Therapeutic Potential of Rivastigmine for Managing Complications Associated with Ischemic Stroke

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A thesis submitted to the National University of Sciences and Technology, Islamabad,

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Supervisor: Dr. Aneeqa Noor

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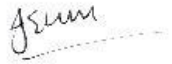
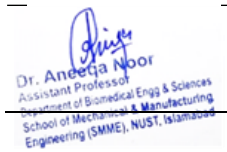
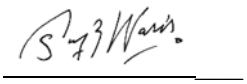
Student Name: Irum Naeem Abbasi Signature: 

Supervisor Name: Signature: 

Name of Dean/HOD: Signature: 

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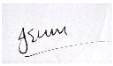

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DEDICATION

Dedicated to my exceptional parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

AChE	Acetylcholinesterase
BuChE	Butyrylcholinesterase
CSF	Cerebrospinal Fluid
Ach	Acetylcholine
ICD-11	International Classification of Disease 11
ICH	Intracerebral Hemorrhage
TIA	Transient Ischemic Attack
AF	Atrial Fibrillation
HDL	High-density Lipoprotein
PCP	Phencyclidine
LSD	Lysergic Acid Diethylamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA	N-methyl-D-aspartate
MCAO	Middle Cerebral Artery Occlusion
MCA	Middle Cerebral Artery
SOD2	Superoxide Dismutase 2
TLR4	Toll like Receptor 4
CCA	Common Carotid artery
ECA	External Carotid artery
ICA	Internal Carotid artery
NOR	Novel Object Recognition
AD	Alzheimer's Disease
PD	Parkinson's Disease
H&E	Haematoxylin and Eosin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
cDNA	Complementary DNA
RT-PCR	Real-time Polymerase chain reaction
qPCR	Quantitative Polymerase chain reaction

ABSTRACT

Stroke continues to be the world's primary cause of morbidity and mortality, frequently with incapacitating consequences like movement dysfunction, cognitive decline, and neurological impairments. It is essential to manage stroke effectively to lessen its negative effects and enhance patient outcomes. The potential neuroprotective benefits of acetylcholinesterase inhibitors may assist maintain the integrity of brain tissue and lessen the damage that neurons may sustain after a stroke, making them a promising option in this situation. This comprehensive thesis explores the neuroprotective benefits of an acetylcholinesterase inhibitor, Rivastigmine, in relation to stroke outcomes in a variety of ways. Using an advanced integrative methodology, this study includes behavioral evaluations, *in silico* analysis, histopathological results, and molecular studies to give a comprehensive picture of the possible mechanisms behind therapeutic effects of Rivastigmine. Within the field of computer modelling, the docking interactions between SOD2 and TLR4, the target proteins of Rivastigmine, provide detailed structural information that directs further experimental validations. Following Rivastigmine treatment, the behavioral tests conducted exhibited improvements in cognitive, motor, and social domains. These results were further corroborated by histopathological analysis, which shows neuroprotective effects in Rivastigmine treated group as opposed to surgery (MCAO) group. Post Rivastigmine treatment, real-time PCR data showed a rise in SOD2 and a decrease in TLR4 levels in surgery (MCAO) rats, exhibiting antioxidant and anti-inflammatory effects of Rivastigmine. These findings provide interesting directions for future neuroprotective approaches and shed light on the possible therapeutic implications of Rivastigmine in reducing neurodegeneration that occurs in stroke.

Keywords: Stroke, rivastigmine, neuroprotection, superoxide dismutase 2, middle cerebral artery occlusion, toll like receptor 4.

CHAPTER 1: INTRODUCTION

1.1 Stroke

1.1.1 Background

Stroke occurs when there is clot in the artery that causes blockage or sudden rupturing of blood vessel in the brain. Blockage in the arteries causes rupturing and swelling of blood vessels and eventually cause bleeding. There is scarcity of oxygen and nutrients supply to brain cells which result in the death of brain cells. When stroke occurs, arteries burst causing brain cells to suddenly die from shortage of oxygen. The consequences include loss of memory, learning and death as well. Since 1995, stroke has been incorrectly classified in ICD-11 which is also known as International Classification of Disease II. This has changed with the release of new ICD-11 which has classified stroke as a neurological disorder (Shakir, 2018).

1.1.2 Epidemiology of Stroke

Stroke is second in the mortality list, killing 5.5 million people every year. It affects 13.7 million people worldwide. The most common type of stroke is ischemic stroke which affects 87% of world population (Roger et al., 2011a). In case of hemorrhagic stroke, first-time hemorrhages commonly occur with an estimation of 85-90%. Following them are second-time hemorrhages resulting in 10-15% of stroke cases. Worldwide statistics state that in financially stable families, stroke occurrence chances reduced by 42% but unfortunately increase two fold in financially less stable families during 1990 and 2016 (Vos et al., 2017).

1.1.2.1 Age-Specific Stroke

With increasing age, the chances of stroke occurrence increase. During 1990 and 2016, Kelly (2010) reported increasing trend in stroke cases among individuals who were not even 55 years of age which was concerning. Number of cases increased from 12.9% to 18.6% (Kelly-Hayes, 2010). Most of the stroke cases were recorded in China with 331-378 cases followed by Eastern Europe with 181-218 cases. Lowest stroke cases were reported Latin America with 85-100 cases (Johnson et al., 2019).

1.1.2.2 Gender-Specific Stroke

Females report highest number of stroke cases in contrast to males. Though age also effects the chances of stroke occurrence, males report low number of stroke cases. Stroke in females occur because of various reasons including pregnancy related complications like preclampsia, by using medications that effect hormones and even the use of contraceptives may result in stroke.

Death rate is higher in females due to cardioembolic stroke which is a very serious type of stroke. Intracerebral hemorrhage is most common in males (Boehme et al., 2017a).

1.1.2.3 Geographic and Racial Variation

Worldwide stroke occurrence causes mainly include physical attributes, behavior, medical history, and laboratory results. It also demonstrated how contact with particulate matter and air pollution affects the risk of stroke (Chen, 2010). In a population-based study, Zhang (2017) discovered that hypertension was statistically significant risk for ischemic stroke (F. L. Zhang et al., 2017). In addition to identifying hypertension as a primary cause of stroke, a US study also noted regional differences in the severity of stroke symptoms in victims. Other risk factors that can lead to stroke included lack of physical activity, unhealthy eating routine and drug abuse (Kiefe et al., 1997).

1.1.2.4 Socioeconomic Variation

The correlation between socioeconomic status and stroke is strong and inverse, leading to adverse healthcare system and stroke after care for financially less stable population (Addo et al., 2012). Studies carried out in Austria linked educational attainments to the use of therapies including speech therapy and echocardiography; however, socioeconomic status had no bearing on medical facilities for stroke patients (Arrich et al., 2008). Kerr (2011) reported equal distribution of healthcare facilities by Scottish medical centers among their people who were suffering from life threatening conditions, not prioritizing their financial backgrounds (Kerr et al., 2011).

1.1.3 Risk Factors for Stroke

Stroke incidence rate increases with age. Males and females who are above 55 years of age, are at a greater risk of stroke. Stroke chances also significantly increase if a person is already suffering from some medical conditions like high blood pressure, hyperlipidemia or atrial fibrillation. If a person is suffering from any sort of coronary heart disease, then the chances of stroke to occur significantly increase. 60% of stroke cases were reported in the patients who were already critically ill and were suffering from TIA, which is called Transient Ischemic Attack. Two stroke risk factors include non-modifiable and modifiable factors as shown in Figure 1.1 (Kuriakose & Xiao, 2020).

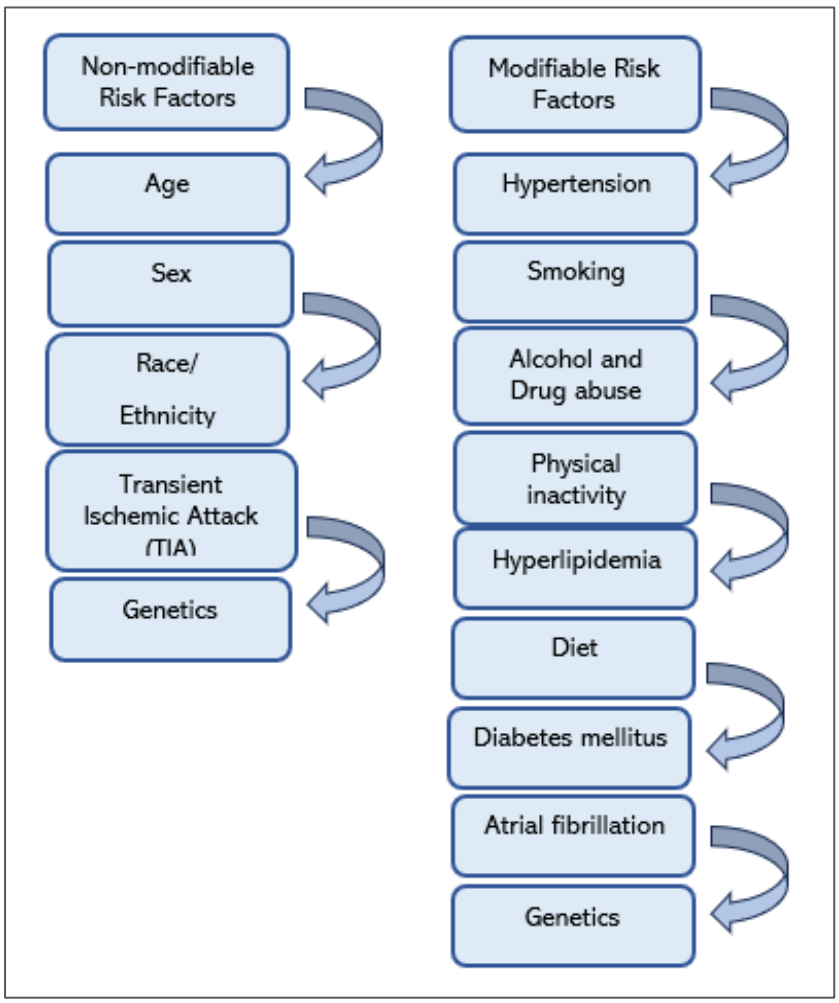

Figure 0.1: Risk factors associated with stroke. There are two types of stroke risk factors: Modifiable and Non modifiable. In both of the stroke risk factors, genetics play a significant role.

1.1.3.1 Non-Modifiable Risk Factors

Ethnicity, sex, TIA, and age contribute to non-modifiable stroke risk factors (Roger et al., 2011b). TIA is categorized as a minor stroke since it shares the same underlying mechanism as a full-blown stroke. A transient interruption in the blood flow occurs to certain areas of the brain. It is an indication that a full blown stroke can occur giving time to modify lifestyle choices and start taking medicines to decrease the chances of stroke occurrence (Ferro et al., 1996). Genetics play a critical role in both types of risk factors associated with ischemia. An individual's age, sex and race all affect their genetic risk (Seshadri et al., 2010)

In addition to these factors, gazillions of other factors also increase the risk of stroke. Initially, a person's chance of having ischemia is heightened if someone in their immediate family also suffered from stroke. Secondarily, in some cases, a gene mutation called cerebral autosomal dominant arteriopathy may cause stroke. Further, a genetic mutation can produce many

diseases, including sickle cell anemia, of which stroke is one of the many repercussions. Additionally, certain frequent genetic variations - like the 9p21 gene polymorphism - are associated with heightened incidence of ischemia (Matarin et al., 2008). Boehme (2008) indicated that genetic research may help us better understand the many types of stroke, manage patients more effectively, and provide earlier and more accurate disease prediction (Boehme et al., 2017b).

1.1.3.2 Modifiable Risk Factors

1.1.3.2.1 Hypertension

Hypertension is one of the main causes of stroke risk. Study conducted by Collins (1990) indicated that if a person suffers from high blood pressure that ranges from 160/90 mmHg, then that individual is at higher risk of stroke. It was recorded in 54% of stroke cases (Collins et al., 1990). Randomized trials demonstrated similar outcomes to control hypertension in adults 60 years of age and above, with a decrease in stroke occurrence signs of 36% and 42% respectively (Staessen et al., 1997).

1.1.3.2.2 Diabetes

Diabetes may also lead to stroke, resulting in a 20% increased mortality rate and increase the chances of ischemic stroke. Diabetic patients have worst disease progression and their recovery time is also very slow as compared to non-diabetic patients. Such patients suffer adverse impairments due to stroke (Banerjee et al., 2012). For diabetics, medical interventions combined with behavioral adjustments may help reduce the severity of stroke; strict control of blood glucose levels alone is unsuccessful (Lukovits et al., 1999).

1.1.3.2.3 Atrial Fibrillation

Atrial Fibrillation (AF) is a major risk factor associated with stroke which doubles the chances of stroke, on the basis of age of affected person (Wolf et al., 1991). Studies have demonstrated that reduced left ventricular blood flow in AF results in brain embolism and thrombolysis. Recent research, however, has refuted this conclusion, pointing to scant evidence of a sequential time relationship between the incidence of atrial fibrillation and stroke, observing that in certain cases, atrial fibrillation is detected post stroke. In other instances, stroke can strike people who have AF- specific genetic abnormalities long before AF manifests (Brambatti et al., 2014).

1.1.3.2.4 Hyperlipidemia

Hyperlipidemia primarily causes heart diseases but its relation with stroke is undeniable. High cholesterol level is directly proportional to stroke incidence rate implying that high cholesterol levels lead to stroke. On the other hand, high-density lipoprotein (HDL) decreases the chances of stroke (Iribarren et al., 1996). Therefore, examination of lipid profile provides an estimate of the chance of stroke. Yaghi and Elkind (2015) research demonstrated that stroke death rate was increased due to low high-density lipoprotein (<0.90 mmol/L), increase blood pressure and high triglyceride (>2.30 mmol/L) (Yaghi & Elkind, 2015).

1.1.3.2.5 Alcohol and Drug Abuse

Stroke risk increases with daily alcohol intake. Stroke risk is increased by excessive liquor consumption. Contrary to this, minute amount of alcohol heightens the chance of hemorrhagic stroke (Gill et al., 1986). Chances of stroke are increased by using illegal drugs like heroin, cocaine, amphetamines, cannabis/marijuana, PCP also called phencyclidine, and LSD also termed as lysergic acid diethylamide (Esse et al., 2011). Alcohol intake of <1 for women and <2 for men decrease the chances of ischemia, whereas high intake increases it.

1.1.3.2.6 Smoking

Smoking is a significant modifiable risk factors of ischemia and adversely affects quality of life. The stroke risk is significantly raised by tobacco use. Smokers are at increased risk of stroke than people who don't actively smoke. Bhat (2008) reported that passive smokers are at increased risk of stroke by 30%. People who quit smoking lowers the incidence of stroke occurrence (Bhat et al., 2008). Quitting smoking is one preferable step one can take to minimize the chances of stroke occurrence.

1.1.3.2.7 Insufficient Physical Activity and Poor Diet

Lack of physical activity increases the chance of ischemia. High blood pressure, obesity and diabetes, are the consequences of lack of physical activity, leading to stroke (Zhou et al., 2007). Certain food items like too much salt consumption can increase the risk of stroke as it may cause high blood pressure. Stroke chances are greatly reduced when a person consumes healthy food items, with less salt and cholesterol like Mediterranean diet (Larsson et al., 2011).

1.2 Pathophysiology of Stroke

Stroke is caused by either blockage of blood vessels or by rupturing or leaking of blood vessels in the brain. Flow of blood towards brain is under control of certain arteries namely internal carotid arteries and vertebral arteries (the circle of Willis). Hemorrhagic stroke is the result of

leaking of blood vessels in the brain that causes blood leakage within the brain tissues causing them to swell and dysfunctional. Contrary to this, ischemic stroke is the result of lack of nutrients to the brain (Musuka et al., 2015a).

1.2.1 Ischemic Stroke

15% of stroke deaths are related to intracerebral hemorrhage. On the other hand, 85% stroke mortalities are result of ischemic stroke. These blockages occur in thrombotic and embolic conditions, which ultimately lead to stress and pre mature cell death in thrombotic and embolic stroke. In thrombotic stroke, arteries become narrow and blood cannot easily flow through vascular chamber in this condition (Musuka et al., 2015b). Ultimately, aggregation of plaque occurs in the arteries that hinder blood flow and cause swelling of arteries as well. In embolic stroke, there is decrease in the blood supply to the brain tissues, making brain tissues deprived of oxygen and nutrients and lead to stress and necrosis, eventually leading to stroke.

Following necrosis is disruption of cell membrane, increase in the size of organelles and all the cellular contents are released into the outer space. It results in loss of neuronal function and cell death (Broughton et al., 2009). Certain factors have a vital role in causation of stroke. Some of them include neuronal inflammation, loss of energy, decline in homeostatic functions, increase in calcium levels between the cells, increased toxicity, oxidative stress, responsible for the progression of ischemia in patients (Gelderblom et al., 2009).

1.2.2 Hemorrhagic Stroke

Death rates are higher in hemorrhagic stroke as compared to ischemic stroke, accounting for 10-15% of death rate. In hemorrhagic stroke, there is leaking and bursting of blood vessels in the brain, which causes swelling of neuronal cells and neuronal death. Toxicity is induced in vasculature, ultimately lead to ischemia (Flaherty et al., 2005). There are two types of hemorrhage: subarachnoid and intracerebral. Aberrant blood accumulation happens in the brain due to ICH, which is brought on by clotting of blood in arteries, increased blood pressure, aberrant vasculature, increase intake of anticoagulants, and thrombolytic medications (Testai & Aiyagari, 2008a).

