# Therapeutic Potential of Galantamine for Managing the Complications Associated with Ischemic Stroke.



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Supervisor: Dr. Aneeqa Noor

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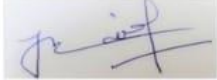
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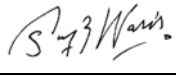
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"Dedicated to my beloved parents Muhammad Ahsan and Kalsoom Ahsan, whose unwavering faith, enduring belief, and ceaseless encouragement have been my guiding light and anchor. To my dearest siblings, your consistent support has been my strength and inspiration."

# ACKNOWLEDGEMENTS

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# LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

| Abbreviation | Full Form |
|---|---|
| AVM | Arteriovenous Malformation |
| AChE | Acetylcholinesterase |
| AD | Alzheimer's Disease |
| AIS | Acute Ischemic Stroke |
| ALDH2 | Aldehyde Dehydrogenase |
| ASP | Aspartic Acid |
| ATP | Adenosine Triphosphate |
| BBB | Blood-Brain Barrier's |
| BChE | Butyrylcholinesterase |
| CCA | Common Carotid Artery |
| ChAT | Choline Acetyltransferase |
| CSF | Cerebrospinal Fluid |
| CYS | Cysteine |
| DAMPs | Damage-Associated Molecular Pattern Molecules |
| DCs | Dendritic Cells |
| dNTPs | Deoxyribonucleotide triphosphate |
| DTT | Dithiothreitol |
| ECA | External Carotid Artery |
| FOXO | Forkhead Box Protein O1 |
| GLN | Glutamine |
| GLY | Glycine |
| GST | Glutathione S-Transferase |
| H&E | Hematoxylin And Eosin |
| HIS | Histidine |
| HMG-Coa | 3-Hydroxy-3-Methylglutaryl Coenzyme A |
| HO1 | Heme Oxygenase 1 |
| ICA | Internal Carotid Artery |
| IL-10 | Interleukin 10 |
| ILE | Isoleucine |
| Kcal/mol | Kilo calories per mole |
| LEU | Leucine |
| LPS | Lipopolysaccharide |
| LRRs | Leucine-Rich Repeats |
| MD-2 | Myeloid Differentiation Factor 2 |
| Meth | Methamphetamine |
| MMP | Matrix Metalloproteinases |
| MRI | Magnetic Resonance Imaging |
| Myd88 | Myeloid Differentiation Primary Response Gene 88 |
| NCBI | National Centre for Biotechnology and Information |
| NF-κß | Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells |
| NK | Natural Killer |
| NLRP3 | NOD-Like Receptor Protein 3 |
| NMDARs | N-Methyl-D-Aspartate Receptors |
| NQO1 | NAD(P)H Quinone Oxidoreductase |

| | |
|---|---|
| NrF2 | Nuclear Factor Erythroid 2–Related Factor 2 |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PBS | Phosphate-Buffered Saline |
| PDB | Protein Data Bank |
| PFA | Paraformaldehyde |
| PHE | Phenylalanine |
| PINK1 | PTEN-Induced Kinase 1 |
| PRO | Proline |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| rpm | Revolution per minute |
| RT-PCR | Real Time-Polymerase Reaction Chain |
| SDF | Structure Data File |
| SIRT | Sirtuin (SIRT1-7) |
| SOD2 | Superoxide Dismutase 2 |
| tBHQ | Tertiary Butylhydroquinone |
| TIA | Transient Ischemic Stroke |
| TIR | Toll/Interleukin-1 Receptor |
| TLR4 | Toll-Like Receptor -4 |
| TRIF | TIR Domain-Containing Adaptor Protein Inducing Interferon-Beta |
| TRP | Tryptophan |
| TYR | Tyrosine |
| UCP2 | Uncoupling Protein 2 |
| VAChT | Vesicular Acetylcholine Transporter |
| VAL | Valine |
| $A\beta$ | Alpha-Beta T Cells |
| $\gamma\delta$ | Gamma-Delta T Cells |

# ABSTRACT

Ischemic stroke remains the leading cause of illness and second leading cause of death worldwide. Previous research has shown that galantamine has neuroprotective properties, reducing neuronal death and damage in neurodegenerative conditions and improving cognitive function in Alzheimer's disease patients. The investigation looked into the possible mechanisms by which this medication could reduce cell death. Wistar rats were subjected to a temporary 30-minute occlusion of the right middle cerebral artery (MCA) and given an oral dose of 5mg/kg for three weeks. After 18 days of surgery, behavioral assessments were carried out. Galantamine enhanced grip strength, motor function, and muscle strength in rats. Spatial memory and object recognition examinations revealed cognitive improvements, thus indicating enhanced cognitive abilities and memory retention. Moreover, rats subjected to galantamine treatment displayed increased sociability and heightened locomotor activity during social interaction and open-field assessments. Molecular analysis of galantamine treatment in rats showed an upregulation of SOD2 expression, suggesting enhanced antioxidant defense, and a downregulation of TLR4 expression, suggesting a reduction in neuroinflammation, supporting the anti-inflammatory properties of galantamine. The histological analysis done with H&E staining of brain tissue revealed enhanced tissue morphology in the galantamine-treated group, signifying the neuroprotective effects of galantamine. The reduction in neuronal damage, edema, and inflammatory cell infiltration further supported this observation. The results demonstrate that galantamine, potentially through the preservation of a functional cholinergic system, mitigated the impairments caused by stroke in a basic learning and memory test by decreasing cellular death.

**Keywords:** Cholinergic System, Galantamine, Ischemic Stroke, Middle Cerebral Artery Occlusion, , Pharmacological Intervention.

# CHAPTER 1:    INTRODUCTION

## 1.1 Stroke

A stroke refers to the abrupt interruption of uninterrupted blood circulation to the cerebral region, leading to the impairment of neurological capabilities. Interruption in blood circulation can result from an obstruction, causing the more prevalent ischemic stroke, or from hemorrhage in the brain, resulting in the more fatal hemorrhagic stroke (Lo et al., 2003). Stroke can happen abruptly, often without any indication, and its consequences can be catastrophic. It is imperative to restore enough blood circulation and oxygenation to the brain promptly. The absence of oxygen and essential nutrients results in the impairment or death of the impacted brain cells within a brief timeframe. Brain cells typically do not undergo regeneration after death, which can lead to severe damage and could cause neurological and mental impairments (Barthels & Das, 2020)

The World Health Organization defines a stroke as a sudden occurrence of focal or global disruption to cerebral function, resulting in clinical signs that last for at least 24 hours or lead to death. This condition is of vascular origin and encompasses cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage (Force, 1989). Despite significant advancements in the field of diagnosis and therapeutic approaches, it is anticipated that the incidence of strokes will increase twofold by the year 2050, and the prevalence of long-term disabilities after stroke is anticipated to equally increase due to demographic changes and the growing number of stroke survivors (Howard & Goff, 2012).

### 1.1.1 Epidemiology

Stroke, a condition that is more common as people get older, will become more prevalent as the age increases in the population. This is supported by the 85% surge observed in the worldwide incidence of occurrence of stroke (King et al., 2020). A study in 2021 found that 70% of stroke patients achieved a three-year survival rate or longer, largely due to the success of acute therapies. However, clinical evidence suggests that some patients continue to experience ongoing functional impairment for an extended period after a stroke (Crichton et al., 2016). The investigation of secondary injury mechanisms that drive the progression of chronic injury may hold a great deal of significance for acquiring greater knowledge about the prevention and treatment strategies for stroke. This is because stroke

is a contributing factor to the growing patient population who are going through chronic cognitive impairment and neurological disorders (Stuckey et al., 2021).

The frequency of stroke significantly rises with advancing age, experiencing a twofold increase with each passing decade after reaching the age of 55 (Chong & Sacco, 2005). Stroke occurs in 30 to 120 out of 100,000 individuals aged 35-44 annually, while 65-74 individuals face an incidence rate of 670-970. Children have a lower prevalence of stroke, with 1 to 2.5 out of 100,000 individuals experiencing it annually (Roger et al., 2011). Stroke affects 15 million people globally annually, causing significant impacts on families and communities. Five million die and five million are permanently disabled, resulting in irreversible effects on families and communities (Abdalla et al., 2022). The total incidence of strokes, on the other hand, is continuing to rise as a result of the growing elderly population (Soto-Cámara et al., 2020).

### 1.1.2 Epidemiology in Pakistan

The population of Pakistan places it in fifth place on the global scale. Around 225 million people are living in Pakistan, and the average age of the population is 22.5 years. Large-scale epidemiological investigations are needed to ascertain Pakistan's actual stroke incidence (Malik et al., 2020). In Pakistan, the anticipated yearly stroke prevalence is 250/100,000 (Farooq et al., 2021). A survey on the aging of individuals aged 18 years and above was conducted in Khyber Pakhtunkhwa, one of Pakistan's four provinces. The study encompassed 22,500 participants, comprising 51.4% females (11,556 individuals) and 48.6% males (10,944 individuals). The average age of participants was 42 ± 12.6 years. Among the study population, 66.4% (14,942 individuals) were below 50 years of age. The majority of participants (74.66%) resided in rural areas, and approximately 10.9% had not undergone formal education. The survey identified 271 cases of stroke, resulting in a stroke prevalence of 1.2% (Jafar, 2006).

A study in Karachi, Pakistan, revealed that 4.8% of adults in the adult Pushtoon community experienced a stroke, with 30% occurring in individuals aged 45 or younger (Farooq et al., 2009). Stroke patients in developing nations like Pakistan are ten years younger than those in Western countries, and the prevalence of stroke risk factors is comparable to South Asian countries. The mortality rate for strokes in Pakistan is between 11% and 30% (Nomani et al., 2017). Poor prognostic factors include being over 60 years

old, having significant hypertension, hyperglycemia, and unconsciousness (Ahmad et al., 2020).

**1.2 Stroke's Type**

The three primary categories of strokes are urgent medical situations that halt or disrupt the circulation of blood to the brain. These categories include:

- Hemorrhagic Stroke.
- Transient Ischemic Attack
- Ischemic Stroke.

*1.2.1 Hemorrhagic Stroke*

When blood flow in the brain suddenly halts, it causes a hemorrhagic stroke, resulting in impaired function. This bleeding may manifest in the brain alongside the cranium or within the brain itself. Approximately 15% of all strokes are hemorrhagic, which are classified according to the site and etiology of the hemorrhage:

*1.2.1.1 Intracerebral Hemorrhage*

Hemorrhaging arises from a ruptured blood artery in the brain. Factors that elevate the likelihood of experiencing this type of hemorrhage include hypertension (high blood pressure), excessive alcohol use, older age, and the usage of cocaine or amphetamines. Various types of stroke have the potential to transform into an intracerebral hemorrhage (Flower & Smith, 2011). A thrombotic or embolic stroke without bleeding can lead to intracerebral hemorrhage, especially in cases of embolic strokes linked to endocarditis, a heart valve infection. This occurs when a cluster of bacteria and inflammation cells from the valve's infection forms an embolus, a mobile mass within the circulatory system. The infected mass can migrate into a cerebral artery and become lodged in that location. Subsequently, the infection has the potential to propagate via the artery. Occasionally, intracerebral hemorrhage may occur as a result of a ruptured arteriovenous malformation (AVM), which is an anomalous blood vessel with a weakened wall that links an artery and a vein. The blood flowing into it can exert significant pressure, leading to the eventual stretching or leakage of the AVM (Rajashekar & Liang, 2024).

*1.2.1.2 Subarachnoid Hemorrhage*

Hemorrhaging from a compromised blood artery results in the accumulation of blood on the outer layer of the brain. Hemorrhage occurs within the intracranial space, where blood intermingles with the cerebrospinal fluid (CSF), providing protection and support to the central nervous system (CNS). When blood flows into the CSF, it applies force to the brain, leading to an immediate onset of a headache. During the initial days after the bleeding, the presence of clotted blood surrounding the brain might lead to chemical irritation, which can result in the constriction of nearby brain arteries. Arterial spasms possess the capacity to inflict damage upon brain tissue. A subarachnoid hemorrhage can be caused by a leak caused by an arteriovenous malformation (Marcolini & Hine, 2019).

*1.2.2 Transient Ischemic Stroke (TIA)*

The American Heart Association and the American Stroke Association in 2009 updated the description of TIA as follows: "a transient episode of neurologic dysfunction caused by focal brain, spinal cord or retinal ischemia without acute infarction"(Arsava et al., 2011). Before this, TIA was commonly characterized operationally as having symptoms that lasted for no more than twenty-four hours, with typical episodes lasting less than 1 hour. The reduced emphasis on length was a result of several investigations that revealed brain injury on magnetic resonance imaging (MRI) in up to 50% of TIAs that were traditionally characterized. The average annual risk of experiencing a future ischemic stroke following a TIA or primary ischemic stroke is approximately 3-4%, (Gupta et al., 2014) with a prevalence of 11% throughout the next 7 days and 24-29% within the subsequent 5 years. A TIA might be regarded as a significant indication of an imminent ischemic stroke. The largest risk occurs within the initial 48 hours after experiencing a TIA. TIA typically manifests as focal neurologic deficits and/or speech disturbances within a specific vascular area, resulting from underlying cerebrovascular illness. A TIA always begins abruptly and without warning. Using imaging and laboratory tests, TIA evaluation must be done quickly to lower the risk of more strokes. (Rothwell & Warlow, 2005).

## 1.3 Ischemic Stroke

An ischemic stroke is a condition when the brain's blood arteries are suddenly damaged by a blockage, leading to long-term brain damage, and reduced neurological function. Around

87% of strokes are ischemic stroke, resulting from embolism or thrombosis (Roger et al., 2011). Stroke often leads to enduring functional and neurological issues, varying in intensity from significant post-stroke dementia to slight motor function abnormalities (Lawrence et al., 2001). Moreover, the occurrence of long-term pathological consequences following a stroke has been documented to reach a staggering 44%, persisting for up to ten years after the initial stroke (Synhaeve et al., 2014). An ischemic stroke is a multifaceted condition with various causes and diverse clinical presentations. Around 45% of ischemic strokes result from the formation of blood clots in either tiny or big arteries, 20% are caused by emboli, and the other cases have an unknown etiology (Hickey et al., 2009).

### 1.3.1 Types of Ischemic Stroke

### 1.3.1.1 Thrombotic Stroke

A **thrombotic stroke,** which is the predominant form, happens when a thrombus, or a clot of blood, obstructs the circulation of blood to certain brain regions. Arteries affected by atherosclerosis have the potential to develop a thrombus. Atherosclerosis is a pathological condition characterized by the thickening and narrowing of the arterial lining due to the accumulation of plaque. Plaque consists of lipids, cholesterol, fibrinogen (a substance involved in blood clotting), and calcium. As atherosclerotic plaque accumulates within the arterial walls, it hinders the normal flow of blood, resulting in reduced velocity and increased likelihood of clot formation. A constricted blood vessel due to atherosclerosis is more prone to obstruction by a blood clot, resulting in the cessation of blood circulation. Thrombotic strokes typically occur during the nocturnal or early morning hours. A TIA sometimes called a "mini stroke," typically precedes a thrombotic stroke (Wendelboe & Raskob, 2016).

### 1.3.1.2 Embolic Stroke

**Embolic stroke** is the result of thrombus that migrates from another location in the body, typically originating from the heart. Subsequently, the blood clot obstructs a cerebral artery. An embolic stroke occurs when an embolus, a clot, becomes detached and is transported through the bloodstream to the brain, specifically to the point where the major arteries divide into smaller blood vessels. The blood clot enters an impasse, unable to progress any further. It becomes lodged, obstructing a tiny cerebral artery and interrupting the blood flow to the brain. Embolic strokes typically result from the formation of a blood clot (embolus) in another part

of the body, which then moves through the bloodstream and reaches the brain. Embolic strokes frequently arise from cardiovascular illness or cardiac surgery and manifest abruptly and without any preceding indications (Toi et al., 2022).

*1.3.2 Morphological Changes in Ischemic Stroke*

During the development of ischemic stroke, various cell types within the CNS undergo distinct morphological transformations due to ischemic damage. Neurons located in the ischemic core exhibit morphological alterations characterized by the disappearance of cell bodies and axons. Neuronal and glial cells frequently exhibit cytoplasmic swelling and removal of the nucleolus. During the penumbra phase, neurons, known as 'ischemic neurons', typically undergo several alterations, while still being reasonably alive. Following ischemia, glial cells, and neuronal cells, including astrocytes and microglia, can go through morphological changes.

Ramified microglia transform into an "activated state" when they become swollen and take on an ameboid-like shape. This transformation is responsible for the delivery of pro-inflammatory chemicals like chemokines, reactive oxygen species (ROS) and cytokines (Yenari et al., 2010), Astrocytes often experience gradual changes in both their molecular expression patterns and morphological characteristics, which function to safeguard neurons in the ischemic penumbra (Sims & Yew, 2017). After ischemia, there is an elevation in the blood-brain barrier's (BBB) permeability, permitting immune cells to enter the areas affected by ischemia. These immune cells release various substances that can either protect or harm the brain tissues affected by ischemia (Jin et al., 2013). The initial event triggers a cascade of processes, including excitotoxicity, inflammation, and oxidative stress. The pathophysiology and changes in spatial and temporal regions are shown in Figure 1.1.
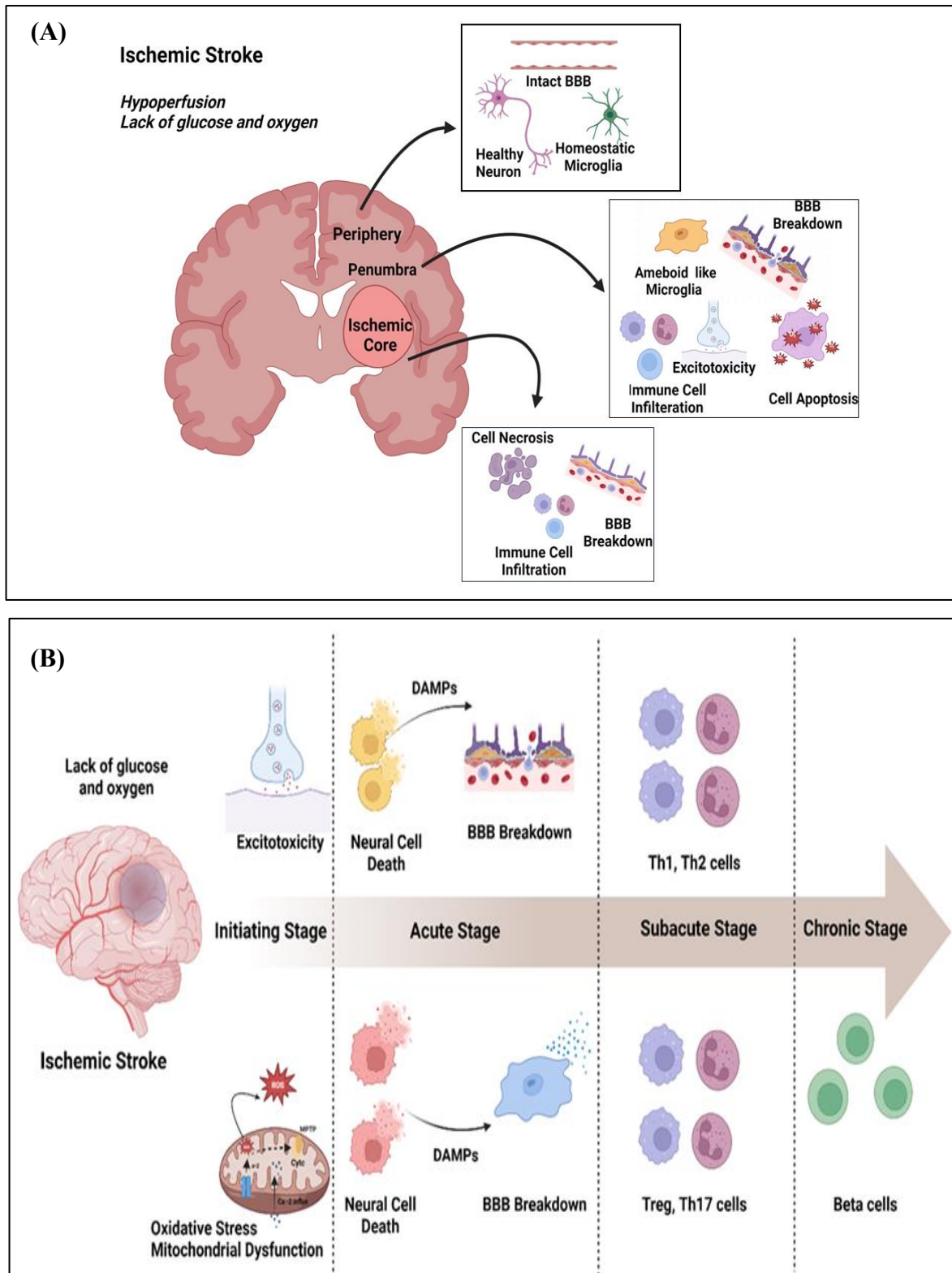
**Figure 1.1**: **Pathology and Stages of ischemic stroke.** A) The spatial and temporal associations of the pathophysiology in ischemic stroke This illustrative representation provides a thorough examination of the spatial and temporal characteristics inherent in the pathophysiological mechanisms of ischemic stroke. B) Stages of Ischemic Stroke. This illustration succinctly outlines the sequential progression of ischemic stroke, providing a concise overview of key events from the initial ischemic insult to subsequent cellular and molecular processes. A brief visual guide for understanding the chronological evolution of this complex neurological condition.

*1.3.3 Pathophysiological Mechanisms Involved in Ischemic Stroke*

Disrupted blood flow to the brain, a hallmark of ischemic stroke, results in the deprivation of essential glucose and oxygen, causing disturbances in critical processes such as adenosine triphosphate (ATP) synthesis, energy production, acid-base balance, and proper ion regulation (Li et al., 2016). These dysfunctions lead to cerebral neuropathological alterations, incorporating edema of the brain, neurological inflammation, and cell death of neurons, ultimately causing significant neurological impairments (Xiong et al., 2018). The progress in science has made it possible to comprehend the development and mechanisms of stroke, such as cell death processes (Datta et al., 2020), cellular excitotoxicity (Lai et al., 2014), BBB impairment (Steliga et al., 2020), neuroinflammation, and mitochondrial dysfunctions (Macrez et al., 2011). Multiple signaling channels are engaged during these pathogenic changes and controlling them specifically could be a viable therapeutic approach. Figure 1.2 shows the pathophysiological overview of ischemic stroke.

Ischemic stroke is a complex neurological condition characterized by the sudden blockage of cerebral blood arteries, primarily due to thrombotic or embolic events, leading to a critical situation where brain regions are deprived of oxygen and glucose, initiating complex processes (Park et al., 2013). Neurons release neurotransmitters, like glutamate, leading to excitotoxicity and calcium ion increase. This causes enzymes like proteases and lipases to activate, causing cell damage, mitochondrial dysfunction, and reduced neuron integrity. The lack of blood flow causes a strong inflammatory reaction, activating microglia and astrocytes. The BBB is compromised, allowing immune cells to enter and worsen tissue damage. Pro-inflammatory cytokines sustain the inflammatory response. Prolonged periods of reduced blood flow trigger apoptotic signaling, leading to programmed cell death, particularly in areas with severely impaired perfusion. If blood flow is restored, the penumbra, the area around the core, may show reversible damage. However, the future of penumbral tissue depends on factors like ischemia duration and intensity (Z. Zheng & Yenari, 2004).

