in charge of cognition and regular memory function. Additionally, acetylcholine plays a role in protecting neurons against glutamate excitotoxicity. The acetylcholinesterase enzyme is responsible for the metabolism of this acetylcholine. When acetylcholinesterase acts on acetylcholine in healthy humans, acetylcholine metabolizes into acetate and choline groups. Through nerve terminals, choline acetyltransferase (ChAT) reabsorbs choline that has been produced during metabolism. After that, this is used with acetyl CoA to create new acetylcholine molecules. When taking into account AD, there is an imbalance between these two enzymes, i.e., an increase in Acetylcholinesterase and a decrease in ChAT, which

eventually leads to lower levels of Acetylcholine as metabolism rises relative to formation. Studies have shown that people with AD experience a reduction of up to 90% in acetylcholine from normal levels[26,27].

Three major discoveries—a reduction in cholinergic indicators, neurodegeneration in the nucleus basalis of Meynert, and the effect of cholinergic medications on memory—supported the cholinergic theory, which transformed research on Alzheimer's disease by emphasizing the significance of synaptic neurotransmission. This notion is the main focus of research on Alzheimer's disease and led to the creation of cholinergic therapies. The presence of neurofibrillary tangles (NFTs) in Ch4 neurons, specifically Ch4al and Ch4p, is linked to the severity of AD, indicating an early involvement of nbM. M1 agonists, such as AF267B, have the potential to reverse cognitive decline by decreasing anomalies related to tau and Aβ. By blocking butyl cholinesterase and acetylcholinesterase (AChE), medications such as galantamine, rivastigmine, and donepezil improve memory. These drugs activate PI3K/Akt signalling via stimulating NAChR, which raises Bcl-2 expression and stops nerve cell death. NAChR hypoactivation results in GSK-3 activation, increasing tau phosphorylation and neuronal death.

2.6.4 Neurofibrillary Tangles (NFTs)

Microtubules comprise the internal support structure of neurons. These phosphorylated microtubules are stabilized by tau protein. Tau protein clusters together to create NFTs when tau mutations or hyperphosphorylation cause microtubules to collapse in dementia. NFTs are segments of paired and helically wound protein filaments, along with the cytoplasm of the neurons' cells. The microtubule-binding domain of the tau protein allows it to combine with developed, stable microtubules are produced by tubulin. In order to create the proper, stable network of microtubules and maintain their connectivity, these microtubules have the ability to stabilize other microtubules and build bridges between neighbouring ones. Since tau protein is hyperphosphorylated, it interacts with the kinase pathway and forms oligomers[28].

The tubule becomes unstable as a result of tubule subunits dissociating, breaking off into large filament fragments that reassemble into NFTs. These NFTs are very intractable areas in the cytoplasm of neurons that are fibrillary and straight. They result in the erroneous loss of signal processing, the inappropriate loss of neural transmission, and ultimately, the death

of individual neurons. Reports state that soluble amyloid controls phosphorylation and cleavage to create NFT. Furthermore, a variety of kinases that regulate phosphorylation include cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3), which are both activated by extracellular A. While the primary kinases involved in hyperphosphorylation are GSK3 and CDK5, Aβ may also activate additional kinases that play significant roles, such as Protein Kinase A, Protein Kinase C, a serine/threonine kinase, ERK2, caspase 3, and caspase 9[23,28].

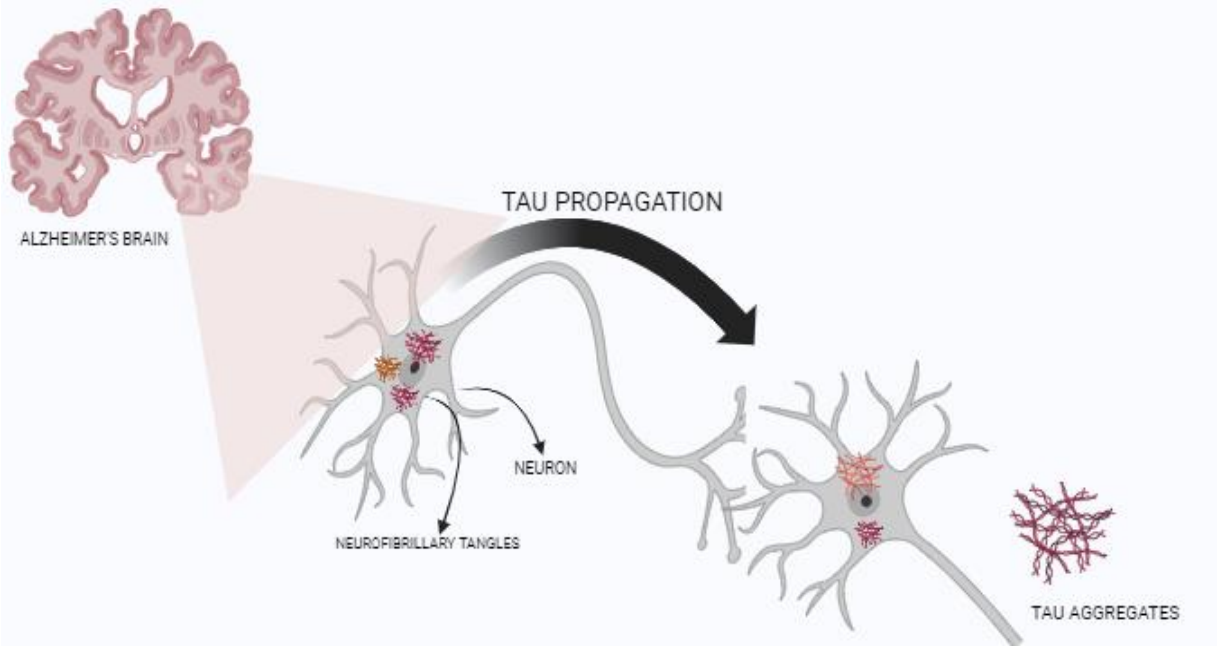


Figure 6: NFT pathogenesis[78]

2.6.5 Oxidative stress

The pathophysiology of AD is either directly or indirectly associated with oxidative stress. In one way or another, AD is mediated by oxidative stress. Reactive oxygen species (ROS) are largely formed as a result of oxidative stress. Oxidative stress is inevitable and increases in direct proportion to age. Oxidative stress rises with age, and this raises the risk of several brain diseases, including neurodegenerative disorders. The formation of Tau neurofibrillary tangles or amyloid senile plaques, or damage or dysfunction to the mitochondria, can all contribute to the reactive oxygen species (ROS) produced by oxidative stress. This creates a vicious cycle of events that makes the neurodegeneration more severe and potent, which advances AD[29].

According to experimental evidence, a major factor in the early stages of Alzheimer's disease (AD) is a change in the redox state, which triggers cell signalling pathways that lead to neurodegeneration. In hippocampus neurons of AD patients, oxidative stress can cause mitochondrial malfunction and metabolic problems. Mitochondria are essential for ATP synthesis and the creation of ROS. A deficiency in cytochrome oxidase leads to increased generation of reactive oxygen species (ROS) and poor storage of energy, exacerbating this oxidative stress. In addition, oxidative brain damage, increased Aβ build-up, and neurofibrillary tangles are seen in AD patients. Toxic Aβ aggregates are a result of high zinc concentrations in AD-affected brain regions. Zinc homeostasis is disrupted by immune/inflammatory responses to non-soluble Aβ plaques, which results in uncontrollably released zinc and oxidative stress. In addition, oxidative stress in Alzheimer's disease can be made worse by other bio-metals like iron and copper.

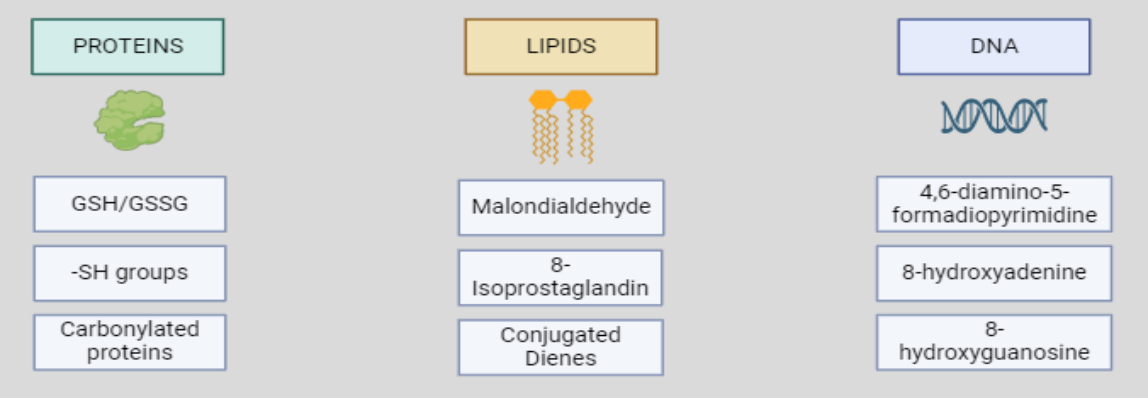


Figure 7: Various oxidative damage markers in brain for Alzheimer's disease[70]

2.7 Approved treatments for AD

2.7.1 Donepezil

It has been suggested that donepezil can cure all forms of AD by changing the way acetylcholine is broken down in the brain. It is the most reversible and selective antagonist against AChE. A single daily dosage is permitted due to the pharmacological profile and extended half-life. In 1998, research published in the Archives of Internal Medicine assessed 468 patients' responses to the treatment. The drug donepezil is a member of the acetylcholinesterase inhibitor class. Since it is non-competitive, this kind of inhibitor is

non-reversible. The half-life of donepezil is 70 hours. For the treatment of AD, the recommended dosages of donepezil are 5 mg and 10 mg[30,31].

2.7.2 Galantamine

Galantamine is a reversible acetylcholinesterase inhibitor with a dual effect, in contrast to donepezil. Through the inhibition of acetylcholinesterase, galantamine raises acetylcholine levels and enhances its effect on nicotine receptors. The recommended effective dose for the treatment of AD is 16–24 mg/kg. Galantamine has a half-life of six to eight hours and a plasma protein binding percentage of 28% to 33% [32,33,34]. Hansen and colleagues performed a meta-analysis of AD treatments and found that galantamine can lessen cognitive impairment with a small number of adverse effects[35].

2.7.3 Rivastigmine

When treating mild-to-moderate AD, this medication is prescribed far less frequently than other cholinesterase inhibitors. Testing was done at various intervals (12, 18, and 26 weeks) for the lowest dose, which was 1 to 4 mg/day, and the highest dose, which was 6 to 12 mg/day. Overall time periods, the group receiving the greater dosage shown the most improvement in their scores on cognitive tests and activities of daily living. After 26 weeks of treatment, the lowest dose showed improvement but was unable to change their everyday routine[36,37].

2.7.4 Memantine

Memantine is a medication that falls under the class of NMDA (N-Methyl D-Aspartate) receptor blockers. Memantine inhibits glutamate impulses at NMDA receptors, which is surprising given that other medications in the same family have negative effects on Alzheimer's disease. According to research, memantine even has neuroprotective properties in addition to being a validated symptomatic treatment with 5-10mg/day[38].

Table1: Pharmacology of available treatments

Drugs	Mechanism of Action	Dose	Toxicity
Donepezil	Acetylcholinesterase Inhibitor	5-10mg/day	"Seizures, insomnia,

			fatigue, chest pain, hypertension, atrial fibrillation, nausea, vomiting, bleeding, weight loss"
Rivastigmine	Acetylcholinesterase Inhibitor	1.5-12mg/day	"Dizziness, confusion, nervousness, paranoia, hypertension, chest pain, oedema, back pain, bone fractures, bronchitis, cough"
Galantamine	Acetylcholinesterase Inhibitor	4-24mg/day	"Depression, dizziness, fatigue, insomnia, bradycardia, diarrhoea, nausea, anorexia, abdominal pain, anaemia"
Memantine	NMDA receptor Blocker	5-10mg/day	"Stroke, aggressiveness, agitation, fatigue, confusion, pain,

			syncope, heart failure, oedema, anorexia, constipation, nausea, vomiting"

Table 2: Drugs in clinical trials for Alzheimer's Disease

Drug	Phase	Year of Completion	Summary	Outcomes
Valacyclovir NCT02997982	Phase II	2020	• Including patients with positive HSV and have Hetero or Homozygote for allele 4 of gene Apolipoprotein E. • Observing the effect of 4 weeks of oral valaciclovir in 36 APOE4 carriers with AD or with amnesic mild cognitive impairment.	• CSF acyclovir concentrations were mean 5.29 ± 2.31 μmol/L. • CSF total tau and neurofilament light concentrations were unchanged; MMSE score and CSF soluble triggering receptor expressed on myeloid cells 2 concentrations increased.