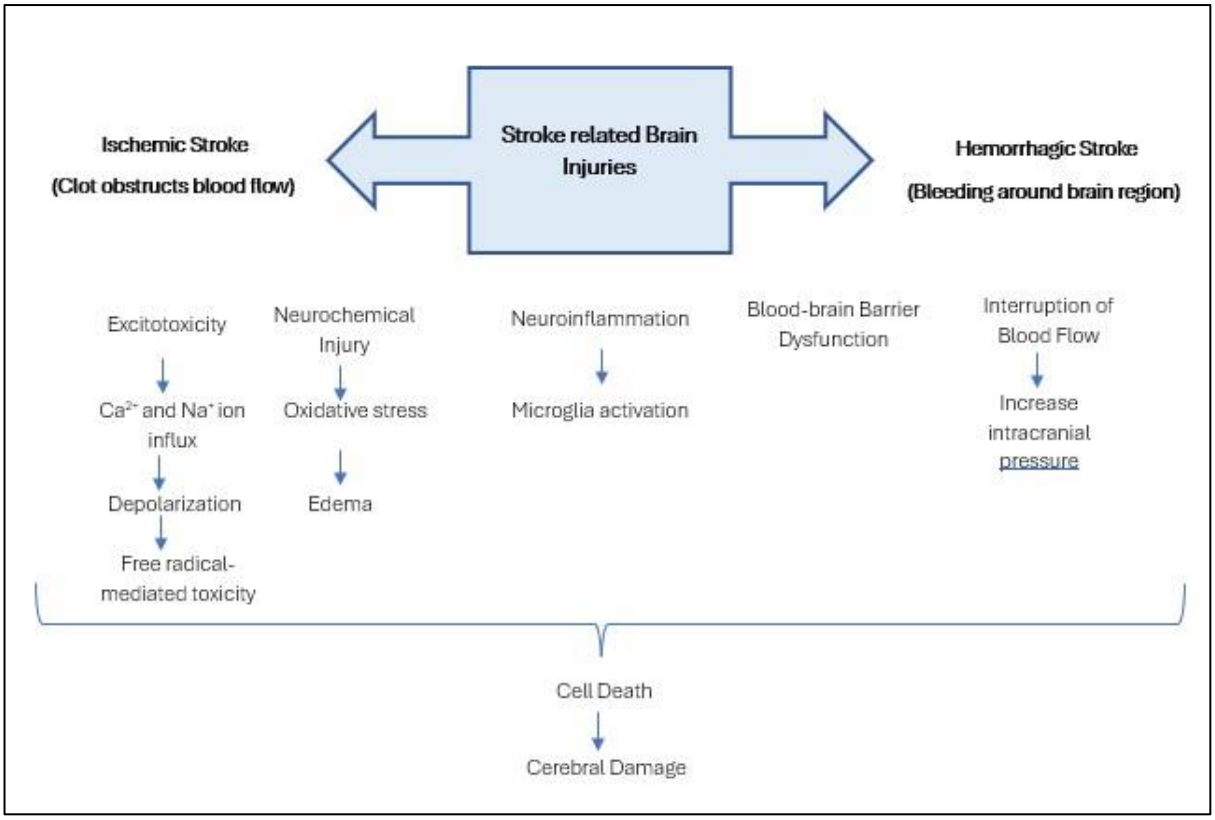

Figure 0.2: Stroke Molecular Mechanism. Ischemic stroke occurs because of the obstruction in the blood vessel resulting in deficient supply of nutrients to the brain. Bursting of blood vessels in the brain cause hemorrhagic stroke.

1.3 Acetylcholinesterase Inhibitors

Acetylcholinesterase inhibitors (AChE) inhibitors are also widely called anti-cholinesterases. These medications boost the total quantity and duration of acetylcholine's activities in the synaptic cleft by preventing it from breaking down normally into by products, choline and acetate. There are many uses of anti-cholinesterases. Primarily, they are used to treat neurodegenerative disorders including Lewy body dementia, Parkinson's disease, and Alzheimer's disease. These neurodegenerative diseases involve several physiological mechanisms that lead to the death of Ach-producing cells and a decrease in cholinergic transmission in various brain areas. By decreasing hydrolysis of acetylcholine into its subsequent products, cholinesterase inhibitor medications suppress AChE activity and preserve Ach levels (Colovic et al., 2013).

Cholinesterase inhibitors, most frequently neostigmine, are given to patients after surgeries to undo the effects of nondepolarizing muscle relaxants such as rocuronium (Ma et al., 2019). If anticholinergic poisoning is suspected, cholinesterase inhibitors must also be given.

Vasodilation, anhidrosis, mydriasis, delirium, and urinary retention are signs of anticholinergic poisoning (Ahmad et al., 2019). Cholinesterase inhibitors are also used less frequently to treat glaucoma by lowering aqueous humor pressure in people with specific psychiatric illness including schizophrenia (Østergaard et al., 1989).

1.3.1 Cholinesterases

Cholinesterase enzyme catalyze the hydrolysis of acetylcholine in to its subsequent products, acetic acid and choline. After activation of neurons, they need to return to their resting state which is achieved by this hydrolysis. This causes cholinergic neurons to come to their resting phase. Cholinesterases can be divided into two types, namely, acetylcholinesterase and pseudocholinesterase.

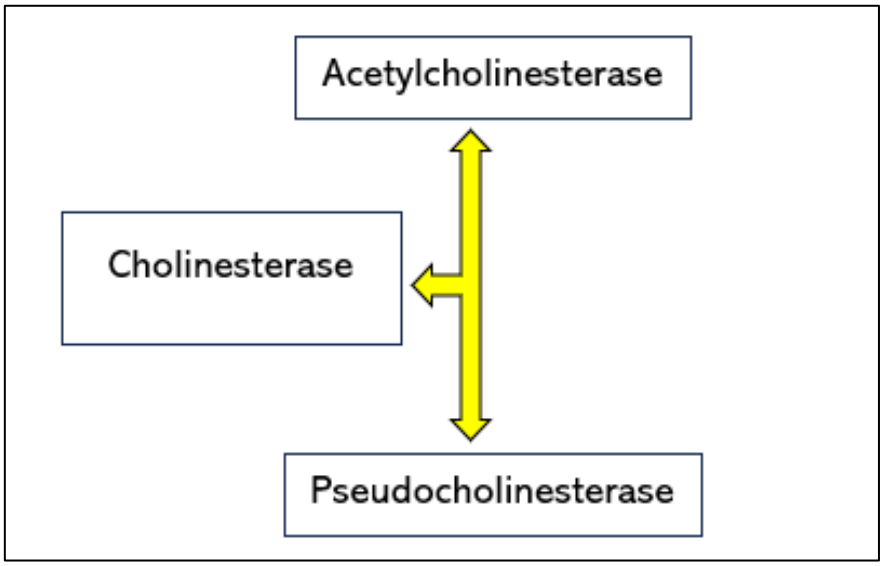

Figure 0.3: Types of Cholinesterases. Cholinesterase catalyzes breakdown of Ach in the brain. It is of two types: Acetylcholinesterase and Pseudocholinesterase.

1.3.1.1 Acetylcholinesterase

Different types of tissues such as muscle tissues, nerve tissues, central and peripheral tissues, sensory fibers, motor fibers, cholinergic and noncholinergic fibers, have the enzyme, acetylcholinesterase. Compared to sense neurons, motor neurons exhibit higher levels of AChE activity (Koelle, 1954). Acetylcholinesterase is also present in the membranes of RBCs, where it is recognized as the Yt blood group antigen. The enzyme exists in a variety of molecular forms, each of which has unique oligomeric assembly and cell surface attachment mechanisms while sharing similar catalytic capabilities. With significantly smaller levels of a monomeric

G1 (4S) form, the majority of AChE in the mammalian brain is found in the tetrameric G4 form (10) (R. Wang & Xi, 2005).

1.3.1.2 Pseudocholinesterase

The liver is the primary location of pseudocholinesterase, which is sometimes referred to as plasma cholinesterase, butyrylcholinesterase, or acyl choline acyl hydrolase. BuChE hydrolyzes butyrylcholine more quickly than AChE does (Huang et al., 2007). BuChE's characteristics and molecular forms are changed, and its cortical levels are elevated in AD. The clinical hallmarks of this condition, plaques and tangles, contain significant levels of BuChE, which may have a role in the development of these lesions. Therefore, inhibiting BuChE may have therapeutic benefits for AD (R. Wang & Xi, 2005).

1.3.2 Acetylcholine as Neurotransmitter

The first neurotransmitter to be identified was acetylcholine (Ach), which is present in the central nervous system at numerous synapses, neuromuscular junction, numerous autonomically innervated organs, and at all autonomic ganglia. Acetylcholine is primarily found in central nervous system interneurons, with significant long-axon cholinergic routes, including the notable projection from Meynert's basal forebrain nucleus to the neocortex and related limbic structures. One of the ailments connected to AD is the degradation of this mechanism (Perry et al., 1999). Acetylcholine is the hallmark for cholinergic neurons that is produced by choline acetyltransferase. Most of acetylcholine is encased in translucent 100 nm vesicles at nerve terminals, and then during transmission of neurons, it is released in the synaptic cleft. This whole process is done after acidification by a pump that is energy dependent.

Transmission of nerve signals is done by acetylcholine receptors which are present on membranes of post synaptic neurons. The post-synaptic membrane contains AChE as well, which hydrolyzes Ach to stop the signal from being sent. The presynaptic neuron absorbs released choline from the Ach breakdown once more, and choline acetyl-transferase facilitates the synthesis of the neurotransmitter by joining it with acetyl-CoA (Perry et al., 1999).

1.3.3 Mechanism of Action

Increase in the amount and mode of action of acetylcholine is done by inhibiting the enzyme, acetylcholinesterase, which is responsible for the hydrolysis of acetylcholine into its subsequent products. This results in the increased function of this neurotransmitter at the post

synaptic neuron. Certain inhibitors such as organophosphates, inhibit the destruction of this neurotransmitter, acetylcholine. It prevents acetylcholine from hydrolyzing in its subsequent products, namely, choline and acetate (Oliveira et al., 2019).

1.4 Rivastigmine

Rivastigmine tartrate is an acetylcholinesterase inhibitor that is pseudo-irreversible and centrally selective in nature (Sugimoto et al., 1995). Long half-life and prolonged dissociation of a carbamoyl derivative from the esteratic region of acetylcholinesterase enzyme plays its role in centrally selective and pseudo-irreversible nature of Rivastigmine. (Gottwald & Rozanski, 1999). The ethyl group of the dimethyl amino ethyl substituent interacts hydrophobically at the major anionic site in addition to crucial N-ethyl carbamate moiety. Rivastigmine does not bind to opioid, dopaminergic or α or β adrenergic receptors (Enz et al., 1994).

Rivastigmine is a unique dual inhibitor of AChE and butyrylcholinesterase (BuChE) which are responsible for acetylcholine hydrolysis in the brain (M. Mesulam et al., 2002). Of them, AChE is the principal cholinesterase and is mostly present at nerve synapses as well as parts of the adult human cerebral cortex that exhibit strong activity (M. -M Mesulam & Geula, 1991). On the other hand, BuChE is primarily found in brain's glial cells and is involved in cholinergic mediation (Wright et al., 1993). Neuronal depletion in the cholinergic cells residing in basal forebrain corresponds to memory loss. The invention of drugs which enhance acetylcholine-mediated neurotransmission is an outcome of this 'cholinergic hypothesis.'

The neurotransmission of acetylcholine is vital for learning, memory, attention, and other cognitive functions. As a result, an inadequate supply of cholinergic neurons may have a significant role in the loss of cognitive functions. AChE serves as a catalyst in the breakdown of acetylcholine into choline and acetic acid (Enz et al., 1994). Gottwald and Rozanski's (1999) preclinical research validated the Rivastigmine's transport into the cerebrospinal fluid (CSF) and at moderate dosages, Rivastigmine enhanced cognition and was generally well tolerated (Gottwald & Rozanski, 1999).

1.4.1 Rivastigmine in the Treatment of Neurodegenerative Diseases

Some of the carbamate inhibitors that block AChE and BuChE are Rivastigmine. Through the formation of a covalent link, Rivastigmine binds to esterases, temporarily deactivating them. It responds to acetylcholinesterase's ester and anionic sites. As a result, Ach generally rises. The symptomatic treatment of moderate to intermediate dementia in people with Alzheimer's

disease and idiopathic Parkinson's disease is the indication for oral Rivastigmine administration (Nguyen et al., 2021).

Rivastigmine, taken orally at a dose of 6 to 12 mg/day or as a transdermal patch containing 9.5 mg/day may provide AD patients with sustained improvements in cognitive function and daily activity performance. Furthermore, in several clinical trials, Rivastigmine slowed the advancement of the disease. In every cognitive ability test that was administered, the patients' cognitive abilities improved (Birks et al., 2015). While Rivastigmine is advised for intermediate AD, it also appears to be successful when used topically as a transdermal patch for severe AD. Additionally, it enhanced these patients' cognitive skills (Ferris et al., 2013).

Furthermore, Rivastigmine exhibits neuroprotective properties, lowers β-amyloid levels and changes the APP processing pathway to a non-amyloidogenic one. In recent trials, Rivastigmine has been demonstrated to enhance gait stability, which may help lower the risk of falls in PD patients (Henderson et al., 2016). Youn's (2016) study has demonstrated the usage of perioperative Rivastigmine in reducing the incidence of surgical delirium in older patients with cognitive dysfunctions (Youn et al., 2017).

According to computerized cognitive evaluation, some studies have also demonstrated that medications such as Rivastigmine and other cholinesterase inhibitors can enhance cognitive function in patients with dementia exhibiting Lewy bodies. Patients on Rivastigmine showed 30% improvement over the placebo group. They also reported reduced anxiety levels overall and fewer hallucination episodes (Hershey & Coleman-Jackson, 2019). As AChE and BuChE activity increase as people get older, their levels are significantly higher than normal in neurodegenerative disorders. Rivastigmine has been proven to bind reversibly and block both of these enzymes, in contrast to other cholinesterase inhibitors, increasing acetylcholine levels overall (Kandiah et al., 2017).

1.5 Prevention and Treatment Strategies for Stroke

1.5.1 Excitotoxicity

Neuronal death in stroke is caused by depolarization of neurons and incapability of cell to maintain potential of plasma membrane. Primarily recognized as neuroprotective drugs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA), now initiate this process of neuronal excitotoxicity. Targeting the metabolic pathways that follow excitotoxicity signaling instead of only concentrating on glutamatergic signaling may help mitigate the detrimental effects of the process (Sutherland et al., 2012).

1.5.2 Gamma Aminobutyric Acid (GABA) Agonists

GABA agonist clomethiazole reduced the signs of ischemia, however it was shown to be ineffective in lowering the toxicity brought on by glutamate receptor activation. Clomethiazole may be a GABAergic agent, although it was ineffective in reducing glutamate-induced toxicity in stroke patients. This demonstrates the intricacy of neuroprotective techniques in stroke care and emphasizes the necessity of additional study into complementary therapy modalities (Wahlgren et al., 1999).

1.5.3 Sodium (Na+) Channel Blockers

In many animal stroke models, Na^+ channel blockers have been used to provide neuroprotection. These blockers lessen white matter destruction and stop demise of neurons (Carter, 1998). Clinical trials examined several voltage-gated Na^+ channel blockers, however most of them were ineffective. Hewitt (2001) conducted a research which demonstrated mexiletine, neuroprotective agent and Na^+ channel blocker, is beneficial in case of gray and white matter ischemia (Hewitt et al., 2001).

1.5.4 Calcium (Ca2+) Channel Blockers

Segura (2008) reported that in ischemic animal models, voltage dependent Ca^{2+} ion channel blockers minimize the destruction caused by stroke. During experimentation, the Ca^{2+} ion chelator DP-b99 was shown to be efficient and safe when given to stroke patients. Similarly, ischemic patients who were given these calcium channel blockers within 12 hours of occurrence of stroke, showed huge improvement (Segura et al., 2008). In an additional trial, Ca^{2+} channel blockers, as opposed to diuretics and beta blockers, decreased ischemic risk by 13.5%.

Various recent and contemporary approaches for stroke prevention and care have been shown in Figure 1.4. These preventive strategies play a vital role in managing stroke symptoms and post-stroke effects as well. Stroke management strategies include stroke acute care to manage risk factors related to stroke, reperfusion, rehabilitation, cognitive decline, and neuroprotection and repair. Reperfusion includes Intra Arterial Thrombolysis (IAT) and Intravenous Thrombolytics (IVT), as shown in Figure 1.4.