Reperfusion is a therapeutic approach to rehabilitating neurons, but it can lead to ischemia and oxidative stress. The brain's inflammatory response and oxidative stress complicate recovery, potentially prolonging the injury's duration. Scars are formed as the brain restructures, and neuroplasticity, the brain's ability to restructure itself may contribute to functional recovery (Mehta et al., 2007).
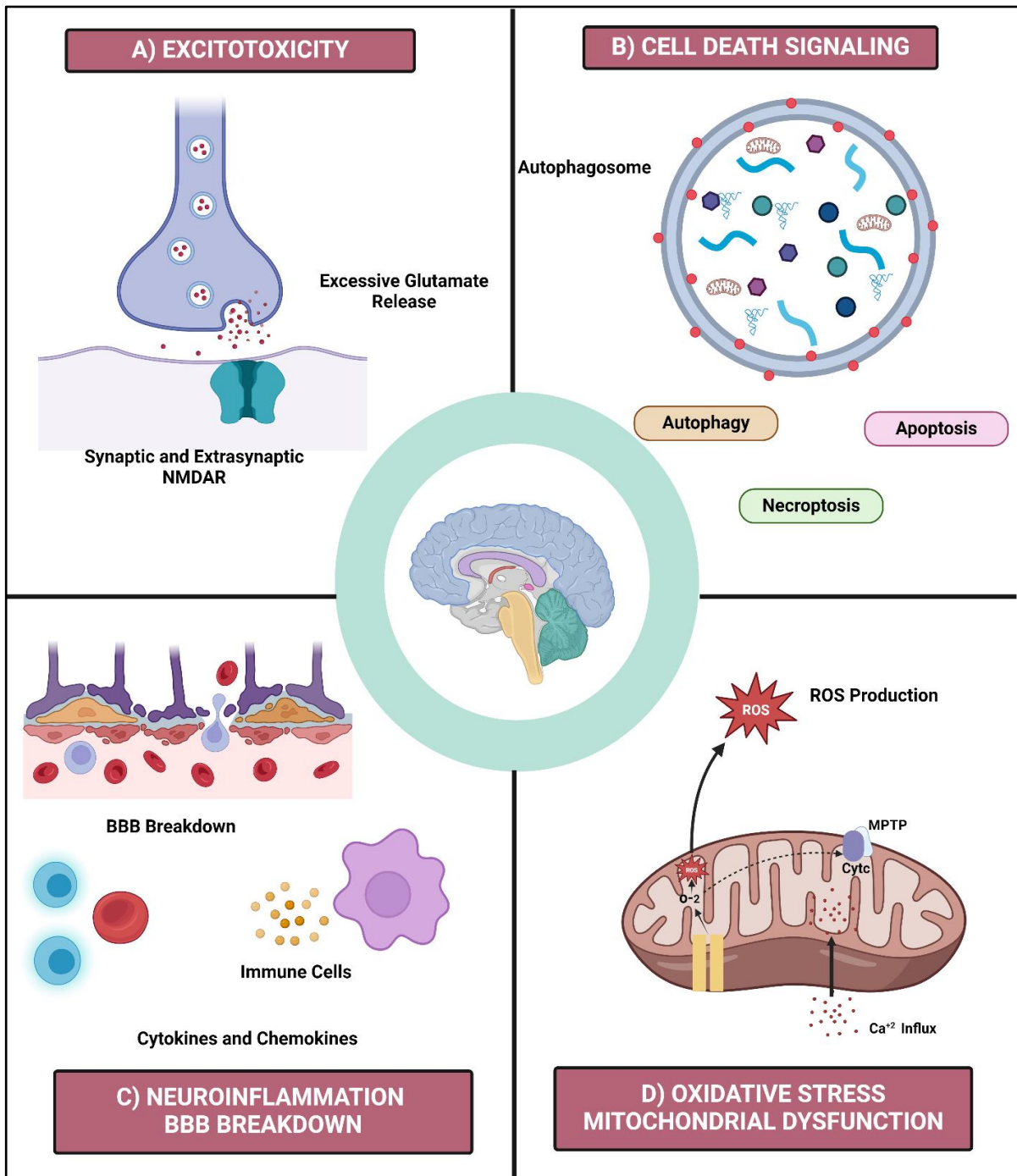
**Figure 1.2: A basic scheme of the pathophysiology of ischemic stroke.** A) Excitotoxicity is brought on by an overabundance of glutamate being released, which triggers the activation of both synaptic and extra-synaptic NMDARs. B) Necroptosis, autophagy, and apoptosis are the main components of the cell death signaling pathway in ischemic stroke. C) BBB breakdown and neuroinflammation. This diagram illustrates how chemokines, different immune cells, and released cytokines contribute to the breakdown of the BBB.  D) In stroke, oxidative stress includes both the influx of Ca2+ into mitochondria and MPTP. Oxidative stress is primarily characterized by the production of ROS and dysfunction of mitochondria.

*1.3.4 Molecular Mechanisms of Ischemic Stroke*

*1.3.4.1 Oxidative stress*

Oxidative stress plays an important role in ischemic stroke, intricately intertwined with disruptions in key cellular processes. The ischemic insult, marked through a substantial blood flow reduction , initiates events that culminate in imbalances in adenosine triphosphate (ATP) synthesis, calcium (Ca2+) homeostasis, and potassium (K+) levels, all while orchestrating the activation of N-methyl-D-aspartate receptors (NMDARs). The reduced ATP synthesis intensifies oxidative stress, as the energy-deficient environment becomes conducive to the generation of reactive oxygen species (ROS). These ROS inflict damage on cellular structures, proteins, lipids, and nucleic acids. The ensuing oxidative stress perpetuates a cascade of molecular responses, leading to protein oxidation, lipid peroxidation, and DNA damage (Manzanero et al., 2013).

Despite the damaging effects, oxidative stress triggers cellular defense mechanisms, activating antioxidant enzymes like superoxide dismutase (SOD) to counteract ROS. However, in the context of ischemic stroke, the overwhelming production of ROS often overwhelms these defense mechanisms, perpetuating oxidative stress and sustaining neuronal injury. The intricate interplay between oxidative stress disrupted cellular processes, and the activation of NMDARs and ion imbalances underscores its pivotal role in the neurodegenerative consequences of ischemic stroke. Understanding these complexities is essential for developing targeted therapeutic strategies aimed at mitigating the impact of oxidative stress on the brain after ischemic stroke (Shirley et al., 2014).

Nrf2, also known as nuclear factor erythroid 2–related factor 2, is a pivotal overseer in the regulation of antioxidant responses. In circumstances of regularity, Keap1, or Kelch-like ECH-associated protein 1, effectively confines Nrf2, thereby subjecting it to degradation. However, oxidative stress disrupts this interaction, as Nrf2 binds with antioxidant response elements (ARE) and initiates the transcription of genes encoding antioxidant enzymes, detoxifying proteins, and cytoprotective factors. PINK1 (PTEN-induced kinase 1) is another crucial player implicated in mitochondrial quality control and oxidative stress response. PINK1, localized on the outer mitochondrial membrane, becomes stabilized under conditions of mitochondrial damage. Stabilized PINK1 recruits Parkin, starting a series of reactions that ends up with the removal of damaged mitochondria through mitophagy. This process acts as a

protective mechanism to mitigate further oxidative stress caused by malfunctioning mitochondria (Cherubini et al., 2005).

PTEN (Phosphatase and tensin homolog) is a phosphatase enzyme with diverse functions. In the context of oxidative stress, PTEN has been shown to modulate Nrf2 activity. PTEN, through its phosphatase activity, can dephosphorylate Nrf2, preventing its nuclear translocation and thereby limiting the activation of antioxidant responses. This interaction adds a layer of complexity to the delicate balance between pro-survival and pro-oxidant signaling pathways.(Manzanero et al., 2013)

*1.3.4.2 Neuroinflammation*

Following an ischemic stroke, neurons and glial cells generate damage-associated molecular pattern molecules (DAMPs), which ultimately results in activation of astrocyte after 28 days. These activated astrocytes undergo rapid proliferation, morphological changes, and functional alterations, along with the secretion of pro-inflammatory molecules. Consequently, this process results in damage to the BBB and an increased infiltration of leukocytes. Moreover, astrocytes contribute positively to the ischemic response by modifying their characteristics and releasing factors that facilitate the growth of neurons. Similarly, microglial cells, akin to astrocytes, form part of the innate immune response within the central nervous system and experience activation shortly after a stroke (Cheng et al., 2023). Microglia undergo morphological changes and acquire the capacity to phagocytose, as well as present antigens to T cells, thereby augmenting T cell response.

Following ischemic events, microglia exhibit both pro-inflammatory and anti-inflammatory effects within the brain. The pro-inflammatory phenotype of microglial cells releases molecules that stimulate inflammation and can permeate the BBB. Conversely, when microglia polarize towards an anti-inflammatory phenotype, they release molecules that mitigate inflammation and activate regulatory T cells, consequently modulating the immune response and reducing inflammation (Rajkovic et al., 2018).

The activation of astrocytes and microglia disrupts the integrity of the BBB, resulting in the migration of leukocytes. Monocytes, which possess both pro-inflammatory and anti-inflammatory characteristics, appear within 24 hours of cerebral ischemia. There is a decline in pro-inflammatory monocytes and an increase in anti-inflammatory monocytes as time

progresses. M2-type macrophages, which are associated with tissue repair, accumulate in the damaged brain tissue following a stroke. These macrophages originate from pro-inflammatory monocytes.

This conversion is essential for the regenerative processes of the tissue. The primary contributors to stroke damage are alpha-beta (αβ) T cells and gamma-delta (γδ)T cells. However, regulatory T cells (Tregs) possess protective properties during the later stages (Ceulemans et al., 2010). Regulatory B cells possess a greater capacity to decrease the influx of pro-inflammatory cells to the site of injury, in comparison to regulatory T lymphocytes. During the acute phase of stroke, B lymphocytes protect against damage to nerve tissue through the release of interleukin 10 (IL-10). This cytokine inhibits the production of pro-inflammatory cytokines (Amruta et al., 2020).

The activation of astrocytes by IL-17 and Tumor necrosis factor-alpha (TNF-α) leads to the infiltration of neutrophils, due to the heightened presence of chemokines in the compromised tissue. Suppression of CXCL-1/CXCR2 activation, or the administration of IL-17-blocking antibodies, results in a decline in the inflammatory reaction and a reduction in the size of the lesion. Furthermore, ischemic stroke prompts the infiltration of natural killer (NK) cells into the cerebral tissue, thereby contributing to the initial stage of the immune response to tissue damage (Yang et al., 2019). Dendritic cells (DCs) contribute to the immune response following cerebral ischemia, exerting influence on local tissue reactions, regardless of their migration to lymphoid organs or their role in antigen presentation. Moreover, mast cells, play a significant role in the causation of cerebral damage, cerebral edema, and neutrophil infiltration (Zheng et al., 2021).

*1.3.4.3 Other Pathways*

The etiology of ischemic stroke is complex and encompasses more than just neuroinflammation and oxidative stress. Excitotoxicity is a significant factor characterized by an excessive release of glutamate, which is a primary neurotransmitter that promotes neuronal excitation. Excessive stimulation overpowers the neurons, causing an increase in calcium ions and initiating a series of events that ultimately lead to cellular harm and demise. Simultaneously, disturbances in the BBB play a crucial role in ischemia damage. The damaged BBB permits the entry of immune cells, such as microglia and leukocytes, into the brain tissue (Woodruff et al., 2011). The arrival of this large number of cells amplifies the body's

inflammatory reaction, making the damage to the tissues worse by releasing substances that promote inflammation and reactive molecules containing oxygen. The complex interaction between excitotoxicity and disruption of the BBB underscores the multifaceted nature of the pathogenesis of ischemic stroke (Kuriakose & Xiao, 2020).

Another crucial factor is the disruption of cerebral blood flow. Ischemic stroke commonly arises from thrombosis, embolism, or systemic hypoperfusion, resulting in inadequate oxygen and nutrition supply to crucial brain areas. Coagulation anomalies, such as the activation of platelets and the initiation of the clotting cascade, have a role in the development of blood clots within the blood arteries of the brain. These blood clots can block the flow of blood, leading to malfunction in the small blood vessels and worsening the lack of oxygen supply to the affected area. The subsequent energy depletion and metabolic impairment initiate a series of intracellular processes that ultimately lead to brain damage. Comprehending the intricacies of excitotoxicity, BBB disruption, and cerebral blood flow abnormalities is essential to fully decipher the pathophysiology of ischemic stroke. This information is essential for creating precise treatment plans (Deb et al., 2010).

**1.4 Symptoms**

Although the signs of a stroke often appear suddenly, this does not necessarily mean that you will not have enough time to act if you experience one. Certain patients will have symptoms such as headaches, tingling, or numbness for a few days before to experiencing a massive stroke (Lecouturier et al., 2010). A study revealed that 43 percent of stroke patients exhibited symptoms of a TIA, often called a mini-stroke, in the week leading up to their occurrence of a major stroke (Sharry et al., 2014).

The mnemonic FAST can be employed to recall the primary symptoms of stroke. If you suspect someone may be experiencing a stroke, assess the following:

- **Face** – a particular side of the face may exhibit a drooping appearance, resulting in the inability to smile or a drooping of the mouth or eye.
- **Arms** — the individual may experience difficulty in raising and maintaining both arms due to muscle fatigue and weakness in one arm.
- **Speech**– individuals may exhibit slurred or incoherent speech, or they may be completely unable to speak despite being conscious.

- **Time** is critical – If you observe any of these indicators, promptly dial and request an ambulance. Prompt intervention has the potential to preserve their lives.

Other possible signs of stroke include:

- Weaknesses or immobility somewhere in the body or on one or both sides
- Visual impairment or blurry eyesight in 1 or both eyes
- Cognitive disorientation
- Impaired comprehension of verbal communication
- Impaired ability to swallow (dysphagia)
- Abrupt and intense headache causing excruciating pain that is unprecedented
- Vertigo
- Lack of equilibrium, or an inexplicable fall
- Impaired ability to swallow
- Lethargy
- Loss of consciousness
- Nausea and Vomiting

## 1.5 Risk Factors for Stroke

Stroke, a condition resulting from prolonged exposition to lifestyle-associated risk factors, can be significantly influenced by modifying these risk factors. This, in turn, can have a substantial impact on the occurrence of stroke and the level of disability associated with it. Ischemic thrombotic stroke is associated with both modifiable and nonmodifiable risk factors. Nonmodifiable factors include :

- Age
- Gender
- Ethnicity and Race
- Genetics
- Family History
- Cardiac factors

Modifiable risk factors include following:

- Hypertension

- Diabetes mellitus

- Smoking

- High Cholesterol

- Hyperlipidemia

- Obesity and inactive lifestyle

- Excessive alcohol intake

- Poor Diet

- Drug Abuse

## 1.6 Diagnosis

The diagnosis of strokes typically involves conducting physical examinations and analyzing brain images obtained from a scan. Various diagnostic tests can be conducted to confirm the diagnosis and determine the cause of the stroke (Yew & Cheng, 2009). These may encompass:

- A blood analysis is conducted to determine the levels of cholesterol and blood sugar in your body.

- Assessing your pulse to detect any abnormal heart rhythm.

- Performing a blood pressure assessment

- Brain scans

Despite the apparent physical signs of a stroke, it is imperative to do brain scans within one hour to ascertain:

- If the stroke is of an ischemic nature, caused by arterial blockage, or hemorrhagic nature, produced by a ruptured blood vessel

- The specific region of the brain that has been impacted

- The degree of severity of the stroke

## 1.7 Treatment

While the management of stroke is dependent on treating the pathophysiology of the condition, stroke prevention entails making changes to risk factors that are present in a population or among individuals. Even though a significant amount of research has been

conducted on stroke over the past two decades, there is still no straightforward method that can be used to treat or prevent all of the clinical reasons for stroke. The overarching goal of the present research being conducted on stroke is to develop novel therapeutics that can modify the variables that lead to both primary and secondary stroke instances. The following is a discussion of recent and current tactics employed for the prevention and treatment of strokes.

*1.7.1 Targeting Oxidative Stress*

In the realm of ischemic stroke, a promising treatment approach revolves around leveraging the Nrf2/ARE signaling system. This intricate pathway serves as a guardian against oxidative stress-induced damage. Key players in this defense mechanism include heme oxygenase 1 (HO1), NAD(P)H quinone oxidoreductase (NQO1), and glutathione S-transferase (GST), all targeted by the Nrf2/ARE system. One potential therapeutic strategy involves the modulation of the Nrf2/ARE pathway to bolster the cellular defense against oxidative stress in ischemic stroke. Tertiary butylhydroquinone (tBHQ), an Nrf2 activator, has demonstrated promising results in experimental cerebral ischemic stroke. The administration of tBHQ has shown relief from symptoms, suggesting its potential as a neuroprotective agent in mitigating oxidative stress-induced damage (Hou et al., 2018).

Metformin, a commonly used medication for diabetes, has also emerged as a candidate for ischemic stroke treatment. Metformin activates the NRF2/ARE signaling pathway, offering a protective effect against damage to the blood-brain barrier (BBB). This finding suggests that Metformin's action on Nrf2 could be harnessed to mitigate the consequences of ischemic stroke, particularly in preserving BBB integrity. Studies involving Nrf2-knockout mice have further emphasized the crucial role of this signaling pathway in neuroprotection. Nrf2-knockout mice displayed heightened susceptibility and exacerbated brain damage, underscoring the significance of Nrf2 in defending against the detrimental effects of ischemic stroke. Consequently, strategies aimed at activating the Nrf2/ARE signaling pathway present a promising avenue for neuroprotective interventions, offering potential relief from the oxidative stress associated with ischemic stroke.

In the intricate landscape of ischemic stroke, the Sirtuin (SIRT1-7) family emerges as a crucial player in combatting oxidative stress. The SIRT/ forkhead box protein O1 (FOXO1) signaling system, a sentinel in preventing oxidative damage during cerebral ischemia-

reperfusion, showcases its efficacy. Specifically, SIRT1 takes the stage, exhibiting antioxidative prowess by activating either the FOXO family or Peroxisome proliferator-activated receptor gamma (PPAR-γ). This unique attribute positions SIRT1 as a promising candidate for therapeutic intervention in the battle against oxidative stress in ischemic stroke. Delving deeper into the Sirtuin family, SIRT3 steps into the spotlight, enhancing the functionality of Superoxide dismutase 2 (SOD2). This enhancement translates to a tangible reduction in reactive oxygen species (ROS) levels, offering a notable protective mechanism. The intricate interplay between SIRT3 and its downstream effector, SOD2, holds promise for alleviating oxidative stress-induced damage in the aftermath of ischemic stroke, as demonstrated by studies (Ahnstedt et al., 2016).

Exploring potential interventions, transsodium crocetinate emerges as a noteworthy contender, displaying protective effects in rats facing oxidative stress induced by brain ischemia-reperfusion injury. The mechanism underlying this protection is linked to the activation of the SIRT3/FOXO3a/SOD2 signaling pathway, revealing a targeted approach to mitigating oxidative damage in the context of cerebral ischemia. In a similar vein, genipin makes its mark by modulating the uncoupling protein 2 (UCP2)/SIRT3 signaling pathway. This modulation results in a tangible decrease in oxidative damage associated with cerebral ischemia, offering another avenue for therapeutic exploration (Chang et al., 2019).

### 1.7.2 Targeting Neuroinflammation

Interventions aimed at modulating the expression of CCL2/CCR2 present potential strategies for alleviating symptoms and pathologies associated with ischemic stroke. Genetic manipulations, such as the disruption, deletion, and knockout of the CCL2 gene, have exhibited promise in reducing the volume of infarction and mortality in mice. Conversely, the inhibition of the CCR2 receptor resulted in a delay in long-term behavioral recovery and a decrease in the expression of anti-inflammatory genes in mice undergoing middle cerebral artery occlusion (MCAO). The regulation of chemokine expression, particularly within the signaling pathway of CCL2/CCR2, emerges as a promising therapeutic approach for ischemic stroke (Hammond et al., 2014).

The signaling of toll-like receptor (TLR) holds a pivotal role in neuroinflammation, rendering it a promising target for treatment. Elevated levels of miR-18a-5p are associated with the downregulation of Toll-like receptor-4 (TLR4) and TLR7, which protect against ischemic

injury. Resveratrol displays efficacy in improving neurological symptoms, while stevioside acts as an inhibitor of the TLR/NF-κB pathway, thereby mitigating neuroinflammation (Lu et al., 2020).

The modulation of the cytokine families IL-1 and TNF emerges as a promising strategy for mitigating injuries caused by stroke. Agents such as XPro1595 or Etanercept demonstrate the ability to alleviate inflammatory reactions and enhance locomotor abilities in focal cerebral ischemia of mouse model. Modified therapies, such as cTfRMAb-TNFR, showcase a reduction in the area of infarction and an improvement in neurological deficits. However, caution is advised when targeting both solTNF and tmTNF, as it may heighten the risk of cardiovascular and demyelinating diseases (Liguz-Lecznar et al., 2015).

Recombinant IL-1Ra remains the exclusive therapeutic intervention for inflammation associated with IL-1. Focusing on the NOD-like receptor protein 3 (NLRP3) inflammasome presents a potential avenue for therapeutic intervention when addressing ischemic stroke. Inhibitors such as Brilliant Blue G and MCC950 demonstrate efficacy in reducing infarctions, alleviating neurological impairments, and modulating the signaling axis of the immunoproteasome/ Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)/NOD-like receptor protein 3 (NLRP3) inflammasome, thereby preventing apoptosis (Chen et al., 2022).

*1.7.3 Targeting Other Pathways*

Excitotoxicity, a contributing factor to ischemic brain damage, involves glutamate-dependent channels (N-methyl-D-Aspartate Receptors, Ca2+ Channel, Phosphatase, and PTEN Receptors) and glutamate-independent Ca2+ channels (Asics, TRP Channels, and NCX). Hypoxia and ischemic stroke induce mitochondrial changes, leading to nitrosative and oxidative stress, involving aldehyde dehydrogenase (ALDH2), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-Coa Reductase), NRF2, and reactive nitrogen species (RNS) /Caveolin-1/ matrix metalloproteinases (MMP). Many antioxidant medications are under investigation for treating ischemic stroke and preventing secondary brain damage (Huang et al., 2018).

Cell death encompasses regulated autophagy, apoptosis, ferroptosis, and necroptosis. Apoptosis, a controlled and ATP-dependent process, contrasts with autophagy, a self-protective mechanism that preserves cellular balance and promotes cell

viability. Cerebral ischemia disrupts both proapoptotic and antiapoptotic signals, presenting potential targets for therapeutic interventions. After an ischemic stroke, various cell types experience the activation of autophagy, which has two purposes (Uzdensky, 2019). Ferroptosis, caused by iron-dependent lipid peroxidation, is linked to ischemic stroke and TNF-α signaling, while necroptosis, primarily due to low ATP, is a potential health risk (Weber et al., 2018).

As far as the landscape of contemporary medical interventions is concerned, the search for innovative and efficient therapeutic options continues to be of the utmost significance. The pharmaceutical chemical galantamine (acetylcholinesterase) , which was developed to treat Alzheimer's disease, is the subject of this thesis, which investigates the potential applications of the substance for stroke. This study aims to provide significant insights into the therapeutic landscape by presenting galantamine as a potentially useful addition to drug therapies for stroke.

