EVALUATION OF NANOFORMULATION OF ANTI-VIRAL DRUG IN ALZHEIMER'S DISEASE INDUCED MODEL OF RATS

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MASTER OF PHARMACY

IN

PHARMACOLOGY

BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Evaluation of nano-formulation of Anti-viral drug in Alzheimer's disease induced model of rats" submitted by Ms. BINA AMARNANI with Roll. No. (18MPH202) in partial fulfillment 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/our 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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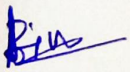
DECLARATION

I hereby declare that the dissertation entitled "Evaluation of nano-formulation of Anti-viral drug in Alzheimer's Disease model of rats", is based on the original work carried out by me under the guidance of Dr. JIGNA SHAH, Professor, Head of Department, Department of Pharmacology, 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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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORM
AD	Alzheimer's disease
Aβ	Amyloid beta
VAL	Anti-viral drug
HSV-1	Herpes simplex Virus
NFT	Neuro-fibrillary tangles
MDA	Malone Dialdehyde
IL-6	Interleukin- 6
SOD	Superoxide dismutase
AchE	Acetylcholinesterase enzyme
OS	Oxidative stress

CHAPTER 1
ABSTRACT

1. ABSTRACT

Background and Objectives:

Alzheimer's Disease is a type of dementia, that currently affects 4 million people worldwide. Being a syndrome of the brain, it is associated with progressive loss of cognitive impairment with time which ultimately affects the day-to day activities of the person. Adding to this, it's causes are multifactorial in nature, has genetic as well as environmental factors that together leads to dementia, inflamed brain and death of the neurons of the brain. Currently there is only symptomatic treatment available, aiming to reduce the symptoms only i.e. Cholinesterase inhibitors, NMDA antagonist and Memantine. There exist multiple causes that affect a variety of neurological disorders either directly or indirectly through multiple pathways, that must be researched and studied. The main objective of this study is to find new targets for Alzheimer's disease that provides an entire treatment by treating the main cause that acts directly or indirectly and contribute to the disease progression. The anti-viral drug is currently used for the treatment for Herpes Simplex Virus. The presence of this virus has also been studied, in the brains of Alzheimer disease patient. The virus travels to the brain probably in middle age, where it remains in a latent state, with very limited transcription and probably very low or zero protein synthesis. During events such as immunosuppression, peripheral infection, and stress, the virus reactivates, causing localized damage and inflammation. Studies have shown that the viral DNA is located very specifically within AD plaques, and that the main component of plaques, beta amyloid (Aβ), accumulates in HSV1-infected cell cultures, and in the brains of HSV1-infected mice subsequently others confirmed and extended these results. Since this drug is available as oral tablet formulation, a nana-emulsion would provide a more targeted drug delivery to the brain. The intranasal route drug delivery forms as an ideal route since maximum bioavailability would be obtained through this route. The main objective of this study is to investigate the pharmacological affect, characterize the nano- emulsion prepared.

Materials and methods:

Formulation Development

For the preparation of the formulation the aqueous phase was added slowly to the oily phase on a magnetic stirrer and by spontaneous technique an emulsion of white milky in nature was formed.

Further the prepared emulsion was ultra-sonicated at 20rpm for 2min. Parameters such as pH, viscosity and particle size were measured using instruments such as Brookfield viscometer, pH meter and particle size analyzer.

Pre-Clinical:

For the study, to create conditions for Alzheimer's disease the well validated model of Aβ model of male Wistar rats was used, wherein Aβ plaques were injected by ICV route. After the disease induction the disease control groups (Aβ model) were treated with standard donepezil (5mg/kg), antiviral drug (2 mg/kg, 4mg/kg and 8mg/kg) by the developed nano-emulsion through intranasal route. To evaluate the disease, neurobehavioral tests Y-maze and Morris water maze were performed prior to induction of AD and after the treatment of anti-viral drug and donepezil. After completion of neurobehavioral parameters animals were sacrificed and brain was removed and subjected for analysis of biochemical parameters like Alzheimer specific parameters, anti-inflammatory parameters and histopathology of brain tissue was done.

Results:

Amyloid-β induced model

In the Y-maze, when compared to the disease group, in the treatment groups the time spent in the novel arm increased. The same was observed for the number of entries in the novel arm by the rodent.in the water maze there was increase in the entries as well as time percentage in the novel arm. There were no much significant results in the anti-inflammatory parameters. In the Alzheimer' specific parameters reduction in the levels of amyloid compared to disease group was observed.

Conclusion:

Our results suggest that the anti-viral drug improved disease condition by reducing the Alzheimer specific parameters as well as anti-inflammatory parameters. However, the formulation showed less efficacy as compared to standard treatment. This study can however be much explored to gain further insights for the same.

CHAPTER 2
INTRODUCTION

2. INTRODUCTION

Alzheimer's disease is a syndrome of the brain associated with progressive loss of cognitive impairment with time which ultimately affects the day-to day activities of the person affected by it, being mostly prevalent among the old aged people (above 65). This also being a type of dementia, currently affects 4 million people worldwide. Researchers have foreseen, that this number would be three times higher by the year 2050. The most common signs include loss of memory at a steady rate, problems related to reasoning, obstacles in the way of thinking and perceiving and alterations of personality and temper. This disease being multifactorial in nature, has genetic as well as environmental factors that together leads to dementia, inflamed brain and death of the neurons of the brain. At the end stages of the disease, the person becomes bed bound and requires complete care. This disease is ultimately fatal. By its pathological studies, neurons of the hippocampus and cerebral cortex are seen to be damaged and is characterized by the presence of tangled fibril proteins and amyloid-β proteins. During the formation of A-β protein, the intermediate substrate formation of oligomers contributes to the pathology of this disease. There are other brain changes such as inflammation and atrophy. Neurobiological hypothesis underlying AD have become a critical to understand its pathology. There have been several other hypotheses postulated to describe the etiology of AD, most important being the amyloid-β theory, glutamate hypothesis, tau proteins. Added on is the, oxidative stress (OS) and calcium involvement in the pathology in AD. (Klein, 2006)Another aspect, of the presence of virus in the brain brings to the viral hypothesis of the 1980's origin, for AD pathology. Herpes Simplex virus type 1 and 2 have been known to cause deterioration of the neurons of the brain, primarily showing the involvement of HSV1 than HSV2.(J. L. Cummings, Isaacson, Schmitt, & Velting, 2015) This forms the basis for the use of anti-viral agents that are effective against herpes simplex virus type 1 (HSV1) and acts as a possible strategy for its use to treat AD. In the verge for creating greater life expectancy, increasing number of patients for dementia, specially the old age people, has resulted in more focused and greater research studies for its treatment drugs as well as prevention drugs that could prevent the disease. The struggle of the scientists to bring up therapeutic strategies for treatment as well as prevention of the disease is still on. The symptomatic treatment available today are, cholinesterase inhibitors and NMDA antagonist. Donepezil, rivastigmine and galantamine are cholinesterase inhibitors that act via inhibiting acetylcholinesterase enzyme that breaks down the

acetylcholine into its various substrates. Memantine, an NMDA antagonist used for the same shows its effect by blocking the glutamate chemical which gets released in abundant amount and is responsible for brain damage. Globally, the search for an alternative treatment has been shifted to herbal system of medication. All of these drugs have major disadvantage of low bioavailability, because of their peripheral route if administration. This route of administration has limited uptake of the drug molecules to the brain because of the presence of the blood brain barrier. The protective layer limits the brain's access to many of the drug substances, protein moieties, phytochemicals and other large molecules.(Kumar, Singh, & Ekavali, 2015) Other routes of administration such as oral and parenteral route causes enzymatic degradation via liver and systemic output respectively. Other factors include binding with the plasma, distribution to the various compartments that leads to delayed delivery to the brain. Thus, these factors open up for the search for an alternative to deliver the drug at the site of the brain with higher bioavailability, lesser drug clearance and also targeted action. Intranasal route acts as a favorable drug delivery having all the above characteristics. Formulations such as microemulsions and nano-emulsions are suitable for delivering both hydrophilic and hydrophobic kind of drugs.(Graham, Bonito-Oliva, & Sakmar, 2017)

The aim of the research work is to repurpose the anti-viral drug for its evaluation to treat AD in the form of microemulsion being delivered by Intranasal route of drug delivery.

CHAPTER 3
REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

3.1 ALZHEIMER'S DISEASE

Alzheimer's disease is a degenerative disease of the brain that progressively occurs affecting old aged people. It is most common form of dementia. Symptoms of this disease includes memory impairment, cognitive impairments interrupting daily activities like eating, drinking, sleeping, reading, writing etc. Worldwide 50 million people suffer from Alzheimer's disease or other forms of dementia. One in four people get diagnosed by Alzheimer's disease. It is most common in the western Europe and least in sub Saharan Africa. By 2050 it is said that 68% in global prevalence and burden would take place in low- and middle-class people. The word dementia and Alzheimer distinctly different from each other's as dementia is only the loss of memory while Alzheimer comprises of a group of symptoms like memory loss, thinking and behavioral disability, cognitive memory impairment, mood swings, difficulty in communication, reading, writing and other daily activities. It is a multifactorial disorder having different pathophysiology according to various theories. The most acceptable theory being the amyloid pathology, others include Tau hypothesis Cholinergic hypothesis, Excitotoxicity hypothesis (glutamate) and Mitochondrial cascade hypothesis. Other factors include genetic factors, inflammatory responses, free radical or oxidative stress, nitric oxide or toxins, environmental factors, etc.

3.2 EPIDEMIOLOGY

Globally, currently 50 million people suffer with Alzheimer's disease or related form of dementia. According to Alzheimer's disease International, 1 in 4 people are diagnosed with Alzheimer's disease. This disease is most prevalent in the western Europe and least in the sub- Saharan African continent.

3.3 STAGES OF ALZHEIMER'S DISEASE

Alzheimer's disease consists of mild, moderate and severe stages based on disease progression.

MILD AD: Patients in the early stage functions normally and independently. There is no effect in the long-term memory but they might experience recent memory lapses such as forgetting objects, familiar words. They do live socially active life but then there are gradual lapses in memory with

new people around him. Symptoms such apathy, anxiety and depression are associated with this stage of disease.

MODERATE AD: This stage is the longest of all the stages for a patient and lasts for years. The symptoms in this stage are more specific and identifiable. The patient deals with problems related to short term memory but retains the essential information of his life events. The person may not be able to express his thoughts or feelings and may require assistance in his daily routine activities. The person may forget his personal history, have mood swings, socially detaches himself, does repetitive, tends to become lost or wander around, remains in confused state etc.

SEVERE AD: Being the final stage of this disease, dementia symptoms are very much prominent and severe as the name says. The patient requires utmost care for undergoing his daily tasks. They completely lose the ability provide any response to the outside world. They speak up short phrases but communication becomes very tough for them. They may also become bedbound and gain complications like immobility, pneumonia infections, become malnutritional and eventually lead to death,

3.4 RISK FACTORS

The major of the risk factors involved in this disease is age, genetics, family history and other factors which mainly involves with an undergoing disease that the person may have.

3.4.1 AGE

It is one of the greatest risk factors but does not directly cause Alzheimer's disease. People above age of 65 are susceptible to this disease and after this age the risk gets doubled every 5 years.

