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RESEARCH ARTICLE

NEUROPROTECTION AND COGNITIVE ENHANCEMENT BY GINKGO BILOBA IN MCAO MICE

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Abstract

This study investigates the neuroprotective and cognitive-enhancing effects of Ginkgo biloba in a mouse model of middle cerebral artery occlusion (MCAO). Different doses of Ginkgo biloba (50 mg/kg, 100 mg/kg, and 200 mg/kg) were administered post-ischemia, with the 100 mg/kg dose showing the most significant neuroprotection, enhancing neuronal survival and improving cognitive outcomes. The optimal dose preserved neurons in the ischemic penumbra, upregulated neurotrophic factors (BDNF and NGF), and improved spatial and recognition memory in behavioral tests. The 200 mg/kg dose, while effective, demonstrated mild toxicity. Overall, Ginkgo biloba exhibited potential as a therapeutic agent for ischemic stroke, offering neuroprotective benefits comparable to nimodipine, with additional neurogenesis-promoting properties.

Introduction:-

Stroke is a leading cause of death and long-term disability worldwide, resulting in significant motor, cognitive, and psychiatric deficits among survivors (Feigin et al., 2020). The primary clinical interventions for stroke focus on restoring blood flow through mechanical thrombectomy or pharmacological clot removal. Recombinant tissue plasminogen activator (rtPA), a widely used clot-busting drug, must be administered within a narrow window of 4.5 hours after stroke onset, limiting its widespread applicability (Gilligan et al., 2005; Krause et al., 2019). Similarly, while mechanical thrombectomy has shown efficacy, it is also constrained to acute phases of stroke (Campbell et al., 2019). Despite advances in stroke care and prevention, the incidence of stroke-related chronic disabilities remains high. Survivors often face long-term cognitive and motor impairments, with these outcomes depending on factors such as the size and region of the brain injury, timing, and type of therapeutic intervention (Iadecola, 2013).

The brain exhibits an intrinsic capability for self-repair following ischemic injury, particularly through processes of neuroplasticity and neurogenesis (Zhao & Willing, 2018). Motor function, in particular, shows spontaneous recovery during the chronic phase, attributed to molecular, cellular, and structural reorganization in the brain (Nakayama et al., 1994; Kwakkel et al., 2003; Cassidy & Cramer, 2017). Stroke triggers neurogenesis in the adult brain's neurogenic niches: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Arvidsson et al., 2002; Parent, 2003). While this neurogenic response promotes recovery by generating new neurons, the ischemic environment's complexity can hinder the maturation and integration of these neurons, limiting the brain's repair capacity (Thored et al., 2006).

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Although motor recovery is often observed, cognitive function tends to deteriorate over time following a stroke (Levine et al., 2015; Mijajlović et al., 2017). Post-stroke cognitive impairment affects more than a third of stroke survivors, manifesting as deficits in attention, memory, and executive function, which are influenced by factors such as the size and location of the infarct (Tatemichi et al., 1994; Pendlebury & Rothwell, 2009). Emerging evidence suggests that stroke impacts brain regions distant from the initial injury, particularly the hippocampus, contributing to memory decline and post-stroke dementia (Prins et al., 2005; Blum et al., 2012). Potential mechanisms for these secondary effects include chronic hypoxia, inflammation, vascular dysfunction, and maladaptive neurogenesis in the hippocampus (Li et al., 2013; Ma et al., 2013).

The role of SVZ and SGZ neurogenesis in post-stroke recovery is under active investigation. Stroke induces neurogenesis in the SVZ, with neural progenitors migrating to the site of injury and differentiating into neurons and astrocytes, contributing to glial scar formation and neuroprotection (Parent et al., 2002; Lindvall&Kokaia, 2015). However, the ability of these newly generated neurons to fully integrate and replace damaged neurons remains limited (Arvidsson et al., 2002). In contrast, hippocampal neurogenesis may be maladaptive, with evidence suggesting that the newly formed neurons in the SGZ integrate abnormally, potentially disrupting hippocampal circuitry and exacerbating cognitive impairments (Nakatomi et al., 2002; Kempermann et al., 2015).

This study aims to explore the mechanisms governing stroke-induced neurogenesis with a focus on understanding the factors that either promote or inhibit functional recovery. Understanding these processes is critical for developing therapeutic interventions that harness neurogenesis to improve post-stroke outcomes.

Research Envisaged

The Role of Borneol in Neuroprotection and Drug Delivery

Borneol, a naturally occurring terpene and bicyclic organic compound found in species of *Artemisia* and *Dipterocarpaceae*, has become the focus of significant research due to its diverse bioactivities. Among these are its anti-inflammatory properties, enhancement of energy metabolism, and particularly its role in neuroprotection against cerebral ischemia/reperfusion injury (Gutiérrez-Fernández et al., 2011; Ehrnhofer-Ressler et al., 2013). Its highly lipophilic nature has been harnessed to improve drug delivery across the blood-brain barrier (BBB), making it an effective penetration enhancer in neuropharmacology.

Mechanism of Drug Delivery Enhancement

The lipophilicity of borneol allows it to interact with the lipid components of cell membranes, affecting enzymes, carriers, ion channels, and receptors. This interaction facilitates the absorption and concentration of co-administered drugs, such as gastrodin and edaravone, in brain tissues (Cai et al., 2008; Wu et al., 2014). Borneol has also been observed to increase the number and volume of pinocytosis vesicles in BBB endothelial cells, promoting the transport of therapeutic agents into the brain (Chen et al., 2010). This property highlights its potential in addressing the challenge of delivering neuroprotective agents across the restrictive BBB.

Neuroprotective Applications

In addition to its penetration-enhancing effects, borneol has demonstrated neuroprotective properties. It is under investigation for its role in treating various neurological diseases, including stroke, Alzheimer's disease, and Parkinson's disease (Liu et al., 2011; Han et al., 2011; Tian et al., 2007). This broad spectrum of applications reflects borneol’s versatility in the field of neuroprotection.

