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The Effects of *Taraxacum officinale* On TNF-alpha Gene Expression and Behavioral Tests in Brain Ischemia-Reperfusion Injury in Male Rats

A Thesis Submitted to the Council of the College of Medicine,
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the Degree of Master in Pharmacology \ Pharmacology and Toxicology

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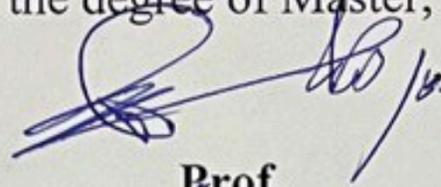
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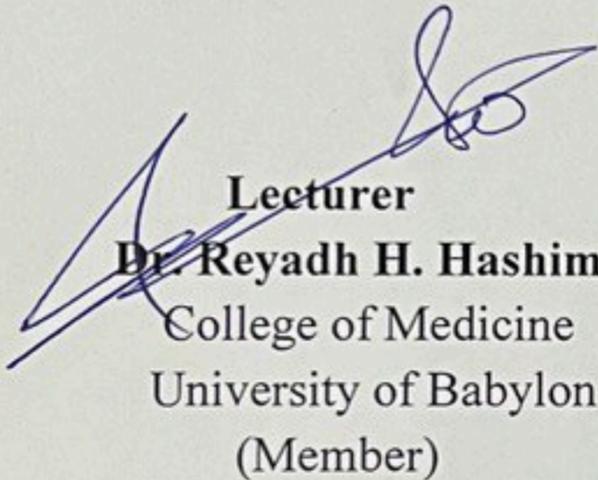
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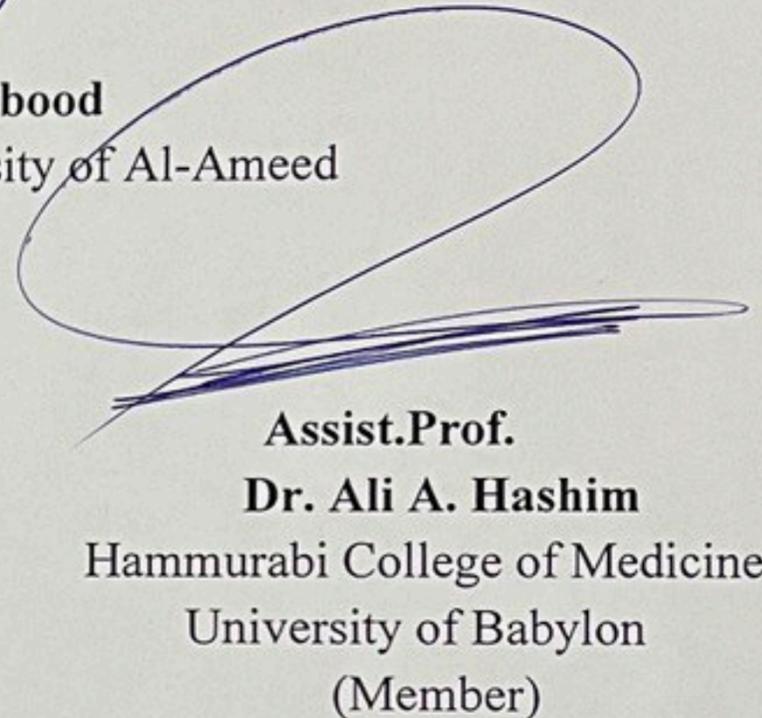
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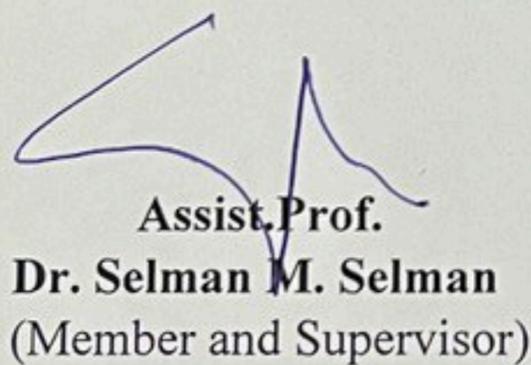
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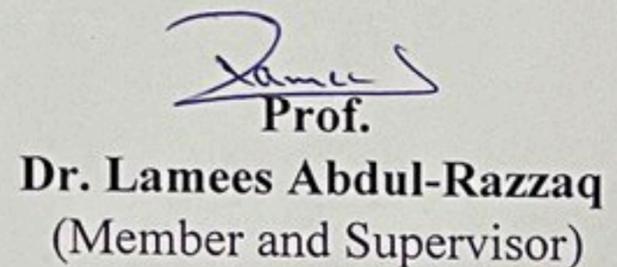
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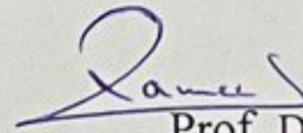


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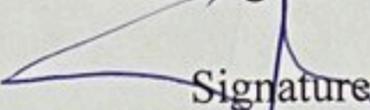
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Summary

A stroke is a sudden and severe brain illness. It is a disorder caused by a hemorrhage or blockage of the cerebral arteries in the brain. Stroke is the second greatest cause of death in the world, as well as one of the top causes of disability. Cerebral ischemia–reperfusion injury (IRI) is a complex process resulting in cellular damage and death. Ischemia and reperfusion in the brain, as in other organs, induces an inflammatory response which may exacerbate initial levels of tissue injury. Tumor necrosis factor-alpha (TNF-alpha) is the major pro-inflammatory cytokine that causes inflammatory reactions in the brain after a stroke. Recently, herbal plants, e.g. *Taraxacum officinale*, are gaining a great deal of attention in stroke because of their safety, efficacy, and cost-effectiveness.

The aim of this study is to investigate the effect of *T.officinale* against cerebral ischemia/ reperfusion injury in male rats brain and to determine the effect of *T.officinale* on behavioral tests and the gene expression of TNF-alpha in cerebral I/R injury in the male rat.

Fifty male rats were divided into five groups, ten rats in each one. Sham group, rats subjected to anesthesia and surgical procedure but ruled out the bilateral common carotid artery occlusion (BCCAO). Control- vehicle group, rats subjected to anesthesia and BCCAO for half-hour in addition to oral administration of vehicle (distilled water) for two hours before (I/R) induction. Groups 3, group 4 and group 5 (treated groups with 200, 300 and 400mg/kg *T.officinale* respectively), these groups will receive 200, 300 and 400 mg/kg for 21 days orally by a Gavage. Then, 24 hours after the last dose, each animal should subject to BCCAO. Following 24 h of I/R induction for five groups, the open field and forced swimming tests should applied for all groups to study all the animals in terms of depressive-like behaviors. Twenty-four hour after behavioral tests, the rats should

sacrificed by decapitation immediately after anesthesia. The brains were then removed for histopathological analysis and the TNF-alpha gene expression measurement.

The results of this study in open field tests show significant decrease in travelled distance, rearing and time spend in central area in control -vehicle group as compared with the sham group. While in treated groups 3,4 and 5 travelled distance, rearing and time spend in central area significantly increased as compared with the control -vehicle group. While in forced swimming test the mean immobility time of the control-vehicle group in forced swimming test was significantly increased ($p\text{-value} < 0.05$) as compared with the sham group. While in treated groups 3,4 and 5 the mean immobility time was significantly decreased as compared with the control -vehicle group. The induction of cerebral (I/R) increased histopathological damage score significantly ($P < 0.05$) in the control-vehicle group comparing with the sham group. While in treated groups 3,4 and 5 significantly ($P < 0.05$) improved the severity of cerebra-histopathological damage as compared with control-vehicle group. RT-PCR shows a significant increase in mean of fold change of TNF-alpha in c-vehicle group as compared with sham group while shows a significant decrease in mean of fold change of TNF-alpha in the groups that received 200 ,300 and 400 mg\kg of *T.officinale* for 21 days as compared with the control- group which received no treatment.

In conclusion, *T.officinale* might have neuroprotective effect via anti-inflammatory mechanism in addition showed decrease in fold change of TNF-alpha gene expression. Also *T.officinale* might have antidepressant and anxiolytic effects.

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List of Abbreviation

Abbreviation	Meaning
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ASK1	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
BBB	Blood brain barrier
Bdnf	Brain-derived neurotrophic factor
BH4	Tetrahydrobiopterin
BMI	Body mass index
°C	Centigrade
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CIRI	Cerebral ischemia-reperfusion injury
COVID-19	Coronavirus disease
Cox-1	Cyclooxygenase 1
CT	Cycle threshold
C-Vehicle	Control-Vehicle
D.W	Distilled water
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FST	Forced swimming test
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPX	glutathione peroxidase
H ₂ O ₂	Hydrogen peroxide
HDL	High density lipoprotein
HK	Housekeeping

HNE	4-hydroxynonenal
I / R	Ischemia/reperfusion
ICAM-1	Intercellular adhesion molecules
ICH	Intracerebral hemorrhage
IDO	Indoleamine 2, 3-dioxygenase
IL-1	Interleukin -1
IL-6	Interleukin-6
IL-8	Interleukin 8
INF	Interferon
iNOS	Inducible nitric oxide synthase
KA	Kainite
kg	kilogram
LPS	Lipopolysaccharide
LSD	Lysergic diethylamide
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDD	Major depressive disorder
mg	Milligram
Mkp-1	Mitogen-activated protein kinase phosphatase-1
ml	Milliliter
MMP	Matrix metalloproteases
MPTP	Mitochondrial permeability transition pore
NF- κ B	Nuclear factor kappa Beta
Ng	Nanogram
Nm	Nanometer
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
O ⁻²	Superoxide anion

OFT	Open field test
OH	Hydroxyl radicals
PCP	Phencyclidine
PSA	Post stroke anxiety
PSD	Post stroke depression
qPCR	The quantitative Real-Time PCR
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROO ⁻	Peroxyl radical
ROS	Reactive oxygen species
RPM	Revolution per minute
RT-PCR	Real-Time Polymerase Chain Reaction
SE	Standard error
SOD	Superoxide dismutase
SOD	Superoxide dismutase
SPSS	Statistical package for the social sciences
T.officinale	Taraxacum Officinale
TGF-b	Transforming growth factor beta
TNF- α	Tumor necrosis factor -alpha
TNFR1	TNFR1-associated death domain
tpa	Tissue plasminogen activator
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
WHO	World Health Organization
μ L	Microliter

Chapter One
Introduction and
Literature Review

1.1 Introduction

Stroke is a major health problem that occurs when a blood vessel carrying blood to the brain becomes clogged or ruptures, causing bleeding. Stroke is the second greatest cause of death in the world, as well as one of the top causes of disability. Stroke can result in muscle weakness, numbness, incontinence, apraxia, difficulties carrying out everyday activities, vision loss, speech loss, and discomfort, depending on the size and location of the lesion on the brain. Recovery after a stroke continues to receive a lot of attention because of its major impact on population health (Gorman and Gustafsson 2020).

Ischemic and hemorrhagic strokes are the two most common forms of stroke. Hemorrhagic strokes are much less common than ischemic strokes. Hemorrhagic stroke caused by bleeding or leaky blood vessels, whereas ischemic stroke caused by a lack of blood and oxygen delivery to the brain (Kuriakose and Xiao 2020).

Ischemic stroke has a different incidence in men and women (according to the burden research, women had 99 cases per 100,000 people per year, whereas men have 133 cases per 100,000 people per year, resulting in a stroke incidence of roughly 25 -30% in men). The protective impact of estrogen on cerebral circulation or hereditary variables may account for the low incidence rate of stroke in women, however, these differences narrowed with age (Feigin, Norrving, and Mensah 2017).

Ischemia and reperfusion is a pathological condition, which is characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of perfusion and concomitant reoxygenation. After a stroke, the inflammatory response is also considered to be responsible for ischemia/reperfusion injury(I/R) (Wang et al. 2018).

In the clinic, restoring blood reperfusion as soon as possible helps to reduce cerebral ischemia injury, but subsequent reperfusion can produce cerebral edema, brain hemorrhage, and neuronal death, resulting in brain injury (Lyu et al. 2018).

Following I/R, neuronal function in the brain is threatened by three major factors. First, excitotoxicity caused by a lack of energy and oxygen, which resulted in an excess of calcium ions inside neurons and the release of excitatory glutamate neurotransmitters into the extracellular area. The second oxidative and nitrosative stress, by which free radicals are constantly created in the mitochondria as a result of oxidative phosphorylation, even though they serve essential functions in physiological quantities. Enzymatic antioxidants such as superoxide dismutase (SOD) and non-enzymatic components such as glutathione control the number of free radicals in the body.

The ratio of oxidants to antioxidant components in favour of oxidant collapses in ischemic stroke, resulting in oxidative stress (J. Ma, Liu, and Shi 2018). Neuro-inflammation, which plays a central role in the etiology of stroke, is the third major hazard to neuronal function after ischemia-reperfusion (I/R). TNF- α is the major cytokine that causes inflammatory reactions in the brain after a stroke (Farhadi Moghadam and Fereidoni 2020). Common carotid artery occlusion causes global ischemia (Rabea et al. 2021).

The World Health Organization (WHO) encourages the use of traditional medicinal herbs for both treatment and prevention. However, To our knowledge no experimental research has been done on *Taraxacum Officinale's* ability to prevent cerebral I/R injury in rats. *T. Officinale* (Dandelion) was chosen for this study because it is a perennial herb belonging to the composite family (Asteracea), a weed that may be found practically anyplace. It provides antioxidant, anti-inflammatory, and free radical scavenging effects. Because of its higher phenolic and flavonoid content, *T. Officinale* has excellent radical scavenging activity, antioxidant capacity, and anti-inflammatory properties (Aremu et al. 2019).

1.2 Aims of the study

This study aimed to :-

1. Investigate the effect of *T.officinale* against cerebral I/R injury in male rat brain.
2. Determine the effect of *T.officinale* on behavioral tests and the gene expression of TNF- α in cerebral in I/R injury in the male rat.

1.3 Stroke

A stroke is a sudden and severe brain illness. It is a disorder caused by a hemorrhage or blockage of the blood arteries in the brain. a scarcity of Brain blood flow promotes brain cell malfunction, oxidative stress, and neurological impairment (Dong et al. 2020).

1.3.1 Epidemiology of stroke

Stroke continues to be one of the major causes of death and disability in the world, accounting for over 113 million disability-adjusted life years. 6.5 million people die every year. A significant increase in the number of people who have had a stroke has occurred around the world (Xing et al. 2021).

The incidence of ischemic stroke in young individuals varies greatly, ranging from 7–8 per 100 000 person-years in Europe to more than 100 per 100 000 person-years in Sub-Saharan Africa (Egger et al. 2018). In comparison to Western Europe, the Americas, and Australasia, Asia has a higher rate of stroke mortality (Venketasubramanian et al. 2017). Ischemic stroke now affects 60% of people under the age of 70 and 7% of people under the age of 44, with 52 percent of men and 48

percent of women (Lindsay et al. 2019). The incidence of stroke rises in lockstep with age, and this rise is dependent on the modifiable factor being recognized earlier, allowing the stroke or at least its complications to be prevented or minimized (Al-Asadi and Habib 2014).

Stroke during pregnancy is uncommon, but it does happen. Hypertensive disorders of pregnancy raise the risk of ischemic stroke, intracerebral hemorrhage, and cerebral venous sinus thrombosis. Pregnancy is a prothrombotic state that increases the risk of stroke. Stroke is expected to affect about 30 every 100000 pregnancies, while studies vary widely (rates range from 10 to 65 per 100000 pregnancies)(George 2020).

1.3.2 Pathophysiology of stroke

Ischemic and hemorrhagic strokes are the two most frequent forms of stroke. Hemorrhagic strokes are much less common than ischemic strokes. According to new numbers from the American Heart Association(J. Yang et al. 2018). Ischemic stroke (brain infarction) is produced by thrombus or emboli occluding the cerebral arteries, denying neuronal cells the oxygen and nutrients they need to function normally (Mustroph et al. 2019). In thrombosis, the blood flow is affected by the narrowing of vessels due to atherosclerosis. Plaque buildup will eventually narrow the vascular chamber and cause clots, resulting in a thrombotic stroke. Reduced blood supply to the brain region creates an embolism in an embolic stroke; blood flow to the brain decreases, producing acute stress and premature cell death (necrosis). Following necrosis, the plasma membrane is disrupted, organelles enlarge, cellular contents leak into the extracellular environment, and neuronal function is lost (Kuriakose and Xiao 2020). While the other type (hemorrhagic

stroke) is caused by intracranial hemorrhage, it has the highest fatality rate with no effective treatments, accounting for just around 15% of all stroke cases (Zille et al. 2017). Blood vessels rupture as a result of the tension on the brain tissue and the internal injuries. It causes vascular toxicity, which leads to infarction. Intracerebral and subarachnoid hemorrhages are the two types of hemorrhages. Blood vessels in the brain rupture with intracerebral hemorrhage (ICH), causing an abnormal accumulation of blood. Hypertension, disturbed vasculature, and the use of anticoagulants and thrombolytic medicines in excess are the main causes of ICH. In subarachnoid hemorrhage, blood accumulates in the subarachnoid area of the brain due to a head injury or cerebral aneurysm (Kuriakose and Xiao 2020).

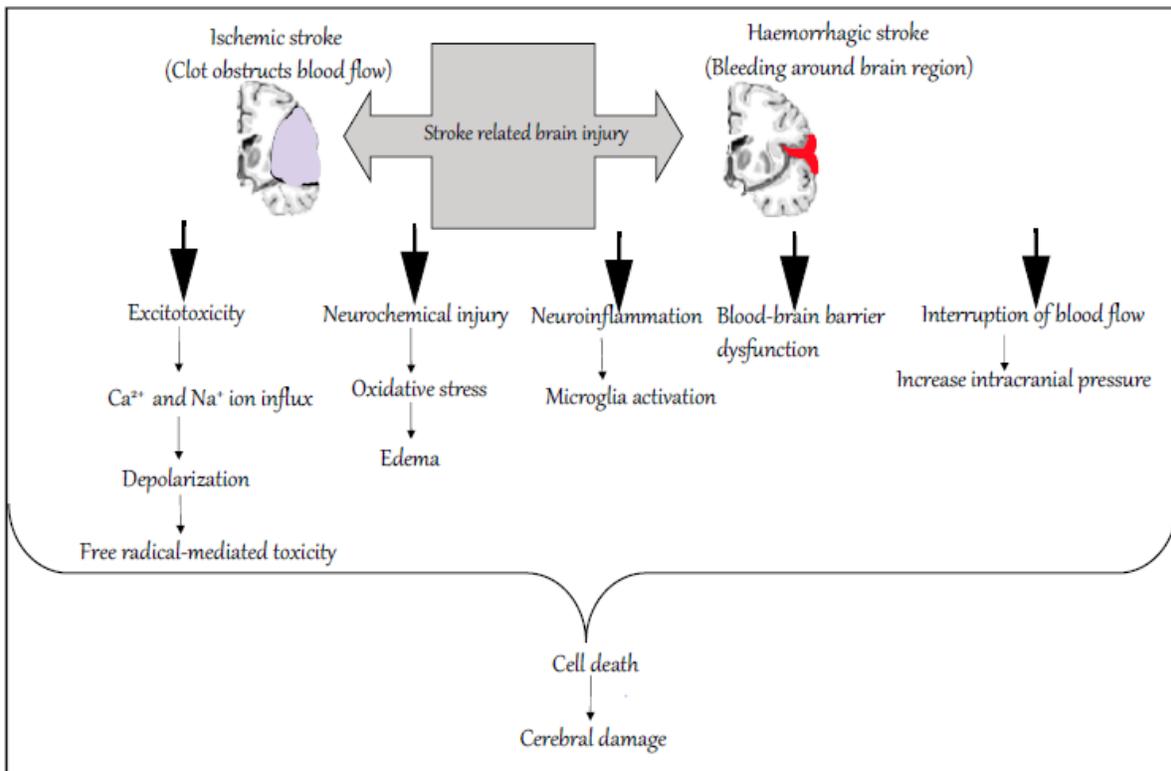


Figure 1.1: Molecular mechanism of stroke (Kuriakose and Xiao 2020)

1.3.3 Major causes of stroke

1. Atherosclerosis: It is include

- Aortic arch or cervical arteries
- Intracranial arteries

2. Cardioembolism: It is include

- Atrial fibrillation
- Akinetic myocardial segment
- Patent foramen ovale
- Endocarditis (Murphy and Werring 2020)

3. Small vessel disease

Other causes:

1. High blood pressure, smoking, obesity, and diabetes
2. Other arterial diseases (eg, dissection, vasculitis)
3. Haematological diseases (eg, antiphospholipid syndrome, polycythemia rubra vera, essential thrombocytosis)
4. Inflammation can also cause a stroke (eg, inflammatory arteriopathy after infection is a major cause of paediatric stroke and can also occur after herpes zoster in adults) (Campbell and Khatri 2020).