3.4.2 FAMILY HISTORY

Another greater risk of AD is the family history where anyone in the family having the disease like brother, sister, parent, the person may easily develop the disease. When the disease becomes heredity, both the genetic facto and environmental factor plays a role in developing of the disease.(Hewes, 1950)

3.4.3 GENETICS

The early onset of AD is said to have a genetic cause. Mutations in the genes of APP, PSEN1 and PSEN2 occurs at this stage, whereas during the late stages of the AD is related to the formation of

the polymorphic form of APOE gene i.e. the apolipoprotein E gene. 80% of the early cases are related to the mutations of the PSEN1 gene, and 5% with PSEN2 genetic mutations. APP and PSEN1 mutations altogether are associated with the increase in the Aβ 42 fragments.

ApoE gene consists of three alleles i.e. ϵ2, ϵ3 and ϵ4 among which ϵ4 allelic form has greater risk of developing AD. ϵ2 and ϵ3 forms of alleles are less involved to aggravate the disease than ϵ4 type, whose involvement in heterozygous and homozygous form increases the risk of the disease by three and four times respectively. Recent studies have also observed that TREM2 gene also aggravates the disease by 2.9%.

3.4.4 OTHER FACTORS

3.4.4.1 DIABETES

Studies have shown that the diabetes and dementia are interlinked, i.e. diabetes does increase the risk that promotes dementia by many biological pathways as well as vascular pathways itself.(Baumgart et al., 2015)

3.4.4.2 HYPERTENSION

Longitudinal studies and other such studies showed that hypertension especially the middle age hypertension is able to increase the risk of developing AD. Hypertension causes many changes in the body such as vascular changes that help in the development of AD. Cerebral ischemia, on the other hand has shown to promote the aggregation of the Aβ proteins as well as APP proteins. They also stimulate the expression of genes involved in the protein synthesis of Aβ protein.

3.4.4.3 OBESITY

The mechanism behind obesity acting as obesity acting as a risk factor for AD is not yet exactly drawn out, but several meta-analysis studies have shown different results regarding its relationship with AD. BMI greater than 30kg/m^2 showed independence regarding AD, whereas another study showed that obesity occurring at the mid age has a risk factor for developing dementia. At later stages it becomes independent. BMI less than 20kg/m^2 is related to have greater risk of incurring dementia. Meta-analysis studies have also shown that together low, over and middle-aged weight have higher risk for the same. (Baumgart et al., 2015)

3.4.4.4 DYSLIPIDEMIA

Higher cholesterol levels have shown to be associated with the risk of developing AD. It is because of hypercholesterolemia that effects the BBB due to which AD risk is increased. Animal studies have shown that increased cholesterol levels are connected with decline of cognitive function, neuroinflammation, increased levels of Aβ aggregation.(Silva et al., 2019) One meta-analysis study has shown that the use of the drug statin that is used to treat cholesterol, may decrease the risk of dementia. In the contrary another study found no proof that the use of statins reduces the risk of dementia.

3.4.4.5 SMOKING HABIT

Many mechanisms have shown to increase the risk of AD by smoking. The activation of the pro-inflammatory markers, immune system, free radicle formation which happen as a consequence of smoking in the body in addition to its ability to cause cerebrovascular diseases increases the risk of AD. There is reduction in the risk of developing AD in those who quit smoking when compared to smokers. One study showed that the middle-aged smokers carry double the risk of developing dementia at later stages of their life.

3.5 PATHOPHYSIOLOGY

3.5.1 AMYLOID HYPOTHESIS

The presence of amyloid-β plaques, that are derived from the altered processing of the amyloid precursor protein when cleaved by the cleaving enzymes have become the widely considered component in AD.(Sanabria-Castro, Alvarado-Echeverría, & Monge-Bonilla, 2017). It is the presence of these Aβ peptides that progressively causes neurodegeneration, thus resulting in dementia typically being AD.(Chen & Mobley, 2019) Other molecular and genetic studies also supported the amyloid type hypothesis. The mutations in the presenilin genes both 1 and 2 have known to cause early onset of autosomal type of AD. These mutations enhanced the Aβ-42 production. In-vitro studies, have shown the toxicity of Aβ-42 in the cells and then further become aggregated. The presence of α-secretase enzyme showed the involvement in APP processing. The APP processing occurs by the breakdown by the enzymes. The α-secretase enzyme cleaves the protein resulting in the formation of peptide APPs soluble in nature and a carboxyl terminal

portion. In AD patient's the enzyme β-secretase cleaves the APP forming a soluble shorter amino acid terminal and fragment of longer carboxyl terminal. ϒ-secretase cleaves a cut at the ϒ-site producing a fragment APP intercellular domain and Aβ peptide that gets accumulated extracellularly because of its low solubility. The transport of this peptide occurs through the BBB by receptors (LRP-1 receptors), multi-ligand AGE (advanced glycation end products) receptors. Decrease in the expression of LRP-1 and increase in the expression of AGE receptors causes accumulation of these peptides in the brain. Those enzymes that carry out the degradation of the Aβ peptides, during ageing the expression of these enzymes decreases (IDE and NEP) especially the cortex and hippocampus regions of the brain that are involved in learning and memory.

Another pathway for APP processing is the endosomal lysosomal pathway. The dysregulation of this pathway that leads to dysregulation of autophagy in the neurons and have thought to be involved in the pathogenesis of AD. This is the widely accepted theory but studies show that the other fragments that are formed during processing contributes to the pathology involved behind AD.

3.5.2 CHOLINERGIC HYPOTHESIS

Being one of the most researched hypotheses, the pathology behind the AD disease is the damaging of the cholinergic neurons (mainly in the areas of frontal cortex, hippocampus, nucleus basalis, and medial septum and those that are involved in learning and cognitive memory). The down-regulation of the acetyl-cholinesterase and acetyltransferase causes the cognitive impairment progression. (Craig, Hong, & McDonald, 2011)Many such alterations like choline uptake, impaired acetylcholine release, shortfalls in the expression of nicotinic and muscarinic receptors, dysfunctional neurotrophin and shortfalls in axonal transport have major contributions to this hypothesis. The neurotransmission of the brain occurs mainly by cholinergic and glutaminergic receptors that significantly also interact with each other. Any change in one transmission effects the other transmission. In AD, the glutaminergic neurotransmission gets affected which causes defects in the areas of hippocampus, parietal cortex, frontal brain and amygdala. Studies have shown in humans and primates, when the cholinergic transmission is blocked by scopolamine, deficiency in memory is seen which is same as that of which that occurs in aged people.(Parihar & Hemnani, 2004) This deficiency when treated with acetylcholine agonists showed reversed affects. Currently drugs that enhance the cholinergic neurotransmission are acetylcholinesterase

inhibitors i.e. donepezil, rivastigmine, galantamine, rivastigmine that are approved for the treatment of AD (mild to moderate). They only provide a symptomatic treatment and not the long-term cure for AD. It was due to the failure to completely cure the disease by making use of acetylcholinesterase inhibitors, that the amyloid hypothesis as well as tau hypothesis were given prime importance and search for molecules was done.

3.5.3 TAU PROTEIN

The τ protein functions as a stabilizing protein that stabilizes the microtubule, that gives rigidity and supports growth of the neuronal cells. This protein exists in six different isoforms that differ on the number of exons and microtubules.(Hamilton, Kofler, Klunk, & Oscar, 2015) During the pathological condition of AD, hyperphosphorylation of this protein occurs resulting on its aggregation with the other cytoplasmic proteins which further leads to fibril formation which ultimately interferes with the neuronal transport of signals from one neuron to another. These fibrils are found mainly in the cortical areas, amygdala and hippocampus of the brain. Tau protein along with amyloid plaques together are the major hallmarks for AD.(Natasa Kablar, 2019)

3.5.4 OXIDATIVE STRESS

Oxidative stress (OS) occurs in situation where there is an imbalance between the ROS and the level of oxidants present in our body. This then results in cell damage. The CNS is highly susceptible to oxidative stress because of the presence of high lipid levels and greater oxygen demand by the brain. In AD patients, the brains showed the presence of greater amount of oxidative proteins, breakdown products of glycation process, 4- hydroxyenol product of lipid peroxidation. This 4- hydroxyenol inhibits the dephosphorylation of tau protein. Therefore, the presence of oxidative stress becomes the characteristic of AD.(Allan Butterfield & Boyd-Kimball, 2018)

ROS chemically interact with biological molecules such as nucleic acids, proteins and lipids, and cell organelles. In addition to the established pathology of senile plaques and neurofibrillary tangles, the presence of extensive ROS is a characteristic of AD brains. The accumulation of free radical damage, alterations in the activities or expression of antioxidant enzymes such as superoxide dismutase and catalase are also present in AD patients. Although ROS is an important factor in AD pathogenesis, the mechanisms by which the redox balance is altered and the sources

of free radicals are not exactly known. It has been demonstrated that abnormal accumulation of amyloid β is capable of promoting the formation of ROS through a mechanism that involves the activation of NMDA receptors, and that ROS may augment amyloid β production and aggregation as well facilitate tau phosphorylation and polymerization, forming a vicious cycle that promotes the initiation and progression of AD. Neuronal mitochondria (essential for cellular metabolism) show metabolic abnormalities in AD models. It has been demonstrated that mitochondria are quite vulnerable to OS, which may directly disrupt its functions (energy production, decrease of antioxidant enzymes, and loss of membrane potential), generating a further increase in ROS levels that finally produce cell death by caspase activation and apoptosis**.**

3.5.5 CALCIUM HYPOTHESIS

Calcium, a ubiquitous intracellular messenger, regulates multiple physiological functions, generating concentration gradients and binding to several proteins, receptors, and ion channels. The regulation of intracellular calcium homeostasis is a very complex mechanism that is vital for several cellular pathways and is thus involved in cell survival and death. Two organelles play a major role in calcium homeostasis, the endoplasmic reticulum (ER) and mitochondria, whereas ATPase calcium pump and the sodium-calcium exchanger are the 2 main systems involved in calcium efflux through the plasma membrane. Ca^{2+} is continuously exchanged between the cytosol and the lumen of the ER. Overload of intracellular calcium due to a blockage or dysfunction of the transport system leads to the cleavage of several proteins and other substrates, OS, perturbs energy production, stimulates protein production (amyloid β and τ protein) , and induces cell death through necrosis and/or apoptosis .In AD, the ability of neurons to regulate the influx, efflux, and subcellular compartmentalization of calcium is compromised. These disruptions involve several mechanisms, such as alterations of calcium buffering capacities, deregulation of calcium channel activities, excitotoxicity or disruption of mitochondrial functions. Alterations resulting from calcium disruption are the result of age-related OS, metabolic impairment in combination with disease-related accumulation of Aβ oligomers and the presence of mutations of genes that encode presenilin. Particularly, Aβ may promote cellular calcium overload by inducing membrane-associated OS and forming pores in the membrane.(Hu, Chrivia, & Ghosh, 1999)

