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Effect of Hypercholesteremic Diet on Level Glutamate, BDNF And Other Biomarkers in The Central Nervous System of Male Rats

A Thesis

Submitted to the Council of the College of Veterinary Medicine, University of Karbala in Partial Fulfillment of the Requirements for the Master Degree of Science in Veterinary Medicine / Physiology

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بسم الله الرحمن الرحيم

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Declaration

I hereby declare that this dissertation is my original work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

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Dedication

To Almighty Allah Creator of the heavens and the earth.

present fragments of my humble research as a gift to, the Awaited Alimam Mahdi (may God bless him and grant him peace).

My husband and best friend Mr. Muhammad Kamel for his eternal support, and love.

My father and mother…. No words could describe my gratitude, thank you for giving me wings to fly and big dreams to follow.

My two little angels Jood & Jana, the miracle that makes my life complete.

To my dears... my brothers and sisters

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I extend my sincere thanks to the Deanship of the College of Veterinary Medicine, University of Kerbala, for help me in postgraduate studies in the branch of physiology, biochemistry, and pharmacology, I also extend my thanks and appreciation to all the teaching staff in the college, including doctors and professors, through their support for me during my postgraduate studies.

Thank you very much to **(Dr. Haider Ali Mohammed**) for helping me in gene expression assay.

Hadeel Ghani Rafash

Summary

The aim of this study is to investigate the physiological changes of the central nerve system which happen from receiving hypercholesteremic diet. Take 20 male rats were randomly divided into two groups(10/group): the control group, which received normal diet and the hypercholesteremic diet (HCD) group, which received a 1% supplement of cholesterol in their food for 4 weeks. After the experiment completed, samples of blood serum collected for the measuring lipid profile, Malnodialdehyde (MDA), Glutathione (GSH), BDNF level and synapsin-1. Rats were sacrificed by chloroform anesthesia, and dissected for brain section for the measuring Malnodialdehyde (MDA), Glutathione (GSH), nitric oxide, glutamate level, gene expression of activity regulated cytoskeleton (ARC), and histopathological examination.

The present study showed a significant increase in the Total cholesterol (TC), Triglycerides (TG), Low density lipoprotein (LDL) in the HCD group compared to the control group. In contrast, a significant decrease in the (HDL-C) in the HCD group wh compared to the control group.

The results indicated that hypercholesteremia had a positive impact on glutamate levels and negative on BDNF level, due to oxidative stress, while synapsin-1 levels in brain tissue remained unchanged. There were also notable decreases in serum and homogenized brain tissue concentrations of NO and GSH, along with increases in MDA, also showed down-regulation in the activity regulated cytoskeleton (ARC) gene expression in the HCD group compared to the control group.

Furthermore the histopathological examination of the tissues section shows the pattern of disruption of nerve tissue cells with varying degrees deposition neurofibrillary tangles on nerve cells is manifested by intracellular and extracellular deposition of beta-amyloid plaques of the central nervous system, which permeate of cerebral layers and lesser than in cerebellum and spinal cord parts .As it turns out, neuronal atrophy nuclei with neuroglial proliferation and inflammatory infiltrations cells of neural tissues in the HCD group are compared with control group.

It can be concluded that hypercholesteremic diet has promote elevated levels of oxidative stress the have been shown to be closely associated with oxidative stress and the biochemical structural integrity of neurons and glial cells in the cerebral cortex in a significant way. Additionally, there was a noticeable decrease in glutamate levels in brain tissue and down-regulation of the Activity Cytoskeleton Association protein gene (ARC gene) expression.

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List of Abbreviations

Abbreviations	Meaning
AD	Alzheimer ‘s disease
(AICD)	APP intracellular domain
DHCR	Dehydrocholesterol reductase
24-OHC	24-hydroxycholesterol
7D	7-dehydrocholesterol
7-DHC	7-dehydrocholesterol
7-DHCR	3β-hydroxy-steroid-Δ7-reductase
ABCA1	ATP-binding cassette transporter A1
ADDLs	Aβ-derived diffusible ligands
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor pens park
Apo-E	Apolipoprotein-E
APP	Amyloid precursor protein
ARC	Activity Regulated cytoskeleton
Aβ	β-amyloid
BBB	Blood brain Barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central Nervous System
CTF-α	C-terminal fragment
CVD	Cardiovascular Disease
CI	cognitive impairment
CR	Conogo red
DMSO	Dimethyl Sulfoxide
EGF	Epidermal growth factor
ELISA Assay	Enzyme-Linked Immunosorbent Assay
EVs	Electric Vehicles
FH	familial hypercholesterolemia
GABA	Gamma-aminobutyric acid
Glu	Glutamate
GSH	Glutathione
H&E	Hematoxylin & Eosin

HAPP	Human amyloid precursor protein
HCD	Hypercholesteremic diet
HFS	High frequency stimulated
Hch	Hypercholesterolemia
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3- methylglutaryl coenzyme A
IEG	Immediate-early gene
LA	Lanosterol
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MDA	Malondialdehyde
mg	Milligram
mg/dl	milligrams per deciliter
mg/kg	Milligram per kilogram
mGluRs	metabotropic glutamate receptors
MetS	Metabolic syndrome
NAC	Nacetly cysteine
NDDs	Neurodegenerative diseases
NFT	Neurofibrillary tangles
NGF	Nerve growth factor

Nm	Nanometer
NMDA	N-Methyl-D-Aspartate
NMDARs	N-Methyl-D-aspartate receptors
OS	Oxidative stress
P3	non-toxic peptide
SOD	superoxide dismutase
TBA	Thiobarbituric acid
TS	Tourette Syndrome
TC	Total cholesterol
TG	Triglycerides
VLDL-C	Very low density lipoprotein -cholesterol

Chapter One
Introduction

Introduction

Cholesterol, a vital constituent for normal functioning of the nervous system, plays a significant part in adult life as well as during the developmental stage **(Hussain *et al*., 2019)**. The primary constituent and essential functional unit of the membrane found in mammals is cholesterol **(Zuo *et al*., 2017)**. Approximately 25% of the body's total cholesterol is found in the brain, making it considered a cholesterol-rich organ **(Jin *et al*., 2019)**. The majority of body cholesterol is stored in the brain as myelin,which makes up over 80% of the total cholesterol in an adult brain **(Qian *et al*., 2022)**.Therefore, it is an essential component of myelin in the central nervous system, which is produced by Schwan and oligodendrocytes, respectively **(Mirzaie *et al*., 2020)**. There are two reservoirs of cholesterol in the brain, one is in the myelin membrane and the other in is the plasma membranes of glial cells and neurons. The overall amount of cholesterol in the body is largely comprised of this organ. It is already known that abnormalities in cholesterol metabolism are associated with neurodegenerative diseases **(Ledreux A *et al*., 2016).**

During the first few weeks after birth, the central nervous system (CNS) actively synthesizes cholesterol. Any disruption in this process during this newborn stage can result in the development of neurodegenerative diseases (NDDs) **(Cunningham *et al*., 2015).**

The synthesis of cholesterol can occur endogenously or exogenously through particular receptor-mediated endocytosis of plasma lipoproteins, including low-density lipoproteins (LDLs) **(Patel & Kashfi, 2022)**. Neurons can only synthesis a very small amount of cholesterol, they mostly rely on lipoproteins produced by astrocytes that contain cholesterol **(Mattos *et al*.,**

2019). In the central nervous system, some lipoproteins released by astrocytes, such as Apolipoprotein-E (Apo-E), are responsible for transporting cholesterol **(Kıray *et al*., 2016; Mahley RW *et al*., 2006)**.

Hypercholesterolemia is a significant risk factor for the onset of neurodegenerative disorders **(Engel *et al*., 2019; van Vliet, 2012)**. Specifically, altered metabolism of cholesterol is thought to play a major role in the etiology of Alzheimer's disease **(Kim *et al*., 2009).** Individuals with familial hypercholesterolemia (FH) have a higher risk of moderate cognitive impairment in later life compared to those with the sporadic variant **(Zambón *et al*., 2010)**. An environment that is susceptible to harm from a high-fat diet is the aging brain **(Ledreux *et al*., 2016**). Evidence suggests that cholesterol is associated with AD and represents promising targets for intervention. However, the causality of these associations is unclear **(Sáiz-Vazquez *et al*., 2020)**.

The association between blood and brain cholesterol levels can be explained by the fact that high-fat diets facilitate the easier transportation of oxidized cholesterol, or oxysterols, to the brain. These cholesterol-related substances are believed too potentially be beneficial. Cerebral cholesterol performs its proper functions in the brain. A study indicates that oxysterols are crucial signaling molecules for brain functions **(Chen *et al*., 2016).**

A member of the neurotrophic family, brain-derived neurotrophic factor (BDNF) is highly expressed throughout the CNS **.**The original function BDNF was to aid in neuronal proliferation, neurogenesis, differentiation, and degeneration, similar to other neurotrophies **(Cesca *et al*., 2010)**. Evidence also points to its role in controlling activity-dependent neuronal changes **(Lin *et al*., 2015)**. However, the mechanisms by which the production of these

neurotrophins could be therapeutically increased in the CNS are poorly understood.The link between high cholesterol and decreased BDNF levels is a complex one, and research is ongoing, while some studies suggest a negative correlation **(Bus *et al*., 2011).**

Synapsin-1 is a protein found in the brain that plays a vital role in regulating communication between nerve cells. It's specifically located in the nerve terminal of axons, also is thought to be important for several brain functions, including learning, memory, and mood regulation **(Mirza *et al*., 2018).** There is a connection between synapsin-1 and cholesterol. The high cholesterol levels can affect the localization and function of synapsin-1in the brain. This may contribute to cognitive decline and neurodegenerative diseases such as Alzheimer's disease **(Lazarevic *et al*., 2022**).

Brain-enriched immediate early gene that affects critical pathways implicated in learning and memory is activity-regulated cytoskeleton-associated protein (ARC) **(VictorRamírez *et al*., 2005).**

ARC is expressed in neurons and synapses, and is therefore tightly associated with the nervous system. The nervous system is a complex network of communicating neurons and glial cells information among themselves, muscles and endocrine glands in the body/organism **(Kandel *et al*., 2000).**

ARC may be implicated in learning and memory as they both are underlined by synaptic plasticity. A positive correlation between ARC mRNA expression in the rat hippocampus and spatial learning in the Morris water maze was observed **(Guzowski *et al*., 2001).**

Aim of study:

The principal objectives of this study effort are to investigate the impact of the hypercholesteremic diet on central nerves system by using the biomarker including serum lipid profile and oxidative stress i.e., oxidant MDA and antioxidant GSH and nitric oxide and using the following parameter BDNF, synapsin-1, glutamate and ARC gene expression with histological study in male rats given 1% w/w cholesterol every day in their diet for four weeks.

Chapter Two
Literatures Review

2.1. cholesterol

In normal cells, cholesterol is a crucial component of biomembranes. It plays a regulatory and signaling role interacting with cholesterol-binding proteins. Cholesterol is the precursor of steroid hormones and bile acid biosynthesis pathways **(Ershov *et al*., 2021)**. There are two main forms of cholesterol in the body produces roughly 70% of the substance, with the remaining 30% coming from diet **(Duan, *et al*., 2022)**. All tissues have a significant amount of cholesterol, with the brain containing roughly 1/4 of it. The majority of brain cholesterol, which is an important lipid class, is found in myelin sheaths, and one-third is found in cellular membranes. Brain cholesterol is mostly found in the unesterified form **(Marchi, 2020).** Cholesterol is crucial for astrocytes and neurons because it controls the fluidity and structural integrity of the plasma membrane **(Lee *et al*., 2021).** Additionally, cholesterol is necessary for the formation of myelin sheaths in oligodendrocytes, which encase axons in electrical insulation and hasten the transmission of electrical signals throughout the nervous system **(Montani, 2021).**

Like other sterols, cholesterol is largely hydrophobic. It is biosynthesized by all mammalian cells and predominantly localizes to cell membranes, where it interacts with the adjacent lipids to regulate rigidity, fluidity and permeability of the bilayer. In addition, cholesterol can bind numerous transmembrane proteins, helping to maintain or alter their conformations. Cholesterol also interacts with numerous sterol transport proteins that facilitate cholesterol trafficking and regulate its subcellular distribution **(Luo *et al*.,2019).**

During the initial weeks after birth, the central nervous system (CNS) of humans and other rodents actively synthesizes cholesterol. Any disruption in this process during this newborn stage might result in the development of neurodegenerative diseases (NDDs) **(Cunningham *et al*.,2015).**

Insufficient or excessive cellular cholesterol results in diseased conditions such as fatty liver, metabolic syndrome, cancer, cardiovascular disease, atherosclerosis, type II diabetes, and Alzheimer's disease **(Duan *et al*., 2022).**

2.1.1. Cholesterol structure and biosynthesis

Cholesterol, a vital structural component of cell membranes, is synthesized within the body through a complex series of enzymatic reactions collectively known as the mevalonate pathway. This pathway involves approximately 30 enzymes and utilizes acetyl-CoA as the starting material **(Nes, 2011).**

A cholesterol molecule is composed of three main components **figure (2.1):** 1) A short non-polar carbon chain attached to ring D figure **(2.1)**, 2) A polar hydroxyl group attached to ring A, and 3) The tetracyclic carbon ring, which serves as the core of steroids. Each of the sterol's four rings cholesterol is a planner molecule since its group is in trans conformation. The cholesterol's stiffness is maintained in part by the double bond between C5 and C6 **(Li *et al*., 2019).**

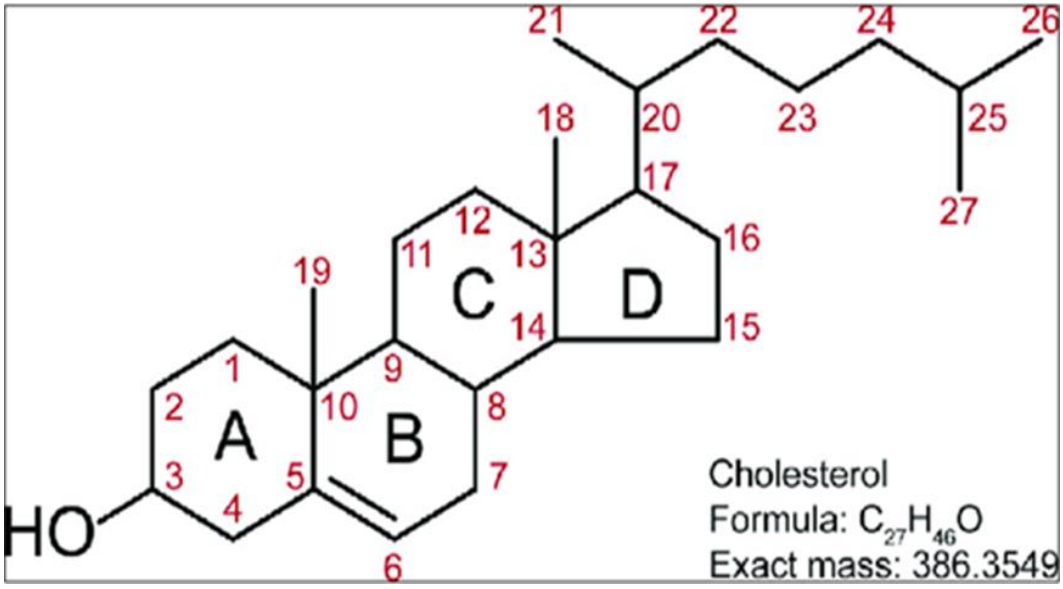

Figure (2.1): Cholesterol structure (Li *et al.*, 2019)