## 1.8 Cholinergic Signaling

Cholinergic systems in the brain are believed to cause neural disorders like schizophrenia, Parkinson's, and Alzheimer's disease, which have pathobiological characteristics like immune dysregulation and abnormal inflammation. Recent discoveries highlight the crucial role of cholinergic signaling (Metz & Pavlov, 2021). The study of cholinergic signaling has enhanced our understanding of its therapeutic benefits, particularly in treating immunological and metabolic disorders like Alzheimer's, Parkinson's, and schizophrenia. Cholinergic signaling regulates inflammation, which is linked to these conditions. The article discusses the ongoing therapeutic use of galantamine and its research on its potential in treating various diseases, emphasizing the importance of understanding brain cholinergic dysfunction and inflammation (Marco-Contelles et al., 2006).

### 1.8.1 The Cholinergic System of Brain

The cholinergic system is a vital network of neuromodulators within the brain, with the basal forebrain cholinergic system (BFCS) serving as its principal component. This system supports attention, learning, and memory regulation by establishing neuronal connections with key brain regions such as the cerebral cortex, hippocampus, amygdala, and olfactory bulb. Cholinergic neurons also contribute to memory regulation. Cholinergic neurons in the

pedunculopontine tegmental (PPT) and laterodorsally tegmental (LDT) regions contribute to the microcircuitry involved in motor activity. The BFCS, overall, plays a crucial role in the brain's function. (Deiana et al., 2011).

Choline acetyltransferase (ChAT) is an integral component of the cholinergic signaling mechanism within the brain. It is responsible for the synthesis of acetylcholine (ACh) by utilizing choline and acetyl coenzyme A. The vesicular acetylcholine transporter (VAChT) is responsible for the encapsulation of ACh prior to its release into the synaptic cleft. Acetylcholinesterase (AChE) is involved in the termination of cholinergic neurotransmission by converting ACh into acetate and choline. Conversely, butyrylcholinesterase (BuChE), predominantly present in glial cells, fulfills a similar function. Neuronal acetylcholine receptors (nAChRs) are influential in regulating and augmenting cholinergic neurotransmission, whereas muscarinic acetylcholine receptors (mAChRs) have a significant impact on the processing of cholinergic neurotransmission. (Bagwe & Sathaye, 2022). Figure 1.3 gives a basic overview of the action mechanism of acetylcholine.

The cortex, hippocampus, and hypothalamus are connected to the dorsal vagal complex, including the dorsal motor nucleus of the vagus. These connections are made through polysynaptic pathways. Recent research shows these connections play a crucial role in physiological inflammation regulation (Abreu-Villaça et al., 2011).Over the past decade, understanding cholinergic signaling in the brain has improved, with a connection between cholinergic pathways and immune function, inflammation, and metabolic processes. Galantamine, a cholinergic drug, has been approved by the FDA for treating neurological problems like Alzheimer's, schizophrenia, and Parkinson's by inhibiting AChE enzyme and modulating nAChRs in the CNS (Halder & Lal, 2021). Studying vagus nerve-based inflammatory reflex and cholinergic signaling can help understand immunity and inflammation regulation. Galantamine's ability to reduce inflammation offers new insights (Bertrand & Wallace, 2020).

Cholinergic medicines with anti-inflammatory properties, approved by the FDA, offer a potential approach for clinical application. A double-blind trial found that administering galantamine significantly reduced inflammatory TNF and leptin concentrations in patients' bloodstreams, demonstrating the potential of preclinical discoveries in translating research findings into clinical applications (Zabot et al., 2021). Galantamine, compound found in food

has been shown to increase anti-inflammatory IL-10 and adiponectin levels, improve oxidative stress, and reduce insulin resistance, and further research is expected to determine its effectiveness in treating metabolic syndrome.
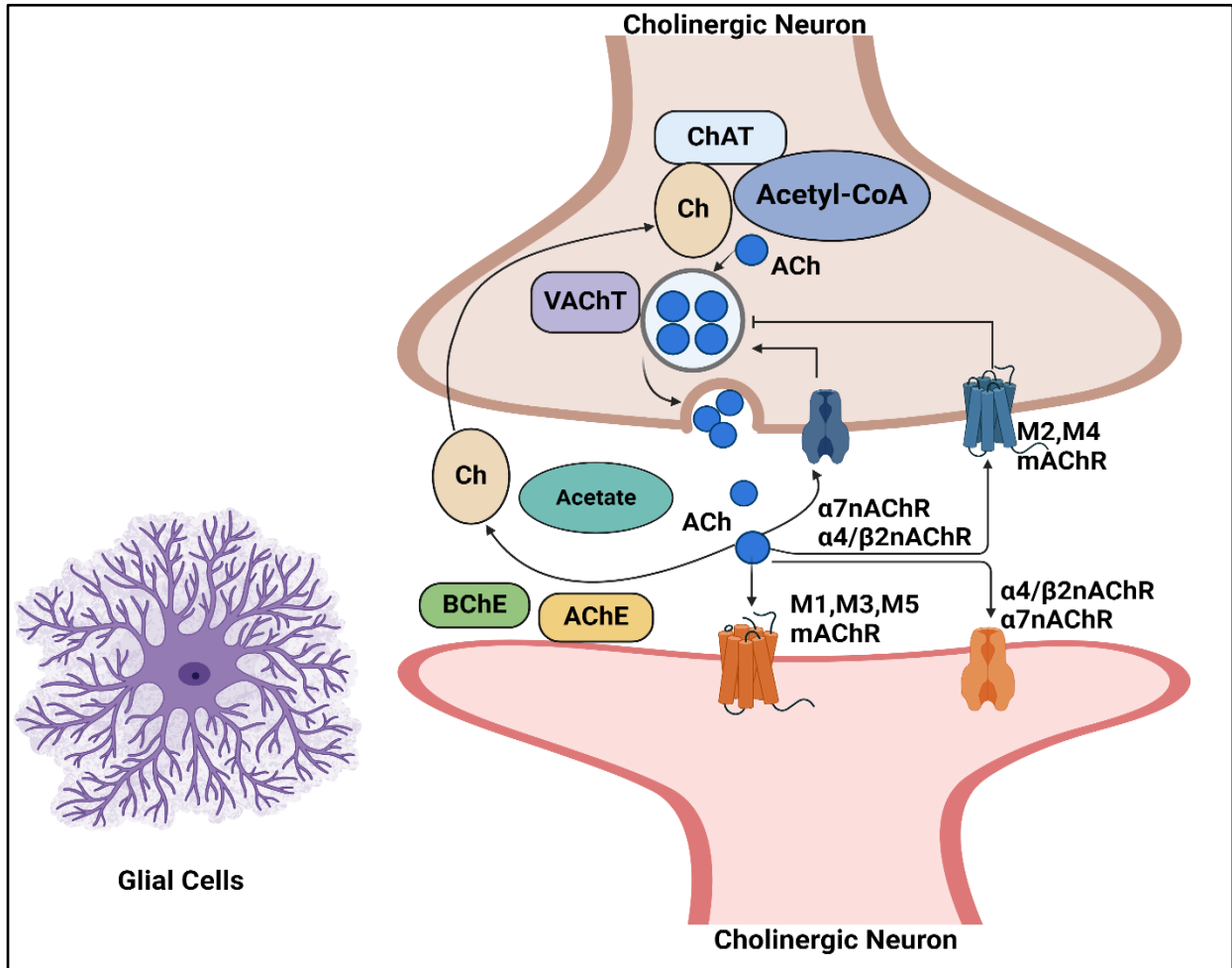


**Figure 1.3: The mechanism of action of acetylcholinesterase involves the synthesis and degradation of acetylcholine (ACh).** Cholinergic neurotransmission: ACh production by ChAT, uptake via VAChT, synaptic release, and interaction with receptors, regulated by presynaptic receptors, and termination through AChE and BChE, ensuring a continuous cycle of choline reuse for ACh synthesis.

## 1.9 Aim of the Study

Research on the potential of galantamine in ischemic stroke, particularly in rodent models such as MCAO in rats, is imperative for uncovering the neuroprotective effects and therapeutic applications of this compound. Galantamine, with its inhibitory activity on acetylcholinesterase and modulation of nicotinic acetylcholine receptors, is a promising candidate for reducing the negative effects of ischemic stroke. Investigations aim to ascertain

whether galantamine can effectively reduce damage to brain tissue, enhance cognitive function following a stroke, and regulate the inflammatory responses and oxidative stress associated with ischemia. Preclinical studies on galantamine's use in treating ischemic stroke can lead to clinical trials, enabling exploration of optimal dosage, timing, and combination strategies, ultimately enhancing stroke management and rehabilitation. Following are the aims and objectives of the research:

- Establishment of MCAO rat model.
- Optimization of galantamine doses to prevent stroke-associated effects.
- Molecular and histopathological analysis for evaluation of stroke.

# CHAPTER 2:    MATERIALS AND METHODOLOGY

## 2.1 Ethical Approval

Prior to commencing the in vivo investigation, ethical approval (IRB number. 07-2023-ASAB-01/01) was obtained from the NUST-IRB committee of the National University of Sciences and Technology, Islamabad.

## 2.2 Animals

This research was conducted with male Wistar Hans rats that were between 12 and 14 weeks old. Up until the moment of the dissection, the rats were housed in plastic cages that allowed them unrestricted access to water and food for rodents. Before beginning the experiment, rats were allowed to become accustomed to the conditions of the laboratory. The rats were divided into three groups (n=15): the Control group (n=5), the MCAO group (n=5), and the MCAO group treated with galantamine (n=5 ).

## 2.3 Experimental Design

The experimental protocol involved acclimatizing a total of fifteen male Wistar Hans rats, aged 8-14 weeks, in the animal house for one week, with each group comprising n=5. During this period, the rats in their cages were given unlimited access to both food and water. On the $9^{th}$ day, the medical procedure called MCAO was performed. Galantamine drug is administered to the treatment group for a total of 18 days, beginning on day 9 and continuing through day 26.

An assortment of behavioral examinations was carried out between the days of 27 and 35. These examinations included the grip strength test, the novel object recognition test, the Y-maze test, the open field test, and the social interaction test. Tissue samples were collected on days 36 and 37 of the experiment. The process of histopathology was carried out from day 38 to day 43, which included the preparation of slides, staining with Hematoxylin and Eosin stain (H & E), and slide examination. After that, samples were taken for molecular analysis, which was done between day 44 to day 54 and included the Real Time-Polymerase Chain Reaction (RT-PCR). From $55^{th}$ to $60^{th}$ day, an analytical analysis of behavior testing, histology, and molecular analysis was conducted. Figure 2.1 gives a graphical timeline and experimental

design of the project.
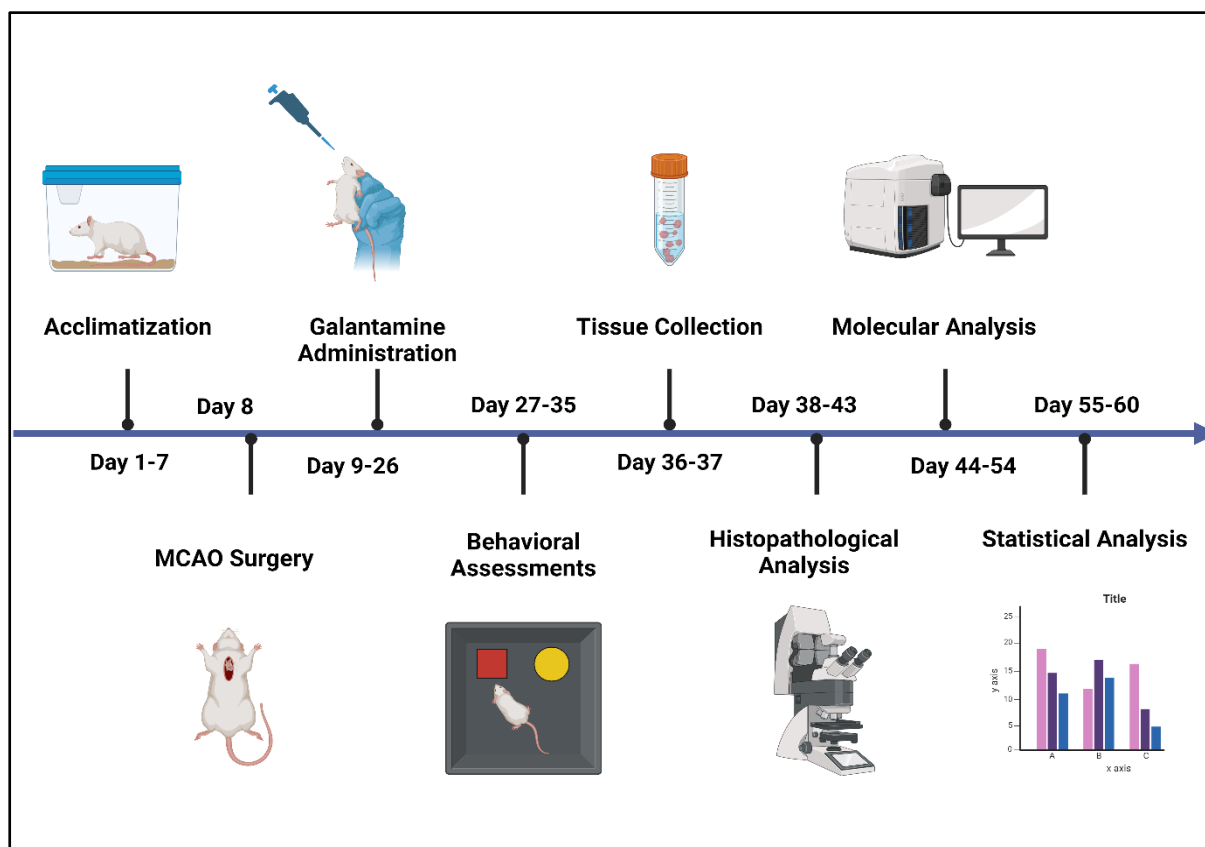


**Figure 2.1:Timeline and Experimental Design.** "Explore the impact of galantamine on Wistar Hans rats post-MCAO. A meticulous timeline encompassed acclimatization, treatment administration, behavioral assessments, and comprehensive histopathological and molecular analyses. This research sheds light on galantamine's effects in a systematic manner."

## 2.4 *in silico* Analysis

Before beginning the experimentation, an *in silico* analysis was performed utilizing various software programs and computational methodologies. When it comes to the development and research of drug molecules at an early stage, as well as validation of safety and the evaluation of older medications, the identification of potential therapeutic targets is both valuable and crucial according to the new use. This study was conducted to gain insights and make predictions on the hypothesis before the actual execution of the experiments. Additionally, the three-dimensional structure of SOD2 (P07895:SODM_RAT) and TLR4 (Q9QX05:TLR4_RAT) was obtained from the PubChem.

Through the use of the PubChem compound database, the chemical structure of galantamine, which has the PubChem CID number 9651, was made available. PyRx was then used to carry out the docking process to understand the structural basis of SOD2 (Duc Nguyen,

2023) and TLR4 (Aryal et al., 2022) with galantamine selectivity, as well as to compute the binding affinity of SOD2 and TLR4 (the target) with galantamine (the ligand). After that, Discovery Studio was utilized to visualize the interactions between SOD2, and TLR4 with galantamine, with a particular emphasis on the main interaction patterns.

## 2.5 Disease Induction by MCAO

Among the arteries that are involved in acute ischemic stroke (AIS), MCA is the most frequently affected (Nogles and Galuska, 2020), it results in serious nerve injury and a dismal prognosis. Considering that the MCA is the site of about 80% of ischemic strokes, a number of animal stroke models have been created that focus specifically on this segment of the artery.

### 2.5.1 Pre-Operative Care

For the surgical procedure, control adult male rats weighing between 200 g and 250 g are chosen. The preference for male rats is predicated upon the recognized neuroprotective properties of estrogen, with a specific interest in its potential impact on the severity of myocardial infarction. The experimental onset entails a methodical preparatory phase wherein all subjects undergo an overnight fasting period, refraining from food consumption. This standardized fasting regimen serves as a strategic maneuver to establish a homogenous metabolic baseline, effectively attenuating variances in nutritional states. The meticulous orchestration of these preparatory measures imparts methodological rigor to the investigation, affording a heightened level of precision and internal validity in discerning the intricate interplay between estrogen, neuroprotection, and myocardial infarction severity within this meticulously selected cohort of male rodents.

### 2.5.2 Protocol for MCAO

To ensure that all surgical instruments and materials are sterile, it is essential to use an autoclave to sterilize them. Additionally, the surgical procedure must be carried out in a sterile environment. The two drugs, xylazine-10 mg/kg ( FX56092-BIOSYNTH, Switzerland ) and ketamine-80 mg/kg (17750-Pipelinepharma, Pakistan), are administered intraperitoneally to animals to induce anesthesia (Zausinger et al., 2002).

After the rat has been positioned in a supine position, adhesive tape is used to attach it to the surgery table. White latex surgical gloves were used. Then a cut is made in the middle

of the neck, and the soft tissues that cover the trachea are carefully pulled aside once the cut has been made. To temporarily disconnect the common carotid artery (CCA) from the vagus nerve, a cotton thread is used. This procedure is performed regardless of whether the CCA is located on the left or right side. The CCA commonly bifurcates into two branches: the external carotid artery (ECA) and the internal carotid artery (ICA), both of which are responsible for supplying blood to the head region. The internal carotid artery subsequently divides to give rise to the MCA and the pterygopalatine artery (Yousuf et al., 2007). After locating the initial division of the CCA, the next step is to meticulously extract the surrounding soft tissues that enclose both the ECA and the ICA without causing any damage to the arteries.

After that, two permanent knots are placed near one another at the opposite end of the ECA to impede the flow of blood in the opposite direction, and the ECA is then severed between the knots. It is feasible to straighten the narrowed section that is linked to the junction of the CCA to facilitate the passage of a filament through the ICA. Subsequently, the second division is meticulously removed to obtain a distinct visual representation of the MCA. To facilitate the insertion of the nylon 4-0 monofilament (SA9151524- Vital Medical Supplies, Australia), the knotted section of the ECA is incised using micro scissors. A knot is tied below the arteriotomy in the ECA once the CCA connection is completed. The ECA stump is orientated toward rats, and the filament is meticulously injected into the MCA, beginning at the junction of the CCA. As a result of this process, the distance that separates the two is anywhere between 17 and 20 millimeters (Carmichael, 2005).

After confirming that the filament has been effectively implanted into the MCA, we wait for thirty minutes to ensure that the filament continues to be in its original position after it has been implanted. When it comes to identifying the severity of the infarction, the length of time that the MCA was blocked is one of the most crucial factors that determines the severity of the infarction. It is required to obstruct the flow of blood for a minimum of thirty minutes to guarantee accurate results when determining the extent of the injury to the brain. 30 minutes, 60 minutes, 90 minutes, and 120 minutes are the durations that are utilized the most frequently to block the middle cerebral artery in rats (Durukan & Tatlisumak, 2007).

Next, the filament is retracted moderately until the tip is close to the arteriotomy. This process continues until the arteriotomy is completed. Following the removal of the filament, the knot is then thoroughly and firmly fixed in the ECA where it was previously located. The incision that was made in the middle of the neck is then closed with surgical silk suture 3-0

(BNSG093221- Vital Medical Supplies, Australia) once it has been confirmed that the blood flow has been restored in the forward direction (anterograde reperfusion).

After surgery, a topical gel that contains lidocaine is administered directly to the wound to decrease the pain and discomfort that the patient is experiencing. Following the conclusion of the experiment, the animals are put to sleep, and a histological examination is carried out to confirm the presence of infarction. An infarction of the brain can typically be identified after 24 hours have passed since the completion of reperfusion or surgery (Rupadevi et al., 2011). Figure 2.2 gives the summary of the MCAO technique.
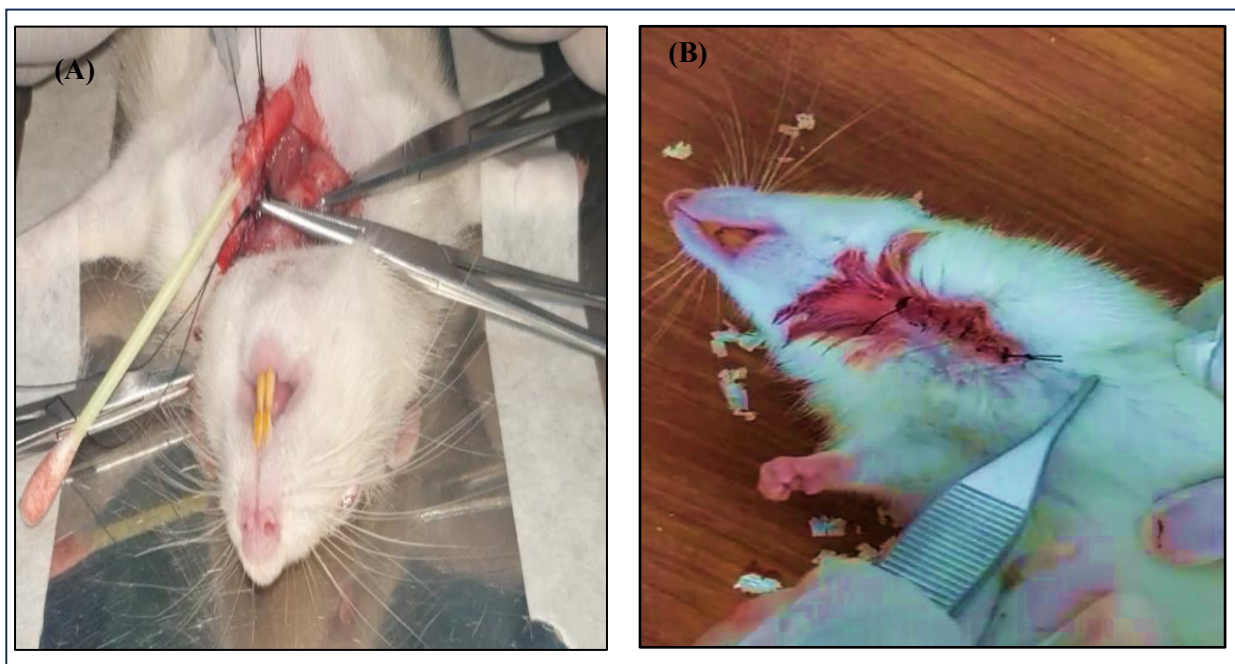


**Figure 2.2: Summary of the MCAO Technique and Reperfusion.** (A) It involves the insertion of a monofilament (a thin nylon monofilament 4-0) into a cerebral artery, typically the middle cerebral artery, to induce focal cerebral ischemia. This technique mimics the blockage of blood flow to simulate conditions similar to an ischemic stroke. This is used to study the pathophysiology of stroke, test potential interventions, and explore neuroprotective strategies in rats. (B) The critical moment of reperfusion captured as the monofilament is carefully withdrawn after MCAO. This meticulous step marks the restoration of blood flow, unraveling insights into the effects of reperfusion injury and paving the way for potential therapeutic strategies in stroke research.

*2.5.1 Drug Preparation*

The commercially available galantamine 8mg (46922, APOTEX, United States), which has the potential to have cognitive effects, was used for this investigation. The material was properly crushed, and a dose of 5 mg/kg of rat body weight was measured (Geerts et al., 2005). To attain a consistently dosed oral solution, the predetermined quantity of the substance was

dissolved in a solution comprising ethanol and distilled water. This precise mixture aimed to ensure uniformity and accuracy in the drug formulation. Subsequently, a precisely measured volume of 2 microliters was employed for the once-a-day oral administration of galantamine to each rat. Figure 2.3 demonstrates the oral administration of galantamine to rats.

Throughout the 18-day experimental duration, this methodological approach was consistently applied. Its purpose was threefold: first, to simplify the process of controlled drug administration; second, to adhere rigorously to predefined procedures; and third, to guarantee the high precision and accuracy of dosage delivery. The systematic implementation of this approach not only facilitated the practical aspects of drug administration but also maintained the experimental integrity, contributing to the reliability and reproducibility of the study's outcomes.