The Role of Herbal Medicine in Neuroprotection and Stroke Recovery

Herbal medicines have been used for centuries in traditional oriental medicine to treat ischemic stroke and vascular dementia. Recent scientific investigations have begun to confirm the neuroprotective potential of these remedies, which act primarily through the inhibition of inflammatory cytokines and the suppression of microglia activation.

Gongjin-dan as a Neuroprotective Formula

One prominent herbal formulation, Gongjin-dan, has gained attention in Korea and China for its antifatigue and antiaging properties. It has also been explored as a potential treatment for neurodegenerative diseases. Studies have shown that Gongjin-dan enhances learning and memory in stress-induced rat models (Moon et al., 2010). However, there is a significant gap in experimental evidence that elucidates the mechanisms through which this multi-herbal formula exerts its effects.

In an ischemic stroke model, Gongjin-dan has been observed to reduce infarct volume and enhance functional recovery. It also affects glucose metabolism, apoptosis, cell survival, and the modulation of inflammatory cytokines (Jung et al., 2011).

Genistein-3'-Sodium Sulfonate (GSS) as a Neuroprotective Agent in Ischemic Stroke

Ischemic stroke remains a leading cause of mortality and long-term disability worldwide, highlighting the urgent need for effective therapeutic interventions. Genistein, an isoflavone found in soy and other plants, has attracted attention for its neuroprotective potential in cerebral ischemia. However, its poor solubility in water has limited its use in clinical settings.

Enhanced Solubility with GSS

To overcome the solubility issue, researchers synthesized genistein-3'-sodium sulfonate (GSS), a more water-soluble compound with enhanced bioavailability. In in vitro studies, GSS was shown to reduce glutamate-induced cytotoxicity in rat cortical neurons by inhibiting apoptosis. In vivo studies with rat models of middle cerebral artery occlusion (MCAO) further demonstrated that GSS effectively reduced ischemic injury by modulating apoptosis-related pathways (Zhao et al., 2013).

Neuroprotective Properties of Selected Plant Extracts

Plant-derived compounds have gained attention in recent years for their potential neuroprotective effects, offering a natural alternative to synthetic drugs with fewer side effects. Below are some notable plant extracts known for their neuroprotective properties.

Bellis perennis (Daisy)

Bellis perennis extract has demonstrated neuroprotective effects by enhancing neuronal cell viability in response to alcohol-induced cytotoxicity. This suggests its potential in protecting neurons from toxic insults (Jiang et al., 2008).

Calendula officinalis (Marigold)

Calendula officinalis has been studied for its neuroprotective effects against neurotoxicity induced by substances like monosodium glutamate (MSG). The extract attenuated behavioral alterations, oxidative stress, and hippocampal damage in animal models exposed to MSG, indicating its role in protecting neurons from excitotoxicity (Aly et al., 2013).

Carthamus tinctorius (Safflower)

Hydroxysafflor yellow A (HSYA), derived from Carthamus tinctorius, has shown neuroprotective effects in models of cerebral ischemia. HSYA reduces infarct size, inhibits neuronal death, and improves neurological outcomes by modulating mitochondrial function and oxidative stress pathways (Zhou et al., 2010).

Cassia occidentalis

Extracts of Cassia occidentalis have shown anxiolytic and antidepressant effects in rodent models. These findings suggest that it could be developed as a natural remedy for mood disorders, though clinical studies are needed to confirm its therapeutic potential (Singh et al., 2011).

Coriandrum sativum (Coriander)

Pretreatment with Coriandrum sativum extract has been shown to enhance antioxidant enzyme levels and reduce cerebral infarct size in ischemia-reperfusion injury models. This neuroprotective effect is attributed to its ability to attenuate oxidative stress and calcium overload (Chithra&Leelamma, 1999).

Crocus sativus (Saffron)

Saffron extract has demonstrated neuroprotective effects in models of neurotoxicity, diabetic encephalopathy, and multiple sclerosis. Its diverse mechanisms of action include restoring antioxidant activity and modulating inflammatory pathways (Nam et al., 2010).

Cyperus rotundus (Nut Grass)

Extracts of Cyperus rotundus have shown neuroprotective effects in Parkinson’s disease and global ischemia models by protecting dopaminergic neurons and improving cognitive function. Its antioxidant properties play a key role in mitigating neuronal damage (Zhang et al., 2009).

Dalbergia sissoo (Indian Rosewood)
Dalbergia sissoo extracts have demonstrated neuroprotective effects in cerebral ischemia by preserving neuronal integrity and reducing oxidative stress in experimental models (Muralidharan et al., 2008).

Research Methodology:-

Animal Selection and Housing

Adult male C57BL6/J mice, aged between 8 to 12 weeks and weighing 25-30 g, were selected for this study. Mice were housed in groups of 4-5 per cage under a 12-hour light/dark cycle with a controlled ambient temperature of 22°C ± 2°C. Standard rodent chow and water were provided ad libitum. Mice were acclimatized to the environment for at least 7 days prior to any experimental procedures to ensure physiological stability.

Preoperative Preparation

Mice were fasted for 12 hours before surgery but had unrestricted access to water. Anesthesia was induced with 4% isoflurane and maintained at 1.5-2% isoflurane in oxygen during surgery. Local analgesia was administered using 0.5% bupivacaine at the incision site to mitigate surgical pain.

Surgical Procedure for Middle Carotid Artery Occlusion (MCAO)

Surgical Setup

The anesthetized mice were placed on a heating pad set to 37°C to maintain body temperature during the procedure. Mice were positioned supine, with limbs secured using adhesive tape. The neck area was disinfected with 70% ethanol followed by povidone-iodine to ensure asepsis.