The two pathways that contribute to the synthesis of cholesterol are the Bloch and the Kandutsch-Russell routes. These pathways split off after lanosterol, sharing the first steps with acetate. Despite not sharing an intermediate beyond lanosterol, these pathways are linked via 24-DHCR. Comparable sterol intermediates in the other method lack a double bond at position 24 on the alkyl side chain, but those in the Bloch route do. With the aid of 24-DHCR, the alkyl side chain of sterols may experience reduction at the 24th double bond. These reduced sterol intermediates are used often in the Kandutsch-Russell pathway, which synthesizes cholesterol **(Singh *et al.*, 2013).**

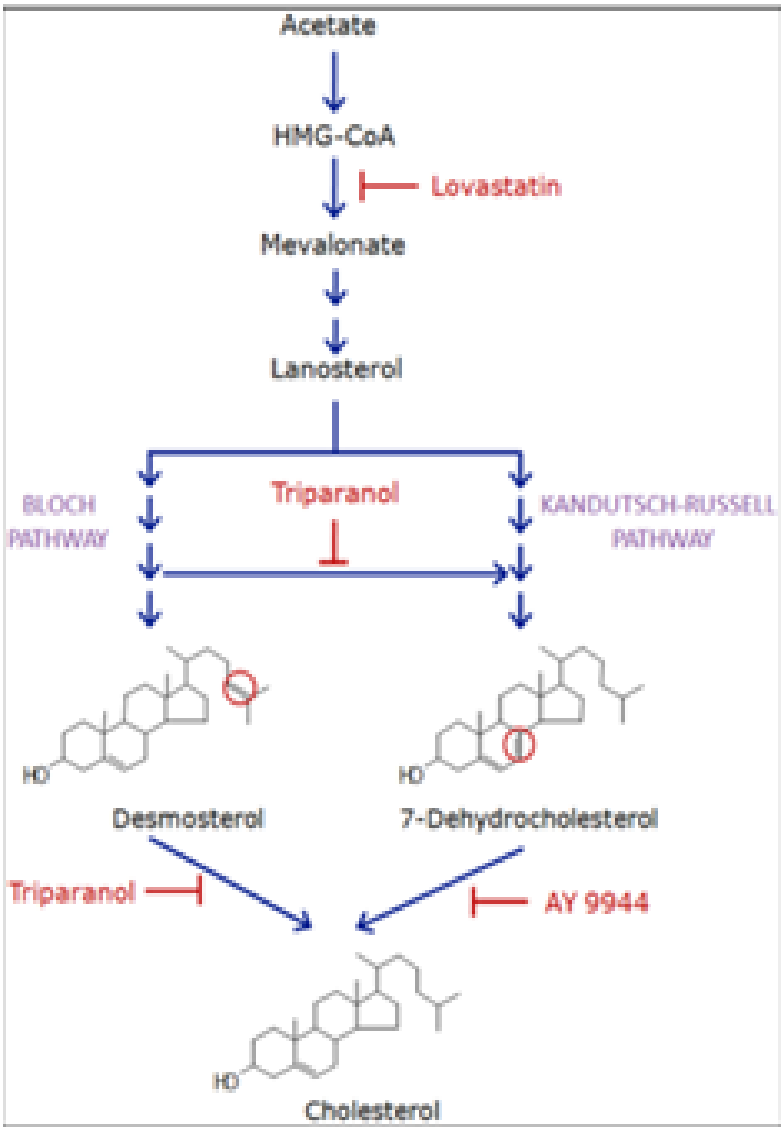

Figure (2.2). A schematic representation of various inhibitors that inhibit cholesterol biosynthesis (Singh *et al*., 2013).

Desmosterol is comparable, but it has an extra double bond at the 24th position on its flexible alkyl side chain **(Singh *et al*., 2007).** 3-hydroxysteroid-7-reductase (7-DHCR) catalyzes the final step of the Kandutsch-Russell pathway, which is the conversion of 7-DHC to cholesterol. Nevertheless, 24-

dehydrocholesterol reductase (24-DHCR) catalyzes the final step in the Bloch route, which is the conversion of desmosterol to cholesterol, by reducing the unsaturation at the 24-position of the flexible alkyl side chain of desmosterol **figure (2.2)**.

The availability of inhibitors that block the formation of cholesterol at different stages has considerably benefited in our understanding of how cholesterol and its precursors regulate multiple physiological functions. We employ lovastatin upstream and learn more about the significance of cholesterol for the advancement of the cell cycle, researchers should look into downstream (trimaran and AY 9944) cholesterol synthesis inhibitors**. Figure (2.2)** displays inhibitors of several vital activities involved in the synthesis of cholesterol. Because statins compete with HMG-CoA reductase, an enzyme that converts HMG-CoA to mevalonate, they reduce the production of cholesterol (**Singh *et al*., 2013).**

The enzyme 24-hydroxycholesterol reductase, which catalyzes the final step in the Bloch route converting desmosterol to cholesterol—is metabolically inhibited by tripranol. According to earlier findings, the Kandutsch-Russell route needs 24 DHCR for it taking place. When cells are treated with trimaran, cholesterol synthesis is inhibited by both the Kandutsch-Russell and Bloch routes.However, the end of the Kandutsch-Russell pathway in cholesterol production is the target of AY 9944, a metabolic inhibitor of 7-DHCR, which effectively blocks its utilization **(Singh *et al*., 2013) Figure (2.2)**.

2.1.2. Transformation of cholesterol to oxysterol

The enzyme cholesterol 24-hydroxylase is responsible for converting cholesterol into 24-hydroxycholesterol (24-OHC), which is the main type of oxidized cholesterol eliminated from the brain **(Zhang & Liu, 2015).** Compared with non-oxidized cholesterol, oxysterols such as 24-hydroxycholesterol is easily able to pass across lipophilic membranes such as the blood brain barrier (BBB) **(Lange *et al*., 1995; Meaney *et al*., 2002).**

About 99.5% of the cholesterol in the central nervous system (CNS) is unesterified, and most of the cholesterol in the CNS is thought to be divided into two pools: one is represented by the plasma membranes of astrocytes and neurons, and the other by the myelin sheaths (i.e., oligodendroglia) **(Björkhem & Meaney,2004)** .One notable feature of myelin is that, in relation to its dry weight, it comprises 70% lipid and 30% protein, which is about the reverse of what is observed in most other cell membranes. Myelin is known to contain phospholipids, cholesterol, and glycosphingolipids (especially glucocerebroside) at molar ratios of 4:4:2, despite the absence of true myelin-specific flag lipids.

But only a small portion of brain cells cerebellar Purkinje cells and frontal cortex pyramidal cells express by cholesterol 24-hydroxylase **(Lutjohann *et al*., 1996; Lund *et al*., 2003).** is main, the cells are more vulnerable to the negative effects of cholesterol. According to **(Ramirez *et al*., 2008)**, immunocytochemical labeling revealed that CYP46A1 was primarily restricted to the soma and dendrites of cultured neurons, rather than the axons or presynaptic terminals**.**

Brain glial cells do not exhibit high levels of expression for cholesterol 24-hydroxylase **(Ramirez *et al*., 2008)**. suggesting that the majority of the cell's cholesterol turnover is likely caused by neurons rather than astrocytes by 40%, the amount of cholesterol produced in the brain was decreased when the murine cholesterol 24-hydroxylase gene was disrupted **(Zhang & Liu, 2015).**

However, a concurrent decrease in the cholesterol mevalonate pathway meant that brain cholesterol levels remained unchanged **(Lund *et al*., 2003).** Based on the information at hand, it appears that a little portion of the synthesis and breakdown of cholesterol is brought about by the 24-hydroxylase pathway. Elderly people's hippocampal tissue has a somewhat lower concentration of 24S-hydroxycholesterol and produces less cholesterol overall **(Thelen *et al*., 2006**; **Zhang & Liu, 2015)**.

2.2. Cholesterol in the Central Nervous System

The major function of neurons, which are made up of an axon and a cell body, is the production and transmission of electrical impulses. A prolonged electrical conductor, the axon bifurcates and diminishes repeatedly until it stops at the nerve terminals, where neurotransmitters are released. The myelin sheath, which is made up of segments of oligodendrocyte plasma membrane that are repeatedly wrapped around the axon with nearly all of the cytoplasm extruded, facilitates the rapid transmission of impulses down the length of the axon. Periodic gaps exist in the sheath because myelin from many oligodendrocytes might encase a single axon. These are the locations where the action potential propagates and are known as the Ranvier nodes. Thus, myelin can be thought of as a discontinuous insulator that permits the action potential to conduct saltatorily **(Snipes & Suter, 1998).**

Research suggests that myelin accounts for as much as 70% of the cholesterol found in the brain. That the brain is the organ in the body with the highest cholesterol content is not surprising, as myelin may make up as much as half of the white matter. Its membrane properties are compatible with an essential function of the brain's cholesterol content, especially in myelin.Hence, myelin that has been enriched with cholesterol has less ion permeability (high resistance and low capacitance). Ultimately, this means that instead of diffusing across the membrane, the current prefers to go down the axon **(Snipes & Suter, 1998)**.

2.2.1. Transport of Cholesterol Within the Brain

One of the main apolipoproteins in plasma is apoE, or apolipoprotein E. This is thought to be the brain's quantitatively most significant cholesterol transport protein under normal circumstances. ApoE is found in the central nervous system (CNS) as discoidal and spherical particles the size of high-density lipoprotein. It is thought that astrocytes have the greatest capacity to generate apoE **(Björkhem & Meaney., 2004)**.

The astrocyte cell can release cholesterol and transport by Apo-E into the other neurons cell in CNS **(Shanmugaratnam *et al.*,1997**) Cerebrospinal fluid contains lipoproteins that contain cholesterol and Apo-E, which indicates that lipoproteins are circulated in the brain, Accordingly, the brain's many components, including neural cells, have LDL receptor mRNA **(Björkhem& Meaney, 2004)**.The multi-ligand receptor LRP (LDL receptor-related protein) may be able to absorb substances instead of the LDL receptor **(Bu *et al*., 1994)**. It is known that the brain synthesizes a number of other lipoproteins, such as apo A, apo D, and apo J **(Björkhem & Meaney, 2004).**

Other receptors expressed in the brain include megalin, apoE receptor 2, and the very low-density lipoprotein receptor. It is currently unknown how important each lipoprotein and receptor are in relation to the other for the transport and recycling of cholesterol inside the central nervous system, and there probably is a lot of redundancy in these systems. In the mouse brain, there do not seem to be any significant functional problems linked to the deletion of the apoE or LDL receptor genes **(Masliah E *et al*., 1995; Osono Y *et al*., 1995)** Nonetheless, lipid accumulation has been observed in astrocytes in some brain regions of elderly ApoE knockout animals **(Mato M *et al*., 1999)** A relatively significant phenotype is produced by deletion of the genes coding for the apoE receptor 2 and the very low-density lipoprotein receptor, which have a deficiency in the neuronal layering **(Björkhem & Meaney., 2004)**.To facilitate the transfer of cholesterol from glial cells to neurons, ATP-binding cassette (ABC) transporters may be crucial in addition to apoE. This is because astrocytes produce the ABCA1 transporter **(Figure 2.3)**. ABCA2 transporter expression is mostly found in neural tissues including the brain, according to a recent investigation (**Zhou *et al*., 2001; Schmitz *et al*., 2002).**

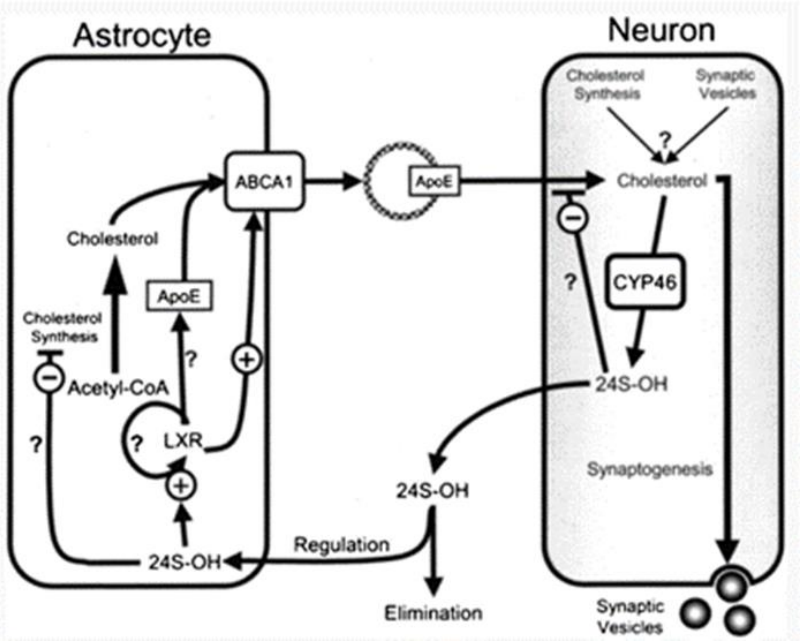

Figure (2.3): Interactions of astrocytes and neurons in cholesterol homeostasis (Pfrieger, 2003)

2.3. Hypercholesterolemia and Blood-Brain Barrier Breakdown

A genetic condition of lipoprotein metabolism, familial hypercholesterolemia (FH) is characterized by significantly raised plasma total cholesterol levels that start to affect the cardiovascular system in childhood. Even though atherosclerosis brought on by FH mainly appears in adults, it can begin as early as the first decade of life **(Varghese, 2014)**. In addition to being a risk factor for vascular dementia **(Appleton *et al*., 2017)**, midlife hypercholesterolemia has been connected to both early- and late-onset Alzheimer's disease (AD) **(Anstey *et al*., 2017; Wingo *et al*., 2019).** The link between familial hypercholesterolemia and cognitive abnormalities **(Zambón *et al*., 2010)**. supports the role of metabolism of cholesterol in the development of neurodegeneration. The development of neurodegenerative illnesses is largely influenced by lifestyle factors **(Lisko *et al*., 2021**). The

brain, however, must create cholesterol from scratch because it cannot cross the blood–brain barrier (BBB) **(Jurevics & Morell, 1995).** However, secondary vascular variables have been found to connect high systemic cholesterol to neurodegenerative alterations, including white matter lesions and atherosclerosis **(De Bem *et al*., 2021)**.