				• Four weeks of high-dose valacyclovir treatment was safe, tolerable, and feasible in early-stage AD.
Sustiva Pill (Efavirenz) NCT03706885	Phase I	2022	• Participants should be carriers of the APOE E4 allele. • Participants will be genotyped for the APOE isoform status (E2, E3, or E4) and presence of the SNPs rs754203 and rs3745274 in CYP46A1 and CYP2B6, respectively.	• In subjects receiving efavirenz, there was a statistically significant within-group increase ($P \leq 0.001$) in the levels of plasma 24HC from baseline. The levels of 24HC in the CSF of subjects on the 200-mg dose of efavirenz were also increased. • Findings suggest efavirenz target

				engagement in human subjects with early AD.
(-)-L-2',3'-dideoxy-3'-thiacytidine (3TC) NCT04552795	Phase I Phase II	2024	• Evaluate the ability of 3TC to engage its intended target, penetrate the CNS, suppress neurodegeneration, and assess safety and tolerability in patients with early-stage AD.	• Glial fibrillary acidic protein (GFAP) (P=0.03) and Flt1 (P=0.05) were significantly reduced in CSF over the treatment period; Aβ42/40 (P=0.009) and IL-15 (P=0.006) were significantly elevated in plasma. • 3TC is safe and well-tolerated among participants, and gains access to the CNS.
Valacyclovir NCT03282916	Phase II	2024	• Patients must test positive for serum	• Ongoing status

			antibodies to HSV1 or HSV2. • Comparing the effect of valaciclovir for 18 months in 65 treated participants and 65 controls, all with mild AD.	

2.8 Diagnostic Tools in AD

Early intervention and successful treatment of neurodegenerative disorders such as dementia depend on the ability to identify patients in the early stages of the ailment. Preventing cognitive decline and brain degradation can be greatly enhanced by early detection and treatment of Alzheimer's disease. The emergence of in-vivo biomarkers along with other recent developments in comprehending the natural progression of AD have significantly enhanced diagnostic capabilities.

The diagnosis of AD is based on neuropathological assessment, which measures disease related alterations. The National Institute on Aging and the Alzheimer's Association revised their guidelines in 2012, emphasizing the use of semi-quantitative measurements for the neuritic plaque score, amyloid phase, and NFT stage. Neuroimaging is used in clinical diagnosis to track biomarkers in CSF, such as Aβ peptides and certain proteins. Although amyloid accumulation can be seen on PET scans, differences between CSF markers and PET scan results are still a cause for concern.

While certain brain regions exhibit damage later in the course of AD, whereas, changes in topographic pattern in the medial temporal lobe specifically the entorhinal and perirhinal cortex and the hippocampus, appears early. Targeting specific brain regions, these unique patterns have prompted the development of imaging tools for early AD diagnosis[39,40].

2.8.1 Magnetic Resonance Imaging

Certain brain regions are initially affected by degenerative changes in AD, while others become significant in later stages. For instance, neuronal damage causes the hippocampus to lose volume, thereby utilizing volumetric MRI scans to assess hippocampal volume has emerged as a commonly accepted technique for diagnosing AD pathology. Projected volumes show a significant association with neuronal counts, confirming the anatomical precision of volumetric MRI measurements[42].

2.8.2 Amyloid-PET Scan

Many specialists believe that the first pathogenic event in the AD is the accumulation that results in the formation of senile plaques. Two markers, amyloid-PET scan and A-42 in the CSF, are frequently used to evaluate AD pathology. This neuroimaging method is used to find the location and amount of Aβ plaques in the brain. A radioactive tracer attaches particularly to the Aβ plaques in the brain during an amyloid-PET scan. The radioactive signals are subsequently detected by the scan, which results in the images that indicate the location and degree of amyloid build-up. It is crucial to remember that amyloid-PET scans are not yet commonly used for screening or tracking the efficacy of treatment; rather, they are largely utilized as a diagnostic tool[43,44].

2.8.3 FDG-PET Scan

Numerous disorders affecting the CNS are linked to reduced absorption of glucose by neurons. As a measure of neuronal activity, FDG-PET, which stands for Fluorodeoxyglucose positron emission tomography, can evaluate how the brain uses glucose at rest. FDG-PET is a radiolabelled glucose analogue used to measure synaptic transmission and metabolic activity. The FDG-PET endophenotype, which is a particular pattern of metabolic abnormalities in AD, can be distinguished by comparing the results of the scans between AD patients and their age-matched healthy counterparts. In addition, FDG-PET is considered more diagnostically useful than volumetry measures using structural MRI[46,47].

2.9 Pharmacology of drug under investigation

2.9.1 DNA Polymerase Inhibitor

2.9.1.1 Mechanism of action

In order to prevent further synthesis and hinder viral replication, DNA Polymerase Inhibitor works as an antiviral drug by integrating itself into viral DNA. It interferes with DNA synthesis upon its conversion to DNA Polymerase Inhibitor triphosphate by both viral and cellular enzymes. In both lab and living organisms, this synthetic purine nucleoside analog exhibits inhibitory effect against varicella-zoster virus and herpes simplex virus types 1 and 2 (HSV-1, HSV-2).

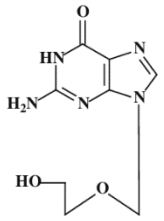
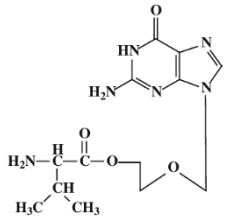
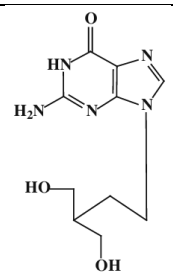
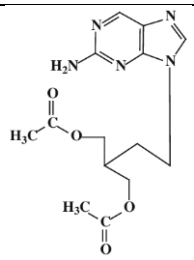
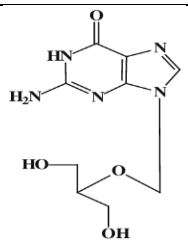
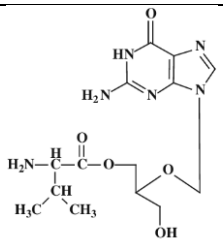
HSV resistant to DNA Polymerase Inhibitor are uncommon in immunocompetent patient; less than 1% of people have this resistance, with the exception of ocular infections. However, DNA Polymerase Inhibitor resistant HSV infections are more common among immunocompromised individuals, such as those receiving hematopoietic stem cell transplants[48].

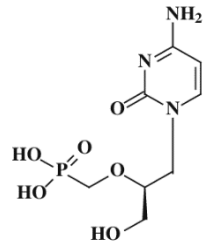
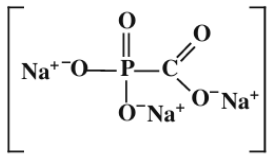
Various steps involved in the mode of action of DNA Polymerase Inhibitor are the following:

- Entry: DNA Polymerase Inhibitor enters infected cells by crossing their membrane transporters when given orally or intravenously. Viral thymidine kinase (TK), and enzyme found in herpesviruses but absent in uninfected cells, then phosphorylates it.
- Phosphorylation: DNA Polymerase Inhibitor is first phosphorylated to form DNA Polymerase Inhibitor monophosphate. Cellular enzymes, specifically guanylate kinase and nucleoside monophosphate kinase, phosphorylate DNA Polymerase Inhibitor monophosphate further to generate DNA Polymerase Inhibitor diphosphate.
- Activation: The active version of the medication, DNA Polymerase Inhibitor triphosphate, is formed when DNA Polymerase Inhibitor diphosphate undergoes an additional phosphorylation process that is catalysed by cellular enzymes.

- Inhibition: Viral DNA polymerase incorporates DNA Polymerase Inhibitor triphosphate and deoxyguanosine triphosphate (dGTP) in competition for inclusion into the viral DNA. Once integrated, it functions as a chain terminator to prevent the viral DNA strand from getting any longer, which then functions as an efficient inhibitor of viral replication[48].

2.9.1.2 Classification of DNA Polymerase Inhibitors[69]

A. Nucleoside Analogues		
Drug	**Prodrug**	**Indications**
 Acyclovir	 Valacyclovir	• Herpesviruses (HSV-1, HSV-2, VZV, HCMV, EBV, HHV-6)
 Penciclovir	 Famciclovir	• Herpesviruses (HSV-1, HSV-2, and VZV)
 Ganciclovir	 Valganciclovir	• Herpesviruses (HSV-1, HSV-2, and HCMV)
B. Nucleotide Analogues		

 Cidofovir	• Herpesviruses (HSV, VZV, HCMV, EBV, etc.), papilloma-, polyoma-, adeno-, and poxviruses.
C. Pyrophosphate Analogue	
 Foscarnet	• Herpesviruses (HSV, VZV, HCMV, etc. and also HIV)

2.9.2 Experimental Animal Models

2.9.2.1 Scopolamine

Scopolamine hydrobromide typically appears as colourless crystals, white powder, or solid, devoid of any discernible odour. It is also referred as hyoscine or Devil's Breath, which is either chemically produced or taken from natural sources. It is an anti-cholinergic drug and a member of the tropane alkaloid family which can be used to reduce motion sickness symptoms, nausea and postoperative vomiting. Even though scopolamine was first synthesized in 1959, the process of synthesis is still less effective than the plant extraction method. Scopolamine's molecular makeup is indicated by its chemical formula, C17H21NO4 with molar mass of 303.358g/mol.

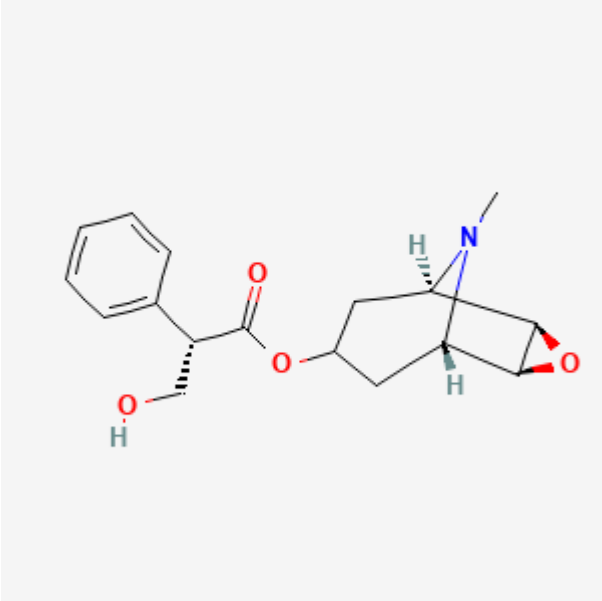


Figure 9: Chemical structure of scopolamine

2.9.2.1.1 Mechanism of action

Scopolamine acts as a non-selective competitive inhibitor of muscarinic acetylcholine receptors (MAChRs) M1-M5, having weaker inhibition towards M5. Based on this property it is categorized as anticholinergic agent. Depending on the dosage, this compound yields both therapeutic and adverse effects. Despite its widespread use, the exact mechanism is still not fully understood. Recent studies reveals that it antagonistically affects M1(and potentially M2) MAChRs, especially at interneurons, which inhibits neurotransmitter release downstream and activates pyramidal neurons as a result. This mechanism is thought to mediate the brain reactions associated with depression and stress.

Moreover, scopolamine's antagonistic action on M4 and M5 receptors is linked to possible therapeutic advantages in the treatment of neurological conditions like schizophrenia and drug abuse disorders. It is unclear, nevertheless, how directly these findings relate to the existing therapeutic indications of scopolamine, particularly in terms of avoiding nausea and vomiting. Scopolamine's anticholinergic action and its capacity to alter CNS signals, however, are thought to be responsible for some of its effectiveness in controlling vomiting.

2.9.2.1.2 Pharmacokinetics

2.9.2.1.2.1 Bioavailability and Absorption

The bioavailability of scopolamine after oral administration is very limited; levels usually recover to baseline, five to six hours after the dose. Certain pharmacokinetics parameters were observed following the oral administration of 0.5mg scopolamine in 150ml water to a group of healthy volunteers. The results showed (geometric mean ± geometric SD) tmax = 23.5 ± 8.2 minutes, Cmax = 0.54 ± 0.10 ng/mL, t½ = 63.7 ± 1.3 minutes, AUC0–24h = 50.77 ± 1.76 ng·min/ml, and F = 13 ± 1%. In contrast, 0.5mg of scopolamine IV administration over a 15 min period resulted in an approximate Cmax = 5.00 ± 0.43 ng/ml, tmax = 5min and AUC = 369.4 ± 2.2 ng·min/mL.Scopolamine is rapidly absorbed from different muscles, one of which is deltoid. The intramuscular injection showed the Cmax of 0.96 ± 0.17 ng/mL, 18.5 ± 4.7 minutes tmax and AUC of = 81.27 ± 11.21 ng·min/ml.