3.5.6 VIRAL PATHOLOGY

Humans have been susceptible to viral infections and their development and involvement in Alzheimer's disease is been described by the viral hypothesis. There have been many viruses that have known to be associated with neurological diseases.(Lövheim et al., 2018) HSV1 being among one of them, remains in the latent stage in the peripheral nervous system. It then enters into the brain at later stages and remains in latent stage especially in elderly people. It then becomes more prevalent in the brain. (Itzhaki, 2014)Further it gets reactivated in the brain and progression of the infection starts. This infection happens in a recurring pattern and causes severe cell destruction. Stress, inflammation suppressed immune system causes this recurring infection. The fact that the HSV1 virus is present in elderly people, its involvement in producing the change in the pathological hallmarks was studied, that revealed that the DNA of the virus gets relocated with the Aβ protein when compared with the controlled brains.(Harris & Harris, 2015) In a longitudinal cohort study, wherein humoral responses were being analyzed on the virus, the presence of both IgG and anti-HSV1-IgM antibodies suggested the presence of either primary or reactivation of the infection and mostly this reactivation occurs in elderly people due to the age related low immune system.(Satpute-Krishnan, DeGiorgis, & Bearer, 2003) Those being the carriers of the gene APOE-e4 gene have greater risk for developing AD. The evidence of causing AD in such carriers is associated with its ability to cause the accumulation of Aβ plaques and tau proteins. This virus also interacts with other processes such as altering DNA transcription, protein synthesis, apoptosis pathway, immune system by binding to these plaques and tau proteins.(Devanand, 2018) In-vitro studies have showed the buildup of $A\beta_{40}$ and $A\beta_{42}$ in the neuroblastoma cells of human brain. The mechanism behind this is said to be dependent upon the calcium activating pathway. Autopsy studies of AD patients have revealed the presence of HSV1 viral DNA in the amyloid plaques (nearly 72%) in compared to brains of normal age (only 24%). At the same time phosphorylation of tau protein takes place at several sites which is the major pathological hallmark of this disease. Other possible link of the virus to this disease is the genetic factor. Those genes involved in the viral transport, using the host cell machinery, entering into the host cells, are upregulated. There are other such factors like neuroinflammation, traumatic brain injury, aging that provides a direct link of HSV1 virus and AD risk factor

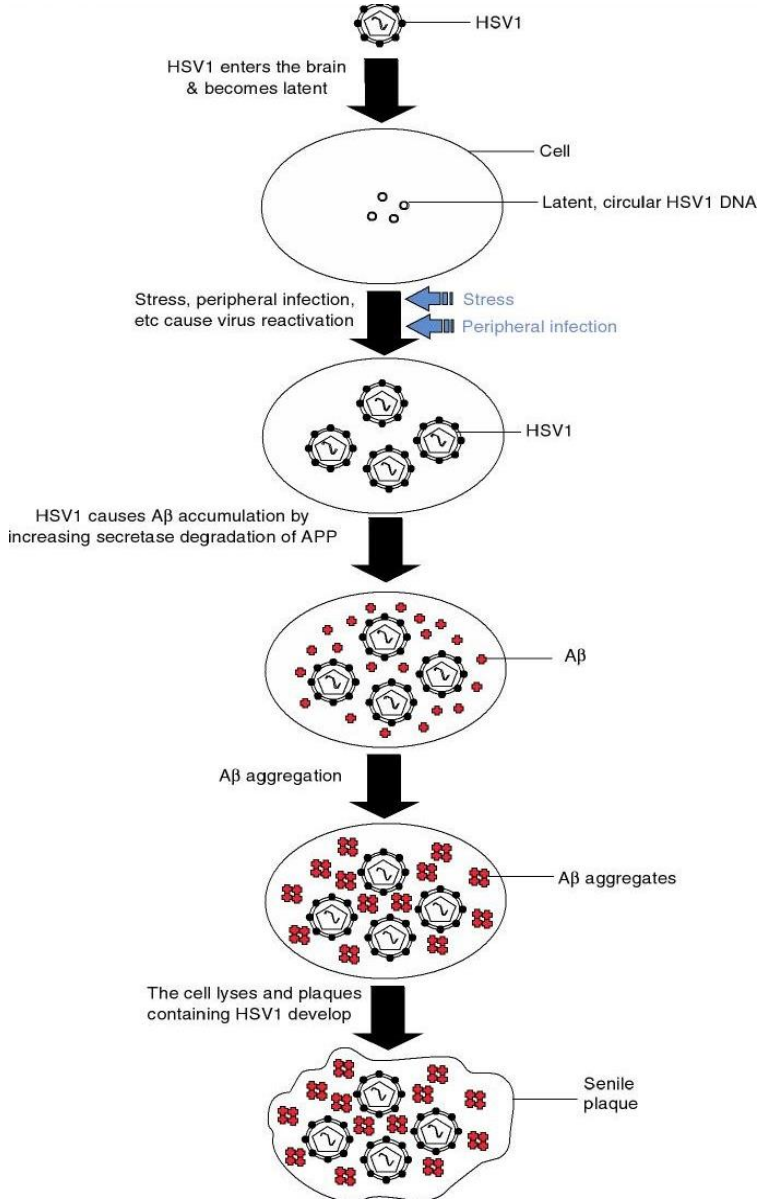

Figure3.1: Mechanism of action of the antiviral drug in causing accumulation of Aβ plaques

3.6 DIAGNOSIS

The most important part of diagnosis is firstly reporting the type of symptoms that one might be suffering from. Accurate and precise information must be provided by family or close friends regarding the symptoms for the same. Other tests are conducted by the doctor to check the thinking and memory ability of the person such as imaging tests help to characterize the disease more efficiently. Diagnostic tools ensure that dementia can be determined with high accuracy.(Arvanitakis, Shah, & Bennett, 2019)

3.6.1 PHYSICAL EXAMINATION

Physical examinations like reflexes, balance, hearing and sight hearing, ability to get up and sit up on the chair.

Lab tests are conducted such as blood tests that give precise information regarding the cause of the disease does not lay in connection with thyroid or vitamin deficiencies.

Neuro-psychological testing and mental status of the patient is assessed by a set of tests. These tests are also beneficial for checking the progression of the disease.

3.6.1.1 BRAIN IMAGING

Brain images shows the visible abnormalities related of brain related diseases. These includes as follows:

- **Magnetic resonance imaging (MRI).** MRI imaging makes use of radio waves and magnetic field that develops a detailed image of the brain and its various areas. They mainly depict the reduced size of the brain due to the undergoing disease, therefore does not play significant role in the diagnosis.
- **Computerized tomography (CT).** An X-ray used technology produces the image of the brain in cross-sectional manner and used in many types of disease diagnosis such as stroke, head injuries or tumors.
- **Positron emission tomography (PET):** In this type of scan a radioactive tracer is injected into the blood, and a specific feature of the is revealed and studied. It includes the following:

- **Fluorodeoxyglucose (FDG) PET:** This type of scan reveals those areas of the brain where degeneration has occurred and the areas where the metabolic activity of the cells are low, therefore differentiating between the Alzheimer's patient and other type of dementia.

Amyloid PET imaging: This imaging technique is used to reveal the amount of amyloid deposits in the diseased brain for those who have early symptoms of dementia. Mostly this being used for research purpose.

Tau PET imaging: This imaging measures the number of neurofibrillary tangles in the brain mostly being used for research purpose only.

In special cases the CSF of the brain is taken to measure amyloid-beta or tau proteins. In exceptional cases, where heredity is doubted to be the cause of the symptoms, or the disease runs in the family history, genetic testing is recommended by the doctor under certain conditions.

3.7 CLINICAL AND LABORATORY MANIFESTATIONS

The appearance of symptoms of the disease varies from person to person. The clinical signs of the disease depend also on the progression of the disease based on which the following symptoms are categorized:(Edition, 2008)

Stage by MMSE scores	Cognitive	Behavioral	Neurological
Mild (Score ≥20)	Relative preservation of some cognitive functions. Cognitive deficits affect IADL, but not ADL	Apathy may be present. Major depression can be the initial symptom. Psychosis, aggression and agitation are rare	The neurological exam is usually normal Mild parkinsonism in some patients Myoclonic movements are uncommon
Moderate (Score 19–10)	All cognitive domains are affected. Impairments in all IADL, and in some ADLs	Apathy is prominent. Depression is less frequent than in the mild stages. Psychosis, aggression, and agitation are more frequent	The neurological exam can be normal More parkinsonism in some patients Myoclonic movements are more frequent, especially at night Paratonia (Gegenhalten or Mitghem)
Severe (Score ≤9)	Severe deterioration of all cognitive domains. May need help with all ADLs	Apathy is prominent. Major depression is less frequent than in other stages. Psychosis, aggression, and agitation are more frequent	The neurological exam is non-focal, but mild to moderate parkinsonism can be present in the majority of the patients Myoclonic movements can be observed during the day

IADL, instrumental activities of daily living (e.g., job performance, managing finances); ADL, activities of daily living (e.g., getting dressed, control of sphincters).

Figure3. 2: Description of the clinical symptoms of AD

3.8 CURRENT TREATMENT AVAILABLE

3.8.1 CHOLINESTRASE INHIBITORS

Since the cholinergic neurons are affected in the early stages of AD causing deterioration in the memory loss as well as other cognitive and non-cognitive functions of the brain, cholinesterase inhibitors have been used as a symptomatic treatment for AD. These agents enhance the cholinergic transmission by delaying the degradation of the Ach. Three drugs have been approved to treat mild to moderate type of AD. Being used as a first line of treatment these drugs are Donepezil from Pfizer, Rivastigmine from Novartis and galantamine from Janssen. Now Donepezil is also approved for the severe conditions for AD. Tacrine previously used, is not being currently used because of liver toxicity. With rivastigmine and galantamine there have beenincidences of adverse effects being related to the gastrointestinal tract like nausea, vomiting,

abdominal irregularities. Donepezil has very less of these effects, however both of these drugs i.e. galantamine and rivastigmine are equal to donepezil in terms of tolerability. The use of these cholinesterase inhibitors also has been related with reduction in the blood pressure. Thus, the risks and benefit ratio must be weighed accordingly before the use of these drugs takes place. Their use is mostly preferred during the as first line treatment, at the initial stages of the disease.

3.8.2 NMDA ANTAGONIST

For the treatment of severe type of AD another therapeutic agent being used is the NMDA antagonist i.e. memantine. Memantine acts on the NMDA receptors. It is noncompetitive type of antagonist having low affinity to the receptor.(Folch et al., 2018) Since it has low affinity to the receptor, it has advantage over higher affinity antagonists that causes negative effects over learning and memory. It also has several different advantages such good tolerability, safer therapeutic margin and it acts on channels when it gets activated during excessive glutamate concentration at the neuronal joining.(Yiannopoulou & Papageorgiou, 2013) Therefore, it protects the neurons from excitotoxicity by blocking the channels. It acts as a therapeutic option for moderate to severe type of AD. The most frequent type of adverse effects seen is dizziness, confusion and headache.

3.8.3 COMBINATION THERAPY

Studies on parallel group of patients on randomized clinical trials showed improvement in language, cognitive functions when combination of memantine and donepezil was used compared to the placebo group. These results were studied on moderate to severe kind of patients and such benefit was not seen in mild to moderate type of AD.

3.9 MANAGEMENT STRATEGIES

The management strategy for a person suffering from dementia involves goals to which the strategy is planned. These goals may be achieved by pharmacological and non-pharmacological approaches.

The goals are listed as follows:

1. Reduction in the suffering due to impairments of memory and cognition.
2. Reducing the symptoms for the same.
3. Slowing down the progression of the disease.