1.3.4 Signs and symptoms of stroke

A brain attack is another term for a stroke. When the brain is damaged as a result of an interruption in blood supply to a specific area of the brain (Campbell

and Khatri 2020), this condition occurs. The following are some of the most prevalent stroke signs and symptoms:

- 1) A sudden weakness of an arm or limb on one side of the body, an uneven smile, or facial numbness.
- 2) Slurred speech, difficulty speaking or understanding.
- 3) Blurry or double vision in one or both eyes, homonymous hemianopia
- 4) Dizziness, vertigo, inability to walk in a straight line, and a loss of balance or coordination.
- 5) Severe headache with no apparent explanation, as well as vomiting or passing out (Poopitukkul, Jokkrajai, and Ruangkhruawong 2018)(Soto-Cámara et al. 2020).

1.3.5 Risk factors for ischemic stroke

There are many risk factors for cerebral ischemia which can be divided into non-modifiable and modifiable factors (Johansson et al. 2021)

1.3.5.1 Non-modifiable risk factors

1. Age

Is the most strong non-modifiable risk factor for ischemic stroke, and older stroke patients had worse mortality, morbidity, and functional recovery than younger stroke patients. (Roy-O'Reilly and McCullough 2018).

2. Sex

Premenopausal women have a stroke risk that is as high as or higher than men's due to the dangers of pregnancy and oral contraceptive use. Stroke rates are slightly greater in men as they get older. Strokes affect more women than males in the United Kingdom (Murphy and Werring 2020).

3. Ethnicity

In the United Kingdom and the United States, African Caribbean people have twice the risk of having an incident stroke as their white counterparts. The risk of intracerebral hemorrhage is twice as high in younger black adults as it is in age-matched white individuals. This could be related to the greater prevalence of stroke risk factors among African Caribbean populations, such as uncontrolled hypertension, obesity, and diabetes (Albright et al. 2018).

4. Genetics

Both modifiable and non-modifiable risk factors for stroke are genetically influenced. The individual's genetic risk is proportionate to his or her age, gender, and race (Seshadri et al. 2010), Stroke risk can be increased by a variety of hereditary causes. To begin with, having a parent or family member who has had a stroke raises the risk of acquiring this neurological illness. Second, in cases where the stroke is the major clinical manifestation, such as cerebral autosomal dominant arteriopathy, a rare single gene mutation can play a role in pathogenesis. Third, stroke is one of several consequences of numerous disorders induced by genetic mutations, such as

sickle cell anemia. Finally, several common genetic variations, such as the 9p21 polymorphism, have been linked to an elevated risk of stroke (Matarin et al. 2008).

1.3.5.2 Modifiable risk factors

1. Hypertension

It's one of the most common causes of stroke. Both hypertensive and nonhypertensive people have a link between blood pressure and the risk of stroke. According to one study, lowering blood pressure by 5–6 mm Hg reduced the risk of stroke by 42%. Randomized trials of interventions to reduce hypertension in people aged 60+ have shown similar results, lowering the incidences of symptoms of stroke by 42% (Kim et al. 2019).

2. Diabetes

It increases the risk of ischemic stroke by double and increases mortality by about 20%. Furthermore, diabetes people have a worse prognosis after a stroke than non-diabetic patients, with a higher incidence of severe impairment and delayed recovery. Glycemic control alone is inadequate; medicinal intervention combined with behavioral changes could assist diabetics to reduce the severity of their stroke (Banerjee et al. 2012).

3. Hyperlipidemia

It is a major cause of coronary heart disease, but the link between it and stroke remains unclear. Total cholesterol is linked to a higher risk of stroke because high cholesterol levels can restrict blood arteries, whereas high-density lipoprotein (HDL) lowers the risk of stroke. Low HDL levels (0.90 mmol/L), high total triglyceride

levels (>2.30 mmol/L), and hypertension were linked to a two-fold increase in the risk of stroke-related death in general population (Kuriakose and Xiao 2020).

4. Smoking

Tobacco use is associated with an increased risk of stroke. A smoker has twice the risk of having a stroke as someone who does not smoke (Hou et al. 2017). Because cigarettes include nicotine, they can produce cerebral vasoconstriction, trigger inflammatory processes through increased quantities of leukocytes, and improve their migration, rolling, and adhesion in the cerebral microvasculature, as well as raise C-reactive protein and fibrinogen levels. Smoking is responsible for 15% of stroke-related deaths. According to research, stopping smoking lowers the relative risk of stroke, however chronic second-hand smoking raises the risk of stroke by 30% (B. Pan et al. 2019).

5. Alcohol and drug abuse

The risk of stroke is connected to the amount of alcohol drunk daily, and the relationship between them follows a curvilinear pattern. Low to moderate alcohol consumption (2 standard drinks per day for men and 1 drink per day for women) lowers the risk of stroke, due to low alcohol intake has neuroprotective via inhibition of ischemia inducing ICAM-1, P-selectin, and L-selectin decreased brain damage, and improved neurological function (McCarter et al. 2019). High intake, on the other hand, raises it (Kuriakose and Xiao 2020). Use of illegal substances such as cocaine, heroin, phencyclidine (PCP), lysergic acid diethylamide (LSD), cannabis/marijuana, or amphetamines regularly is linked to an elevated risk of stroke in all subtypes (Esse et al. 2011).

6. Body mass index and physical exercises

Increased body mass index is linked to other risk factors like hypertension and diabetes, making it a risk factor for stroke. Lack of exercise has been linked to an increased risk of stroke, according to research (Murphy and Werring 2020).

7. Inflammation

Increased inflammatory biomarkers are linked to a higher risk of arteriosclerosis and stroke. Infection can cause a stroke, and there is evidence that people who have been vaccinated against influenza have fewer strokes. Large artery occlusions have been connected to Coronavirus disease (COVID-19), which is associated with a hyperinflammatory and hypercoagulable state (Murphy and Werring 2020).

1.3.6 Cerebral ischemia-reperfusion

Although the brain only accounts for roughly 2% of total body weight, it requires about 20% of oxygen and 25 to 50% of the body's glucose. The largest energy demands are found in brain neurons, which necessitate continual oxygen and glucose delivery from cerebral blood flow. During cerebral ischemia, however, the blood supply of oxygen and glucose to the brain tissue is significantly reduced (L. Li et al. 2018). As a result, ischemia affects the brain more than any other organ. Only 5 minutes of complete ischemia causes susceptible neurons in various brain regions to die, whereas cardiac myocytes or kidney cells take 20–40 minutes of ischemia to die (Lee et al. 2000). The hypoperfusion of brain tissues is known as cerebral ischemia. (M.-Y. Wu et al. 2018). It is caused by artery narrowing, which causes neurological impairments as well as increased morbidity and mortality. Lack of oxygen and nutrient supply leads to BBB disruption, inflammation, and ATP

depletion (Liao et al. 2020). There are two types of cerebral ischemia: localized and global cerebral ischemia. The reduction of cerebral blood flow in a specific region of the brain is known as focal ischemia, whereas the reduction of blood flow in the entire brain or forebrain is known as global ischemia (Dai et al. 2018). Cardiac arrest, severe hypotensive shock, hypoxia, and difficult cardiac surgery are all common causes of global ischemia (L. Li et al. 2018). The first phase of cerebral injury, known as "ischemic injury," occurs when there is an imbalance between metabolic demand and oxygen and glucose delivery. This is followed by the "reperfusion phase," in which the cerebral blood supply is restored to the ischemic area either through thrombolysis or mechanical thrombectomy (Stegner, Klaus, and Nieswandt 2019).

Reperfusion can restore blood flow, oxygen delivery, and many other energy materials (J. Yang et al. 2018). But may cause cerebral ischemia-reperfusion injury this process bears risks of worsening the original brain damage caused by ischemia through different mechanisms including the release of free radicals, activation of an inflammatory cascade, promotion of cellular apoptosis, calcium overload, and the release of excitatory amino acids, all of which are far more complex than ischemia alone (J. Yang et al. 2018).

The pathogenic mechanisms of cerebral ischemia and cerebral ischemia-reperfusion are distinct. cerebral ischemia is mainly a hypoxic-ischemic primary injury, in which a small decrease in blood flow does not lead to obvious functional or metabolic disorders; as the ischemic time advances, the ischemic core gradually expands to the ischemic penumbra, and the infarct volume reaches its maximum(Hossmann 2008). The infarct core injury can be reversed if blood flow is restored promptly; otherwise, intervention therapy only slows the expansion of the infarct core. Furthermore, if reperfusion is delayed (by more than 3 hours), the

reversal effect is limited, resulting in a more serious ischemia-reperfusion injury. Ischemia-reperfusion in the brain primarily results in a secondary (nonischemic) kind of cell death (R. Ma et al. 2020).

1.3.6.1 Pathophysiology of ischemia/reperfusion injury

Cerebral ischemia-reperfusion injury (CIRI) is a complex pathophysiological process that can cause severe damage to brain functioning. , the major Mechanisms of cerebral reperfusion injury include Energy metabolism abnormalities, cellular acidosis, synthesis or release of excitotoxic amino acids, intracellular calcium homeostasis, free radical generation, and activation of apoptotic genes (L. Lin, Wang, and Yu 2016). These many elements interact to build a complex regulatory network, resulting in a series of pathogenic cascades that cause apoptosis/death of nerve cells, disruption of the blood-brain barrier, brain edema, and ultimately neurological impairments (Xiong, Liu, and Yang 2018). Oxidative stress occurs during cerebral ischemia-reperfusion, and a considerable number of inflammatory cytokines are present in the center and periphery of the ischemic focus.

The activation and infiltration of inflammatory cells and the synthesis and secretion of adhesion molecules are cascade reactions that reinforce and promote one another. Through certain oxidative stress and inflammatory signaling pathways, brain tissue may suffer from ischemic and oxidative stress-inflammatory injuries. Therefore, oxidative stress-inflammation plays an important role in CIRI (L. Wu et al. 2020).

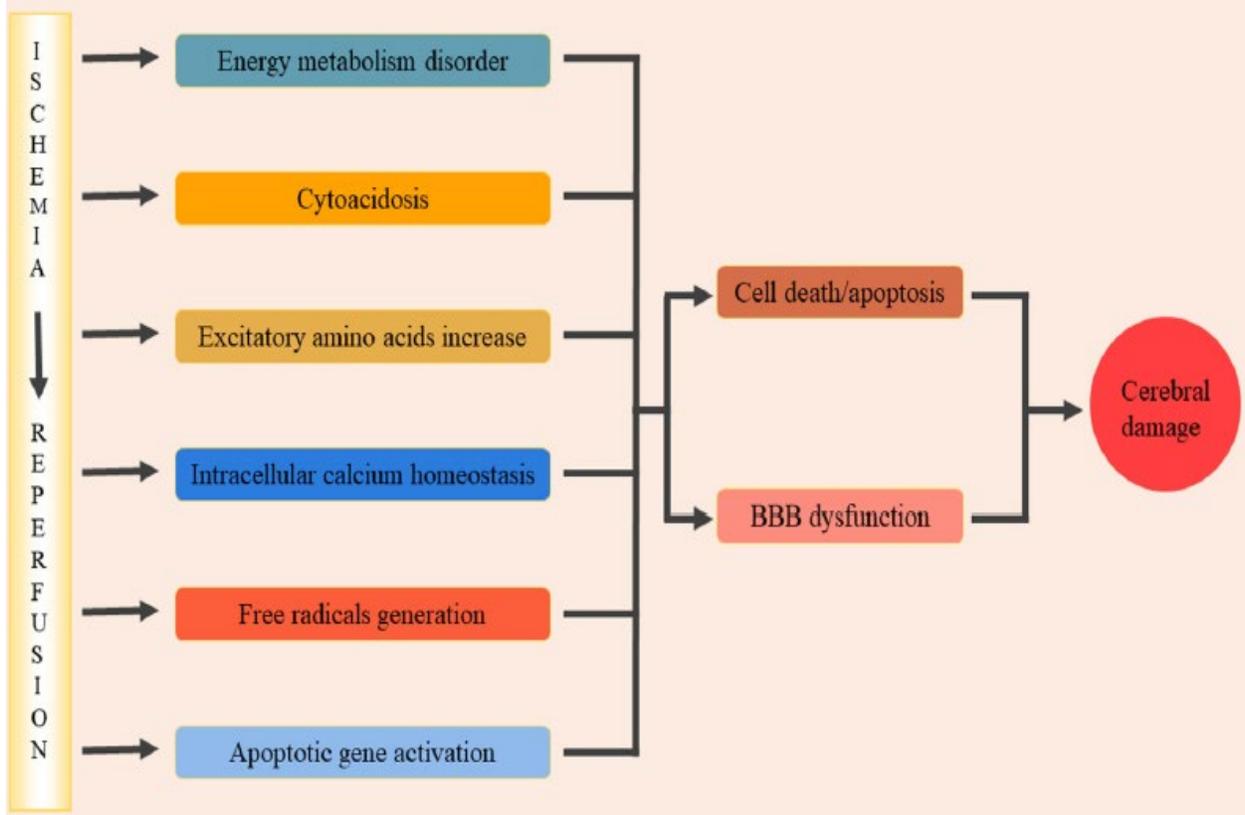


Figure 1.2 The pathophysiological mechanisms of cerebral ischemia/reperfusion injury (L. Wu et al. 2020)

1.3.6.2 Events Triggered by I/R

1.3.6.2.1 Inflammatory response after ischemia/reperfusion injury

During an ischemic stroke, inflammation is a significant factor. Several inflammatory cascades are triggered by cerebral ischemia injury and blood flow reperfusion, including inflammatory cell infiltration and the production of toxic inflammatory mediators, which lead to nerve tissue damage and cell death. The inflammatory response involves both native tissue immune cells (such as microglia) and peripheral immune cells. Understanding the effects of inflammation on the

neurological system following ischemia and reperfusion will open up a new direction for stroke treatment (Mo, Sun, and Liu 2020). Ischemia and reperfusion in the brain, as in other organs, cause an inflammatory response that may aggravate original tissue injury levels. Reactive oxygen species and proinflammatory mediators (cytokines and chemokines) are rapidly released from injured tissue during the early phase (minutes to hours) of ischemic stroke.

These mediators stimulate the adherence and transendothelial migration of circulating leukocytes by inducing the production of adhesion molecules on cerebral endothelial cells (neutrophils and monocytes). These cells may build up in capillaries, obstructing cerebral blood flow even more, or they may extravasate into the brain parenchyma, releasing neurotoxic compounds such as proinflammatory cytokines, chemokines, and oxygen-nitrogen free radicals (Amantea et al. 2009).

The blood-brain barrier is disrupted, cerebral edema occurs, and neuronal cells die as a result of post-ischemic neuroinflammatory alterations. As a result, therapeutic targeting of neuroinflammatory pathways in acute ischemic stroke has been identified as a key field of research in translational medicine (Lakhan, Kirchgessner, and Hofer 2009).

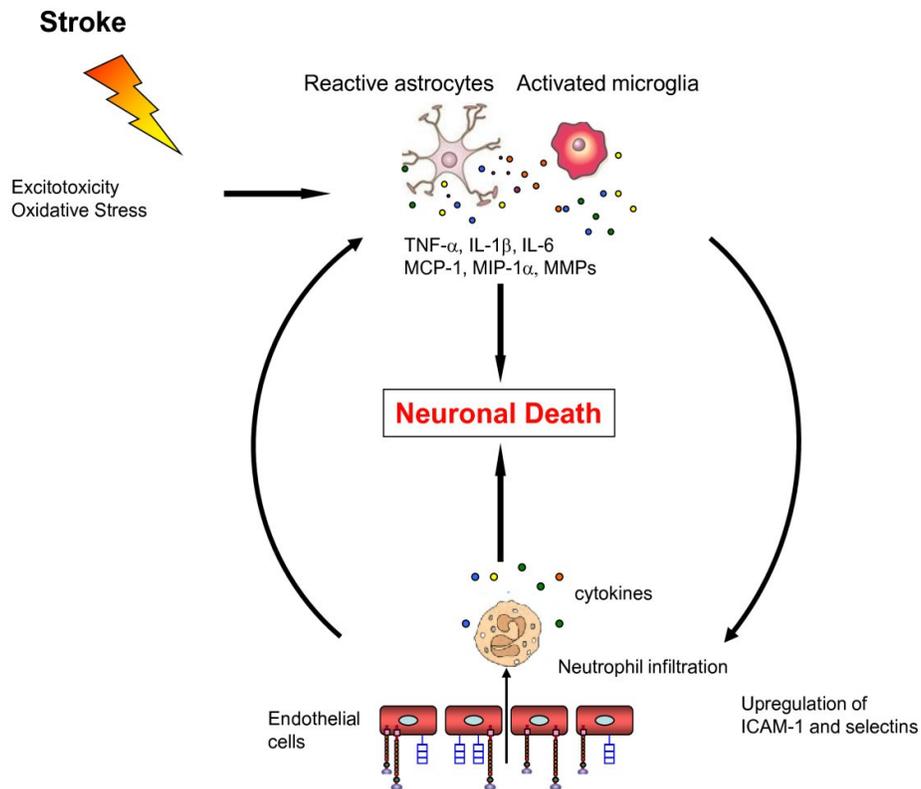


Figure 1. 3 Postischemic inflammatory response(Lakhan, Kirchgessner, and Hofer 2009).

Excitotoxicity and oxidative stress caused by the initial ischemic event activate microglia and astrocytes which react by secreting cytokines, chemokines, and matrix metalloproteases (MMP). These inflammatory mediators lead to an upregulation of cell adhesion molecules on endothelial cells, allowing blood-derived inflammatory cells, mainly neutrophils, to infiltrate the ischemic brain area. Neutrophils themselves also secrete cytokines which cause further activation of glial cells. These processes all result in neuronal cell death and enhance the damage to the ischemic brain.

1.3.6.2.1.1 Inflammatory Cells

1. Leukocytes

Neutrophils, monocytes, and lymphocytes make up the majority of the leukocyte population, and each can contribute to inflammation following ischemia (Duan et al. 2019). Neutropenia, lymphocytopenia, and an increase in circulating monocytes are all symptoms of a stroke. Neutrophils are among the first to infiltrate the ischemic brain (30 minutes to a few hours after cerebral ischemia), peak sooner (Days 1–3), and then fade away or decline significantly with time. However, infiltrating neutrophils may remain in the ischemic brain for up to 3 days following focal cerebral I/R, although their presence is most likely hidden by the large-scale buildup of activated microglia/macrophages in the inflammatory region after 3 days. After cerebral ischemia, neutrophil infiltration into the brain increases after one day, peaks after three days, and then declines for seven to fifteen days (Jin, Yang, and Li 2010).

Monocytes enter the ischemia focus after an acute ischemic stroke from three different sources: circulatory monocytes, splenic monocytes, and bone marrow monocytes. In the early stages of ischemia, the spleen's monocytes can rapidly enter the circulation. The BM's neonatal monocytes, on the other hand, take a long time to enter circulation. During the early stages of AIS, monocytes circulating in the blood vessels migrate to the brain parenchyma and differentiate into tissue macrophages, which remove necrotic cell debris and produce a variety of cytokines, including interleukin-1 (IL-1), interleukin-6 (IL6), and TNF- α (Han, Liu, and Gao 2020). Diverse T-lymphocyte subtypes play different functions in a stroke. CD4+ TH1 cells may contribute to stroke by generating proinflammatory cytokines such as IL-2, IL-12, IFN, and TNF- α , but CD4+ TH2 cells may protect against stroke by

releasing anti-inflammatory cytokines including IL-4, IL-5, IL-10, and IL-13. (Chiba and Umegaki 2013).

2. Microglia

Microglia are central nervous system resident immune cells that can be activated by local and systemic infections, neurodegenerative diseases, and damage. Microglia can change their morphology from resting (ramified) to active (amoeboid) phase (Vidale et al. 2017). Microglial activation is enhanced by disruption of brain homeostasis. Following ischemia, inflammation is triggered by several factors such as reactive oxygen species (ROS), necrotic cells, and damaged tissues, all of which activate inflammatory cells such as microglia. Activated microglia have been found in diverse forms in previous research. Microglia of the M1 type (classical) are proinflammatory, releasing TNF- α , IL-1 β , IL-18, and IL-6. M2-type (alternative) microglia, on the other hand, are the healing cells essential in neuroprotection and repair, generating TGF- β , nerve growth factor, and IL-4. The M1 type is present in the ischemic core during the first 24 hours, and its number increases over the first two weeks after the ischemia injury. The M2 type was discovered at the end of the first 24 hours, and it entered the area through the first week before declining in number (Qin et al. 2019).