A key risk factor for neurodegenerative disorders is hypercholesterolemia. Specifically, changed metabolism of cholesterol is thought to play a significant role in the etiology of Alzheimer's disease **(Engel *et al*., 2019).** Research has demonstrated that people with elevated serum cholesterol levels in middle age have a higher chance of developing AD in later life **(Mancini *et al*., 2021)**. Recently, however, there is evidence that hypercholesterolemia may impair BBB function, upsetting the equilibrium of cholesterol between the brain and the peripheral, which may facilitate degenerative processes inside the central nervous system **(Tóth *et al*., 2020).**

Chronic exposure to high serum cholesterol levels promotes functional degradation of the vascular endothelium leading to cerebrovascular dysfunction and blood-brain barrier (BBB) collapse (**Grammas *et al*, 2011)**, the vascular hypothesis of AD rationalizes the causal relationship between vascular impairments and the development of sporadic AD. It suggests that long-term cerebrovascular dysfunction causes brain hypoperfusion, which in turn causes a neuronal energy crisis and insufficient BBB clearance of amyloid-(A). In-depth epidemiological research has been done on the connection between cholesterol and AD. After reviewing 17 studies, researchers conducted a meta-analysis which revealed that persons with high midlife total cholesterol (TC) have an increased risk of AD **(Anstey *et al*., 2017).**

A crucial component of healthy neuronal functioning is the regulation of chemical transport into and out of the central nervous system (CNS) via the highly selective semipermeable blood-brain barrier (BBB) **(Cai *et al*., 2018)**. When the blood-brain barrier breaks down, neurotoxic chemicals can enter the brain and damage neurons. They can also cause amyloid deposits, which hasten the progression of Alzheimer's disease (AD) **(Sweeney *et al*., 2018)**. It has been determined that BBB disruption, which is independent of Aβ and tau, is an early indicator of human cognitive impairment **(Nation *et al*., 2019)**. The expression of BBB tight junction proteins was down-regulated and IgG was elevated in the hippocampus of rabbits fed a hypercholesteremia diet. This suggests that the BBB is disturbed because IgG is absent from normal brain parenchyma **(Chen *et al*., 2008)**. Simvastatin, a cholesterol-lowering medication, can lessen the increase in BBB permeability brought on by HCD **(Jiang *et al*., 2012)**. Decreased levels of claudin-5 and occludin mRNA and higher permeability to sodium fluorescein in the hippocampus are indicative of BBB disruption in wild-type and LDLR−/− mice **(de Oliveira *et al*., 2020)**. Additionally, the permeability of the blood-brain barrier was also increased by cholesterol supplementation in rats during hypertension **(Kalayci *et al*., 2009)** and diabetes paired with hypercholesterolemia **(Acharya *et al*., 2013)**. Measuring the rate of water exchange across the blood-brain barrier revealed elevated BBB permeability in patients with hypercholesterolemia **(Shao *et al*., 2019)**.

2.4. Correlation of hypercholesteremia with Alzheimer disease pathogenesis:

The most frequent cause of dementia is Alzheimer's disease (AD), a neurodegenerative illness that spreads widely and impairs cognition. AD is acknowledged as a growing global health concern, affecting over 55 million individuals worldwide at present **(Mancini *et al*., 2021)**. Neurofibrillary tangles (NFT) and β-amyloid (Aβ) plaques are widely regarded as the primary pathological characteristics of Alzheimer's disease (AD) **(Busche & Hyman, 2020)**. Scientists are devoted to creating medications that specifically target tau and Aβ in the hopes that eradicating pathology would result in a novel cure for AD. New avenues for AD research are desperately needed, yet they are largely left disappointed. A mixture of hereditary and environmental risk factors contributes to the multifaceted and complicated disease known as AD **(Mancini *et al*., 2021)**. Risk factor management has been suggested as a successful strategy to halt the progression of AD in recent years. Later-life cognitive decline is linked to modifiable risk factors that are present in midlife, such as hypertension **(Lennon *et al*., 2019),** diabetes **(González *et al*., 2020),** and hypercholesterolemia **(Anstey *et al*., 2008; Knopman *et al*., 2018**).

2.5. Beta amyloid

A prolonged high-cholesterol diet sadly has the potential to cause a number of pathological alterations in the brain, including synaptic degeneration, neuroinflammation, reactive gliosis, Tau hyperphosphorylation, Aβ buildup, and neuronal death **(Chen *et al*., 2016).** These pathogenic alterations have intricated internal relationships with one

other, contributing to the pathophysiology of AD and impairing memory **(Chen *et al*., 2016).**

The primary constituent of the amyloid plaques discovered in the brains of Alzheimer's patients are beta amyloid peptides, which range in length from 39 to 43 amino acids **(Hamley, 2012)**. The primary constituent of amyloid plaques, which are extracellular deposits seen in the brains of Alzheimer's patients, is beta amyloid. Additionally, in cases of cerebral amyloid angiopathy, it can create the deposits that border cerebral blood vessels **(Sadigh-Eteghad *et al*., 2015; Röhr *et al*., 2020)**. The production of beta amyloid the brain expresses high levels of the single-pass transmembrane protein known as amyloid precursor protein (APP), which is processed quickly and intricately during synthesis **(O'brien *et al*., 2011)**.

2.5.1. non-amyloidogenic processing

Distinguished by the absence of Aβ peptide synthesis. The α-secretase enzyme cleaves APP within the Aβ domain, releasing the soluble ectodomain of APP (sAPP-α) and inhibiting the synthesis of Aβ peptide. As illustrated in **Figure (2.4)**, the APP intracellular domain (AICD) and the non-toxic peptide P3 are released when the remaining C-terminal fragment (CTF-α) is cleaved by γ-secretase **(Grimm *et al*., 2013; Dawkins & Smalll, 2014)**.

2.5.2. Amyloidogenic processing Distinguished by the synthesis of the Aβ peptide. The soluble ectodomain of APP (sAPP-β) is released when the enzyme β-secretase cleaves APP at position 671 within the extracellular domain. As illustrated in **Figure (2.4)**, the remaining C terminal fragment (CTF-β) is then broken down by γ-secretase, which releases the AICD fragment and the Aβ peptide **(Grimm *et al*., 2013).**

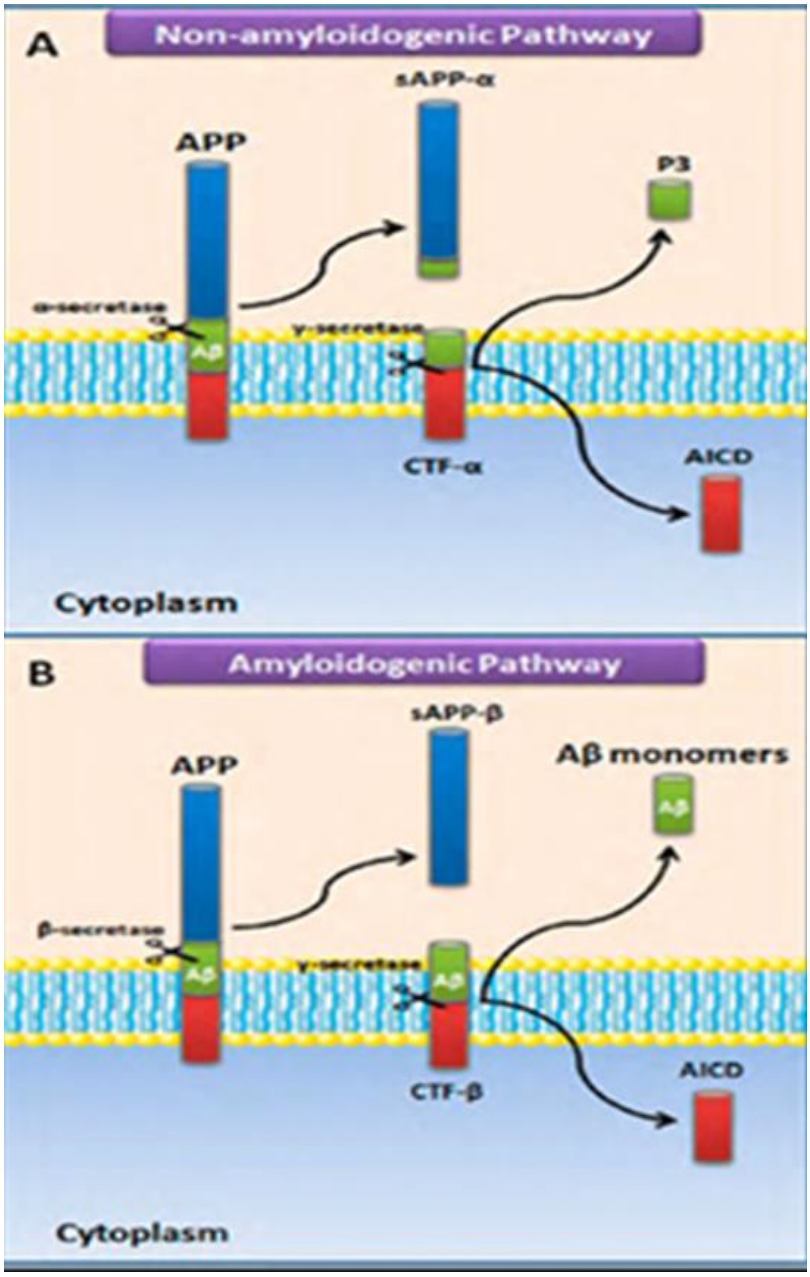

Figure (2.4): Proteolytic processing pathways of APP (Bernabeu-Zornoza *et al.*, 2019) (A) The nonamyloidogenic pathway is where α- and γ-secretases work together to produce the proteolytic products p3, AICD, and sAPP-α. (B) The amyloidogenic route, which produces the proteolytic byproducts sAPP-β, Aβ, and AICD, is where β- and γ-secretases function. Aβ stands for amyloid-β, APP for amyloid precursor protein, sAPP for souble amyloid amyloid precursor protein, CTF for C-terminal fragment, and AICD for amyloid precursor protein intracellular domain.

2.6. Brain Derived Neurotropic Factor (BDNF)

As a constituent of the nerve growth factor family, BDNF is crucial for the control of nutrition and brain metabolism **(Błaszczyk *et al*., 2021).** BDNF is thought to be anorexigenic and influences the metabolism of glucose, lipids, and energy **(Chaldarov *et al*.,2009)**. Its concentration is thought to play a role in aging, neuroprotection, and cardioprotection, and it inversely correlates with body weight and age. Moreover, plasma BDNF levels are markedly lower in patients with acute coronary syndrome or MetS**(Chaldarov *et al*., 2009)**, It suggests that metabolic diseases may develop more quickly in people who don't have enough neurotrophins. A Limited study has been conducted on the function and plasma level of BDNF in TS patients. Brain cells produce a number of chemicals, including growth factors, neurotrophic factors, and anti-inflammatory cytokines, to help them survive stressful situations. These molecules can also help neurons survive. Brain-derived neurotrophic factor (BDNF) is one of the neurotrophins that has been investigated the most **(Lima Giacobbo *et al*., 2019)**. Brain diseases are typically linked to a downregulation of BDNF production, which lowers blood and brain levels of BDNF. Given that the majority of existing treatments result in a notable shift in blood BDNF levels, BDNF has been proposed as a potential biomarker of pathological disorders and therapy success. But there's still a hole in our knowledge of the physiological processes underlying pathological diseases that alter BDNF levels **(Lima Giacobbo *et al*., 2019)**.

2.6.1. BDNF Synthesis and Secretion

According to the BDNF-mediated activity-dependent synaptic modulation, the release of the neurotrophin is modulated by neuronal activity. BDNF and

the other neurotrophins are synthesized in the endoplasmic reticulum, as pre-pro-neurotrophins. Pre-proBDNF is then converted into pro-BDNF by removal of the signal peptide and further cleaved to generate the mature form of the neurotrophin. Whether BDNF is released in the precursor and/or in the mature form remains controversial **(Leal *et al*., 2017).**

The adult mouse hippocampal excitatory presynaptic terminals exhibit colocalization of the precursor and mature forms of neurotrophin in large dense core vesicles, according to electron microscopy studies employing immunogold staining and antibodies against the pro- and mature forms of BDNF **(Dieni *et al*., 2012).** This implies that furin, in the trans-Golgi, or pro-protein convertase 1/3, in secretory granules, cleaves pro-BDNF within the cell, at least in part **(Arango-Lievano *et al*., 2015)**, as previously proposed in light of research on biochemistry **(Matsumoto *et al*., 2011)**. On the other hand, other study revealed that a sizable portion of BDNF might be released as a precursor **(Le Blanc *et al*., 2020).** Here, proBDNF is further broken down into mature neurotrophin in the extracellular compartment by metalloproteinases and plasmin. Consequently, extracellular buildup of proBDNF was seen in response to low-frequency electrical stimulation in investigations conducted in cultured hippocampal neurons employing epitope-tagged BDNF. Additionally, HFS triggers the production of tissue plasminogen activator (tPA), which in the extracellular compartment cleaves proBDNF to produce mature BDNF **(Leal *et al*., 2017)**. The controlled secretory pathway vesicles, which are preferentially transported to the dendritic compartment in cultured hippocampal neurons, are the target of BDNF generated in the cell body **(Leßmann & Brigadski, 2009)**. Additionally, following the movement of the appropriate mRNAs along

dendrites, BDNF is locally produced at the synapse. Before reaching the presynaptic region, large dense core vesicles containing BDNF are also carried anterogradely along the axons **(Matsuda *et al*., 2009).** The pre- and postsynaptic regions release the vesicles containing BDNF/pro-BDNF through an activity-dependent mechanism. Once glutamatergic synapses are activated, the dendritic compartment releases BDNF in response to Ca2+ entering through NMDA receptor channels and/or postsynaptic voltage-gated Ca2+ channels being activated **(Matsuda *et al*., 2009).** In cultured hippocampal neurons, much higher levels of neuronal spiking are necessary to release BDNF from the axonal compartment **(Matsuda *et al*., 2009),** indicating that neuronal activity is more likely to induce the exocytosis of BDNF stored in the postsynaptic compartment under physiological conditions. The molecular processes controlling the maturation and release of BDNF in the adult brain are still largely unknown to us, though.