3.9.1 PHARMACOLOGICAL APPROACH

Pharmacological Approach makes use of the approved drugs used for the treatment for the disease. These drugs are approved from the USFDA that provide symptomatic treatment for AD. Till now 5 drugs are being used for the treatment i.e. acetylcholinesterase inhibitors: rivastigmine, galantamine, donepezil, NMDA antagonist memantine and lastly the combination of memantine and donepezil. These drugs are available for oral use only except rivastigmine that is also available as a transdermal patch. A dose regimen of initial 4-8 weeks is given slowly so that the targeted dose is reached and the adverse effects become minimal. Depending on the adverse effects that a drug has different maintenance doses of the drugs are prescribed. For an example donepezil 5mg is the maintenance dose, higher dose of which will cause poor tolerability, 10mg dose is a typically targeted dose once daily. With slow dose regimen adverse may still occur such as nausea, vomiting, diarrhea. This incidence can be reduced by reducing he dose of the drug temporarily for may be weeks or days. If the incidence still occurs another drug from the same class could be prescribed. Assessments are then carried out may be annually or every 6 months once the patient becomes tolerated to the drug. Mainly the clinicians rely in the observation or reports generated by the care giver in assessing any improvement or any change seen in the day-to-day life of the patient. Cognitive tests on the other hand give precise information of the progression of the disease.

Memantine is prescribed for moderate to severe kind of dementia, and is also used as a first line drug. Those patients who are less tolerant to acetylcholinesterase inhibitors, this drug can be used. Headache, constipation are the adverse effects of this drug. Other management strategy includes:

1. Comorbidity medical condition must be considered.
2. Reducing the other brain diseases (stroke, brain injury) risk by treating the vascular factors. (diabetes, hypertension, thrombosis).
3. Dementia in addition causes non-cognitive symptoms such as apathy, depression. In such condition anti-depressant can be prescribed.
4. Agitation and aggression are seen is such patients and becomes difficult to manage. Anti-psychotic drugs like atypical agents should be given and monitored very carefully and must be given for a limited period of time.

3.9.2 NON-PHARMACOLOGICAL APPROACH

Benefits from this kind of approach is limited, but it is considered safe and inexpensive.

1. Training activities i.e. reading, playing games that improve the cognitive functioning. Those activities that provide much of stress must not be done. Other activities such as music or art may help in improving the cognitive functioning and also the quality of life.
2. Reminiscence therapy along with psychic therapy which uses the person's life history stories may improve wellbeing of the person.
3. Physical interventions such as exercise that maty be both aerobic or anaerobic improve one's cardio-vascular health effects the cognitive function positively.
4. Activities such as holidaying, attending various occasions, support groups or dog therapy is suggested. Brain healthy diet is also recommended.
5. To those having severe dementia (moderate to severe) have difficulty in participating in such activities and thus should be restricted when no productivity is seen
6. Centers especially that assist such patients may be helpful to them.
7. Creating awareness and educating the caregivers to have constant care and communication with the patient is necessary. Family members should also be educated for the same, on addition to the type of behaviors they would be likely to be encountered with the patient and how to cope up with those behaviors avoiding any agitation or anger.(Arvanitakis et al., 2019)

3.10 PRECLINICAL MODELS FOR AD

3.10.1 NORMAL/WILD TYPE

For the purpose of preclinical research on dementia mostly rodents and non-primates are used. Other species such as frog, rabbits, lemurs, sheep, chimpanzee, squirrel, chicken, black bear have also been an option as an experimental model to mimic the human type of dementia.(Mullane & Williams, 2019) Depending upon various factors such as cost, supply, ethical issues its use is limited. Two types of wild-type of animal models are categorized i.e.

1. Spontaneous models and
2. Chemically induced model

TYPE OF MODEL		CHARACTERISTICS
SPONTANEOUS	Senescence accelerated mouse (SAM) model	Behavioral and Aβ impairment increases due to accelerated senescence.
	Normal aged models' rodents	Normal ageing
CHEMICALLY INDUCED MODELS	Scopolamine	Muscarinic degradation
	Mecamylamine	Nicotinic degradation
	STZ (streptozotocin)	Memory loss
	Aβ injection	ICV route of Aβ42 or Aβ40 causes cognitive impairment
	Okadaic acid	Increases Aβ and tau
	Ibotenic acid, quinoline, kainic acid	This in combination with Aβ causes loss of cholinergic neurons, cognitive impairment.
	192-IgG saporin	Reduces the cholinergic function and causes permanent impairment
	AF64A	Reduces Ach, AChE, ChAT, HACht and K^+
	Stroke	Memory impairments, vascular dementia

Table 3.1: Types of pre-clinical model for AD

3.11 IN VITRO ASPECTS

In vitro models give detailed study at molecular and cellular level changes regarding the pathogenesis of the disease.(Li, Bao, & Wang, 2016)

3.11.1 TISSUE MODELS

To develop a more targeted therapeutic pathology of the Alzheimer's disease and to study the molecular mechanisms, a model was developed undergone where the neurons were cultured in-vitro. Adding on to this, other factors such as enzyme involvement, cell homeostasis, inflammatory reactions were employed, that provided a controlled environment.

Brain slices that consisting of neurons mimic the physiological environment is another accurate model of AD. The presence of extracellular matrix, neuron interactions, connection between the

neurons provide made this as an ideal model for study of AD. One study that involved the same, used okadaic acid to induce AD, observed hyperphosphorylated tau in many regions of the brain (hippocampus and cortex). Another study with memantine showed its effect by inhibiting the hyperphosphorylated tau proteins and reversed the neuron degradation.

3.11.2 CELL MODELS

For drug discovery purpose and drug modelling induced pluripotent stem cell lines have been developed from patients of sporadic or familial type AD. When these stem cells were collected from familial type AD having mutations of PS1 and PS2, showed increase in the levels of Aβ42 in all the stem cell lines. In another study the stem cells from both familial and sporadic type AD were collected, that contained higher levels of phosphorylated tau, GSK-3β & endosomes.
For AD pathology model human neuroblastoma cells are also used. The amyloid cascade pathway was observed to be activated when the APP pathway was processed in the neuroblastoma cells.
In another study carbonic acid was used to treat the neuroblastoma cells when was exposed to amyloid beta. This acid reduced the cell apoptosis by inhibiting the activated caspase pathway. The blockage of adenosine receptors by this acid in the neuroblastoma cells when exposed to aluminum chloride was seen. Thus, the AD pathology can be explored by multiple mechanisms by modifying the neuroblastoma cell lines.

3.11.3 SIMULATION MODELS

To speed up the drug development process, researchers have simulated AD pathology in vitro. An NADPH oxidase-nitric oxide system (as a bio machinery) was created by a researcher to mimic the inflammatory process that occurs in AD. This system induced neuronal death by activating the inflammatory pathway. Another model consisted of Aβ40 peptide that interacted with beta-sheet breakers. These breakers mimicked the region of Aβ40 and inhibited its aggregation. Therapeutic compounds can easily be screened at a faster speed and with greater accuracy by such mechanistic approach at a molecular level studies that involved the major pathways of AD.

3.12 CLINICAL TRIALS IN PIPELINE

In the latest review of 2019 pipeline, monoclonal antibodies like crenezumab and aducanumab that targeted amyloid-beta proteins failed to show difference between drug and placebo at larger clinical studies.(J. Cummings, Lee, Ritter, Sabbagh, & Zhong, 2019)

BACE inhibitor Verubecestat, the development of this drug was halted while being tested for on prodromal AD because analysis showed that the agent would not succeed. The fate of lanabecestat ended the same as above. In the same class of agent Atabaecestat trial was not continued further because in some participants it caused elevated liver enzymes.(J. Cummings et al., 2019)

Intranasal insulin showed no drug placebo difference when it was being assessed for mild to moderate type of AD. Pioglitazone trials were discontinued at pre-clinical trials cause of its futility. A multi-transmitter agent ITI-007, because of lack of drug- placebo difference was rejected.

In China, GV-971 multi-target drug molecule completed phase 3 trial in 2018, showed benefits over placebo. Moreover, no impact on behavior and functioning was seen and the requirements being met with the Chinese criteria is now under review.(J. Cummings, Lee, Ritter, & Zhong, 2018)

The Alzheimer's Association have established biomarkers for diagnosis of AD as well as characterization. Currently in the AD pipeline 52 amyloid imaging or CSF are being used to assess that could support diagnosis of this disease i.e. 20 are amyloid imaging, 10 tau imaging as an outcome. Compared to 2018, in 2019 more agents are observed, 28 in phase 3, 74 in phase 2 and 30 agents in phase 1. Mostly the agents fail at having no drug-placebo differences while coming to phase 2. (J. Cummings et al., 2019)

3.13 NOSE TO BRAIN DRUG DELIVERY

3.13.1 MECHANISM OF ACTION

The nasal cavity consists of three regions where the absorption of the drug molecules takes place. Firstly, the vestibular region consists of mucus and ciliary hairs that helps in the clearance of the air by trapping the foreign particles.(Bourganis, Kammona, Alexopoulos, & Kiparissides, 2018) Then comes the respiratory region which is the largest area of the nasal cavity made up of four types of cells i.e. ciliated and non-ciliated columnar epithelium cells, basal cells and goblet cells. This part of the nasal cavity is highly vascularized consisting of trigeminal neurons that transport the drug molecules to the brain (cerebrum and pons) and part of it goes into the systemic circulation

comes the respiratory region. Next comes the olfactory region present at the upper part of the nasal cavity below the cribriform plate which is also made up of four type of cells supporting cells, microvillar cells, basal cells and the olfactory neurons and also little number of trigeminal neurons. The drug molecules after the mucociliary clearance, comes in contact with the olfactory and respiratory epithelium from where further the transport takes place by olfactory nerve pathways or trigeminal nerve pathway, lymphatic and vascular pathway and the CSF.(Shinde, Bharkad, & Devarajan, 2015) The movement of the drug molecule through which pathway depends upon the various formulation related factors. Among the olfactory nerve pathway and trigeminal nerve pathway that leads to the brain, it is through the olfactory pathway that the drug gets transported to the brain via lamina propria to the brain by extracellular and intracellular pathways into the brain.(Agrawal et al., 2018) The drug molecules then get distributed by perivascular transport and the clearance of the drug from the brain to the peripheral tissue takes place by the same transport. Once the drug enters the brain, it is rapidly distributed throughout the CNS via perivascular spaces. (Bourganis et al., 2018)(Erdő, Bors, Farkas, Bajza, & Gizurarson, 2018)

3.14 NANO-DRUG DELIVERY SYSTEM

Nano-formulations of different kinds are used in pharmaceutical industry such as micelles, polymers, dendrimers, emulsions, liposomes.(Karthivashan, Ganesan, Park, Kim, & Choi, 2018) Dendrimers are macromolecules polymeric nature kind, synthetically made, branched severely forming structures like a branched tree that has the ability to carry various drugs. Nano-spheres, nano-cap are also used as drug carriers that carry the drug at the site of action. They belong to the category of polymeric nanoparticles.(Fonseca-Santos, Gremião, & Chorilli, 2015) It also has the property of having a controlled release rate. Nano-emulsions consists of droplet range between 10-200nm, thermodynamically stable in nature and have higher shelf stability. Next liposomes, occurs naturally or synthetically containing phospholipids, bilayer composed, entrapping the drug in a spherical type of vesicle. Hence, they are stable and act as carrier for delivery of various kinds of drugs.(Khan, Yang, Fu, & Zhai, 2018) Lastly micelles being amphiphilic molecule they consist of hydrophobic core and hydrophilic carrier particle. This enables them to circulate more time in the biological system.