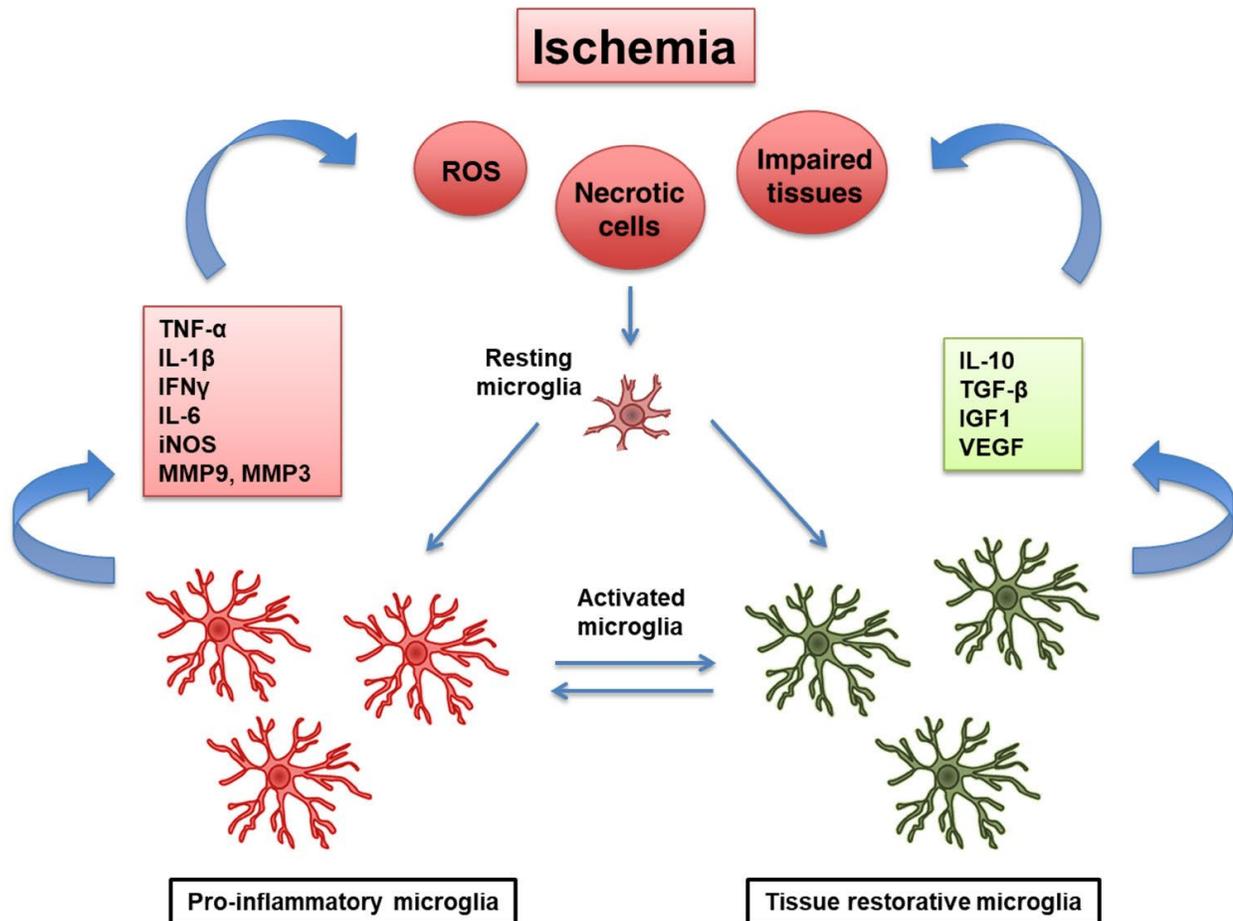


Figure 1.4: Activation of Microglia Following Ischemic Stroke (Qin et al. 2019)

In ischemic stroke, resting microglia are activated and polarized into functionally distinct phenotypes that range between two extremes. Classical microglia produce pro-inflammatory mediators including tumor necrosis factor- α (TNF- α), interleukin 1 beta (IL-1 β), interferon- γ (IFN γ), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and proteolytic enzymes (MMP9, MMP3), identified as pro-inflammatory. Alternative microglia are characterized by the production of IL-10, transforming growth factor β (TGF β), insulin-like growth factor (IGF1), and vascular endothelial growth factor (VEGF), which are pro-angiogenic and anti-inflammatory. Alternative microglia are associated with tissue repair and remodeling, immunity against parasites, and growth stimulation.

3. Astrocytes

The establishment and maintenance of the blood-brain barrier, brain homeostasis, structural support, control of cerebral blood flow, and production of neuroprotective substances are all significant functions of astrocytes. According to new research, astrocyte activation has both helpful and harmful consequences after an ischemic stroke (Sofroniew and Vinters 2010). Because they enhance extracellular glutamate absorption and sodium/potassium ATPase activity, activated astrocytes provide neuroprotection and are crucial for BBB repair and neuroprotection in the acute stages of ischemic stroke. However, Astrocyte activation also causes the release of proinflammatory mediators (e.g., IL-6, IL-1, and TNF- α) as well as the production of inflammatory mediators such as cytokines, chemokines, and cytotoxic molecules such as ROS, proteases, and inducible nitric oxide synthase (iNOS) this causes the ischemic lesion to worsen. Furthermore, astrocyte-derived inducible nitric oxide synthase (iNOS) has been found to enhance ischemia-like damage to neurons (Patabendige et al. 2021).

1.3.6.2.1.2 Role of Cytokines in cerebral I/R:

Cytokines are a diverse group of tiny glycoproteins generated from different cells (Zhang et al. 2016). They control numerous biological functions, such as innate and acquired immunity, proliferation, inflammation, and repair through extracellular signaling. Cytokines are pro- and anti-inflammatory proteins that play a key role in the progression of stroke-related inflammation. The synthesis of cytokines and the expression of their receptors are restricted to a low basal level in normal conditions. The inflammatory response is propagated following cerebral ischemia, resulting in the release of a high level of proinflammatory cytokines and up-regulation of their receptors. After an ischemic stroke, immune system cells and resident brain cells such as neurons and microglia generate cytokines (Ramiro et al.

2018). IL-6, IL-1, and tumor necrosis factor(TNF- α and TNF-beta) are the primary pro-inflammatory cytokines involved in the inflammatory response.(Pawluk et al. 2020) .

A variety of active cells, including microglia, astrocytes, and endothelial cells, produce and secrete IL-1, which is a pro-inflammatory cytokine. It has two different structures, one intracellular IL-1 α , and the other secreted IL-1 β . IL-1 β is the main form in tissue fluid, plasma, and brain tissue. It not only synergizes with other cytokines to enhance the activation of B lymphocytes and T lymphocytes, but also causes the production of other inflammatory mediators, strengthens the adhesion of leukocytes to endothelial cells, and can induce IL-1 β production, upregulate ICAM-1 expression, enhance leukocyte adhesion to vascular endothelial cells, increase neutrophil adhesion to endothelial cells, and cause leukocytes to accumulate in the ischemic area and promote inflammation. Ischemic damage to the brain is worsened by this reaction (L. Wu et al. 2020).

1.3.6.2.2 Oxidative stress

Any chemical molecule with an unpaired electron in an atomic orbital is referred to as a free radical. The existence of an unpaired electron causes most radicals to have certain features in common. Many radicals are highly reactive and unstable. They can either give or absorb electrons from other molecules, causing them to act as oxidants or reductants (Lobo et al. 2010).

There are two types of free radicals: reactive oxygen species and reactive nitrogen species. During I/R , ROS and RNS play important roles in a variety of pathogenic processes (Sun et al. 2018).

The pathological process of tissue damage induced by excessive production or diminished scavenging capability of reactive oxygen species (ROS) during

metabolic activities, resulting in an imbalance between oxidative and antioxidant systems, is referred to as oxidative stress (L. Wu et al. 2020).

The brain is a target for a variety of reasons, including high lipid concentrations, low amounts of protective antioxidants, high oxygen consumption, and dopamine and glutamate oxidation processes (Allen and Bayraktutan 2009). Radiation, chemicals, hypoxia, inflammatory cells, cell aging, and I/R are all variables that lead to the creation of free radicals inside the cell. All of these factors eventually lead to cell injury and death by necrosis, apoptosis, or necroptosis (Kumar, Abbas, and Aster 2018).

Superoxide anion ($O_2^{\cdot-}$) hydroxyl radicals (OH), and hydrogen peroxide (H_2O_2) are the three most harmful kinds of ROS. Superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, and other antioxidant enzymes can protect brain tissues from ROS cytotoxicity by catalysis in the physiological condition, maintaining a neutral balance. However, in ischemia-reperfusion, free radicals are highly generated (i.e. in numbers that exceed the antioxidant system's ability), resulting in an accumulation of ROS, especially during the reperfusion phase (Sun et al. 2018).

ROS are produced in excess through four different routes: the mitochondrial chain respiratory chain; NADPH oxidases; the reaction of arachidonic acid catalyzed by cyclooxygenase 2; and xanthine and hypoxanthine via xanthine oxidase (Margail, Plotkine, and Lerouet 2005).

ROS bind to DNA and generate passive DNA damage, which results in base alteration and single-strand breaks, triggering apoptosis. Peroxyl radical ($ROO\cdot$) is formed when OH reacts with unsaturated fatty acids, which can induce passive DNA damage. Protein dysfunction can be caused by lipid peroxidation products such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein. Furthermore, lipid peroxidation causes mitochondrial enlargement by increasing membrane

permeability. P53 activated by ROS can potentially promote mitochondrial swelling by opening the mitochondrial permeability transition pore (MPTP) via P53-cyclophilin interaction (Cyp D). By reacting with Bcl-2 family proteins, P53 causes cytochrome c (Cyt C) to be released from mitochondria, resulting in a caspase cascade that causes apoptosis. Furthermore, ROS can activate the apoptosis signal-regulating kinase 1 (ASK1)-activated c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, which lead to apoptosis (Sun et al. 2018).

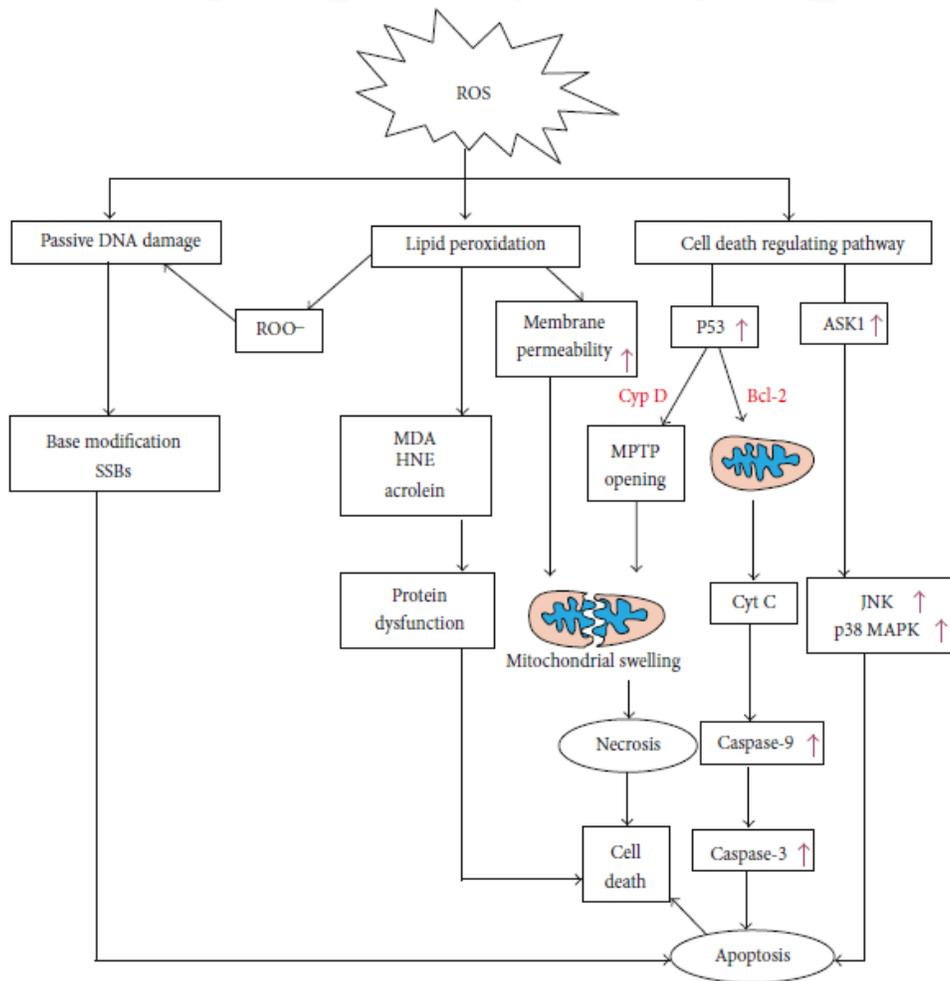


Figure 1.5: ROS damage in ischemia-reperfusion injury (Sun et al. 2018)

1.3.6.2.3 Glutamate Excitotoxicity and role of Ca²⁺ ion in I/R injury

Glutamate is a main excitatory amino acid in the central nervous system that plays a role in a variety of physiological functions of neurons and synapses, including cell survival and death, motor function, learning, and memory, as well as a variety of diseases such as neurodegenerative disease, epilepsy, and stroke. (Mayor and Tymianski 2018). The control of neurotransmitter homeostasis is one of the most important tasks of astrocytes in the CNS, as they absorb synaptically produced neurotransmitters like glutamate, metabolize them, and release their precursors back to neurons (Mahmoud et al. 2019).

As with a stroke, occlusion of a primary cerebral artery lowers blood supply to the brain and produces cerebral ischemia. As a result, neuronal damage occurs due to a lack of glucose and oxygen. The injury occurs when oxidative phosphorylation is inhibited, resulting in a decrease in adenosine triphosphate (ATP) generation, inactivation of the Na⁺/K⁺-ATPase, and an excessive release of glutamate, among other alterations (Pineda-Ramírez et al. 2020).

On the postsynaptic receptors of vulnerable neurons, there are two types of glutamate receptors: ionotropic type receptors (ligand-gated ion channels) and metabotropic type receptors (mGluR) (coupled to cellular G-protein via GTP-binding proteins). Based on which ligand preferentially activates the receptor, the ligand-gated ionotropic type is further split into three categories (NMDA), (AMPA), and kainate (Scheefhals and MacGillavry 2018).

Although activation of mGluRs also causes a release of calcium from the endoplasmic reticulum to the cytosol through activation of several second messengers, excitotoxic damage is mostly mediated by the NMDA subtype of ionotropic receptors. Collectively, When glutamate activates both NMDA and mGluR, a large amount of calcium is released into the more sensitive neurons, activating enzymes like oxidase, endonuclease, and protease, phospholipase that can

hydrolyze lipids, proteins and destroy the cytoskeleton. Besides, A large amount of calcium stimulates protein kinase C, which changes the activity of several ion channels (Sanganalmath et al. 2017).

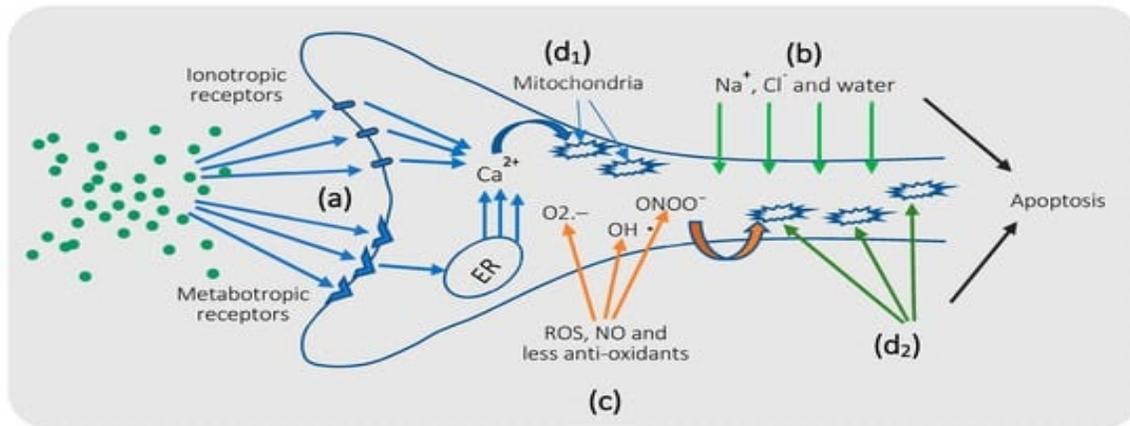


Figure 1.6: Molecular mechanisms of glutamate excitotoxicity (Mahmoud et al. 2019)

(a) Glutamatergic receptor hyperactivation: (a) Extracellular glutamate overstimulates ionotropic (NMDA, AMPA, or KA) and metabotropic (type I and V) receptors in neurons, causing intracellular Ca²⁺ entry through ionic channels and Ca²⁺ release from the ER. The first symptom of apoptosis is an increase in intracellular Ca²⁺ levels, (b) high ion influx: NMDA receptor hyperstimulation causes a rapid influx of Na⁺, Cl⁻, and water into neurons, causing a cell membrane rupture, and (c) oxidative stress: In neurodegenerative illnesses, excessive generation of ROS and NO, as well as impaired antioxidant defenses, cause damage to cellular nucleic acids and intracellular components, leading to neuronal cell death, (d) mitochondrial dysfunction: Overstimulation by high [Ca²⁺] concentration (d1) and oxidative stress (d2) cause mitochondrial transition pores to open and apoptotic factors to be released into the cytoplasm, resulting in neuronal apoptosis.

1.3.7 Treatment of stroke

1.3.7.1 General Management

Physiological Homeostasis (Oxygen, Temperature, Blood Pressure, Blood Glucose)

1. Supplemental Oxygen therapy

If a patient's oxygen saturation falls below 95%, supplementary oxygen should be administered.

2. Management of Body Temperature:

It's important to take your temperature:

- Paracetamol should be used to treat a fever of more than 37.5°C.

A search for an infection (both site and etiology) should be conducted.

- Temperature below 34.6°C can cause coagulopathies, electrolyte imbalances, infection, and heart arrhythmias, hence it should be treated with treatments like:

1. Take off any wet clothing.
2. Use blankets around the victim.
3. Provide hot beverages
4. Apply warm, dry compresses to the affected area (do not apply direct heat.)
5. Medical treatment: blood rewarming, warm intravenous fluids, airway rewarming, and so on, depending on the severity of the situation (“Guidelines for Prevention and Management of Stroke” 2019).

1.3.7.2 Pharmacological management of stroke

A-Vascular agents

In the treatment of stroke, vascular agents play an important role. Thrombolytics, which break down clots that have already formed, and platelet aggregation inhibitors, which limit the risk of clot formation, are two drugs that interrupt distinct stages of the clotting process that cause ischemic events.

Antihypertensives and oral anticoagulants, for example, modify factors that predispose patients to such occurrences.

1. Thrombolytic drugs

Ischemic stroke develops when a clot made up of activated platelets bound by a fibrin mesh obstructs a cerebral blood artery. Tissue plasminogen activator (tPA) is a serine protease that cleaves the plasminogen to plasmin, a broad-spectrum protease. Plasmin can break down fibrin, causing the clot to dissolve and blood flow to return. The standard of therapy for ischemic stroke is intravenous tPA administered within 4.5 hours of the beginning of symptoms. Alteplase, reteplase, and tenecteplase are examples of these medicines (Gravanis and Tsirka 2008).

2. Platelet aggregation inhibitors

The avoidance of secondary ischemic episodes is important in the treatment of cerebral ischemia in the elderly. Platelet aggregation inhibitors can reduce the risk of ischemic events by reducing the likelihood of clot formation. Aspirin is the first-line and most cost-effective medication for preventing recurrent ischemic stroke among these drugs. Aspirin suppresses thromboxane A₂ formation by acetylating a serine residue on COX-1's active site, rendering the enzyme permanently inactive. (Oza, Rundell, and Garcellano 2017). Cilostazol is an oral antiplatelet medication that also has a vasodilator effect. Cilostazol and its active metabolites inhibit phosphodiesterase type III, which prevents cAMP from being degraded, resulting in higher cAMP levels in platelets and vascular tissues. Increased cAMP inhibits platelet aggregation and promotes blood vessel vasodilation, respectively (Kannan et al. 2021).

3. Antihypertensives

Hypertension is connected to a worse prognosis during the acute phase of stroke, suggesting that blood pressure management during this period could improve results. Candesartan is an angiotensin II AT1 receptor blocker (ARB) that works by antagonizing this receptor in numerous organ systems, lowering blood pressure by vasodilation, and lowering aldosterone and vasopressin production. (Abraham, White, and White 2015). Perindopril is an angiotensin-converting enzyme (ACE) inhibitor that stops angiotensin I from being converted to angiotensin II, preventing the latter's blood pressure-raising effects. Perindopril medication was observed to protect against recurrent major vascular events, as expected (Czernichow et al. 2010).

B-Immunomodulatory agents

Inflammatory processes and the immune system play a major part in the pathogenesis of AIS, according to an increasing body of research. An array of inflammatory and immunological cascades are activated following a disruption in blood flow to a brain region, contributing to cell malfunction and death. Inflammatory responses increase in intensity with age, so immunomodulation may be particularly useful in elderly patients (Amantea et al. 2018).