**Figure 2.3: Administration of oral dosage of Galantamine.** Rats received a once-daily oral dose of Galantamine at 5mg per kg for 18 days. The drug was meticulously dissolved in ethanol and distilled water, with each rat receiving a precisely measured 2 microliters.

## 2.6 Behavior Testing

The animals underwent a pre-test preparation routine, arriving at the behavioral assessment area 30 minutes before the initiation of behavioral tests. To assess neurological decline post-MCAO, a battery of behavioral tests was systematically employed. These included the Grip Strength Test, Novel Object Recognition Test, Open Field Test, Y-Maze Test, and Social Interaction Test. Each test evaluates different aspects of neurological function and

behavior. Following the MCAO procedure, the behavioral performance of the rats was meticulously recorded during the execution of these tests. This comprehensive approach allowed for a multifaceted evaluation of the impact of MCAO on various behavioral parameters, providing valuable insights into the neurological consequences of cerebral ischemia in the experimental subjects.

*2.6.1 Grip Strength Test*

Conducting the grip strength test in MCAO-induced stroke research is crucial for understanding neurological and motor consequences. This assessment provides a quantitative measure of limb strength and coordination, enabling the evaluation of neuromuscular function. Scrutinizing grip strength symmetry helps identify potential neurological impairment or focal deficits resulting from MCAO. Monitoring temporal changes and comparing baseline with post-MCAO measurements aids in quantifying stroke impact and tracking motor deficit progression or recovery. The grip strength test is pivotal for assessing intervention efficacy, contributing to a comprehensive understanding of motor outcomes in the context of MCAO-induced stroke research.

A specialized mesh board designed for grip strength assessment was intricately arranged in the context of MCAO stroke research. This test, integral to evaluating the impact of cerebral ischemia on neuromuscular function, meticulously scrutinizes multiple factors. The horizontal placement of the mesh serves as the testing platform for assessing forelimb strength and coordination, crucial for identifying motor deficits. The testing procedure involves gently lifting the animal above the mesh and lowering it towards the platform, ensuring alignment of the tail, body, and left forelimb at a right angle. As the rats instinctively clung to the mesh, their grip strength was assessed by noting the precise moment of release, representing the maximum force endured. This detailed approach provides a physiological indicator of neuromuscular function and contributes valuable data to the broader understanding of motor outcomes in stroke research. Figure 2.4 shows the rat clinging upside down to the mesh board before falling down.

Conducting multiple grip strength trials addressed biological variability among rats, capturing diverse range of responses for nuanced understanding of each animal's grip strength. The calculated mean value from these trials provided a representative measure of the rats' average grip strength, ensuring a comprehensive assessment and mitigating the impact of

individual fluctuations. This strategy increased precision in grip strength measurements, enhancing the reliability and validity of the behavioral assessment (Wang et al., 2008).



**Figure 2.4: Grip Strength Test for motor function.** A rat, depicted hanging upside down on a mesh board, engages in a grip strength assessment. The figure captures the unique experimental setup where the duration of the rat's secure clinging to the mesh is timed before it falls. This illustration provides a representation of the rat's neuromuscular endurance and limb strength, offering valuable insights into motor function and potential neurological impacts in research studies.

*2.6.2 Y-Maze Test*

The Y-maze test plays a pivotal role in MCAO stroke research involving rats, offering unique insights into cognitive function and spatial memory. As a widely employed behavioral assessment tool, the Y-maze allows researchers to determine the effects of cerebral ischemia on the rats' potential to navigate and remember spatial configurations. The test involves the exploration of a Y-shaped apparatus, requiring the rats to make spontaneous choices between alternate arms. In the context of MCAO-induced stroke, the Y-maze provides valuable data on cognitive deficits, alterations in exploratory behavior, and potential recovery patterns post-stroke. By assessing the rats' spatial working memory and spontaneous alternation behavior, researchers can gain a deeper understanding of the neurological consequences of ischemic events, aiding in the development of targeted therapeutic interventions and contributing to the overall comprehension of post-stroke cognitive impairments.

This equipment is made up of three arms that are 80, 30, and 15 centimeters in length, and they are angled at a 120-degree angle. The rats were given free movements in two arms, the arm that they knew well (Familiar arm) and the arm that they started with (Starting arm),

for ten minutes. The third arm, which stood in for the novel arm, was to be closed. The rats roamed in each of the three arms for five minutes during the second phase. When doing the Y-maze, it is assumed that rats will switch between the three arms, moving starting with the "familiar arm" towards the "novel arm" and finally arriving at the "start arm," all the while keeping in mind the previous study of the arms that came before them. In contrast, rats that are subjected to pathological conditions are unable to switch among the three arms, demonstrating an inability to recall the previous experiment. To determine the percentage alteration, the subsequent formula is utilized (Kitanaka et al., 2015). Figure 2.5 shows the rat at the starting position at the start of the test.

$$Percentage\ of\ Alteration = \frac{Number\ of\ Alterations}{Total\ Number\ of\ Entries} \times 100$$



**Figure 2.5: Y – Maze Test for spatial memory and exploratory tendency.** This figure portrays a rat transitioning from the initial arm to either a familiar arm or a novel arm in the Y-Maze Test. The percentage of modification is determined by calculating the amount of time spent in the old arm and the new arm. This percentage is indicative of the rat's spatial memory and exploratory tendencies, providing valuable insights into its cognitive abilities.

*2.6.3 Novel Object Recognition Test*

During the novel object recognition test, the rats are tested on their capacity to identify a new object that is present in their environment. It is a procedure that evaluates the inherent preference for novel things that rodents exhibit. The procedure for the task is broken down into three stages: the habituation phase, the familiarization phase, and the test phase. Every rat has

five minutes to wander around the open-field arena unhindered by objects during the habituation phase. Subsequently, the rat is removed from the arena and returned to its original holding cage. When the familiarization phase is being carried out, a rat is positioned in the open-field arena that contains two sample objects that are identical to one another (A + A) for five minutes (Gaskin et al., 2010). Figure 2.6 gives the Novel Object recognition test being carried out on rats.

The experimental situation does not considerably differentiate itself from one another during the familiarization phase and the test phase of the experiment. A retention interval is followed by the test phase, which consists of the rat being returned to the open-field arena with two objects. One of the objects is the same as the sample, and the other is new (A + B) for five minutes. In both the familiarization phase and the test phase, items are placed in the arena that are opposite to one another and symmetrical. Additionally, the location of novel things is counterbalanced with the location of familiar objects (Broadbent et al., 2010).
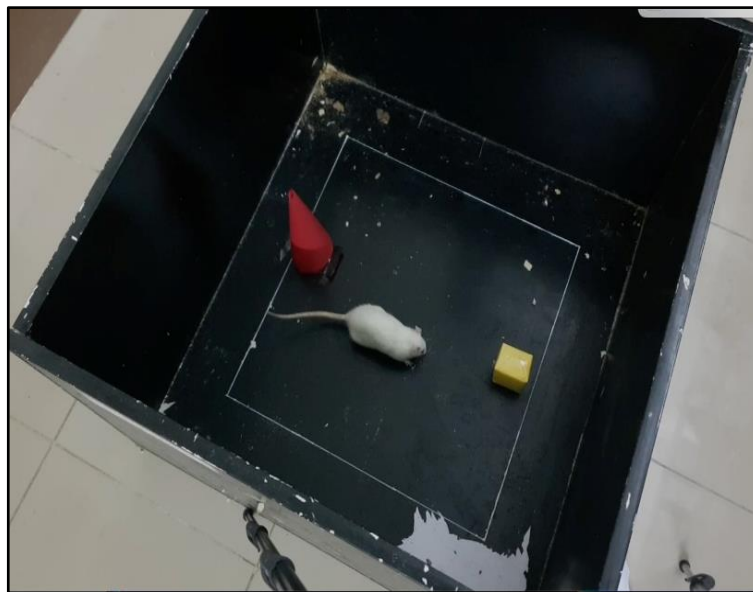


**Figure 2.6: Novel Object Recognition Test for cognitive processes**. This figure depicts the movement of the rat between the two objects, one familiar and one novel object. The time spent with each object is calculated. The preference for the novel object, as indicated by a greater amount of time spent with it, suggests that the rat remembers the familiar object and perceives the novel object as new. This test is particularly valuable in the field of neuroscience for studying cognitive processes such as memory and attention in rodent models.

*2.6.4 Open Field Test*

The open field test is crucial in behavioral neuroscience, particularly when studying the effects of an MCAO stroke in rat models. This test gives information about the rat's motor function, activity levels, exploratory behavior, and anxiety levels, all of which can be impacted

by a stroke. For instance, changes in the rat's movement or movement patterns can indicate the severity of the stroke's effects on its physical abilities. Similarly, the rat's behavior in the open field, such as spending more time in the corners or edges (thigmotaxis), can signal increased anxiety levels, potentially resulting from the stroke. Open Field Test primarily focuses on motor and anxiety-related behaviors. Figure 2.7 shows the grip strength test carried on rat.

The open field apparatus had been constructed using a wooden enclosure, measuring $60 \times 60$ cm, with walls that were 60 cm in height. The entire equipment was coated in black paint, except the white floor. The floor was partitioned into 16 squares of equal size, each measuring $15 \times 15$ cm. For the duration of the experiment, the animals' movements were recorded for five minutes using a video camera that was placed some distance away from the arena. For every rat, the following behaviors were observed: how much time is spent in the central region and how much time is spent outside of it (Zimcikova et al., 2017).



**Figure 2.7: Open Field Test for exploratory behavior and anxiety level.** This figure shows the movement of the rat across the squares. The time a rat spends in the central region and time spent in peripheral region is calculated. These measurements are crucial as they can provide insights into the rat's exploratory behavior and anxiety levels, both of which can be affected by a stroke.

*2.6.5 Social Interaction Test*

The social interaction test emerges as a crucial component in stroke research employing the MCAO rat model. Stroke-induced alterations in the brain can extend to social cognition, affecting the way rats interact with their conspecifics. Through systematic observation of social

dynamics in both familiar and novel contexts, the test provides quantitative data on parameters such as social exploration, proximity, and responsiveness, allowing for a comprehensive evaluation of the impact of stroke on social dynamics.

For our experiment Figure 2.8 , we utilized an acrylic container with dimensions of 44 cm in length, 33 cm in breadth, and 20 cm in height. In order to promote adaptation to the cage, rats were individually placed in cages of comparable size for 5 minutes before being brought in pairs to the test cage. Each examination had a duration of 10 minutes and was recorded on camera for subsequent analysis. The two parameters that were considered were sociability and social novelty. Following each trial session, the test cage underwent meticulous cleaning using a cleaning solution. To facilitate the diffusion of the fragrance, the boxes were permitted to desiccate for 5 minutes before commencing a fresh experiment (Whittaker et al., 2016).



**Figure 2.8: Social Interaction Test for social cognition and behavior.** The figure shows the Social Novelty. This test carries two-part sociability and social novelty. The test assesses sociability and social novelty. In the first part, the subject's interactions within a familiar social setting are observed, gauging innate sociability. The second part introduces novelty, examining adaptability to unfamiliar social stimuli. This test provides insights into the intricacies of social cognition and behavior."

## 2.7 Histopathological Analysis

Histopathology is crucial in animal research as it enables the microscopic examination of tissues, offering significant insights into disease causes, treatment effectiveness, and the influence of experimental factors. By analyzing changes in cellular and structural characteristics, scientists can identify and categorize diseases in animals, assess the efficacy of

treatments, and identify biomarkers to monitor disease progression. Histopathology plays a vital role in toxicology studies by evaluating the impact of toxins and drugs on tissues. Moreover, it facilitates the understanding of disease causes, validates the reliability of animal models, and enables the conduction of long-term investigations.

## 2.7.1 Dissection and Tissue Fixation

The rats were dissected by administering deep inhalation of chloroform. To conduct a histological investigation, the circulatory system was washed out with a fixative solution containing 4% paraformaldehyde during transcranial perfusion. By circulating through the bloodstream and displacing blood. The fixative ensured thorough fixation of the tissue, preserving its integrity. Afterwards, the rats were carefully dissected. The skull was then dissected along the midline using scissors and a surgical knife to expose the brain. The brain was delicately extracted from the skull using little forceps. To eliminate excess fixative and blood from the sample, the tissues were subsequently rinsed with phosphate-buffered saline-PBS (A9162.0100, Avantor, America). Subsequently, the brain tissue was carefully submerged in a solution containing 4% paraformaldehyde-PFA (E-IR-R114, Elabscience, USA), which serves as a fixative.

## 2.7.2 H and E Staining

Hematoxylin and eosin (H&E) staining is used in histology that provides detailed information about the cellular and structural composition of tissues. This staining method involves the use of two dyes, hematoxylin, and eosin, to visualize different cellular components under a light microscope. Hematoxylin, a basic dye, stains acidic components in the tissue, particularly the cell nuclei, giving them a purplish-blue color. This is because hematoxylin forms complexes with acidic compounds, such as nucleic acids in the cell nuclei. As a result, the nuclei of cells become easily distinguishable in the stained tissue sections.

On the other hand, eosin, an acidic dye, imparts a pink hue to the extracellular matrix and cytoplasm of cells. Eosin binds to basic structures in the tissue, such as proteins, providing contrast to the purple-stained nuclei. This allows pathologists to differentiate between the nucleus (which appears blue) and the cytoplasm and extracellular matrix (which appears pink). In the context you provided, the use of H&E staining in examining a stained brain region is described. The light microscope is a crucial tool for visualizing the tissue at a microscopic

level, allowing the observation of individual cells and their relationships.

The information gathered from the stained sections, specifically photomicrographs of the cortex, aids in comparing and contrasting differences among three groups. The comprehensive representation of the structural makeup of the tissue sample obtained through H&E staining allows researchers and pathologists to draw insights into the composition, quantity, and orientation of cells within the examined region. Figure 2.9 encompasses the steps in histological analysis.
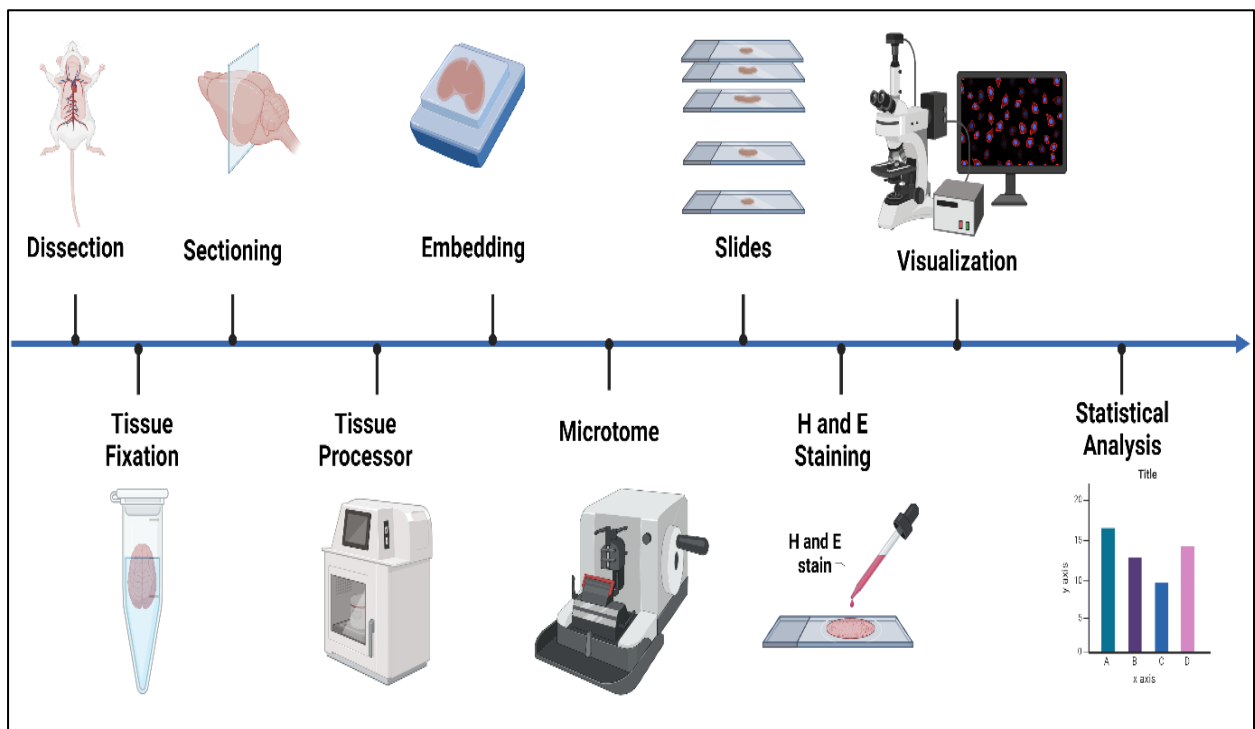


**Figure 2.9: Steps for evaluation of histopathological aspects.** Histopathology unveils cellular stories through key steps: tissue collection, fixation, embedding with a tissue processor, microtome sectioning for slides, staining, and visualization.

## 2.8 Analysis of Gene Expression

### 2.8.1 Dissection

The rats were subjected to profound anesthesia by chloroform inhalation. Subsequently, the rats were properly decapitated using sharp scissors. The cranium was subsequently incised using precise scissors and a knife along the central axis to reveal the brain. The brain was delicately extracted from the skull using little forceps. The sample was rapidly frozen using dry ice and preserved at a temperature of -80°C for further processing.

### 2.8.2 RNA Extraction

The use of TRIzol (FTR 100, Fine Biotech Life Sciences, China) isolation reagent for RNA extraction is a common and widely employed method in molecular biology and biochemistry. Initially, a volume of 1000µl of TRIzol reagent was introduced to the sample and subsequently mixed thoroughly by centrifugation at 12000 rpm at 4˚C for 10 minutes. After the process of centrifugation, the liquid portion above the sediment was moved to a separate tube. After that , 200 µl of chloroform was added to the sample, then vigorous stirring was done  for 30 seconds. Following the mixture preparation, centrifugation was conducted at a speed of 12,000 rpm for 10 minutes at a temperature of 4˚C.

Next, carefully transfer the RNA-containing aqueous phase to a fresh tube. Next, carefully mix the contents of the tube thoroughly by adding 500 µl of isopropanol. Let the mixture sit at room temperature for ten minutes. After incubation, the supernatant was discarded, and centrifugation was carried out for 10 minutes at 4˚C and a speed of 12,000 rpm. After being cleaned with 75% ethanol, the pellet was centrifuged for two minutes at 4˚C at 12000 rpm. After carefully removing the ethanol, the pellet was air dried for five to ten minutes. Ultimately, 20–50 µl of nuclease-free water were added to the pellet to resuspend it.

### 2.8.3 Assessment of RNA Quality and Quantity

The quality and amount of collected RNA were assessed by the use of Colibri Nanodrop instrument manufactured by Titertek Berthold in Germany.

### 2.8.4 cDNA Synthesis (Reverse Transcription)

The RNA extraction was later followed by cDNA transcription with RevertAid Reverse Transcriptase (EP0441, Thermo Fisher Scientific, Lithuania). The reaction mixture was subsequently produced, comprising the reaction buffer, dNTPs, reverse transcriptase, oligodts, dithiothreitol (DTT), and the RNA sample. The reaction mixture was incubated in the thermal cycler at a particular temperature of 42˚C for 60 minutes.

## 2.9 Polymerase Chain Reaction (PCR)

### 2.9.1 Designing of Primer

The selection of primers was based on information found in previously published scientific publications. Following that, primer BLAST was conducted on NCBI (National Centre for Biotechnology and Information) to confirm the specificity and accuracy of the chosen primer with the target indicated in Figures 2.10 and 2.11 before their utilization in PCR. The primers possessed an annealing temperature of 66°C, as determined through calculations.



**Figure 2.10: SOD2 Primer's NCBI BLAST Analysis.** The SOD2 primer's specificity was verified by doing a primer BLAST analysis in NCBI.



**Figure 2.11: TLR4 Primer's NCBI BLAST Analysis.** The TLR4 primer's specificity was verified by doing a primer BLAST analysis in NCBI

**Table 2.1: Characteristics of Primers.**

| GENE | Sequences' Direction | Sequence | Product Length | Temperature °C |
|------|---------------------|----------|----------------|----------------|
| ß-actin | Forward | CATCCCCCAAAGATTCTAC | 347 | 57°C |
| ß-actin | Reverse | CAAAGCCTTCATACATC | 347 | 57°C |
| SOD2 | Forward | CAGACCTGCCTTACGACTATGG | 113 | 62°C |
| SOD2 | Reverse | CTCGGTGGCGTTGAGATTGTT | 113 | 62.5°C |
| TLR4 | Forward | GTGGGTCAAGGACCAGAAAA | 506 | 61.1°C |
| TLR4 | Reverse | GAAACTGCCATGTCTGAGCA | 506 | 59.6°C |

*2.9.2 Gradient PCR*

A sample was created using gradient PCR to optimize the primer and estimate the annealing temperature. The gradient PCR profile is as stated below. The procedure begins with a 3-minute first denaturation stage at a temperature of 94°C. Subsequently, there are 35 cycles, with each cycle comprising a denaturation phase at 94°C lasting 30 seconds. Following the denaturation phase, the SOD2 and TLR4 are annealed for 30 seconds at 58 to 68°C in temperature range. Gradient temperatures were then maintained for 45 seconds in the next phase at 72°C, and 7 minutes in the final phase at 72°C. After PCR, bands were detected in the resultant product by gel electrophoresis.

**Table 2.2: Gradient temperatures.** The table displays the range of annealing temperatures used for gradient PCR.

| Gradient Temperature °C | | | | | |
|---|---|---|---|---|---|
| 58°C | 60°C | 62°C | 64°C | 66°C | 68°C |

*2.9.2.1 Reaction Mixture*

The PCR tube was filled with a total volume of 25 µl. This volume consisted of 12.5µl of PCR master mix (W1401-2, Wizbio Solutions, South Korea), 8.5 µl of Nuclease-free water, 1 µl of forward primer, 1 µl of reverse primer, and 2 µl of cDNA template.

**Table 2.3: List of PCR ingredients.** The table gives the components along with their quantities to make 25 ul PCR mix.

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

*2.9.3 Agarose gel Electrophoresis*

To confirm the occurrence of annealing at the correct temperatures, gel electrophoresis was conducted using a 2% agarose gel (39346, Sigma Aldrich, USA) and 10X TBE buffer (T1051, Solarbio, China). The positions of the bands were compared to the DNA ladder, which ranged from 100 to 1500 base pairs, to ascertain if annealing had taken place or not. The gels were subsequently examined using a Benchtop 2UV transilluminator (LM-20 | P/N 95044902, UVP Co., USA).

**Table 2.4: qPCR Master Mix Preparation.** The table shows the components of qPCR master mix preparation along with their quantities to make 20µl of PCR mix.

| | Component | Quantity (ul) |
|---|---|---|
| 1 | cDNA Template | 1.0 ul |
| 2 | Forward Primer | 1.0 ul |
| 3 | Reverse Primer | 1.0 ul |
| 4 | SYBR Green Master Mix | 4.0 ul |
| 5 | Nuclease Free Water | 13.0 ul |
| | Total Reaction Volume | 20 ul |

*2.9.4 RT-PCR*

The expression levels of SOD2 and TLR4 in brain tissues were measured using RT-PCR, also referred to as qPCR. The qPCR was conducted on a real-time PCR detection system (Biorad),

employing specific primers for SOD2 (sense 5′-CAGACCTGCCTTACGACTATGG-3′, anti-sense 5′-CTCGGTGGCGTTGAGATTGTT-3′) and TLR4 (sense 5'-GTGGGTCAAGGACC AGAAAA-3′, anti-sense 5′-GAAACTGCCATGTCTGAGCA-3′), following the cycling parameters outlined in Figures 2.10 and 2.11. Additionally, rat ß-actin was used as a control, and its qPCR was conducted with primers (sense 5′-CATCCCCAAAGATTCTAC-3′,anti-sense 5′-CAAAGCCTTCATACATC-3′), involving denaturation, annealing and  elongation cycles.