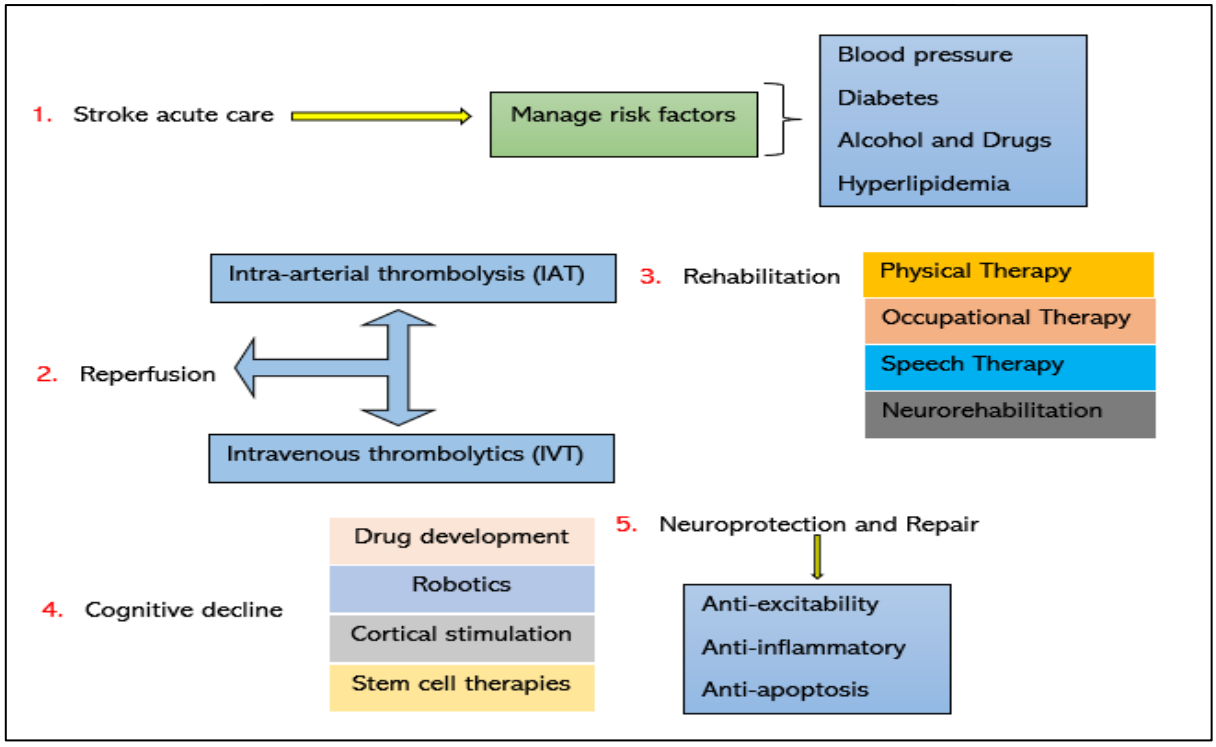

Figure 0.4: Stroke Therapy. Stroke management strategies include acute care, reperfusion, rehabilitation, cognitive decline, and neuroprotection and repair**.**

1.6 Trends in Stroke Research

Since new medications were created to treat several potential targets. In addition to that, our awareness of the pathophysiology of stroke has also been improved, the incidence of cerebrovascular-related emergencies has significantly decreased. Death and morbidity rates have decreased because of technological developments (Akbik et al., 2017). Consequently, post-stroke care facilities should be a part of stroke management systems. Hospitals should create standard processes for responding to emergencies quickly to minimize fatalities and stop subsequent stroke (Adeoye et al., 2019). Physiotherapists' roles have become more prominent in the management of post-stroke care recently. Many physiotherapists have already started doing different types of rehabilitation therapies to relieve the symptoms of stroke (Arienti et al., 2019).

One extending study conducted by Bonini-Rocha (2018) demonstrated the use of circuit class treatment, electromechanical device therapy, and treadmill exercise to improve mobility and control handicap (Bonini-Rocha et al., 2018). Physiotherapists and other specialists gather in Stroke Recovery and Rehabilitation Roundtables to suggest research directions and develop guidelines for recovery after stroke. The coming era of stroke healthcare will involve better

access to rehabilitative treatments and the implementation of stroke care systems (Eng et al., 2019).

The effects of stroke in human beings are not fully reflected in animal models employed in research. Furthermore, the amount of research that can be produced by tests carried out in a single laboratory is frequently limited. To be as relevant as possible, not only young animal models but also old animal models should be included to develop in-vivo animal models following ischemia. To eliminate gender biasness, studies on stroke should include both male and female participants.

Additionally, other variables such as obesity, diabetes, and hypertension should also be included. Stroke research is expensive and complex due to all these problems, which suggests that it should be conducted cooperatively across several labs. In a perfect environment, a global multicellular clinical trial platform would be devised to improve the validity of research findings. The current obstacles in the process of turning laboratory data into stroke treatments will be addressed with the aid of this approach (Kalladka et al., 2016). Regenerative therapy has been made possible by innovations in stem cell technologies and genes, which can be used to re-establish brain working and mend neuronal damage resulting from stroke (Macrae & Allan, 2018). As a regulator of Wnt signaling, the WIP1 gene presents a viable target for therapeutic intervention (Qiu et al., 2018). Several natural substances have shown potential in the prevention and treatment of stroke. They are competitively safe and efficacious and their composition costs much lesser than artificial substances

The Utstein methodology is a strategy that identifies the key components of management tools, standardizes, and publishes research on stroke that occurs outside of a hospital. The Utstein community has created extensive initiatives to enhance stroke sufferer's early detection and care on a global scale (Rudd et al., 2020). Future clinical trials ought to focus on characterizing recovery and clinical outcomes in addition to determining the safety and efficacy of medications (Chollet et al., 2014). All things considered, stroke management research has flourished quickly in past years and will surely generate further important findings to limit stroke adverse effects.

1.6.1 Translational Obstacles in Contemporary Stroke Therapeutic Approaches

Recent years have seen major improvements in the field of stroke research. Numerous pharmacological targets and therapeutic treatments have been made possible by advancements in methodological development, imaging techniques, and animal model selection. Despite this,

pre-clinical outcomes were not validated in the succeeding clinical trials. Although recanalization therapy produced some encouraging outcomes in clinical trials, its benefits were limited to a small subset of stroke patients (Khandelwal et al., 2016). Obstacles like endpoint selection, gender and age effects, confounding illness models, co-occurring disorders, impede potential of research of stroke (Boltze & Ayata, 2016).

The potential for multiple causation of the stroke is another issue that often goes unrecognized. Homogenous stroke models are essential to capture the diverse range of stroke causes associated with stroke injury, cortical damage, or intracerebral damage. As a result, stroke animal models that focus on stroke causes ought to be used. Finding the latent connection between impairments and stroke treatment will improve the clinical outcome's safety and effectiveness (Boltze et al., 2017). As short-term experimental studies frequently provide false-negative results in clinical settings, they frequently fail to develop successful therapeutics (Fisher et al., 2009).

In clinical studies, it might be challenging to interpret the functional and behavioral outcomes that could mislead about authentic recovery because animal models are better able to minimize the functional improvements (Boltze et al., 2014). This has an impact on how well the research can be translated. For effective transition, a coordinated approach to model rehabilitation and recovery must be adopted. Inadequate data management is one of the other issues with the stroke clinical studies. There should be a standardized process to manage the massive amounts of data produced by multiple clinical trials due to their overwhelming influence. Additionally, for ease of access, this data ought to be placed in a publicly accessible data repository.

To increase the translational value of stroke research, industry and academic collaborations are essential (Boltze et al., 2016). A successful transition requires collaboration between academic and corporate interests. Industry collaborations are sometimes motivated by financial gains and time restrictions, which may jeopardize the design of pre-clinical study protocols, adequate sample sizes, and treatment effect measurements. Translational barriers in stroke research are likely to be advanced by a multicenter strategy, extended cooperation, using cutting-edge methodology, and formulation of practical objectives (L. Wang et al., 2015).

1.7 Animal Models of Stroke

Research often uses induced, spontaneous, negative, and orphan animal models. An animal selected for the spontaneous model has a similar disease state that is, needless to say, existing already in it. However, disease is induced in animal in the induced model in order to study the

effects. To investigate the underlying resistance mechanisms of a certain illness situation, negative animal models are employed. To comprehend the biology of a recently identified illness in human patients, orphan models are utilized (Rollin & Kesel, 1990). In contrast to animal models, which are extremely dependable and foreseeable, ischemia in humans is unforeseeable in nature, with a wide range of clinical manifestations and locations. Easy access to the brain's tissue is frequently necessary for pathophysiological research, but it is not feasible in human beings as compared to animal models (Fluri et al., 2015).

1.7.1 Middle Cerebral Artery Occlusion

Over the years, a number of animal models have been established to investigate the pathophysiology of post-ischemic reperfusion (Shigeno et al., 1985). The middle cerebral artery occlusion (MCAO) procedure in rodents, known as the "suture model", has gained preference recently due to its comparatively atraumatic nature and ease of reversibility to allow for reperfusion, setting it apart from many other models (McAuley, 1995). Commonly implicated, MCAO has recently been expanded to the mouse in genetic research examining the pathways behind cell death. As compared to craniotomy models, this model generates focal blockage of a major cerebral artery as observed in human strokes, can be extensively used, and is more amenable to reperfusion research (Carmichael, 2005). Variability is decreased by strict control over temperature, physiological parameters, and occlusion assessment using noninvasive techniques (such as Laser Doppler).

1.7.2 Intraluminal Filament Technique

One proven technique for creating repeatable infarcts in the middle cerebral artery (MCA) region is suture or filament occlusion. This technique mimics the therapeutic process of mechanical thrombectomy without requiring a craniectomy and permits reperfusion upon removal of the occluding filament. Consequently, this technique is being used excessively to imitate stroke patients in animals (Lopez & Vemuganti, 2018). The benefit of this approach is that permanent or temporary occlusion can be achieved by precisely controlling the reperfusion duration through filament removal.

There is noticeable damage from the ischemic penumbra, although not as many as during the craniotomy surgery. On the other hand, its drawbacks include limited visibility, a potential for subarachnoid hemorrhage and non-applicability for thrombolytic research. The intracranial filament approach can be used to mimic the removal of endovascular thrombosis and has been extensively utilized in the research of reperfusion injury, ischemic penumbra, and the time

window of thrombolytic therapy (Durukan & Tatlisumak, 2007). Two major classes of ischemic animal models include Global Ischemia Model and Focal Ischemia Model. Global Ischemia Model can be induced either by complete ischemia or incomplete ischemia. If we talk about Focal Ischemia Model, it can also be induced by two ways namely focal ischemia and multi focal ischemia. In complete ischemia, there is aortic occlusion and cephalic artery. In incomplete ischemia, 4-vessel occlusion occurs in animal models. Focal ischemia causes MCAO and multi-focal ischemia results in embolism as shown in Figure 1.5.

1.8 Aims and Objectives

To fully understand the neuroprotective properties and possible therapeutic uses of Rivastigmine, more research on the compound's potential in ischemic stroke is necessary, especially in animal models like MCAO in rats. Rivastigmine is a unique dual inhibitor of AChE and BuChE and it can be a workable choice for lessening the detrimental

consequences of ischemia because of its dual inhibitor activity. Studies are being conducted to determine whether Rivastigmine can successfully lessen brain damage, improve cognitive function post stroke, and control stroke related oxidative and inflammatory responses.

1. Establishment of rat model of stroke.
2. Optimization of drug doses to overcome post-stroke effects.
3. Molecular and histopathological analysis for stroke evaluation.

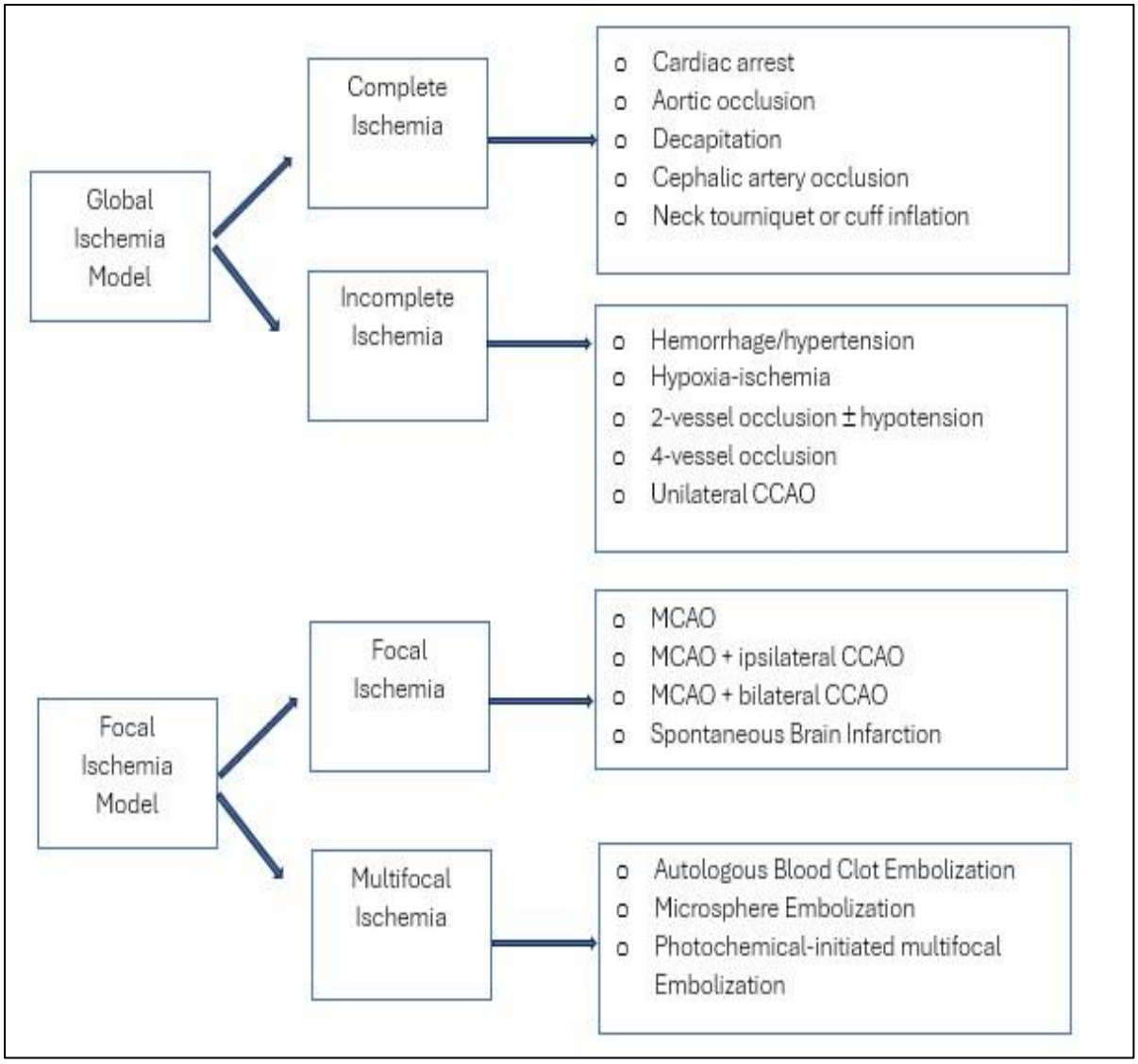

Figure 0.5: Stroke Animal Models. Global Ischemia Model includes Complete and Incomplete Ischemia. Focal Ischemia Model includes Focal and Multi-Focal Ischemia**.**

CHAPTER 2: MATERIALS AND METHODS

This chapter discusses the complete materials and methodology used in the entire course of this research study.

2.1 Animals

Male Wistar adult rats (age 8-12 weeks) were used in this study. The rats were kept in plastic cages with open access to water and rodent food till the time of dissection. Prior to experimentation, rats were acclimated to the laboratory environment. The rats were divided into three distinct experimental groups: Healthy group, Surgery group (MCAO) and Treatment group (Rivastigmine treated).

2.3 Ethical Considerations

The project was reviewed by the NUST Institutional Review Board (IRB number 07-2023-ASAB-01/01) prior to initiate the experimentation and received approval. The study followed all ethical considerations and was administered by the Institutional Animal Care Guidelines.

2.2 Experimental Design

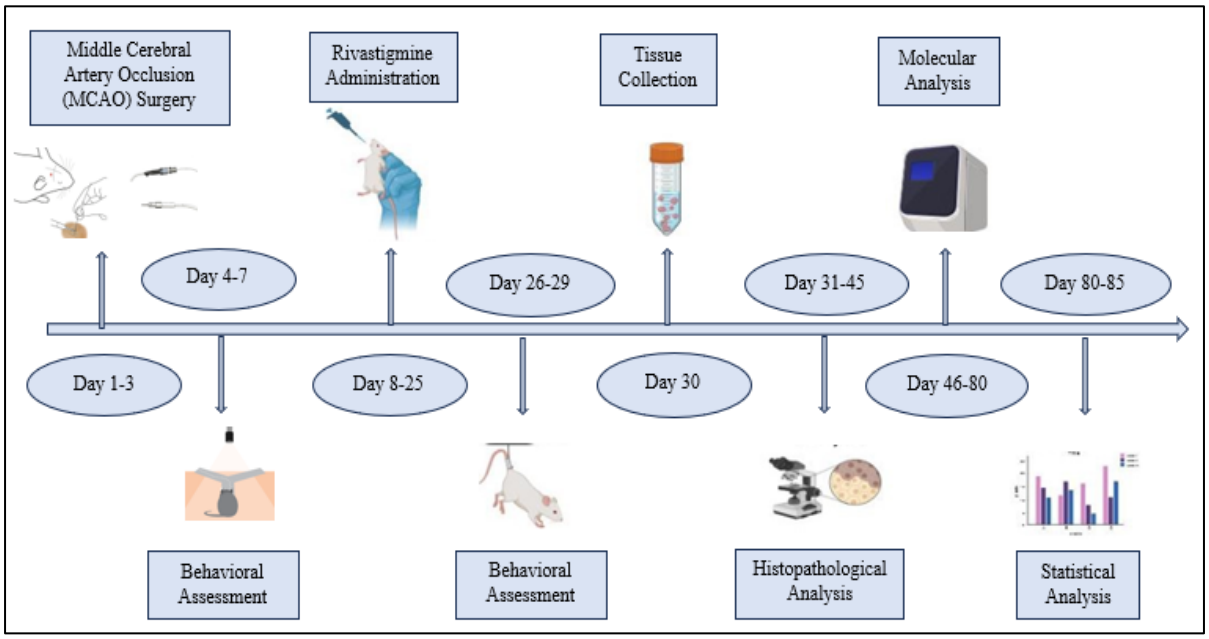

Figure 0.1: Experimental Design. This figure illustrates the timeline and experimental design throughout the research study.