MCAO Induction

A midline cervical incision was made to expose the common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The CCA and ECA were ligated using 6-0 silk sutures, and a microvascular clamp was temporarily placed on the ICA. A silicone-coated nylon monofilament (0.18-0.20 mm in diameter) was introduced into the ICA via a small incision in the ECA stump. The filament was advanced approximately 9-10 mm to block the middle cerebral artery (MCA), inducing occlusion for 60 minutes. The filament was secured in place for the designated occlusion duration.

Reperfusion

After the occlusion period, the filament was carefully withdrawn to restore blood flow, and the microvascular clamp was removed from the ICA. The incision was closed using 6-0 polypropylene sutures.

Post-Surgical Care and Monitoring

Recovery

Mice were placed in a recovery chamber set to 28-30°C until they regained consciousness. To prevent dehydration, subcutaneous saline (0.5-1 ml) was administered. Post-surgery, analgesic buprenorphine (0.1 mg/kg) was provided every 12 hours for 48 hours. Mice were monitored for pain, distress, and neurological deficits, including hemiparesis and circling behavior.

Physiological Monitoring

Body temperature was continuously monitored during surgery using a rectal probe. In a subset of mice, blood pressure and blood gas measurements were taken intermittently to ensure stable physiological parameters throughout the procedure.

Postoperative Assessment

Neurological evaluations were conducted using the Bederson score at 24, 48, and 72 hours post-MCAO. Body weight was recorded daily, and mice were monitored for signs of dehydration or infection.

Validation of Ischemic Injury

Histological Analysis

Mice were euthanized 24 hours after reperfusion using carbon dioxide asphyxiation followed by decapitation. Brains were harvested, sliced coronally into 2-mm sections, and immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution in PBS at 37°C for 20 minutes. Infarct size and volume were assessed through imaging and analyzed using image analysis software.

Behavioral Assessments
Motor function recovery was evaluated using the rotarod and grip strength tests at 48 and 72 hours post-MCAO.

Ginkgo biloba Administration and Evaluation
Ginkgo biloba Treatment
Ginkgo biloba extract, standardized to 24% flavonoids and 6% terpene lactones, was administered via oral gavage at doses of 50 mg/kg, 100 mg/kg, or 200 mg/kg daily. Treatment commenced 24 hours after MCAO and continued for 14 days. Control groups included vehicle-treated mice (saline) and those receiving standard treatment with nimodipine (10 mg/kg).

Evaluation of Neuronal Survival
At the end of the treatment period, mice were euthanized, and their brains were extracted for histological analysis. Nissl staining was performed on brain sections to assess neuronal survival in the cortex and hippocampus. Neuronal viability in the ischemic penumbra was quantified using microscopy and image analysis software.

Measurement of Neurotrophic Factors
Brain tissues from the cortex and hippocampus were extracted for protein analysis. Levels of neurotrophic factors such as BDNF and NGF were quantified using ELISA or western blotting. Results from Ginkgo biloba-treated groups were compared with those of control groups.

Cognitive Function Assessment
Cognitive function was assessed at 14 days post-MCAO using the Morris water maze test. Mice were trained for 5 consecutive days, and escape latency was recorded. On day 6, a probe trial was conducted to evaluate memory retention. The novel object recognition test was also used to assess recognition memory by measuring exploration times for familiar and novel objects.

Optimization and Comparative Analysis
Dose Optimization
The effects of different doses of Ginkgo biloba on neuronal survival, cognitive function, and neurotrophic factor levels were assessed to determine the optimal dose for neuroprotection with minimal adverse effects.

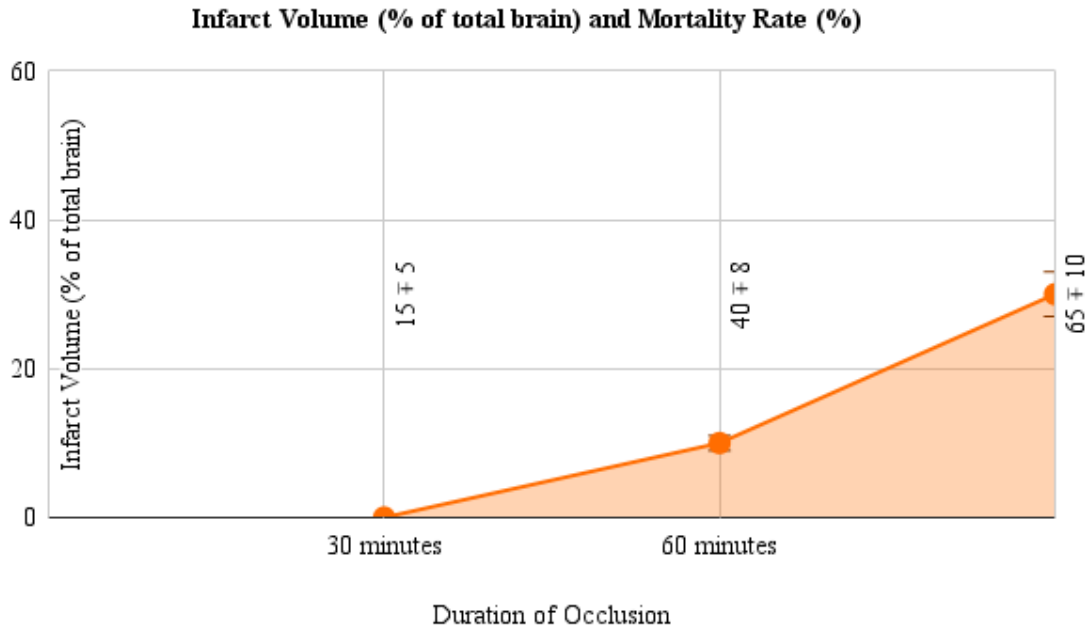
Comparison with Standard Treatments
Outcomes such as neuronal survival, cognitive function, and neurotrophic factor expression were compared between Ginkgo biloba-treated groups, vehicle controls, and standard treatment groups. Statistical analysis, including ANOVA with post hoc tests, was performed to evaluate the efficacy of Ginkgo biloba relative to established treatments.