2.6.2. Physiological context of regulated release of BDNF

High levels of total cholesterol and low-density lipoprotein (LDL) cholesterol have been associated with reduced BDNF expression in the brain. This reduction may contribute to cognitive decline and increased risk of dementia **(De Rooij, 2020).** Diverse triggers are known to cause the controlled release of BDNF, just as there are various cell types that express and release BDNF. The neuronal electrical activity patterns that cause BDNF release in both developing and adult neurons, such as sustained depolarization, high-frequency stimulation (HFS), or theta-burst stimulation (TBS), are the best-known stimuli **(Edelmann *et al*., 2014)**. Naturally, the primary release stimuli in neuronal cells are not the same as those in electrically non-excitable cells **(Brigadski & Leßmann, 2020)**. Extracellular nucleotide binding, pro-

inflammatory factors, or neuropeptides to their corresponding receptors has been shown to induce release of BDNF from astrocytes and microglia **(Lopez-Benito *et al*., 2018)**. In addition to the various physiological stimuli that release BDNF, understanding the subcellular locations where BDNF secretion can occur is crucial for comprehending the functions of specific cell types and release sites of BDNF, such as during neuronal development, synaptic plasticity processes, or in disease situations **(Edelmann *et al*. 2014).**

2.6.3. Release of BDNF by microglia

Like astrocytes, microglia, the brain's primary immune cells, mediate a number of CNS processes, including neuroprotection and synaptic spine sculpting **(Stratoulias *et al*., 2019)**. There are various microglia phenotypes that can be converted into one another through intercellular contacts and various chemokines, leading to varied roles in the brain **(Orecchioni *et al*., 2019)**. The M1/M2 concept, which was first introduced to simplify the in vitro classification of microglia, divides the various overlapping activated microglial phenotypes into two extremes: the neuroprotective M2 phenotype caused by IL-4 and the proinflammatory M1 phenotype induced by lipopolysaccharide (LPS) **(Werry *et al*., 2019)**. It has been demonstrated that microglia of the non-activated M0 phenotype as well as the activated M1 and M2 phenotypes express and secrete BDNF **(Zhou *et al*., 2020)**. For the neuroprotective phenotype, extracellular accumulation of BDNF was shown after polarization of microglial cultures with IL-4 (**Zhou *et al*., 2020)**. Furthermore, Merlo et al. demonstrated an Amyloid-β 42-induced release of BDNF thereby mediating a neuroprotective effect in cultures of a neuroblastoma cell line **(Merlo *et al*., 2018).**

2.7. Synapsin-1

Synapsin-1 is a protein that plays a critical role in regulating neurotransmitter release at synapses in the brain. It binds to synaptic vesicles, which are small sacs that store neurotransmitters, and helps to cluster them near the presynaptic membrane. When an action potential reaches the presynaptic terminal, synapsin-1 is phosphorylated, which causes it to dissociate from the synaptic vesicles. This allows the vesicles to move closer to the presynaptic membrane and fuse with it, releasing neurotransmitters into the synaptic cleft **(Cardozo *et al*., 2019**).

Synapsin-1 is also involved in the recycling of synaptic vesicles. After neurotransmitters have been released, the empty vesicles must be recycled back to the presynaptic terminal for reuse. Synapsin-1 helps to this process by coating the vesicles and preventing them from fusing with the plasma membrane **(Rocchi *et al*., 2019).**

Synapsin-1 is an important protein for synaptic plasticity, the ability of synapses to change in strength. When a synapse is repeatedly activated, synapsin-1 is phosphorylated, which increases the likelihood that vesicles will be released. This can lead to long-term potentiation (LTP), strengthening of the synapse. Synapsin-1 also plays a role in long-term depression (LTD), a weakening of the synapse **(Tavares *et al*., 2019**).

Defects in synapsin-1 have been linked to a number of neurological disorders, including Alzheimer's disease, Parkinson's disease, and schizophrenia. For example, people with Alzheimer's disease have lower levels of synapsin-1 in their brains. This may contribute to the cognitive decline seen in Alzheimer's disease. Overall, synapsin-1 is a critical protein

for synaptic function and plasticity **(Meng *et al*., 2022).** It plays a role in a number of important brain functions, including learning, memory, and movement. Defects in synapsin-1 have been linked to a number of neurological disorders, understanding the function of synapsin-1 may lead to new treatments for these disorders. In AD patients, the expression of synapsin-1 decreased in the brain, which is accompanied by synaptic dysfunction **(Meng *et al*., 2022),** indicating that the dysfunction of synapsin-1 may contribute to synaptic dysfunction in AD.

2.8. Glutamate

The brain's most prevalent excitatory neurotransmitter, glutamate, is involved in a number of processes, including learning, memory, and cognition. It facilitates signal transmission between nerve cells **(Chen *et al*.,2021).** Because glutamate cannot cross the blood–brain barrier, neurons and astrocytes work together to synthesize it in the brain. These cells use the intermediate metabolites of the tricarboxylic acid cycle as precursors **(Chen *et al*., 2021).**

The actions of glutamate, the most prevalent excitatory neurotransmitter in the human central nervous system, are mediated via the activation of glutamate receptors. This system's malfunction could lead to specific types of seizures or exacerbate existing ones, issues with the central nervous system since these receptors are necessary for plasticity, synaptogenesis, appropriate synaptic transmission, and neuronal development **(Traynelis *et al*., 2010)**.

Ionotropic and metabotropic Glu receptors are the two main kinds of Glu receptors. Ionotropic glutamate receptors (iGluRs) in rapid neurotransmission are divided into three classes based on which agonist causes the most

activation. Kainate (KA) receptors. The receptors for amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) **(Traynelis *et al*., 2010)**, and receptors for N-methyl-d-aspartic acid (NMDA). All of these receptors are favorable to Na+ and K+, however the selectivity of the channel they generate depends on the composition of their subunits. Depending on their NMDARs, Ca2+ may travel through them, Subunit composition, specific AMPARs, and KARs **(Traynelis *et al*., 2010).**

The G-protein-coupled ion channels known as metabotropic glutamate receptors (mGluRs) are involved in "slow" neurotransmission. They are classified into three classes and eight subtypes (mGluRs 1-8): The mGluR1 and mGluR5 receptors in group I are phospholipase C (PLC) positively coupled; group II includes mGluRs 2–8, and group III includes mGluRs 4-6 **(Niswender & Conn, 2010). Figure (2.5)**

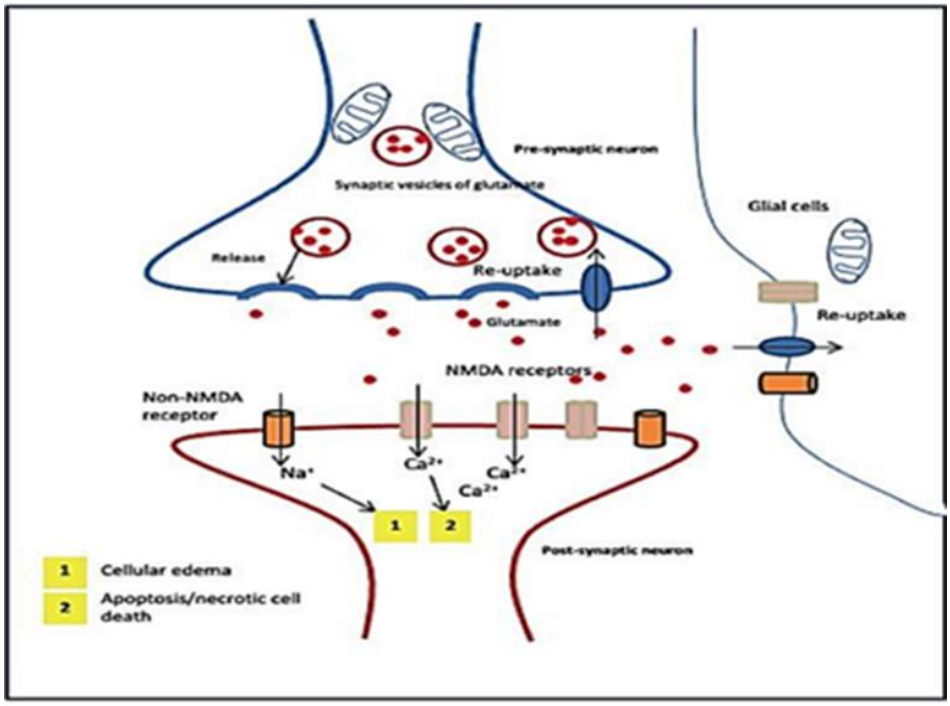

Figure (2.5): Mechanisms of glutamate (Niswender & Conn, 2010)

2.9. Activity-regulated cytoskeleton-associated protein (ARC) gene:

A plasticity protein that is believed to derive from a retrotransposon; The protein is found in the neurons of tetrapod's and other animals where it can form virus-like capsids that transport RNA between neurons **(Chen *et al*., 2021).** ARC mRNA is localized to activated synaptic sites in an NMDA receptor-dependent manner **(Steward *&Worley*, 2001a**) where it is thought that the newly translated protein is essential for molecular processes connected to learning and memory **(McIntyre *et al*., 2005)**. Because of its location, ability to regulate activity, and usage as a marker for alterations in brain plasticity, arc protein is widely regarded as significant in the field of neurobiology. A number of neurological illnesses, such as forgetfulness, Alzheimer's disease, autism spectrum disorders, and fragile X syndrome, have been linked to dysfunction in the synthesis of ARC protein **(Gautam *et al*., 2013)**. The immediate-early gene (IEG) family, which includes ARC, was initially described in 1995 **(Lyford *et al*., 1995)**. This class of rapidly activated genes is functionally defined by its ability to be transcribed in the presence of inhibitors of protein synthesis **(Vazdarjanova *et al*., 2002)**. In rats, the ARC gene is found on chromosome 7, the ARC transcript requires the activation of the mitogen-activated protein kinase, or MAP kinase (MAPK) cascade **(Waltereit *et al*., 2001)**. This route is crucial for controlling cell growth and survival **(Lmpey *et al*., 1999).** Via a range of signaling molecules, including mitogens like epidermal growth factor (EGF), extracellular signaling to neuronal dendrites activates postsynaptic sites to raise ARC levels. Nerve growth factor (NGF) (**Lyford *et al*., 1995)** and brain-derived neurotrophic factor (BDNF) **(Giorgi *et al*., 2007),** glutamate binding to NMDA receptors **(Steward & Worley 2001 B**), dopamine through

activation of the D1 receptor subtype **(Granado *et al*., 2008)** and dihydroxyphenylglycine (DHPG) **(Bloomer *et al*., 2008**).

ARC is a single-copy gene expressed in the cortical and hippocampal glutamatergic neurons. It is highly conserved in vertebrates and expressed in the nucleus, dendrites, and postsynaptic density. ARC protein is a master regulator of synaptic plasticity, involved in learning, memory consolidation, and behavior **(Lyford *et al*., 1995; Fila *et al*.,2021).**

Numerous behavioral changes, such as cued fear conditioning, contextual fear conditioning, spatial memory, operant conditioning, and inhibitory avoidance, are associated with changes in ARC mRNA and/or protein. The mRNA is massively and universally induced by maximal electroconvulsive shock (MECS) and is significantly elevated after electrical stimulation in LTP-induction procedures such as high frequency stimulation (HFS) **(Chen *et al*., 2021).**

2.9.1. ARC and memory consolidation

Intrahippocampal infusion of ARC inhibits LTP consolidation and long-term spatial memory but has no effect on acquisition or short-term memory function **(Guzowski *et al*., 2000)**. The selective requirements for ARC in long-term memory for a range of hippocampal-dependent and hippocampal-independent tasks, such as spatial learning in the Morris water maze, auditory and context-dependent fear conditioning, conditioned taste aversion, and object recognition, were established in a recent thorough behavioral analysis of homozygous ARC knockout mice **(McIntyre *et al*., 2005; Plath *et al*., 2006)**. Inhibitory avoidance memory also requires ARC production in the dorsal hippocampus, as demonstrated by ARC infusion experiments.

Furthermore, the activation of β-adrenoreceptors in the basolateral complex of the amygdala affected both hippocampal ARC synthesis and memory performance, suggesting a significant role for extrinsic inputs in regulating ARC function in the hippocampus. Pavlonian fear conditioning increases the production of ARC in the lateral amygdala; this protein is selectively needed for the establishment of long-term memory and is dependent on ERK **(Ploski *et al*., 2008).**

Alzheimer's disease (AD) is linked to a decrease in hippocampal and cortical network connection, which causes memory loss. ARC expression is mainly decreased in the granule cells of the dentate gyrus in transgenic mice overexpressing human amyloid precursor protein (HAPP) and HAPP-derived amyloid-β (Aβ) **(Palop *et al*., 2005).** Even at sublethal doses of Aβ, BDNF-induced ARC expression is inhibited in cultured cortical neurons **(Wang *et al*., 2006)**. Other researchers found that when dissociated hippocampal neurons are exposed to Aβ oligomers that are neurologically active, called Aβ-derived diffusible ligands (ADDLs).They lengthen their dendritic spines and upregulate Arc, which is followed by a decrease in the density of synapses **(Wang *et al*., 2002; Lacor *et al*. 2004; Viola *et al*., 2008)**. Together, this work suggests that dysregulation of ARC expression could contribute to cognitive impairment and memory loss in AD.

Chapter Three
Methodology

3. Methodology

3.1. Materials

3.1.1. Instruments and Equipment:

All the devices utilized and equipment as a part of this study are summarized in table **(3.1)**

Table 3.1. Apparatus and equipment's with their manufactures.

NO	Apparatus & Equipment	Company	Manufactures
1.	Anatomical set (Scissors, Forceps, Scalpel)	Chemo lab	China
2.	Beakers (100, 250, 500, 1000)	Chemo lab	India
3.	Cold Eppendorf Centrifuge	Hermle	Germany
4.	Colony flask	Chemo lab	India
5.	Conventional thermocycler PCR	BioRad	USA
6.	Cotton	Entrepreneur	India
7.	Digital balance	Denver	Germany
8.	Digital camera	Canon	China
9.	Eppendorf 's tubes	Chemo lab	India
10.	Filter paper	Chemo lab	India
11.	Freezer	Hitachi	Japan
12.	Gel tube	Chemo lab	India
13.	High speed centrifuge	Ohaus	Germany
14.	Insulin syringe	Eldawlia	Egyp
15.	light microscope with camera	MEIJI	Japan
16.	Liquid nitrogen	Rockefeller	USA
17.	Micropipettes	Eppendrof	Germany
18.	Microtome	Leica RM	USA
19.	Mince machine	Hitachi	Japan

20.	Pipette tips (10 – 1000) µl volume	Chemo lab	India
21.	qPCR tubes	Kirgen	Korea
22.	Real time qPCR machine	BioRad	USA
23.	Slide & cover slip lab	Chemo	China
24.	Spin centrifuge	Oxford	UK
25.	Syringe (1 ml, 5 ml)	Chemo lab	India
26.	Surgical gloves	Chemo lab	India
27.	Test tubes	Chemo lab	India
28.	Water bath	labtech	Korea

3.1.2. Chemicals and Kits

All the chemicals and the standard kits used in this study are shown in table **(3.2).**

Table 3.2: Chemicals and Kits with their suppliers.

NO	Chemicals & Kits	Company	Suppliers
1.	Acetic anhydride	SdfcL	India
2.	Activity Regulatory Cytoskeleton Associated Protein (ARC) kit	ADDBio	Korea
3.	Brain Derived Neurotropic Factor (BDNF) kit	Elabscience	USA
4.	cDNA synthesis kit	ADDBio	Korea
5.	Cholesterol powder	Avonchen	UK
6.	Chloroform	Scharlau	Spain
7.	Congo red stain	Himedia Lab	India
8.	Dimthylsulphoxid DMSO	LOBA	China
9.	Eosin Stain	Himedia Lab	India
10.	Ethanol (molecular grade)	Sigma	Germany
11.	Formalin 10 %	chemano	SA
12.	Glacial acetic acid	SdfcL	India
13.	Glutamate kit	Elabscience	USA

14.	Glutathione (GSH) kit	laboratorio ct	Italy
15.	Hematoxylin Stain	Himedia Lab	India
16.	Lipid profile kit	Biolabo	Italy
17.	Malondialdehyde (MDA) kit	Elabscience	USA
18.	Nitric oxide	Elabscience	USA
19.	Normal saline	Chemo lab	India
20.	Paraffin wax	Citotest	China
21.	Perchloric acid	SdfcL	India
22.	RNA extraction kit	Genaid	Korea
23.	Synapsin 1 kit	Elabscience	USA

3.2. Examination methods

3.2.1 Experimental protocol

Twenty male rats' weight of the rat (178 -200) gm. were used in the current study taken from college of veterinary university of Karbala –Iraq the animals lived in clean, specialized plastic cages, and their ages varied from (10 -12 weeks). We used a light cycle of 12 hours and a humidity level of 55%. They stayed there for two weeks to be used to the testing environment. The temperature stayed between (22-25℃). Animals were kept comfortable with the help of a thermostat and a ventilation hoover, and they were given pellets of freshly made food, with approval from a moral standpoint.