2.4 Grouping of Animals for Treatment and Drug Schedule

Rivastigmine (1 mg/kg) was administered orally in rodents to examine its neuroprotective response. The present study used a total of three groups namely healthy, MCAO and Rivastigmine treated group. Each group contains five rats. Healthy group contains normal rats that did not undergo any surgical procedure or drug treatment. Surgery group comprised of five rats that underwent middle cerebral artery occlusion surgery and did not receive any drug treatment. Rivastigmine treated group comprised of five rats that underwent middle cerebral artery occlusion surgery and later, also received rivastigmine treatment.

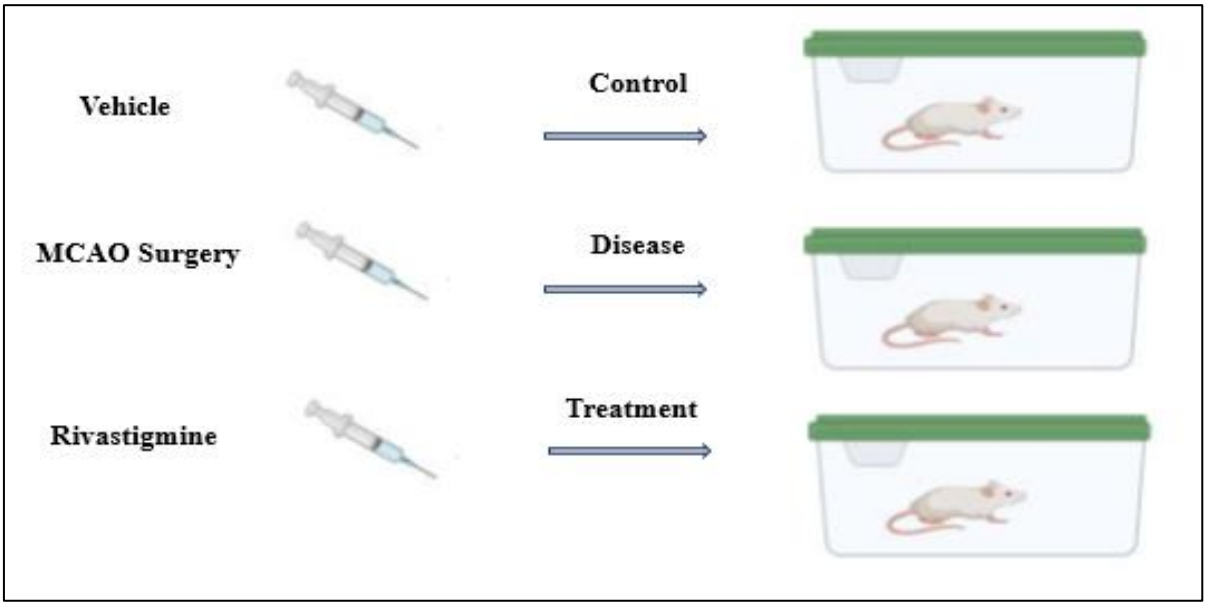

Figure 0.2: Group Design for Drug and Treatment Administration. The figure shows the corresponding groups with their specific drug and treatment.

2.5 Induction of Stroke Model

Rats were anesthetized by the administration of xylazine-10 mg/kg (FX56092-BIOSYNTH) and ketamine-80 mg/kg (17750-Pipelinepharma) intraperitoneally. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were then exposed via a midline incision made in the neck region as shown in Figure 2.3 (a). For isolation of vessels, common carotid artery was separated from the surrounding tissues. To prevent blood flow from external carotid artery, it was ligatured. Nylon 4-0 monofilament (SA9151524- Vital Medical Supplies) was used to create a temporary blockage, inducing ischemia in the rats. The filament was then pushed through the isolated CCA and then into ICA, then it finally reached MCA. Incision was then closed with silk suture 3-0 (BNSG093221- Vital Medical Supplies). Occlusion was maintained for about thirty minutes to induce transient ischemia. After the

desired duration, nylon filament was withdrawn to allow reperfusion of the MCA. Then restoration of blood flow was allowed as shown in Figure 2.3 (b). The incision was sealed with sutures. Finally, rats were allowed to recover from anesthesia in a warm and monitored environment.

Figure 0.3: Stroke Induction in Rats. (a) MCAO surgery. (b) Withdrawal of nylon filament to allow reperfusion.

2.5.1 Rivastigmine Dose Preparation

The total volume of Rivastigmine solution needed for the experiment was calculated and this was derived by measuring the weights (in grams) of all the rats that will be given rivastigmine solution orally. Stock solution of 0.2 mg/ml was prepared by dissolving 1 mg of rivastigmine powder in 5 ml of distilled water.

2.6 Behavioral Assessments

Five behavioral tests were conducted following the induction of stroke in animal models assessing symptom development and evaluating treatment efficacy. These behavior tests played a crucial role analyzing the role of Rivastigmine in the improvement of spatial and cognitive memory in the rats. After the Rivastigmine treatment phase, behavioural evaluations to assess cognitive and motor deficits were carried out. To assess cognitive and motor functions, five behaviour tests were carried out such as Novel Recognition Test (NOR), Y-Maze Test, Social Interaction Test, Open Field Test and Grip Strength Test.

2.6.1 Novel Object Recognition Test

NOR is a commonly employed behavior test in rodents as it is very helpful in studying short-term memory, intermediate-term memory, and long-term memory. It is accomplished by adjusting the retention interval, which is the amount of time that animals have to remember the sample objects that were offered to them during the familiarization phase before a new object replaces the familiar one during the test phase (Taglialatela et al., 2009). The test was comprised of three stages: habituation phase, familiarization phase, and test phase. Before commencement of the test, it was completely made sure that rats were not perplexed or hyperactive. Rats were relaxed once they were put into testing environment. A completely sterile environment was made sure for behavior tests. Special care was given to hygiene. When a test was performed on one rat, testing instruments were properly sterilized before entry of the second rat.

In the habituation phase, each individual rat was allowed to explore empty box. Then two identical red colored cylinders were put at opposite corners of the box and each rat was allowed to explore these objects for about six minutes as shown in Figure 2.4 (a). It was a familiarization phase. Then the rats were removed and placed in their cages. In the testing phase, new object replaced the known object, which was a yellow-colored cylinder in this experiment. Then each rat was individually allowed to explore the novel object for about six minutes (Ennaceur, 2010) as shown in Figure 2.4 (b).

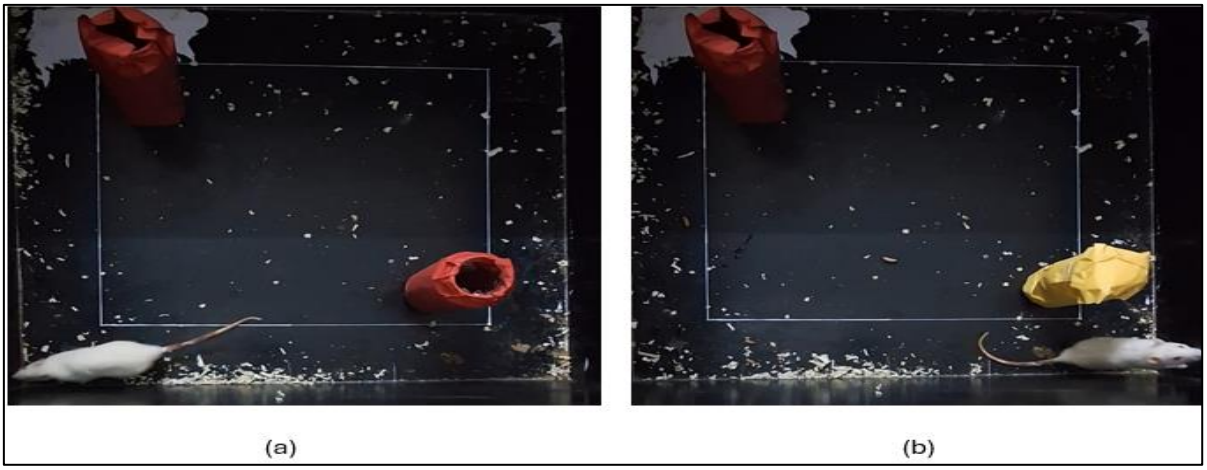

Figure 0.4: Novel Object Recognition Test (NOR). a) Familiarization phase. (b) Testing phase with novel object. In familiarization phase, two identical (red) objects were placed in the arena. In testing phase, the red colored object at the extreme right corner was replaced by novel (yellow) object. Recognition memory was assessed by calculating total time rats spent with new object post-Rivastigmine treatment in stroke models.

A twelve-minute session was analyzed and videotaped. After the test phase, the behavioral parameters were measured by analyzing the recorded videos. The total duration during which the rats explored novel object was calculated (Ennaceur & Delacour, 1988). To examine the behavioral variations between the rivastigmine treated and surgery groups, a healthy control group of rats who did not go through MCAO surgery were included and the behavior differences were compared.

2.6.2 Y-Maze Test

Y-maze is used to test short-term memory of rats. Allowing rats to explore all three y-maze arms allows for the assessment of spontaneous alternation, a measure of spatial working memory that is motivated by rodent's natural eagerness to investigate locations they have not visited before (Kraeuter et al., 2019). On experiment day, each rat was habituated to y-maze for about ten minutes as shown in Figure 2.5 (a). Y-maze consisted of three arms namely S, O and N. During habituation phase, entry to N-arm was closed and each rat was allowed to explore the rest of the two arms freely. Then rats were removed from the Y-maze and were placed in their cages back. For the testing phase, each individual rat was placed at the center of the maze and allowed to explore three arms (S, O, N) of the maze freely for 10 minutes as shown in Figure 2.5 (b). After the test phase, the behavioral parameters were measured by analyzing the recorded videos. The arm entry sequence was used to calculate number of alternations (Prieur & Jadavji, 2019). To examine the behavioral variations between rivastigmine treated and surgery (MCAO) groups, a healthy control group of rats who did not go through MCAO surgery were included and behavior differences were compared.

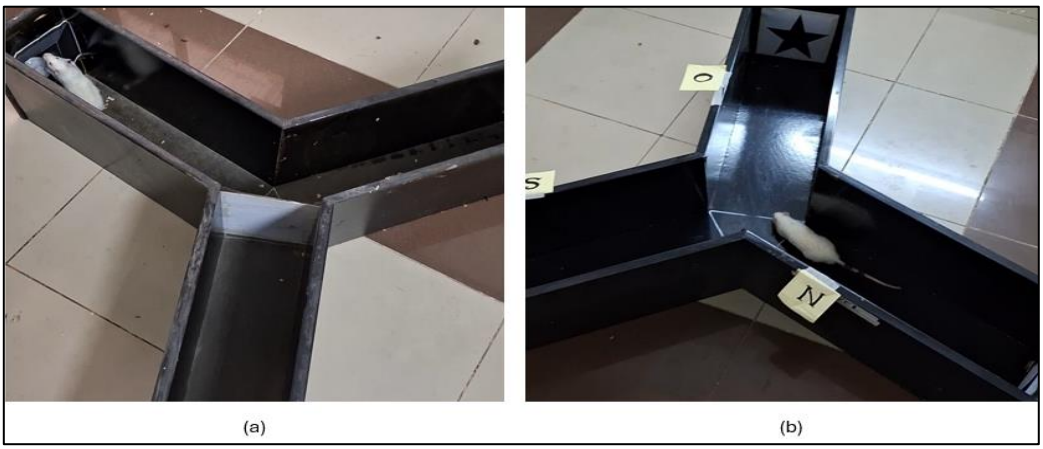

Figure 0.5: Y-Maze Test. (a) Habituation phase; (b) Testing phase. Novel arm was temporarily blocked during habituation phase and rats explored two arms of y-maze arena only. During testing phase, previously blocked novel arm was opened and rats were allowed to explore all three arms of y maze freely.

2.6.3 Grip Strength Test

Assessing muscle strength is a crucial first step in the study of neuromuscular disorders (Cabe et al., 1978). Each rat was gently held by the base of its tail and allowed to hold the bar with its forelimbs as shown in the Figure 2.6 (Takeshita et al., 2017). After the test phase, the behavioural parameters were measured by analysing the recorded videos. Time taken by rats to hold the bar before falling was measured. To examine the behavioural variations between the rivastigmine treated and surgery (MCAO) groups, a healthy control group of rats who did not go through MCAO surgery were included and behavior differences were compared.

Figure 0.6: Grip Strength Test. Rats were gently put on the grid and total time before their falling was recorded to assess their muscular grip strength.

2.6.4 Open Filed Test

Open field test was developed with the purpose of assessing anxiety-like behavior in rodents following neurodegenerative disorders (Hall, 1934). Open field maze that was used for behavior test was enclosed by walls high enough to keep rats from running away. Rats were brought to the experiment room approximately thirty minutes before the beginning of the testing phase. Each rat was individually placed in the center of the open filed maze by gently holding its tail. Then the rat was freely allowed, in the open field maze, to explore the quadrants for about seven minutes as shown in the Figure 2.7 (Seibenhener & Wooten, 2015). After the test phase, the behavioral parameters were measured by analyzing the recorded videos. Number of entries in the center and periphery were calculated. To examine the behavioral variations between the rivastigmine treated and surgery groups, a healthy control group of rats who did not go through MCAO surgery were included and behavior differences were compared.

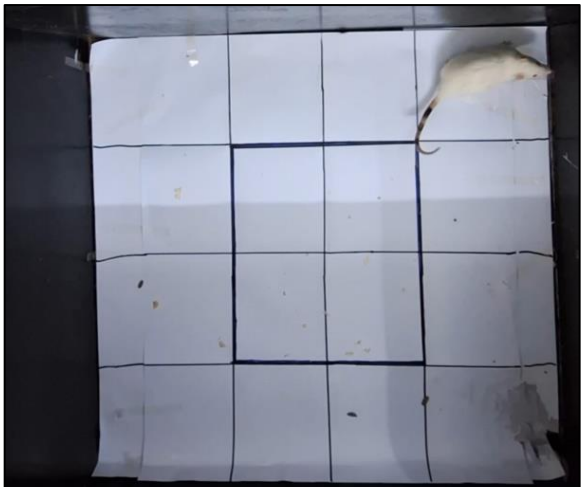

Figure 0.7: Open Field Test. Rats were placed in an open filed box. Inner and outer zone were separated by making boxes. Four boxes in the center represent central region. The outer twelve boxes represent peripheral region.

2.6.5 Social Interaction Test

A range of neuropsychiatric conditions are distinguished by abnormalities in social cognition and behavior (Robinson et al., 2005). Crawley's sociability procedure was used in this test which is basically a three-chamber paradigm test that has been effectively used to investigate social memory and social connections in rodent models (Clapcote et al., 2007). During habituation or adaptation phase, the subject rat was placed in the central chamber for about 3 minutes as shown in Figure 2.8 (a). Then during session 1 of the test, one of the control rats (Stranger I) was placed in the left chamber and the subject rat was allowed to explore three chambers for about five minutes as shown in figure 2.8 (b).

During session 2 of the test, the second control rat (Stranger II) was placed in the right chamber and the subject rat was allowed to explore three chambers for about five minutes as shown in figure 2.8 (c). After the test phase, the behavioural parameters were measured by analysing the recorded videos. Total duration of contact of subject rat with stranger I and stranger II was calculated. To examine the behavioural variations between the rivastigmine treated and surgery groups, a healthy control group of rats who did not go through MCAO surgery were included and behavior differences were compared.

Figure 0.8: Social Interaction Test. Three different compartments were built inside a large box. In the left compartment, stranger I was placed and in the right one, stranger II was placed. Middle one was empty to allow free movement of rats.

2.7 Rivastigmine Treatment Protocol

A Rivastigmine treatment regimen was designed for rats who went through MCAO, taking rats' weights and desired dosage into consideration. The dosage was administered orally once in a day for a period of 18 days. Rivastigmine treatment regimen for MCAO rats was based on their weights keeping a standard dosage of 1mg/kg.

2.8 Histopathological Analysis

Histopathological analysis was done to examine the tissues at a microscopic level and study the morphological changes and histological patterns in the cellular structures of the brain.