3.15 MICROEMULSIONS

Microemulsions are dispersion systems consisting of two immiscible liquids that are stabilized using surfactants or cosurfactants.(L. Shinde, B. Jindal, & V. Devarajan, 2011) They have a droplet size usually in nanometer range. A number of surfactants can be used which could be either ionic, non-ionic or cationic in nature. They can further be categorized into:

- Water in oil (water dispersed in oil)
- Oil in water (oil dispersed in water)
- Bi-continuous (water and oil inter-dispersed within them)

Nano-emulsions have advantages than ordinary emulsions such as;

1. Higher rate of absorption
2. Higher bioavailability
3. Can incorporate both lipophilic and hydrophilic drugs.
4. Enhances the efficacy of drug delivery(Shinde et al., 2015)

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3.15.1 STRUCTURE OF MICROEMULSION

The micelle is spherical in shape and the structure forms aggregates at the surface of oil and water interface. The structure is as follows:

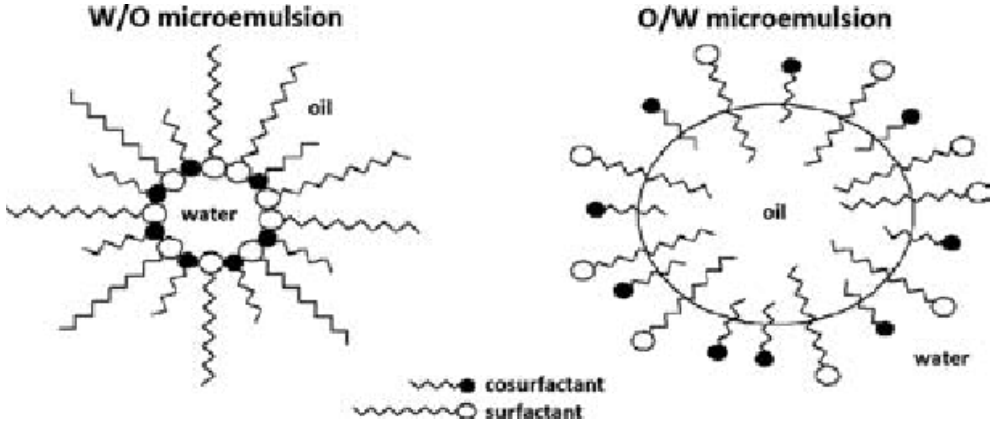

Figure 3.2: Structure of microemulsion

3.15.2 METHODS OF PREPARATION:

a) Ultrasonic emulsification

This emulsification technique reduces the droplet size by sonicator probe containing a quartz crystal by ultrasonic sounds. This produces vibrations and the formation of cavities occurs in the liquid. This process is known as cavitation. 0.2 micrometer size of the droplets are produced by this technique.

b) **High-pressure homogenization**

This technique makes use of high-pressure piston to obtain droplet size range upto 1nm. Its preparation takes place at a very high pressure.

c) **Microfluidization**

This technique uses a device known as microfluidizer that uses high pressure for reducing the droplet size. The drug product is forced into a chamber at a high-pressure producing droplet size in submicron range. To obtain nano range droplet size this step is repeated many times, thereby producing a uniform emulsion.

d) **Phase inversion temperature**

This technique makes use of high temperature producing a phase change that results in the formation of microemulsion in the desirable nano-range particle size.

e) Spontaneous emulsification

In this technique of preparation organic phase and aqueous phase are mixed together under magnetic stirring, formation of O/W emulsion. Therefore, a spontaneous emulsion is formed.

3.16 ANTIVIRAL DRUG

The anti-viral drug is an ester of the drug acyclovir. This drug acyclovir has a very poor bioavailability through oral route. So, the anti-viral drug is a pro-drug of acyclovir having greater bioavailability, gets converted to acyclovir by liver enzymes. It has been indicated for the following treatment conditions:

Cold Sores, Genital Herpes treatment of, Genital herpes lesions in immunocompetent patients Reduction of viral transmission, Herpes Zoster Cold Sores, Chickenpox(Science et al., 1996)

3.16.1 MECHANISM OF ACTION

The anti-viral drug is a prodrug of acyclovir (nucleoside analog) used as an anti-viral drug. This anti-viral drug gets converted to acyclovir by the enzyme antiviral drug hydrolase. This acyclovir drug has activities against HSV, CMV, EBV, HH-6 viruses. Acyclovir gets converted to the active form (i.e., triphosphate form) after it has been formulated. It then inhibits the DNA synthesis. The thymidine kinase enzyme is necessary for the phosphorylation to occur which is present in the viral infected cells only. The tri-phosphate form inhibits the DNA polymerase leading to DNA termination and further resulting in blocking of virus replication.(GlaxoSmithKline, 2005)

3.16.2 DOSAGE

2g of drug dosage is considered with 12hrs of difference. Side effects includes headache, abdominal pain and nausea. Less than 10% of the subjects includes renal failure, thrombocytopenia and some CNS side effects.(GlaxoSmithKline, 2005)

3.17 HYPOTHESIS

Literature searches have shown that there is no drug available for the treatment of AD and those drugs which are available in the market are only providing symptomatic treatment for the disease. There is greater need for exploration of various pathways related to AD and to apply new strategies that contribute to the development of various aspects of treating AD. Evidences have shown that infectious agents, have been known to cause AD, and herpes simplex virus 35 type 1 (HSV1) in particular.

The HSV1 infectious cycle is an intricate process that starts with the virus being attached to the heparan sulphate proteoglycan cell surface molecules. Through fusion, virus enters the cell by binding to specific receptors. Gene expression of virus occurs, when the virus uncoats within the cell and the DNA of virus circulates to move inside the nucleus. Protein complexes are held responsible for expression of gene which consist of two cellular proteins (host cell factor 1 and octamer-binding protein and a viral protein (virion protein).(Satpute-Krishnan et al., 2003) HSV1 travels to the brain probably in middle age, where it remains in a latent state, with very limited

transcription and probably very low or zero protein synthesis.(Cribbs, Azizeh, Cotman, & LaFerla, 2000) During events such as immunosuppression, peripheral infection, and stress, the virus reactivates, causing localized damage and inflammation .Studies have shown that the viral DNA is located very specifically within AD plaques, and that the main component of plaques, beta amyloid (Aβ), accumulates in HSV1-infected cell cultures, and in the brains of HSV1-infected mice subsequently others confirmed and extended these results. Taken together, the data suggest that HSV1 is a cause of Aβ products and plaques.(Devanand, 2018) Others have shown too that the main component of tangles an abnormal form of the protein called tau (P-tau) accumulates in HSV1-infected cell cultures.(Kukhanova, Korovina, & Kochetkov, 2014) Further, in HSV1-infected cells in culture, treatment with various types of antiviral have been found to decrease the level of Aβ and particularly, that of P-tau. Usage of antivirals such as acyclovir (ACV), which inhibits viral DNA replication, showed that P-tau formation depends on viral DNA replication, whereas Aβ formation does not do so; inhibition of the latter by such agents probably occurs via inhibition of virus spread.(Everett, n.d.)

And also, to provide more targeted drug delivery to the brain nano-emulsion in the nano-range size of antiviral drug is taken to evaluate its novelty in this direction. The intranasal route drug delivery forms as an ideal route since maximum bioavailability would be obtained through this route compared to the other routes of administration.

3.18 OBJECTIVES:

- To find out the pharmacological effect of antiviral drug of action in amyloid-β induced model of Alzheimer's disease.
- To develop and characterize nano-formulation for the treatment of Alzheimer's disease. To evaluate the neuroprotective effect and mechanism of action of nano-formulation of antiviral drug in amyloid-β induced Alzheimer's disease model

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CHAPTER 4
MATERIALS AND MEHODS

MATERIALS AND METHODS

PART-1: FORMULATION DEVELOPMENT

4.1 Preparation of Nano emulsion

By spontaneous emulsification technique the emulsion was prepared. This involved the following steps:

1. Firstly, the oily and aqueous phase and oily phase were prepared separately.
2. The water phase contained DMSO, drug, and water in the ratio as shown in the table and the oily phase contained the oil Isopropyl alcohol, and co-surfactant tween80.
3. Mixing of the water phase with the oily phase on magnetic stirrer at 300rpm was then done.
4. The resulting emulsion was probe sonicated at 20rpm for 2min for size reduction.

FORMULATION BATCH	OIL (ml)	TWEEN 80(ml)	WATER (ml)	DMSO (ml)
F1	1	0.3	1	0.3
F2	1	0.3	0.5	0.3
F 3	0.8	0.3	0.5	0.3
F4	0.8	0.15	0.5	0.3

Table 2.1: Formulation batches of the drug

4.2 EVALUATION PARAMETERS

4.2.1 Particle size, pH and viscosity analysis

For characterization F3 was found thermodynamically stable and hence was for selected for the same.

1. To measure the droplet size, Nanopartica SZ-100 series (Horiba, China) was used after dilution with double distilled water at 1:10 ratio.
2. For pH analysis, pH meter () was used.
3. For viscosity analysis Brookfield viscometer was used

PART-2: PRE-CLINICAL

4.2 EXPERIMENTAL ANIMALS

6-7 weeks old Wistar rats of either male or female were taken. The animals were then maintained under the appropriate conditions like temperature (22 ± 2 C), humidity (55 ± 5%) and 12h/12h light dark cycle. The animals were acclimatized under standard laboratory conditions providing water and food. The experimental protocol was approved by institutional animal ethics committee of Institute of Pharmacy, Nirma University, Ahmedabad (IP/PCOL/MPH/25/2019/002)

4.3 AMYLOID- BETA INDUCED ALZEHIMER'S MODEL:

4.3.1 Experimental protocol:

For further study, the acclimatized rats were divided into 7 groups. During the first week, behavioral parameters were conducted. Followed by that, disease was induced by amyloid-beta plaques which were injected through ICV route in the brain. Lastly, all the rats were sacrificed by giving them high dose of diethyl ether and the brain tissue was isolated for further biochemical parameters and histopathological studies (stored brain in 10% formalin solution)

4.3.2 Grouping of animals

The Wistar rats weighing 250-450 gm was randomly divided into 7 groups i.e.

1) NC: Normal Control,
2) DC: Disease Control Animals treated with amyloid-β plaques,
3) SC: Sham Control
4) Disease treated with Donepezil (3mg/kg)
5) VAL 2: Disease treated with antiviral drug (2mg/kg).
6) VAL 4: Disease treated with antiviral drug (4mg/kg)
7) VAL 8: Disease treated with antiviral drug (8mg/kg).

4.4. BEHAVOURAL PARADIGMS

4.4.1 **Morris water maze test for the spatial memory:**

A 5-day protocol for Morris water test was performed. A round maze having dimensions of 110cm diameter and 60cm height was used. Four equal size quadrants were made using a thread tied above the edge of the maze. A platform of 30cm height square in shape was placed in one of the quadrants, after which food color was added to make the platform opaque (invisible to the animal). The animals were then acclimatized for 15min prior to the starting of the trial. The platform was placed in such a way that it remained 2cm below the water level. The trials were conducted in dark, silent rooms since these were the neurobehavioral aspects of observing the animals.

Training period: The trial period consisted of 4 trials per day for each animal. The animal was kept in the circular maze for a time period of 60 sec. Once the animal found the platform, within these 60 secs, 15 sec after the platform was removed. If the platform was not found by the animal within 60 sec, the animal was guided to the platform after 15 sec. The time required to reach the platform was measured in this training period.