3.2.2. Experimental Design

Twenty male rats were divided equally for two groups (10/group) and given the following cholesterol (4 weeks), as shown in figure **(3-1).**

1-Control group: made up of this group were given a normal diet (pellets of freshly made food contain from carbohydrate, protein, vitamin and mineral).

2- Hypercholesteremic diet group (HCD): Rats in this group were given a pellets of freshly made food contain from carbohydrate, protein ,vitamin and mineral with cholesterol orally for 4 weeks, comprising 1% cholesterol (w/w) **(fiddle *et al*., 2017).**

3.3. Blood samples collection

After four weeks of the experiment, blood samples were taken after fasting the animals. the animals were first given a chloroform anesthesia to make them more controllable and docile before the blood was taken. Blood sample were taken by sterile medical syringes of 5 ml were accustomed to extract 5 ml of cardiac blood by using the heart puncture method, The blood was then put into gel tube and the blood was centrifuged in at a speed of 4000 revolutions per minute for 5 minutes. Once the serum had been separated, it was put in Eppendorf tubes for storage and kept in the fridge at (-20 ° C) until the assays were working.

3.4. Brain tissues collection

Rats were sacrificed by chloroform anesthesia after completion of the experiment, and the animals were dissected for brain sampling. After taking brain samples, MDA, GSH nitric oxide and glutamate examination (take about 100 mg of brain tissue), and gene expression (take about 50mg of brain tissue).

3.4.1. Estimation of the MDA, GSH, Nitric oxide and Glutamate

Brain samples homogenized for 150 seconds (to ensure the tissue was well dispersed) in 0.1 N perchloric acid (200 µl), using Squishers. The homogenates were then centrifuged at a speed (5000) in a cold room (4ºC) for 30 minutes, and the supernatants drawn off carefully. At this stage the

supernatants could be frozen (-80ºC) or taken directly for analysis. Take 500ml from anhydral glacial acetic acid and Add 25ml from acetic anhydride and Add about 8.5 ml from perchloric acid and put in cool (24h). Complete the mixture to1000ml from Anhydral glacial acetic acid.

3.4.2. Gene expression Activity Regulated cytoskeleton (ARC)

Liquid nitrogen (5 liter) was used to preserve the brain tissue until it could be extracted and studied in a lab.

3.4.3. Histopathological examination

The brain tissue they preserved in 10% formalin in sterile plastic containers for 24 hours then subjected to histological technique, H&E stain and congo red special stain.

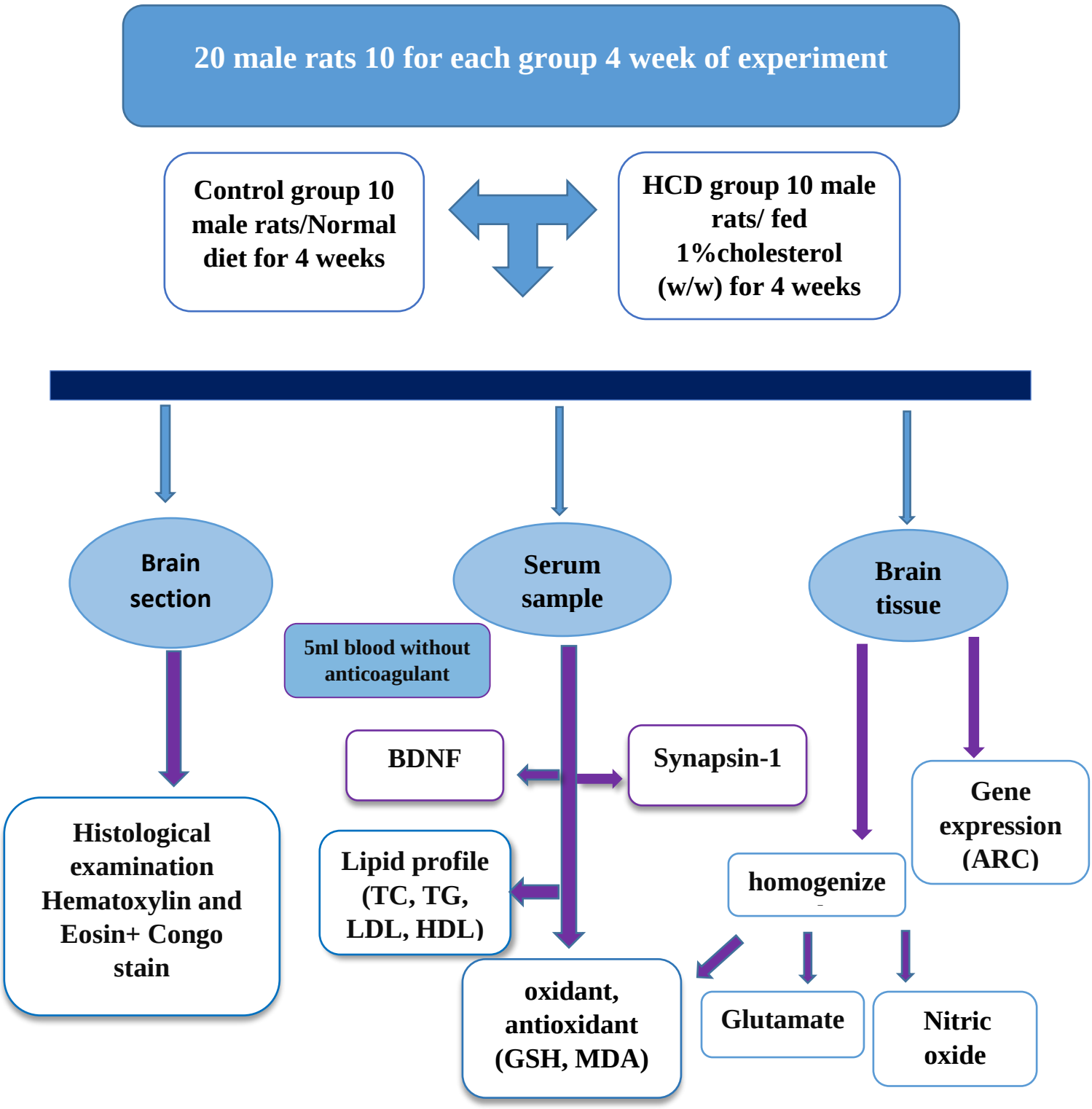

Figure (3.1) Experimental Design

3.5. Determination of Serum Lipid profile:

3.5.1. Determination of Serum Total Cholesterol (TC) Concentration(mg/dl):

Total Cholesterol (TC) was detected by using Enzymatic method described by **(El Shahat *et al*., 2018),** as illustrated in **Appendixes I.**

3.5.2. Determination of Serum Triglyceride (TG) Concentration (mg/dl):

Total Serum TG was detected by using Fossati and Principle method associated with Trinder reaction. Reaction scheme **(Taneja& Girhotra, 2006)**, as illustrated in **Appendixes II**.

3.5.3. Determination of Serum High Density lipoprotein-cholesterol (HDL-C) Concentration (mg/dl):

Serum HDL-C concentration was measured enzymatically by utilizing HDL-C kit, according to **(Rahman *et al*., 2014)**, as illustrated in **Appendixes III.**

3.5.4. Determination of Serum Low Density lipoprotein-cholesterol (LDL-C) Concentration (mg/dl):

Serum low density lipoprotein-cholesterol concentration was measured depending on equation **(Sung *et al*., 2020),** as illustrated in **Appendixes IV.**

3.6. Evaluation of Biomarkers: -

3.6.1 Determination of homogenized brain tissue and Serum Malondialdehyde (MDA) concentration (µ mol /L):

Malondialdehyde was estimated by Thiobarbituric acid (TBA) assay method of **(De Leon & Borges, 2020).** on spectrophotometer, as illustrated in **Appendixes V.**

3.6.2. Determination of homogenized brain tissue and serum Reduced Glutathione (GSH) concentration (µ mol /L):

Reduced glutathione was measured following the method of **(Dhouib *et al*., 2014)**, as illustrated in **Appendixes VI.**

3.7. Determination of brain tissue nitric oxide (NO) concentrations (µ mol /L):

Nitric oxide metabolite concentration in the supernatant was measured using the Griess reaction as the basis for a colorimetric technique **(Tsikas., 2004),** as illustrated in **Appendixes VII.**

3.8. Neruomarker concentration examination

The neruomarkers concentration were measured by using a special Enzyme Linked Immunosorbent Assay (ELISA) kit, and it was measured by the steps included in the kits: -

3.8.1. Estimation of serum brain derived neurotropic factor (BDNF) concentration

An ELISA kit was used to determine the amount of BDNF in the serum of rats. Sandwich ELISA is the underlying technology for these ELISA kits. These kits include a micro-ELISA plate pre-coated with an antibody against Rat BDNF, as shown in **Appendixes VIII.**

3.8.2. Estimation of serum Synapsin-1 concentration

A commercially available ELISA kit was utilized to quantify the concentration of synapsin-1in rat serum. This kit employed the sandwich ELISA principle, featuring a microplate pre-coated with a synapsin-1 specific antibody, as shown in **Appendixes IX.**

3.8.3. Estimation of tissue glutamate:

The ELISA kit was used to measure the brain tissue of the rat (Table 3.2). Sandwich-ELISA is the principle used in this ELISA kit. This kit includes a micro-ELISA plate that has been pre-coated with a rat-specific antibody., as illustrated in **Appendixes X.**

3.9. Determination Gene expression Activity Regulatory Cytoskeleton associated protein (ARC):

This approach was carried out according to the comparative Ct approach (ΔΔCt) with normalization to the level of the control group in the presence of the transcript levels to those of GAPDH mRNA. This was achieved according to the recommendation of **(Schmittgen & Livak, 2008)**. **Appendixes XI.**

For this purpose, amplifying of the ARC gene was carried out using the following primers in the **table (3-3)**

Table (3.3): primers of activity regulatory cytoskeleton protein (ARC) gene

Primer name	Sequence (5'->3')	Target
ARC-Forward	GACAAGGCAGAGGAGAGTGTC	Gene of Interest
ARC-Reverse	CCGGAGTGACTAATGTGCTCT	
B- actin-F	GTGGCACCACCATGTACCCAGGCAT	Housekeeping gene
B-actin-R	ACTACAGGGCTGACCACACCCCACT	

3.10. Histopathological study

3.10.1. Brain sections and fixation

All samples were fixed with formalin 10% concentration, then submitted to graded alcohol concentrations of 70%, 90% and 100%, then xyline and paraffin in an automated processor. The blocks were sliced using the 5µm thickness by microtome and stained by hematoxylin and eosin stain used for general structure tissue of the research **(Bancroft, 2013).**

3.10.2. Congo red stain

The tissue sample portions were sliced using a rotary microtome, mounted onto glass slides, and heated at 37 °C until they were completely dry. The textile slides were then stained using the technique described in **(Miguel *et al*., 1998),** as illustrated in **Appendixes XII.**

3.11. Statistical analysis:

The statistical program Graph Pad Prism 8.0 the t-test and correlation was used, P≤0.05 was chosen as the standard of significance. The data points were shown as mean± SD **(Wang *et al*., 2017)**.

Chapter Four
Results & Discussion

4.1. Effect of hypercholesteremic diet on serum Lipid profile in male rats:

4.1.1. Effect of hypercholesteremic diet on total cholesterol (TC) level in male rats:

The results of this investigation demonstrated a high significant ($P < 0.0001$) rise in serum TC in the HCD group (65.53±0.25) mg/dl relative to the control group (30.35 ± 0.16) mg/dl , as seen in **figure (4-1).**

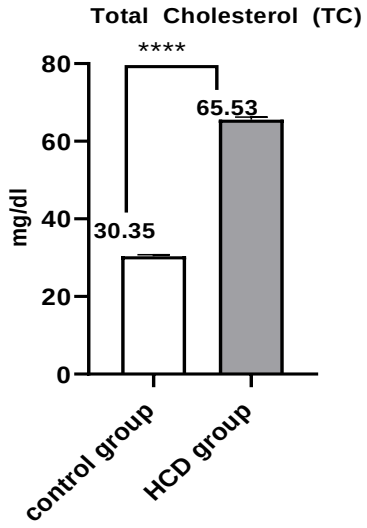

Figure (4-1): Effects of 1% hypercholesteremic diet for 4 weeks on the serum total cholesterol concentration in the male rats

4.1.2 Effect of hypercholesteremic diet on serum triglyceride (TAG) level in male rats:

The current study showed a significant ($P \leq 0.0001$) increase in serum TAG in the hypercholesteremic diet group (87.02±0.59) as compared with the control group (39.9±0.60) mg/dl shown in **figure (4-2).**

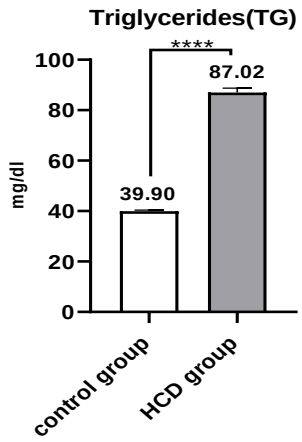

Figure (4-2): Effects of 1% hypercholesteremic diet for 4 weeks on on the serum triglycerides (TG) concentration in the male rats

4.1.3 Effect of hypercholesteremic diet on serum Low-density lipoprotein (LDL)level in male rats:

This study showed a high significant ($P \leq 0.0001$) increase in serum LDL in the hypercholesteremic diet group (19.17±0.11) mg/dl as compared with the control group (11.168±0.21) mg/dl as shown in **figure (4-3).**

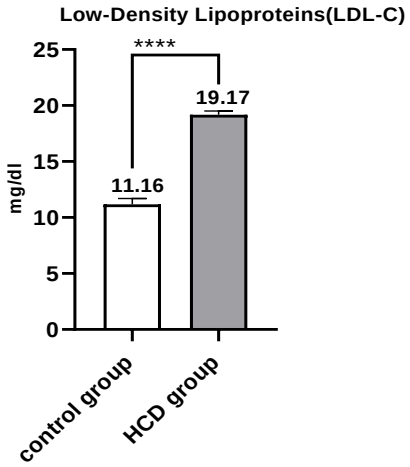

Figure (4-3): Effects of 1% hypercholesteremic diet for 4 weeks on the serum (LDL-C) male rats' levels

4.1.4 Effect of hypercholesteremic diet on serum High-density lipoprotein (HDL) in male rats:

This study showed a high significant ($P \leq 0.0001$) decreased in serum HDL of the hypercholesteremic diet group (24.08±1.27) mg/dl as compared with in the control group (37.24±0.24) mg/dl as shown in figure **(4-4).**

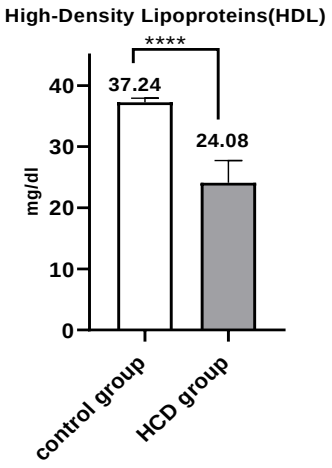

figure (4-4): Effects of 1% hypercholesteremic diet for 4 weeks on the serum (HDL) concentration in the male rats

Cholesterol is a cellular component that is crucial for cellular homeostasis and transmembrane communication (the movement of information across cell membranes) **(Liu *et al*., 2010; Hussain *et al* 2023))**. The brain, nerves, and other tissues contain large amounts of cholesterol. Blood cholesterol levels above normal can lead to lipid plaque formation, which can impair target organ blood flow and result in tissue ischemia and hypoxia. Tissue structure and function are thereby compromised **(Zhang *et al*., 2024)**. The current study found that adding 1% cholesterol to the diet daily for four weeks increased TC, TAG, and LDL while reducing HDL in group of the cholesterol compared to group of the control and this result agreement with **(Tolani et al., 2013;**

Diao *et al*., 2018; de Oliveira *et al*., 2020; Mokhtarzadeh Bazargani *et al*., 2021; Keser *et al*., 2023; Raheem *et al*., 2023).