2.8.1 Tissue Fixation and Dissection

The rats were euthanized under deep chloroform inhalation. For histopathological analysis, the transcardial perfusion was performed by using the fixative solution of 4% paraformaldehyde flushing through the circulatory system. By flushing through the bloodstream and displacing blood, the fixative ensured complete tissue fixation. After that the rats were carefully decapitated. The skull was then removed using scissors and a scalpel along the midline to expose the brain. By using little forceps, the brain from the skull was removed gently. To remove extra fixative and blood from the sample, the tissues were then washed with phosphate buffered saline (PBS). The brain tissue was then carefully immersed in the fixative solution of 4% paraformaldehyde.

2.8.2 H&E Staining

For Hematoxylin and eosin (H&E) staining the brain was then dehydrated by immersing the perfusion-fixed brain in ethanol at progressively higher concentration of 100%. Then transferred to a clearing agent of xylene to remove ethanol. Then thin sections of the tissue were cut down. The segments were then tinted with H&E dye to visualize the cellular structures.

2.8.3 Microscopic Examination

The stained sections of the brain were then examined under the light microscope and the tissue morphology, cell count, and cellular patterns were analysed. The photomicrographs of cortex were taken to analyse the changes between the three groups and to understand the effects of the treatment and disease processes.

2.9 Gene Expression Analysis

2.9.1 Reverse Transcription Polymerase Chain Reaction

2.9.1.1 Dissection

Rats were deeply anesthetized under chloroform inhalation. After that, rats were carefully decapitated by using sharp scissors. The skull was then cut using fine scissors and a scalpel along the midline to expose the brain. By using little forceps, the brain from the skull was removed gently snap-frozen on dry ice and kept at -80°C for later processing.

2.9.1.2 RNA Extraction

The total RNA from the tissues was isolated using the TRIzol isolation reagent (FTR100, Fine Biotech Life Sciences, China). In this first step, 1000 µl trizol reagent was added to the sample and then homogenized followed by centrifugation at 12000 rpm for 10 minutes at 4˚C. After centrifugation, the supernatant was transferred to a new tube and 200 µl of chloroform was added to the sample. After that, the tube was vigorously shaken for 30 seconds and then centrifuged at 12000 rpm for 10 minutes at 4˚C. Then carefully transfer the aqueous phase (containing RNA) to a new tube and add 500 µl of isopropanol to it, mix well and, incubate at room temperature for 10 minutes. Then again centrifugation was done at 12000 rpm for 10 minutes at 4˚C which was followed by removing the supernatant. The next step was to wash the pellet with 75% ethanol and then centrifuge at 12000 rpm for 2 minutes at 4˚C. The ethanol was discarded carefully and then the pellet was air dried for 5-10 minutes followed by resuspending the pellet with 20-50 µl nuclease-free water.

2.9.1.3 Assessment of RNA Quality and Quantity

The extracted RNA's quality and quantity were assessed using Colibri NanoDrop (TitertekBerthold, Germany).

2.9.1.4 cDNA Synthesis (Reverse Transcription)

The RNA extraction was then followed by cDNA transcription using RevertAid Reverse Transcriptase (EP0441, Thermo Fisher Scientific, Lithuania). The reaction mixture was then prepared including the reaction buffer, dNTPs, reverse transcriptase, oligodts, diathiothreitol (DTT) and, RNA sample. The thermal cycler was then used to incubate the reaction mix under specified conditions of 42˚C for 60 minutes.

2.10 *In silico* Analysis

Before staring the experimentation *in silico* analysis was carried out using different software's and computational methods to gain insights and predict the hypothesis before conducting them physically. For that the three-dimensional structure of Rivastigmine, PubChem ID (77991), was downloaded from PubChem in SDF format. The 3-D structures of Toll-like receptor 4 (TLR4) and Superoxide dismutase 2 (SOD2) were obtained from Protein Data Bank in PDB format. These structures were then cleaned by using the software LigPlot. The docking was then carried out using the software of PyRx to comprehend the structural basis of Rivastigmine, TLR4 and SOD2 selectivity and to calculate the binding affinity of Rivastigmine (ligand) with TLR4 and SOD2 target proteins. Rivastigmine, TLR4 and SOD2 interactions emphasizing key interaction patterns were then visualized using the Discovery Studio software.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 Primer Designing

The primers, TLR4 and SOD2, were selected from the published literature. Then primer BLAST was done in NCBI (National Center for Biotechnology and Information) to verify the specificity and accuracy of the selected primer with the target shown in Figure 2.9 (a) and 2.9 (b) before using them for Polymerase Chain Reaction (PCR). The primers had the calculated annealing temperature of 66.0. The primers were ordered from Bionics (Islamabad, Pakistan). Three primers namely Beta actin, SOD2 and TLR4 were used in PCR. Their specific characteristics are mentioned in Table 2.1. Beta actin was used as a house keeping gene against SOD2 and TLR4. Annealing temperature for beta actin was 57°C. SOD2 and TLR4 had the annealing temperatures of 66°C

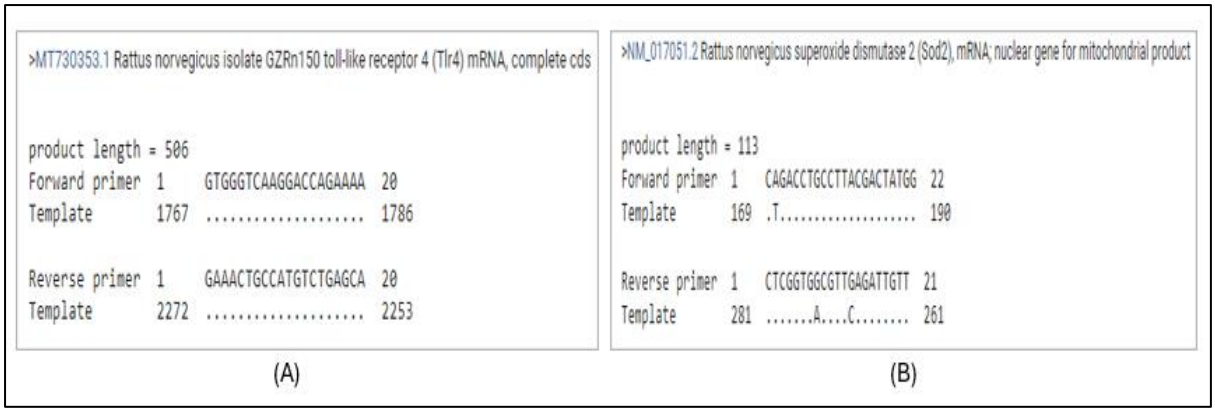

Figure 0.9: Blast for TLR4 and SOD2. The details of the primer BLAST were done in NCBI to verify the specificity of the primer.

Table 0.1: Primer Characteristics. The table shows the primers used with their specific length, sequence, and optimized annealing temperature.

GENE	DIRECTION	LENGTH	SEQUENCE (5' to 3')	ANNEALING TEMPERATURE (°C)
ß-actin	Forward	20	GCCTTCCTTCTTGGGTATGG	57
ß-actin	Reverse	19	CAGCTCAGTAACAGTCCGC	57
Toll like receptor 4 (TLR4)	Forward	20	GTGGGTCAAGGACCAGAAAA	66.0
Toll like receptor 4 (TLR4)	Reverse	20	GAAACTGCCATGTCTGAGCA	66.0
Superoxide dismutase 2 (SOD2)	Forward	22	CAGACCTGCCTTACGACTAGG	66.0
Superoxide dismutase 2 (SOD2)	Reverse	21	CTCGGTGGCGTTGAGATTGTT	66.0

2.11.2 Gradient PCR

Using Gradient PCR, a sample was prepared for primer optimization to determine the annealing temperature. Gradient PCR profile is as follows. A three minutes initial denaturation step at94°C, followed by 35 cycles at 94°C for 30 seconds, and an annealing step at temperatures between 58°C and 68°C for 30 seconds. Gradient temperatures were then followed by an

extension step lasting 45 seconds at 72°C and a final extension lasting 7 minutes at 72°C. After the PCR, the resultant product was analyzed for bands on a gel electrophoresis.

Table 0.2: Gradient Temperatures. The table displays the range of annealing temperatures used for gradient PCR.

GRADIENT TEMPERATURES					
58.0 °C	60.0 °C	62.0 °C	64.0 °C	66.0 °C	68.0 °C

2.11.2.1 Reaction Mixture

The PCR tube was filled to a total capacity of 25 µl with 12.5 µl of PCR master mix (W1401-2, Wizbio Solutions, South Korea), Nuclease-free water: 8.5 µl, forward primer: 1 µl, reverse primer: l µl, cDNA template: 2 µl

Table 0.3: List of PCR Ingredients.

	COMPONENTS	QUANTITY (µl)
1.	PCR Master Mix	12.5
2.	Nuclease Free Water	8.5
3.	Forward Primer	1.0
4.	Reverse Primer	1.0
5.	cDNA Template	2.0

2.11.3 Agarose Gel Electrophoresis

To validate whether annealing had occurred at the appropriate temperatures or not, gel electrophoresis was performed by using 2% of agarose (39346, Sigma Aldrich, USA) and 10X TBE buffer (T1051, Solarbio, China). The bands' locations were compared to the DNA ladder (ranging from 100 to 1500bp) to determine whether annealing had occurred or not. The gels were then analyzed using a Benchtop 2UV transilluminator (LM-20 | P/N 95044902, UVP Co., USA).

2.11.4 Real-Time PCR

Real-Time PCR also known as the qPCR was used to measure TLR4 and SOD2 expression levels in brain tissues on a real-time PCR detection system (Biorad) using TLR4 (sense 5'-

GTGGGTCAAGGACCAGAAAA-3' anti-sense 5'-GAAACTGCCATGTCTGAGCA-3') and SOD2 (sense 5'-CAGACCTGCCTTACGACTATGG-3' and anti-sense 5'-CTCGGTGGCGTTGAGATTGTT-3') primers, by using the cycling parameters described in Figure 2.10. Mouse beta actin (control) qPCR was also conducted employing the primers (sense 5′- GCC TTC CTT GGG TAT GG-3′ and sense 5′- CAG CTC AGT AAC AGT CCG C -3′) Denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 30 seconds. 35 cycles. The reaction mixture was made using WizPure™ qPCR Master (SYBR) (W171, Wizbio, Korea). The PCR reaction mix consists of a cDNA template, nuclease-free water, forward primer, reverse primer, and SYBR green master mix making a total of 20 µl of the reaction mixture as described in table 5. To assess the quality of the PCR product, amplification curves, and agarose gel electrophoresis were employed.

Table 0.4: qPCR Master Mix Preparation. The table shows the components of qPCR master mix preparation along with their quantities to make 20 µl of PCR mix.

	COMPONENTS	QUANTITY (µl)
1.	cDNA Template	1.0
2.	Forward Primer	1.0
3.	Reverse Primer	1.0
4.	SYBR Green Master Mix	4.0
5.	Nuclease Free Water	13.0
	Total Reaction Volume	**20.0**

2.11.4.1 Cycling Parameters for Real-Time PCR

Figure 2.10 displays the Real-time PCR cycling parameters. PCR circumstances: 35 cycles of denaturation at 94 °C (3 min), annealing at 66°C (30 s), and elongation at 72 °C (45 s).

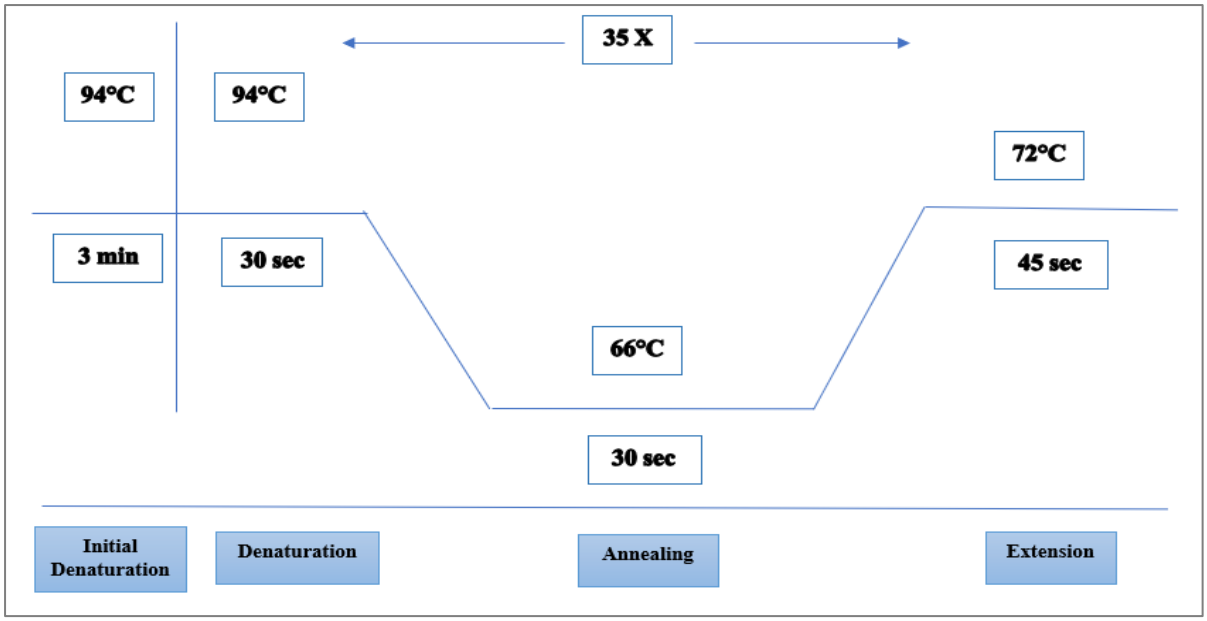

Figure 0.10: Cyclic Parameters for qPCR. The figure shows thermocycling profile for TLR4 and SOD2.

2.12 Statistical Analysis

The distribution of all data sets was evaluated for normality before any statistical analysis. Statistical analysis was performed to analyze the differences between the control, surgery, and Rivastigmine-treated groups. To ascertain whether there were any significant differences between the groups, statistical tests such as Tukey's test, one-way ANOVA and t-test were utilized. The graphs were created using Graph Pad Prism version 10.0, at $P < 0.05$. Data and outcomes were expressed using ±SEM.

CHAPTER 3: RESULTS

3.1 Behavioral Assessment Results After Rivastigmine Treatment

3.1.1 Novel Object Recognition Test (NOR)

NOR is a measure to analyze recognition memory and exploratory behavior in rodents. Figure (A) exhibited that surgery group spent maximum time exploring familiar object, indicating poor memory and least exploratory abilities. The healthy group spent least time with familiar object followed by Rivastigmine treated group. It is a symbol of intact recognition memory and exploratory abilities.

Healthy group spent maximum time exploring novel object as shown in Figure (B) Rivastigmine treated group spent comparatively less time exploring novel object but more than MCAO group. It depicts robust recognition memory in rivastigmine treated group which was significantly higher than surgery group. The surgery group spent minimum time with novel object indicating weak recognition memory and cognitive abilities.

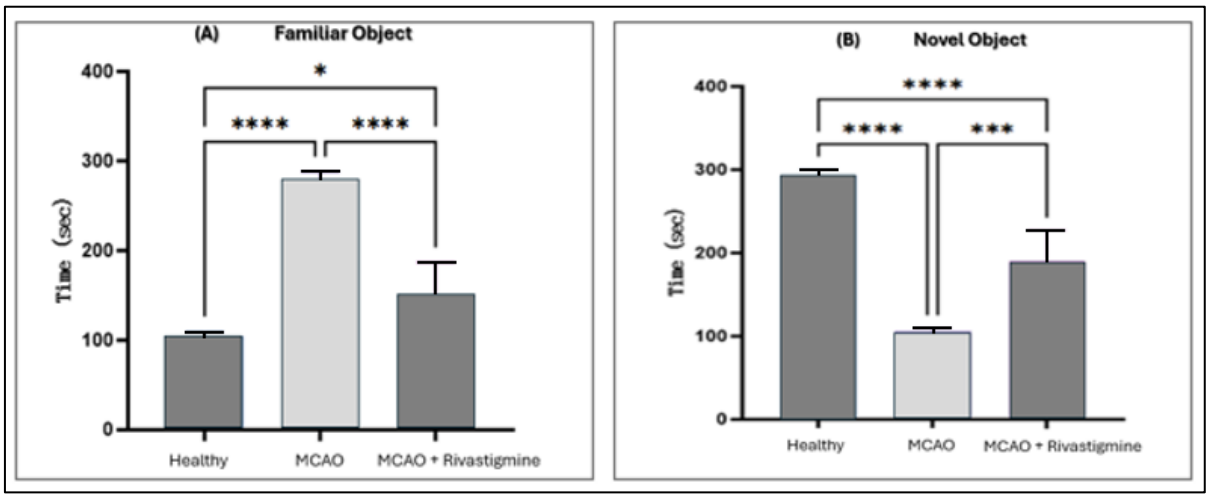

Figure 0.1: Experimental Set up for Novel Object Recognition (NOR) Test. (A) Surgery group depicted a significant preference for spending time with familiar object compared with the control group and the Rivastigmine treated group. This indicates that the MCAO group had a stronger inclination towards familiar object. (B) The control group spent most time with novel object as they preferred exploring it. They were followed by the Rivastigmine treated group, and then MCAO group. For statistical study, Tukey's multiple comparison test and one-way ANOVA were used. ±SEM at ***$p < 0.001$ and *$p<0.05$.