2.9.2.1.2.2 Distribution

Scopolamine's volume of distribution (Vd) has been investigated by IV infusion, specifically by administering 0.5mg of drug over a 15-minute period. The resulted Vd of around 141.3±1.6 L was obtained using this method. It's important to note that, Vd of scopolamine might not be exactly characterized due to various factors such as its complex pharmacokinetics and individual variability.

2.9.2.1.2.3 Metabolism

Although several metabolites have been identified in animal research, little is known about the metabolic routes of scopolamine in humans. Scopolamine is normally metabolized in the liver, where it mostly generates different conjugates of glucuronides and sulphides. Conducted lab researches have shown that oxidative demethylation, linked to the activity of CYP3A subfamily, might be linked in the metabolism of drug.

Scopolamine metabolism has been shown to entail oxidative demethylation, which may be related to CYP3A activity, according to the in vitro studies. This notion is supported by clinical observations that indicates significant changes in drug pharmacokinetics when grapefruit juice, a known inhibitor of CYP3A4, is co-administered. These results imply that CYP3A4 enzyme may be involved in at least some of the oxidative demethylation of scopolamine.

2.9.2.1.2.5 Excretion

Approximately 2.6% of scopolamine that is administered orally is eliminated unaltered in urine. On the other hand, less than 10% of the whole dose, which includes both the unmodified drug and its metabolites, is recovered in urine over a 108-hr period when it's administered via transdermal patches. Specifically, less than 5% of the total dose is found unchanged in urine.

2.9.2.1.2.6 Toxicity

Many symptoms, such as disorientation, hallucinations, dry mouth, lethargy, visual disturbances, high B.P, retention of urine, faeces and irregular heart rhythm can result from scopolamine overdose. Sometimes these symptoms can be mistaken for those that occur after drug withdrawal.

2.9.2.2 Streptozotocin

Streptozotocin (STZ) is produced from glucosamine nitrosourea and is naturally found in Streptomyces achromogenes. ICV administration of STZ in rodents has been shown to elicit significant modifications on brain structure, biochemical, metabolic and functional characteristics. These effects include decreased uptake of glucose and energy expenditure, elevated oxidative stress in tissues, changes in cholinergic activity, and compromised cognitive performance. The induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is linked to the hyperphosphorylation of tau protein and neuronal damage caused by STZ treatment. Research conducted on rats administered 3mg/kg of STZ ICV has shown decreased levels of Ach and increased AChE enzyme in the brain. Furthermore, STZ's regulation of GSK α/β has been linked to the activation of amyloid beta peptides aggregates[9].

2.9.2.3 Aluminium Chloride

Aluminium is one of the heavy metals that are known to present serious health hazards, especially when exposed to them for an extended period of time. Specifically, Al is present in many industries and environmental settings, and its effects on biological systems have been well-researched. Numerous health problems, including anaemia, osteomalacia, encephalopathy and neurological abnormalities, are linked to it. Interestingly, elevated

amounts of Al have been found in the brain of people suffering from AD, which has been linked to harmful consequences. According to recent research, oral Al administration causes oxidative stress, cholinergic dysfunction and build-up of neurofibrillary tangles along with amyloid beta in the rat's brain[9].

2.9.2.4 Colchicine

Colchicine has been found as a possible drug for producing dementia by damaging cholinergic neurons, thanks to recent breakthroughs in modelling Alzheimer's disease. It is believed to inhibit cholinergic pathways, which reducing cholinergic activity, especially in the hippocampus, and impairs cognition. Reduced levels of serotonin, dopamine, and norepinephrine in regions such as the caudate nucleus, hippocampus, and cerebral cortex may lead to memory impairment as a result of this neurotoxic effect. Colchicine ICV administration (7.5 g in 10L) has been demonstrated to replicate cognitive memory deterioration in rats and mice, with notable abnormalities evident within two weeks of induction. Mice can experience impairment in their spatial memory even at lower doses (3g). this model is useful for research because it mimics some of the symptoms of sporadic AD, including time-dependent behavioural changes and alterations in biological markers[9].

2.9.2.5 Okadaic acid

Okadaic acid (OKA) is known for its protein phosphatase 2A (PP2A) inhibition and cause tau protein hyperphosphorylation. An important feature of the pathogenesis of AD is the hyperphosphorylated tau protein, which plays a role in the development of intraneuronal neurofibrillary tangles. In animal models, ICV injection of OKA causes neurotoxicity characterized by mitochondrial dysfunction and oxidative stress. However, it's important to note that this model does not replicate the Aβ pathology as observed in AD. Administration of 200ng OKA through ICV bilaterally into hippocampus of male Wistar rats, followed by exposure to hypoxic conditions (10%) for 3 days. This combination model showed an increase in Aβ and an induction of tau hyperphosphorylation.

2.9.3 Mechanism of DNA Polymerase Inhibitor in Alzheimer's treatment

DNA Polymerase Inhibitor is used in the treatment of viral infections and are recognized as DNA polymerase inhibitors. Its application in Alzheimer's disease is, however, speculative and has not been shown in clinical practice. The mechanism works on the cholinergic theory of AD, where Ach neurotransmitter levels are reduced due to the increased action of enzyme AChE. The drug may block the enzyme from breaking down the Ach and thereby increasing its concentration in the synapse. Medications known as cholinesterase inhibitors are already in current use and are quite popular line of drugs for AD.

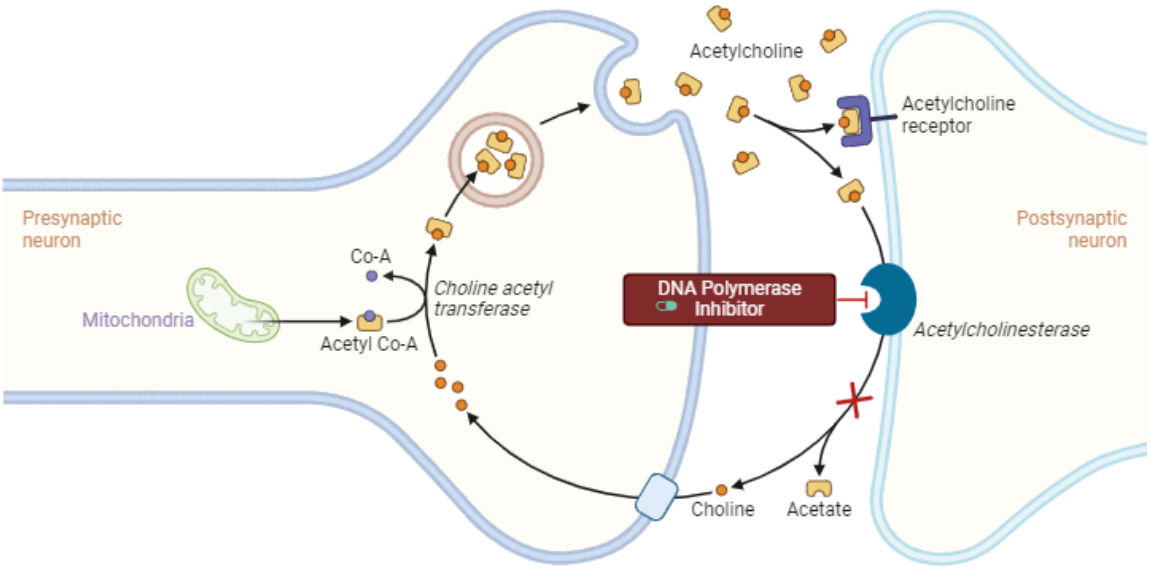


Figure 10: Mechanism of DNA Polymerase Inhibitor in treatment of AD

CHAPTER 3

AIM AND OBJECTIVES

3.1 Aim

The present study aims to evaluate the neuroprotective activity of DNA polymerase inhibitor against scopolamine-induced amnesia model in rats.

3.2 Objectives

- To evaluate the neuroprotective action of DNA Polymerase Inhibitor in AD rat model.
- To investigate the mechanism of action of DNA Polymerase Inhibitor against scopolamine-induced AD in rats.

CHAPTER 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Experimental animals

Healthy Female Sprague Dawley rats were used for experiment protocol. Rats with weight range 175-275 gm were kept under carefully controlled conditions of temperature (20±2°C), humidity (60±5%) and 12-12h light and dark cycle. Standard laboratory rat chew and unlimited access to filtered water were provided. As mandated by the CCSEA, Good Laboratory Practise (GLP) standards for animal care and housing have been meticulously followed throughout the study. Rats were divided in to 6 groups of 5 rats equally.

4.2 Drug, chemicals, and materials:

Scopolamine was received as a gift sample from Zydus lifesciences, Donepezil from Intas Pharma, and DNA Polymerase Inhibitor from Cipla.

4.3 Ethical statement for protocol:

According to guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Social justice and Empowerment Government of India, the institutional animal ethics committee (IAEC) of Nirma University, Ahmedabad approved the experimental protocol number IP/PCOL/MPH/35/2023/08.

4.4 Experimental protocol:

Rats were kept in isolated surroundings and acclimatised before being used in the experiment. The rats were initially (Day -7 to -1) trained for neurobehavioral paradigms i.e., Morris Water Maze, and Novel Object Recognition. The disease was induced from Day 0 to Day 15 by administering scopolamine 2 mg/kg intraperitoneally (I.P) which was followed by 28 days treatment with standard treatment (donepezil) and test drug (DNA polymerase inhibitor) orally along with scopolamine intraperitoneally (IP). At the end of the protocol, the neurobehavioral paradigms were evaluated and finally, the rats were sacrificed and brain samples were isolated for biochemical estimations, histopathology, and immunohistochemistry.

4.5 Treatment protocol:

The Sprague Dawley female rats weighing 175-275 gm were divided into 6 groups randomly. Normal control groups were treated with saline. Disease control group treated with scopolamine (IP) (2mg/kg) for 42 days. DPI-1 treatment: Disease treated with DNA Polymerase Inhibitor at the dose of 87mg/kg PO; DPI-2 treatment: Disease

treated with DNA Polymerase Inhibitor at the dose of 130mg/kg PO; DPI-3 treatment: Disease treated with DNA Polymerase Inhibitor at the dose of 173mg/kg PO. The disease was induced from Day 0 to Day 15 by administering scopolamine which was followed by 28 days of treatment with standard treatment (donepezil) and test drug (DNA polymerase inhibitor) orally along with scopolamine IP.

4.6 Behavioural parameters:

4.6.1 Morris Water Maze Test

The Morris water maze is a popular tool for hippocampal-dependent memory and is used to test an animal's spatial memory. The calm, enclosed, and controlled light room was used for this test. In a test room, rats were trained to swim in a pool for four days. The pool of clear water is kept at a temperature of 25 ± 1 °C, and a platform is positioned around its perimeter using visual cues. Rats were carefully positioned with their backs to the water pool in four different positions for each trial. There are four beginning points for the maze: North, South, East, and West. Every rat was kept on the same platform for every trial. Each animal is allowed 60 seconds to locate a platform. Rats were permitted to remain on the platform for 10 seconds. Four experiments were carried out at 24-hour intervals. Following the first experiment, water was dyed blue or green to make it opaque, and the rat was given a platform that was different from the one used on the first trial. Rats were given a four-day trial period during which they had to become familiar with the changing placement of the platform. Note how long it takes each rat to get to the platform. In the event that the rats were unable to locate the platform, the quantity of paths they took was noted. The amount of time spent on platform and the mean escape delay were noted[49].