Probe trial: On the of the after the training period, the platform was removed and the rat was allowed to swim for 120 sec. This trial performance was known as probe trial. The following parameters were measured during this trial:

1) Time to reach the target area
2) No. of entries from passing the target area
3) Time spent in target quadrant

4.4.2 Modified Y-maze:

The purpose of this test is to measure the spontaneous alteration as a behavioral parameter in the animals, therefore measuring the short-term memory in the animals. The Y-maze consists of three arms having the dimensions of 40cm long, 3cm wide and 18cm height. On the surface of the arms corn husk was used as bedding to so that the animals are nor prone to anxiety environment.

Training period: The animals were allowed to explore any two arms, for 10min while one of the arms was blocked. This was the training period of the animals.

Probe trial: Probe trial was for the animals took place for 5min where in the animals were allowed to explore all the three arms. The rats have the tendency to explore the novel arm. Therefore, the following parameters were measured during the trial:

1) Total no. of entries in all the three arms
2) Time spent in each arm
3) First preferred arm

4.5 ALZHIEMER SPECIFIC PARAMETERS

4.5.1 Amyloid β 1-40:

The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Rat amyloid beta peptide 1-40, Aβ1-40 present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Rat amyloid beta peptide 1-40, Aβ1-40 in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

4.5.2 Amyloid β 1-42:

The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Rat amyloid beta peptide 1-42, Aβ1-42 present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Rat amyloid beta peptide 1-42, Aβ1-42 in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

4.5.3 Butylcholinestrase:

The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated on microwells. Samples and standards are pipetted into microwells and Rat Butyrylcholinesterase, BCHE present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Rat Butyrylcholinesterase, BCHE in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

4.6 ANTI-INFLAMMATORY PARAMETERS

4.6.1 Nuclear Factor NF-ΚB:

The ELISA kit for the determination for NF- KB(in-vitro) concentrations of rat in serum, plasma etc. was used. The principle behind this is the Sandwich ELISA method. The ELISA plate provided in the kit consists of an antibody coated at the bottom of each plate, that ar specific to NF-κB. Samples or standard solutions are added into the micro-plate and combined with specific antibody. The detection antibody specific to the NF-κB and Avidin-Horseradish Peroxidase (HRP) conjugate were added to each micro-plate and then incubated. The unattached components are washed away and the substrate solution was added to each well. Those wells containing the NF-κB, biotinylated detection antibody and Avidin-HRP conjugate will show blue color. Sulphuric acid solution was added to terminate the reaction and the color turns yellow. The optical density parameter is measured using spectrophotometer at wavelength 450± 2 nm. The OD value is directly proportional to the NF-κB. The conc of the samples was found using the standard curve plot. Detection Range: 0.156-10ng/ml Sensitivity>0.094 pg/ml.

4.6.2 Interleukin-6 estimation:

ELISA kit for IL-6 measurement commonly used for body fluids like serum, plasma, buffered solutions etc. was used. Natural as well recombinant type of IL-6 can be estimated by this method. The ELISA kit consists of a microtiter plate coated with a specific antibody. Standard of different concentrations (in duplicates as well as control duplicates) and test samples were added. Biotinylated specific monoclonal IL-6 antibody was added to the microtiter plates and incubated for a particular period of time. Washes were given after which HRP enzyme was added that specifically binds to the biotinylated antibody. The color produced after adding the substrate was detected by the ELISA plate reader. The intensity of the color produced is directly proportion to the con of IL-6 present in the samples. Range:1.56 pg/ml - 50 pg/ml Sensitivity< 0.8 pg/ml

4.6.3 IFN-γ estimation

Interferon gamma is a type of cytokine that is produced in the body in response to any bacterial or viral infection. It is one of the most important inflammatory markers. This marker is detected by the principle of sandwich ELISA. The kit containing all the reagents were bought to room temperature before use. The sample was centrifuged, thawed before the assay procedure to be followed. Each and every reagent was mixed thoroughly. Standard solutions were added as well as the samples. The plate was then incubated for 90min at 37°C. The plate was then removed from the incubation after which the liquid from each well was removed. 100 µL of Biotinylated Detection Ab/Ag solution was immediately added mixed, and then again, the plate was allowed for incubation for 1 hour at 37°C. Further the solution from each well was decanted and 350 uL of wash buffer was added to each well. The wells were again decanted and tis step was repeated 2-3 times. 100 µL of HRP solution was then added to each well. The plate was incubated for 30min again. The solution from each plate was decanted and 90 µL of substrate was added to each well. The plate was further incubated for 15min at 37°C. 50 µL of stop solution was added. The OD (optical density value) of each well was determined at 450nm using the micro-plate reader.

4.7 HISTOPATHOLOGY

Histological examination of the rat brain sections was done. The brains of the animals were dissected out after euthanizing them and stored in 10% formalin solution. Then a section of the brain about 10mm from the posterior part as well as the hippocampus portion was separated. Small slices were made using a microtome. Thin section was stained by HE stains (hematoxylins and eosin) and then examined under microscopic for the visible microscopic features.

CHAPTER 5
RESULTS

5. RESULTS

PART 1: FORMULATION DEVELOPMENT

5.1 Solubility Studies

Table 5.1: Table showing the solubility of drug tested on various lipids/oils.

Lipids Used	Solubility
Rice Bran Oil	Insoluble
Olive Oil	Sparingly Soluble
Coconut Oil	Sparingly Soluble
Isopropyl Myristate (IPM)	Soluble

- After testing the solubility of 1:1 ratio of oil is to drug, the maximum solubility was obtained with isopropyl myristate.
- Based on this solubility study the oil was further taken for the formulation development of the drug.

5.2 Miscibility Studies

Table 5.2: Table showing the miscibility study of isopropyl myristate along with various surfactants in the ratio of 1:1.

	Surfactants	Result
Isopropyl Myristate (IPM) (Ratio 1:1)	PEG 400	Immiscible
	SPAN 80	Miscible
	TWEEN 20	Immiscible
	DMSO	Immiscible
	TWEEN 80	Miscible

- When IPM was tested for its miscibility, among all the surfactants used, Tween 80 was found to be most promising in the results. Tween 80 showed greater miscibility when

compared to other surfactants, therefore was further taken for the formulation preparation i.e. nano-emulsion,

5.3 Characterization Studies

Table 5.3: Characterization of anti-viral nano-emulsion

DOSES	Particle size(nm)	Viscosity(cp)	pH
2mg	193	241	6.13
4mg	163	211	5.25
8mg	198	220	5.12

- The average particle size was found to in the ideal range for the nano-emulsion (Ideal range: 10-1000nm)
- The pH was also found to be in the ideal range for the intranasal route drug delivery (Ideal range: 5.1-6.1)

PART 2: PRECLINICAL

AMYLOID-β INDUCED ALZHEIMER'S DISEASE MODEL

5.4 BEHAVIOUR PARAMETERS:

1. **WATER MAZE**

Table 5.4: **Effect of anti-viral nano-emulsion on spatial memory of rats in amyloid beta induced Alzheimer's disease model**

GROUPS	Time(secs) spent in the target quadrant	Time(secs) taken to reach the target quadrant
NC	16.3±3.215	2.3±0.5774
SC	14±1.0	8±0.32
DC	7±0.575**	17±1.0**
STD	15±1.0###	7±2.57##
VALA 2	13±1.0##	12±2.0##
VALA 4	13.1±1.52##	10±0.5##
VALA 8	14±1.52##	6±0.2##

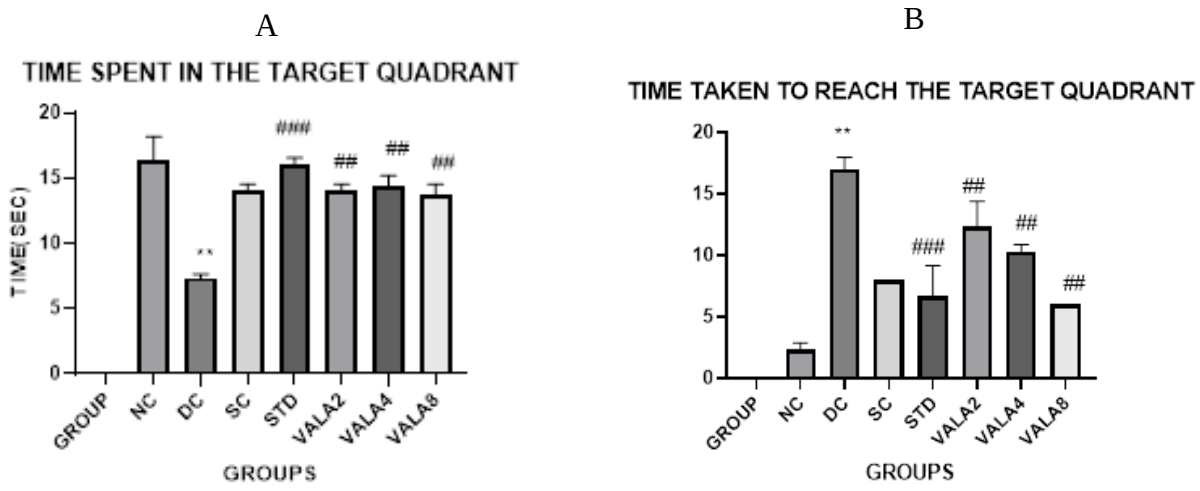


Figure 5.1: Effect of anti-viral nano-emulsion on spatial memory of rats on amyloid beta induced diseased model (A) Shows the time(sec) spent in the target quadrant. (B) Shows time (sec)taken to reach the target quadrant

n= 6, the values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with anti-viral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg, SC= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

**=Significantly different as compared to normal control; **=p<0.01,
=Significantly different compared to disease control (DC); ##=p<0.01
=, Significantly different compared to disease control (DC); ###=p<0.001 one-way ANOVA followed by Tukey's post-test

Our data suggests that the time spent in the quadrant decreased when compared to the control and treatment groups. The treatment groups did not show greater effect than standard. And also, the time taken to reach the target quadrant was less in the treatment groups and control groups when compared to disease control group.

2. Y-MAZE

Table 5.5: Effect of antiviral drug nano-emulsion on short term memory in amyloid induced Alzheimer's disease model in rats.