1. Edaravone

Edaravone is an antioxidant molecule that has been synthesized. It freely crosses the blood-brain barrier and is hypothesized to work by scavenging free radicals, reducing endothelial cell and neuron inflammatory responses in the following of ischemia. Edaravone may also reduce neuronal cell death by lowering long-term inflammation and vascular endothelial cell damage.

Edaravone protects the brain by scavenging free radicals like superoxide and peroxynitrite and preventing lipid peroxidation. In animal models of permanent and transient cerebral ischemia, the neuroprotective effect is demonstrated by a significant reduction in infarct volume and neurological impairments. (Kannan et al. 2021).

2. Anakinra

Anakinra is an IL-1 receptor antagonist that prevents IL-1 from doing its job. The expression of pro-inflammatory substances, such as the interleukin 1 (IL-1) family of cytokines, has a role in the immunological response to stroke. IL-1 is an endogenous cytokine that plays a role in neuronal death and injury after ischemia (Pawluk et al. 2020).

3. Natalizumab

Natalizumab is a monoclonal antibody that targets a subunit of alpha-4 integrin (CD49d) and has been approved to treat autoimmune disorders such as Crohn's disease and multiple sclerosis, however, it has failed to treat acute ischemic stroke. Natalizumab prevents activated lymphocytes from adhering to endothelial cells and migrating to ischemic parenchyma. Treatment with natalizumab was thought to be able to control the inflammatory response after acute ischemic stroke. (Delbue, Comar, and Ferrante 2017). Integrins control cellular growth, proliferation, migration, signaling, and cytokine activation and release, and so play a key part in cell proliferation and migration, apoptosis, tissue healing, and all other inflammation and infection-related processes.

1.4 Cytokine in the study

1.4.1 Tumour Necrosis Factor-alpha

TNF- α is a multifunctional cytokine that is most frequently described as a potent pro-inflammatory cytokine released by macrophages/monocytes, neutrophils, T cells, mast cells, and other cells during acute inflammation. It is responsible for a variety of signaling events within cells, including necrosis and apoptosis (Holbrook et al. 2019). TNF- α is a cytokine that plays an important part in the central nervous system's homeostatic and pathologic states. TNF regulates critical physiological processes such as synaptic plasticity, learning and memory, sleep, and food and water intake when it is in a healthy state. When astrocytes and, in particular, microglia are in a pathologic state, they generate a large amount of TNF α , which is a key component of the neuroinflammatory response that is seen in a variety of neurological illnesses. Neuroinflammation is a protective reaction in the brain initially, but highly inappropriate inflammatory responses are harmful, and in reality, they reduce neuronal regeneration, leading to neurodegenerative illnesses and other neurological problems (Muhammad 2020).

When a TNF- α -producing stimulation is delivered, TNF is first produced as a 26 kDa (233-amino-acid) transmembrane protein (mTNF) that is expressed on the cell surface, where it either remains or is actively cleaved by TNF-converting enzyme to produce a 17 kDa (157-amino-acid) soluble TNF (sTNF) form, which is then released and detectable in the blood plasma. Both mTNF and sTNF have receptors that mediate biological functions: TNFR1, which is found in all human tissues, and TNFR2, which is found largely in immune cells and neurons (Muhammad 2020). Both membrane TNF (mTNF) and soluble TNF (sTNF) activate TNFR1. TNFR1 contains a death domain that interacts with the TNFR1-associated death domain (TRADD). Depending on the ubiquitination state of receptor-interacting serine/threonine-protein kinase 1 (RIPK1), the cell undergoes apoptosis

(via complexes IIa and IIb), necrosis (via complex IIc), or cell survival (via complex I). The creation of complexes IIa and IIb leads to the cleavage of pro-caspase-8 to form caspase-8 and trigger apoptosis. The mixed lineage kinase domain-like protein (MLKL), which is activated when complex IIc forms, causes necroptosis. Nuclear factor-kappa B (NF- κ B) and AP1 transcription factors are made active upon the formation of complex I as a result of RIPK1 ubiquitination. Although TNFR2 lacks an intracellular death domain and is largely activated by mTNF, it interacts directly with TNFR-associated factors (TRAF) 1 and 2 to cause the development of complex I and the production of homeostatic signals (Holbrook et al. 2019).

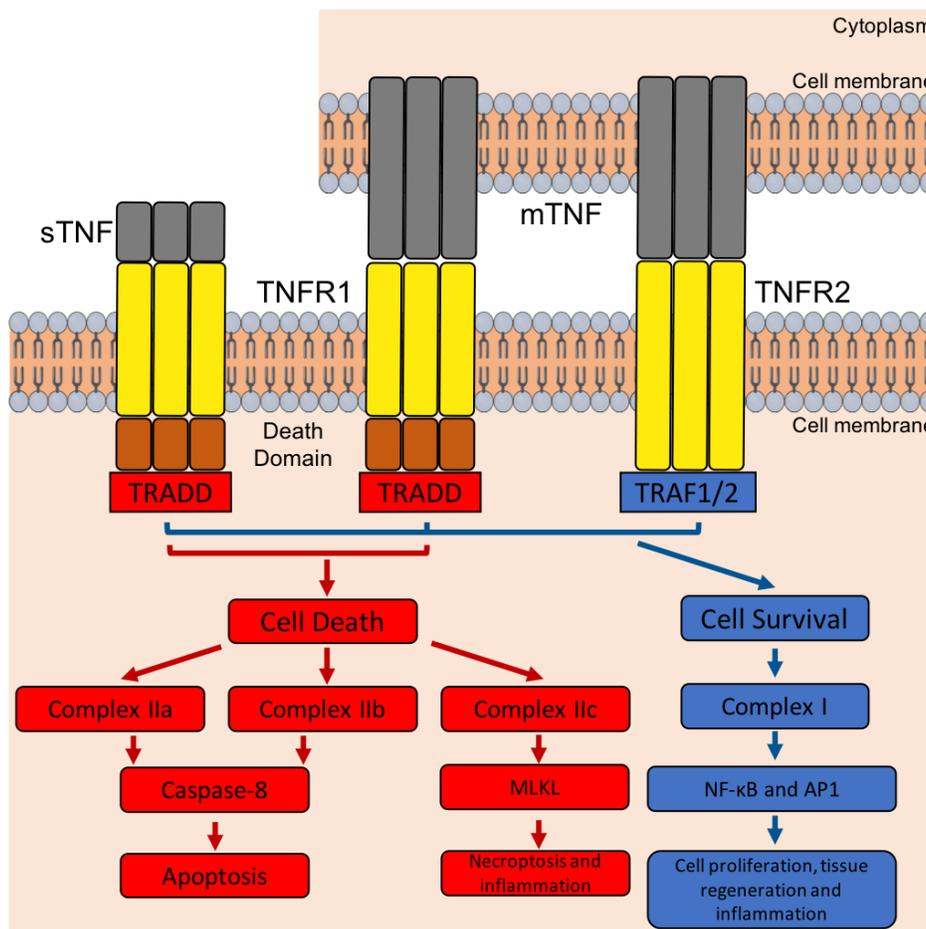


Figure 1. 7: Overview of tumor necrosis factor (TNF): TNF receptor 1/2 (TNFR1/2) signaling pathways (Holbrook et al. 2019)

1.4.2 Regulation of TNF α expression in the CNS after stroke

One of the first indications that TNF- α is an important mediator of stroke is the correlation of its expression with stroke damage. Gene expression levels of TNF- α which are regulated by the redox-sensitive transcription factor nuclear factor (NF)- κ B, have been shown to rapidly accelerate the early stage of cerebral IR, encourage an inflammatory response, and further aggravate cerebral IR. Accordingly, reducing the inflammatory response may thereby improve the prognosis of ischemic diseases (J. Li et al. 2017). Before the cytokine protein itself, increased TNF mRNA can be found in the injured rat brain, and it has been demonstrated that upregulation of TNF- α occurs before leukocyte infiltration to the site of injury. Early elevations in TNF- α expression may be beneficial as a diagnostic or prognostic indicator in the clinical setting (Woodcock and Morganti-Kossmann 2013). Also, TNF- α affects BBB permeability by alterations in cytoskeletal organization and tight junction protein expression as well as the production of serine proteases involved in BBB disruption, tissue remodeling, and neural plasticity (W. Pan and Kastin 2007).

1.4.3 Association between higher levels of serum TNF- α and increased probability of major depressive disorder

Major depressive disorder (MDD) is a growing public health concern due to the high prevalence, longer duration, or recurrence that can strongly spoil an individual's personal and work life. Genetic, biological, social, environmental, and psychological hypotheses are postulated for the understanding of the pathogenesis of MDD (Vilagut et al. 2016). Several studies reported that the altered levels of some pro-inflammatory and anti-inflammatory cytokines are responsible for the pathogenesis of depression one of these pro-inflammatory cytokines is tumor necrosis factor-alpha. TNF- α is produced by the actions of macrophages, natural

killer cells, and T-lymphocytes. Therefore, the hypothalamus pituitary axis is activated by the inflammatory response system (IRS) and the neurotransmitter serotonin (5-HT) can be destroyed by the elevated level of TNF- α . Moreover, TNF- α increases dopamine metabolism which results in depressive symptoms. Moreover, the pro-inflammatory cytokines affect the immune systems by modulating corticotropin-releasing hormone (CRH) thus increases in ACTH and cortisol levels in depression (R. Das et al. 2021).

1.5 Housekeeping genes

Housekeeping (HK) genes have been classically defined as genes that are necessary for the maintenance of basic cellular activities, and vital for the existence of any cell type. Hence, they are expected to be constitutively expressed in all cell types of the organism in normal physiological conditions regardless of particular cell function, cell cycle step, or developmental stage. In molecular biology and computational research, as well as in our understanding of many structural and functional genomics and evolutionary features, HK genes are beneficial as references for gene expression because of these properties (Hounkpe et al. 2021).

Additionally, several biotechnological applications and genomic investigations use HK genes as a calibration tool. The number of HK genes that have been found has gradually risen as a result of improvements in our ability to measure RNA expression. The most well-known HK genes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal proteins (RPL), β -actin, β -tubulin, and ubiquitin (UBQ), phosphoglycerate kinase (PGK), and ribosomal protein (18S rRNA) (Eisenberg and Levanon 2013).

1.5.1 glyceraldehyde-3- phosphate dehydrogenase

Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) is a special enzyme that, besides its major role in glycolysis, is a cycle in which glucose is transformed into pyruvate (catalysis of glyceraldehyde-3-phosphate oxidation), possesses several non-glycolytic functions. It is among the most popular HK genes. It continues to have a role in the control of transcription and DNA repair. GAPDH is also used as one of the most crucial reference genes for normalizing gene expression data and is employed as an endogenous control in RT-qPCR quantitative analysis since its expression is very constant in several experimental analyses (Muronetz et al. 2020).

1.6 The plant used in the study

1.6.1 *Taraxacum Officinale*

T. Officinale, sometimes known as Dandelion, is a herbaceous perennial in the Asteraceae (Compositae) family and Cichorioideae subfamily of the Asteraceae (Compositae) family. *Taraxacum* is an Arabic word that means "bitter herb." Dandelion is an edible plant that is extensively spread in the warmer temperate zones of the Northern Hemisphere, as well as in various Central and South American countries, as well as Australia and New Zealand (Lis and Olas 2019). It has been scientifically proven to treat liver problems, and inflammations, and has antibacterial, cytotoxic, antiviral, antiparasitic, antifungal, and antidepressant properties, as well as research, to confirm its anticancer and carcinogenic preventive properties. *T. officinale* appears to have low acute toxicity, with LD50 values of 36.8g/kg for the root and 28.8g/kg for the whole plant, respectively (Faria et al. 2019).



(a) *Taraxacum officinale* herb

(b) Flower

(c) Ripe fruits

Picture 1.1 A *Taraxacum officinale* herb, B, its flower, and C, ripe fruits

1.6.2 Constituents of *T. Officinale*

Medicinal plants often include a variety of chemical components that work together to promote health, either individually, additively, or synergistically. Bitter chemicals are believed to stimulate digestion, while phenolic components are responsible for plant extracts' anti-inflammatory and antioxidative properties (Schütz, Carle, and Schieber 2006). Phytochemical studies have demonstrated that dandelion contains phenylpropanoids, flavonoids, polysaccharides, hydroxycinnamic acids, coumarins, and chlorogenic acid. Dandelion leaves are rich in vitamins such as β carotene, choline, and trace elements including iron, silicon, magnesium, potassium, sodium, zinc, copper, and phosphorus. On the other hand, the roots are rich in bitter glycosides, sterols, tannins, taraxacin volatile oils, taraxacerins, asparagine, and triterpenes.

The flavonoids and coumaric acid derivatives found in *T. Officinale* flowers were found to have high antioxidant activity (Benalia and Djjebar 2020). The constituents of *T. Officinale* with their antioxidant and anti-inflammatory effects can be stated as follows:

1-Root components

Taraxasterol (anti-inflammatory properties), Ixerine (anti-inflammatory), Caffeic acid (anti-oxidant), Chicoric acid (anti-inflammatory),

Ainsloside, Tetrahydroridentin B (anti-inflammatory), Monocaffeoyltartaric acid, 11,13-dihydrolactucin, Taraxacolide-D glucoside.

The roots of *T. officinale* contain carotenoids (e.g. lutein), minerals, carbohydrates(e.g. inulin), sugars (e.g. glucose, sucrose, and fructose), vitamins, choline, and fatty acids (e.g. myristic acid) and pectin (PIERZAK-SOMINKA, n.d.).

2-Flower components

Caffeic acid (anti-oxidant), chrysoeriol, Chlorogenic acid(strongest anti-oxidant), chicoric acid (anti-inflammatory),Luteoline 7-O-glucoside (anti-oxidant) and Monocaffeoyltartaric acid. These compounds are antimutagenic, anti-inflammatory, and can activate the body's endogenous antioxidant systems (Wirngo, Lambert, and Jeppesen 2016).

3-Leaf and stem components

β -sitosterol, stigmasterol (anti-inflammatory), α -amyrin, , quercetine glycosiders (flavonoid) (anti-oxidant), sesquiterpene lactones (anti-inflammatory), mono-caffeoyl tartaric acid and chicoric acid (anti-inflammatory). Bitter sesquiterpene lactones, polyphenols, and coumarins are the primary chemical components found in the leaves. Furthermore, *T.officinale* leaves are strong in vitamins, particularly vitamin A, which is even greater in concentration than carrots (PIERZAK-SOMINKA, n.d.).

1.6.3 Antioxidant Activity of *T. Officinale*

Extracts of *T. officinale* have been found to include flavonoids and phenolic substances such as luteolin, chlorogenic acid, and caffeic acid. These compounds, which are generally found in plants, these extracts are effective hydrogen donors, hydrogen peroxide scavengers, and reducing agents. These compounds protect cells from oxidative stress by inhibiting free radical generation or detoxifying free radicals, preventing several pathological events (Colle et al. 2012). *T. Officinale*

increased glutathione reduction and antioxidative enzyme activity, such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase(Fulga et al. 2021).

1.6.4 Anti-inflammatory Activity of *T. Officinale*.

Proinflammatory cytokines and chemokines, such as interleukin IL-1, TNF- α , and IL-8, are produced by activated macrophages and play a role in the inflammatory process. TNF- α is thought to have a key function in the inflammatory cascades. Nuclear factor (NF) κ B, which is found throughout the cytoplasm, regulates the expression of iNOS, COX-2, and TNF- α . The p50 and p65 subunits of this inflammatory transcription factor are linked to an inhibitory protein called I κ B α . I κ B α is phosphorylated and released from Nf κ B in response to inflammatory stimuli generated by the bacterial endotoxin lipopolysaccharide (LPS). The p50 and p65 subunits of active NF κ B move into the nucleus and upregulate inflammation-related genes (Park, Cho, and Song 2014).

Polysaccharides from *Taraxacum officinale* have anti-inflammatory properties, reducing the expression of inducible nitric oxide synthase (iNOS), PGE₂, IL-1, and TNF- α . Phosphorylation of the inflammatory transcription factor nuclear factor (NF) κ B was reduced by *T.officinale* polysaccharides therapy (Park, Cho, and Song 2014).

Two flavonoid compounds (luteolin and lutein-7-O-glycoside) rich in the ethyl acetate fraction of *T. officinale* were shown to decrease the generation of nitric oxide NO and prostaglandin E₂ in LPS-activated macrophages cells.

This is due to the inhibition of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)-induced synthase(Faria et al. 2019).

1.6.5 *T. Officinale* Pharmacological investigations

Because of their chemical compositions rich in phenolic compounds, minerals, and numerous vitamins, *T. officinale* is employed as adjunctive therapy in specific illnesses all over the world. Several health-promoting properties have been attributed to the use of dandelion extracts or the plant itself throughout history, including antidepressant, laxative, diuretic, anti-rheumatic, antiviral, pulmonary protective, hepatoprotective, anti_carcinogenic, immunomodulatory, and hypoglycemic activities. The extract of *T. Officinale* has been demonstrated to have anti-obesity and anti-hyperlipidemic properties (Schütz, Carle, and Schieber 2006).

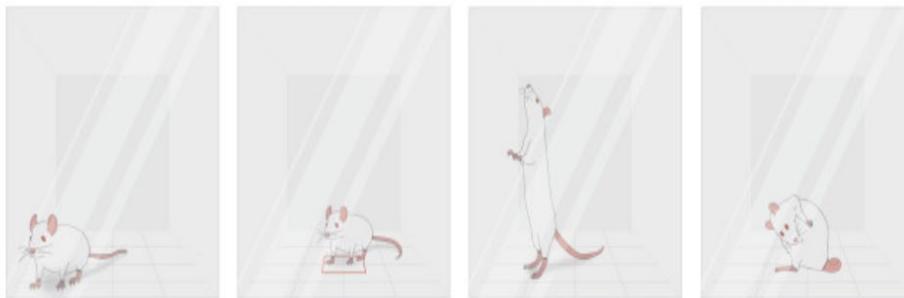
1.7 The Behavioral Tests after cerebral ischemia

1.7.1 The Open Field Test (OFT)

In scientific research, the OFT is an experimental test designed to assess general locomotor activity, anxiety, and desire to explore in animals (typically rats) (Zeldetz et al. 2018). The discovery of a novel environment in the open field was first prescribed by Hall & Ballachey, 1932. the apparatus consists of an arena encircled by high barriers, with the open field's floor separated into squares. By placing each rat in the center of the apparatus for around 10 minutes, the activity of each rat was measured. Travelled distance and rearing behaviors are employed to determine hyperactivity. Travelled distance refers to the total number of square crossings made throughout the test period, which is used to assess the animals' locomotor activity. The frequency with which the rat stands on its hind legs in the field throughout the test time is referred to as rearing. Risk-factor behavior is measured by the total number of visits to the open field's center. The term grooming relates to total number of grooming throughout the test period (Chang et al. 2018).



Picture 1.2: Open field test



(a) travelled distance (b) risk behavior (c) rearing (d) grooming

Figure 1.8: open field test show travelled distance, risk behavior, rearing and grooming

1.7.2 Forced swimming test (Porsolt test):

One of the most often used animal models for measuring depression-like behavior in the forced swim test (FST). and it was described for the first time by (Porsolt et al., 1977). The rodent is placed in a water-filled tank from which it cannot escape during the FST. The animal will try to flee at first, but will eventually become immobile (i.e. floating with the absence of all movement except for those necessary to keep the nose above water). During the forced swimming test (FST), each animal was placed individually within the forced swimming cylinder for 5 minutes, and immobility and floating behaviors were recorded. Immobilization refers to the animal's inactive behavior in a cylinder, which is defined as merely keeping the head

out of the water, whereas swimming refers to active behavior in water other than keeping the head out of the water (for example, swimming around the cylinder) (Falahieh et al. 2020).

The main benefits of this operation are that it is reasonably easy to perform and that the results can be examined easily and rapidly, making it a good screening test (Yankelevitch-Yahav et al. 2015).

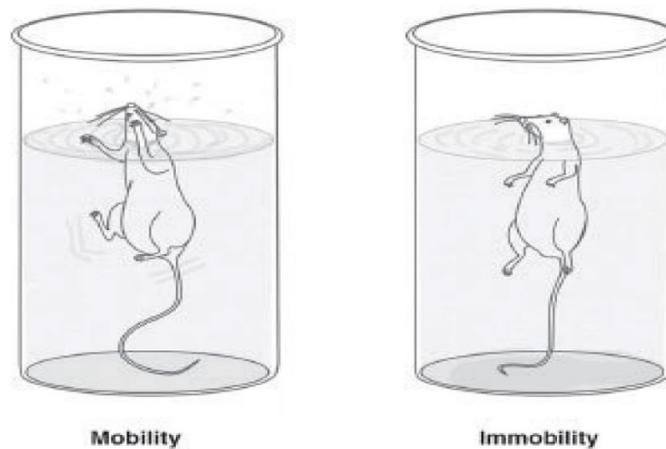


Figure 1.9 illustration of mobility and immobility in a Forced swimming test

Chapter Two

Materials and

Methods

2.1 Materials

2.1.1 Animals

In this study, fifty male adult albino rats were enrolled. Their weights ranged from 200 to 250 g. The rats were kept in the Animal House of the College of Medicine/University of Babylon at a temperature of 25°C, a room humidity of 60–65 percent, and a 14–10 h light-dark cycle with free access to water and food. According to the experiment, the animals were randomly divided into five groups after two weeks of acclimatization. Ten experimental rats have been allotted to each group. From 1/10/2021 to 1/2/2022, a study was conducted at the College of the Medicine/University of Babylon.