3.1.2 Y-Maze Test

Y-maze test has significant importance in assessing spatial working memory. The effects of Rivastigmine in Y - maze test is shown in Figure 3.2. Rats which have intact spatial memory and a functional prefrontal cortex, usually spend more time exploring novel arm that was blocked during previous exploratory session. Results demonstrated in the graph depict highest spatial memory in healthy group. They encounter highest number of successive entries in three arms of y-maze arena as they had a functional prefrontal cortex and intact memory.

This trend was followed by Rivastigmine treated group as they also showed intact working memory but comparatively less than healthy group. The surgery group spent least time exploring novel arm as they had weak spatial memory. They encountered least number of successive entries in three arms of y-maze. This suggests that rivastigmine administration has a positive impact on spatial working memory, enhancing the rats' ability to alternate between y-maze arms.

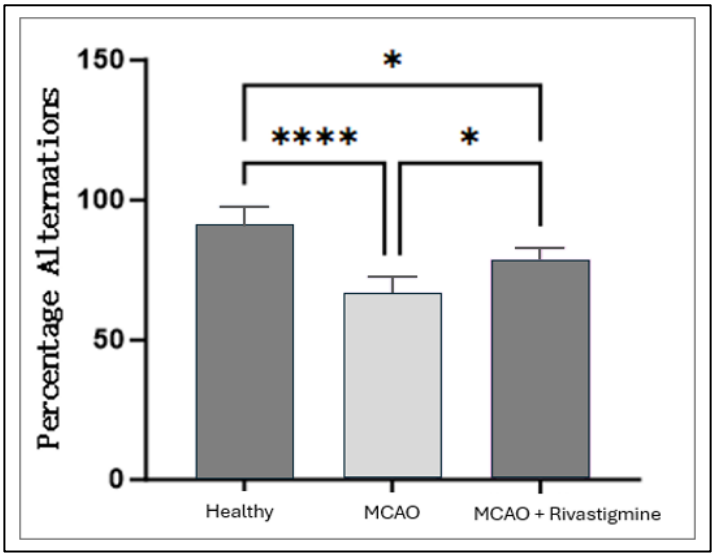

Figure 0.2: Experimental Set up for Y-Maze Test. The graph shows the effects of Rivastigmine and the proportion of successive entries of rats into different arms of y-maze in Y-maze test. Exploring ability was highest in healthy group, followed by Rivastigmine treated group. MCAO group recorded least exploration of arms in y-maze arena. The statistical analysis comprised of conducting a one-way ANOVA, followed by Tukey's multiple comparison tests. The error bars represent the standard error of the mean (SEM) with a significance threshold of *$p < 0.05$.

3.1.3 Grip Strength Test

Grip strength test plays vital role in assessing muscular strength of rodents. The effect of rivastigmine in grip strength test is shown in Figure 3.3. Healthy group had robust muscular grip strength as they spent comparatively more time hanging with the grid using their forelimbs than surgery and Rivastigmine treated group. The treatment group showed improvement in muscular strength of rats as compared to surgery group. Time spent by Rivastigmine treated group to hold grid with their forelimbs was significantly more than surgery group.

The surgery group quickly fall because of diminished muscular grip strength compared to healthy group and Rivastigmine treated group. When assessing the possible neuroprotective effects of Rivastigmine post stroke, grip strength test and the results which are obtained from it, plays a pivotal role. These results add to our understanding of the possible therapeutic effects of Rivastigmine in the setting of neurological rehabilitation.

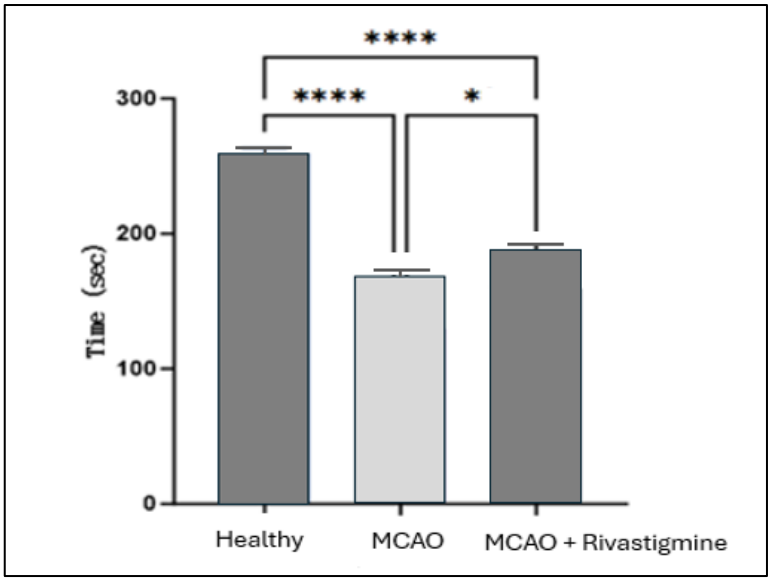

Figure 0.3: Experimental Set up for Grip-Strength Test. This graph depicts the effects of Rivastigmine on rats that underwent MCAO as well as the duration of rats' grip on a mesh grid when inverted. It is crucial to note that among surgery and healthy group, there is significant difference, as well as between the healthy group and the Rivastigmine treated group. The statistical analysis included one-way ANOVA, followed by Tukey's multiple comparison tests. ±SEM at *$p < 0.05$.

3.1.4 Open Field Test

Open field test is one of the significant behavioral tests which is used to assess and analyze rats' anxious behavior. The effect of Rivastigmine in open field test is shown in Figure 3.4 (A) and (B). The results demonstrated increased number of entries by healthy group in central region which is the sign of more exploratory and less anxious behavior, followed by Rivastigmine treated group. On the other hand, MCAO group exhibited most entries in the peripheral region which is the indication of trauma and anxious behavior. Healthy group entered peripheral region the least as they were less anxious, followed by Rivastigmine treated group.

The surgery group encountered maximum number of entries in the peripheral area indicating anxious behavior as they stayed still and exhibited less to no motion. It was followed by Rivastigmine treated group and then healthy group. Figure (B) demonstrated maximum number of entries by healthy group in central region indicating less anxious and exploratory behavior. It was followed by Rivastigmine treated group and then surgery group.

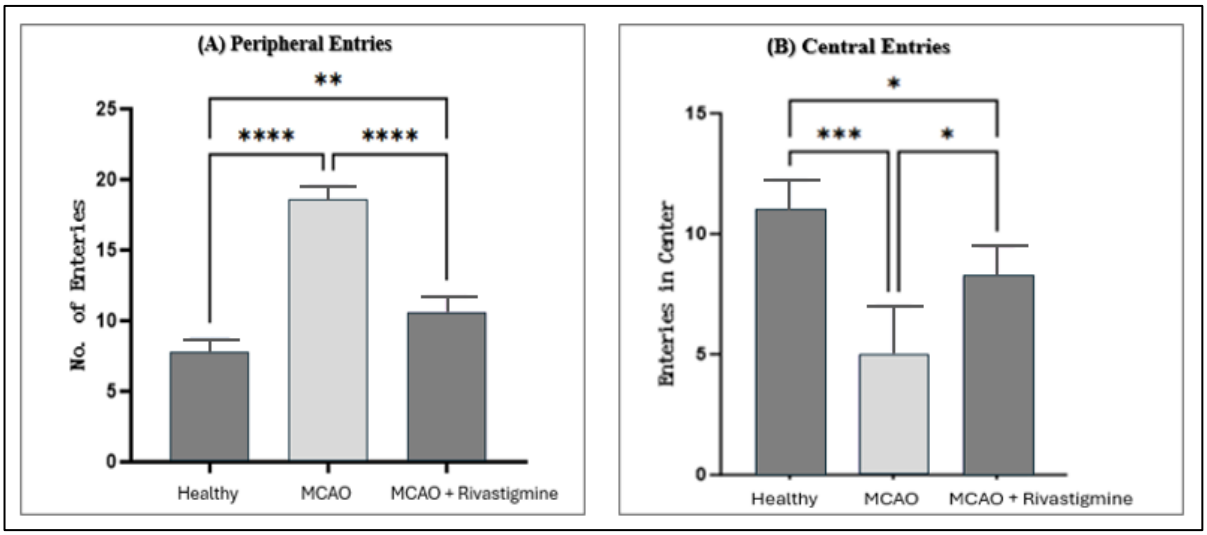

Figure 0.4: Experimental Set up for Open Field Test. (A) MCAO group showed maximum number of entries in the peripheral region (outer zone), indicative of less exploratory and more anxious behavior. It is then followed by Rivastigmine treated group and healthy group. (B) Healthy rats recorded largest number of entries in the central region (inner zone) of the open field arena, followed by Rivastigmine treated group. MCAO group showed least number of entries in central region and indicate less exploratory behavior. Highest ability of exploration was recorded in healthy group and then by Rivastigmine treated group. The statistical analysis included one-way ANOVA, and then Tukey's multiple comparison tests. ± SEM at *$p<0.05$.

3.1.5 Social Interaction Test

The effects of Rivastigmine in social interaction test are shown in Figure 3.5 (A) and (B). The healthy group spent maximum time with stranger I followed by Rivastigmine treated group and lastly by MCAO group. It symbolizes more social behavior in healthy rats as they spent maximum time exploring stranger I rather than empty box. MCAO rats turned out as antisocial as they interacted most with empty box. After the introduction of stranger II in the testing arena, healthy rats exhibited highest sociability. It was then followed by a Rivastigmine treated group as they spent more time with stranger II but it was comparatively less than healthy group but more than MCAO group. MCAO group exhibited anti-sociality and least interaction. They spent more time with stranger I than stranger II as they became familiar with stranger I during first session of the test.

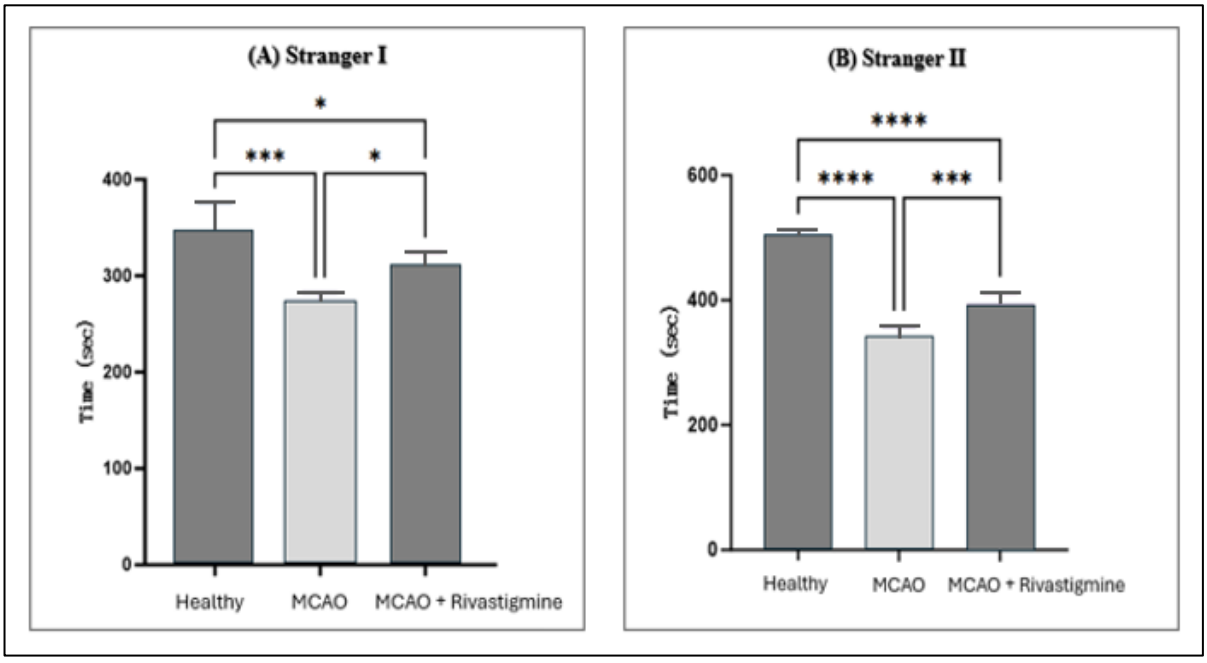

Figure 0.5: Experimental Set up for Social Interaction Test. (A) The healthy group spent maximum time with stranger I followed by Rivastigmine treated group and lastly by MCAO group. It symbolizes more social behavior in healthy rats, followed by Rivastigmine treated group**.** MCAO group exhibited anti-social behavior. (B) Rivastigmine treated group spent more time with stranger II but comparatively less than healthy group. MCAO group exhibited anti-social behavior. For statistical analysis, Tukey's multiple comparison test and one-way ANOVA were used. Standard error of the mean (SEM) at a significance level of *** $p<0.001$ and *$p<0.05$.

3.2 Histopathological Results

3.2.1 Effects of Rivastigmine on Histology

3.2.1.1 Cortex

The effects of Rivastigmine on cortex of the MCAO rats were stained with H&E. Cell quantification was achieved by ImageJ software in order to calculate number of viable cells and for comparative analysis. Electron microscope (Binocular NSL – CX23 Olympus (Japan) was used to obtain images at 10X magnification power. The histological findings are presented in Figure 3.6.

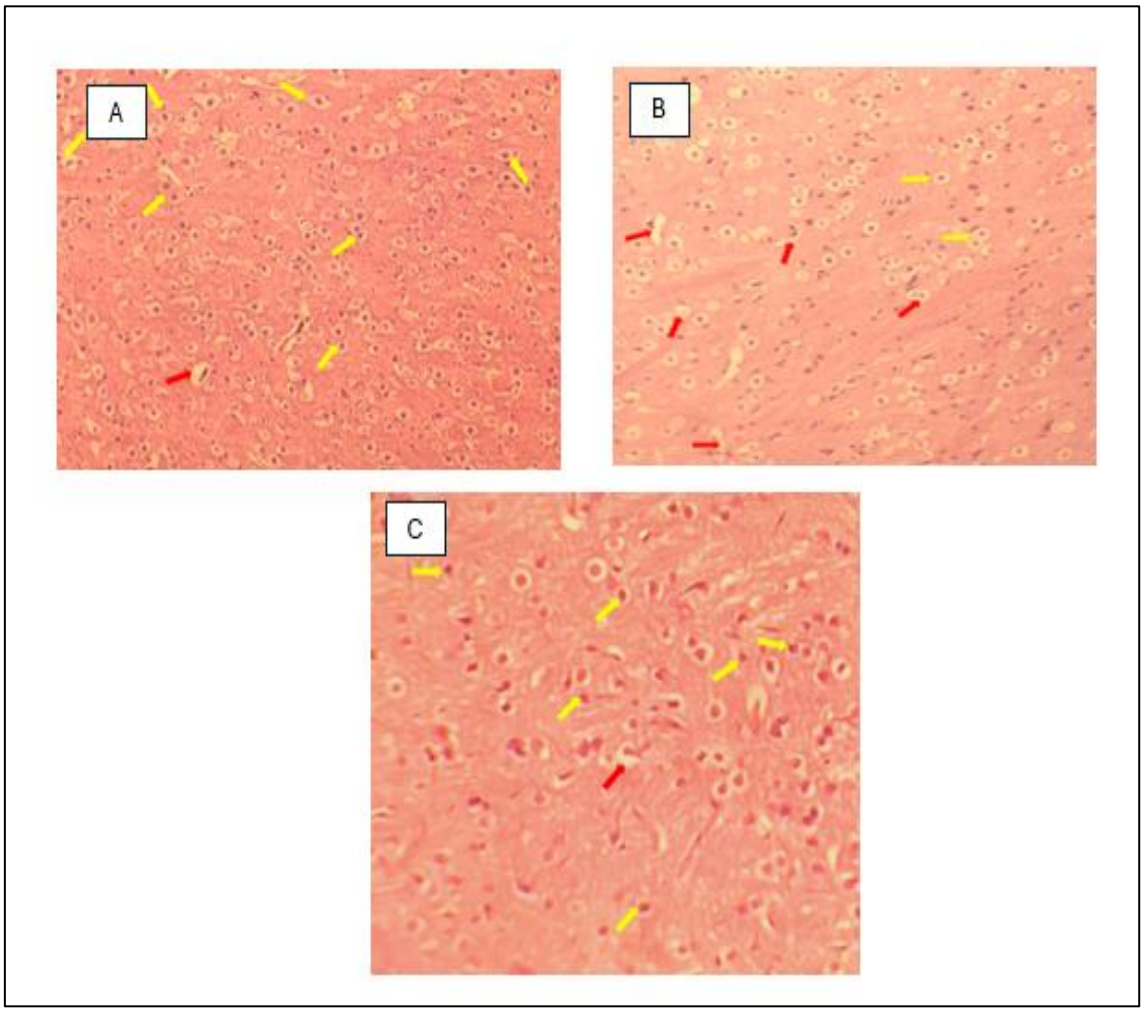

Figure 0.6: The Section of Cortex stained with H & E. (A) Control rats showed viable neurons with distinct nuclei. (B) Surgery group (MCAO) exhibited deformed and disfigured neuronal cells. (C) Rivastigmine-treated group showed more viable neuronal cells with distinct nuclei, exhibiting neuroprotection. Red arrows show non-viable neurons and yellow arrows represent viable neurons.

3.2.1.2 Morphometric Results

3.2.1.2.1 H&E Neuronal Cell Count in the Cortex

Using Image J's software, the cells in the digital photomicrographs were counted, and the results are shown in Figure 3.7. The surgery group MCAO exhibited deformed and disfigured neuronal cells than the control group (vehicle-treated) or treatment group (Rivastigmine-treated).