A significant contributing factor to the onset and progression of cognitive impairment is increased TC. The following reports detail the fundamental pathways via which increasing TC leads to cognitive impairment. First, elevated TC levels impair cerebral blood flow and cause lipid plaques in the carotid artery, raising the possibility of plaque rupture. Concurrently, elevated TC levels lead to increased amyloid β-protein (Aβ) deposition, which further affects cerebral blood flow and impairing cognitive performance **(Hua *et al*., 2020)**. Second BBB is harmed by elevated TC levels Cholesterol normally cannot cross the BBB **(Wang *et al*., 2016).** Third, oxidative stress brought on by elevated TC levels damages the membranes of neurons **(Chiesa & Charakida, 2019).**

TG is a lipid that is used in the transportation and storage of energy. Serum TG is primarily produced by liver synthesis and intestine absorption; in a healthy condition, it is less than 1.70 mmol/L. Atherosclerosis, coronary heart disease, and other ischemic cardiovascular disorders can be brought on by excessive levels of TG **(Liu C *et al*., 2018).** TG's current role in the development of cognitive impairment is still up for debate **(Dimache *et al*., 2021)**. It has been proposed that TG is closely correlated with inflammatory indicators and may contribute to cognitive impairment in addition to being linked to the breakdown of the blood-brain barrier **(Ridker *et al*., 2003).** According to an animal study, TG may impair long-term increased N-methyl-d-aspartic acid in the hippocampus, which could lead to cognitive deterioration **(Farr *et al*., 2008)**. Lower TG levels have the potential to reverse cognitive impairment and lessen oxidative stress in the brain.

Approximately 2/3 of the lipoproteins in plasma while fasting are made up of LDL-C it derived from (VLDL-C) and serves as the primary means of delivering cholesterol to tissues outside the liver. Furthermore, there is a danger of cognitive impairment developing and occurring as a result of elevated LDL-C. elevated low-density lipoprotein (LDL-C) may be a key pathogenic component for atherosclerosis and the trigger for persistent carotid artery inflammation **(Liu *et al*., 2020).** The risk of brain microvascular damage and cerebral hypoperfusion is positively linked with higher LDL-C levels. Furthermore, increasing LDL-C levels results in lipid peroxide accumulation, which can worsen platelet aggregation and raise the risk of cerebral hemorrhage, increased blood viscosity, and reduced cerebral blood flow **(Zhang *et al*., 2022)**. There is a positive correlation between the density of nerve plaques and elevated serum levels of TC and LDL-C **(Chen *et al.*, 2019)** Interestingly, when LDL-cholesterol levels are too high, the condition referred to as hypercholesterolemia **(Ibrahim *et al*., 2023)**

HDL-C has the ability to carry cholesterol from extrahepatic tissues to the liver, where it is either immediately excreted from the intestines through the bile or transformed into bile acids by the liver. Additionally, HDL-C has the ability to absorb cholesterol from the cell membrane and use lecithin cholesterol acyltransferase to catalyze the formation of cholesterol ester, which is subsequently transferred to (LDL-C) & (VLDL-C) **(Bruckert & Hansel, 2007).**

The factors support HDL-C's protective impact against cognitive decline are: First, by preserving cerebral blood flow and preventing the development of lipid plaques in the carotid artery, HDL-C can lessen the cognitive impairment brought on by ischemia and hypoxia **(Yun *et al*., 2019).** Second,

HDL-C has a fibrinolytic impact that lowers the risk of cerebral ischemia by preventing the development of atherosclerotic plaques, controlling blood rheology, and lessening the occlusion of cerebral blood arteries brought on by lipid buildup **(Bowman *et al*., 2018**). Third, HDL-C regulates nitric oxide (NO) and platelet-activating factors, which relaxes the vascular smooth muscle and has an antithrombotic impact. Furthermore, it inhibits leukocyte adhesion and platelet aggregation in the vascular endothelium, which is advantageous for the prevention of carotid artery thrombosis as well as the defense of neurological and cognitive processes **(Yun *et al*., 2019).**

There is a correlation between plasma cholesterol levels and cognitive decline, i.e., higher cholesterol levels were related to a lower discriminatory score in the novel object recognition task. Increased BBB permeability in the hippocampus was negatively correlated with the rat's memory performance **(de Oliveira *et al*., 2020)**.

The function of elevated LDL-C and decreased HDL-C as separate risk factors for beta-cell dysfunction **(Kruit *et al*., 2010; Bardini *et al*., 2012; Raheem *et al*., 2023; Mahdi et al., 2021).**

Blood-brain barrier (BBB) leakage, hippocampal apoptosis (**de Oliveira *et al*., 2020)**, cortico-cerebral mitochondrial dysfunction, oxidative damage, neuroinflammatory process **(De Oliveira *et al*., 2011)**, and neuronal and synaptic dysfunctions are among the biological underpinnings that underlie the cognitive impairments associated with LDL-C **(Mulder *et al*., 2007; Wang &Eckel, 2014; Engel *et al*., 2019; Rodrigues *et al*., 2023)**. Additionally, there is evidence that high-cholesterol diets, methylmercury

(MeHg), and amyloid-β (Aβ) produce neurotoxicity more readily in LDL-C **(de Oliveira *et al*., 2020).**

There are two main ways that cholesterol is transported to the brain the Local Synthesis. Astrocytes, a type of glial cell, is the main source of cholesterol in the brain. They synthesize cholesterol themselves and then transport it to neurons and other brain cells via apolipoproteins **(Zhang& Liu, 2015**), and Blood-Brain Barrier Transport a small amount of cholesterol can cross the BBB from the bloodstream. This cholesterol is carried by lipoproteins, particles that package cholesterol and other fats for transport throughout the body. However, the BBB tightly regulates this transport, allowing only certain types of lipoproteins to pass through **(Rhea& Banks, 2021).**

The lipid profile, which includes total cholesterol, triglycerides, and LDL, is rising, but the levels of high-density lipoprotein are declining. This could be because of the action of cholesterol, which is rising in the liver cells' internal process of producing cholesterol. Additionally, by emulsifying lipids and facilitating their absorption, it can encourage the absorption of lipids from the gastrointestinal system **(Raheem *et al*., 2023).**

4.2 Effect of hypercholesteremic diet on serum & brain tissue anti-oxidant parameters:

4.2.1. Effect of hypercholesteremic diet on the serum and brain tissue Malondialdehyde (MDA) concentration in male rats:

The current study showed a high significant ($P \leq 0.0001$) increase in brain MDA in the hypercholesteremic diet group (7.91±0.17) as compared with the control group (5.84±0.19), and showed a high significant ($P \leq 0.0001$) increase in serum MDA in the hypercholesteremic diet group (0.344 ± 0.008)

nmol/mg as compared with the control group (0.14±0.004) nmol/mg , as shown in **figure (4-5).**

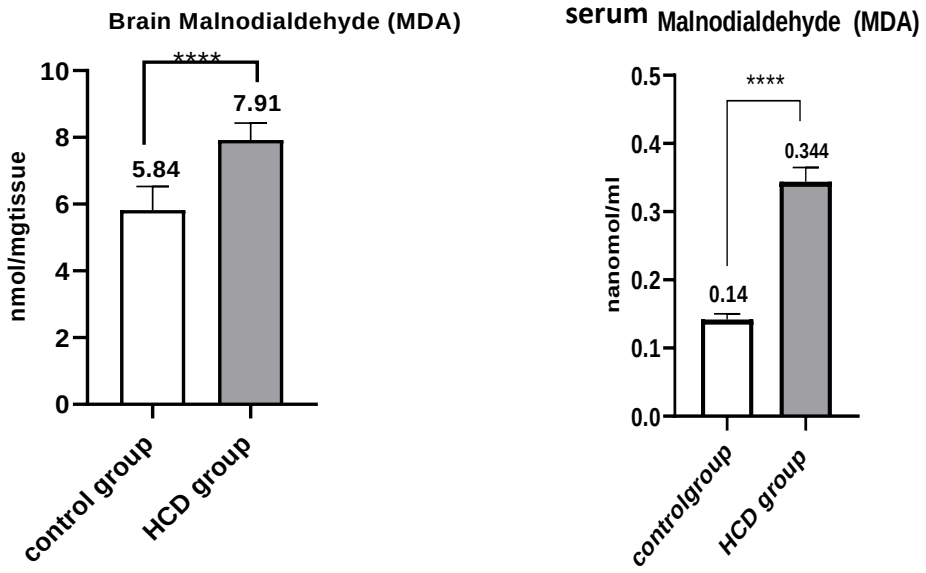

Figure (4-5): Effect of 1% hypercholesteremic diet for 4weeks on the serum and brain tissue Malondialdehyde (MDA) concentration in the male rats.

Hypercholesterolemia is characterized by oxidative stress caused by elevated malondialdehyde (MDA) levels. MDA, on the other hand, it is an oxidative damage marker of physiological cell damage. A study showed that the hypercholesterolemia had higher plasma MDA concentrations, this indicates the correlate between oxidative stress and hypercholesterolemia progression **(Widhiantara *et al*., 2021).**

Oxidative stress is a major factor in the genesis of numerous disorders. The association between oxidative stress and hypercholesterolemia in causing endothelial dysfunction in brain arterioles, even in the absence of atherosclerotic lesions, was observed **(Sezgin *et al*., 2023)**. Higher MDA concentrations in both brain tissue and serum in hypercholesterolemic group

compared with control group, these result agreement with **(Mahdi *et al*., 2021; Higashi *et al*., 2022; Raheem *et al*., 2023).**

Indicators of oxidative stress in the body brought on by free radical toxicity, such as high-cholesterol diets, include brain tissue and plasma MDA levels **(Banc *et al*., 2022; Bati *et al*., 2023).** Additionally, MDA which is the end result of polyunsaturated fatty acid peroxidation in cells, is a proof of lipedperoxidation. An excess of MDA is produced due to a rise in free radicals **(Kurutas, 2015).**

Numerous experimental studies have reported that cholesterol overload markedly induces ROS accumulation and redox imbalance in tissues. Lipid peroxidation of cellular membranes has also been implicated as a causative mechanism **(Alsaad *et al*., 2020; Mahdi *et al*., 2021; AlHasnawi *et al*., 2022).** Moreover, studies have revealed links between oxidative stress and inflammation that were closely correlated with tissue necrosis and cellular apoptosis during hypercholesterolemia **(Alsaad *et al*., 2020).**

Oxidative stress and oxidative damage resulting from aging are associated with a lot of diseases, including diabetes, heart disease, cancer, iron overload, iron deficiency anemia, reproductive issues, Alzheimer's disease, and aging itself, creation of lipid peroxidation markers **(Park & Choi, 2016)**.

4.2.2. Effect of hypercholesteremic diet on serum and brain tissue Glutathione (GSH) concentration in male rats:

This result showed a high significant ($p \leq 0.0001$) decrease in the serum GSH concentration (2.90±0.14) nmol/mg in the hypercholesteremic diet group as contrast with the control groups (4.02 ± 0.02), and the current investigation indicates a significant ($p \leq 0.001$) decrease in the brain tissue

GSH in the hypercholesteremic diet group (22.07±0.31) mol/ml as compared with the control group (52.36±0.48) mol/ml as shown in **figure (4-6).**

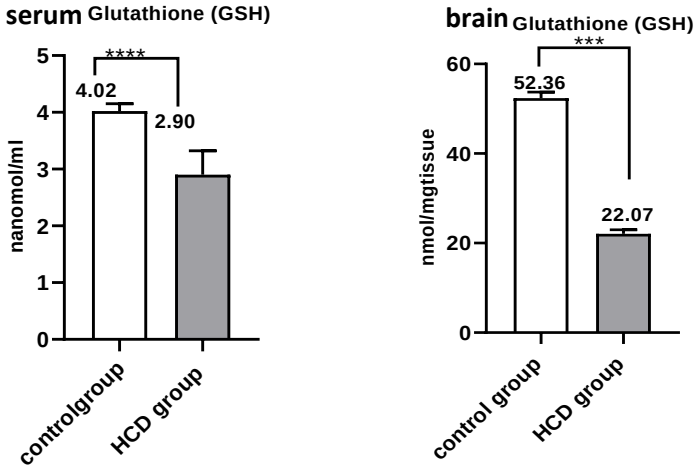

Figure (4-6): Effect of 1% hypercholesteremic diet for 4weeks on the serum and brain tissue GSH concentration in the male rats.