Toxicity Evaluation
Throughout the treatment period, mice were monitored for signs of systemic toxicity, including weight loss, behavioral changes, and overall health status.

Results and Discussion:-

Middle Cerebral Artery Occlusion (MCAO) Optimization
Optimal Duration of Ischemia
Mice subjected to different durations of MCA occlusion were evaluated to determine the optimal ischemic duration. A 60-minute occlusion consistently resulted in infarcts localized to the ipsilateral cortex and striatum, as confirmed by TTC staining. In comparison, the 30-minute occlusion group exhibited smaller, less defined ischemic areas, while the 90-minute group presented with larger infarcts, increased mortality, and severe neurological impairments.

Infarct Volume and Mortality Rate
The 60-minute occlusion group exhibited an infarct volume of 40% ± 8% with a manageable mortality rate of 10%. By contrast, the 30-minute group showed a significantly smaller infarct volume (15% ± 5%) with no mortality, while the 90-minute group had the highest infarct volume (65% ± 10%) and a mortality rate of 30%.



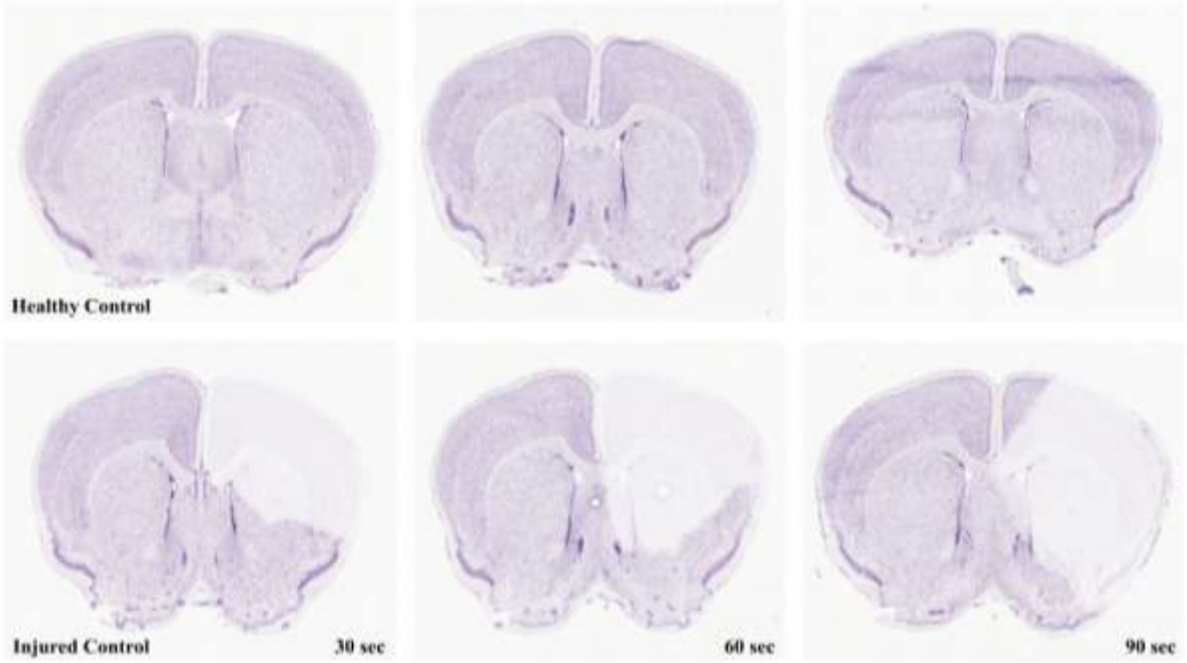
Neurological Deficits

Neurological outcomes were assessed using the Bederson score. The 60-minute group showed moderate deficits (average score: 2.5 ± 0.5), while the 90-minute group exhibited severe impairments (average score: 4.0 ± 0.7). The 30-minute group had milder deficits (average score: 1.0 ± 0.3).