2.1.2 Instruments and Equipment:

Table 2.1 show the instruments and equipment which are used in the study.

Table (2-1): The following are the instruments and equipment that were used in this study

No.	Instrument / equipment	Company / Country
1	Disposable 1cc syringe	Shanachuan/china
2	Disposable 3cc syringe	Shanchuan/china
3	Disposable 5cc syringe	Shanchuan/china
4	Disposable gown	CHINA
5	Distilled water	IRAQ
6	Eppendorf tubes	Sigma(England)
7	Exispin vortex centrifuge	Bioneer/ Korea

8	Face mask	CHINA
9	Fine surgical tools	Biotechno/Germany
10	Gloves	Top glove/ Malaysia
11	High speed Cold Centrifuge	Eppendorf/ Germany
12	Hot plat stirrer	Labtech /Korea
13	Incubator	Memmert (Germany)
14	Micropipettes (different volumes)	Eppendorf / Germany
15	Miniopticon Real Time PCR	Bio-Rad/ USA
16	Nanodrop	Thermo Scientific/ UK
17	Refrigerator	Concord/ lebanon
18	Sensitive balance	Sartorius (Germany)
19	Sensitive electrical balance	Sartorius/Germany
20	Sterile gauze	INDIA
21	Thermocycler apparatus	Bioneer/ Korea
22	Tube rack	CHINA
23	Vascular clamp	Biotechno/Germany
24	Vortex	CYAN/ Belgium
25	Water bath	PolyScience (USA)

2.1.3 Kits

Table 2.2 show the kits which are used in the study.

Table (2-2): The following are the kits utilized in this investigation, along with their manufacturers and countries of origin:

No.	Kit	Company	Country
1	Total RNA Extraction Kit AccuZol™	Bioneer	Korea
	Trizol reagent 100ml		
2	DNase I enzyme kit	Promega	USA
	DNase I enzyme		
	10x buffer		
	Free nuclease water		
	Stop reaction		
3	M-MLV Reverse Transcriptase kit	Bioneer	Korea
	M-MLV Reverse Transcriptase (10,000U)		
	5X M-MLV RTase reaction buffer		
	dNTP		
	100mM DTT		
	RNase Inhibitor		
4	GoTaq® qPCR Master Mix	Promega	USA
	qPCR Master Mix, 2X SYBER green dye, Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP) and 10X buffer		
	DEPC water		

2.1.4 Primers

The primers for TNF- α target genes and housekeeping gene (GAPDH) were designed by using the NCBI-Gene Bank database and Primer 3 design online. These primers were provided by (Bioneer company, Korea) as follows in table (2.3)

Table 2.3: Primers, their sequences, and their sizes are shown below.

Primer	Sequence (5'-3')		Product Size	NCBI Reference
TNF-alpha	Forward	TCCCAGAAAAGCAAGCAACC	107bp	NM_012675.3
	Reverse	TCATGCTTTCCGTGCTCATG		
GAPDH	Forward	ATGCCCCCATGTTTGTGATG	136bp	NM_017008.4
	Reverse	TCCACGATGCCAAAGTTGTC		

2.1.5 Chemicals

The lists of chemical and biological ingredients employed in this study are in Table (2-4) below:

Table (2-4): Chemical materials with their remarks

No.	Chemicals	Company	Country
1	Absolute Ethanol	Labort	India
2	Alcohol spray	ALKAFEEL	IRAQ
3	Chloroform	Labort	India
4	DEPC water	Bioneer	Korea

5	Iodine spray 10%	AQUA	TURKEY
6	Isopropanol	Labort	India
7	Ketamine	alfasan	HOLLAND
8	RNase free water	Bioneer	Korea
9	Xylazine 2%	kepro	HOLLAND

2.1.6 Preparation of plant

With the support of the College of Agriculture/Medicinal Plant Department/ Al-Qasim Green University, the dried leaves and roots of *T. officinale* were approved as *T. officinale* on 4/4/2022, according to the document No 1465.



Picture 2.1: *Taraxicum officinale*

The dried leaves and roots were cleaned, sorted, ground into a powder with a pestle and mortar, then weighed to measure the extract yield. The powdered leaves and roots were extracted with hydro ethanol (a 70% ethanol solvent) and then agitated

for 72 hours on an automated shaker. The mixes were filtered using a Buchner funnel and Whatman No. 1 filter paper. A rotary evaporator at 40°C and reduced pressure was used to extract ethanol from the filtrates. The water was removed in a fan oven set to 40°C. The experiment was carried out with a dry extract. Shortly before the experiment, the extracts were diluted with water to the final concentration. (Aremu et al. 2019), (Mišek, Marcinčáková, and Legáth 2019). Then 5 gm of the dried extract were dissolved in 10 ml D.W.

2.1.7 Induction of brain ischemia

Global ischemia Induced by bilateral common carotid artery occlusion BCCO (Rabea et al. 2021) (Hadi et al. 2014) All animals were anesthetized with ketamine and xylazine (80 mg/kg and 5 mg/kg intraperitoneally, respectively)(Al Mudhafar et al. 2021). This procedure was carried out under a light bulb at 37°C. The anesthetized rat has been placed in a supine posture on a back plate. The two common carotid arteries were then carefully identified and clamped using vascular clamps to produce ischemia (picture 2.2). The ischemia lasted 30 minutes before the reperfusion phase began (Tang et al. 2014). Following the I/R induction for 24 hours, all groups should be subjected to forced swimming and open field tests to examine all animals for depressive-like behavior. Twenty-four-hour after behavioral tests, the rats should be sacrificed immediately after anesthesia.



Picture 2.2: Induction of brain ischemia

2.2 Methods

2.2.1 Ethical approval

This study was approved by the committee of publication ethics at the College Of Medicine, University of Babylon, Iraq.

2.2.2 Animals

The animals were randomly divided into 5 groups, with ten rats in each group

2.2.3 Study design and Experimental protocols:

Group-1: Sham group

This group has been subjected to the same surgical procedure (anesthesia and identical period) but ruled out BCCAO (Rabea et al. 2021)

Group-2: Control- vehicle group

This group has been subjected to anesthesia and BCCAO for a half-hour in addition to oral administration of vehicle (distilled water) for two hours before (I/R) induction.

Groups 3

This group received 200mg/kg for 21 days orally by a Gavage. Then, 24 hours after the last dose, each animal was subjected to BCCAO.

Group 4

This group will receive 300mg/kg (Baqir, Selman, and Mohammed, n.d.) for 21 days orally by a Gavage. Then, 24 hours after the last dose, each animal was subjected to BCCAO.

Group 5

This group received 400mg/kg (Baqir, Selman, and Mohammed, n.d.) for 21 days orally by a Gavage. Then, 24 hours after the last dose, each animal was subjected to BCCAO. Following 24 h of I/R induction for five groups, the forced swimming and open field tests were applied to all groups to study all the animals in terms of

depressive-like behaviors. Twenty-four hours after behavioral tests, the rats were sacrificed immediately after anesthesia. The brains were then removed for histopathology and the gene expression of TNF- α measurement.

2.2.4 Open Field Box:

The open field consists of a researcher-made wooden box (100 cm x 100 cm) with a square floor divided into 100 equal squares by thin white lines. The open-field test is recorded using a video camera suspended about 200 cm above the field (Poveda et al. 2020). By placing each rat in the center of the box for around 10 minutes, the activity of each rat was measured. A video camera was used to record all of the actions (Samsung, Korea). Crossings and rearings behaviors are used to assess hyperactivity in the open-field apparatus. The total number of square crossings throughout the test period is referred to as crossings, and it is used to determine the animals' locomotor activity. The total number of erect postures adopted by the rodent to explore during the test period is referred to as rearings. Time spent in central area is evaluated through the measurement of the total time of visits to the center of the open field. The term grooming refers to total number of grooming throughout the test period (Valvassori, Varela, and Quevedo 2017).



Picture 2.3: Open field box



(a) Rearing Behavior



(b) Line-crossing

Picture 2.4: (a) Rearing Behavior within open field test, (b) Line-crossing within open field test

2.2.5 Forced swimming box

A cylindrical glass box (30cm*30cm*70cm) was made by the researcher as described by (Cryan, Valentino, and Lucki 2005). Individual rats were forced to swim for 5 minutes in a cylindrical glass container filled with tap water (25 ± 1 °C) to a depth of 30 cm. On the 23rd day, the total time of immobility during the first 5 minutes of a swimming exercise was recorded. Swimming time refers to the amount of time an animal spends moving around in the water. Until the end of the test, this behavior tends to diminish. While immobility time refers to the amount of time the animal spends doing nothing or moving to maintain its head above water while not intending to move about the device (C. Wu, Zhang, and Chen 2015).



Picture 2.5: Forced swimming test

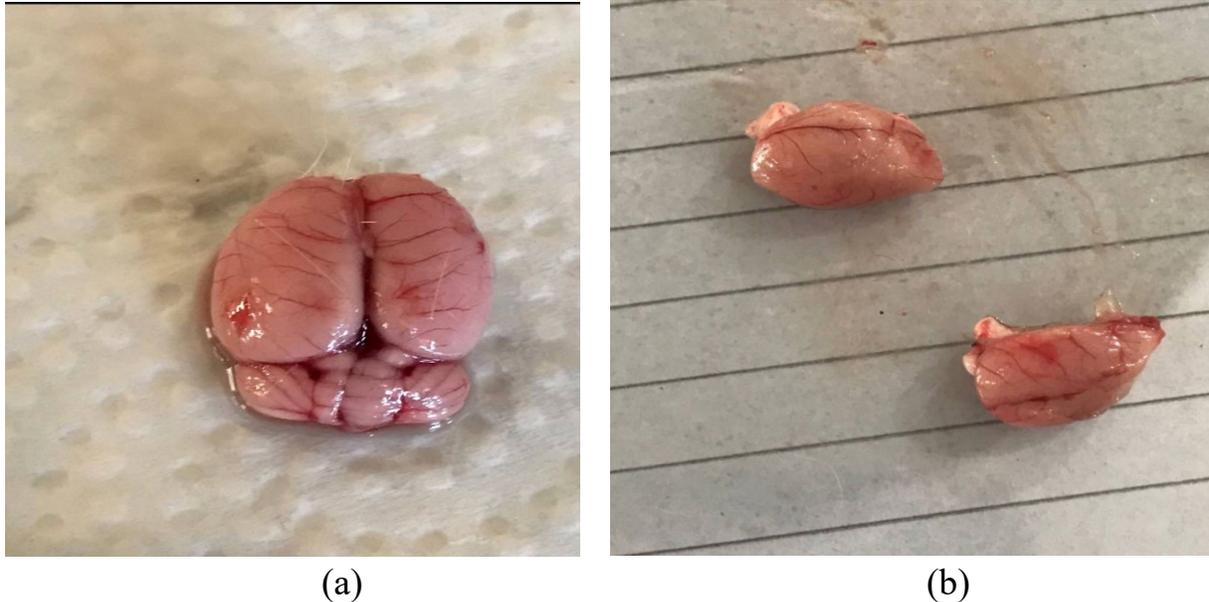
2.2.6 Preparation of Sample

2.2.6.1 Isolation of the Brain

On the twenty-fourth day, the animals were sacrificed by decapitation 24 h after the behavioral tests. The brains were removed after dissection of a skull from the foramen magnum posteriorly. Olfactory pulbs and cerebellum were cut and the brain was removed gently from the skull and prepared for sampling. The brains of each rat were placed in an ice container, each brain sliced into second coronal sections. The first has been kept in 10% formalin and then in paraffin wax for histopathological analysis. And the second part was kept in an Eppendorf tube containing 0.5 ml of accuzol reagent and was frozen on dry ice for tumor necrosis factor-alpha gene expression measurement



Picture 2.6: Skull dissection of the rat



Picture 2.7: Rat brain :(a) Isolation of the Brain, (b) left and right hippocampus

2.2.6.2 Preparation of Samples for Histopathology

The brain coronal sections have been set in 10% formalin for at least 24-48 hours followed by tissue processing by automated tissue processor for 20 hours, then dehydration in different concentrations of ethanol alcohol until absolute alcohol, followed by clearing in xylene, then impregnation in wax, embedding, and formation of paraffin blocks. For histopathological analysis preparation, they have been cut down to 5 μm with the help of a microtome and stained with Hematoxylin and Eosin (H&E) stains (Liaquat et al. 2019).

2.2.6.2.1 Histological analysis and damage scoring of brain

Histological findings (examined by a pathologist that used a double-blind method) have been graded using a scale of pathological scoring as follows:

Score (0) refers to normal appearance (no signs of brain damage).

Score (1) refers to mild changes, there is the presence of edema, pyknotic (dark neurons), eosinophil, and/or dark (shrunk) cerebellar Purkinje cells.

Score (2) refers to moderate changes in histological tissues (at least two small hemorrhage areas).

Score (3) refers to severe changes in histological tissues (clearly infarcted foci, i.e., local necrosis) (Alblesh and Raghif 2021).

2.2.6.3 Preparation of samples for tumor necrosis factor_ alpha gene expression measurement

2.2.6.3.1 Total RNA extraction

Total RNA was extracted from frozen brain tissue samples by using (a TRIzol® and RNA purification kit) and done according to company instructions in the following steps:

1. 100mg tissue sample was homogenized by adding 750 µl of TRIzol® reagent.
2. 200µl chloroform was added to each tube and shaken vigorously for 15 seconds.
3. The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4°C, for 15 minutes.
4. Supernatant was transferred into a new Eppendorf tube, and 500µl isopropanol was added. Then, the mixture was mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm, 4°C for 10 minutes.
5. Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4°C for 5 minutes.
6. The supernatant was discarded and the RNA pellet was left to air to dry.
7. 100µl free nuclease water was added to each sample to dissolve the RNA pellet, then, the extracted RNA sample was kept at -20.

2.2.6.3.2 Estimation of extracted total RNA yield

The extracted total RNA was assessed and measured by a Nanodrop spectrophotometer (THERMO. USA), There are two quality controls were performed on extracted RNA. The first one is to determine the quantity of RNA (ng/ μ L), and the second is the purity of RNA by reading the absorbance in a spectrophotometer at 260 nm and 280 nm in the same Nanodrop machine as follows:

- 1- After opening up the Nanodrop software, choose the appropriate application (Nucleic acid, RNA).
- 2- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2 μ l of free nuclease water and put it on the surface of the lower measurement pedestal to blank Nanodrop.
- 3- After that, the pedestals are cleaned and pipet 1 μ l of total RNA sample for measurement.

2.2.6.3.3 DNase I Treatment

The extracted RNA was treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to the method described by Promega company, USA instructions as follows

Table (2-5) DNase I Treatment

Mix	Volume
Total RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total	20ul

After that, the mixture was incubated at 37C° for 30 minutes. Then, a 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

2.2.6.3.4 cDNA synthesis

DNase-I treated RNA samples were also used in the cDNA synthesis step for TNF-alpha and GAPDH gene by using the **M-MLV Reverse Transcriptase kit** and done according to company instructions as following tables:

Step 1

Table (2-6) Table 2.6: cDNA synthesis step 1

RT master mix	Volume
Total RNA 100ng/ul	8ul
Random Hexamer primer	1ul
DEPC water	1ul
Total	10ul

Then RNA and primer were denatured for 10 min at 65 °C, and after that immediately cool on ice.

Step 2

Table (2-7) cDNA synthesis step 2

RT master mix	Volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

Then the tubes were placed in the vortex and briefly spun down. The RNA is converted into cDNA in the thermocycler under the following thermocycler conditions:

Table(2-8) RT step thermocycler conditions

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.2.6.3.5 Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of target gene TNF- α expression analysis that normalized by housekeeping gene (GAPDH) in control and treated tissue samples by using Real-Time PCR technique and this method was carried out according to the method described by Promega company, USA and include the following steps:

1- qPCR master mix preparation

qPCR master mix was prepared by using GoTaq® qPCR Master Mix kit based on SYBER green dye detection of TNF- α and GAPDH gene amplification in a Real-Time PCR system and including the following:

Table (2-9) qPCR master mix preparation

qPCR master mix	volume
cDNA template (100ng)	5 μ L
Forward primer(10pmol)	1 μ L
Reverse primer (10pmol)	1 μ L
qPCR Master Mix	12.5 μ L
DEPC water	5.5 μ L
Total	25 μ L

After that, these 96 plate master mix components mentioned above are placed in qPCR tubes or plates and mixed by Exispin vortex centrifuge for 3 minutes, then placed in MiniOpticon Real-Time PCR system.

2- qPCR Thermocycler conditions

After that, the qPCR plate was loaded, and the following thermocycler protocol is in the following table:

Table (2-10) qPCR Thermocycler conditions

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extention	60 °C	30 sec	
Detection(scan)			

2.2.7 Data analysis of qRT-PCR

The data results of q RT-PCR for TNF-alpha and housekeeping genes were analyzed by the relative quantification of gene expression levels (fold change) (The Δ CT Method Using a reference gene) described by (Livak and Schmittgen 2001) as the following equation:

$$\Delta\text{CT (Test)} = \text{CT (target gene, test)} - \text{CT (HKG gene, test)}$$

$$\Delta\text{CT (Control)} = \text{CT (target gene, control)} - \text{CT (HKG gene, control)}$$

$$\Delta\Delta\text{CT} = \Delta\text{CT (Test)} - \Delta\text{CT (Control)}$$

$$\text{Fold change (target / HKG)} = 2^{-\Delta\Delta\text{CT}}$$

2.2.8 Statistical analysis

Statistical analysis was carried out using SPSS version 27. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means \pm SEM). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. Fisher-freeman-halton Exact Test was used to find the association between categorical variables. *A p-value of ≤ 0.05 was considered significant.*

Chapter Three

Results

3.1 Effect of global cerebral I/R and *T.officinale* on behavioral tests

3.1.1 Open field test

3.1.1.1 Travelled distance

The travelled distance was significantly decreased (P value <0.05) in the control-vehicle group as compared with the sham group while in treated groups the no. of squares significantly increased (P value <0.05) as compared with the control-vehicle group (Table 3.1 and figure 3.1).

Table 3.1: The mean differences of travelled distance \pm SEM (cm) according to study groups, N = 10 rats for each group.

Table 3.1: The mean differences of travelled distance \pm SEM (cm) according to study groups

Study variable	Study groups	N	Mean \pm SE	P-value
Travelled distance (cm)	sham	10	219.00 \pm 1.93	
	c-vehicle	10	90.70 \pm 3.17	<0.001*
	200mg/kg <i>T.officinale</i>	10	122.00 \pm 2.78	<0.001#
	300mg/kg <i>T.officinale</i>	10	117.30 \pm 6.24	0.002#
	400mg/kg <i>T.officinale</i>	10	124.60 \pm 4.83	<0.001#

P -value ≤ 0.05 was significant.

*Vs. sham, # Vs. C-vehicle.