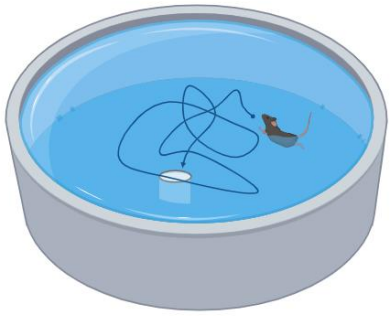


Figure 11: Morris water maze test[64]

4.6.2 Novel Object Recognition

The novel objective recognition test (NOR), also known as the objective recognition test (ORT), is a rapid and somewhat efficient way to assess working memory and learning in addition to visual perception. A rectangular box of 100 cm in width, 100 cm in enclosing depth, and 35 cm in height was used for this test. Three parts make up the test protocol: familiarization, testing, and habituation. During the familiarization phase, each animal is exposed to two familiar objects for five minutes, whereas in habituation, each animal is permitted to wander the arena for ten minutes without an object. The rats were given permission to spend five minutes in the testing arena after a day in which one familiar object was swapped out for a new one. The rats were given permission to spend five minutes in the testing arena after a day in which one familiar object was swapped out for a new one. The amount of time spent touching or sniffing an object determined the recognition object's score. The ratio of time spent on unfamiliar things to time spent on familiar objects is used to measure the performance of recognition. The formula [time in novel object / (time in novel object + time in old object) ×100] was used to determine the discrimination index[50]

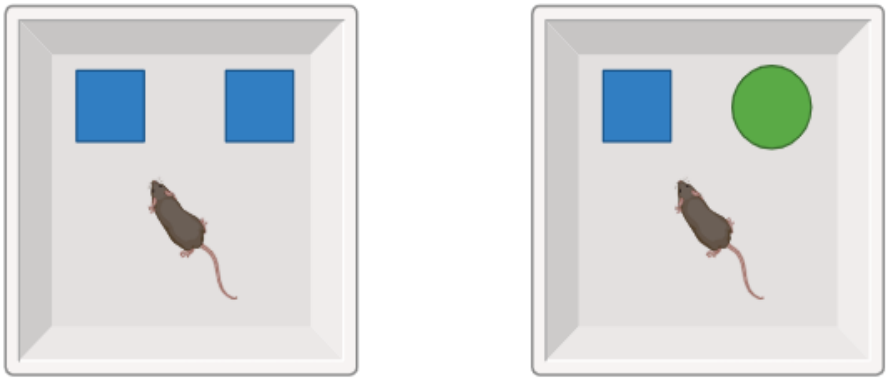


Figure 12: Novel object recognition test[65]

4.7 Biochemical Parameters

4.7.1 Brain homogenate preparation

During sacrifice, brains of all the female rats were collected, weighed, and promptly stored at -80ºC to ensure optimal preservation. The brains were then homogenized using a homogenizer in PBS that had been adjusted to 7.4 pH. Following homogenization, the resultant mixture was centrifuged at 4000 rpm for a duration of 30 minutes. After centrifugation was completed, the supernatant was carefully collected and divided into aliquots for subsequent analysis of various parameters[51]

4.7.2 Total Protein Estimation

Proteins emits an intensive violet-blue complex when they are exposed to copper salts under an alkaline medium. Iodide is used as an antioxidant in this test. The intensity of the resultant colour formed is directly correlated to the total protein concentration present in the sample[77].

Reagents Required-

Reagent I	Biuret Reagent
Protein Standard	6g/dl (stored at 2-8°C)

Procedure:

- Firstly, all reagents, standard, samples were bought to the room temperature 18-28°C, prior to analysis.

	Blank	Standard	Test
Reagent I	1000microL	1000microL	1000microL
Standard	-	20microL	-
Sample	-	-	20microL

- Mix well, incubate for 10 min at temperature of 20-25°C. Measure absorbance of sample and standard against reagent blank.
- Calculation- Total protein g/dl = [Abs. of sample (AT) / Abs. of standard (AS)] *Standard value (6 g/dl)
- Limit of detection: The limit of detection for Total protein is 0.1 g/dl[77].

4.7.3 Acetylcholinesterase assay

The technique utilized to measure the activity of AChE in the homogenate is termed as photometric method, in which enzymatic activity is tracked by identifying the increased yellow colour produced by the reaction of thiocholine with dithiobisnitrobenzoate ion. The key steps involved in this approach are the hydrolysis of acetylthiocholine by AChE to produce thiocholine and acetate, and the subsequent interaction between thiocholine and dithiobisnitrobenzoate ion generates yellow coloured product. Using a photometer, the rate of colour development is measured spectrophotometrically at 412 nm. It has been demonstrated that the thiol reaction proceeds rapidly enough to not impose limitations on the rate of the enzyme's activity measurement, and the concentrations employed do not impede enzymatic hydrolysis. Comprehensive assay records can be obtained by continuously recording photometer outputs[52].

Reagents Required:

Phosphate Buffer Solution	0.1 M
Solution A	5.22gm K2HPO4 + 4.68gm NaH2PO4 in 150ml of DW
Solution B	6.2g NaOH, dissolved in 150ml DW Solution B then mixed in solution A to get the desired pH (pH 8.0 or 7.0) and volume adjusted to 300ml with DW
DTNB Reagent	39.6mg of DTNB + 15mg NaHCO3 in 10 ml of 0.1M PBS (pH 7.0)
Acetylthiocholine	21.67 mg of acetylthiocholine, in 1ml PBS

Procedure:

- To prepare the sample, PBS was adjusted to the pH 8 by dissolving 6.2 gm of NaOH in 150ml of distilled water. 0.05 ml of supernatant was diluted with 3ml of PBS and mixed with 0.1ml of acetylthiocholine.
- Lastly, 0.1 ml of Ellman's reagent was added, which is frequently used in assays to measure substances that include thiols.

- Blank was also prepared with the same compositions except tissue homogenate.
- Spectrophotometric analysis using microplate method at 412nm was used to measure the rate of Ach hydrolysis at 1 min intervals during a 5 min period. The quantity of acetylthiocholine (ATCL) hydrolysed per minute per milligram of protein was used to quantify and express the activity of acetylcholinesterase. The amount of ATCL hydrolysis every minute per mg of protein was used to measure the process's activity [51].

4.7.4 Lipid Peroxidation (MDA) estimation:

Malondialdehyde (MDA), a byproduct of lipid peroxidation, was evaluated in this estimation. A pink chromogen is formed when one MDA molecule combines with two molecules of thiobarbiturate acid (TBA) in a slightly acidic condition. Using colorimetric measurement, the intensity of this chromogen was determined at 535 nm[54].

Reagents Required:

Sodium Dodecyl Sulphate – 8.1%
Glacial acetic acid (pH 3.5) – 20%
Thiobarbituric acid – 0.8%
n-butanol and pyridine mixture – (15:1, v/v)

Procedure:

- 0.2ml of SDS was mixed with1.5ml of glacial acetic acid.
- Above prepared solution was added with 1.5ml of 0.8% aqueous solution of TBA.
- Then, this reaction mixture was combined with .1ml of supernatant.
- Later, that mixture was made up to 4ml distilled water and incubated for 1 hr at 95ºC in a water bath.
- The resulted combination was cooled by tap water, now 5ml mixture of n-butanol and pyridine (15:1, v/v) was added and centrifuged at 4000 rpm for 10min.
- Absorbance of organic pink layer was recorded at 532 nm for 5 min.
- Using malondialdehyde bis- (dimethoxy acetyl) as standard, a calibration curve was plotted.

- Result unit were presented in nmol MDA/mg[51].

4.7.5 Superoxide dismutase (SOD) estimation:

The enzyme superoxide dismutase is in charge of catalysing the transformation of superoxide radicals in oxygen molecules and hydrogen peroxide. The test technique for measuring SOD activity indirectly depends on blocking the reaction between superoxide radicals and adrenaline. In this mechanism oxygen is created during the oxidation of adrenaline, and SOD uses it as a substrate, especially in alkaline pH environment. Adrenochrome is created when O2 combines with adrenaline in excess, but when all of the available adrenaline is used up, less adrenochrome is formed, which leads to the precipitation of insoluble brown chemicals in the mixture. The rate and total amount of adrenochrome production are slowed by SOD's interaction with the oxygen generated during oxidation. As a result, SOD activity changes the pace at which adrenochrome is formed by impeding the oxidation of adrenaline[55].

Reagents Required

EDTA solution	0.0001 M – 9.3mg/250ml
Carbonate Buffer	9.7pH – 8.4g NaHCO3 + 10.6gm Na2CO3 in 500ml DW
Epinephrine	0.03M- 50mg/100ml in HCl of pH 2

Procedure:

- Blank and test were prepared using 100 microliters of EDTA, 500 microliter of carbonate buffer along with 1000 microliter of epinephrine. Test contains 200 microliter of brain homogenate's supernatant whereas blank contains distilled water.
- After mixing the reagents well, absorbance measurements were taken at 480 nm every 60 seconds for 5 min, using blank as a reference.
- The determination of SOD activity required using a standard curve that was produced with known SOD concentrations [68].

4.7.6 Reduced glutathione (GSH) level estimation:

GSH level was estimated as per the reported method (Moron). Glutathione consists of sulfhydryl group 5,5-dithiobis 2-nitro benzoic acid (DTNB), a disulphide compound, gets readily attacked by these sulfhydryl group and forms a yellow coloured which was measured calorimetrically at 412nm[56].

Reagents Required-

Ellman's Reagent	10mM DTNB +15mM NaHCO3

Procedure:

- 160ul of supernatant was mixed with freshly prepared 2ml of Ellman's reagent.
- The mixture containing tubes were incubated for 5 minutes at room temperature.
- The absorbance of consequent yellow colour solution was taken at 412 nm against blank reagent through spectrophotometric analysis using a microplate method [51].

4.7.7 Catalase (CAT) activity estimation:

CAT activity was estimated as per the reported method. The UV absorption of hydrogen peroxide can be measured at 240nm, the absorbance reduces when it is degraded by the enzyme catalase. By quantifying decrease in absorbance, the enzyme activity can be calculated[57].

Reagents Required-

Phosphate buffer Solution (50mM)	3.872gm of Disodium phosphate 1.457 of Monosodium phosphate
Hydrogen peroxide (5.9mM) in distilled water	67ul in 100ml distilled water

Procedure

- 50mM of PBS was prepared by adding 3.872gm of Disodium phosphate and 1.457 of Monosodium phosphate in 400ml of distilled water. Adjust up to 500ml with HCl/ NaOH to reach 5 pH.
- Hydrogen peroxide (5.9mM) was prepared by mixing 67ul of 30% of H2O in 100ml distilled water.
- Then 2.5ml of PBS was taken with 0.4ml H2O2 and mixed with 0.1ml of supernatant.
- Absorbance was recorded every min for total of 5min at 240nm using UV spectrophotometer [67].

4.7.8 Histopathological analysis

The animals were sacrificed, and their entire brain was dissected out and rinsed with saline solution. Prior to specific staining process, tissue preparation was done through several steps, including fixation, processing, embedding, and sectioning. Fixation process involves treating the sample with chemicals in order to maintain its structural integrity. It prevents degradation by cross-linking proteins irreversibly. Although there are several fixatives available, Neutral Buffered Formalin is the most commonly used. It is an important step as it hardens the tissue, facilitating subsequent sectioning processes by maintaining its chemical composition. Then further processing was done by ethanol to dehydrate the tissue and strengthening for microscopy. Following dehydration, xylene was used to remove the excess residual ethanol. The next step was embedding, where the sample was placed in paraffin wax or a plastic resin to enhance its cellular structure extraction. Sectioning entails mounting the specimen on a microtome and then slicing it into thin sections. The typical 4-5 micrometres thick sections are suitable for staining and microscopic examination. For staining purpose, Haematoxylin, a basic dye was used to impart blue colour to the proteins, while Eosin, an acidic counterstain which targets basic structures and gives pink colour. These stains are commonly used to delineate intracellular organelles and different proteins in the cell. The hippocampal region's neuronal density was then measured under a 40x magnification by Research microscope and further analysed using ImageJ software[58]. Under a microscope, the slides containing hippocampus tissue sections from several experimental groups were inspected, and the neurons in the CA1 region were visible at magnifications

of 10x and 40x. Image Fiji was used to analyse the images after they were taken using the ImageView software. To achieve precise measurements, a 100µm scale bar was placed before analysis. The photos were then stored in specified folders as JPEG files in preparation for additional processing. The photos from every experimental group were then assembled into a PowerPoint presentation. Annotations and arrows identifying regions of interest were inserted on each slide, with a focus on neuronal degeneration. The visual emphasis on the effects of DPI treatment on neuronal integrity was achieved by comparing photographs from the control and treatment groups. This method made it easier to explain the histopathological results succinctly and clearly, which allowed for a thorough comprehension of the neuroprotective effects of DPI therapy on the hippocampal CA1 region.

4.7.9 Immunohistochemistry

For the immunohistochemistry analysis, Glial Fibrillary Acid Protein (GFAP) and synaptophysin were performed. Immunohistochemistry is done in order to determine the distribution of causative factor in the brain and its action/reactivity/effect on the brain tissues. It determines the degree of damage that has been done to the brain tissues. Synaptophysin is a synaptic membrane protein which is involved in neurotransmission in the hippocampal region therefore, this stain indicates the synaptic plasticity of the neurons.

GFAP, a kind of intermediate filament protein, controls astrocyte structure and function by influencing their response to aging. It is a reliable marker of brain aging since it tends to become more expressed as organisms age, both in rodents and humans.

The animals were sacrificed, and their entire brain was dissected out and rinsed with saline solution. Prior to specific staining process, tissue preparation was done through several steps, including fixation, processing, embedding, and sectioning. Fixation process involves treating the sample with chemicals in order to maintain its structural integrity. It prevents degradation by cross-linking proteins irreversibly. Although there are several fixatives available, Neutral Buffered Formalin is the most commonly used. It is an important step as it hardens the tissue, facilitating subsequent sectioning processes by maintaining its chemical composition. Then

further processing was done by ethanol to dehydrate the tissue and strengthening for microscopy. Following dehydration, xylene was used to remove the excess residual ethanol. The next step was embedding, where the sample was placed in paraffin wax or a plastic resin to enhance its cellular structure extraction. Sectioning entails mounting the specimen on a microtome and then slicing it into thin sections. The typical 4-5mm thick sections are suitable for staining and microscopic examination. These sections were further incubated for a number of hours with primary antibody (GFAP 1:100 and Synaptophysin 1:200) in PBS-BSA solution. The slices were then washed with PBS and again incubated for half an hour with secondary antibody. Then, the sections were treated for 20 minutes with a solution containing 0.05% DAB and 0.01% H2O2 in 0.05M Tris-HCl (Ph 7.4). The immunostained slices were dehydrated and cover slipped for additional examination after several rinses with water. They were observed under a 40X magnification by Research microscope and further analysed using ImageJ software[60].