Ginkgo biloba Treatment

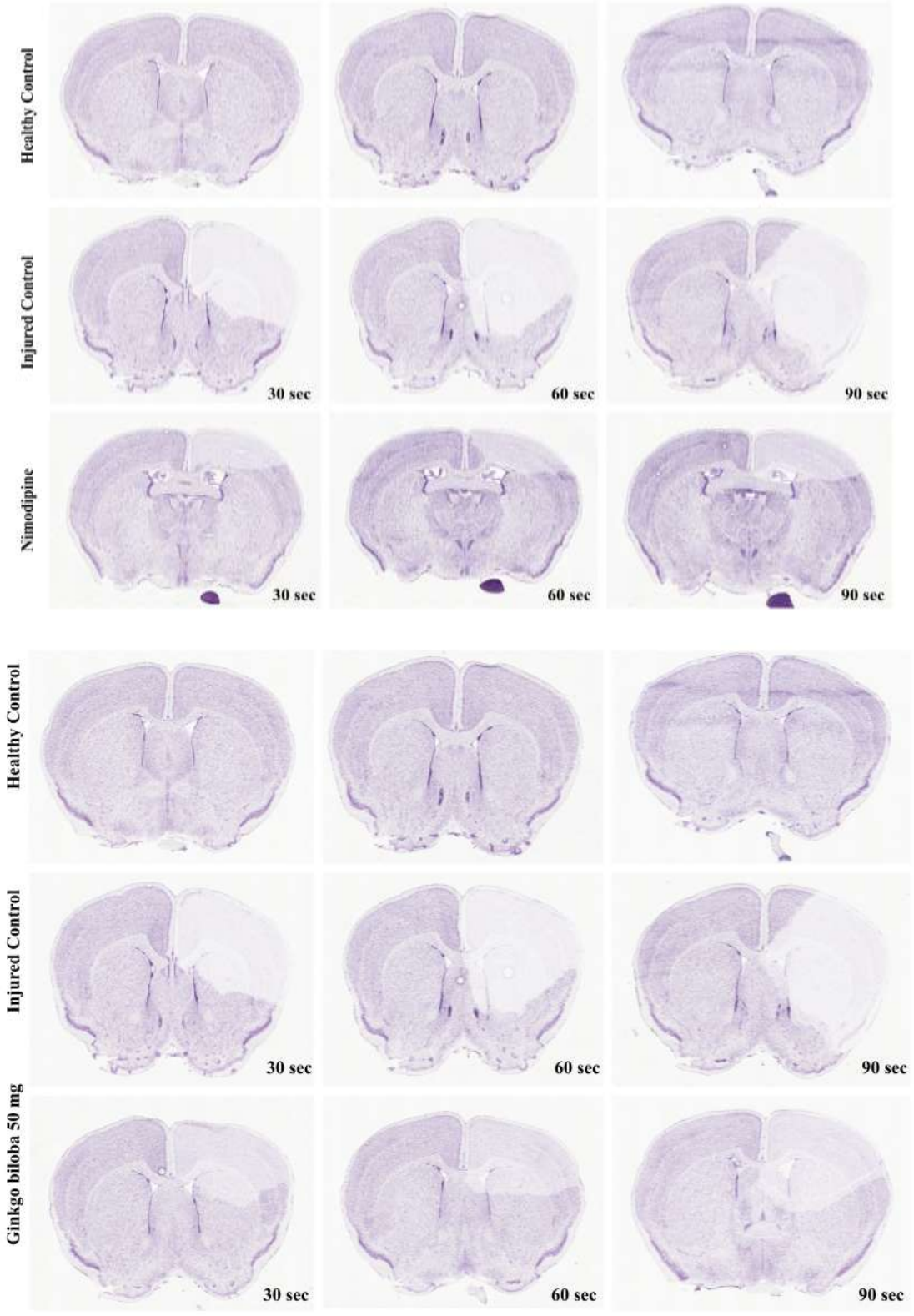
Neuronal Survival

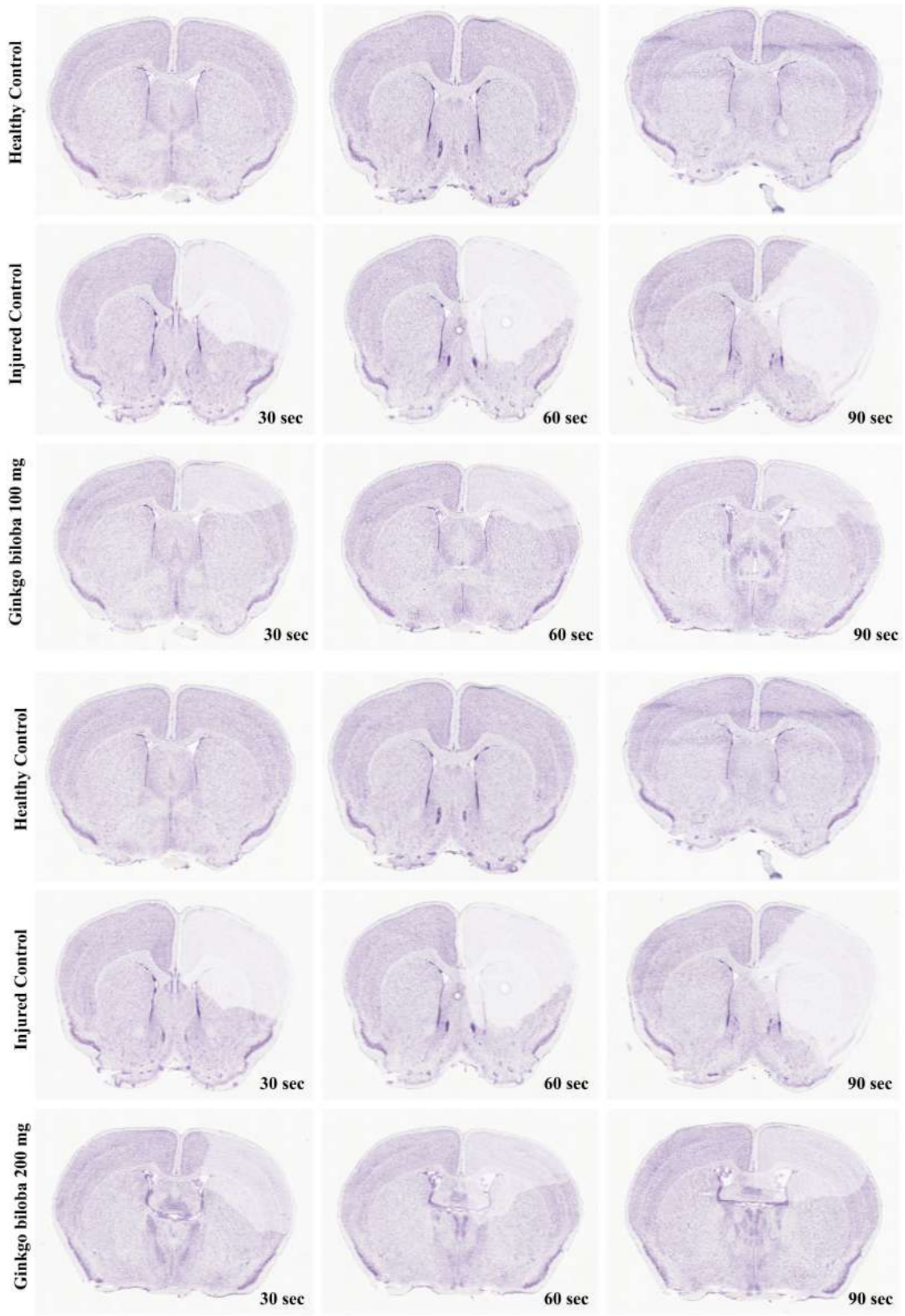
Nissl staining revealed that Ginkgo biloba treatment significantly preserved neuronal survival in the cortex and hippocampus, particularly at the 100 mg/kg dose. The 100 mg/kg group had the highest number of viable neurons in the ischemic penumbra, indicating a dose-dependent neuroprotective effect. The 50 mg/kg dose provided moderate protection, while the 200 mg/kg group exhibited mild toxicity, reflected in behavioral lethargy and reduced weight gain.