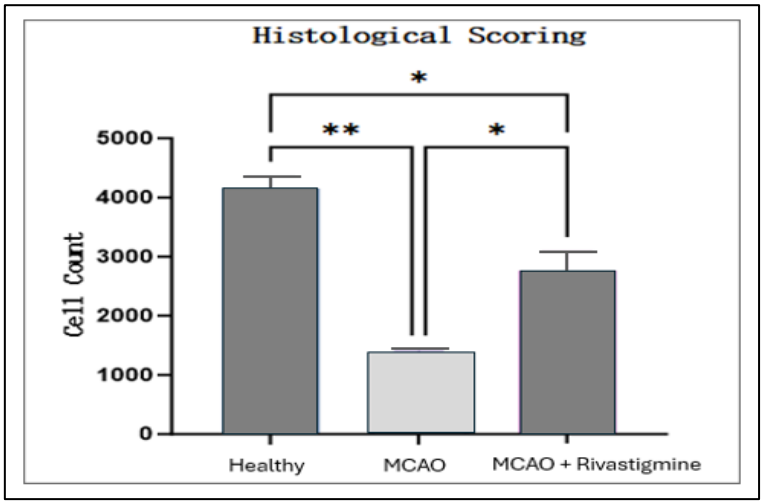

Figure 0.7: Effects of MCAO and Rivastigmine on Cortex Histology (H&E -stained tissue sections). Treatment group depicts higher cell count in contrast to MCAO group. Data is represented as mean ± SEM, *p<0.05, **p<0.01.

3.3 *In Silico Results*

3.3.1 Drug and Ligand Structures

The structure of Rivastigmine was obtained in Structure Data File (SDF) format retrieved from PubChem. The proteins of interest, SOD2 and TLR4 chemical structures were obtained in PDB format from Protein Data Bank. These structures provide thorough illustration on spatial organization of proteins of interest. SDF file procured from PubChem provides in-depth information regarding chemical structure, molecular composition, and conformation of the ligand Rivastigmine.

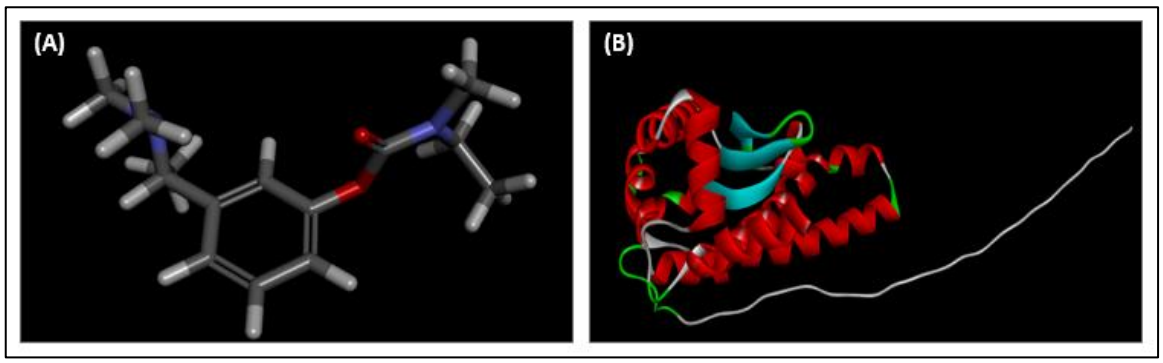

Figure 0.8: 3-D Structures of Rivastigmine and SOD2. (A) Structure of Rivastigmine in SDF format retrieved from PubChem. (B) Structure of SOD2 obtained in PDB format from Protein Data Bank. These structures exhibit spatial organization of ligand and target proteins.

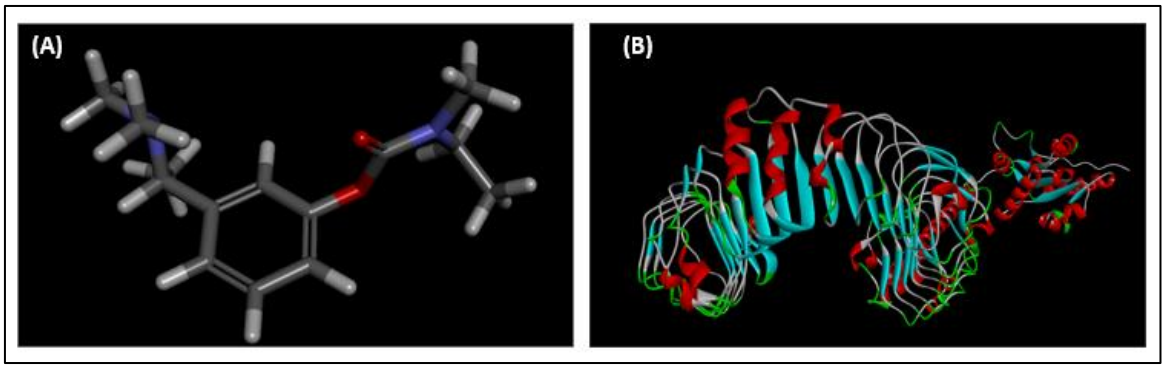

Figure 0.9: 3-D Structures of Rivastigmine and TLR4. (A) Structure of Rivastigmine in SDF format retrieved from PubChem. (B) Structure of TLR4 obtained in PDB format from Protein Data Bank. These structures exhibit spatial organization of ligand and target proteins.

3.3.2 Molecular Docking Analysis

Using a ligand-target docking technique, the structural complex of SOD2 and TLR4 (target) with Rivastigmine (ligand) was examined. Results are demonstrated in figure 3.10. The results of this docking experiment offer vital structural and energetic details regarding Rivastigmine's probable affinities and binding mechanism with SOD2 and TLR4. Examining the molecular connections among Rivastigmine, SOD2 and TLR4 will yield important information about the role Rivastigmine plays in neuroprotective pathways.

Molecular docking provides information regarding spatial organization as well as about the binding that occurs when Rivastigmine binds with target proteins, SOD2 and TLR4. The interactions between Rivastigmine and target proteins suggest stable interactions between ligand protein complex. Various binding sites of Rivastigmine showed favorable binding with target proteins.

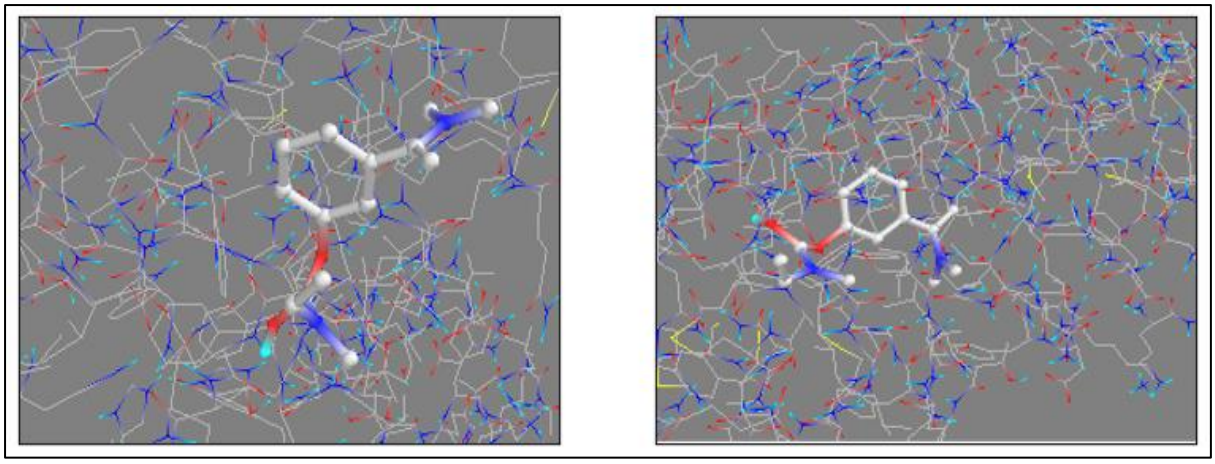

Figure 0.10: Visuals of Docking Interactions of Rivastigmine with (left) SOD2; (right) TLR4.

It shows a computationally projected snapshot of the interactions between SOD2, TLR4 and Rivastigmine provided by the docking analysis carried out with PyRx.

3.3.3 Binding Affinity

The minimal binding energy as shown in Figure 3.11 shows that the ligand i.e. rivastigmine, was successfully docked with SOD2 and TLR4. The binding affinities, which are shown on the vertical axis, represent the degree of interaction between the ligands and the target and are commonly expressed in energy units (for example, kcal/mol). It also evaluates SOD2 and TLR4 potential as protein targets for rivastigmine, to comprehend the stability of the ligand-target complex and, eventually, its biological consequences in the context of neuroprotection. *In Silico* Analysis exhibits interactions of SOD2, TLR4 and Rivastigmine. with binding energies that range between -4.4 to -4.7 kcal/mol and -4.1 to -4.7 respectively. The graph shows how effectively target proteins, SOD2 and TLR4, bind to ligand, Rivastigmine. Energy values, that express binding affinities, give information about how strongly these molecules interact.

Each data point on the graph represents a particular computational docking or binding simulation experiment. Lower values on y-axis in the graph signal that SOD2 and TLR4 have a better binding affinity for ligand, which may indicate a positive interaction. Higher values, on the other hand, can suggest weaker binding. The amino acid residues PRO 169 and SER 99 of SOD2 protein plays role in the creation of carbon hydrogen bond. TRP 147, ILE 100 and LEU 170 participate in alkyl interactions. For TLR4, the amino acid residues LYS 475 forms conventional hydrogen bonding. SER 453 participates in the formation of carbon hydrogen bond and HIS 429 forms pi-alkyl interactions with rivastigmine demonstrated in figure 3.12.

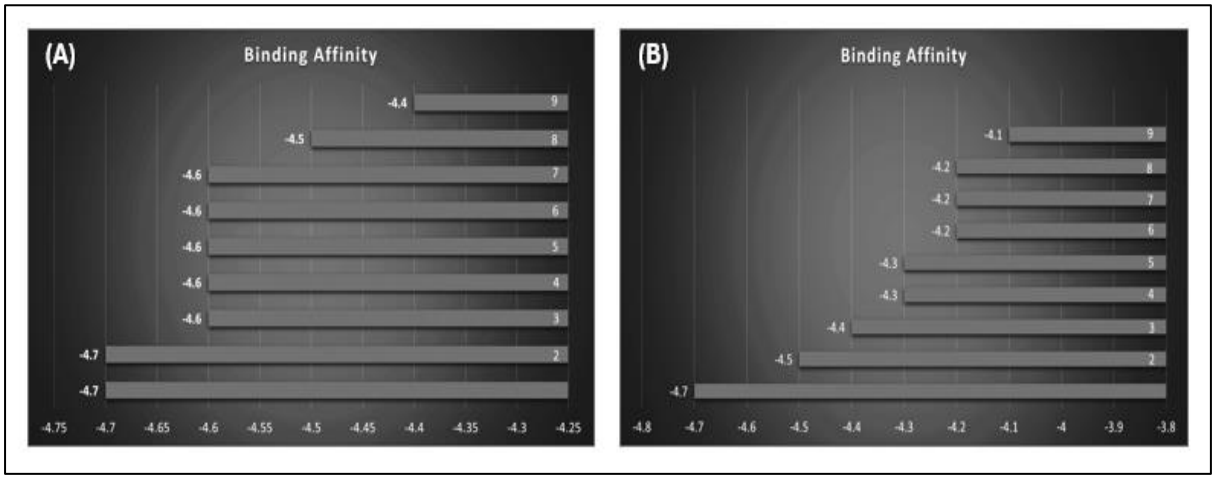

Figure 0.11: In Silico Analysis showing interactions of SOD2, TLR4 and Rivastigmine. with binding energies that range between -4.4 to -4.7 kcal/mol and -4.1 to -4.7 respectively. The graph shows how effectively target proteins, SOD2 and TLR4, bind to ligand, Rivastigmine. Energy values, that express binding affinities, give information about how strongly these molecules interact. Each data point on the graph represents a particular computational docking or binding simulation experiment. Lower values on y-axis in the graph signal that SOD2 and TLR4 have a better binding affinity for ligand, which may indicate a positive interaction. Higher values, on the other hand, can suggest weaker binding.

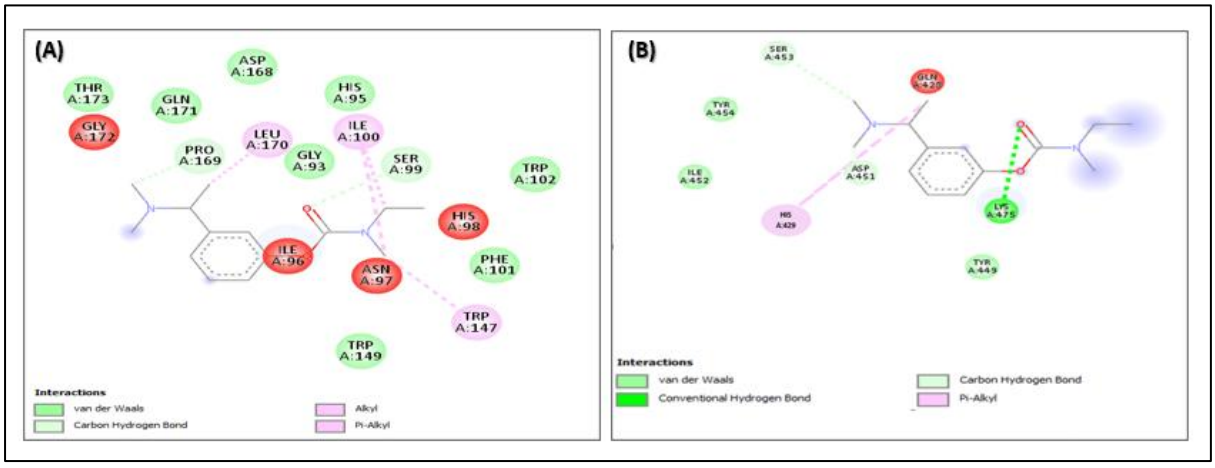

Figure 0.12: Binding locations for SOD2 and TLR4. (A) The amino acid residues PRO 169 and SER 99 of SOD2 protein plays role in the creation of carbon hydrogen bond. TRP 147, ILE 100 and LEU 170 participate in alkyl interactions. (B) For TLR4, the amino acid residues LYS 475 forms conventional hydrogen bonding. SER 453 participates in formation of carbon hydrogen bond and HIS 429 forms pi-alkyl interactions with Rivastigmine.

3.4 PCR Results

3.4.1 Gradient PCR Results

Gradient PCR results demonstrated single band of size 113 bp and 506 bp for SOD2 and TLR4, respectively. It indicates that Rivastigmine mRNA is widely expressed in the brain.

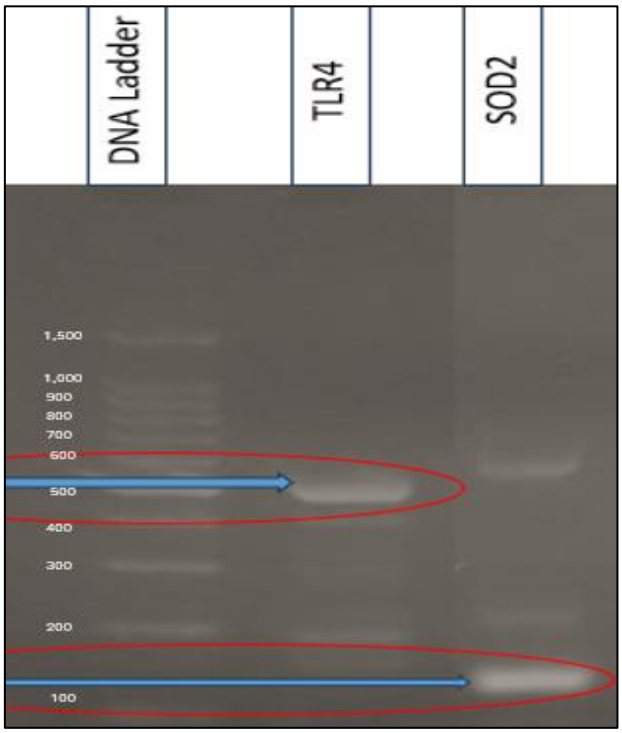

Figure 0.13: Gel Electrophoresis Results. Results for Optimization. PCR analysis of SOD2 and TLR4 expression in rat's brain. Note a single band at approximately 113 bp and 506 bp respectively at 66°C.

3.4.2 Real-Time PCR Results

The relative expressions of SOD2 and TLR4 are shown in Figure 3.14 (a) and (b) respectively. The relative mRNA expression of genes of interest was measured and normalized to the expression of ß-actin as a housekeeping gene. The results showed that SOD2 and TLR4 mRNA expressions were up-regulated in rats treated with rivastigmine and down-regulated in surgery group MCAO.