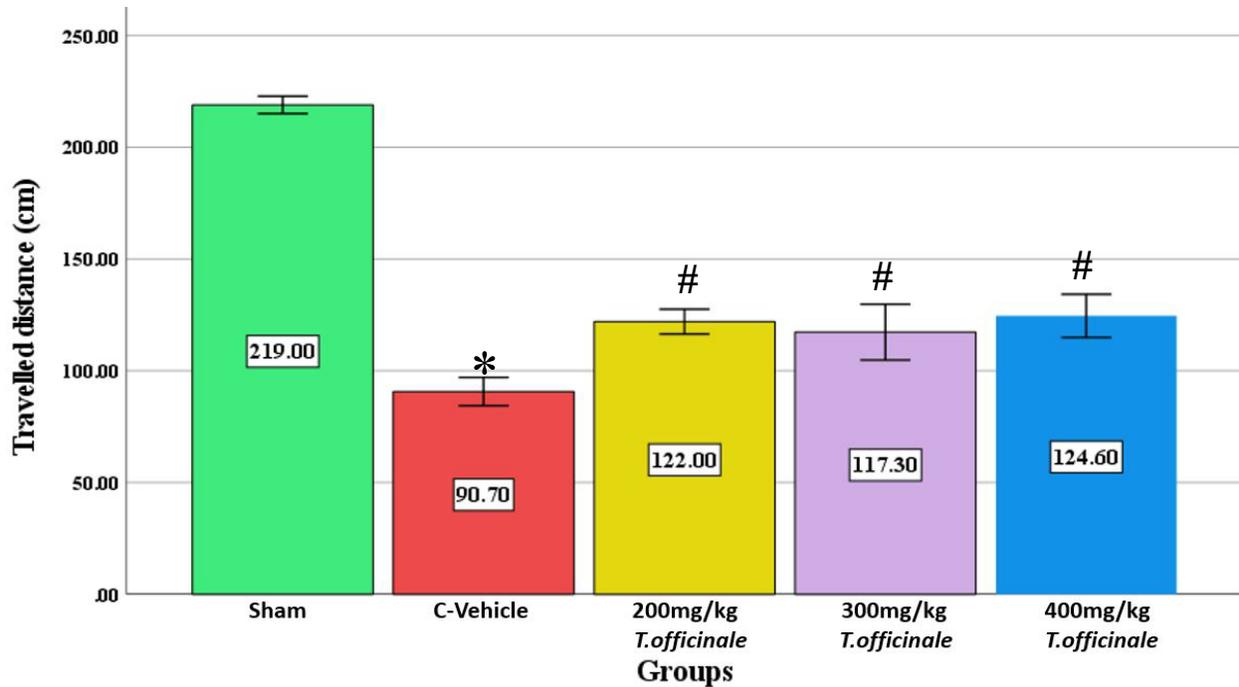


Figure 3.1: The mean differences of Travelled distance (cm) \pm SEM according to study groups

* = significantly decrease (p -value < 0.05) as compared with sham group .

= significantly increase (p -value < 0.05) as compared with c-vehicle group .

3.1.1.2 Rearing

The number of rearings in the control-vehicle group was significantly decreased (p -value < 0.05) as compared with the sham group. While in treated groups, the number of rearings was significantly increased as compared with the control vehicle group. (table 3.2 and figure 3.2).

Table 2.3: The mean differences of rearing \pm SEM according to study groups, N = 10 rats for each group.

Table 3.2: The mean differences of rearing \pm SEM according to study groups

Study variable	Study groups	N	Mean \pm SE	P-value
Rearing	sham	10	71.80 \pm 1.40	
	c-vehicle	10	27.30 \pm 1.14	<0.001*
	200mg/kg <i>T.officinale</i>	10	41.50 \pm 1.04	<0.001#
	300mg/kg <i>T.officinale</i>	10	47.30 \pm 0.67	<0.001#
	400mg/kg <i>T.officinale</i>	10	42.80 \pm 1.12	<0.001#

P-value ≤ 0.05 was significant.

*Vs. sham, # Vs. C-vehicle.

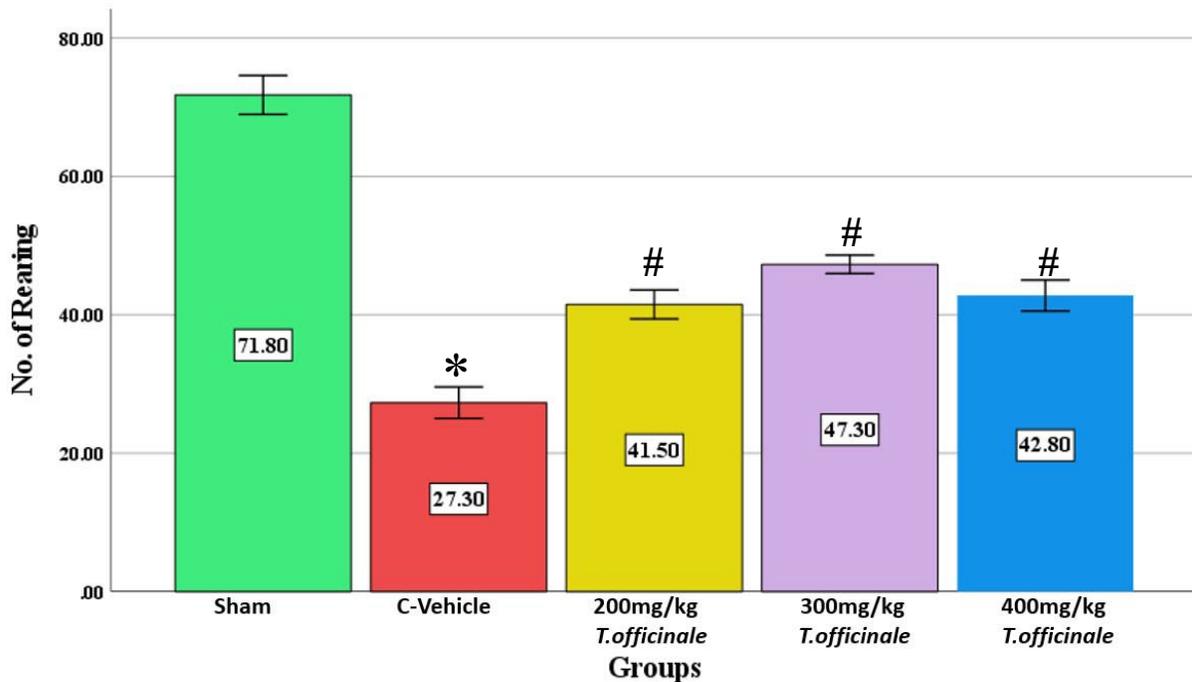


Figure 3. 2: The mean differences of rearing according to study groups

* = significantly decrease (*p*-value <0.05) as compared with sham group .

= significantly increase (*p*-value <0.05) as compared with c-vehicle group .

3.1.1.3 Time spent in the central area

The time of rats remaining in the center area in the control-vehicle was significantly decreased (*p*-value <0.05) as compared with the sham group. while

in treated groups, the time of rats remained in the center area was significantly increased as compared with the control vehicle group. (Table 3.3 and figure 3.3).

Table 3.3: The mean differences of time spend in Central Area± SEM (second) according to study groups, N = 10 rats for each group.

Table 3.3: The mean differences of time spend in Central Area± SEM (second) according to study groups

Study variable	Study groups	N	Mean ± SE	P-value
Time spend in Central Area (second)	sham	10	37.30 ± 1.20	
	c-vehicle	10	26.10 ± 1.06	<0.001*
	200mg/kg <i>T.officinale</i>	10	34.40 ± 0.93	<0.001#
	300mg/kg <i>T.officinale</i>	10	35.90 ± 0.85	<0.001#
	400mg/kg <i>T.officinale</i>	10	37.00 ± 0.76	<0.001#

P-value ≤ 0.05 was significant.

*Vs. sham, # Vs. C-vehicle.

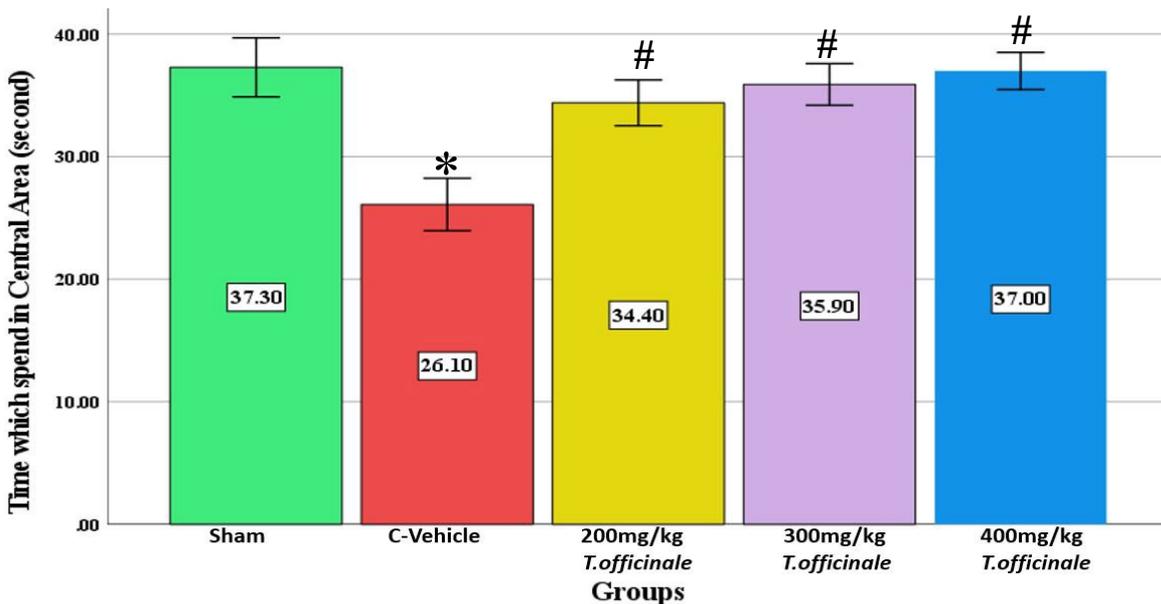


Figure 3.3: The mean differences in Time which spend in Central Area (second) according to study groups

* = significantly decrease ($p\text{-value} < 0.05$) as compared with sham group .

= significantly increase ($p\text{-value} < 0.05$) as compared with c-vehicle group .

3.1.1.4 Grooming

The number of grooming significantly decreased ($P\text{ value} < 0.05$) in the control-vehicle group as compared with the sham group. Within in treated groups, there was no significant difference ($p\text{-value} > 0.05$) in the number of grooming as compared with the control-vehicle group (Table 3.4 and figure 3.4).

Table 3.4: The mean differences in number of grooming \pm SEM according to study groups, N = 10 rats for each group.

Table 3.4: The mean differences in number of grooming \pm SEM according to study groups

Study variable	Study groups	N	Mean \pm SE	P-value
No.of Grooming	sham	10	7.50 \pm 0.27	
	c-vehicle	10	2.80 \pm 0.20	<0.001*
	200mg/kg <i>T.officinale</i>	10	2.80 \pm 0.25	1.000
	300mg/kg <i>T.officinale</i>	10	2.60 \pm 0.22	0.511
	400mg/kg <i>T.officinale</i>	10	2.90 \pm 0.31	0.791

$P\text{-value} \leq 0.05$ was significant.

*Vs. sham, # Vs. C-vehicle.

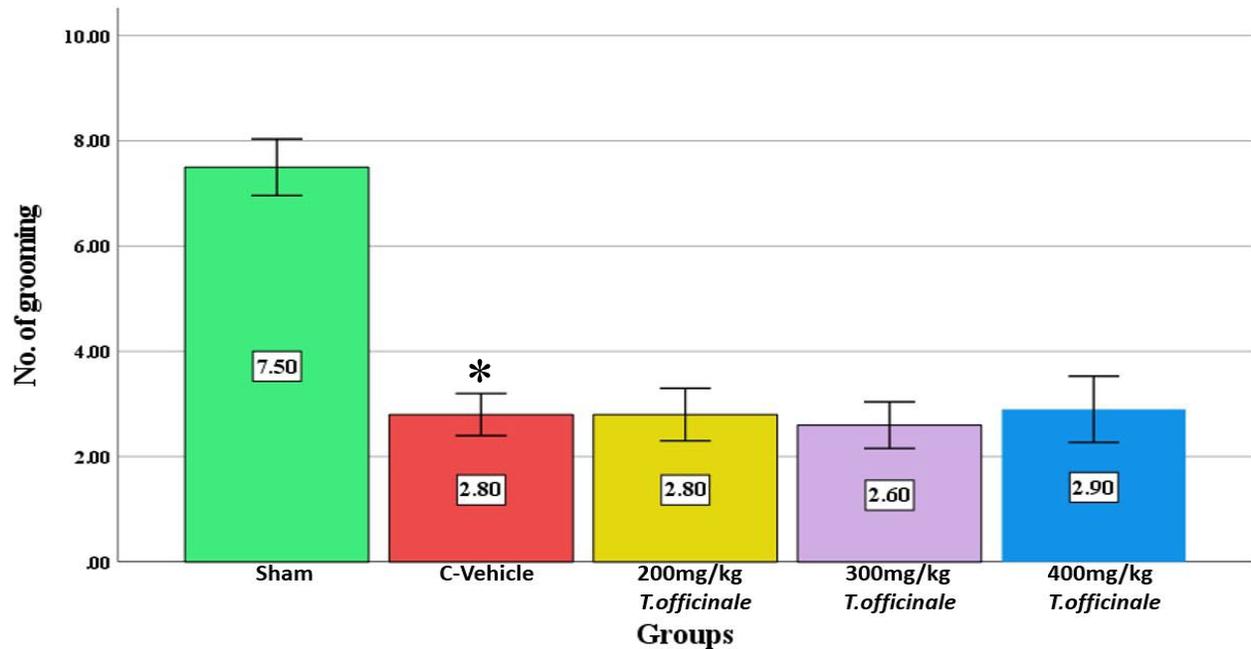


Figure 3.4: The mean differences in number of grooming according to study groups
 * = significantly decrease ($p\text{-value} < 0.05$) as compared with sham group.

3.1.2 Forced swimming test

The mean immobility time of the control-vehicle group was significantly increased ($p\text{-value} < 0.05$) as compared with the sham group. While in treated groups, the mean immobility time was significantly decreased as compared with the control vehicle group (table 3.5 and figure 3.5).

Table 3.5: The mean differences in immobility time \pm SEM (second) according to the study group , N = 10 rats for each group.

Table 3.5: The mean differences in immobility time \pm SEM (second) according to the study group

Study variable	Study groups	N	Mean \pm SE	P-value
Forced Swimming Test (second)	sham	10	50.00 \pm 1.44	
	c-vehicle	10	161.30 \pm 1.28	<0.001*
	200mg/kg <i>T.officinale</i>	10	84.50 \pm 3.99	<0.001#
	300mg/kg <i>T.officinale</i>	10	78.40 \pm 3.29	<0.001#
	400mg/kg <i>T.officinale</i>	10	83.20 \pm 3.34	<0.001#

P-value ≤ 0.05 was significant.

*Vs. sham, # Vs. C-vehicle.

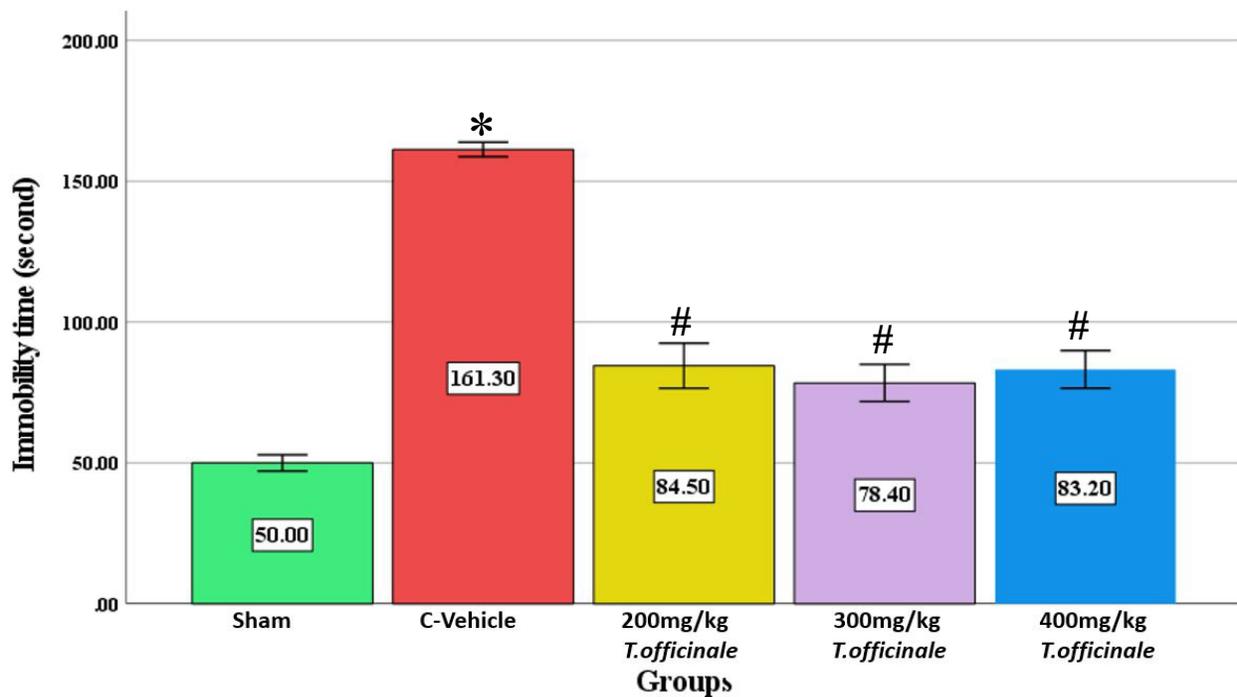


Figure 3.5: The mean differences in immobility time (second) according to study groups

* = significantly increase (*p*-value <0.05) as compared with sham group.

= significantly decrease (*p*-value <0.05) as compared with c-vehicle group.

3.2 Effect of global cerebral I/R and *T.officinale* on Histopathological findings

3.2.1 Sham group

A cross section of sham rats' brains showed a normal appearance (100%) of rats in this group and also showed normal brain appearance. So, it scored 0 scores as shown in Table 3.6, figure 3.6, and picture 3.1.

Table 3.6 Association between Histopathological score and study group. (Normal: Refers to normal appearance (no signs of brain damage). Slight: Refers to mild changes, there are the presence of edema, pyknotic (dark neurons), eosinophil, and/or dark (shrunken) cerebellar Purkinje cells. Moderate: Refers to moderate changes in histological tissues (at least two small hemorrhage areas). Severe: Refers to severe changes in histological tissue (clearly infarcted foci, i.e., local necrosis) and study groups including (Group one: Sham Group. Group two: Control Vehicle. Group three: treated with 200 mg/kg *T. officinale* extract for 21 days. Group four: treated with 300 mg/kg *T.officinale* extract for 21 days. Group five: treated with 400 mg/kg *T. officinale* extract for 21 days. There was a significant association between Histopathological scores and the study group.

Table 3.6 Association between Histopathological score and study group

Study variable	Study group					P-value
	Group 1	Group 2	Group 3	Group 4	Group 5	
Histopathological score						
Normal	10 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001*
Slight	0 (0.0)	0 (0.0)	2 (20.0)	3 (30.0)	3 (30.0)	
Moderate	0 (0.0)	2 (20.0)	3 (30.0)	3 (30.0)	4 (40.0)	
Severe	0 (0.0)	8 (80.0)	5 (50.0)	4 (40.0)	3 (30.0)	
Total	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	

P-value ≤ 0.05 was significant. Fisher-Freeman-Halton Exact Test.