Additionally, rat ß-actin was used as a control, and its qPCR was conducted with primers (sense 5′-CATCCCCCAAAGATTCTAC-3′anti-sense5′-CAAAGCCTTCATACATC-3'), involving denaturation, annealing, and elongation cycles. The reaction mixture, composed of cDNA template, nuclease-free water, forward primer, reverse primer, and SYBR green master mix (WizPure™ qPCR Master Mix (SYBR)), totaled 20 µl. The PCR procedure comprised denaturation at 94°C for 30 seconds, followed by annealing at 57°C for 30 seconds, and elongation at 72°C for 30 seconds. This cycle was iterated 35 times. Amplification curves and agarose gel electrophoresis were employed to assess the quality of the PCR product. The resulting values were analyzed for gene expression using their ΔCt values, normalized to those obtained for ß- actin.

### 2.9.5 Cycling parameters for Real-time PCR

Figure 2.12 displays the RT-PCR cycling parameters. In this PCR cycling profile, 35 cycles are executed to achieve targeted DNA amplification. The process begins with a denaturation step at 94°C for 3 minutes. During denaturation, the double-stranded DNA template is heated, causing the separation of the two strands, and generating single-stranded DNA molecules. Following denaturation, the temperature is lowered to 66°C for a 30-second annealing phase. In this stage, the short DNA sequences known as primers can attach to complementary sequences on the single-stranded DNA template. It is a critical phase that determines the specificity of the PCR reaction, ensuring that the primers attach only to the desired target sequences.

During the elongation step in the polymerase chain reaction (PCR), set at 72°C for 45 seconds, the DNA polymerase enzyme plays a pivotal role in synthesizing a new DNA strand. This phase follows the denaturation step where the DNA template is unwound, exposing the single-stranded regions. Utilizing the primed template, the DNA polymerase reads the

sequence and facilitates the addition of complementary nucleotides, thereby extending the DNA strand. The choice of 72°C is critical as it ensures the enzyme's stability, especially when using heat-resistant polymerases like Taq polymerase, derived from thermophilic bacteria.

The elongation step completes the synthesis of the target DNA strands, essentially doubling the DNA content in the reaction. This meticulous control of temperature in PCR allows for the specific amplification of the desired DNA sequence. The completed elongation phase prepares the DNA template for subsequent denaturation, annealing, and further cycles, making PCR an indispensable tool in molecular biology for tasks such as gene analysis and DNA manipulation.
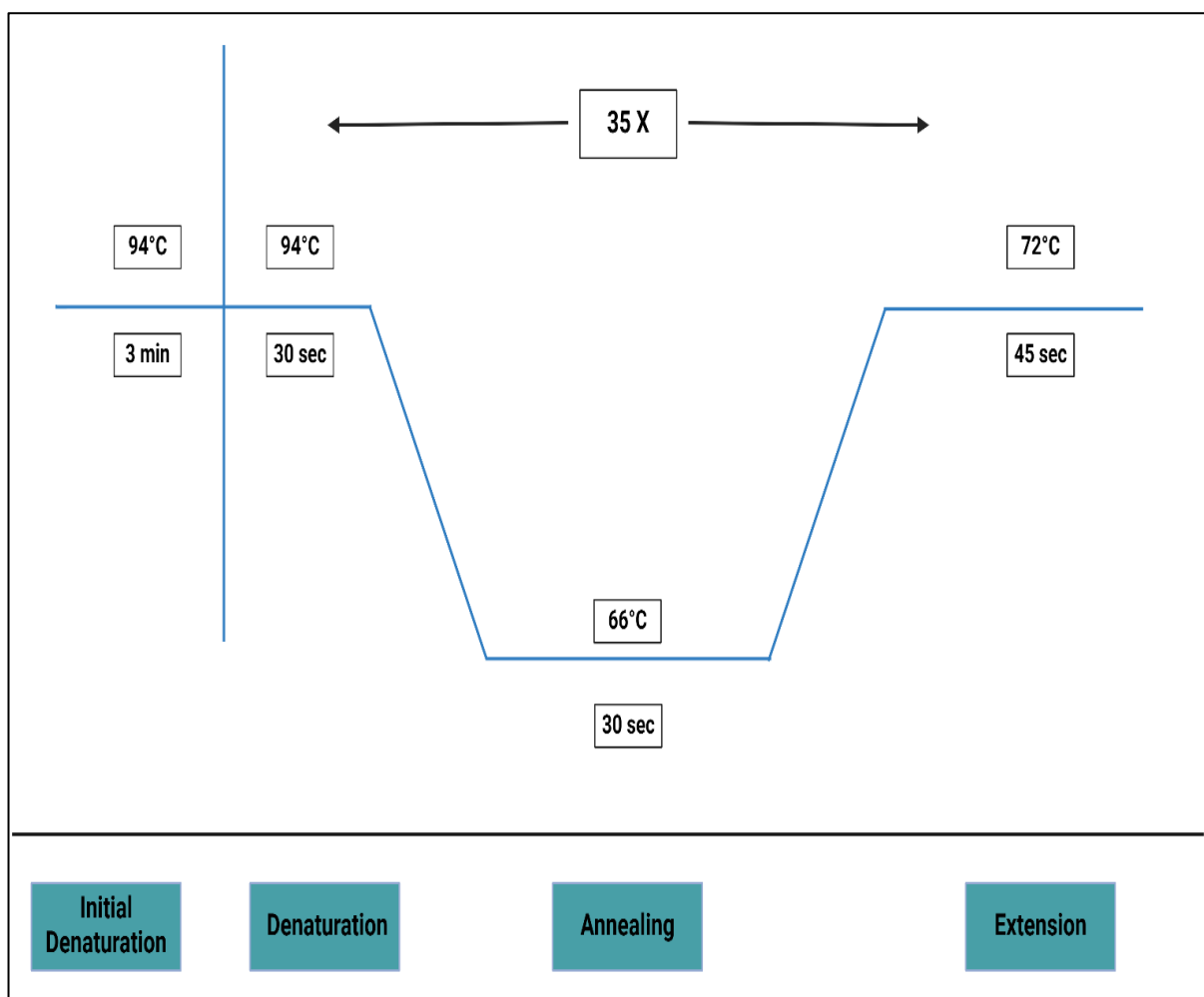


**Figure 2.12: Cycling parameters for qPCR**. The figure shows the thermal cycling profile for SOD2 and TLR4. PCR circumstances: 35 cycles of denaturation at 94°C (3 min), annealing at 66°C (30 s), and elongation at 72°C (45 s).

## 2.10 Statistical Analysis

Before conducting any statistical analysis, the normality of the distribution of all data sets was assessed. A statistical study compared the disparities among the control, MCAO, and the MCAO group treated with galantamine. To determine if there were any notable disparities between the groups, statistical analyses such as the T-test and one-way ANOVA were employed, after this Tukey's test was also performed. The graphs were created using Graph Pad Prism version 10.0, and statistical significance was assessed with a threshold of $P < 0.05$. The data and results were quantified using the standard error of the mean, commonly referred to as SEM.

# CHAPTER 3:    RESULTS

## 3.1. *in silico* Results

### 3.1.1  Structure of Protein and Ligand

In the realm of protein structural analysis, the Protein Data Bank (PDB) format serves as a critical repository, offering comprehensive information on the spatial arrangement and organizational intricacies of proteins such as SOD2 and TLR4. These structural blueprints lay the foundation for in-depth investigations into the functional aspects and intermolecular interactions of these proteins. Concurrently, the compound of interest, galantamine, is procured in the Structure Data File (SDF) format from PubChem, providing a detailed account of its chemical structure, molecular composition, and conformation.

Figure 3.1 visually encapsulates the molecular features of SOD2, TLR4, and galantamine, synthesizing their structural nuances. Figure 3.1 A shows the structure of galantamine. It has a chemical formula represented as $C_{17}H_{21}NO_3$. The hexagonal structure indicates the existence of a benzene ring. The combined ring includes benzofuran and benzazepine components. The nitrogen (N) atom is part of a tertiary amine group, with oxygen (O) present in the methoxy ($OCH_3$) and hydroxyl (OH) functional groups. Carbon (C) atoms form the basic structure of this compound. The structure includes an aliphatic chain and a carbon-carbon chain without aromatic rings. Galantamine is a chiral molecule with specific stereochemistry due to the presence of stereocenters. This structure reveals the complex arrangement of atoms in galantamine, contributing to its pharmacological properties as an acetylcholinesterase inhibitor.

Figure 3.1 B depicts the structure of SOD2. SOD2 enzymes is responsible  in the protection against oxidative stress due to their ability to facilitate the transformation of superoxide radicals into hydrogen peroxide and molecular oxygen. It is constituted of 222 amino acids and contains a metal cofactor, usually manganese (Mn), at its active site. The metal ion is essential for the catalytic activity of the enzyme, and it is coordinated by specific amino acid residues, such as histidine and aspartate. It is a homotetramer, meaning it is composed of four subunits. Each subunit contributes to the formation of the active site, where the metal cofactor is located.

Within the protein structure of SOD2, disulfide bonds could potentially form between cysteine residues, enhancing the protein's overall stability. Hydrogen bonds are instrumental in maintaining the enzyme's tertiary and quaternary structure. SOD2 plays its role by neutralizing superoxide radicals and preventing cellular damage. The presence of a metal cofactor and specific structural features empowers SOD2 to effectively catalyze the dismutation reaction.

Figure 3.1 C showcases the configuration of TLR4, which serves as a transmembrane protein playing a pivotal role in the innate immune system. TLR4's encompasses leucine-rich repeats (LRRs), which are responsible for the detection of pathogen-associated molecular patterns (PAMPs). TLR4 is particularly recognized for its ability to detect lipopolysaccharide (LPS), a constituent of the outer membrane in Gram-negative bacteria. Spanning cell membrane, TLR4 possesses a transmembrane domain, ensuring its anchoring within the membrane. Within the cell, TLR4 houses an intracellular region containing a Toll/interleukin-1 receptor (TIR) domain. This domain is actively engaged in signaling processes and interacts with downstream signaling molecules. TLR4 establishes connections with downstream adaptor molecules, such as MyD88 (Myeloid differentiation primary response gene 88) and TRIF (TIR domain-containing adaptor protein inducing interferon-beta), through TIR-TIR interactions.

Myeloid differentiation factor 2, or MD-2, is a co-receptor that TLR4 forms a complex with. This complex is essential for the recognition of lipopolysaccharides (LPS). A soluble protein called MD-2 attaches itself to the lipid A component of LPS to help it interact with TLR4. The recognition of LPS is dependent on the complex that TLR4 forms with the co-receptor MD-2. A soluble protein called MD-2 attaches itself to the lipid A component of LPS to help it interact with TLR4.

TLR4 can form homodimers, and dimerization is important for its activation. The interaction between two TLR4 molecules enhances the binding of LPS and initiates downstream signaling events. Disulfide bonds may contribute to the stabilization of the overall structure of TLR4. These bonds form between cysteine residues. Understanding the structural details of TLR4 and its interactions with ligands and downstream signaling molecules is crucial for unraveling the mechanisms by which the immune system recognizes and responds to pathogens.
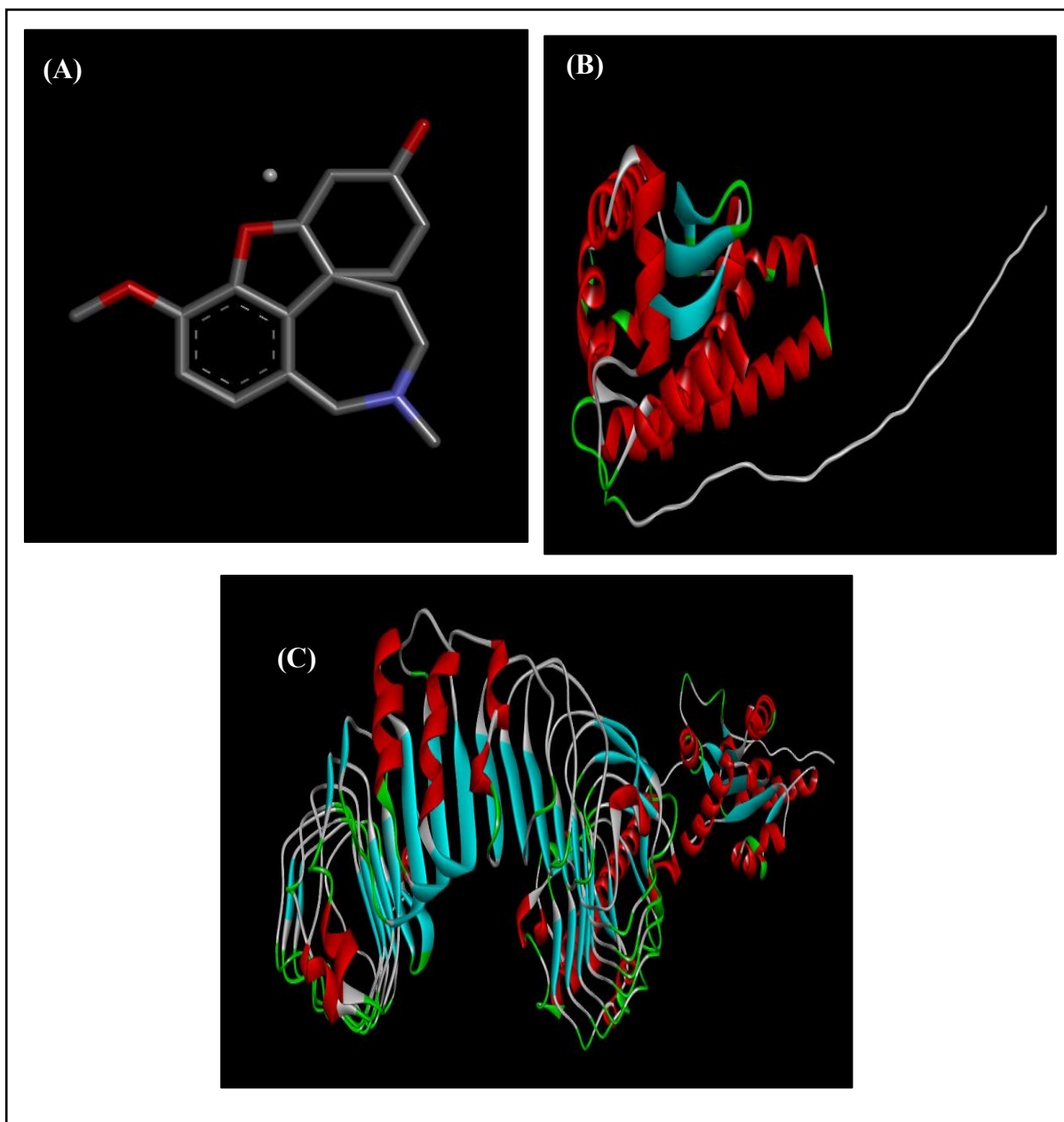
**Figure 3.1: 3D Structures of Galantamine, SOD2 and TLR4.**(A) The protein structures of SOD2 and (B) TLR4 in PDB format, obtained from the Protein Data Bank, (C) The structure of galantamine in SDF format, retrieved from PubChem, provide comprehensive information on the molecular composition and arrangement of these molecules.

*3.1.2 Molecular Docking Analysis*

The structural complex of SOD2 and TLR4 (Target) with galantamine (Ligand) was analyzed using a ligand-target docking approach. The findings of this examination are presented in Figure 3.2. The findings of this docking experiment provide crucial structural and energetic information about the likely affinities and binding mechanism of galantamine with

SOD2 and TLR4. Investigating the molecular-level interactions between galantamine, SOD2, and TLR4 will provide valuable insights into galantamine's involvement in neuroprotection pathways.

In this research endeavor, the structural interaction between two key biomolecules, SOD2 and TLR4, and the pharmaceutical compound galantamine was systematically examined through a ligand-target docking approach. The resulting insights from this investigation are presented in Figure 3.2, encapsulating critical details about the molecular-level interactions and potential binding mechanisms involved in this complex.
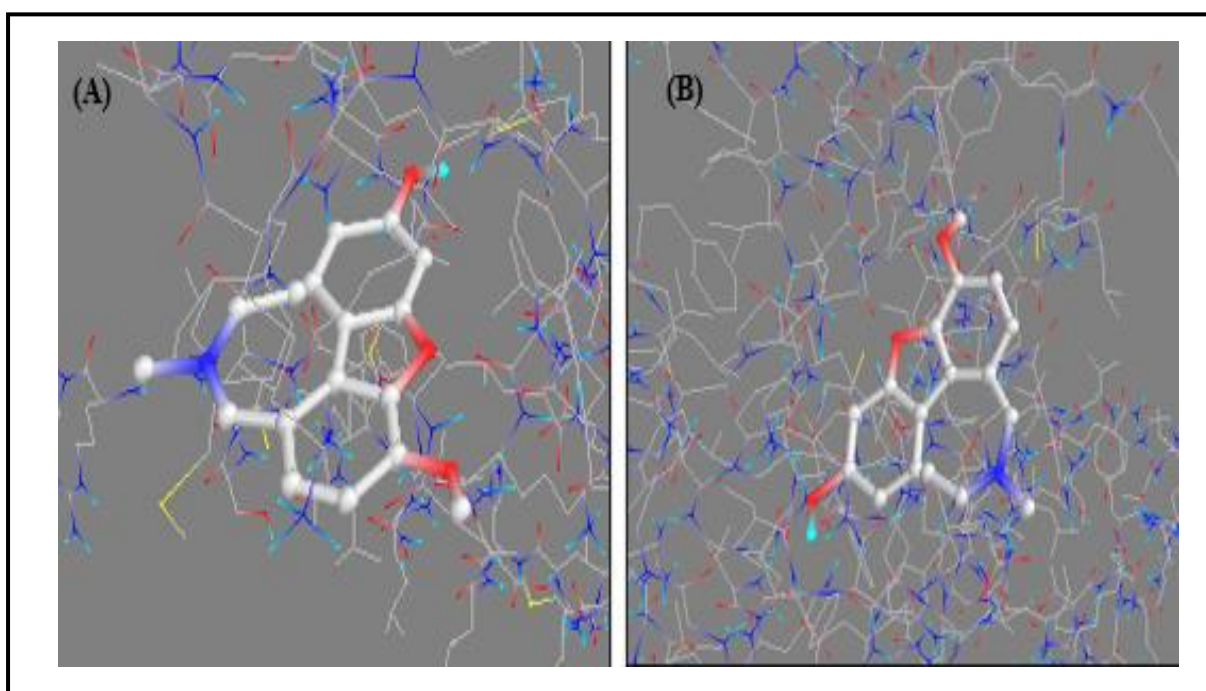


**Figure 3.2: Visuals of Docking Interaction of Galantamine and SOD2 and TLR4.** This is a computational representation of the interactions between galantamine, SOD2, and TLR4. The representation was derived using a docking analysis performed using PyRx.

### 3.1.3. Binding Affinity

In the context of this study, the interaction between the proteins SOD2 and TLR4 with the pharmaceutical compound galantamine was robustly characterized through a quantitative analysis of binding energies, as depicted in Figure 3.3. The observed low binding energy values signify an effective binding between galantamine and both SOD2 and TLR4, pointing towards a stable and favorable interaction. The graphical representation in Figure 3.3 serves as a visual elucidation of the precise binding affinities exhibited by galantamine for these distinct target proteins. The vertical axis of the graph quantifies these binding affinities, often expressed in

energy units such as kcal/mol. This numerical representation provides a quantitative measure of the strength of the interactions between galantamine and SOD2 as well as TLR4.

In Figure 3.3 A, the binding affinities resulting from molecular docking simulations between galantamine and SOD2 are presented. The obtained binding affinity scores are as follows: -5.7, -5.8, -5.8, -5.8, -5.8, -5.9, -6.2, and -6.2 kcal/mol. in the context of molecular docking studies, the binding affinity scores serve as numerical indicators of the strength of the interaction between a ligand (in this case, galantamine) and a target protein (SOD2). Importantly, lower binding affinity scores are conventionally interpreted as indicating stronger binding interactions The negative sign associated with the binding affinity scores is significant. In molecular docking, a negative binding affinity indicates that the energy associated with the interaction is favorable. This means that the ligand, galantamine in this case, is energetically stable in the binding site of SOD2.

In the provided scores, the range of -5.7 to -6.2 kcal/mol suggests strong binding interactions between galantamine and SOD2. The more negative the value, the more energetically stable the ligand-receptor complex is predicted to be. Therefore, the -6.2 kcal/mol scores are indicative of a particularly stable and favorable binding between galantamine and SOD2. These results suggest that galantamine forms stable complexes with SOD2 in the predicted binding poses. The lower binding affinity scores, especially those approaching -6.2 kcal/mol, signify a high likelihood of a stable complex formation, highlighting the potential strength of the interaction between galantamine and SOD2.

The binding affinity score -6.2, represents the most energetically favorable binding modes, suggesting strong interactions between galantamine and SOD2. The affinity scores -5.7 to -5.9 indicates favorable binding, these scores are relatively less favorable than the -6.2 poses. They may represent alternative binding modes or slightly less stable conformations. In summary, the docking results suggest that galantamine has favorable binding interactions with SOD2, with the poses having scores around -6.2 being the most energetically favorable.

Figure 3.3 B gives the binding affinities -4.1,-4.2,-4.2,-4.2, -4.3,-4.3,-4.4,-4.5 and-4.7 kcal/mol, representing the predicted strength of the interaction between TLR4 and galantamine for different docking poses. A more negative binding affinity generally indicates a stronger binding interaction. The most negative binding affinity, -4.7 kcal/mol, suggests an exceptionally strong interaction in that specific docking pose. This indicates a highly stable

and energetically favorable binding between galantamine and TLR4 in this particular conformation. Affinities in this range are also favorable, indicating strong interactions, although slightly less intense than the -4.7 kcal/mol pose. Despite being slightly less negative, these affinities still suggest robust and stable binding interactions between galantamine and TLR4 in these respective docking poses.

In Figure 3.4, a compelling visual representation is provided, showcasing the remarkable capability of galantamine to bind intricately to the active sites of both SOD2 and TLR4. This graphical depiction sheds light on the specific molecular interactions occurring at the binding sites, unraveling the nuanced mechanisms through which galantamine engages with these crucial proteins. For SOD2, the active sites become a focal point of interaction, with key protein residues contributing to diverse binding forces. Notably, residues tryptophan (TRP) 102, valine (VAL) 184, glycine (GLY) 181, and cysteine (CYS) 164 collaborate to establish Van Der Waals Interactions, fostering a proximity between galantamine and the protein.

Additionally, aspartic acid (ASP) 168 and leucine (LEU) 170 engage in Conventional Hydrogen Bonding, while histidine (HIS) 95, isoleucine (ILE) 96, proline (PRO) 169, and tyrosine (TYR) 200 form Carbon Hydrogen Bonds. Further complexity is introduced by ASP 183 facilitating Pi Anion interactions, PHE 101 involved in Pi Sigma interactions, and ILE 182 and TRP 147 contributing to Alkyl and Pi Alkyl interactions, respectively. This intricate network of interactions illustrates the diverse forces orchestrating the binding of galantamine within the active sites of SOD2.