Neuronal Count in the Penumbra

The 100 mg/kg dose resulted in a 60% increase in neuronal count compared to the vehicle control. This dose was the most effective in preserving neurons, followed by the 200 mg/kg and 50 mg/kg doses, which showed 50% and 24% increases, respectively. Nimodipine-treated mice showed a similar neuronal count to the 100 mg/kg group.

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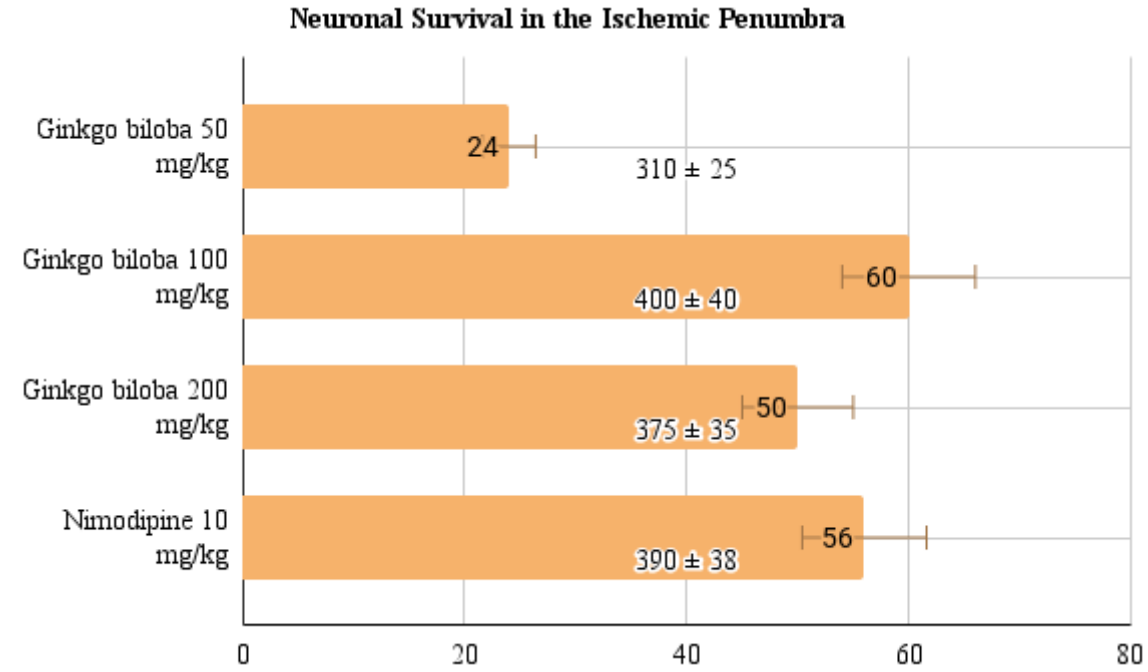
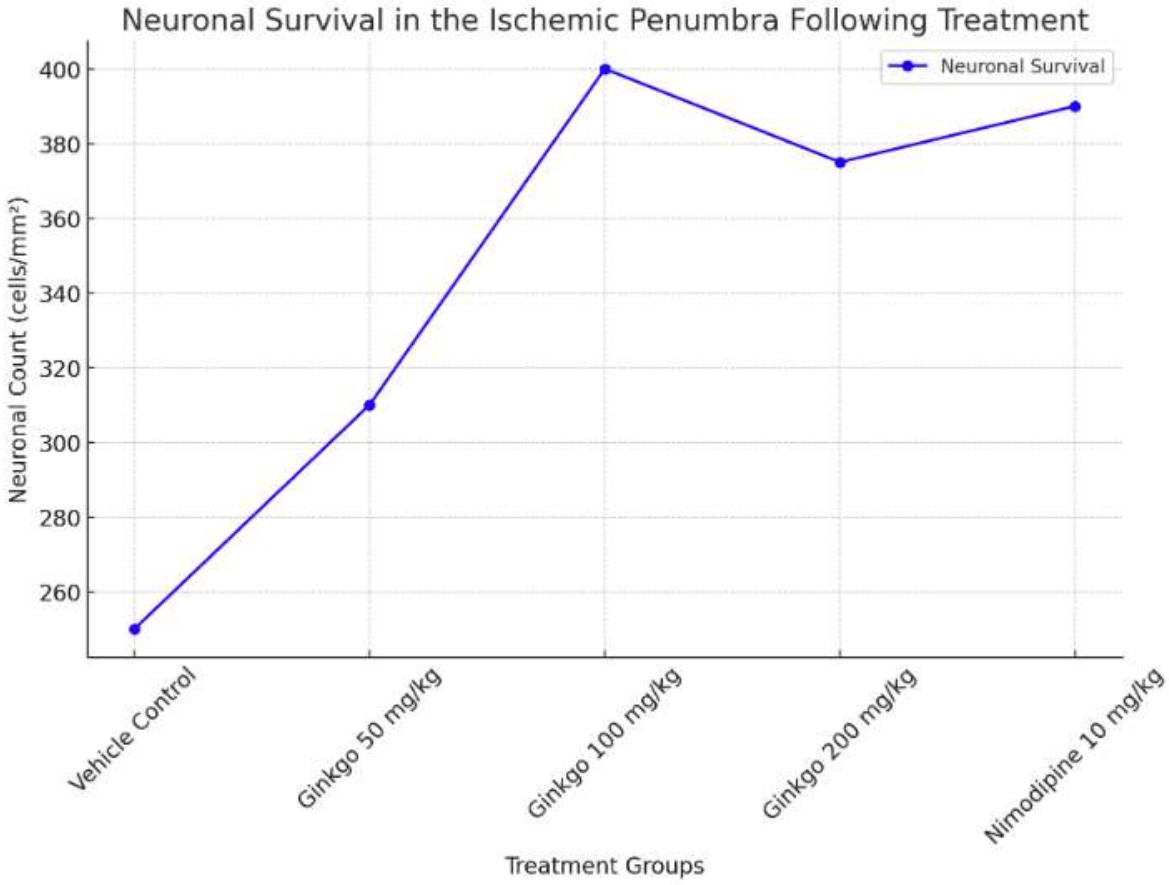
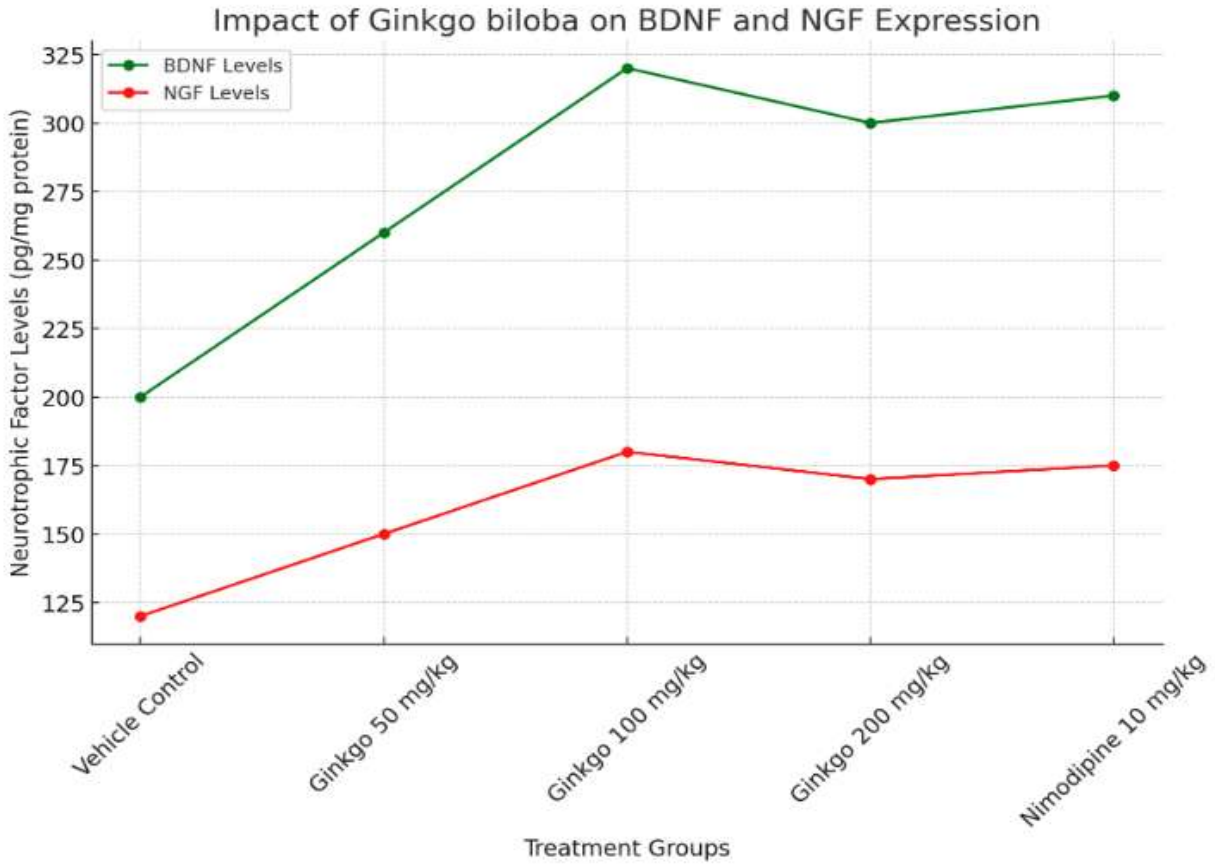