Glutathione (GSH) is a major antioxidant that maintains the homeostasis of redox states in cells, and plays important roles in maintaining the physiological functions of all cells in vivo, its tripeptide molecule composed of three amino acids: glutamate, cysteine, and glycine. It is found in nearly every cell of the body, including the brain, and plays a crucial role in various physiological processes **(Aoyama, 2021).**

Cholesterol is a strong indicator of lipid peroxidation, a known mechanism of oxidative stress, as shown by a considerable decrease in brain and serum glutathione (GSH) concentrations in hypercholesteremia diet group compared to the control group. For this reason, reduced GSH levels is referred to as a free radical productive, the result agreement with **(John *et al*., 2005; Albazi., 2014; Contreras-Duarte *et al*., 2021).** Cholesterol-mediated reduction of mitochondrial glutathione (GSH) is worsened by elevated cholesterol levels

caused by poor mitochondrial GSH transfer **(Al-Bajari *et al*., 2019; De Dios *et al*., 2023; Roca-Agujetas *et al*., 2021).** This, in turn, alters mitochondrial function and increases ROS generation, by amplifying the mitochondrial oxidative damage induced by Aβ **(Roca-Agujetas *et al*., 2021).**

Alzheimer's disease, which includes neuro-inflammation, is accelerated and made worse by brain cholesterol enrichment **(Mahdi *et al*., 2021).** When molecular oxygen goes through an incomplete reduction, it produces radicals and molecules known as reactive oxygen species (ROS). They are essential for cellular homeostasis and play a critical function in communication **(Swerdlow, 2018).**

Numerous studies have shown that oxidative stress, which is defined as an imbalance between the oxidative and antioxidant systems that favors the oxidative system, contributes to the course of AD **(Butterfield & Halliwell, 2019)**. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the most representative oxidants that have a major impact on redox biology and induce oxidative stress **(Ezraty *et al*., 2017).** Oxidative stress is considered as a detrimental condition for normal brain functioning. Since the brain uses chemically diverse reactive species for signal transmission, it is susceptible to oxidative stress **(Lee *et al*., 2020)**. The mitochondria in the brain use the inspired basal oxygen to reduce O2 to H2O to support adenosine triphosphate (ATP) synthesis **(Bailey *et al*., 2009).**

4.3. Effect of hypercholesteremic diet on brain tissue nitric oxide (NO):

The hypercholesteremic diet group showed a significant ($p \leq 0.0001$) decrease in brain nitric oxide (2.78±0.26) nmol/mg as compared with the control group (5.86±0.17) nmol/mg as shown in **figure (4-7).**

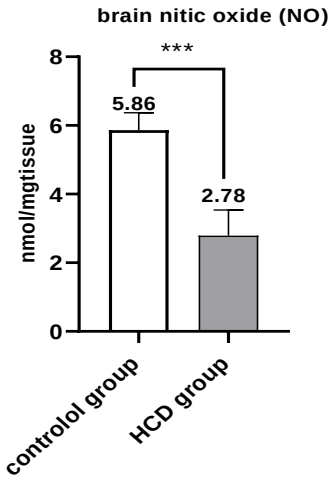

Figure (4-7): Effect of 1% hypercholesteremic diet for 4weeks on the brain nitric oxide (NO) concentration in the male rats.

Hypercholesterolemia group shows reducing the brain nitric oxide production and a marked increase in malondialdehyde (MDA) levels relative to the control group. For this reason, anything that can reduce MDA levels is referred to as a free radical scavenger. The result agrees with **(Lin& Deng, 2019; Mancini *et al*., 2021; Tekeli *et al*., 2023).**

Nitric oxide was established as a novel type of signaling molecule in the central nervous system **(Ivanova *et al*., 2020)**. And works as a retrograde neurotransmitter in synapses, allows the brain blood flow and also has important roles in intracellular signaling in neurons from the regulation of the

neuronal metabolic status to the dendritic spine growth (**Picón-Pagès *et al*., 2019).** NO is involved in the molecular mechanisms of memory formation **(Sase *et al*., 2016).** In particular NO is an anti-inflammatory molecule under resting conditions **(Kanwar *et al*., 2009).**

NO is a molecule with pleiotropic effects in brain favors synaptic functions by LTP maintenance and protein translation at dendritic spines. It is also critical to guarantee the proper blood supply to neurons and it has been demonstrated to be an antiapoptotic molecule and a regulator of neuronal function by nitrosylation. However, when NO is produced in a pro-oxidant environment, as during aging or in AD, reacts with O2 producing ONOO, which nitrotyrosinates proteins damaging brain cells growth (**Picón-Pagès *et al*., 2019).** Therefore, a decrease in brain NO due to hypercholesterolemia might contribute to several negative consequences like Endothelial dysfunction: High cholesterol can damage the endothelium, the lining of blood vessels. This impaired endothelium might have reduced capacity to produce NO through the enzyme endothelial nitric O **(De Oliveira *et al*., 2011).**

Increased cholesterol levels can lead to the formation of free radicals that scavenge and inactivate NO, further reducing its availability in the brain **(Martemucci *et al*., 2022**). Cholesterol can be involved in various cellular signaling pathways that influence NO production. Disruption of these pathways due to hypercholesterolemia might lead to decreased NO synthesis **(Song *et al*., 2021).**

The hippocampus, the center responsible for memory and learning, is one of the first centers to be affected in AD and it degenerates dramatically. APP,

whose function is related to membrane adhesion and cell recognition, is highly expressed in neuronal synapses, allowing synaptic plasticity **(Priller *et al*., 2006)**. Since hippocampus is rich in APP expression, the Aβ production increased favoring its aggregation in an environment where glutamatergic signaling releases NO, making hippocampus especially susceptible to nitrative damage **(Hensley *et al*., 1998).**

4.4. Effect of hypercholesteremic diet on Brain Derived Neurotropic Factor (BDNF) in male rats:

The HCD group showed a significant ($P \leq 0.001$) decrease in BDNF (9.17± 0.28) ng/ml as compared with the control group (19.42±1.48) ng/ml as shown in figure **(4-8).**

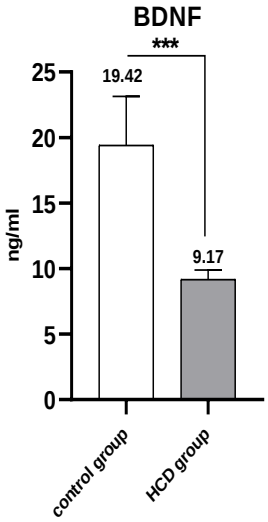

Figure (4-8): Effects of 1% hypercholesteremic diet for 4 weeks on the serum BDNF concentration in the male rats.

Cholesterol is vital for cell maintenance, neural transmission, and synapse formation, and it is found in the brain 23% of total body cholesterol **(Hussain *et al*., 2019).** Myelin sheath, which insulates axons, and dendritic spines are

both heavily enriched with cholesterol. Studies have demonstrated that BDNF expression, neurogenesis, and lipid peroxidation are all negatively impacted by high-fat diets **(Park *et al*., 2010).**

Cholesterol is a precursor for building myelin, which insulates nerve cells and promotes their function. Hypercholesterolemia might disrupt myelin production, hindering the growth and survival of new neurons (neurogenesis). BDNF plays a crucial role in neurogenesis, so its levels might decrease due to fewer new neurons being formed **(Saher *et al*., 2005).** BDNF is a neurotrophic required for the normal development and functioning of the nervous system **(Silakarma & Sudewi., 2019).** In addition to the nervous system, as an energy metabolism regulator BDNF is also synthesized in tissues associated with energy metabolism, such as the liver, muscle and adipose tissue **(Di Rosa *et al*., 2021).** Decreased BDNF level in HCD group compared with control group in this study agreement with **(Hu *et al*., 2014; Keser *et al*., 2023).**

Cell survival depends on brain-derived neurotrophic factor, differentiation, maturation, learning, and memory, among many other brain activities. Many neurological illnesses have also been linked to abnormal BDNF expression. **(Fei Zheng *et al*., 2012; Bodur *et al*., 2019).**

Hypercholesterolemia, in which elevated cholesterol levels cause an increase in the infusion of 27-OH into the brain, can cause neuronal damage in the neocortex; the amount of this damage may be dependent on the BDNF levels **(Loera-Valencia *et al*., 2019).**

Cholesterol and its oxidised metabolites, called oxysterols, are unable to cross the blood-brain barrier (BBB), but oxysterols can enter the brain and

play a vital role in brain regulation cholesterol metabolism **(Raúl Loera-Valenciaa *et al*., 2019)**. The link between blood and brain cholesterol levels may be explained by the oxysterols (oxidised forms of cholesterol) that are more abundant in hypercholesteremic diets **(Guo *et al*., 2014).** And it may impair the BBB ability to function, limiting the brain's access to vital nutrients and development factors. This can decrease BDNF levels and reduce neurogenesis **(Cheon, 2023).**

Although cholesterol is one of the main components of the cell membrane, a high-cholesterol diet causes inflammation and loss of neuronal function. BDNF is an endogenous factor with neuroprotective effects against neuroinflammation and oxidative stress **(Keser *et al*., 2023**).

Neuroinflammation is a factor that mechanistically links high cholesterol and impaired neuronal function. It has been demonstrated that neuroinflammation and blood-brain barrier dysfunction plays a role in cognitive disorders caused by hypercholesterolemia **(de Oliveira *et al*., 2020).** A high-cholesterol diet was reported to lead to a decrease in the number of pyramidal cells and an increase in neuroinflammation in the hippocampus in aged rats **(Rui *et al*., 2017).**

Therefore, the results implied that a high-cholesterol diet triggers neuroinflammation in the presence of BDNF, which is noteworthy, as neuroinflammation and mitochondrial dysfunction were previously reported to play a role in many neurodegenerative diseases such as Alzheimer's disease **(Giordano *et al*., 2013**). Since oxidative stress and neuroinflammation cause a decrease in the mitochondrial activity, they damage cellular structures and

disrupt these processes **(Mecocci *et al*., 2018**). Specifically, oxidative stress and inflammation could influence BDNF levels **(Zhang *et al*., 2020).**

4.5. Effect of hypercholesteremic diet on serum Synapsin-1 in male rats:

The results of this study showed no significant difference in serum synapsin-1 in the hypercholesteremic diet group (0.41±0.03)ng/ml as compared with the control group (0.38±0.01)ng/ml as shown in figure **(4-9).**

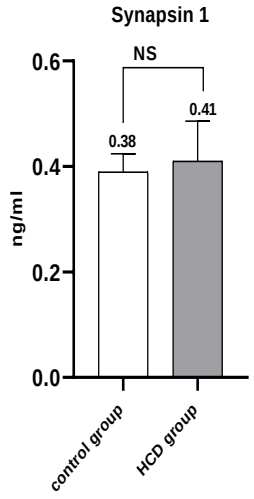

Figure (4-9): Effect of hypercholesteremic diet for 4 weeks on the serum synapsin-1 concentration in the male rats.

According to the current study synapsin-1 shows no significant difference between control group & hypercholesteremic diet group as shown in **figure (4-9)** , this result agreement with **(Petrov *et al*., 2016; Cemal, O *et al*., 2022;)** and disagreement with other studies **(Fei Zheng *et al*., 2012; Saito *et al*., 2018; Wahid *et al*., 2021; Saieva & Taglialatela, 2022).** This result may have appeared due to a difference in the concentration and time of the

experiment, unlike the other experiments, which led to not showing a significant difference between the control group and the group that was fed a diet containing a high amount of cholesterol

About 6% of the synaptic proteins are a phosphoprotein named Synapsin-1 which performs synapse plasticity, vesicle maintenance, and neurotransmitter release. Synapsins are capable of increasing various glutamate, Gamma-aminobutyric acid (GABA), and dopamine neurotransmitters **(Song *et al.*, 2015).**

Synapsin-1 is a protein crucial for regulating neurotransmitter release at synapses communication points between neuron, while a direct link between 1% cholesterol in synapsin-1 and its function is not established, A study suggests cholesterol levels can influence synapsin-1 activity **(Krivoi & Petrov, 2019)**.

Hypercholesteremia can trigger neuroinflammation, a chronic inflammatory state in the brain. Inflammation can disrupt various cellular processes, potentially impacting the production or function of synapsin1**(Crispino *et al.*,2020)**. HCD can promote oxidative stress, damaging cells and their components. This damage could potentially affect the proteins involved in synapsin 1 production or function **(Petrov *et al.*, 2016).** The creation of several cerebrosterols that control neuronal metabolism, as well as synaptic activity, which involves the organisation of proteins in the synaptic bouton, both require cholesterol **(Koudinov *et al.*, 2001;Mokhtarzadeh Bazargani *et al.*, 2021**).An age-related decline in neuronal cholesterol levels may play a significant role in synapse loss and, ultimately, neuronal death **(Michikawa *et al.*, 1999)**.The high cholesterol

level in other brain regions, such the myelin sheath, makes it difficult to examine brain cholesterol fluctuations. However, there are significant implications for neuro-degeneration that might be explored by analysing 24-OH levels in neurons to learn more about cholesterol control **(Raúl Loera-Valenciaa *et al*., 2019).**

On the other hand, diseases where there is loss of either neuronal structure or function as a result of cell death or degeneration are collectively referred to as neurodegenerative disorders (NDD). The most prevalent of them are Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease **(McFerrin *et al*., 2017).** While the underlying mechanisms causing this NDD are still being investigated, a number of underlying genetic variables are closely associated with the modification of synapse physiology **(Bae & Kim 2017)**. Since these proteins are crucial for synaptic communication between neurons, covalent posttranslational modification, or sumoylation, is extremely delicate in the central nervous system. These proteins, which control appropriate synaptic function and synaptic plasticity that results in learning and behaviors, are determined by sumoylation. NDD is a novel and fascinating field that is still being researched. It is believed that perturbation in the sumoylation of these synaptic proteins causes NDD **(Hendriks *et al*., 2018)**.

4.6. Effect of hypercholesteremic diet on the brain tissue glutamate:

The current study showed a significant ($P \leq 0.0001$) increase in brain glutamate in the hypercholesteremic diet group (228.07±1.14) nmol/mg as

compared with the control group (14.03±0.44)nmol/mg as shown in **figure (4-10).**

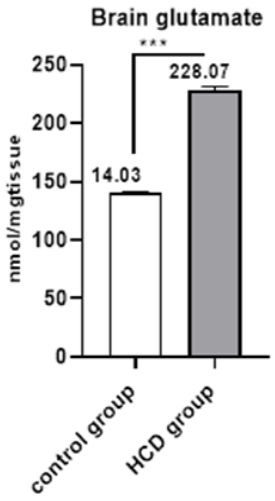

Figure (4-10): Effect of hypercholesteremic diet for 4 weeks on the brain tissue glutamate concentration in the male rats.

Glutamate (Glu) uptake is a pivotal process regulating excitatory transmission within the central nervous system, and its efficacy accounts for the time that released Glu remains in the extracellular space and, consequently, the duration of postsynaptic receptor activation **(Pita-Almenar JD *et al*., 2006; Tzingounis &Wadiche, 2007; Malik & Willnow, 2019).**

The present study shows that hypercholesteremia diets increased glutamate level in HCD group compared control group and this agreement with **(Valladolid-Acebes *et al*., 2012; Al-Rejaie *et al*., 2013; Mbikay *et al*., 2018; Labban *et al*.,2020; Poddar, R, 2021;),** and disagreement this study with **(Deloncle *et al*., 2001; Sodero *et al*., 2012)**, These studies suggest that the relationship between hypercholesterolemia and glutamate levels in the brain may be more complex than initially thought. It's possible that the effects of hypercholesterolemia on glutamate metabolism vary depending on factors

such as the duration of the diet, specific brain regions, or individual differences in cholesterol metabolism.