Simultaneously, for TLR4, the active site interactions are characterized by HIS 429, LYS 475, and phenylalanine (PHE) 406 engaging in Van Der Waals Interactions, ensuring a close association with galantamine. The glutamine (GLN) 428 residue takes part in conventional hydrogen bonding, further stabilizing the ligand-protein complex. These specific residues delineate the molecular landscape of galantamine binding to TLR4, emphasizing the specificity and diversity of forces involved. In conclusion, Figure 3.4 provides a detailed snapshot of the molecular dialogue between galantamine and the active sites of SOD2 and TLR4, unraveling a spectrum of interactions ranging from Van Der Waals forces to hydrogen bonding and aromatic interactions. This visual elucidation is pivotal in understanding the molecular intricacies of galantamine's binding, providing a foundation for interpreting its potential therapeutic effect on neuroprotective pathways.
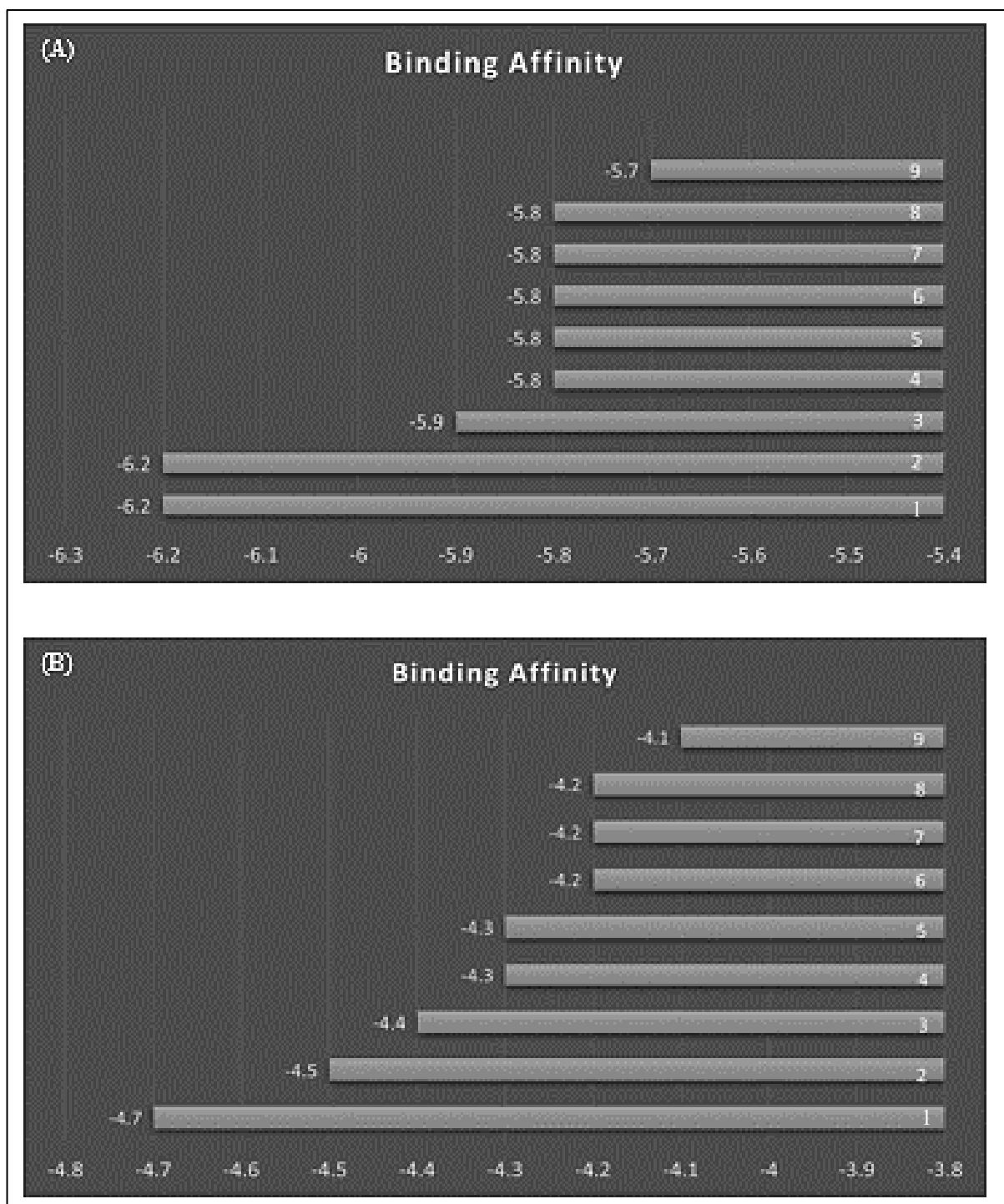
**Figure 3.3: In silico analysis showing the interaction of galantamine with SOD2 and TLR4.**
The binding energies of SOD2 and TLR4 range between – 5.7 to – 6.2 kcal/mol and between -4.1 to -4.7 kcal/mol respectively. The graph illustrates the binding efficacy of the ligand, galantamine, to its target proteins, SOD2 and TLR4. Binding affinities are quantified by energy values, which provide insight into the strength of interaction between two molecules. Every data point on the graph corresponds to a specific computational docking or binding simulation experiment. Decreased values on the y-axis of the graph show that galantamine has a higher affinity for binding to the target protein, suggesting a potentially favorable interaction. Conversely, higher levels may indicate a less strong connection
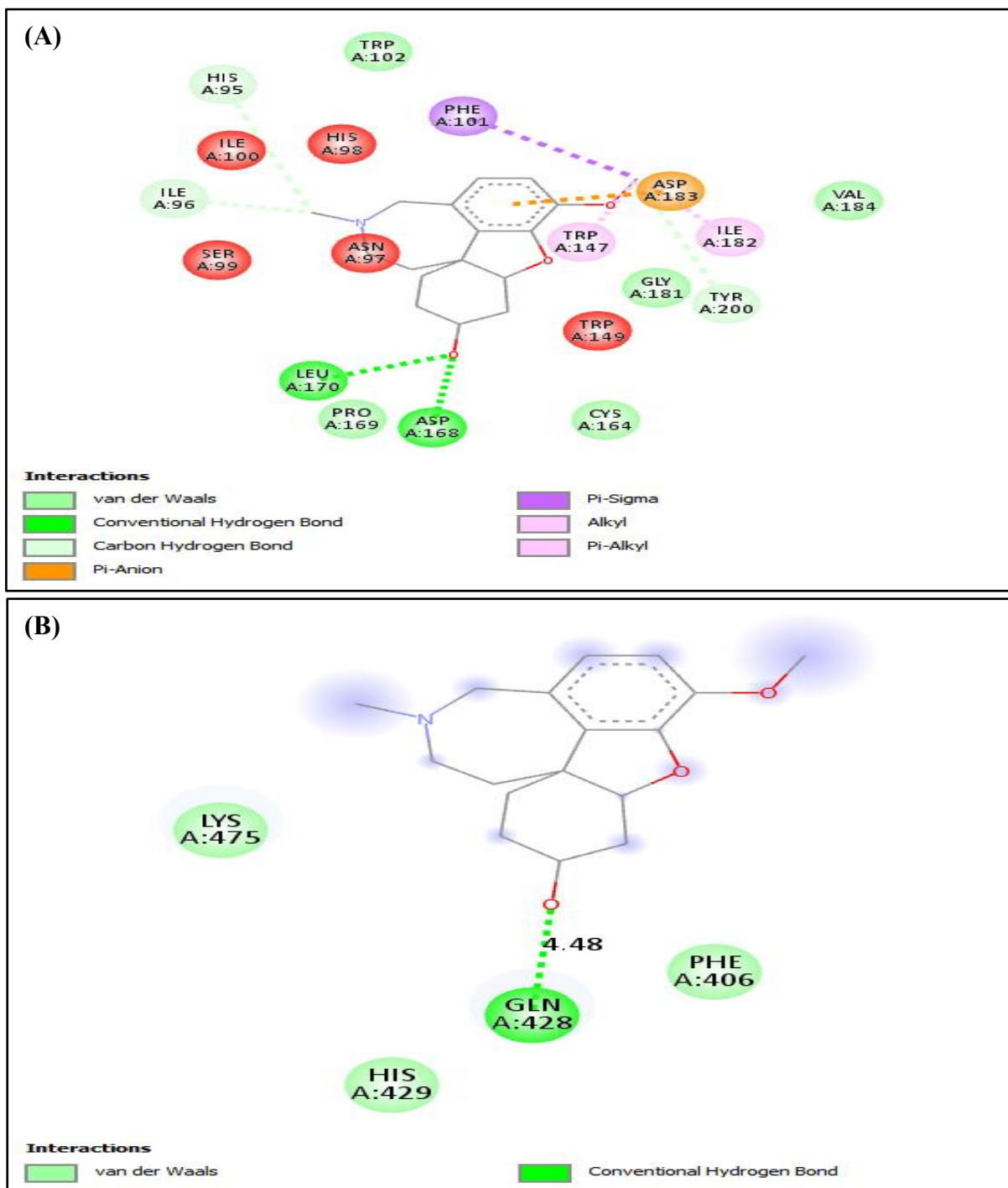
*Figure 3.4:* **The possible locations where SOD2 and TLR4 can bind.** (A) The amino acid residues TRP 102, VAL 184, GLY 181, and CYS 164 of the SOD2 protein play a role in the creation of Van Der Waals Interactions. ASP 168 and LEU 170 participate in Conventional Hydrogen Bonding. The amino acids HIS 95, ILE 96, PRO 169, and TYR 200 engage in Carbon Hydrogen Bonding. ASP 183 is accountable for the generation of Pi Anions, PHE 101 is responsible for Pi Sigma production, while ILE 182 and TRP 147 contribute to the formation of Alkyl and Pi Alkyl, respectively.(B) For TLR4, the amino acid residues HIS 429, LYS 475, and PHE 406 engage in Van Der Waals interactions, while GLN 428 forms a conventional hydrogen bond.

## 3.2. Behavioral Assessment Results

### 3.2.1 Grip Strength Test

In Figure 3.5, the graph provides a detailed illustration of muscle grip strength patterns, presenting the duration of maximal upside-down clinging against the number of repeats at specified time intervals. A notable distinction emerges when comparing the control group to the MCAO group treated with galantamine. Rats in the control group showcase a remarkable ability to cling upside-down on the grid for an extended duration, emphasizing their muscle grip strength. Conversely, the MCAO group exhibits weakened grip strength, evidenced by a shorter duration of clinging and a tendency to fall more quickly, it indicates a potential influence of the neurological condition on muscular function. This suggests a diminished capacity for sustained muscle grip compared to both the control group and the MCAO group treated with galantamine. This highlights the significance of grip strength as an indicative measure in neurological studies and suggests that galantamine treatment may not fully restore grip strength in the context of MCAO-induced neurological challenges, but it does show significant positive results.
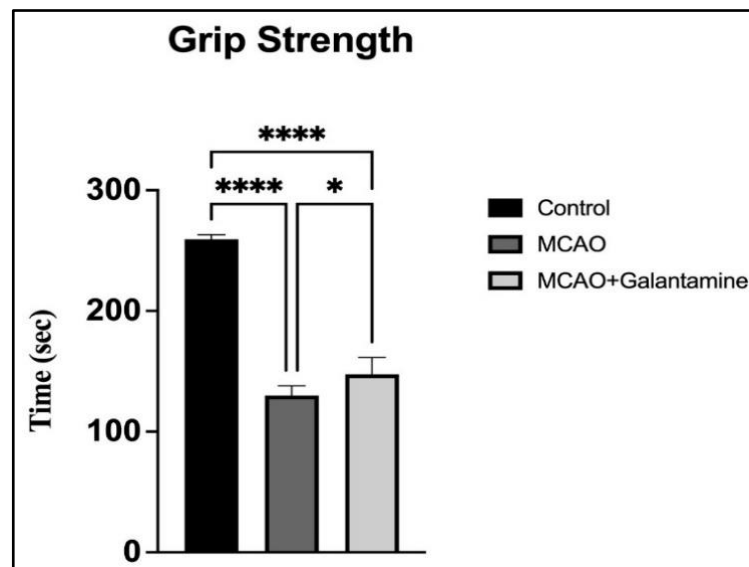


**Figure 3.5: Grip Strength Test Results.** The effect of galantamine on rats that were given MCAO is depicted in this graph, which focuses on how long the rats were able to hold onto an inverted mesh grid. Notable differences between the MCAO group and the healthy group, as well as between the MCAO group and the group treated with galantamine, should be emphasized. Furthermore, a slight degree of significance is noted in the relationship between the MCAO group and the MCAO group that is administered galantamine. One-way ANOVA was used in the statistical analysis, and Tukey's multiple comparison tests were the next step. At a significance level of $p < 0.05$, error bars were used to represent the standard error of the mean (SEM).

*3.2.2 Open Field Test*

According to Figure 3.6 of the open field test, distinctive patterns emerge, revealing significant differences among the experimental groups. The control group displays a notably higher average number of entries into the central region compared to the MCAO group treated with galantamine. This observation implies distinct exploratory behavior in the control rats, as increased entries into the central region often correlate with heightened exploration and reduced anxiety-like behavior in rodents. On the contrary, the MCAO group exhibits the least significant average movement into the central region, suggesting a potentially diminished exploratory trait following cerebral artery occlusion. The reduced willingness of rats in the MCAO group to explore the central area may indicate altered behavior linked to the neurological impact of the occlusion.

Analyzing peripheral entries further explains the behavior. The control group displays fewer average entries in the periphery, suggesting a more focused and centralized exploratory pattern. Conversely, the MCAO group records the highest average number of peripheral entries, reflecting heightened peripheral exploration. Notably, the MCAO group treated with galantamine falls between these extremes. This group's average peripheral entry count falls between that of the untreated MCAO group and the control group. The rats from the MCAO group that received galantamine treatment appear to have moderated exploratory behavior based on their intermediate positioning. The galantamine treatment appears to exert a partial influence on peripheral exploration, aligning the behavior of this group between the more focused exploration of the control group and heightened peripheral exploration of untreated MCAO group.

These findings underscore the impact of galantamine treatment on exploratory behavior in the aftermath of MCAO. The test outcomes suggest that, compared to the untreated MCAO group, rats treated with galantamine exhibit a behavioral profile with intermediate characteristics, hinting at a potential modulation of exploratory patterns. The open field test thus provides an understanding of the effects of galantamine on overall activity and exploratory behavior, contributing valuable insights into the galantamine's role in the post-stroke recovery process.
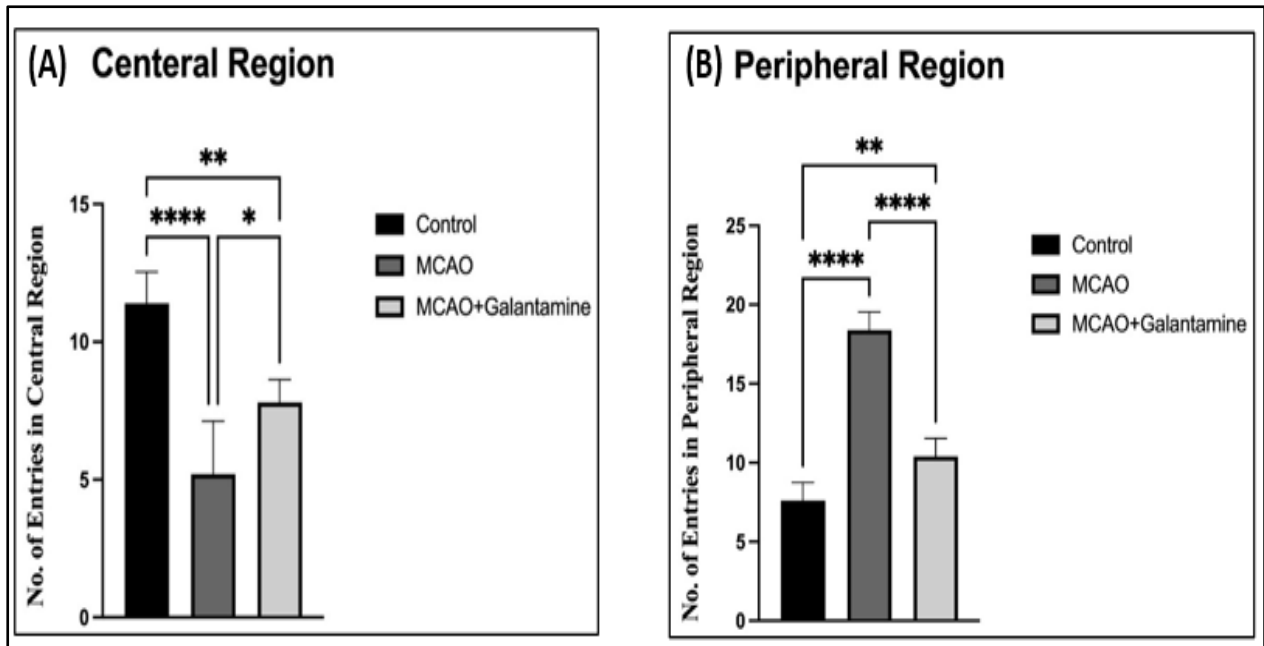
**Figure 3.6: Open Field Test Results.** (A) The number of entries within the Central Region. The graph unequivocally shows that there are substantially more entries in the healthy group than in the MCAO group that received galantamine treatment and then in the MCAO group. The number of entries in the central area reflects exploratory character of these entries. (B) The number of entries in the outer area. The graph shows that, in comparison to the other groups, the MCAO group had a significantly higher number of entries in the periphery region. In the periphery, rats usually stay still because their motor abilities deteriorate, their anxiety levels rise, and their exploratory tendencies disappear. One-way ANOVA was employed for statistical analysis and succeeded by carrying out Tukey's multiple comparison tests. The error bars demonstrate the standard error of the mean (SEM) at a significance level of $p < 0.05$.

*3.2.3 Y-Maze Test*

The control group exhibits a notably higher percentage of alterations than the other two groups, as shown in Figure 3.7-A. This finding implies that the control rats exhibit a stronger propensity for spontaneous alternation, a sign of a robust spatial working memory, due to their intact cognitive function. As opposed to the untreated MCAO group, the galantamine-treated MCAO group exhibits a statistically significant rise in the percentage of alterations. This suggests that the rats' ability to switch between the arms of the maze is improved by the galantamine treatment, which also has a positive effect on spatial working memory.

According to Figure 3.7-B, the control group rats exhibit improved memory performance by correctly identifying the familiar arm, while the MCAO group treated with galantamine shows a less favorable outcome. The MCAO group without treatment displays the

least favorable results, indicating a notable impairment in cognitive memory. This contrast underscores the potential cognitive benefits of galantamine treatment for post-ischemic stroke.

Figure 3.7-C provides additional insights into the rats' exploratory behavior, specifically their inclination to enter the novel arm. This finding implies that the control rats exhibit a stronger propensity for new arm exploration, due to their intact cognitive function and strong exploration trait. As opposed to the untreated MCAO group, the galantamine-treated MCAO group exhibits a statistically significant rise in the exploration of new arm. This suggests that the rats' ability to explore new arms is improved by the galantamine treatment and it has enhanced cognitive functions evident by the behavioral traits.
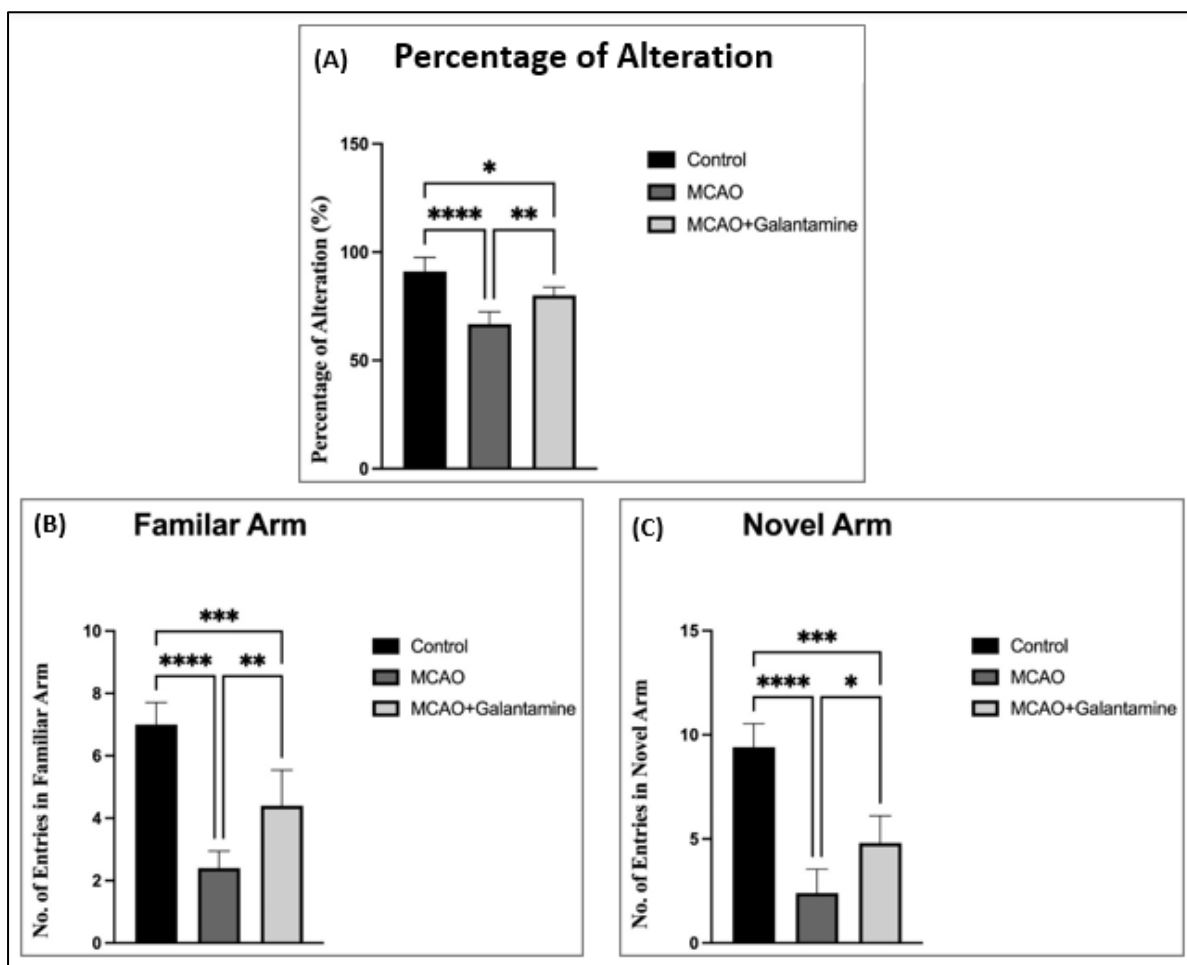


**Figure 3.7: Y-Maze Test Results.** A) The MCAO group that received galantamine treatment showed a rise in the percentage of alteration. B) When comparing the control and galantamine-treated MCAO groups with the MCAO group, the number of entries in the familiar arm is significantly higher. C) Rats' ability to explore was also shown to have improved in the galantamine-treated MCAO group, but when compared to the other two groups, the MCAO group showed no discernible change in response to the novel arm. Tukey's multiple comparison tests were performed after a one-way ANOVA as part of the statistical analysis. The error bars are used to represent the standard error of the mean (SEM) when a significance threshold of $p < 0.05$ is applied.