Neurotrophic Factor Expression

Western blot analysis demonstrated that Ginkgo biloba treatment elevated brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels, particularly at the 100 mg/kg dose. These increases were comparable to those

observed in the nimodipine group, supporting the role of Ginkgo biloba in promoting neuroprotection and neurogenesis.



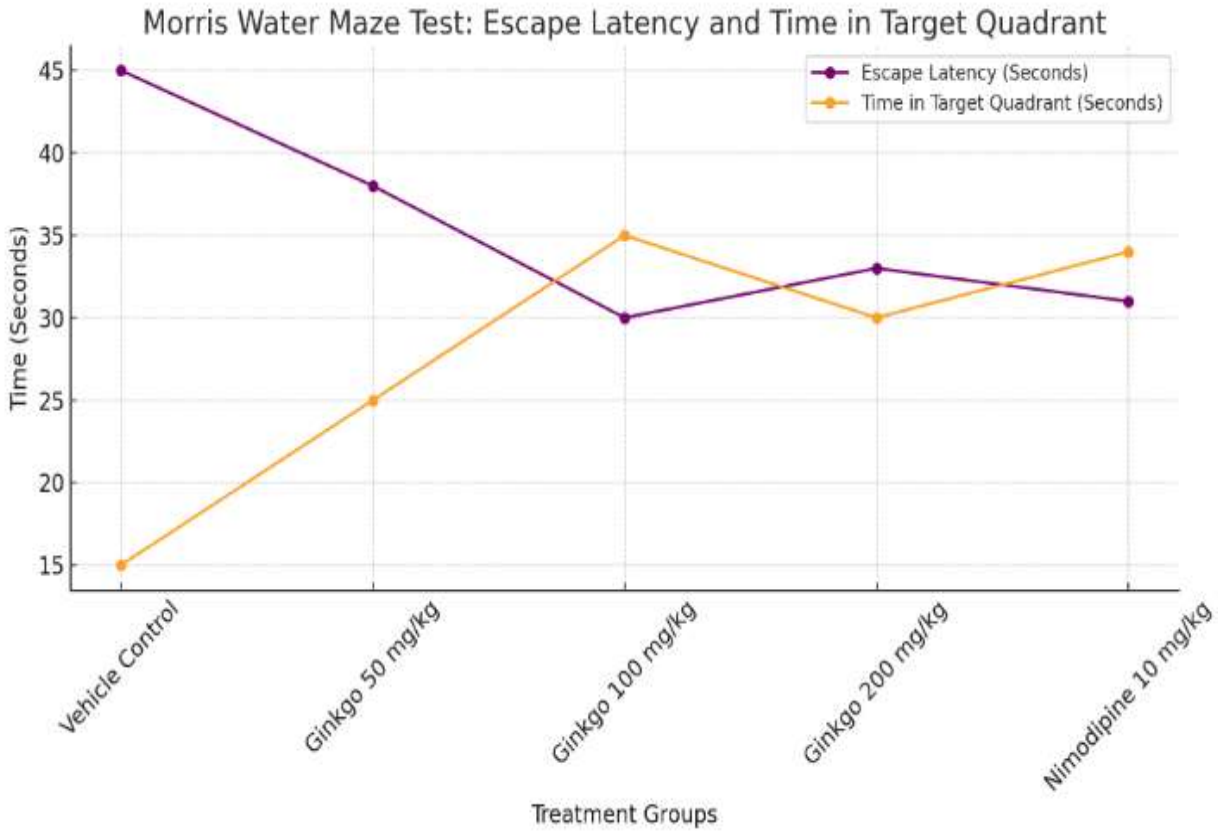
Cognitive Function

Morris Water Maze (MWM)

Mice treated with 100 mg/kg of Ginkgo biloba demonstrated improved spatial learning and memory, evidenced by a 30% reduction in escape latency compared to vehicle controls. During the probe trial, these mice spent significantly more time in the target quadrant, indicating enhanced memory retention. The 50 mg/kg and 200 mg/kg groups also showed improvements, though less pronounced than the 100 mg/kg group.

Novel Object Recognition (NOR)

In the NOR test, the 100 mg/kg group achieved the highest discrimination index (DI = 0.65), showing a marked preference for the novel object, compared to the vehicle control group (DI = 0.40). This suggests enhanced recognition memory with Ginkgo biloba treatment.



Discussion:-

MCAO Optimization

The 60-minute occlusion duration was identified as optimal, providing consistent infarct volumes and a manageable mortality rate. This duration reliably induced ischemia in the cortex and striatum, as confirmed by histological and neurological assessments. The MCAO model was validated as a robust platform for evaluating neuroprotective therapies, with physiological monitoring ensuring stable intraoperative conditions.

Neuroprotective Effects of Ginkgo biloba

Neuronal Survival

Ginkgo biloba significantly increased neuronal survival, particularly at the 100 mg/kg dose. The preservation of neurons in the ischemic penumbra indicates that Ginkgo biloba mitigates cell death pathways such as oxidative stress and apoptosis. The 100 mg/kg dose emerged as optimal, providing the most neuroprotection without the mild toxicity observed at the higher 200 mg/kg dose. The neuroprotective effects of Ginkgo biloba were comparable to nimodipine, a standard treatment for ischemic stroke.

Neurotrophic Factor Expression

Ginkgo biloba treatment upregulated BDNF and NGF levels, which are crucial for neuronal survival, synaptic plasticity, and cognitive function. The significant increase in neurotrophic factors at the 100 mg/kg dose corresponds with improvements in neuronal survival and cognitive function, highlighting Ginkgo biloba's dual role in neuroprotection and neurogenesis.

Cognitive Function

Improved cognitive outcomes in Ginkgo biloba-treated mice, as demonstrated in the Morris water maze and novel object recognition tests, further support its neuroprotective efficacy. Enhanced spatial and recognition memory in the 100 mg/kg group suggests that Ginkgo biloba improves synaptic plasticity, likely through modulation of neurotrophic factor pathways.

Dose Optimization and Comparison with Standard Treatments

The 100 mg/kg dose was identified as the optimal dose for neuroprotection, providing a balance between efficacy and safety. Although the 50 mg/kg dose showed moderate neuroprotection, the effects were less pronounced. The 200 mg/kg dose, while effective, was associated with mild toxicity, indicating diminishing returns at higher doses. Comparisons with nimodipine demonstrated that Ginkgo biloba offers comparable neuroprotective effects, with the additional benefit of enhancing neurotrophic factor expression and promoting neurogenesis.

Toxicity Evaluation

The mild toxicity observed at the 200 mg/kg dose underscores the importance of dose optimization. Behavioral lethargy and reduced weight gain were noted, highlighting the need for careful dose selection in future studies. Monitoring of general health and behavioral assessments was essential for early detection of toxicity, ensuring accurate interpretation of neuroprotective efficacy.

Conclusion:-

This study successfully optimized the MCAO model in C57BL6/J mice and demonstrated that Ginkgo biloba provides significant neuroprotection, particularly at the 100 mg/kg dose. Ginkgo biloba enhances neuronal survival, cognitive function, and neurotrophic factor expression, with minimal toxicity at the optimal dose. These findings support further exploration of Ginkgo biloba as a potential therapeutic agent for ischemic stroke, either as a standalone treatment or in combination with existing therapies such as nimodipine.

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