Under a microscope, the slides containing hippocampus tissue sections from several experimental groups were inspected, and the astrocytes and synaptic plasticity in the CA1 region were visible at magnifications of 10x and 40x. Image Fiji was used to analyse the images after they were taken using the ImageView software. To achieve precise measurements, a 100μm scale bar was placed before analysis. The photos were converted into deconvoluted images, thresholds were set up to 170, 190 and % area was measured in excel sheet. Images were then stored in specified folders as JPEG files in preparation for additional processing. The photos from every experimental group were then assembled into a PowerPoint presentation. Annotations and arrows identifying regions of interest were inserted on each slide, with a focus on neuronal degeneration. The visual emphasis on the effects of DPI treatment on neuroinflammation and synaptic plasticity was understood by comparing photographs from the control and treatment groups. This method made it easier to explain the histopathological results succinctly and clearly, which allowed for a thorough comprehension of the neuroprotective effects of DPI therapy on the hippocampal CA1

CHAPTER 5

RESULTS

RESULTS:

5.1 Physical Parameters

5.1.1 Weight Variation

The most often used parameter to check for any disorder in a living organism is the weight variation test. An abnormal rise or fall in the levels can indicate to a medical issue. For total of 6 weeks, the percentage weight variation of the rats was assessed in the current study, and significant differences were found between the three groups of oral treatment of DPI as compared to DC group.

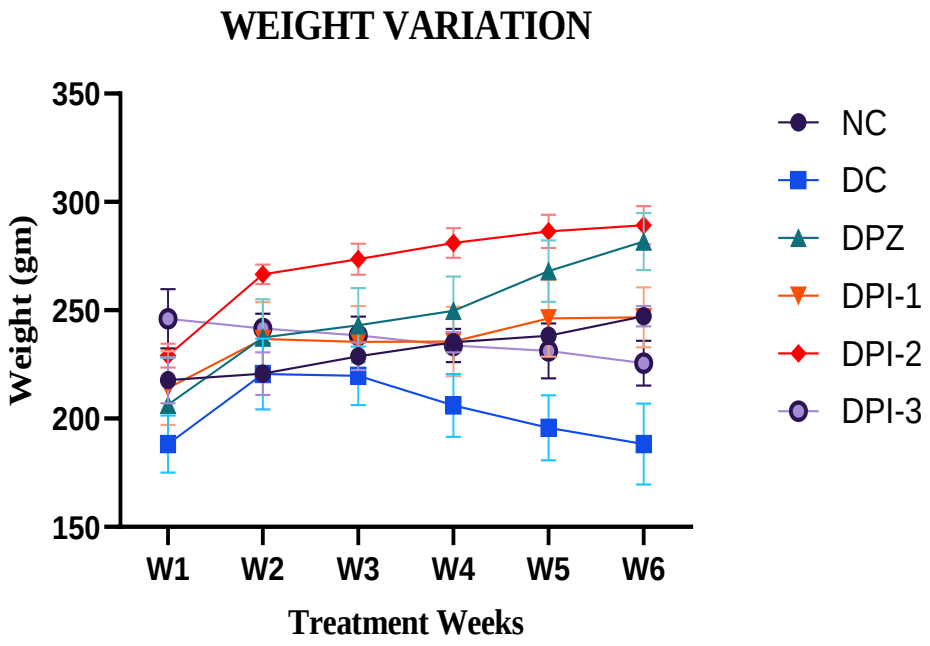


Figure 13: Effect of DNA Polymerase Inhibitor treatment on weight variation levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.2 Neurobehavioral Parameters

5.2.1 Morris Water Test

Morris water Maze test evaluates the spatial memory. Once animals were trained, they were able to found the hidden platform with ease. It was observed that the escape latency was lowered in treatment groups as compared to the DC and was higher in DC when compared with Normal control (NC). Overall, the conclusive results of the test were found to be significant. Treatment groups with 130mg/kg ($p<0.01$) and 173mg/kg ($p<0.05$) showed reduction in escape latency as compared with the DC group.

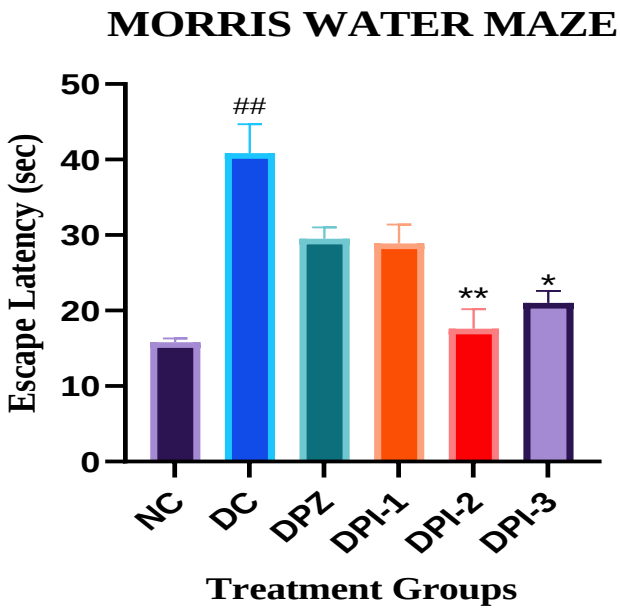


Figure 14: Effect of DNA Polymerase Inhibitor treatment on Morris Water Test levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ when compared to NC, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to DC and $ $p<0.05$, $$ $p<0.01$ and $$$ $p<0.001$ when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.2.2 Novel Object Recognition Test

NOR test evaluates the recognition memory in the animals. Before the disease induction, during training phase animals were accustomed with similar objects without the exposure to the novel objects. Then, after 6 weeks of disease induction as well as treatment, animals still were not able to identify novel object in comparison to similar object, therefore no significance in the results were observed.

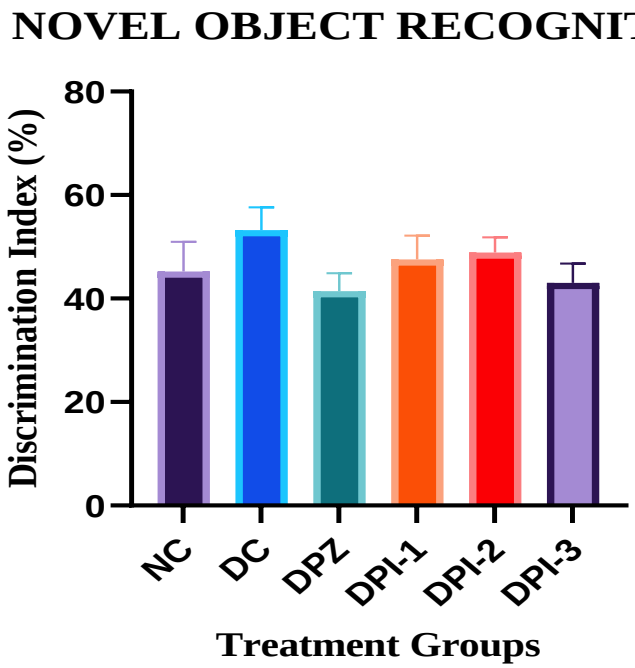


Figure 15: Effect of DNA Polymerase Inhibitor treatment on Novel Object Recognition Test levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.3 Neuroprotective and Anti-Oxidant Parameters

5.3.1 Acetylcholinesterase Estimation

The enzyme acetylcholinesterase destroys the Ach in the synapse, leading to the memory impairment in the animals and causing AD. After 6 weeks of disease induction and treatments, the level of AChE was found to be higher in DC ($p<0.05$) group of animals as compared to the NC group. Treatment dose 130mg/kg gave significantly higher level of enzyme when compared to DC, NC and DPZ groups. Other treatments were not showing significant decrease as compared to DC group. Observation shows 87mg/kg and 130mg/kg has reduced the levels of AChE enzyme but the difference was not significant.

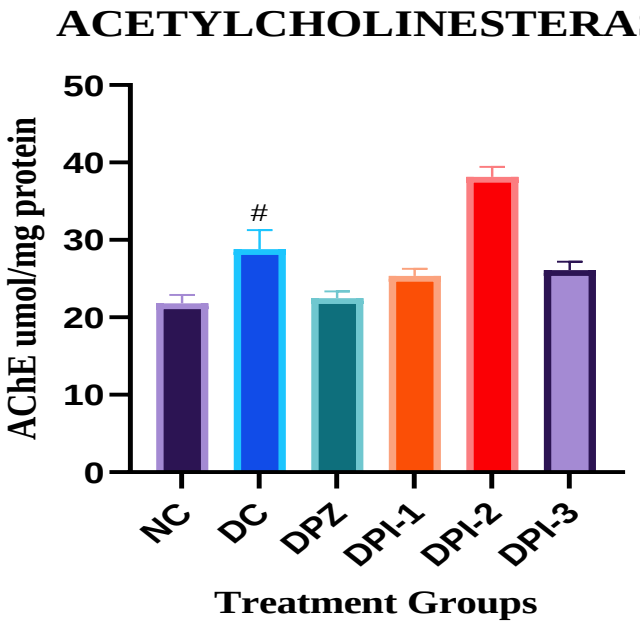


Figure 16: Effect of DNA Polymerase Inhibitor treatment on AChE levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ when compared to NC, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to DC and $ $p<0.05$, $$ $p<0.01$ and $$$ $p<0.001$ when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.3.2 Catalase Test

Levels of enzyme catalase in the disease control group was found to be significantly lowered as compared to NC ($p<0.001$). All the treatment groups showed increased catalase levels as compare with the DC group. The treatment group 87mg/kg ($p<0.05$) and 130mg/kg ($p<0.01$) had significant difference in levels as compared with DC group.

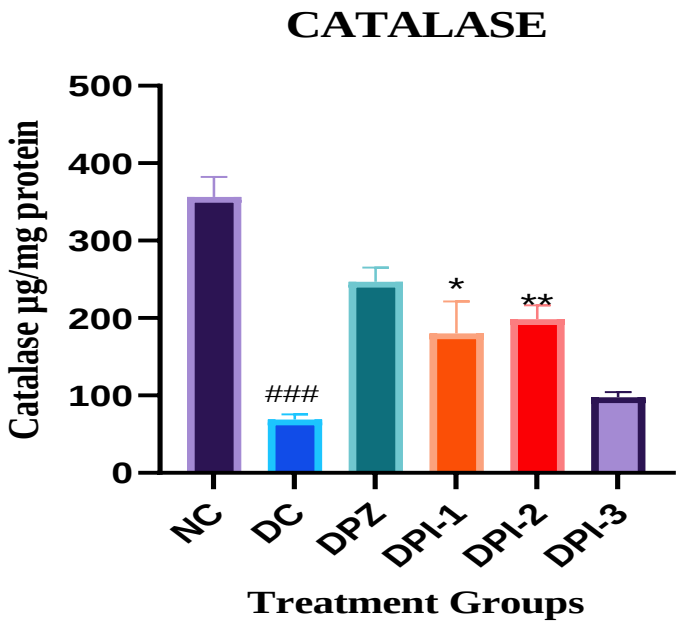


Figure 17: Effect of DNA Polymerase Inhibitor treatment on Catalase levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ when compared to NC, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to DC and $ $p<0.05$, $$ $p<0.01$ and $$$ $p<0.001$ when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.3.3 Glutathione Test

Reduced glutathione level was found to be reduced insignificantly in DC group of animals as compared to NC group. Whereas, the glutathione levels of treatment groups showed significantly increased levels of GSH as compared to the DC ($p<0.001$ and $p<0.01$) group.

All the treatment groups raised the levels of GSH; therefore, we can confer that the oxidative stress in the brain is getting reduced.