*3.2.4 Novel Object Recognition Test*

In Figure 3.8-A, the data presents the duration of time that rats from 3 groups spent interacting with an old object in the novel object recognition test. Remarkably, MCAO group demonstrated the longest time spent with the old object, indicating reduced cognitive abilities and diminished exploration tendencies. This prolonged interaction with the familiar object may suggest a failure to recognize the object as familiar, possibly indicative of cognitive impairment in the MCAO rats.

In contrast, both the control group and the MCAO group treated with galantamine allocated a certain amount of time to the old object. This behavior implies that these groups, particularly the galantamine-treated MCAO group, retained intact or potentially improved cognitive and exploratory capabilities. The willingness of these groups to spend time on the old object suggests an ability to distinguish between familiar and novel objects, demonstrating normal or enhanced cognitive function. These observations in the novel object recognition test highlight the potential cognitive benefits of galantamine treatment in the context of neurological challenges induced by MCAO, emphasizing its role in preserving or improving cognitive abilities and exploratory tendencies in rats.

Figure 3.8-B digs into the exploration times for new objects. The control group exhibits the longest exploration time, indicating strong recognition memory and curiosity in exploring both familiar and novel items. The MCAO group treated with galantamine follows, spending a significantly greater amount of time examining the novel item compared to the MCAO rats. This distinction suggests that galantamine treatment may have a positive impact on the rats' ability to recognize and show interest in new stimuli, potentially mitigating cognitive deficits associated with CNS disorders. The observation that the MCAO rats spend an increased amount of time in the periphery or moving around the old object in the novel object recognition task serves as a crucial indicator of altered exploratory patterns in the untreated MCAO group. The tendency to focus more on the periphery and less on novel stimuli aligns with the notion of reduced cognitive abilities in the untreated MCAO rats, emphasizing the importance of galantamine treatment in reducing these cognitive deficits. In summary, the novel object recognition task, illustrated in Figures 3.8-A and 3.8-B, becomes a key tool in unraveling the cognitive effects of galantamine treatment in the context of CNS disorders. The differential exploration times for old and new objects not only provide insights into recognition memory

but also shed light on the potential therapeutic benefits of galantamine.
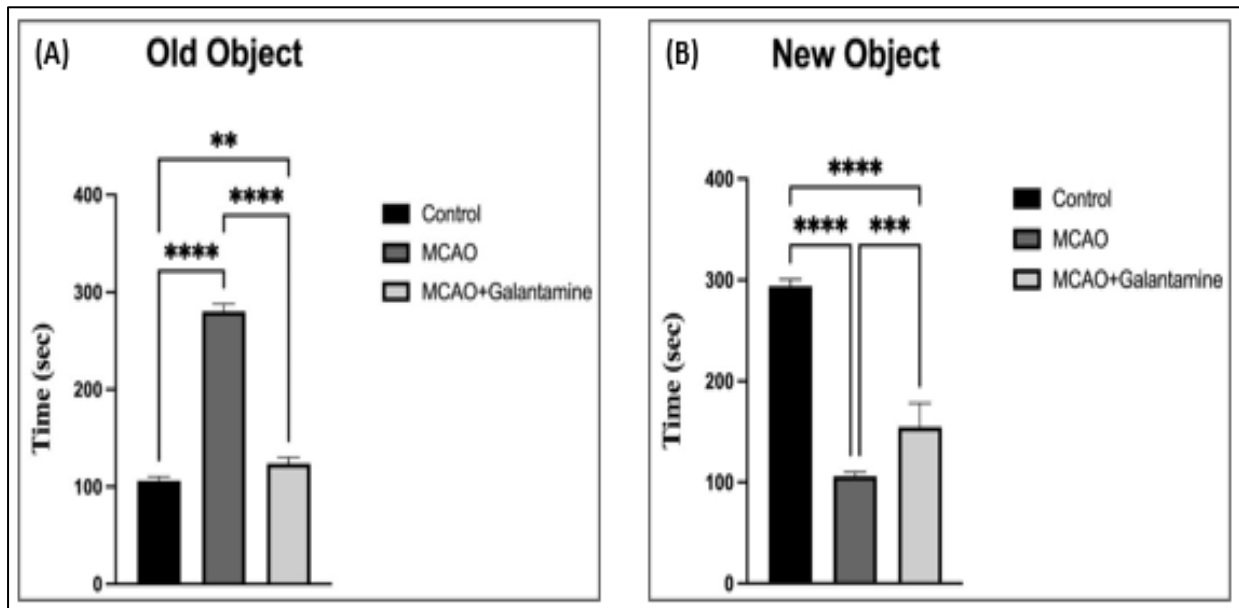


**Figure 3.8: Novel Object Recognition Test Results.** A) When compared to both the control group and the MCAO group that received galantamine treatment, the MCAO group showed a statistically significant preference for spending time with an antique object. This suggests that familiar objects were more appealing to the MCAO group. B) The control group allocated the highest amount of time to the newly introduced object due to their inclination toward exploring it. The MCAO group treated with galantamine followed suit, and lastly, the MCAO group. The statistical analysis employed for the study involved the utilization of both one-way ANOVA and Tukey's multiple comparison test. The error bars depicted the standard error of the mean (SEM) with a level of significance set at $p < 0.01$.

*3.2.5: Social Interaction Test*

In Figures 3.9-A and B, the results of the test highlight distinct sociability patterns within the experimental groups. The control group stands out with the highest level of sociability, spending more time actively engaging with other rats. In contrast, the MCAO group demonstrates a preference for anti-social behavior and a decline in exploratory tendencies. This inclination towards reduced social interaction in the untreated MCAO group suggests potential deficits in sociability and diminished interest in social engagement following MCAO. Importantly, the MCAO group treated with galantamine presents a more favorable outcome compared to the untreated MCAO group. The rats receiving galantamine exhibit improved sociability spending more time interacting with the rat. This suggests a potential ameliorative

effect of galantamine on sociability deficits induced by MCAO.

In Figures 3.9-C and D, the control group exhibits comparable levels of interest in both the rats they are familiar with and the stranger rat, indicating intact social novelty preferences. This suggests that the control rats can distinguish between familiar and novel social stimuli, showcasing normal social recognition and exploratory behavior. In contrast, the MCAO group displays reduced time spent with the familiar group and minimal social interaction with the stranger rat. This observation implies impairments in social novelty recognition, indicating a diminished ability of the MCAO rats to differentiate between familiar and novel social stimuli. The reduced interest in both familiar and unfamiliar rats suggest a general decline in social interaction and recognition abilities after MCAO.

Interestingly, the MCAO group treated with galantamine shows improvement in spending time with the familiar rat, suggesting a partial restoration of social interaction with familiar individuals. However, there is limited progress in terms of exploring and socializing with the stranger rat. This suggests that while galantamine may have a positive impact on familiar social interactions, its effectiveness in enhancing social novelty recognition or exploration of unfamiliar social stimuli may be limited in the context of MCAO-induced challenges.

In summary, in the Social Interaction Test (Figures 3.9-A, B, C, and D), distinct sociability patterns are evident. The control group displays the highest sociability, contrasting with the untreated MCAO group, which exhibits anti-social behavior. Galantamine treatment in the MCAO group appears to enhance sociability, suggesting potential therapeutic benefits. When examining social novelty recognition, the control group effectively distinguishes between familiar and stranger rats, while the MCAO group shows impaired recognition. Notably, galantamine treatment partially restores interaction with familiar rats but exhibits limited effectiveness with strangers. These findings underscore the potential of galantamine to address sociability deficits post-cerebral artery occlusion, offering valuable insights into its therapeutic role in the context of the social interaction test.
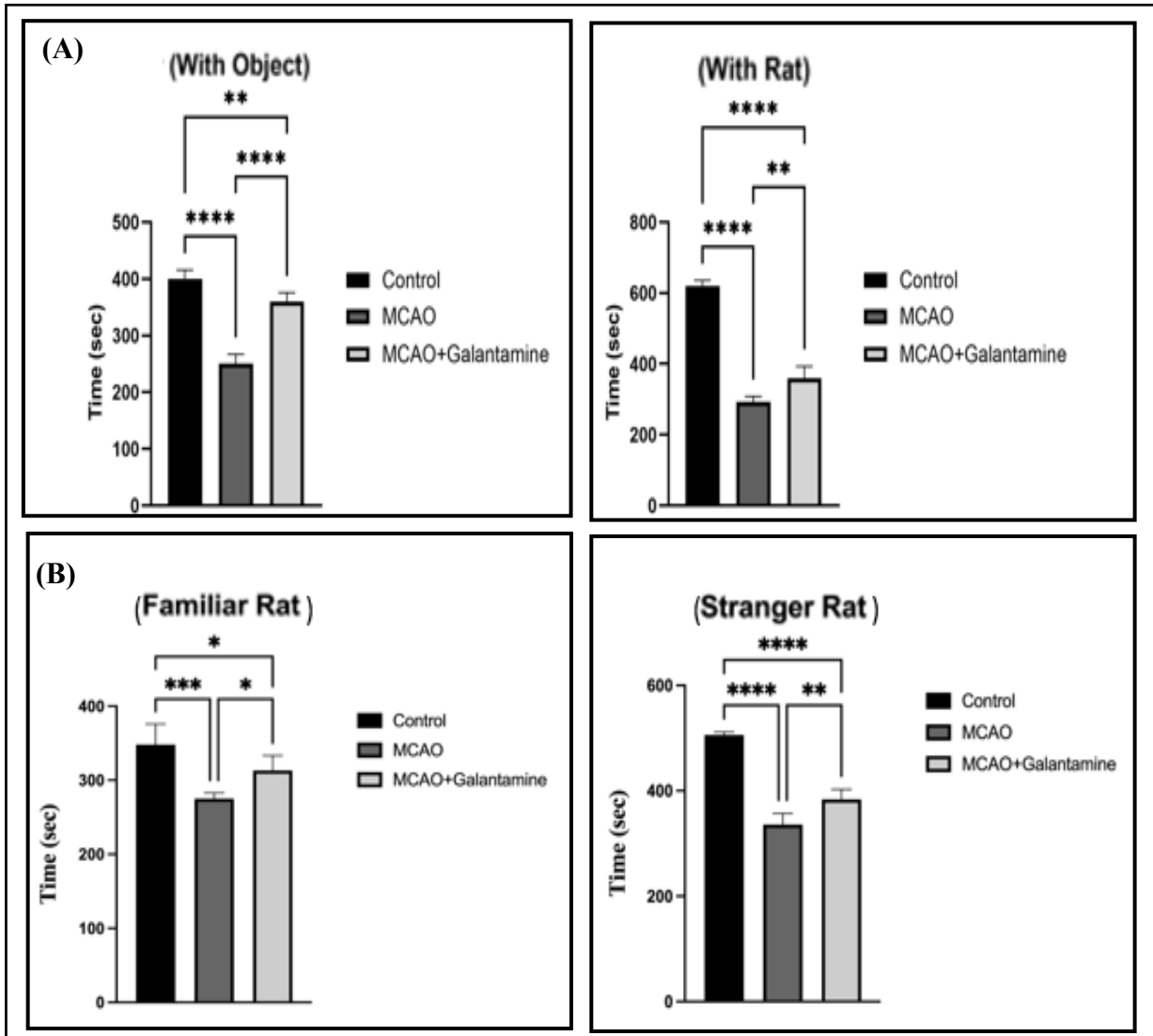
**Figure 3.9: Social Novelty and Sociability Results.** A) Significant results are shown by the control group in socializing with the rat rather than the object, the MCAO group treated with galantamine spent comparatively more time with rat as compared to the object but less than the healthy group, whereas the MCAO group spent time with object or else being antisocial. B) The control group interacted with the new rat the most, followed by the MCAO group that received galantamine treatment, and finally the MCAO group that interacted with the new rat less but spent a lot of time with the old rat. Tukey's multiple comparison test was employed after one-way ANOVA in the statistical analysis. SEM error bars are shown (*p < 0.01).

### 3.3 Histopathological Results

Ischemic stroke poses a substantial risk of impairment, particularly affecting the motor cortex, which can lead to compromised motor coordination, sensory function, and cognitive abilities in both the limbs and face. The analysis aimed to shed light on the histological changes within the cerebral hemisphere, specifically focusing on the right hemisphere. The assessment involved the examination of coronal histological slides under light microscopy, allowing for a

detailed examination of the structural alterations resulting from the ischemic insult.

*3.3.1 Effects of MCAO And Galantamine on Histology*

*3.3.1.1 Cortex*

The impact of galantamine on the cortex of rats subjected to MCAO was assessed through histological staining with H&E and subsequent evaluation. The histological findings are visually represented in Figure 3.10, focusing on the cortex section stained with H&E. The detailed observations reveal distinct morphological characteristics under different experimental conditions. In Figure 3.10 A, the control group showcases pyramidal cells with clearly visible nuclei, indicating the presence of life and control neuronal cells. This serves as a baseline representation of normal cortical histology in the absence of experimental manipulations.

Contrastingly, Figure 3.10 B illustrates the cortex of the MCAO group, where the histological staining reveals deformed and disfigured neuronal cells. The altered morphology is indicative of the adverse effects of the ischemic insult, resulting in structural damage to the cortical neurons. In Figure 3.10 C, the MCAO group treated with galantamine presents a contrasting picture. Galantamine treatment appears to confer neuroprotection, as evidenced by the presence of pyramidal neuronal cells with distinct nuclei, akin to the control group. The distinct cellular architecture indicates a potential mitigating effect of galantamine on the histopathological consequences of MCAO.

This neuroprotective outcome is highlighted by the yellow arrows, which signify the presence of control neural cells. Conversely, the red arrows point to deceased neuronal cells, emphasizing the contrast between the treated and untreated MCAO groups. This histological analysis offers a visual representation of the protective effects of galantamine on cortical neurons in the context of ischemic insult. The distinct improvement in cellular morphology, as indicated by the preservation of clear nuclei, suggests a potential therapeutic role for galantamine in mitigating histopathological consequences of ischemic events.
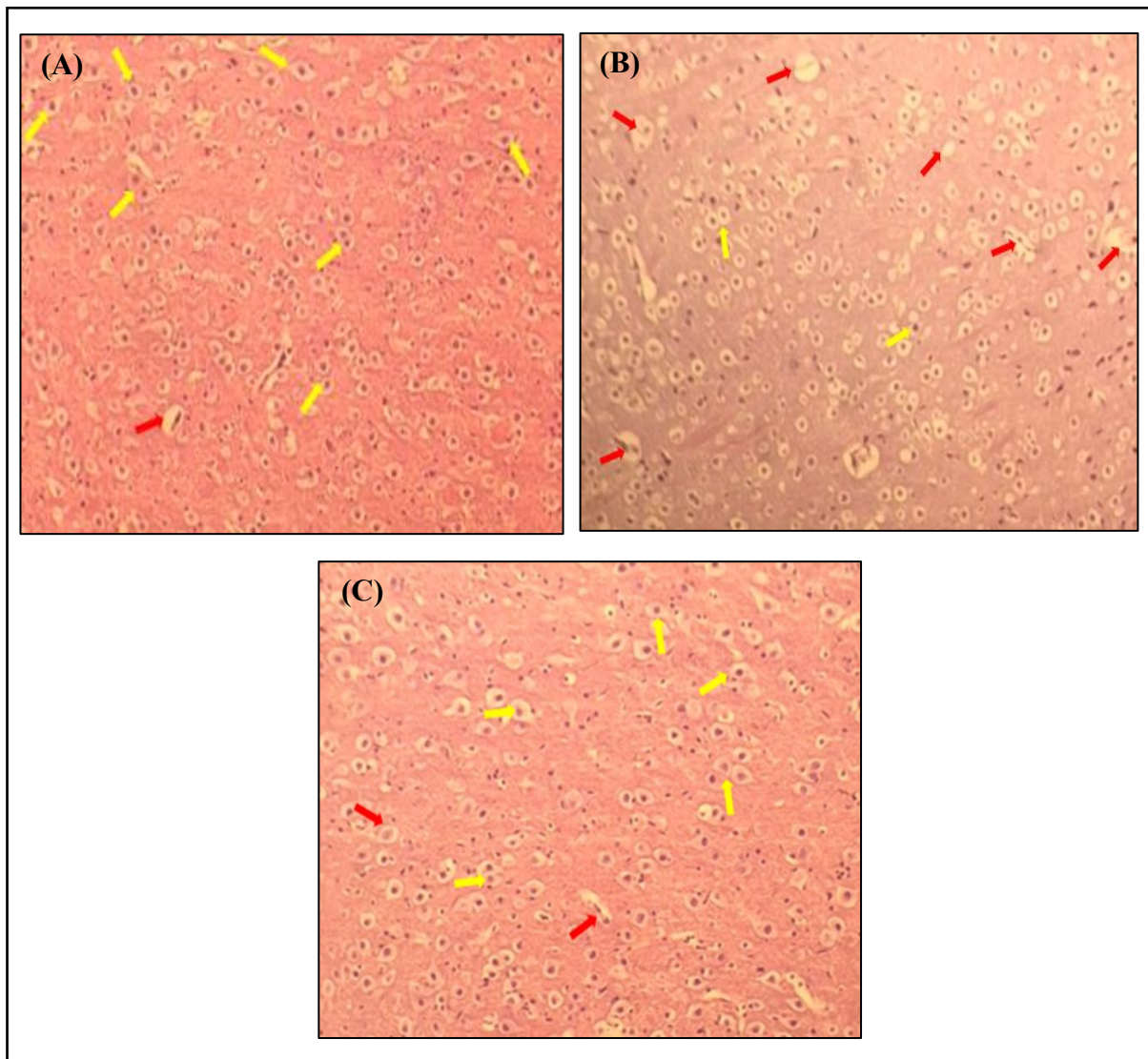
**Figure 3.10: The section of cortex stained with H and E staining**. A) The control rats exhibited pyramidal cells with clearly visible nuclei, indicating the existence of live neuronal cells. B) MCAO group revealed deformed and disfigured neuronal cells. C) MCAO group treated with galantamine The MCAO group that received galantamine treatment exhibited neuroprotection, as evidenced by the presence of pyramidal neuronal cells with distinct nuclei, indicating the presence of live neuronal cells. The yellow arrows represent healthy neural cells, while the red arrows represent deceased neuronal cells.

*3.3.2 Morphometric Results*

*3.3.2.1. H&E Neuronal Cell Count in the Cortex*

Through the utilization of Image J software, cell counting was performed on digital photomicrographs, and the outcomes are depicted in Figure 3.11. When compared to the control group and the MCAO group that received galantamine treatment, the MCAO group's

cortex showed a reduced number of neurons. This quantitative analysis underscores the detrimental impact of MCAO on neuronal populations within the cortex. Moreover, it highlights a potential mitigating effect of galantamine treatment, as evidenced by a comparatively higher neuron count in the treated MCAO group. These findings contribute valuable quantitative insights into the neuroprotective effects of galantamine in the context of ischemic insult.
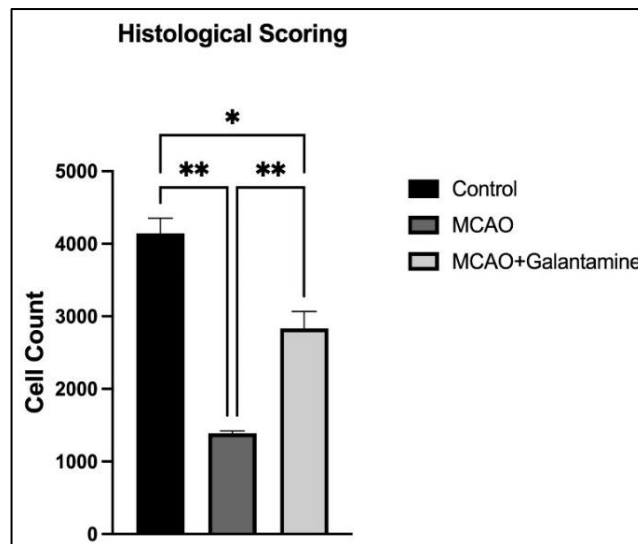


**Figure 3.11*: Effect of MCAO and galantamine on the cortex histology.** (Tissue sections stained with H&E). The histological effects of galantamine and MCAO on cortical tissue, as seen by H&E staining. The data, which are shown as mean ± SEM, show that the MCAO group's cortical neuron count was significantly lower than that of the control and MCAO groups that received galantamine treatment. The utilization of error bars is employed as a means to depict the standard error of the mean (SEM) in instances where a significance threshold of $p < 0.05$ is implemented. These results shed quantitative light on galantamine's neuroprotective properties after ischemia injury.

## 3.4 PCR Results

### 3.4.1. Gradient PCR result

In Figure 3.12, the gel electrophoresis results depict the amplification of SOD2 and TLR4 mRNA using gene-specific primers. Multiple distinct bands are evident, aligning with the expected sizes of 113 base pairs for SOD2 and 506 base pairs for TLR4. This indicates a widespread expression of both genes in the brain tissues under investigation. The bands were observed across a temperature gradient ranging from 58°C to 68°C, with incremental 2°C temperature changes. This series of temperatures reflects the sensitivity of the PCR assay to varying annealing temperatures. Among these, the optimal temperature of 66°C was chosen due to its ability to consistently generate well-defined bands. Importantly this temperature selection serves a dual purpose, aligning with the overlapping temperatures for both SOD2 and

TLR4 primers. By ensuring the PCR amplification's specificity, this optimization sets the stage for later molecular analyses and offers insightful information about the expression patterns of these genes concerning the brain.
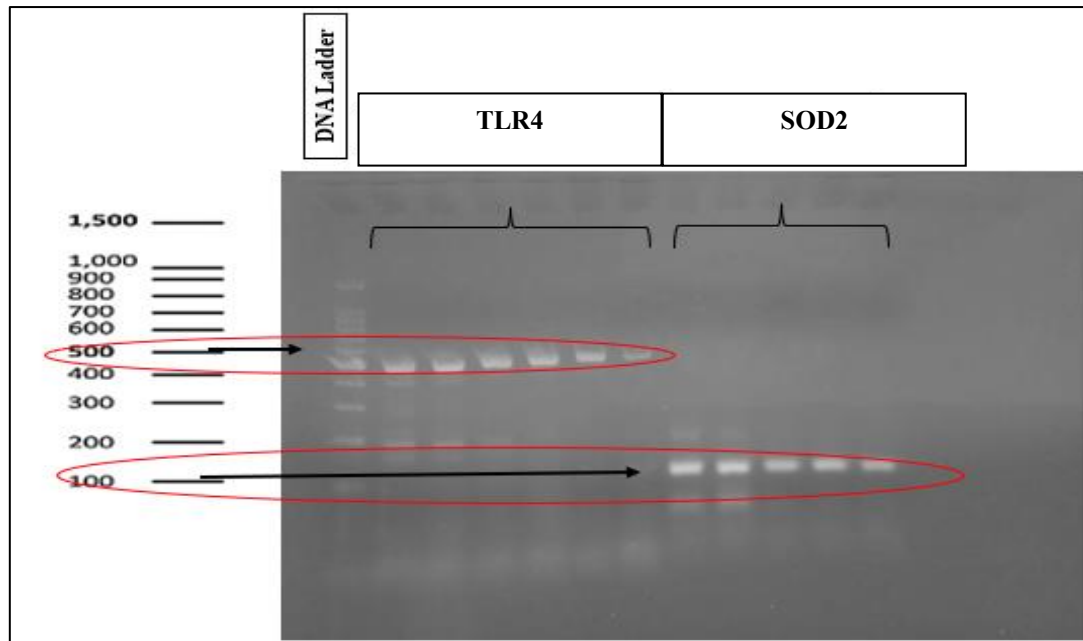


**Figure 3.12: Electrophoresis Results for Optimization.** The Polymerase Chain Reaction (PCR) analysis conducted on the rat brain aimed to assess the expression of two specific genes, SOD2 and TLR4. The resulting gel electrophoresis distinctly exhibits bands corresponding to the expected sizes of the amplified DNA fragments. Notably, at an optimized temperature of 66°C, a prominent band emerges at approximately 113 base pairs, signifying the successful amplification of SOD2. Simultaneously, bands at 506 base pairs appear at the same temperature, indicating successful amplification of TLR4.