GROUP	% Time spent in the novel am(A)	Number of entries in the novel arm(A)
NC	90±3.4	4.3±0.5
SC	57±6.55	2.16±0.28
DC	25±4.2**	1±0.2**
STD	77±2.5##	3.16±0.28##
VALA 2	64.6±1.52##	2.5±0.5##
VALA 4	73.3±2.5##	2.5±0.5##
VALA 8	75±3.51##	2.6±2.66##

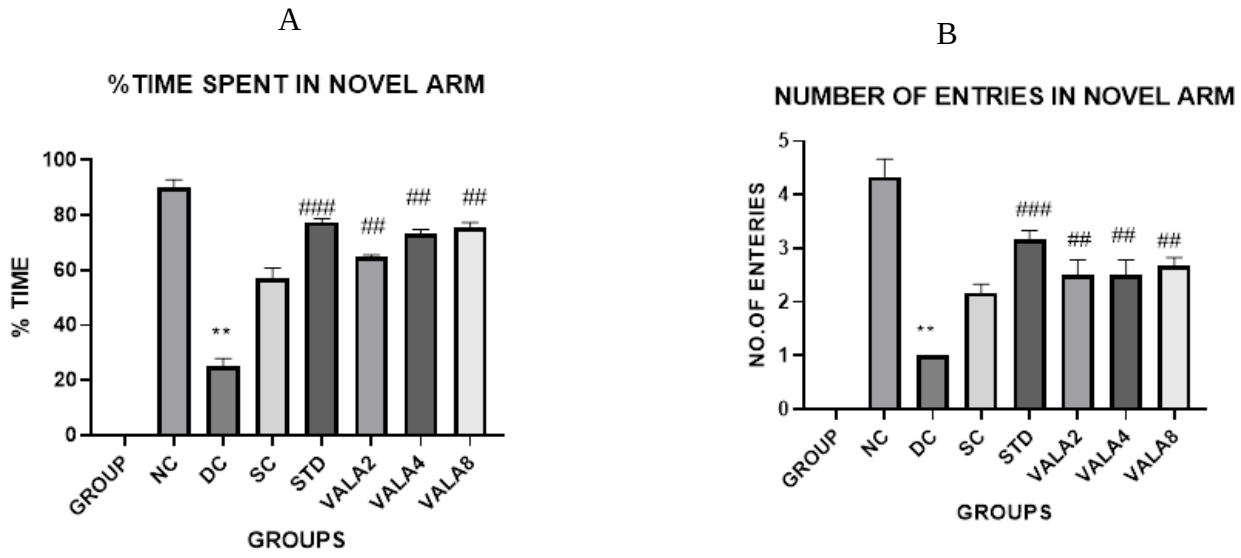


Figure 5.2: Effect of anti-viral drug nano-emulsion on short term memory in Aβ induced Alzheimer's disease model in rats. (A) % time spent in novel arm (B) Number of entries in novel arm.

n= 6, The values are denoted as mean±SEM , NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SC= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

**=Significantly different as compared to normal control; **=$p<0.01$,
##=Significantly different compared with DC; ##=$p<0.01$,
###= Significantly different compared to disease control (DC); ###=$p<0.001$ one-way ANOVA followed by Tukey's post-test

Our data suggests that the %time spent in the novel arm was decreased in disease control due to lack of cognition. When compared to treatment groups the time spent in the novel arm increased. The same was observed for the number of entries in the novel arm by the rodent.

BIOCHEMICAL PARAMETERS
5.5 ALZHEIMER SPECIFIC PARAMETERS:

Table 5.6: Effect of antiviral drug nano-emulsion on Alzheimer specific parameters

GROUPS	PARAMETERS		
	AMYLOID beta 1-42	AMYLOID beta 1-40	BUTYLCHOLINESTRASE
NC	228.5±2.5	1104±5.0	2.324±0.05
SC	300±0.9	1110±4.73	2.67±0.08
DC	522±2.1*	1327±1.0$^{\#\#\#\#}$	3.63±0.02$^{\#\#\#\#}$
STD	225±2.5*	1314±1.35****	2.54±0.01****
VALA 2	467.7±2.51*	1304±1.69***	3.433±0.01****
VALA 4	442±2.11****	1305±0.95**	2.573±0.01*
VALA 8	435±2.37****	1103±2.64**	2.54±0.01****

1. **AMYLOID-β 1-42**

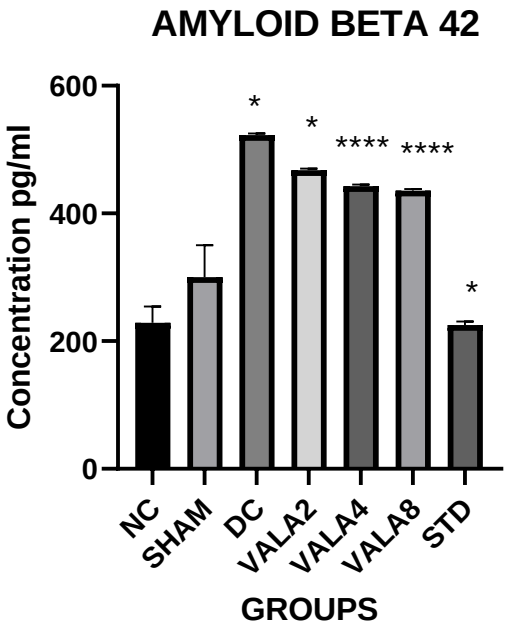


Figure 5.3: Effect of anti-viral drug nano-emulsion on concentration of amyloid beta 42 levels rat brain in amyloid beta induced Alzheimer's disease.

n= 6, The values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

****= significantly different compared to DC; ##=p<0.0001, one-way ANOVA followed by Tukey's post-test

*= significantly different compared to DC; ##=p<0.05, one-way ANOVA followed by Tukey's post-test

The data suggests that the concentration of amyloid 40 was highest in the disease group. When disease group is compared to that of the treated groups reduction in the levels is observed, but at the same time when compared to standard group none of the treated groups showed same or reduced levels in the results obtained.

2. AMYLOID β 1-40

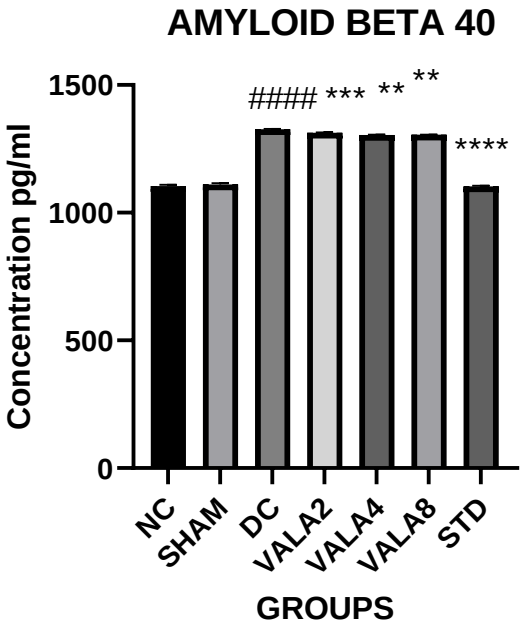


Figure 5.4: Effect of antiviral drug nano-emulsion on concentrations of amyloid beta 40 levels of rat brain in amyloid beta induced Alzheimer's disease.

n= 6, The values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

***= Significant different than DC (Disease control) ($p<0.001$),
****= Significantly different compared to disease control (DC);($p<0.001$)
####= Significant different than NC Normal control ($p<0.0001$)

The data suggests that the concentration of amyloid 42 was highest in the disease group. When disease group is compared to that of the treated groups not much reduction in the levels is observed, at the same time when compared to standard group none of the treated groups showed same or reduced levels in the results obtained.

3. BUTYLCHOLINESTRASE ACTIVITY

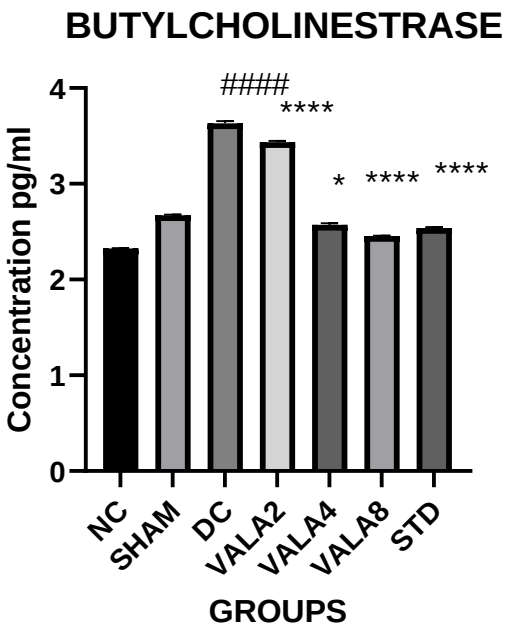


Figure 5.5: Effect of antiviral drug nano-emulsion on concentrations of butylcholinestrase levels of rat brain in amyloid beta induced Alzheimer's disease.

n= 6, The values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

****= Significant different than DC (disease control) ($p<0.0001$)

####= Significant different than NC (normal control) ($p<0.0001$)

*=Significantly different compared to disease control (DC) ;($p<0.05$)

The data suggests that the concentration of butylcholinestrase was highest in the disease group. When disease group is compared to that of the treated groups reduction in the levels is observed, at the same time when compared to standard group 4mg/kg as well as 8mg/kg showed equal levels of butylcholinestrase showing its effect in this ray.

5.6 ANTI-INFLAMMATORY PARAMETERS

Table 5.7: Effect of antiviral drug on anti-inflammatory parameters i.e. Nuclear factor kappa, Interleukin 6 and interferon gamma parameters

GROUPS	PARAMETERS		
	NFKB	IL-6	IFN-ϒ
NC	0.91±0.02	105±0.03	75.9±0.03
SC	0.91±0.02	116±0.02	76.1±0.03
DC	2.10±0.36$^{\#\#}$	175±0.03**	783.3±0.07####
STD	1.42±0.03***	148±0.02*	300±0.05****
VALA 2	1.72±0.03**	135±0.03*	253±0.06****
VALA 4	1.65±003**	126±0.03*	268±0.01****
VALA 8	1.52±0.03**	130±0.02*	164±0.02****

1. **NFKB**

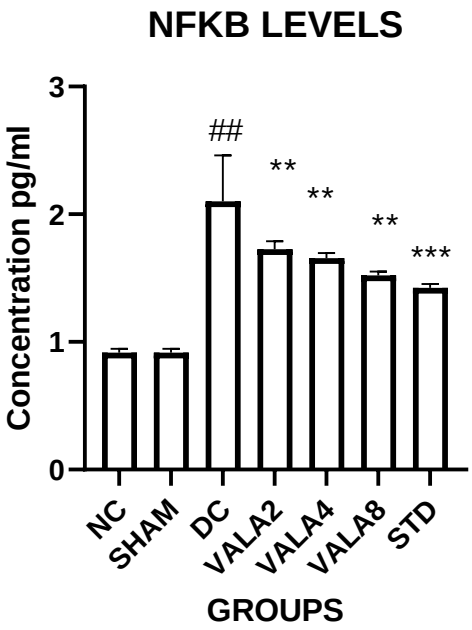


Figure 5.6: Effect of antiviral drug nano-emulsion on NF-ķβ levels of rat brain in amyloid beta induced Alzheimer's disease model

n= 6, the values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

**= Significant different than DC (disease control) ($p<0.01$)

***= Significantly different than disease 05control (DC) ($p<0.001$)

##= Significant different than NC (normal control) ($p<0.01$)

NF-ķβ is transcription factor that activates B or T cell receptors which is responsible for both adaptive and innate immune response. NF-ķβ levels was seen much elevated in disease control group as compared to normal control group. The treated groups when compared to the disease group significant reduction in the levels of NF-ķ is observed, but at the same time none of them could match with the standard levels.

2. IL-6 LEVELS

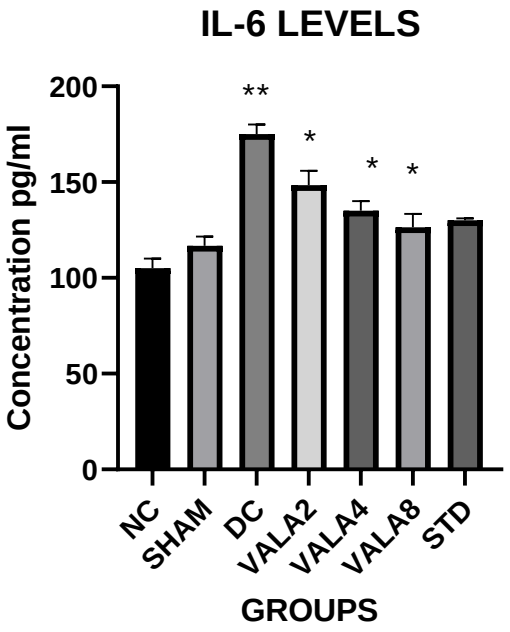


Figure5.7: Effect of antiviral drug nano-emulsion on IL-6 levels of rat brain in amyloid beta induced Alzheimer's disease model

n= 6, the values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

*= Significantly different than disease control (DC) ($p<0.05$)

**= Significant different than NC (normal control) ($p<0.01$)

The IL-6 was elevated in disease control group as compared to normal control group. When compared to disease control group the treatment receiving group did show significant difference in the levels of IL-6 but at the same time did not show normal levels as that when compared to the normal group. VALA8(8mgkg) group showed comparable levels with that of the standard group.