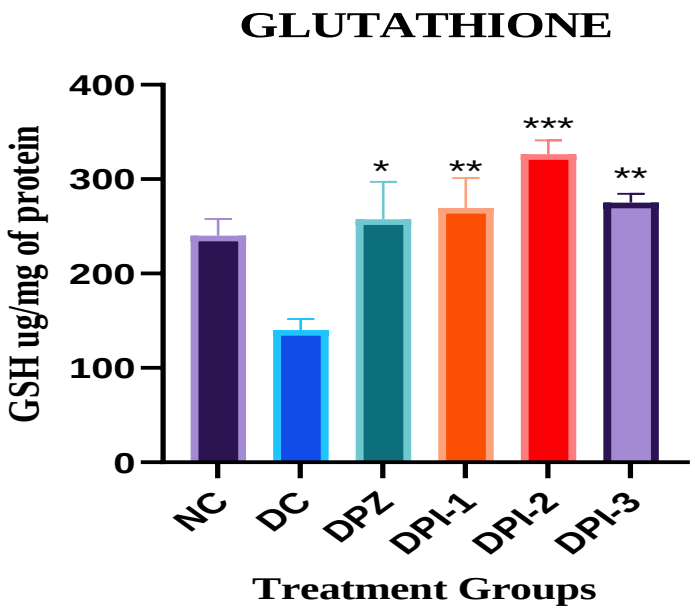


Figure 18: Effect of DNA Polymerase Inhibitor treatment on Glutathione levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.3.4 Superoxide Dismutase

The levels of enzyme superoxide dismutase (SOD) were found to be significantly decreased in DC (p<0.001) when compared to NC. The treatment group 130mg/kg (p<0.001) showed significant rise in SOD levels as compared with DC group. Observation shows 87mg/kg and 173mg/kg have also increased the levels of SOD enzyme as compared with DC group.

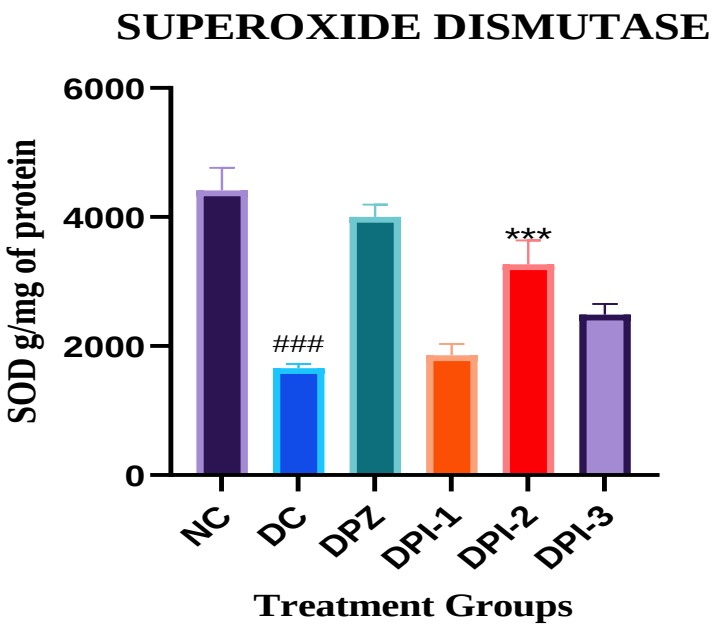


Figure 19: Effect of DNA Polymerase Inhibitor treatment on Superoxide dismutase levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.3.5 Malondialdehyde Test

The level of Malondialdehyde (MDA), on observation was found to be higher in DC when compared with NC. The levels in treatment groups except in 173mg/kg dose were reduced as compared to DC group. Overall, the result was not significant in any of the groups. Levels of MDA in 87mg/kg and 130mg/kg on observation were reduced on comparison with DC, but were not significant.

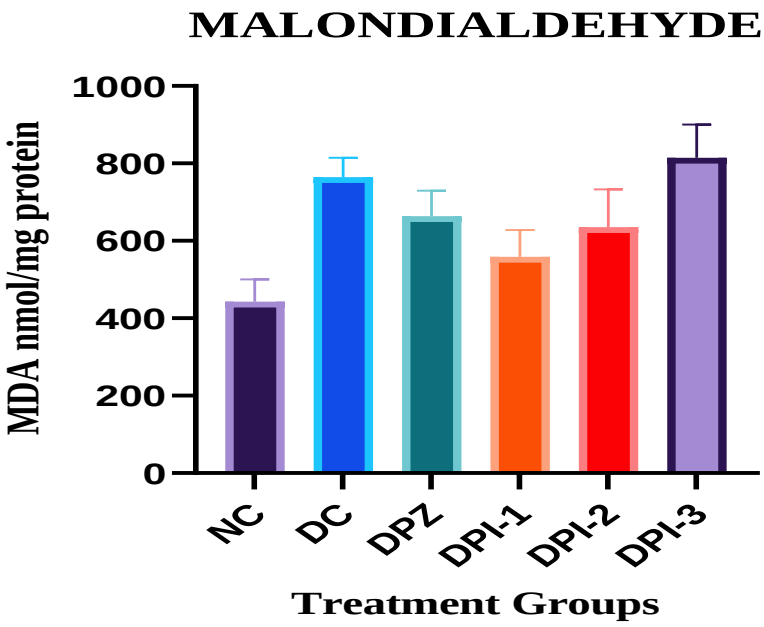


Figure 20: Effect of DNA Polymerase Inhibitor treatment on Malondialdehyde levels in scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.4 Histopathological Examination

5.4.1 Brain

For histopathological examination of the brain samples, Haematoxylin and Eosin staining was performed. HE staining has been done for the Histopathological examination as it is efficient in clearly visualizing the pyramidal neurons in the hippocampus region. We observed the neurons in the dark purple stain. On comparison with NC group, the DC group images shows the higher amount of degeneration, whereas normal neurons are found in higher density in all the three treatment groups.

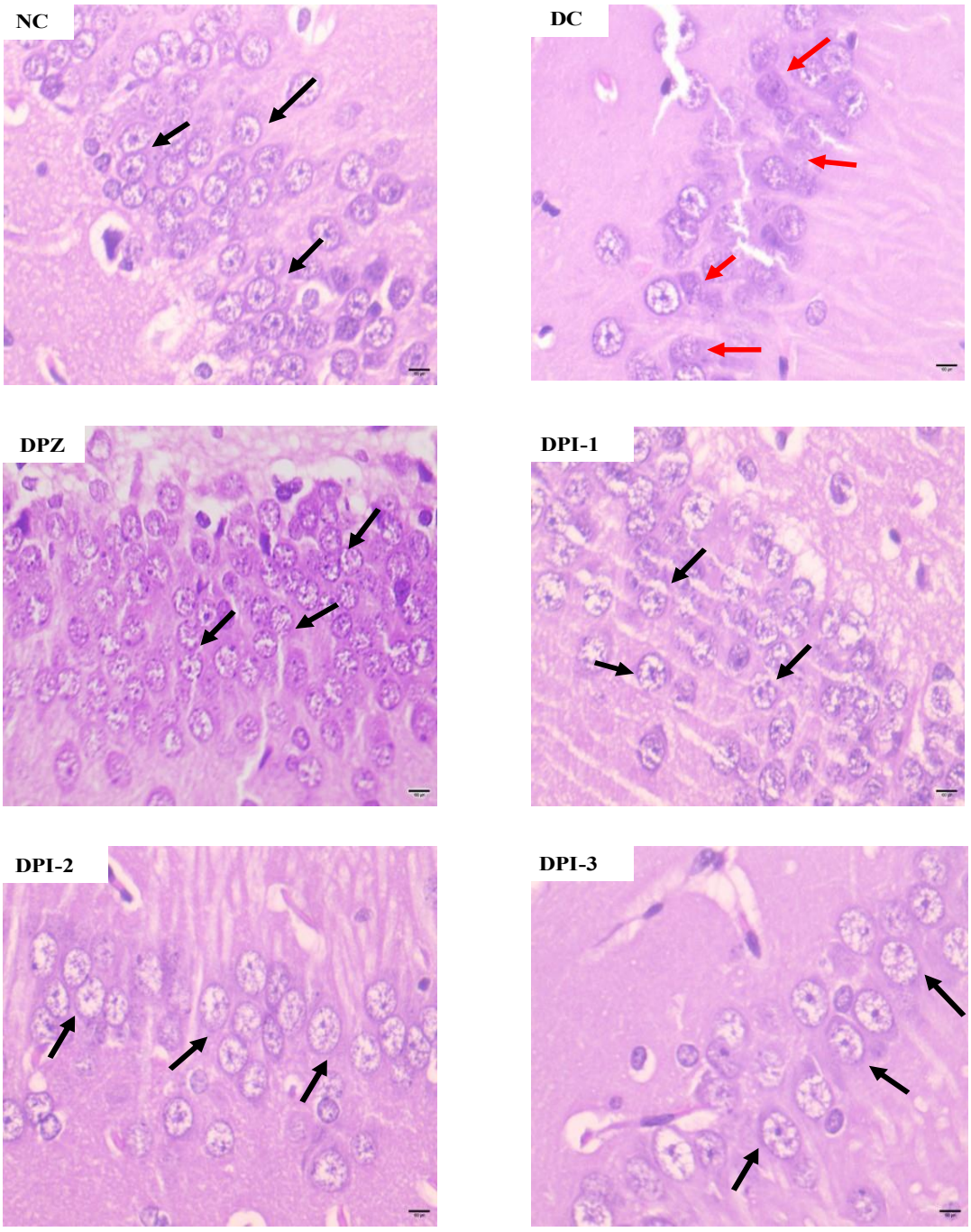


Figure 21: Histopathological analysis of brain in scopolamine induced amnesia model. Microscopic Haematoxylin and Eosin-stained images of rat hippocampus. The black arrow indicates the normal neurons and red arrows indicate degenerated neurons (magnification × 40). Quantitative analysis was performed with the help of Image J Fiji software.

5.5 Immunohistochemistry

5.5.1 Glial Fibrillary Acidic Protein (GFAP) Staining

For immunohistochemistry evaluation of the brain samples, GFAP staining was done. It is reported that GFAP staining indicates the presence of astrocytes in the taken brain section. In the images DC group shows higher area covered with GFAP stain as compared with NC group. All the other treatment groups show less density of astrocytes.

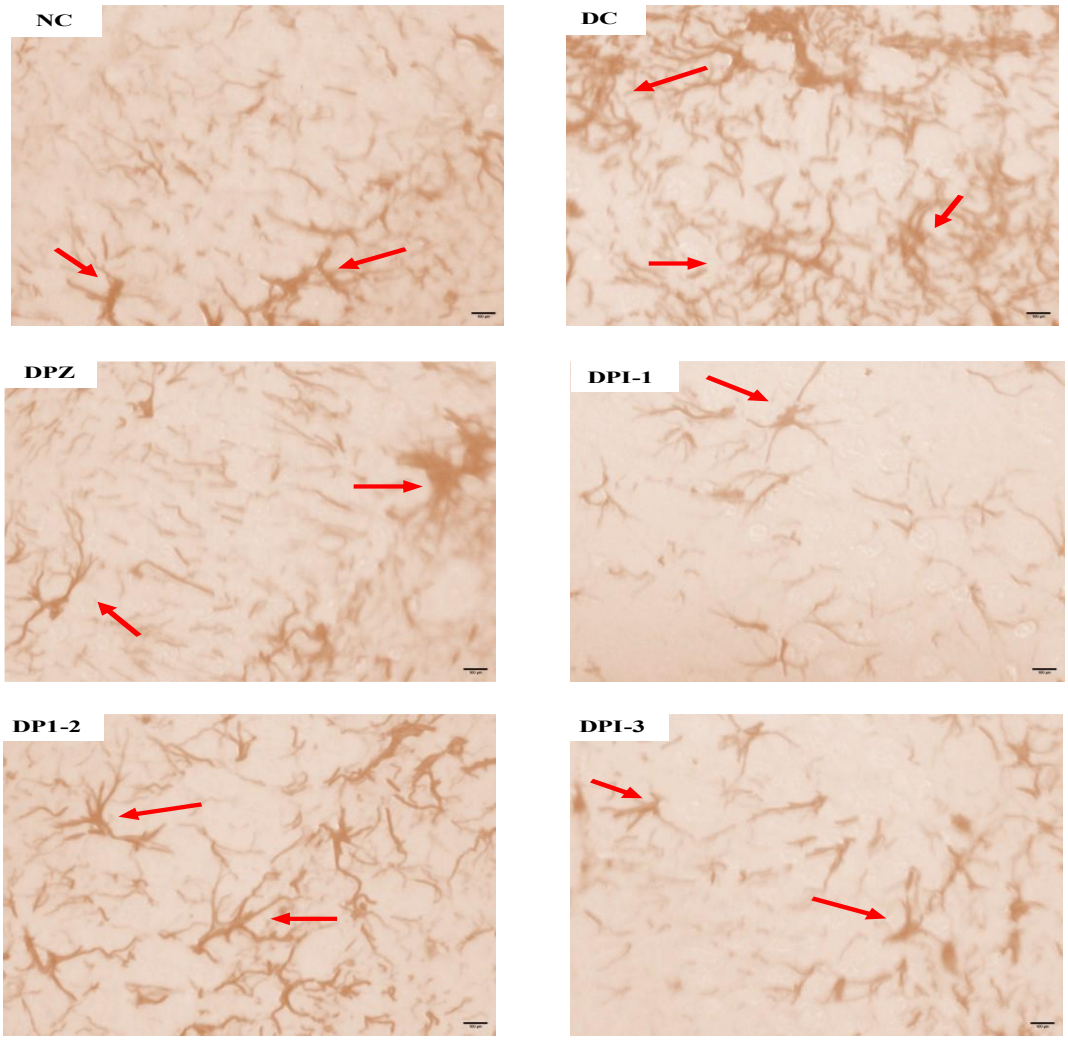


Figure 22: Microscopic GFAP immunostained images of rat hippocampus. The red arrow indicates the astrocytes density area (magnification × 40). Quantitative analysis was performed with the help of Image J Fiji software.