*3.4.2. Real-time PCR Result*

In Figure 3.13, the relative expression levels of SOD2 and TLR4 are presented. The assessment involved measuring and normalizing the expression of these genes to ß-actin, serving as a housekeeping gene for internal control. The results indicate a distinct modulation in gene expression patterns. Specifically, the expression of SOD2 mRNA in the rats subjected to MCAO was found to be down-regulated, indicative of a reduced transcription of this gene under ischemic conditions. In stark contrast, treatment with galantamine resulted in an up-regulation of SOD2, suggesting a potential ameliorative effect of galantamine on the down-regulated SOD2 expression associated with cerebral ischemia. Conversely, TLR4 mRNA expression in the MCAO rat group exhibited up-regulation, reflecting an increased transcription of the gene in response to ischemic insult. Intriguingly, the administration of galantamine demonstrated a down-regulation of TLR4 mRNA expression, indicating a potential regulatory effect of galantamine in mitigating the up-regulation seen in MCAO group.

These results provide important new understandings of the molecular reactions linked to ischemic stroke and the possible modulatory effects of galantamine on the expression of important genes involved in immune response (TLR4) and oxidative stress (SOD2). Figure 3.3 gives relative expression of SOD2 and TLR4.
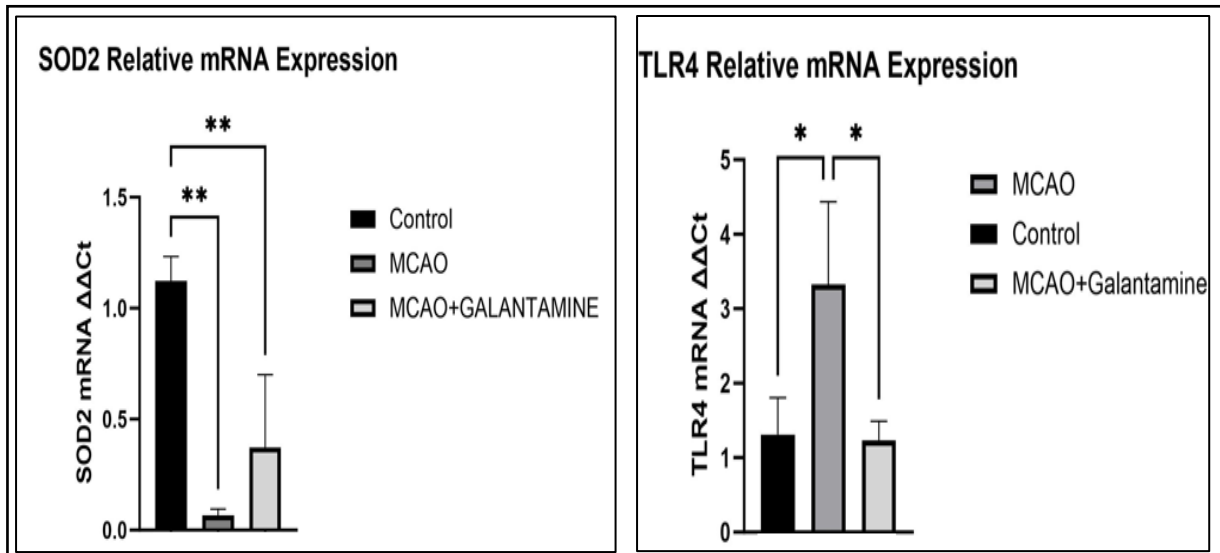


**Figure 3.13: SOD2 and TLR4 mRNA Relative Expression** (Normalized to Beta-actin). SOD2 mRNA expression in the MCAO rat for down-regulation in contrast to the treatment with galantamine which showed up-regulation. TLR4 mRNA expression in the MCAO rat was found to be up-regulated, as opposed to down-regulated following galantamine treatment. Data are displayed as ±SEM. The non-parametric one-way ANOVA and the Tukey multiple comparison test were used in the statistical analysis.

# CHAPTER 4:     DISCUSSION

Stroke, which is widely recognized as a primary contributor to both morbidity and mortality on a global scale, underscores the pressing and crucial necessity to explore and investigate novel and innovative therapeutic interventions. It is crucial to acknowledge that the incidence of stroke is continuously on the rise, thereby imposing an escalating burden on healthcare systems worldwide. Furthermore, it is imperative to acknowledge that the neurological ramifications of stroke extend far beyond the acute stage, thereby necessitating a more profound examination of pharmaceutical avenues that possess the potential to mitigate post-stroke cognitive impairments and neurodegenerative processes (Yang et al., 2020). It is noteworthy that tissue plasminogen activator stands as the sole category of drugs sanctioned by the Food and Drug Administration (FDA) (Bindal et al., 2023).

Any substance that can prevent or lessen the effects of ischemic brain injury without increasing cerebral blood flow is referred to as a "neuroprotector." Neuroprotective medications are devised to impede the cellular, biochemical, and metabolic mechanisms leading to tissue impairment during or after ischemia (Naqvi et al., 2020). They encompass an extensive assortment of pharmacological therapies that are currently expanding at a rapid pace.

The intricate network of neurotransmitter systems in the brain, including acetylcholine (ACh), noradrenaline, dopamine, gamma-aminobutyric acid, serotonin, and glutamate, plays a pivotal role in maintaining cognitive equilibrium. Among these, ACh holds particular significance for learning and memory processes. In the realm of adult-onset cognitive impairment, notably in disorders like Alzheimer's disease (AD), an imbalance in cholinergic neurotransmission emerges as a central player. The absence of proper cholinergic signaling is closely linked to the manifestation of learning and memory deficits observed in AD. A pivotal turning point in understanding this association occurred in the early 1970s when researchers identified a premature decline in basal forebrain cholinergic neurons within the brains of Alzheimer's patients. This groundbreaking revelation laid the foundation for the cholinergic theory of geriatric memory failure, providing crucial insights into the neurochemical basis of cognitive decline in aging brains (Bartus et al., 1982). After the discovery, the theory was substantiated by the identification of a decrease in the synthesis enzyme choline ChAT in brain regions associated with cognition, with Alzheimer's disease (Amenta et al., 2001).

At present, the enhancement of cholinergic neurotransmission stands as a primary approach in the treatment of cognitive and behavioral symptoms in the early and middle stages of AD. Various compounds were utilized by this treatment approach, including linopirdine to boost hippocampus ACh release, xanomeline as a muscarinic ACh receptor agonist, and physostigmine and tacrine as AChE inhibitors. In the brain, acetylcholine is broken down by two enzymes: AChE and BuChE. In the brain tissue of AD patients, AChE is more prevalent than BuChE, leading to the breakdown of acetylcholine in the hippocampus and cerebral cortex (Bertrand & Wallace, 2020).

Multiple studies have provided evidence of the neuroprotective effects of galantamine, which include the reduction of neuronal damage, the promotion of neurogenesis, and the improvement of cognitive function. Galantamine absorbs quickly after being administered orally. The peak plasma drug concentrations (Cmax) varied significantly from 49.2 to 1150 µg/L after a single 10mg oral tablet dose and were achieved in an average of 0.88 or 2 hours in healthy participants across two experiments. Galantamine exhibits a significant volume of distribution after being taken orally, indicating its extensive non-specific absorption (Scott & Goa, 2000). In the context of stroke MCAO, these properties make galantamine a potential candidate for managing the condition and improving patient outcomes. In the context of AD, galantamine, a widely recognized inhibitor of acetylcholinesterase and modulator of nicotinic acetylcholine receptors, has emerged as a potential candidate for neuroprotection and the restoration of cognitive abilities following a stroke (Metz & Pavlov, 2021). However, despite the promising results of galantamine in Alzheimer's disease, its application in addressing cognitive sequelae after a stroke remains an area that has not been thoroughly explored.

One of the key advantages of galantamine is its extended therapeutic window compared to tPA. Tissue plasminogen activator is a thrombolytic agent that works by dissolving blood clots, but its administration is time sensitive. tPA must be given within a narrow time window after the onset of stroke symptoms to be effective. Galantamine, on the other hand, offers a longer time frame for administration (Barfejani et al., 2020). This extended therapeutic window makes galantamine a more flexible option for stroke management, potentially allowing for treatment initiation beyond the critical early hours of a stroke

Mitochondrial SOD2 emerges as a key player in cellular defense against oxidative stress. In the literature review, the role of SOD2 in regulating intracellular reactive ROS levels is underscored, especially in the context of a study involving brain injury and the therapeutic

application of galantamine. Research extends this understanding by investigating SOD2 levels following controlled brain impact traumatic injury (bITON) and subsequent galantamine intervention. (Bernardo-Colón et al., 2018).

However, our research introduces a significant insight. Rats treated with galantamine post-MCAO exhibited SOD2 levels comparable to control shams, showcasing the potential of galantamine in mitigating the decline in SOD2 levels observed after brain injury. The statistical significance ($p < .001$) reinforces the robust impact of galantamine in preserving SOD2 levels. Furthermore, our study delves into the molecular aspects, suggesting that galantamine treatment may induce an upregulation of SOD2 mRNA expression. This anticipated upregulation aligns with the enhanced antioxidant response observed in the context of ischemic stroke. Collectively, the literature (Naguib et al., 2020) and our research converge on the crucial role of SOD2 in antioxidant defense, with galantamine demonstrating its potential as a neuroprotective agent by preserving SOD2 levels and potentially enhancing the cellular defense against oxidative damage induced by neurological injuries.

TLR4 is involved in the inflammatory response, and its modulation can influence the overall neuroinflammatory profile. PCR analysis of TLR4 mRNA expression in galantamine-treated rats may demonstrate a downregulation. This downregulation indicates a potential suppression of the inflammatory response, aligning with galantamine's anti-inflammatory properties observed in previous studies (Durán-Laforet et al., 2021). The molecular data obtained from PCR analysis provides a dual perspective on galantamine's effects. The upregulation of SOD2 suggests an enhancement of antioxidant defenses, while the downregulation of TLR4 implies a reduced inflammatory response. These molecular changes, when correlated with behavioral and histopathological outcomes, contribute to a comprehensive understanding of galantamine's potential neuroprotective mechanisms in the aftermath of ischemic stroke.

Drug behavior studies in stroke-stricken rats MCAO play a critical role in evaluating the efficacy of potential stroke treatments. These tests provide valuable insights into the impact of drugs on the behavior and cognitive function of rats following a stroke, to assess the potential benefits of these drugs in human stroke patients. The MCAO model is widely used in preclinical stroke research as it closely mimics the pathophysiological processes of human stroke (Sommer, 2017) . By subjecting rats to MCAO and then administering potential drugs, researchers can observe and measure the behavioral changes in the rats, such as motor function,

sensory perception, and cognitive abilities (Ruan & Yao, 2020). These tests allow for the evaluation of the drugs' effects on functional recovery and neurological deficits, providing important data for the development of potential stroke treatments.

Behavioral tests in rats also play a crucial role in determining the optimal dosage and timing of drug administration. By assessing the rats' behavior at different time points and drug concentrations, researchers can determine the most effective treatment regimens for stroke (Boboc et al., 2023). This information is essential for the translation of preclinical findings to clinical trials, as it helps to guide the dosing and administration of drugs in human patients. The Y-maze test results exhibited a 39% increase in percentage alternation, emphasizing the positive impact of galantamine on cognitive function in comparison to the Streptozotocin (STZ) group (Rajkumar et al., 2022). On the other hand, our findings in the context of stroke-related damage revealed that rats treated with galantamine exhibited enhanced spatial memory during the Y-maze test, indicating improved cognitive function and spatial working memory. Both studies highlight galantamine's potential to positively influence cognitive aspects, addressing different neurological conditions and emphasizing its versatile neuroprotective effects.

In the Grip strength test it is evident from the research study that a decline in hand grip strength is associated with an increased risk of AD in middle-aged adults (Camargo et al., 2016), the galantamine-treated group in stroke-related research showed improved motor coordination and enhanced grip strength. The positive impact on motor functions observed in the galantamine-treated group suggests its potential to mitigate stroke-induced motor deficits. These contrasting findings highlight the dual role of grip strength assessments – as a potential risk indicator for AD in the aging population and as an indicator of improved motor function in the context of galantamine treatment for stroke-related issues (Okuda et al., 2018).

The behavioral effects observed in open-field tests demonstrate distinct responses in different experimental settings. In a dose-dependent manner, the behavior of both transgenic and non-transgenic mice treated with galantamine has returned to normal, especially in terms of their preference for corners and avoidance of the center (Bhattacharya et al., 2014). Contrastingly, in the context of stroke-related research, galantamine-treated rats exhibited heightened exploratory behavior, indicative of improved overall activity levels and a positive impact on their willingness to explore the environment. These findings underscore the diverse behavioral outcomes associated with galantamine treatment in distinct experimental models.

The findings from the current study reveal that galantamine exhibits a multifaceted cognitive-enhancing effect. Specifically, it not only significantly ameliorates cognitive impairments induced by methamphetamine (Meth) in the novel object recognition test but also extends its positive impact to address learning and memory deficits caused by repeated Meth treatment in mice (Noda et al., 2010). Furthermore, these findings resonate with the observed cognitive improvements in galantamine-treated stroke rats, as evidenced by enhanced performance in the novel object recognition test. The ability to recognize and interact with novel objects in the stroke-related context signifies an enhancement in recognition memory, further supporting galantamine's potential to ameliorate cognitive deficits associated with stroke. The parallel cognitive benefits observed in distinct experimental models underscore the versatility and efficacy of galantamine in addressing varied neurological challenges.

In a comprehensive evaluation of galantamine's impact, it has been demonstrated that, in addition to countering the amnesic effects induced by scopolamine and facilitating social recognition in social memory models, galantamine aligns with the facilitatory role of D1 receptors in social cognition through its cholinergic effects. This highlights its potential relevance in conditions involving disrupted cholinergic transmission and impaired social cognition (Di Cara et al., 2007). Moreover, social interaction tests conducted on MCAO group treated with galantamine revealed a notable increase in sociability and exploratory behaviors. This dual influence on social memory and interaction becomes particularly significant in the context of stroke recovery, where social engagement contributes to overall well-being.

In summary, the multifaceted effects of galantamine, as evidenced by various behavioral tests in stroke models, emphasize its capacity to positively influence cognitive, motor, and social aspects. These collective findings underscore the potential of galantamine as a therapeutic intervention for addressing deficits associated with stroke-related recovery. Through the use of a clinical 3.0 Tesla MRI scanner and standard neurohistopathology, the study verified that guinea pigs exposed to soman had experienced neuronal death. Intriguingly, pre-treatment with galantamine emerged as a promising intervention, effectively preventing soman-induced brain damage. Both MRI and histopathological examinations supported the neuroprotective impact of galantamine in mitigating the consequences of acute soman exposure (Gullapalli et al., 2010).

In our study of the galantamine-treated group, distinct features indicative of potential neuroprotection became apparent. The presence of well-defined pyramidal cells with visible

nuclei suggested the preservation of neuronal structure, portraying a notable neuroprotective effect. Quantitative analysis, facilitated by specialized software such as ImageJ, revealed an increased cell count in the galantamine-treated group, hinting at potential neuron preservation compared to the untreated stroke group. This numerical data offered a quantitative measure of galantamine's impact on cellular survival, providing substantial support for the concept of neuroprotection.

Delving into morphological details, an examination of cellular architecture in the galantamine-treated group unraveled well-defined pyramidal cells with characteristic shapes and clear nuclei, resembling those in the control group. This morphological preservation contrasted with the MCAO group, which exhibited irregularities, deformities, and disfigurement in cell shape, underscoring the detrimental impact of ischemic damage. Together, these observations reinforce the neuroprotective potential of galantamine, emphasizing its significance in preserving cellular integrity and structural stability in the context of acute soman exposure.

# CHAPTER 5:    SUMMARY OF RESEARCH WORK

Ischemic stroke, a devastating cerebrovascular event, stands as a major global health concern, accounting for a significant burden of mortality and morbidity. Ischemic stroke, which is characterized by an abrupt interruption of blood flow to the brain as a result of arterial blockage, presents significant difficulties for healthcare systems all over the world. Its prevalence continues to rise, underscoring the critical imperative to develop effective therapeutic strategies. With limited treatment options and the persistent quest for interventions that can mitigate the profound neurological damage inflicted by ischemic stroke, research endeavors are increasingly focused on uncovering novel therapeutic potentials. In this context, exploring the therapeutic potential of drugs, such as galantamine, emerges as a promising avenue to navigate the complex pathophysiological landscape of ischemic stroke and pave the way for innovative and effective treatment modalities.

The study initiated with a week-long acclimatization period for fifteen male Wistar Hans rats, aged 8-14 weeks, in the animal facility, 3 groups, each group consisting of n=5. Subsequently, MCAO surgery was performed to induce ischemic stroke-like conditions. The treatment group received an 18-day regimen of galantamine starting from the day of the MCAO procedure. A battery of behavioral tests, encompassing the Y-maze, grip strength, open field, novel object recognition, and social interaction assessments, was conducted to discern the impact of galantamine on motor coordination, memory, spatial recognition, exploratory behavior, and social interactions.

Post-behavioral testing and dissection was carried out for molecular analysis, involving RT-PCR targeting genes SOD2 and TLR4. Simultaneously, histopathology procedures were executed using H&E staining for microscopic examination of tissue samples. Additionally, in silico work was undertaken to validate binding interactions. This methodological approach ensures a comprehensive exploration of galantamine's therapeutic potential, ranging from behavioral dimensions to molecular and structural analyses.

These comprehensive results underscore the multifaceted therapeutic effects of galantamine in managing ischemic stroke, encompassing behavioral improvements, neuroprotection at the histological level, and molecular alterations indicative of enhanced antioxidant and anti-inflammatory mechanisms. Galantamine administration significantly improved behavioral outcomes in treated rats. Enhanced spatial memory was observed in the

Y-maze test, along with improved motor coordination in grip strength assessments. Open-field tests showed heightened exploratory behavior, and novel object recognition tests indicated improved cognitive performance. Social interaction tests demonstrated increased sociability and exploratory behaviors in the galantamine-treated group. Histological examination of brain tissue using Hematoxylin and Eosin staining revealed neuroprotective effects of galantamine. The treated group exhibited preserved neuronal morphology and reduced cellular damage compared to the untreated MCAO group.

Molecular analysis through RT-PCR demonstrated notable modulations in gene expression. Galantamine treatment led to an upregulation of SOD2, indicative of enhanced antioxidant defense mechanisms. Simultaneously, there was a downregulation of TLR4, suggesting a mitigated inflammatory response. The *in silico* analysis further corroborated the molecular findings, elucidating potential binding interactions between galantamine and relevant targets associated with neuroprotection.

Galantamine has demonstrated positive outcomes across various analyses, including behavioral assessments (such as spatial memory and motor coordination), histopathological examination, and molecular analyses revealing enhanced antioxidant defenses and reduced inflammation. The consistent and promising results observed in diverse aspects underscore the potential of galantamine as a comprehensive therapeutic approach. This multi-dimensional efficacy positions galantamine as a promising candidate for addressing the complexities associated with neuroprotection and cognitive enhancement in the context of ischemic stroke.

# CHAPTER 6:  CONCLUSION AND FUTURE RECOMMENDATIONS

Galantamine's impact on MCAO-induced stroke reveals promising neuroprotective effects across multiple domains. In behavioral assessments, rats treated with galantamine exhibit significant improvements, reflecting enhanced spatial memory, improved motor coordination, heightened exploratory behavior, and superior cognitive performance. These behavioral results imply that galantamine may be an important therapeutic benefit for stroke recovery, potentially helping to mitigate the neurological deficits caused by MCAO.

Histopathological analysis of brain tissue provides crucial insights into the impact of galantamine in MCAO-induced stroke. Staining with H&E reveals distinct differences between control, MCAO, and galantamine-treated MCAO groups. Control rats exhibit control pyramidal cells with visible nuclei, indicating live neuronal cells. In contrast, MCAO rats without galantamine treatment display deformed and disfigured neuronal cells, reflecting the detrimental effects of stroke. Galantamine-treated MCAO rats show neuroprotection, preserving pyramidal neuronal cells and distinct nuclei, suggesting its potential to mitigate ischemic damage and promote neuronal survival. This histological evidence complements behavioral findings, providing a comprehensive understanding of galantamine's neuroprotective effects in stroke.

Molecular analyses, particularly the examination of gene expression, provide mechanistic insights into galantamine's effects. PCR analysis targeting key genes, such as SOD2 and TLR4, unveils a potential molecular framework underlying galantamine's neuroprotective mechanisms. Upregulation of SOD2 indicates an enhanced antioxidant response, while downregulation of TLR4 suggests a dampened inflammatory reaction. These molecular changes align with the observed improvements in behavior and signify a comprehensive neuroprotective potential for galantamine in the context of MCAO-induced stroke. Additional investigation and confirmation of these results could aid in the creation of focused treatment plans for the treatment of strokes.

The future recommendation for this study would be that further studies could delve into determining the most effective and safe dosage of galantamine for stroke treatment. Different concentrations may be tested to establish a dose-response relationship, ensuring therapeutic benefits without adverse effects. Further studies can encompass investigation of the impact of the timing of administration post-stroke. Studies exploring the optimal time window for

administering galantamine concerning the onset of stroke symptoms may provide insights into its efficacy in acute and delayed treatment scenarios.

It would be beneficial to evaluate the long-term impacts of galantamine therapy on stroke survivors' functional recovery, cognitive function, and general quality of life. This could involve extended behavioral testing and monitoring beyond the immediate post-treatment period. Considering the multifaceted nature of stroke pathology, combining galantamine with other potential neuroprotective agents or rehabilitation strategies may enhance therapeutic outcomes. Exploring synergistic effects with existing stroke interventions could be an avenue for further investigation.

It is imperative to gain a deeper comprehension of the molecular mechanisms that underpin the neuroprotective effects of galantamine after stroke. To get a better understanding of galantamine's mode of action, more studies might concentrate on particular signaling pathways, neurotransmitter systems, or cellular processes that are affected by it. A critical step is transferring promising results from preclinical research to clinical trials. Performing carefully planned clinical trials to assess galantamine's safety and effectiveness in stroke patients would close the knowledge gap between experimental data and possible therapeutic uses.

Expanding research to include other stroke models beyond MCAO, such as embolic or lacunar strokes, would provide a more comprehensive understanding of galantamine's applicability across diverse stroke subtypes. Integrating advanced imaging technologies like MRI or PET scans could offer real-time visualization of structural and functional changes in the brain. This could enhance the ability to track the impact of galantamine treatment on neurovascular dynamics. Investigating the potential of galantamine in promoting neuroplasticity and neuro-regeneration post-stroke could open avenues for therapies focused on functional recovery and repair of damaged neural circuits. Subsequent investigations may expand upon the present comprehension of galantamine's capacity for managing stroke and furnish significant insights for the creation of efficacious treatment approaches.

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