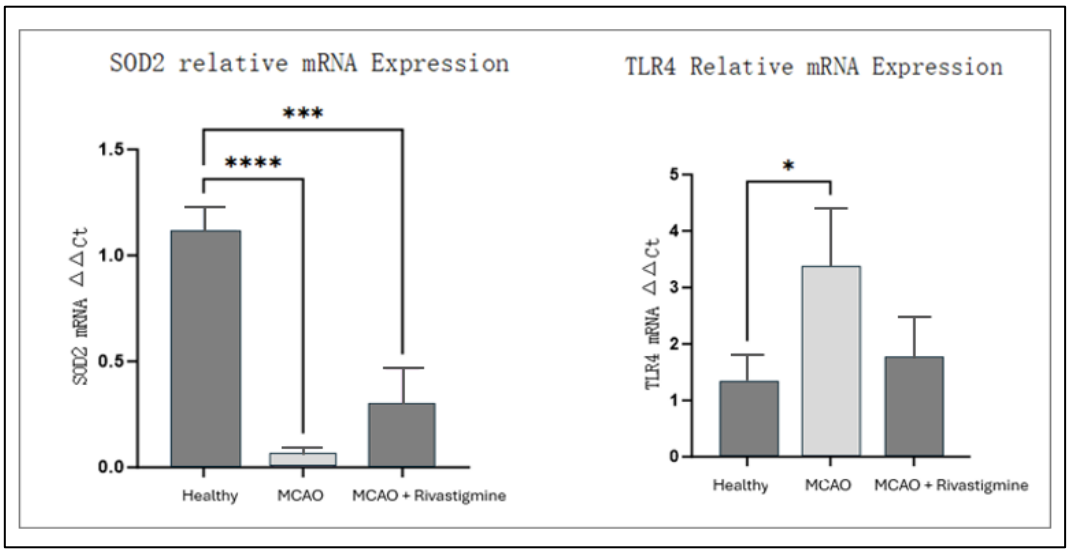

Figure 0.14: SOD2 and TLR4 mRNA Relative Expression (Normalized to ß-actin). (a) SOD2 mRNA relative expression (Normalized to ß-actin). (b) TLR4 mRNA expression (Normalized to ß-actin). SOD2 and TLR4 expression in rats treated with Rivastigmine for eighteen days showed up-regulation in contrast to surgery (MCAO) group which showed down-regulation. Data is shown as mean ± SEM. For statistical analysis, the non-parametric one-way ANOVA was used, followed by the Tukey multiple comparison test.

CHAPTER 4: DISCUSSION

Stroke continues to be the world's primary cause of morbidity and mortality, frequently with incapacitating consequences like movement dysfunction, cognitive decline, and neurological impairments. Most strokes are caused by primary hemorrhages, with secondary hemorrhages accounting for an estimated 10-15% of cases (Vos et al., 2017). Pre-existing medical conditions like hypertension and hyperlipidemia boost the chances of stroke occurrence in patients, where TIA accounts for about 60% of stroke cases (Kuriakose & Xiao, 2020).

Stroke accounts for around 85% of deaths, with ICH accounting for the remaining 15% as they create thrombotic and embolic environments in the brain (Musuka et al., 2015b). Roughly 10-15% of all strokes are hemorrhagic strokes. Blood vessels burst because of internal injuries and stress on the brain tissue leading to its high death rate. It produces toxicity in the vascular system, which results in infarction (Flaherty et al., 2005). It is essential to manage stroke effectively to lessen its negative effects and enhance patient outcomes.

The potential neuroprotective benefits of acetylcholinesterase inhibitors may assist maintain the integrity of brain tissue and lessen the damage that neurons may sustain after a stroke, making them a promising option in this situation. By lowering the rate at which Ach is broken down, cholinesterase inhibitor medications suppress AChE activity and preserve Ach levels (Colovic et al., 2013).These medications boost the amount and duration of acetylcholine's activities in the central and peripheral neurological systems by preventing it from breaking down normally into acetate and choline. Acetylcholinesterase inhibitors have a variety of applications. The neurological illness that they are most commonly used to treat include PD, AD, and Lewy body dementia.

This study presents a comprehensive analysis of the neuroprotective benefits of rivastigmine, an acetylcholinesterase inhibitor, across a wide range of complex stroke outcomes. Rivastigmine is a unique dual inhibitor of AChE and BuChE which are responsible for acetylcholine hydrolysis in the brain (M. Mesulam et al., 2002). Through the formation of a covalent link, rivastigmine binds to esterases, temporarily deactivating them. It responds to AChE's ester and anionic sites. As a result, Ach generally rises.

The symptomatic treatment of moderate to intermediate dementia in people with AD and idiopathic PD is the indication for oral Rivastigmine administration (Nguyen et al., 2021).

Gottwald and Rozanski's (1999) preclinical research validated the Rivastigmine's transport into the CSF and at moderate dosages, rivastigmine enhanced cognition and was generally well tolerated (Gottwald & Rozanski, 1999). By means of an intricate combination of computational modeling, behavioral evaluations, histological inspections, and molecular studies, a thorough comprehension of the possible mechanisms behind the therapeutic effects of Rivastigmine becomes apparent.

Five behavior tests, namely NOR, y-maze, social interaction, grip strength and open filed tests, were conducted to analyze alterations in cognition, memory, and social behavior of rats' post Rivastigmine treatment. Potential gains in cognitive functions may be indicated by the observed increase in the amount of time spent investigating the unfamiliar object in the group receiving Rivastigmine treatment. This is consistent with cholinesterase inhibitors' predicted neuroprotective effects and highlights the drug's potential benefit in reducing cognitive deficits following a stroke (Sambeth et al., 2007). The Y-maze test was performed to assess rats' spontaneous alternation behavior and spatial working memory. Rats were put in a y-shaped maze and their natural tendency to explore different arms was observed. The results exhibited improved memory and cognitive function in Rivastigmine treated group which is supported by the higher number of consecutive entries into distinct y-maze arms, which is indicative of improved cognitive function (Ohara et al., 1997).

The NOR test was performed to evaluate rodents' recognition memory and their ability to distinguish between novel and familiar object. Results showed that Rivastigmine treated group spent more time exploring novel object versus familiar object. Together, these results highlight Rivastigmine's capacity to improve cognition and establish it as a versatile treatment tool. Positive effects on motor function are suggested by the observed increase in grip strength in the Rivastigmine treated group (Takeshita et al., 2017). Each rat was gently held by the base of its tail and allowed to hold the bar with its forelimbs. Time taken by rats to hold the bar before falling was measured. This development adds to a more thorough comprehension of the effects of rivastigmine on behavioral outcomes following stroke, encompassing both the motor and cognitive domains. In open field test, Rivastigmine treated group exhibited heightened exploratory behavior or decreased anxiety, as indicated by the higher entry in the center of the open field labyrinth (Seibenhener & Wooten, 2015). This change in behavior is consistent with the general increase in locomotor activity, indicating a favorable reaction to the treatment with rivastigmine. The Rivastigmine treated group showed improvements in social behavior, exhibited by the longer time spent with stranger II (Clapcote et al., 2007). This provides a more

comprehensive understanding of Rivastigmine's effects on quality-of-life following stroke by highlighting not only cognitive benefits but also suggesting possible emotional improvements.

One important technique in histopathological analysis for assessing anatomical alterations and pathological anomalies in tissue sections is H&E staining (Preet et al., 2022). Cells' quantification and comparative analysis was achieved by histopathological analysis. It offers significant insights into the histological features of brain tissues. In this study, H&E staining was done, and a section of cortex was examined under microscope. Later, ImageJ software was used to count no of cells. Results demonstrated a greater number of viable cells in Rivastigmine treated group, which effectively illustrates the neuroprotective benefits of Rivastigmine. Rivastigmine may be able to preserve neuronal integrity after stroke, as evidenced by this clear contrast with the malformed cells in the surgery MCAO group.

qRT-PCR gene expression analysis is a significant method in molecular biology that sheds light on the transcriptional control of particular genes (Valasek & Repa, 2005). In this study, variations in SOD2 and TLR4 expression under specified experimental settings were evaluated by examining the relative expression of SOD2 and TLR4 mRNA normalised to the housekeeping gene beta-actin.

In qRT-PCR investigations, beta-actin is frequently used as a housekeeping gene or reference for normalisation. Beta-actin is normally expressed at very consistent levels across a variety of cell types and situations and is involved in key cellular activities. The precision and dependability of gene expression measurements are ensured by normalisation with beta-actin, which allows for the adjustment of any changes in RNAinput and cDNA synthesis effectiveness (Ruan & Lai, 2007). Under the investigated experimental settings, a significant change in SOD2 and TLR4 expression was observed. Results demonstrated upregulation of SOD2 levels and downregulation of TLR4 levels in Rivastigmine treated group as compared to MCAO group, validating oxidative and anti-inflammatory roles of Rivastigmine.

Effective docking interactions in *in-silico* analysis offer a three-dimensional view of possible binding sites and conformational dynamics between Rivastigmine and ligands, SOD2 and TLR4. Rivastigmine structure was obtained from PubChem in PDB format. On the other hand, SOD2 and TLR4 structures were obtained from Alpha fold protein data bank. This computational effort highlights the structural details of these molecular relationships and provides a basis for understanding that will direct future experimental validations. The minimum binding energies that range between -4.4 to -4.7 kcal/mol for SOD2 and -4.1 to -4.7

kcal/mol for TLR4, indicate that rivastigmine has firmly docked with SOD2 and TLR4, indicating a positive interaction (He et al., 2023). The possibility of SOD2 and TLR4 functioning as ligands for Rivastigmine is supported by the lower binding energies, which show a stronger potential for ligand binding.

LIMITATIONS

- It is important to recognize the inherent limitations of this study. The results of behavioral assessments and molecular analysis may not be as generalizable due to variability introduced by the relatively small sample sizes used.
- The results' wider relevance is constrained using a single animal model and the exclusion of both sexes.
- Potential areas of investigation include brief duration of Rivastigmine administration and the only dependence on in-silico forecasts.
- The entire range of Rivastigmine's actions might not be covered by the molecular targets that were investigated.

CHAPTER 5: SUMMARY OF RESEARCH WORK

One of the leading causes of mortality worldwide is stroke, showing severe after affects like movement dysfunction, cognitive decline, and neurological impairments. Most strokes are caused by primary hemorrhages, with secondary hemorrhages accounting for an estimated 10-15% of cases (Vos et al., 2017). If a person is already suffering from some health conditions like hypertension, coronary artery disease, or hyperlipidemia, enhance their risk of stroke. Patients who have already suffered from a mini stroke account for about 60% of ischemic cases. Certain stroke risk factors can be changed, while others cannot (Kuriakose & Xiao, 2020). It is necessary to get required help when necessary to cope with the destructive after effects of stroke in an efficient manner.

Neuroprotective ability of acetylcholinesterase inhibitors may help in maintaining the integrity of brain tissue and reduce destruction that neurons suffer after a stroke, making them a promising option in this situation. By lowering the rate at which Ach is broken down, cholinesterase inhibitor medications suppress AChE activity and preserve Ach levels (Colovic et al., 2013). This comprehensive thesis explores the neuroprotective benefits of an acetylcholinesterase inhibitor, Rivastigmine, in relation to stroke outcomes in a variety of ways. Rivastigmine is a unique dual inhibitor of AChE and BuChE which are responsible for acetylcholine hydrolysis in the brain (M. Mesulam et al., 2002). Gottwald and Rozanski's (1999) preclinical research validated the Rrivastigmine's transport into the CSF and at moderate dosages, rivastigmine enhanced cognition and was generally well tolerated (Gottwald & Rozanski, 1999).

Five behavior tests, namely NOR, y-maze, social interaction, grip strength and open filed tests, were conducted to analyze alterations in cognition, memory, and social behavior of rats' post Rivastigmine treatment. Results exhibited potential gains in cognitive functions by the observed increase in the amount of time spent investigating the unfamiliar object. These results were backed cholinesterase inhibitors' predicted neuroprotective effects and highlights the Rivastigmine's potential benefit in reducing cognitive deficits following a stoke (Sambeth et al., 2007).

Cells' quantification and comparative analysis was achieved by histopathological analysis (Preet et al., 2022). H&E staining was done, and a section of cortex was examined under microscope. Later, ImageJ software was used to count no of cells. Results demonstrated a

greater number of viable neural cells which effectively illustrate the neuroprotective benefits of rivastigmine. Rivastigmine may be able to preserve neuronal integrity after stroke, as evidenced by this clear contrast with the malformed cells in the surgery (MCAO) group.

qRT-PCR was performed for gene expression analysis of SOD2 and TLR4 in healthy, MCAO and Rivastigmine treated groups (Valasek & Repa, 2005). Beta-actin was used as a housekeeping gene. Under the investigated experimental settings, a significant change in SOD2 and TLR4 expression was observed. Results demonstrated upregulation of SOD2 levels and downregulation of TLR4 levels in Rivastigmine treated group as compared to surgery group, validating oxidative and anti-inflammatory roles of Rivastigmine.

Effective docking interactions in *in-silico* analysis offer a three-dimensional view of possible binding sites and conformational dynamics between Rivastigmine and ligands, SOD2 and TLR4. The minimum binding energies that range between -4.4 to -4.7 kcal/mol for SOD2 and -4.1 to -4.7 kcal/mol for TLR4, indicate that rivastigmine has firmly docked with SOD2 and TLR4, indicating a positive interaction (He et al., 2023).

CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATIONS

Stroke continues to be the world's primary cause of morbidity and mortality, frequently with incapacitating consequences like movement dysfunction, cognitive decline, and neurological impairments. It is essential to manage stroke effectively to lessen its negative effects and enhance patient outcomes. This can be achieved by using Rivastigmine, an acetylcholinesterase inhibitor, in managing post-stroke complications. This study presents a comprehensive analysis of the neuroprotective benefits of Rivastigmine, an acetylcholinesterase inhibitor, across a wide range of complex stroke outcomes. Rivastigmine is a unique dual inhibitor of AChE and BuChE which are responsible for acetylcholine hydrolysis in the brain (M. Mesulam et al., 2002). Through the formation of a covalent link, rivastigmine binds to esterases, temporarily deactivating them. It responds to AChE's ester and anionic sites. As a result, Ach generally rises.

The combination of behavioral evaluations, histological analysis, in silico predictions, and genetic analysis offers a thorough overview of Rivastigmine's neuroprotective effects in the context of stroke outcomes. Five behavior tests, namely NOR, y-maze, social interaction, grip strength and open filed tests, were conducted to analyze alterations in cognition, memory, and social behavior. Behavioral tests' results indicated improvement in spatial memory and cognition post Rivastigmine treatment. Cells' quantification and comparative analysis was achieved by histopathological analysis exhibiting increase in the number of viable cells, which were comparatively less deformed in Rivastigmine treated group than MCAO group. qRT-PCR gene expression analysis demonstrated upregulation of SOD2 levels and downregulation of TLR4 levels in Rivastigmine treated group as compared to MCAO group, validating oxidative and anti-inflammatory roles of Rivastigmine. *In silico* analysis depicted minimum binding energies that range between -4.4 to -4.7 kcal/mol for SOD2 and -4.1 to -4.7 kcal/mol for TLR4, indicating firm docking of Rivastigmine with SOD2 and TLR4, indicating a positive interaction (He et al., 2023).

In conclusion, Rivastigmine provides a substantial basis for future research considering its oxidative and anti-inflammatory traits and can initiate new trials of therapeutic interventions regarding stroke. Future studies in this field ought to focus on delving more deeply inti the molecular mechanisms underlying rivastigmine's neuroprotective benefits and how does it affect oxidative stress and inflammatory pathways. Examining positive effects of Rivastigmine

on molecular targets may reveal new therapeutic approaches for treating stroke. Additionally, to confirm rivastigmine's place as a common supplementary treatment in clinical practice, longitudinal studies evaluating the drug's long term safety profile and efficacy in stroke survivors are necessary. Additionally, investigating Rivastigmine's possible synergistic effects with already used pharmacotherapies or rehabilitation techniques may improve treatment outcomes and promote functional recovery post stroke. A more thorough knowledge of how Rivastigmine medication affects brain recovery process may be possible by incorporating modern imaging methods like diffusion tensor imaging or functional magnetic resonance imaging. These techniques may reveal insightful information about the structural and functional alterations linked to Rivastigmine treatment.

In conclusion, Rivastigmine shows potential as neuroprotective medication in the treatment of post-stroke problems. This provides a foundation for additional research and the creation of novel therapeutic approaches targeted at boosting patient satisfaction and stroke outcomes.

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APPENDICES

APPENDIX A

Rivastigmine Treatment Calculations

The rivastigmine treatment regimen for surgery (MCAO) rats based on their weights and a standard dosage of 1mg/kg is as follows:

Table 0.1: Rivastigmine Treatment Regimen.

	MOUSE WEIGHT (kg)	RIVASTIGMINE DOSAGE (mg/kg)	RIVASTIGMINE DOSAGE (mg)	TOTAL STOCK SOLUTION (ml)	RIVASTIGMINE DOSAGE (µl)	TREATMENT FREQUENCY
1.	0.132	1	0.6	0.66	6.6	Once daily
2.	0.180	1	0.90	0.9	9.0	Once daily
3.	0.135	1	0.67	0.675	6.75	Once daily
4.	0.125	1	0.62	0.625	6.25	Once daily
5.	0.120	1	0.60	0.6	6.0	Once daily

The calculations done for the dosing of rivastigmine treatment are as follows:

1. Calculate Rivastigmine Dosage: weight of rat × standard dosage (i.e. 1mg/kg)

2. Prepare rivastigmine stock solution: Dissolving the calculated rivastigmine dosage in distilled water to get the stock solution of 5mg/ml.