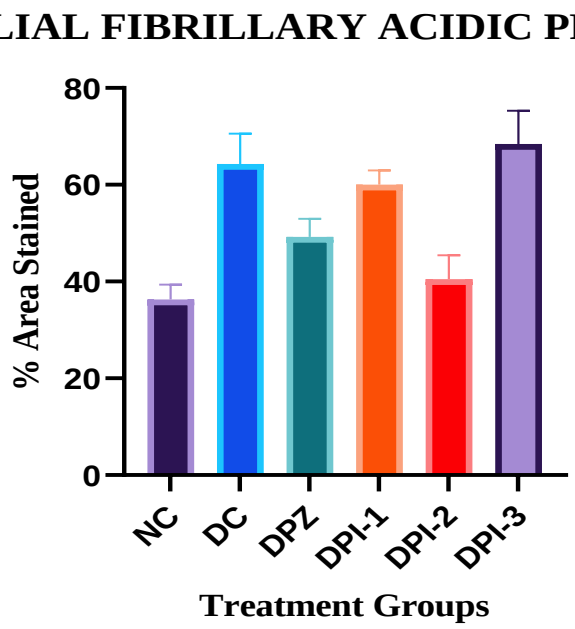


Figure 23: % Area GFAP positive in immunohistochemistry of scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O.

5.5.2 Synaptophysin Staining

Synaptophysin is a synaptic membrane protein which is involved in neurotransmission in the hippocampal region therefore, this stain indicates the synaptic plasticity of the neurons. In the images it can be observed that the stain was denser in all treatment groups except in 130mg/kg. Donepezil (standard) group shows the significantly increased staining as compared to DC group. The 87mg/kg and 173mg/kg treatment doses on observation were found to be higher with DAB staining as compared with DC group which means the synaptic plasticity was improved.

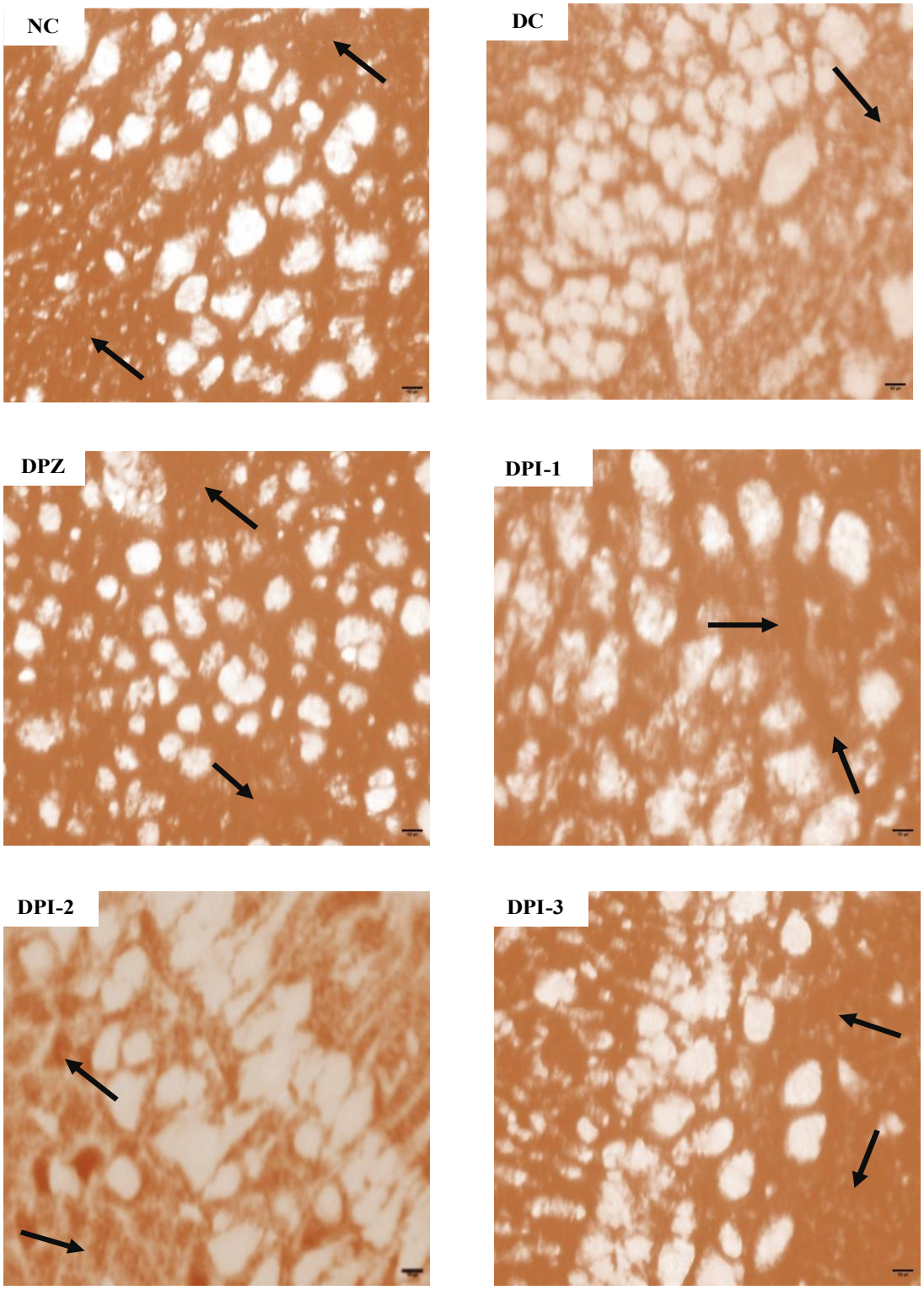

NC
DC
DPZ
DPI-1
DPI-2
DPI-3

Figure 24: Microscopic synaptophysin immunostained images of rat hippocampus. The black arrow indicates the positive area (magnification × 40). Quantitative analysis was performed with the help of Image J Fiji software.

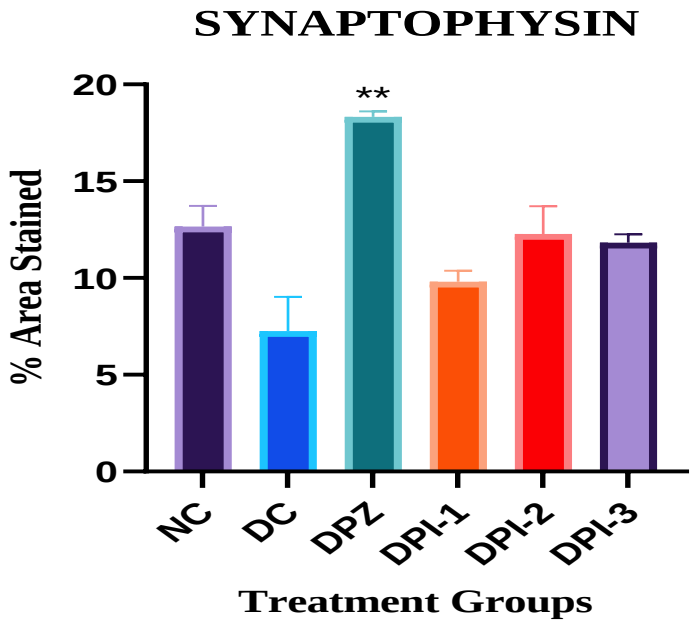


Figure 25: % Area Synaptophysin positive in immunohistochemistry of scopolamine induced amnesia model in rats

Values are expressed as mean ± SEM. # p<0.05, ## p<0.01 and ### p<0.001 when compared to NC, * p<0.05, ** p<0.01 and *** p<0.001 when compared to DC and $ p<0.05, $$ p<0.01 and $$$ p<0.001 when compared to DPZ. NC: Normal Control; DPZ: Donepezil Group; DC: Disease Control; DPI-1: 87mg/kg P.O; DPI-2: 130mg/kg P.O; DPI-3: 173mg/kg P.O

CHAPTER 6

DISCUSSION

6. Discussion

The development of senile plaques and neurofibrillary tangles within neurons, together with neuronal loss in particular brain regions, are characteristics of Alzheimer's disease that results in profound dementia. Amnesia/dementia is the end consequence, followed by behavioural abnormalities, cognitive decline and progressive memory impairment. Our knowledge of the course of AD is based on various theories where central to its pathogenesis is the accumulation of amyloid β plaques, then cholinergic insufficiency, tau protein, oxidative stress and inflammation. There are two different forms in which AD presents itself: sporadic and genetic. The sporadic variant accounts for more than 95% of cases and usually affects individuals aged 80-90 years. On the other hand, a genetic susceptibility characterizes the familial early-onset variant. It is associated with three autosomal dominant genes, which are encoded by mitochondrial proteins, they are named as PSEN1, PSEN2 and APP[72].

Currently, only four drugs have received approval for the treatment of AD, leaving the desperate need for the development of innovative therapeutic agents. The quest for novel medications involves examining a broad range of targets that may be useful against AD, reflecting the variety of research directions pursued with the goal of finding successful therapies for this condition[72].

In this study, the DNA polymerase inhibitor is used for treatment drug against scopolamine induced amnesia in rats. Scopolamine is evident that it causes impairment in brain by disrupting cholinergic pathway. Ach, a neurotransmitter involved in cognition and memory is destroyed by scopolamine by increasing the AChE concentration and reducing Ach level in the synapse.

We conducted a scientific investigation to assess the impact of different doses of DNA Polymerase Inhibitor through oral route of administration (87mg/kg, 130mg/kg, and 173mg/kg) on Alzheimer's disease (AD) induced by intraperitoneal scopolamine administration for 6 weeks. The evaluation involved the assessment of various neurobehavioral parameters, including the Morris Water test and Novel Object Recognition tests. Additionally, we measured several biochemical parameters, such as total protein levels, AChE estimation, oxidative parameters (glutathione estimation, catalase assay,

superoxide dismutase test and malondialdehyde assay), as well as histopathological and immunohistochemical examinations.

We examined the impact of DNA Polymerase Inhibitor on the body weight of animals. Body weight is widely recognized as a fundamental parameter for assessing potential disorders and evaluating the efficacy of treatments. Previous studies have suggested that Alzheimer's disease (AD) does not exert a significant effect on the body weight of animals. However, our study demonstrated a notable reduction in body weight following the induction of AD. The DPZ and treatment groups showed increased body weight when compared to DC.

The decline in cognition is progressive and associated to hippocampus dependent functions, like spatial memory and learning in cognitive functions. These features are applicable for animal models of AD due to spatial disorientation and memory impairments are primary characteristics of the progressive cognitive decline that is observed in AD patients. As revealed from the literature, scopolamine successfully impaired the memory and learning abilities of the rats[74]. This was evaluated by the MWM test where DC grouped animal's escape latency was higher than NC and treatment groups. Additionally, the fact that the treatment groups displayed a reduction in escape latency suggests that the drug is improving the spatial learning ability. This observation is significant because it shows how effective the treatment is at enhancing the cognitive functions in relation to the AD. In terms of its effect on the study, this observation strengthens the hypothesis that the drug has positive effect on cognition which is one of the endpoints in evaluating AD model.

A quick and efficient behavioural test for assessing different facets of rat cognition and learning, as well as visual deficits resulting from AD, is the novel object recognition test. It is possible to determine a rat's preferences by observing how well it can discriminate between novel and familiar objects. Rats in this study were first introduced to a particular object, and then they were shown both known and unfamiliar objects in order to determine their preferences, which were then noted[74]. The NOR test didn't give the significant results as the DC rat's discrimination index was higher for novel and similar objects. The NOR test didn't give the significant results as the DC rat's discrimination index was higher for novel and similar objects. The treatment groups showed decreased discrimination index when compared with DC. This test has significant implications to the study such as,

comprehending the cognitive capabilities of the DC group animals are necessary for evaluating the impact of treatments on cognition. Higher discrimination index means the disease was induced in the animals, as they found it more difficult to differentiate between similar and novel objects. Treatment efficacy can also be understood through this parameter, which indicates potential efficacy of treatment in reducing cognitive and visual deficits related to AD.