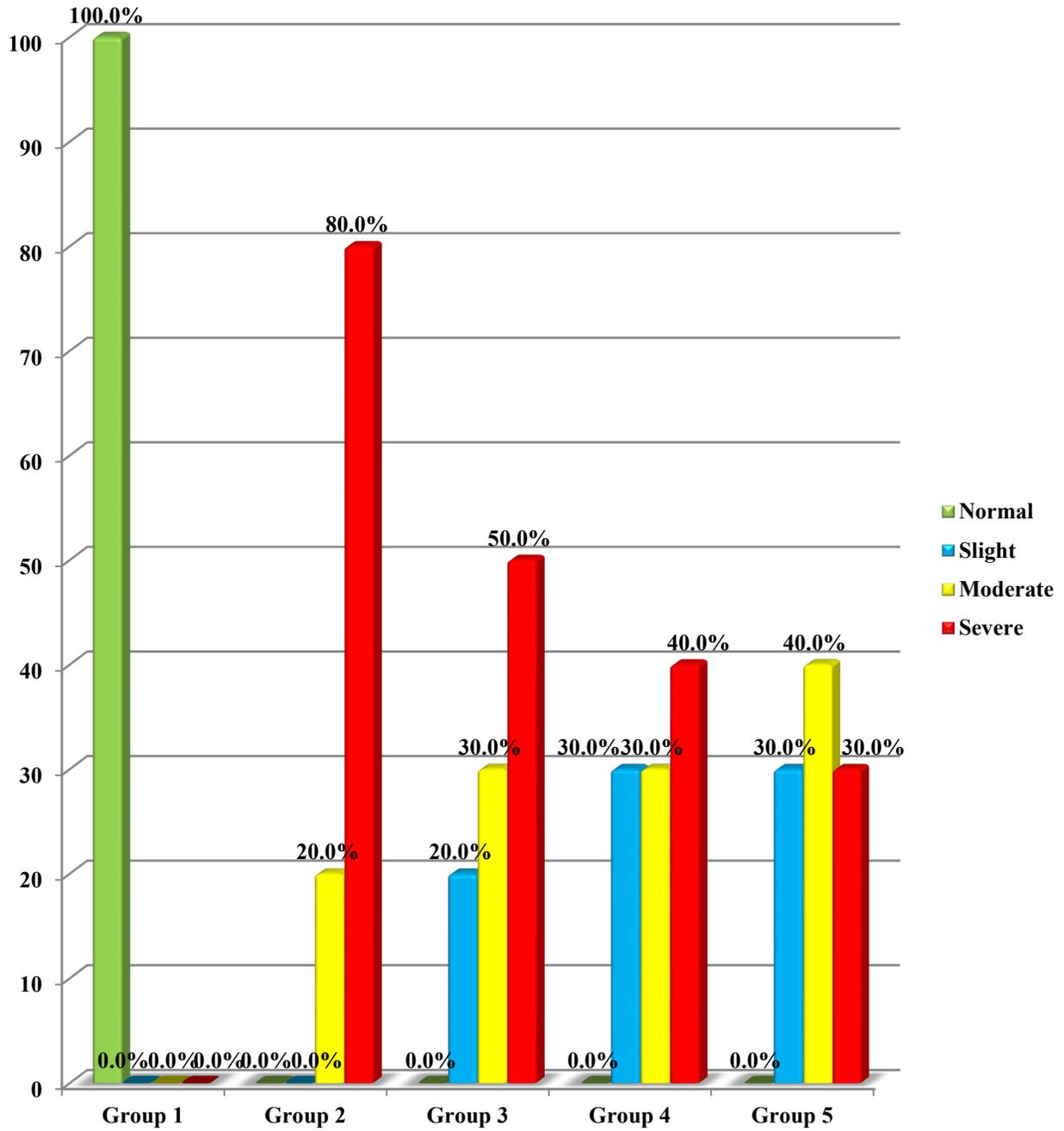


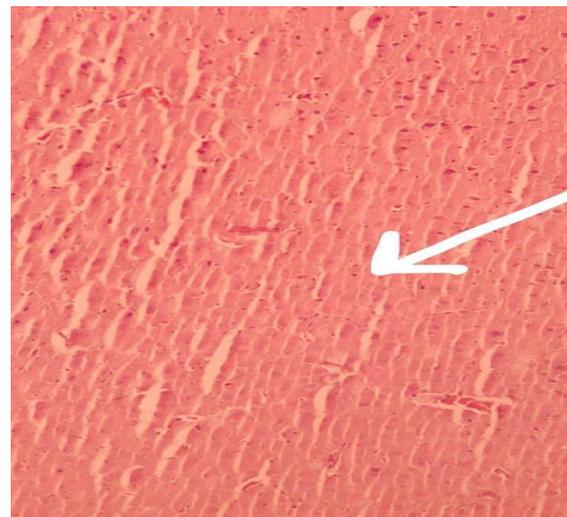
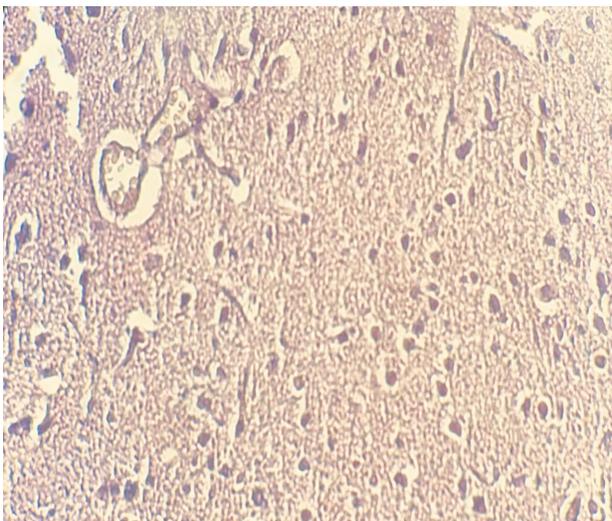
Figure 3.6: Association between Histopathological score and study group ($P < 0.001^*$)



Picture 3.1: Photomicrograph of the normal rat's brain section of the sham group that shows normal tissue, with histopathological score = 0

3.2.2 Control-vehicle group

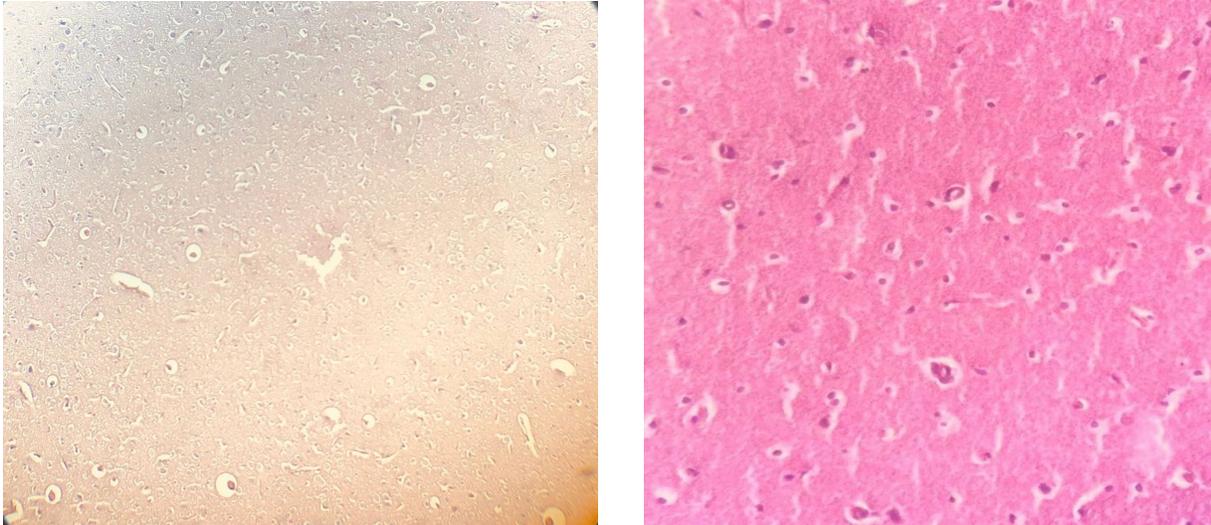
In the control-vehicle group, the result is significantly different from the sham group. Where found a moderate to severe cerebral injury as explained in table 3.6, figure 3.6, and picture 3.2.



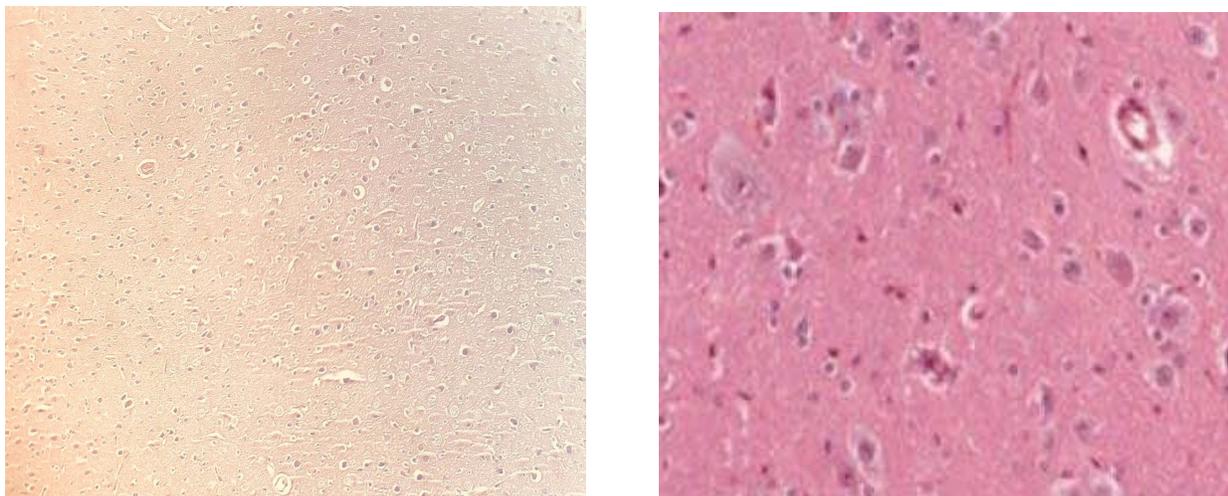
Picture 3.2: Photomicrograph of a section of rat's brain of C-vehicle group showed severe injury expressed as necrosis and edema in the tissue with histological score 3

3.2.3 *T.officinale* treated groups

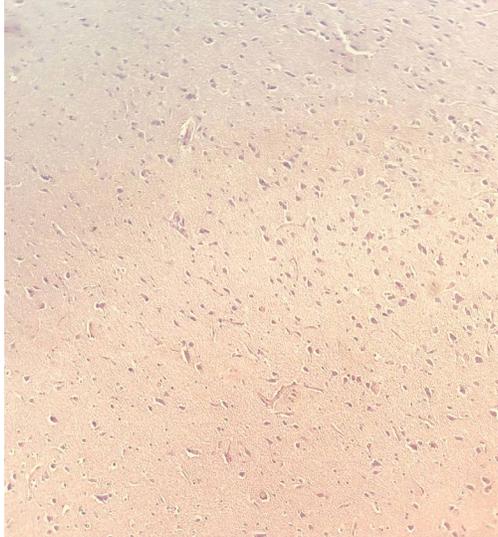
The study shows that the rats were treated with three doses of *T.officinale* (200,300,400 mg/kg) significantly improved the cerebral injury score compared with the C-vehicle group as shown in table 3.6, figure 3.6 and picture 3.3– 3.5.



picture 3.3: Photomicrograph for brain tissue of rats treated with *T.officinale* showing mild injury with the histopathological score =1



Picture 3:4: Photomicrograph for brain tissue of rats treated with *T.officinale* showing moderate injury with the histopathological score =2.



Picture 3.5: Photomicrograph for brain tissue of rats treated with *T.officinale* showing severe injury with the histopathological score =3.

3.3 Effect of global cerebral I/R and *T.officinale* on TNF –alpha gene expression

To evaluate the deep effect of *T.officinale* on the inflammatory response, Real-time –qPCR analysis was used to assess the mRNA expression of TNF- α in the ischemic brain. As shown in table (3.7) and figure(3.14) the mRNA expression of TNF- α has significantly increased ($P<0.05$) in the C-vehicle group as compared with the sham group. In comparison with the C-vehicle group, Treated groups with *T.officinale* (200,300 and 400 mg/kg) significantly decreased ($P<0.05$) the mRNA expression of TNF- α in the ischemic brain. Furthermore, there are no significant differences between treated groups. The change in the mean of fold changes of TNF- α has been summarized in Table (3.7) and Figure (3.14). Table 3.7: The mean differences in TNF-alpha Gene expression according to study groups. N = 10 rats for each group.

Table 3.7: The mean differences of fold change of TNF- α according to study group

Study variable	Study groups	N	Mean \pm SE	P-value
Fold change of TNF-alpha	sham	10	1.59 \pm 0.41	
	c-vehicle	10	8.24 \pm 1.25	<0.001*
	200mg/kg <i>T.officinale</i>	10	5.43 \pm 0.40	0.046 #
	300mg/kg <i>T.officinale</i>	10	5.20 \pm 0.39	0.033 #
	400mg/kg <i>T.officinale</i>	10	3.55 \pm 0.77	0.005 #

P-value ≤ 0.05 was significant.

*Vs. sham, # Vs. C-vehicle

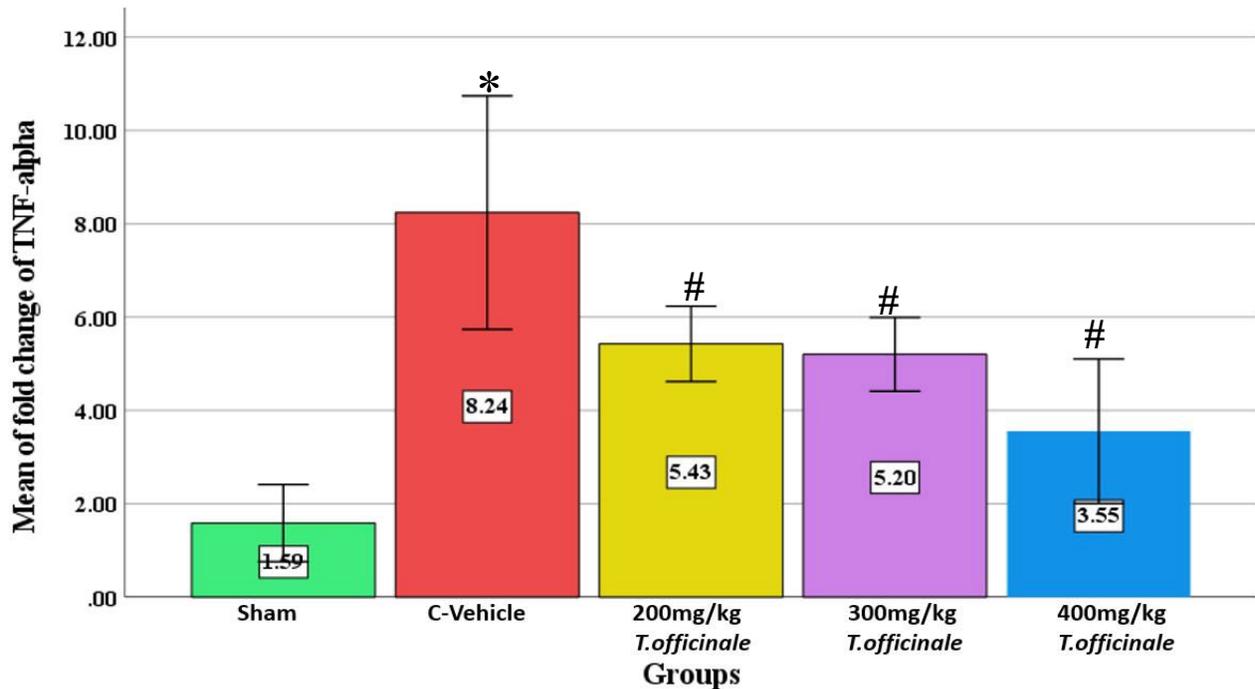


Figure 3.7: The mean differences of fold change of TNF-alpha according to study groups

* significantly increased (*p*-value <0.05) as compared with sham group.

significantly decreased (*p*-value <0.05) as compare with c-vehicle group.

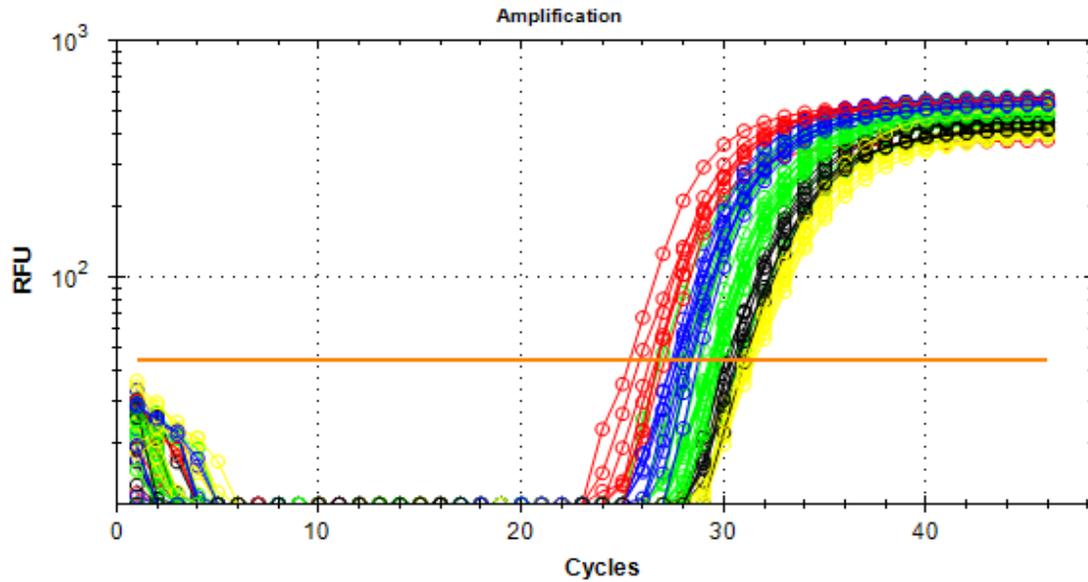


Figure 3.8: Real-time PCR amplification plot of TNF- α gene in experimental samples That showed a clear difference in threshold cycle numbers (Ct value) between treatment, control-vehicle, and sham groups. Where, the red plots: group 2 (control vehicle group), the Blue plots: group 3 (treated with 200 mg/kg *T.officinale* extract for 21 days), the Green plots: group 4 (treated with 300 mg/kg *T.officinale* extract for 21 days), the black plots: group 5 (treated with 400 mg/kg *T.officinale* extract for 21 days), and the Yellow plots: group 1 (sham group). n=10 for each group.

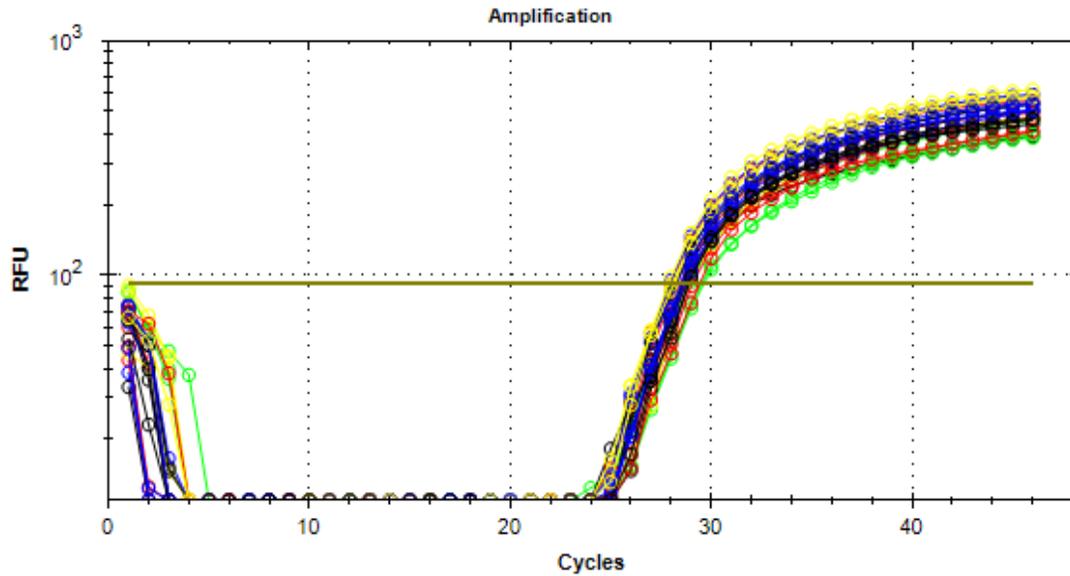


Figure 3.9: Real-time PCR amplification plot of housekeeping GAPDH gene in experimental samples that showed less difference in threshold cycle numbers (Ct value) between treatment, control-vehicle, and sham groups. Where, the red plots: group 2 (control vehicle group), the Blue plots: group 3 (treated with 200 mg/kg *T.officinale* extract for 21 days), the Green plots: group 4 (treated with 300 mg/kg *T.officinale* extract for 21 days), the black plots: group 5 (treated with 400 mg/kg *T.officinale* extract for 21 days), and the Yellow plots: group 1 (sham group). n=10 for each group.

3.3.1 Gene Expression according to histopathological score

According to Table 8, the mean differences in Gene expression according to Histopathological score. Normal: Refers to normal appearance (no signs of brain damage). Slight: Refers to mild changes, there are the presence of edema, pyknotic (dark neurons), eosinophil, and/or dark (shrunken) cerebellar Purkinje cells. Moderate: Refers to moderate changes in histological tissues (at least two small hemorrhage areas). Severe: Refers to severe changes in histological tissues (clearly infarcted foci,

i.e., local necrosis). There were significant differences between means of Gene expression according to the Histopathological score.