3. IFN-ϒ LEVELS

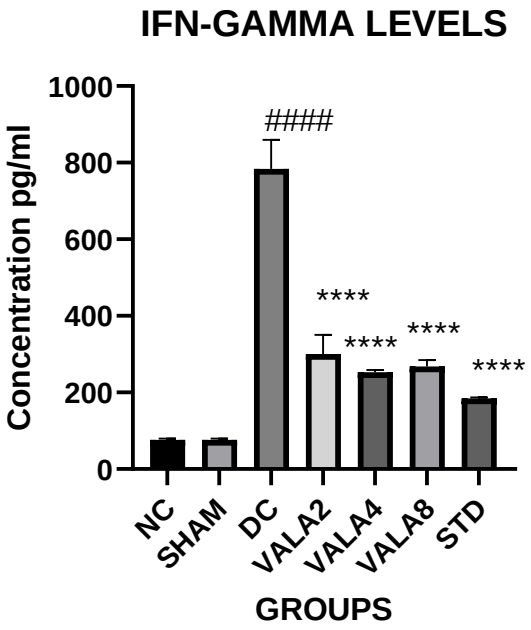


Figure 5.8: Effect of antiviral drug nano-emulsion on IFN-ϒ levels on rat brain in amyloid beta induced Alzheimer's disease model.

n= 6, the values are denoted as mean±SEM, NC= normal control, DC= disease control, VALA 2= disease control treated with antiviral drug 2 mg/kg, VALA 4 = disease control treated with antiviral drug 4mg/kg, VALA 8 = disease control treated with antiviral drug 8mg/kg SHAM= sham control, STD= disease controlled treated with donepezil 2 mg/kg,

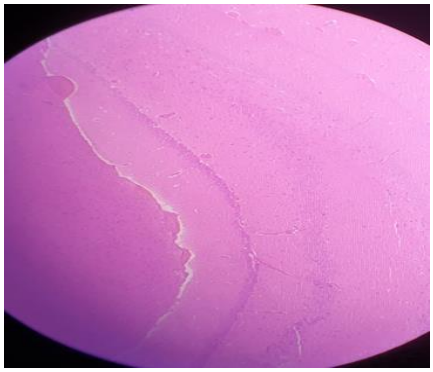
****= Significant different than DC (disease control) ($p<0.001$)

####= Significant different than NC (normal control) ($p<0.001$)

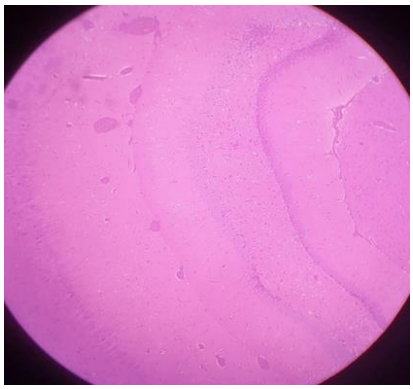
IFN-γ is important modulator of adaptive and innate immunity against bacterial and viral infection. IFN-γ activates MHC class-2 molecules Thus, the levels in the disease control group animals was higher than in the normal control group animals. When compared with the treated groups, due to anti-inflammatory activity of the drug, level of IFN-γ decreased. Between the three doses of the drug, there was no significant difference in the levels of IFN-ϒ. None of the treated groups could match the standard treatment levels of IFN-ϒ.

5.7 HISTOPATHOLOGICAL STUDIES

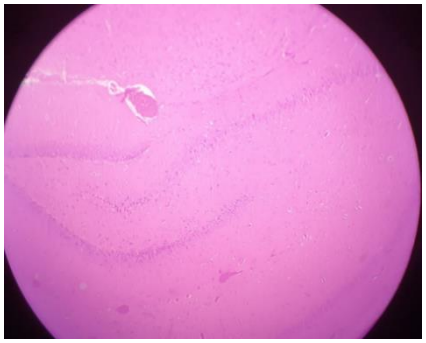
Figure 5.9: Representative images (Magnification 10X) of effect of antiviral drug on histology of rat brain tissue in amyloid beta induced disease model of Alzheimer's disease.



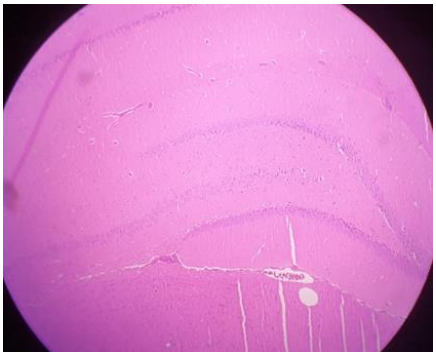
NORMAL CONTROL



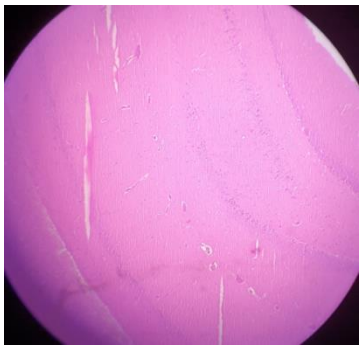
DISEASE CONTROL



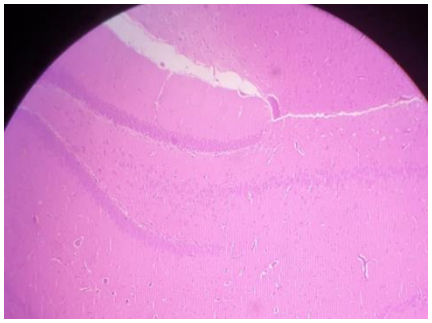
STANDARD CONTROL



VALA 2mg/kg



VALA 4mg/kg



VALA 8mg/kg

CHAPTER 6
DISCUSSION

6. DISCUSSION

HSV-1 has been emerged as a risk factor for AD for which studies to find the treatment in this aspect are being investigated and tested for many years up till now. A more recent retrospective study undergone in a large Taiwanese cohort showed that HSV infection is associated with an increased risk of dementia and that this risk decreased after treatment with anti-herpetic drugs. Added on many studies have also shown that the connection of dementia at older age is due to the herpes virus reactivation at this age. (Front Aging Neurosci. 2014). Hence the anti-viral treatment can reduce the CNS inflammation by causing the reduction in the release of pro-inflammatory markers, Aβ proteins and hyperphosphorylated tau proteins. So, in this ray of hope to find newer therapeutic approaches to treat AD, the repurposing of the anti-viral drug to treat the AD has been investigated or tested for.

In the present study the antiviral drug in its nano-emulsion form was administered through intranasal route, and investigated for its mechanism of action in Aβ induced Alzheimer's disease rats (dose 2mg/kg, 4mg/kg, & 8mg/kg) via ICV route. Results have shown improvement in the behavioral as well as some of the biochemical parameters when compared to treatment group with the diseased group.

Morris water test is the most widely accepted and used parameter to evaluate the learning memory in rodents. They particularly have relationship with the long-term memory of the brain hence used for testing the same. (Nat Protoc, 2006). The latency time was measured which was found to be higher in diseased control compared to normal control. In the treatment groups, there was significant decrease in the latency time compared to disease control, suggesting that the treatment improved the spatial memory as well as learning.

Y-maze test which is used to evaluate the short-term memory in rodent's performance. (Methods Mol Biol, 2019) In the treatment groups there were more entries in the novel arm. With the increase in the dose of the drug the percentage time spent in the novel arm increased. Hence this showed that treatment increased the short-term memory in the animals.

β- and γ-secretase. β-secretase, belonging to the family of endoproteases metabolize the APP protein into its fragments forming filaments containing neurofibrillary tangles made up of microtubules of the tau protein and plaque deposits of amyloid beta peptide protein. These two collectively form the major hallmark of the disease. Cleavage by γ-secretase is less precise in nature that results in different kinds of peptides being formed at the C- terminal. Hence, numerous

different Aβ species exist, those ending at position 40 (Aβ40) are the most abundant that constitute 80-90%, followed by 42 Aβ42 that constitute 5-10% of the peptides formed. The levels of these peptides in the brain forms as a diagnostic tool to detect Alzheimer's disease. There was more or less significant change in the levels of amyloid 40 as well as 42 type levels in the brain of rat when compared to disease-controlled group.

Acetyl cholinesterase is the major enzyme present in the human brain. But it also known that butyl cholinesterase has more wide spread distribution and located in all the regions of the cholinergic regions. BuChE is mainly found in the glial cells and the endothelial cells. The thalamic nuclei of the cortex region of the brain show great BuChE activity. Also, the mediodorsal nucleus being rich in BuChE neurons has an important role to play in the complex behaviors, planning working memory and all the deficits of AD. Pulvinar sub-nuclei that is involved in visual attention whose function is affected in the early stages of AD. (Mesulam et al., 2002a). The levels of BuChE showed reduction in the levels as the dose of the treated drug was increased suggesting that the drug also affects this enzyme.

Neuroinflammation acts as a body defense mechanism against stress or any viral infection. It is essential for cell repair and cell injury. In AD these inflammatory biomarkers act as sign for the disease progression and hence these are evaluated for the same. Inflammatory markers such as IL-6, NF-β, IFN-ϒ was seen increased in the disease group. During acute or chronic type of inflammation the pro-inflammatory cytokine, IL-6 is released acting as a biomarker. In the treated rats there was decrease in this marker showing that the drug also might have anti-inflammatory action..(Heneka et al., 2018)Interferon gamma is another cytokine that plays a vital role in the cellular immune response in response to viral infections in the body. Gamma type interferon is produced by the T type of cells mainly involved in the microglial activation. These cytokines are present in higher levels in the AD brain (mild type). In the diseased group the levels were found to be higher and when treated with the drug, the treated group showed lower levels of this cytokine. (Bagyinszky et al., 2017) Nerve growth factors have major role in cell differentiation, survival and development of the neuronal cells. These factors get activated by the release of pro- inflammatory markers and generally produced in higher levels due to stress and inflammation. The expression of this factor is seen elevated in the hippocampus of the AD brain. In the diseased group these levels are higher while in the treated groups the levels were found to be lower. (Clark, 2007) Aβ induces degeneration of the neurons in the CA1 region.

Histopathology data in the treated groups showed lesser number of the degenerated neurons. There was intact CA1 region in the treated group as well. (Scheltens et al., 1995).

Our results do comprehend with the results obtained from other studies conducted for Alzheimer's disease showing positive affect in the selected area of parameters that we have chosen for our study.

CHAPTER 7
CONCLUSION

CONCLUSION:

From the results obtained we can conclude that the anti-viral nano formulation improved the disease condition by reduced Alzheimer's specific parameters as well as inflammatory biomarkers. Further adequate development of the nano-formulation can yield much better results which could be comparable to that of the standard drug treatment. Much more exploration would be required for the same especially for those patients who have a history of Herpes Simplex Virus infection.

CHAPTER 8
REFERENCES

REFERENCES

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