Cholinergic transmission is linked with the performance of memory and prompts detection and may also play a key role during morphogenesis and neurodegenerative disorders. AChE is responsible for the acetylcholine degradation, ceasing its physiological function and therefore the use of AChE inhibitors is encouraged to improve memory. Numbers of studies reported an increased AChE activity in the brain and consequently associated with cognitive impairments in Alzheimer's condition. Scopolamine has been shown to enhance AChE activity in cognitive deficits sensitized rodent brains[73]. In the present study, AChE activity was also estimated. A significant increase was found in AChE activity in DC rats as compared to DNA Polymerase Inhibitor and donepezil treated rats, and this increase in AChE activity leads to diminished cholinergic transmission due to a decrease in the ACh level. Both DNA Polymerase Inhibitor and DPZ-treated groups showed a significant decrease in AChE activity as compared to the DC group, except for (DPI-2) 130mg/kg dose of DNA Polymerase Inhibitor. Overall, this finding emphasises on the importance of cholinergic dysfunction is to the AD model and shows how the DPI treatments affect AChE activity and possibly enhances cognitive performance. It provides valuable insights about the mechanism of the drug DPI as well as their therapeutic advantages in neurodegenerative diseases like AD that are associated with cholinergic deficiencies.

It is evident from previous studies that oxidative stress has a critical role in neurodegenerative disorders. Oxidative stress may result in the neuronal damage and the memory loss. Oxidative stress occurred because of the imbalance between ROS formation and the body's ability to remove these ROS, as ROS can lead to tissue damage that has been a suggested mechanism for cognitive disabilities because of aging and neurodegenerative disorders. The capacity of antioxidant to improve cognition depends upon its ability to reach the brain.

Catalase, SOD, MDA and GSH were used as indicators of lipid peroxidation and free radical generation in the brain, respectively. The main building blocks of the neural membrane, brain phospholipids, are rich in polyunsaturated fatty acids (PUFA). PUFA are among the most delicate targets for lipid peroxidation and free radical lipid degradation[75]. Lipid peroxidation indicates degeneration of neuronal membrane, this study indicates that MDA levels were significantly increased in the hippocampus of rats of DC group. Except for (DPI-3) 173mg/kg dose, all groups showed reduced levels of MDA when compared with DC. The end product of lipid peroxidation is MDA, which is a critical criterion for oxidative stress and is inversely correlated with antioxidant characteristics. The results of this study indicated reduction in the hippocampal tissue in the DPI treatment group. The results support the hypothesis that drug shows neuroprotective action from oxidative stress involved in AD.

The antioxidant enzyme catalase quenches or scavenge reactive oxygen species such as H2O2, reactive hydroxyl free radicals. In this study, the levels of enzymes were lower in the DC on comparison with NC and was increased in DNA Polymerase Inhibitor treatment groups, compared with DC. Aβ contributes to hydrogen peroxide accumulation in various ways. It has been observed that catalase activity decreases when Aβ binds directly to the enzyme. The finding that DC has reduced levels of catalase emphasis the possible involvement of oxidative stress in the AD aetiology. It implies that methods for enhancing antioxidant defence mechanisms, such as DPI may be therapeutically useful in reducing the course of disease and maintaining neuronal functions[59].

As an essential mitochondrial anti-oxidant enzyme that scavenges superoxide, superoxide dismutase is involved in AD. SOD is the cell's most potent antioxidant and the first enzyme involved in the detoxification. As a part of first line of defence against ROS, it is a significant endogenous antioxidant enzyme. Through its catalytic dismutation of two superoxide anion molecules into hydrogen peroxide and molecular O2, consequently rendering the potentially harmful anion less hazardous[59]. According to reports, SOD levels fall as AD worsens, but they can rise with a successful treatment. In the current study animals treated with DNA Polymerase Inhibitor indicated elevated SOD levels, whereas levels were reduced in DC group compared to the NC. This study results signifies the role

of SOD in pathogenesis as well as neuroprotective action in the case of AD. It also explains the potential of treatment drug in increasing the antioxidant level.

Glutathione is an anti-oxidant found endogenously in brain which scavenges ROS and reduces down the oxidative stress[59]. In the current study, Glutathione levels were greatly reduced in the animals of DC group. Animals treated with DNA Polymerase Inhibitor were able to significantly increase up the levels of Glutathione as compared with the animals in DC group. The result of this test highlights the dual role that GSH plays in AD, emphasising its involvement in both disease progression and neuroprotection. Moreover, it elucidates the ability of treatment drug to elevate antioxidant levels. Monitoring the changes in the GSH levels may provide important insights into the disease aetiology and efficacy of treatment approaches, offering a valuable means of evaluating therapy options.

The Histopathological and immunohistochemistry analysis is performed to confirm the presence of hallmarks of AD. H&E staining is required to analyse the neurofibrillary tangles present in the brain which are marked as purple small clusters in the stained slides of thin sections of brain. Immunohistochemistry done with GFAP demonstrates the presence of Astrocytes with a characteristic brown thread like clusters in the brain section slides.

In current study, the HE stained DC group indicated more degenerated neurons compared to NC and treatment groups. The significance of these results is that it allowed the visualization and examination of all the histopathological changes occurring in the hippocampal region of brain. It enabled the evaluation of neuronal damage and degeneration involving cell shrinkage, nuclear condensation etc. These results affect the study in positive way by providing another evidence for neuroprotective action of treatment drug in AD cases.

A unique but widespread response to disruptions in the CNS, which are frequently brought on by trauma, is Astrogliosis. Astrocytes become reactive during this process, changing in form, function, and genetic composition. The extent and type of changes observed depend on the particular nature and severity of underlying disruption. The primary constituent of astrocyte intermediate filaments, GFAP has evolved into a standard indicator of reactive astrocytes.

Immunohistochemistry analysis of the brains by GFAP staining was done, where GFAP is a marker of astroglial injury is a type 3 intermediate filament that forms of the cytoskeleton of mature astrocytes and other glial cells but not found outside the CNS. Animals in DC group indicated an increase in density of Astrocytes. Treatment groups like 87mg/kg and 173mg/kg showed decrease in astrocytes density compared to DC and other treatment groups. The study result indicates that astrocytes can emit free radicals and harmful inflammatory mediators under oxidative stress and chronic inflammation, which speeds up the microglia activation and neurodegeneration. Thereby, the treatment findings support the hypothesis of neuroprotective action of the drug.

As a novel kind of neurosecretory marker—that is, an integral membrane component whose expression is independent of the synthesis of other indicators of neuroendocrine differentiation—synaptophysin can be utilized to characterize and identify neuroendocrine tumours. Additionally, synaptophysin antibodies should be helpful in the differential diagnosis of tumours, especially when it comes to ruling out malignant melanomas, which often exhibit certain neuroendocrine markers including neuron-specific enolase and specific neuropeptides. Neuronal synaptic plasticity is shown by synaptophysin, a synaptic membrane protein essential for neurotransmission in the hippocampus area. Examining the photos shows that all treatment groups—aside from 130mg/kg dose group—have denser staining. Compared to DPZ, the DC group shows noticeably less staining. The importance of this analysis is that it gives early maker for AD pathology, allowing the detection of synaptic abnormalities prior to severe neurodegenerative changes. It provides a relationship between the synaptic integrity and cognitive functions for understanding the mechanism and therapeutic benefits of the treatment[76].

Overall, the observations can be highlighted as for neurobehavioral paradigms, animals in Morris water maze test showed a decrease in escape latency in DNA Polymerase Inhibitor groups, but no significant changes were seen in the discrimination index of novel object recognition test. In biochemical assays, acetylcholinesterase level was reduced in treatment groups, but the difference was not significant. A significant increase in the levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were observed in the treatment groups in comparison to the disease control group. Decreased malondialdehyde (MDA) level was observed in the treatment groups in comparison to the disease control

group. Histopathological studies revealed that the treatment ameliorated neuronal degeneration and increased neuronal density in the hippocampus. The immunohistochemistry studies showed that scopolamine administration increased glial fibrillary acidic protein (GFAP) immunoreactivity and decreased synaptophysin immunoreactivity in the CA1 region of the hippocampus which was reversed by the DPI treatments.

These results underscore the therapeutic potential of DNA Polymerase Inhibitor to treat AD and warrant further research into its mechanisms of action and clinical applications.

CHAPTER 7

SUMMARY

7. Summary

In summary, the current results indicate that definite dementia was induced in the Scopolamine model, reflected by AD pathology occurred concomitantly with a decrease in hippocampal neurogenesis. Numerous neurobehavioral and biochemical tests were performed to examine the effects of DPI treatment.

Animals given the drug treatment showed reduced escape latency in the MWM test, indicating enhanced spatial learning. The NOR test's discrimination index, however did not show significant difference, indicating no clear effect on recognition memory in the treatment groups.

Biochemical assays revealed a reduction in AChE levels, though this difference was not statistically significant. On the other hand, DPI resulted in significant increase in antioxidant enzyme levels, including GSH, SOD, CAT and MDA, indicating enhanced mechanisms for antioxidant defence.

A neuroprotective effect was shown by histopathological analysis of brain tissue, which showed that DPI reduced neuronal degeneration and enhanced neuronal density in the hippocampus. These results were supported by immunohistochemistry investigations, which showed that DPI administration reversed scopolamine induced changes in GFAP and synaptophysin immunoreactivity.

These results emphasise the potential of DPI treatment as therapeutic intervention for neurodegenerative disorders by indicating that it improves antioxidant status, synaptic function, spatial learning and memory, and neuronal integrity in an amnesia model.

CHAPTER 8

CONCLUSION

8. Conclusion

The results of the present study showed that the DNA polymerase inhibitor drug inhibited acetylcholinesterase enzyme and thus in-turn increased the available stores of acetylcholine and thus improved memory and cognitive functions. It decreased oxidative stress by increasing GSH, SOD and CAT levels and decreasing the MDA levels. Further it reduced neuroinflammation in the brain as observed from GFAP immunohistochemistry and enhanced synaptic plasticity as shown by increased immunoreactivity to synaptophysin. The results showed that DNA Polymerase Inhibitor drug conferred neuroprotection by decreasing neuroinflammation and oxidative stress and improved synaptic plasticity. Further studies to elucidate the mechanistic pathway in advanced animal models are warranted.

CHAPTER 9

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9. REFERENCES

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ANNEXURES

ANNEXURES

Annexure I – Certificate of IAEC of Institute of Pharmacy, Nirma University approved on 08/09/2023 for "Evaluation of neuroprotective activity of DNA Polymerase Inhibitor".

Annexure II – Certificate of NIPiCON 2024, 7th Nirma Institute of Pharmacy International Conference on "NextGen Therapeutics: Multidisciplinary Research Approaches for Drug Development and Delivery" during February 7-9, 2024.

Annexure III – Certificate of 2nd NCIC 2023 on "Recent Advances in Nanotechnology: Drug Discover & Therapeutics" during January 24-25, 2023

Annexure IV – Certificate of summer internship from Marico Limited as an Intern in Technical Regulatory Affairs from 5th June 2023 to 30th November 2023.

Annexure V – Turnitin similarity report.

Item No: NP-8
Protocol Number: IP/PCOL/MPH/35/2023/08

CERTIFICATE

This is to certify that the project proposal no. IP/PCOL/MPH/35/2023/08 entitled Evaluation of neuroprotective activity of DNA polymerase inhibitor submitted by Ms. Prateeksha Sharma under the guidance of Dr Jigna Shah has been approved/recommended by the IAEC of Institute of Pharmacy, Nirma University in its meeting dated 08/09/2023 and has been sanctioned 54+16* (rats) under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman	Dr.Tejal Mehta		8/9/23
Member Secretary	Dr. Bhagawati Saxena	Bhagawat	8/9/23
Main Nominee of CCSEA	Dr. Shrikalp Deshpande		8/9/23

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CERTIFICATE OF ATTENDANCE

This is to certify that

Ms Prateeksha Sharma

has participated and attended the 7th NIPiCON International Conference
In recognition of your commitment to professional development and contribution to the international conference discussions, we hereby acknowledge your valuable presence at

NIPiCON 2024

NextGen Therapeutics: Multidisciplinary Research Approaches for Drug Development and Delivery

"Bridging the Gaps: From Drug Discovery to Patient Care"

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January 24 - 25, 2023

CERTIFICATE

Prof./Dr./Mr./Ms. Prateeksha Sharma *has participated as a Delegate in the* ***2nd Nirma e-Conference for International Connect (NCIC) – 2023*** *on* ***"Recent Advances in Nanotechnology: Drug Discovery & Therapeutics"*** *organized by* ***Institute of Pharmacy, Nirma University*** *during January 24-25, 2023.*

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This is to certify that **Miss Prateeksha Sharma,** has worked as an Intern in Technical Regulatory Affairs department of Technology function from 5th June 2023 to 30th November 2023

Her performance was satisfactory, and she has shown high level of commitment throughout her time with our company.

We wish her success in her future endeavors.

For Marico Limited

Raja Suganth G
Business HR – Technology

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