Table 3.8: The mean differences of fold change of TNF-alpha according to Histopathological score

Study variable	Histopathological score	N	Mean ± SE	P-value
Fold change of TNF-alpha	Normal	10	1.59 ± 0.41	<0.001*
	Slight	8	4.77 ± 0.78	
	Moderate	12	5.15 ± 0.64	
	Severe	20	6.22 ± 0.78	

P-value ≤ 0.05 was significant

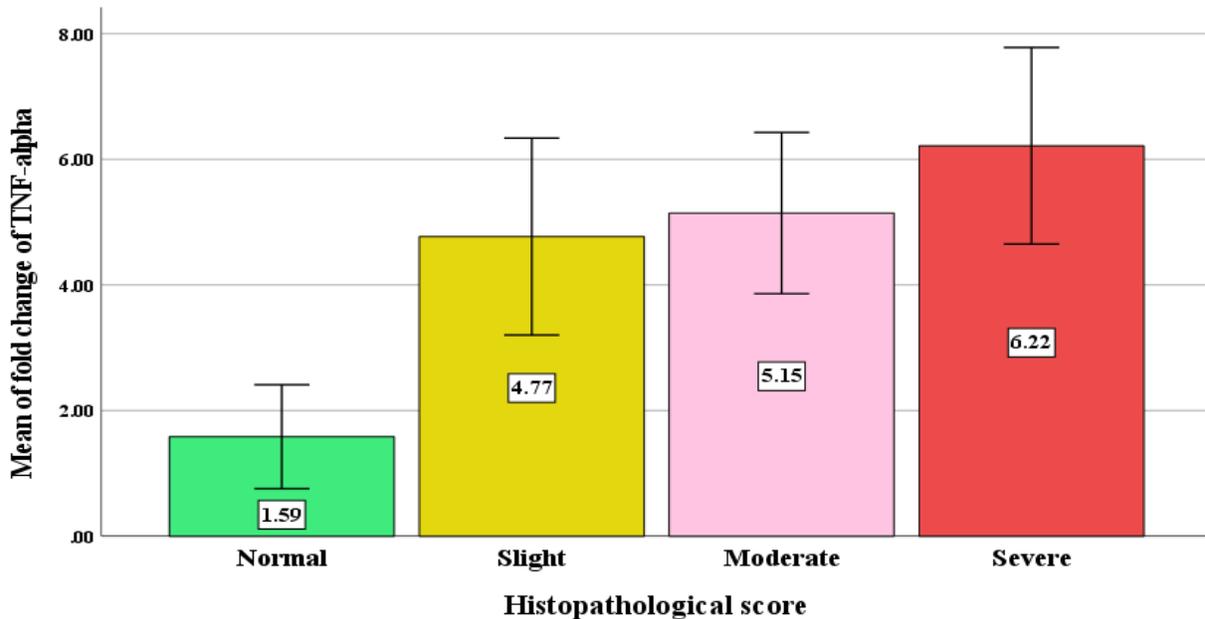


Figure 3.10: The mean differences of fold change of TNF-alpha according to Histopathological

Normal: Refers to normal appearance (no signs of brain damage). Slight: Refers to mild changes, there are the presence of edema, pyknotic (dark neurons), eosinophil, and/or dark (shrunk) cerebellar Purkinje cells. Moderate: Refers to moderate changes

in histological tissues (at least two small hemorrhage areas). Severe: Refers to severe changes in histological tissues (clearly infarcted foci, i.e., local necrosis). There were significant differences between means of Gene expression according to the Histopathological score.

Chapter Four

Discussion

4.1 The Effect of I/R injury and Treatments on Study Parameters

4.1.1 The Effect of I/R injury and *T. officinale* on behavioral tests

After a stroke, post-stroke depression (PSD) is an affective disorder. Depression is common after a stroke, and it has been suggested that depression can worsen disability by inhibiting effective physical therapy and recovery (Blöchl, Meissner, and Nestler 2019). In (Spalletta et al. 2006) proposed a hypothesis that pro-inflammatory cytokines such as IL-1 and TNF-alpha cause depression-like behaviors in rats. These cytokines have also been shown to upregulate the enzyme indoleamine 2, 3-dioxygenase (IDO), which catalyzes greater tryptophan metabolism, hence restricting the availability of tryptophan required for serotonin production. Based on these facts, the “cytokine hypothesis” was proposed that attempted to explain the pathophysiology of PSD. This theory is based on the fact that increased production of pro-inflammatory cytokines (TNF-alpha, IL-1, and IL-8) leads to an increase in an inflammatory state, which is followed by the activation of the IDO enzyme, which leads to a decrease in serotonin production (J. Das and Rajanikant 2018). Also In ischemia-reperfusion injury, reactive oxygen and nitrogen species are generated, Inflammatory cytokines have been reported to reduce the availability of tetrahydrobiopterin (BH4), a critical enzyme co-factor in the production of all monoamines and a highly sensitive to oxidative stress (Miller and Raison 2016).

4.1.1.1 Open field test (OFT)

OFT is used to assess locomotive and behavioral activity levels. Because ischemia-reperfusion injury reduces motor activity, the mean cross line, rearing, and grooming time in the control–vehicle group was considerably lower than in the sham group in the current investigation (Blöchl, Meissner, and Nestler 2019).

The reduction in motor activity in the control-vehicle group compared to the four groups also may be due to PSD. PSD is related to increased disability, and cognitive impairment, and, in the end, it impedes the rehabilitation and recovery process. The pre-treatment of three groups (200mg/kg, 300 mg/kg, and 400 mg/kg) with *T. officinale* led to a significant increase cross line and rearing time as compared with the C-vehicle group. The impact of *T. officinale* may be attributed to *T. officinale* enhancing motor activity and function recovery since no prior studies have been done. Furthermore, *T. officinale's* impact on travelled distance and rearing time may be a result of its antidepressant effect on lowering PSD. Reduction in PSD is associated with a decreased disability, decrease cognitive impairment, and finally, improve quality of life.

The pre-treatment of three groups (200mg/kg, 300 mg/kg, and 400 mg/kg) with *T. officinale* led to no significant increase in grooming time as compared with the C-vehicle group. This could be because the open field test has some disadvantages, such as being very changeable and affected by a range of external and internal factors. As a result, open field activity monitoring is fraught with problems: Animal strain, sex, age, and circadian rhythm all influence open field activity assessments. In addition, variables such as room temperature, humidity, lighting, noise, and even odor may have an impact on assessment outcomes (Tatem et al. 2014).

The present study has found a statistically significant ($P<0.05$) decrease in the mean central area time of the control-vehicle group as compared with the sham group. Reduction in central area time due to anxiety. Anxiety is common after a stroke, and the pathophysiological mechanisms that cause post-stroke anxiety (PSA) are unknown (W. Li et al. 2019). It's still unclear how oxidative stress influences PSA's processes. Multiple lines of evidence show that oxidative stress-induced mitochondrial malfunction, neuroinflammatory response, related signaling, and

neurodegeneration may play a critical role in the pathophysiology of anxiety (Liu et al. 2018).

Treatment of three groups with *T.officinale* for 21 days led to a significant increase in mean central area time as compared with the control–vehicle group. Because there has been no prior research, the potential anxiolytic effect of *T. officinale* may be the reason of the mean central area time increase (Mendelson, n.d.).

4.1.1.2 Forced swimming test (FST)

The Porsolt swim test, often known as the FST, is gradually becoming one of the most widely used assays for the investigation of depressive-like behavior in animal models (Chen et al. 2020). From the experimental results, due to PSD, the mean immobility time in the control vehicle group was significantly longer than in the sham group. PSD is linked to increased disability, cognitive impairment, and the risk of falling in clinical studies. Finally, PSD impedes the recovery process, and rehabilitation and compromises the quality of life. PSD could indicate a failure of neuroprotective mechanisms to activate (Ifergane et al. 2018).

The decrease in immobility time in the groups that received *T. officinale* for 21 days in all doses as compared with the control –vehicle group due to antidepressant effects of *T. Officinale*.

T. officinale extract works by increasing dopamine, noradrenaline, and adrenaline levels in the brain. Furthermore, the extract increased the expression of brain-derived neurotrophic factor (Bdnf), which was linked to a significant decrease in the expression of mitogen-activated protein kinase phosphatase-1 (Mkp-1), suggesting that *T. officinale* has antidepressant properties.

The active constituents of the alcoholic extract, which include isoetin, naringenin, hesperidin, sinapinic, Kaempferol, and gallic acid, were also identified, which could be responsible for its antidepressant effects (Gao et al. 2019).

Also, the antidepressant effect of *T.officinale* may be due to the anti-inflammatory effect of *T.officinale* in reducing proinflammatory cytokines (TNF- alpha) according to the “cytokine hypothesis” and the role of pro-inflammatory cytokines in the pathophysiology of PSD.

4.1.2 The Effect of I/R injury and *T. officinale* on Brain Histopathology

The effect of I/R injury on brain tissues was assessed by histopathological scoring. Histopathological examination can reveal any alterations that occur at the cellular level, such as nuclear pyknosis and morphological changes, or at the tissue level, such as necrotic tissues and intercellular spaces. According to (Shalavadi, Chandrashekhar, and Muchchandi 2020), marked a neutrophil invasion has been observed in the ischemic group, cytoplasmic space expanded and cell density decreased, cell structure modified, and hemorrhage and neuronal cell death were also observed after 30 minutes of global cerebral ischemia and reperfusion.

In this study, global cerebral I/R injury resulted in mild to severe cerebral damage, as compared to the sham group, which had normal brain tissue appearance. This study highly agreed with (Khaleq 2020) study, It was confirmed that 30 minutes of global cerebral ischemia and reperfusion in rats resulted in significant morphological changes in brain tissue, including blood vessel congestion, neutrophil infiltration, edema, pyknotic neurons, hemorrhagic areas, and necrotic cells, when compared to normal tissue from the sham group.

The current study found that pre-treatment with *T. officinale* 21 days before ligation of both common carotid arteries reduces the histopathological score caused by I/R injury because *T. Officinale* has a neuroprotective impact and may help protect the brain from oxidative damage by acting as an antioxidant and anti-inflammatory. This study observation is in line with (Huang et al. 2018) explored *T. officinale* was found to reduce glutamate-induced cytotoxicity and the production of ROS, implying that *T.officinale* could be used as a neuroprotective agent. *T.officinale* has several biological properties in vitro, including anti-cancer, hepatoprotective, pulmonary protective, anti-obesity effects, anti-inflammatory, and antidepressant properties (Huang et al. 2018)(Koh et al. 2010)(Hfaiedh, Brahmi, and Zourgui 2016).

4.1.3 The Effect I/R injury and *T.officinale* on TNF-alpha gene expression

Global cerebral ischemia and reperfusion are well-known damaging agents that disrupt brain tissue integrity, elicit an inflammatory response, and alter brain homeostasis. The cornerstone of the inflammatory response is the central release of cytokines. TNF-alpha has received a lot of attention as a pro-inflammatory cytokine. Therefore, it is worth assessing their level in brain tissues as indicators of inflammation and brain injury. In addition, Treatments can also be assessed based on their capacity to prevent or diminish the release of pro-inflammatory cytokines (Solaroglu et al. 2009).

The pathophysiology of ischemic stroke and other types of ischemic brain injury is complicated by inflammation. The dynamic equilibrium between pro-inflammatory and anti-inflammatory responses could be disrupted by cerebral

ischemia. Inhibition of inflammatory responses appears to reduce brain injury and enhance neurological outcomes in preclinical stroke trials (Jin et al. 2013).

The Real-Time Polymerase Chain Reaction (RT-PCR) is a common method in quantitative molecular biology and bioinformatics research. It gives incredibly important technology for assessing gene expression. In RT-PCR experiments, Fluorescence techniques such as SYBR green dye are used in RT-PCR investigations to identify target gene amplification and analyze their expression levels (Kuang et al. 2018). For each target gene, a cycle threshold (CT) can be calculated by monitoring the fluorescence in individual PCR wells or tubes. A CT is a relative value that denotes the cycle number at which the amount of amplified DNA reaches a threshold level. The CT value and gene expression level have an inverse linear relationship, with a low CT value indicating upregulation and a high CT value indicating downregulation of gene expression (Rao et al. 2020),(Fu et al. 2006).

Based on the results shown in this study, the CT value of the control-vehicle group was the lowest, and fold changes of gene expression were significantly up-regulated, whereas CT values of treated groups with *T.officinale* (200mg/kg, 300 mg/kg, 400 mg/kg) were high, and fold changes of gene expression were significantly down-regulated.

In the control-vehicle group (group 2) the present study has demonstrated there was a significant increase ($p\text{-value} < 0,05$) in fold changes of TNF –alpha gene expression as compared with the sham group (group 1). This increment in TNF-alpha gene expression is due to Inflammatory response and is one of the important pathological mechanisms that aggravate CIRC. After an injury, activated white blood cells and endothelial cells emit a substantial number of pro-inflammatory substances like TNF-alpha, triggering an inflammatory response and exacerbating the severity of brain injury (Cheng et al. 2019).

RT-qPCR revealed that the expression levels of TNF- α were significantly upregulated after CIRI. However, treatment with *T.officinale* significantly downregulated the expression level of TNF-alpha. This finding indicated that the inflammatory mediator (TNF α) was largely produced in brain tissue and promoted the inflammatory response after CIRI; however, *T.officinale* effectively reduced the expression of TNF-alpha and inhibited the inflammatory response, thus, ultimately contributing to the improvement of neurological function following CIRI. Proinflammatory cytokine (TNF-alpha) is a key factor in the early stages of cerebral I/R, as it promotes the inflammatory response and initiates the inflammatory process, as well as the expression of cell adhesion molecules and the infiltration of peripheral leukocytes, aggravating brain tissue damage caused by ischemia-reperfusion.

During ischemia, changes in the brain include the release of glutamate, the generation of reactive oxygen species (ROS) that produce oxidative stress, and the activation of microglia, which can increase the secretion of proinflammatory cytokine. Oxidative stress and inflammatory response have bidirectional effects on the whole stroke process. When blood vessels are blocked, the immune response begins near the ischemic parenchyma and then spreads throughout the ischemic zone, eventually affecting the entire body, and microglia become activated, promoting TNF-alpha production (Xue et al. 2022). TNF- plays a significant role in cerebral ischemia by acting as a chemotactic factor for leukocytes and inducing the production of adhesion molecules in a variety of cellular subtypes, including endothelial cells, other target cells, and various leukocyte subsets. This leads to an increase in inflammatory processes in the cerebral setting (Tuttolomondo, Pecoraro, and Pinto 2014). Also, TNF-alpha is a key mediator in modifying BBB permeability during postischemic reperfusion. However, the mechanism by which postischemic reperfusion causes blood brain barrier disruption is unknown (Xue et al. 2022).

TNF-alpha mRNA expression in the rat increases from 30 minutes to 5 days following brain ischemia. Following cerebral ischemia in rats and mice, early TNF α mRNA expression was found in neurons, microglia, and astrocytes (Esposito and Cuzzocrea 2009) and (G.-Y. Yang et al. 1999). Using a rat model of cerebral ischemia, inhibition of TNF- α offered protection against post-stroke neurological impairments, brain infarction, edema, and oxidative stress. Also suppression of TNF- α provides neuroprotective and anti-inflammatory benefits (S.-Y. Lin et al. 2021).

Interestingly in groups 3,4 and 5, which were pretreated with *T.officinale* Extract of *T.officinale* 21days before ligation of both common carotid arteries induces a significant downregulation in fold changes of TNF- α gene expression levels as compared with the control-vehicle group (p -value < 0.05). Due to a lack of prior research, it is possible that *T. officinale's* ability to block TNF-alpha production and its ability to have an anti-inflammatory effect on the central nervous system are the causes of this drop in the fold change of TNF- gene expression. The main components, which are responsible for the anti-inflammatory effect of *T.officinale*, are luteolin and cichoric acid by inhibiting phosphorylation of nuclear factor kappa B (NF- κ B) pathways. The third component, taraxasterol, has an anti-inflammatory impact by blocking the NF- κ B pathway, which up-regulates pro-inflammatory cytokines like TNF- α and IL-1 β when NF- κ B is activated. NF- κ B is thought to be a critical target for anti-inflammatory Therapies (Baqir, Selman, and Mohammed, n.d.).

Conclusions and Recommendations

Conclusions:

The findings of the present study concluded the followings:

1. The findings of this study have shown that 200mg, 300mg, and 400mg doses of *T. officinale* for 21 days reduced the depression-like behavior and anxiety in male rats after I/R induction.
2. *T.officinale* ameliorates histopathological changes damage that occurred as a sequence of I/R.
3. *T.officinale* had a beneficial role as neuroprotective via anti-inflammatory effects.
4. This study supported the role of pro-inflammatory cytokine (TNF- α) in the evolution of cerebral injury after cerebral I/R induction.
5. This study reveals decreases in the cerebral level of TNF- α gene expression after cerebral I/R injury establishing its anti-inflammatory action of *T.officinale*.

Recommendations:

1. Further studies are needed for measuring the antioxidant activity of *T.officinale*.
2. Further studies are needed for measuring cerebral inflammatory mediators like IL-1 and matrix metalloproteinase-9 (MMP-9).
3. Clinical trials are needed to study the effect of *T.officinale* on the patient with stroke.

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عدم الحركة لمجموعة مركبات التحكم في اختبار السباحة القسري بشكل كبير ($P < 0.05$) مقارنة بالمجموعة الشام. بينما في المجموعات المعالجة 3،4 و 5 انخفض متوسط زمن عدم السباحة بشكل ملحوظ مقارنة بمجموعة مركبات التحكم. أدى تحريض الدماغ (I/R) إلى زيادة درجة الضرر النسيجي المرضي بشكل كبير ($P < 0.05$) في مجموعة المركبات الضابطة مقارنة بالمجموعة الشام. بينما في المجموعات المعالجة 3،4 و 5 تحسن معنويًا ($P < 0.05$) من شدة الضرر المرضي للمخ مقارنة بمجموعة مركبات التحكم. يظهر تفاعل RT-qPCR في الوقت الحقيقي انخفاضًا كبيرًا في جين (TNF-alpha) أضعاف الجين في المجموعات التي تلقت 200 و 300 و 400 مجم / كجم من الهندباء البرية لمدة 21 يومًا مقارنة بمجموعة التحكم التي لم تتلق أي علاج. في الختام الهندباء البرية له تأثيرًا على الاعصاب من خلال آلية مضادة للالتهابات بالإضافة إلى انخفاض في تغيير أضعاف التعبير الجيني (TNF-alpha) كما أن الهندباء البرية له تأثيرات مضادة للاكتئاب ومزيل للقلق.

الخلاصة

إقفار الدماغ - إصابة ضخه (I/R) هي عملية معقدة تؤدي إلى تلف الخلايا والموت. يؤدي نقص التروية وضخه في الدماغ ، كما هو الحال في الأعضاء الأخرى ، إلى استجابة التهابية قد تؤدي إلى تفاقم المستويات الأولية لإصابة الأنسجة. عامل نخر الورم ألفا (TNF-alpha) هو السيتوكين الرئيسي المؤيد للالتهابات والذي يسبب تفاعلات التهابية في الدماغ بعد السكتة الدماغية. في الأونة الأخيرة ، النباتات العشبية ، على سبيل المثال الهندباء البرية تكتسب قدرًا كبيرًا من الاهتمام في السكتة الدماغية بسبب سلامتها وفعاليتها من حيث التكلفة ، والهدف من هذه الدراسة هو التحقيق في تأثير الهندباء البرية ضد نقص التروية الدماغية في دماغ الجرذ الذكري. تحديد تأثير الهندباء البرية على الاختبارات السلوكية والتعبير الجيني لـ (TNF-alpha) في إصابة (I/R) الدماغية في ذكور الجرذ. لذلك ، تم تقسيم خمسين فأرًا إلى خمس مجموعات ، كل منها عشر جرذان. مجموعة الشام ، خضعت الفئران للتخدير والعملية الجراحية لكنها استبعدت انسداد الشريان السباتي الثنائي (BCCAO) مجموعة مركبات التحكم ، الجرذان التي خضعت للتخدير و (BCCAO) لمدة نصف ساعة بالإضافة إلى تناول الماء المقطر عن طريق الفم لمدة ساعتين قبل تحريض (I/R) والمجموعات 3 والمجموعة 4 والمجموعة 5 (المجموعة المعالجة بـ 200 و 300 و 400 مجم / كجم من الهندباء البرية على التوالي. سوف تتلقى هذه المجموعات 200 و 300 و 400 مجم / كجم لمدة 21 يومًا عن طريق (Gavage) ثم ، بعد 24 ساعة من آخر جرعة ، يجب أن يخضع كل حيوان لـ (BCCAO). بعد 24 ساعة من تحريض (I/R) لخمس مجموعات ، يجب تطبيق اختبارات المجال المفتوح والسباحة القسرية على جميع المجموعات لدراسة جميع الحيوانات من حيث السلوكيات الشبيهة بالاكنتاب. بعد أربع وعشرين ساعة من الاختبارات السلوكية ، تم التضحية بالجرذان عن طريق قطع الرأس مباشرة بعد التخدير. ثم تمت إزالة الأدمغة لتحليل الأنسجة وقياس التعبير الجيني (TNF-alpha) بواسطة RT-qPCR. تظهر نتائج دراستنا في اختبارات المجال المفتوح انخفاضًا كبيرًا في المسافة المقطوعة ، والتربية والوقت الذي يقضيه في المنطقة المركزية في مجموعة المركبات الضابطة مقارنة بمجموعة الشام . بينما في المجموعات المعالجة 3،4 و 5 المسافة المقطوعة ، زادت التربية والوقت الذي يقضيه في المنطقة المركزية بشكل ملحوظ مقارنة بمجموعة مركبات التحكم. أثناء اختبار السباحة القسري ، زاد متوسط وقت



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل\ كلية الطب

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والاختبارات السلوكية على الاصابة بنقص التروية واعادة الارتواء في ذكور
الجرذان**

رسالة

مقدمة الى مجلس كلية الطب ولجنة الدراسات العليا / جامعة بابل
كجزء من متطلبات نيل درجة الماجستير في الادوية / أدوية وسموم

من قبل:

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بكالوريوس صيدلة

بإشراف

الأستاذ

الدكتورة لميس عبد الرزاق

دكتوراه في الاحياء المجهرية / علم الاحياء الجزيئي

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