The hypercholesteremia diet triggers significant changes in Glu transmission that are linked to the alteration of Glu uptake and metabolism **(Valladolid-Acebes *et al*., 2012).** Rats' hippocampus influence by hypercholesteremic diet on ***1*)** Glu uptake kinetics, ***2*)** The density of Glu carriers and Glu-degrading enzymes, ***3*).** The density of Glu receptor subunits, and ***4*)** Synaptic transmission and plasticity. Because high fed diets have been shown to evoke leptin resistance within the brain **(Munzberg *et al*., 2005; Mancini *et al*., 2021)**, and leptin might theoretically account for neurochemical and functional changes in the hippocampus **(Irving *et al*., 2006; Shan *et al*., 2023)** .

Upregulation of activity and expression of glial glutamate transporters is a well-characterized event integral to physiological mechanisms involved in the homeostasis of Glu transmission **(Valladolid-Acebes *et al*., 2011**) that can be triggered by pharmacological manipulations, leading to the increase of synaptic glutamate **(Munir *et al*., 2000; Begni *et al*., 2005; Du, X *et al*., 2020).**

The hypercholesteremia diets might impair Glu metabolism by triggering a downregulation of Glu-degrading enzymes. Nevertheless, this possibility does not fit adequately with a more efficient uptake process that would rather suggest an increase in glutamate-degrading enzymes **(Ribeiro *et al*., 2009).**

The relevance of nutritional imbalance as a source of neuropsychological disorders, including a deficient ability to perform learning and memory tasks, is supported by a number of studies suggesting that diet composition would

affect limbic-mediated responses **(Vucetic Z *et al*., 2010; Goldstein, 2020)**. A number of studies have shown evidence that nutritional imbalance also accounts for cognitive deficits **(Fanjiang & Kleinman., 2007; Kanoski *et al*., 2010).** In particular, a recent research has shown evidence that HCD are deleterious for hippocampus structure and function **(Kanoski *et al*., 2007; Mancini *et al*., 2021)** and induce changes in synaptic plasticity and neurogenesis **(Farr SA *et al*., 2008)**. Moreover, previous studies carried out that HCD impair hippocampal-dependent learning and memory processes **(Valladolid-Acebes *et al*., 2011).** All of these research's suggests that changes triggered by HCD within the brain could have a deleterious impact on glutamate neural pathways.

4.7. Result of Gene expression of Activity -Regulated Cytoskeleton associated protein (ARC):

1- Analysis of the RT-qPCR gene expression data (activity -regulated cytoskeleton associated protein (ARC)**,**

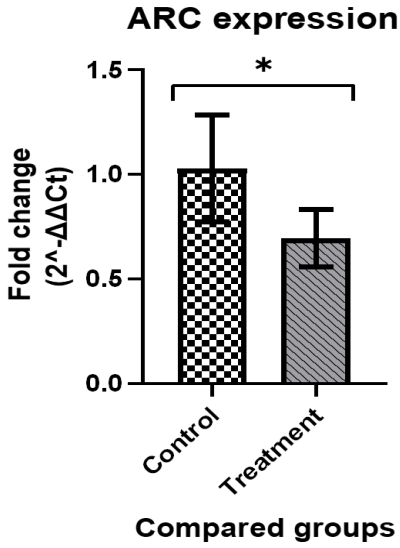

Figure (4-11): Fold change comparison between the compared groups for the expression of ARC for 4 weeks.

This shows a significant down-regulation ($p < 0.05$) of the group treated with hypercholesteremia diet (0.6943 ± 0.06) compared with control group (1.028 ± 0.114), as shown in **figure (4-11).**

2-Efficiency of the assay's amplification (activity -regulated cytoskeleton associated protein (ARC) gene**,** figure **(4-12).**

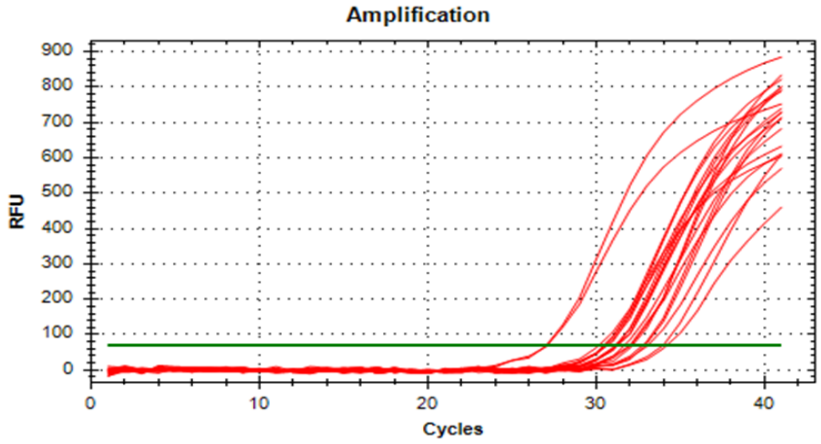

Figure (4-12): amplification curve of the tested samples for expression of gene of interest (ARC).

As shown, the successful amplification curves with corresponding crossing threshold (CT) as the number of cycles with the round forming unit (RFU). The samples were tested in duplicates**.**

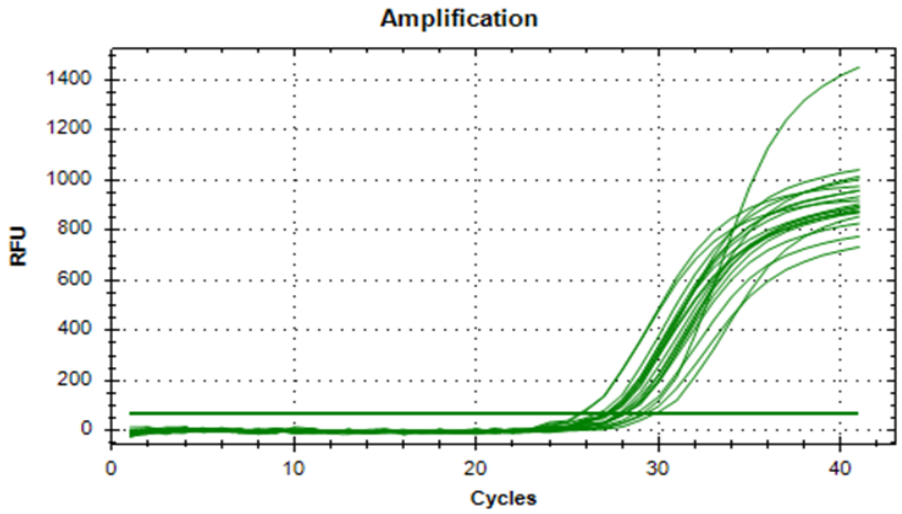

Figure (4-13): amplification curve of the tested samples for the housekeeping gene (b-actin).

As shown, the successful amplification curves with corresponding crossing threshold (CT). The samples were tested in duplicates.

This study finds a significant down-regulation ($p < 0.05$) level of ARC gene in the group treated with hypercholesteremia diet compared with control group, this agreement with **(Bannerman *et al*., 2004; Mateos *et al*., 2009; Ettcheto *et al*., 2015)**, oxidative stress may be the cause of the hypercholesteremic-induced ARC gene decrease process in the central nervous system (**Gella& Durany, 2009).** hypocholesteremia due to the buildup of free radicals in the central nervous system **(Huff *et al*., 2023).** The interactions that take place between those free radicals and DNA molecules result in damage. This damage may cause the ARC gene to decrease **(Gella& Durany, 2009).** and disagreement this result with **(Mayagoitia, K. *et al*., 2020; Pakiet.*et al*., 2020)**. The link between a hypercholesterolemic diet and ARC gene downregulation in the brain is yet to be definitively established. However, the current research suggests a potential connection between

hypercholesterolemia and changes in brain gene expression that warrants further investigation.

They demonstrated that long-term hypercholesteremia diet decreased the number of ARC-positive neurons in cerebral cortex, and observed that hypercholesteremia diet reduced the intensity of ARC staining and also changed the pattern of ARC expression **(Bannerman *et al*., 2004**).ARC down-regulation, as result of hypercholesterolemia, could result in synaptic impairment of neuronal populations, involved in memory, thereby contributing to AD pathology. Additionally, it is possible that in AD individuals with high blood cholesterol, the memory deficits would occur earlier **(Mateos *et al*., 2009**).

In response to intense synaptic activation, ARC (Activity-Regulated Cytoskeletal-Associated Protein), the immediate early gene's protein and mRNA swears+ are swiftly transported into dendrites and localized in locations of active synapses. Consequently, to trace the neural substrates attracted by various stimuli, ARC mRNA and protein are proposed as a neuronal reactivity marker **(Li, Mu *et al*., 2009)**. ARC, commonly known as ARG, is essential for activity-induced synaptic plasticity and specific behavioral memory types **(Lambert *et al*., 2009; Loera-Valencia *et al* 2018**). Synaptic plasticity is accompanied with a decrease in ARC gene expression. Animals with hypercholesteremia ARC mRNA are downregulated in neuronal dendrites, where it binds to cytoskeletal proteins; furthermore, Dendrites in areas of recent synaptic activation lose both its mRNA and protein., suggesting that ARC plays a unique function in synaptic plasticity **(Kivipelto *et al*.,2005; Veloz Castillo *et al*., 2021; Ezkurdia *et al*., 2023).**

ARC is one of the chemicals related to memory consolidation that has been examined the most **(Steward *et al*., 1998)**, it plays a significant role in the brain-derived neurotrophic factor (BDNF), glutamatergic, dopaminergic, and serotonin signaling pathways **(Lyford *et al*., 1995).** Dendrites that are enriched in ARC get ARC mRNA **(Steward *et al*., 2015)**.

A number of mental illnesses, such as major depressive disorder, psychosis, and schizophrenia, have been linked to ARC changes **(Pine *et al*., 2020; Crisafulli *et al*., 2021)**. For several of these illnesses, as discussed in **(Gallo *et al*., 2018)**, ARC is seen as a therapeutic target and a prognostic marker, despite the fact that a cause-effect relationship has not been shown. In cognitive illnesses, ARC decreased synaptic strength **(Zhang *et al*., 2015)**. metabotropic glutamate receptor-mediated long-term depression (mGluR LTD) and fragile X mental retardation disease both have reduced ARC expression **Pine *et al*., 2020)**. Numerous studies indicate a correlation between AD and ARC **(Bi *et al*., 2018; Postu *et al*., 2022).** ARC enhanced the interaction between γ-secretase and trafficking endosomes, which converted the amyloid precursor protein into Aβ peptide and caused amyloid deposition **(Wu *et al*., 2011)**. In addition to the neurological system, ARC plays a role in immune response modulation **(Zhang *et al*., 2021**). It controls T cell activation and migration in skin-migratory dendritic cells, where it is exclusively expressed **(Ufer *et al*., 2016).**

ARC was released from neurons in EVs that mediated the transfer of ARC mRNA to target neurons, where it could undergo activity-dependent translation.ARC capsids can be endocytosed, and ARC mRNA can be released from EVs into the cytoplasm and trafficked into the target sites in recipient neurons **(Dicker *et al*., 2021)**. The interaction between GAG and

RNA in viruses is controlled by host proteins, including Staufen that is involved in regulating ARC mRNA trafficking in neurons **(Dicker *et al*., 2021).**

4.8. Histopathological examination to the effect of hypercholesteremia on CNS tissue

The study after examination the histological slides perpetration from experimental animals; in control groups showed the normal design architect nervous tissues (neuron and neuroglia) of vital organs of aimed research, involve the brain (cerebral, cerebellum) and spinal cord which are composed from neo cortex and neo medulla. The cortex was formed six strata variable thickness that are blended with each other, in cerebral involve (molecular, external granular, external pyramidal, internal granular, internal pyramidal and multiform cells layers. Furthermore, the cerebellum has three well demarcated layers, that as molecular, purkinje and granular cells layers, whereas gray and white matter make up the spinal cord, in **figures (4-16: A, B) and (4-17).**

As for the hypercholesterolemic rats (treatment group), showed a disorder pattern of nerve tissue cells evident by different degree neurofibrillary tangles on neurons (Nero necrosis) by deposits a beta-amyloid plaque leads in neural and morphological degeneration in the neural tissues, specified from dense to mild, that permeates or spreads through the strata of the brain (cerebrum) and cerebellum, spinal cord represented mild, as it turns out, deeply stained a pyknotic nuclei, necrotic karyorrhexis of degenerated neurons and purkinje cells. inflammatory markers infiltration cells and proliferation of neuroglial cells (gliosis), **Figure (4-18) (4-19) and figure (4-20).**

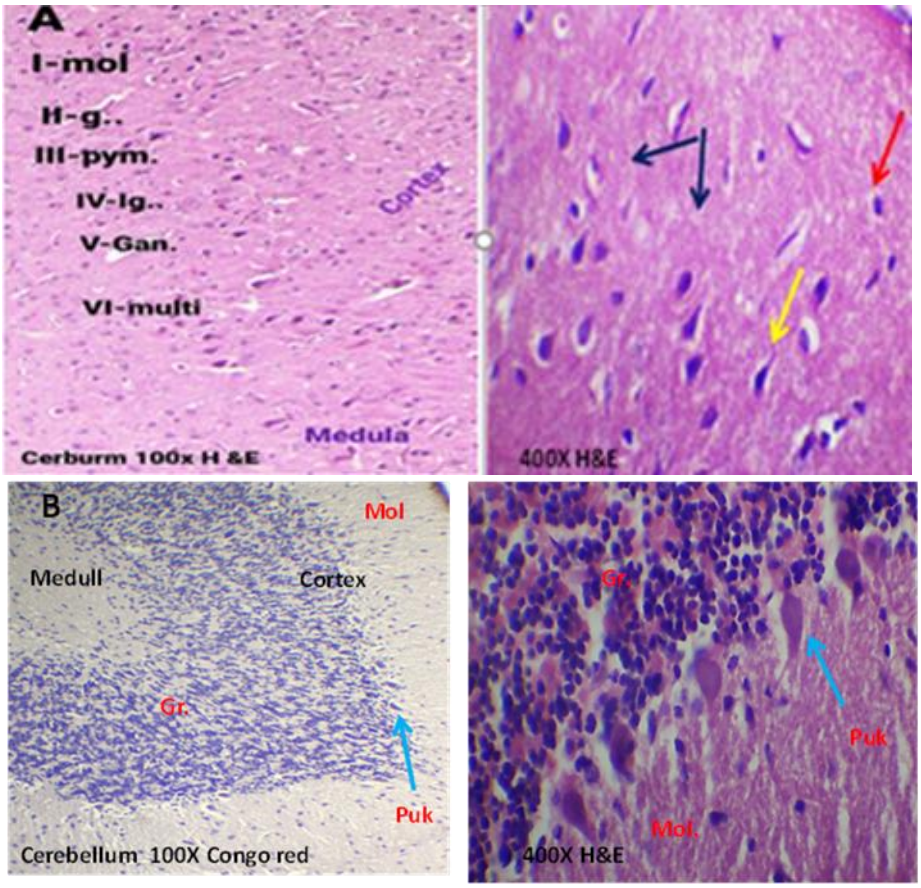

Figure (4-16): (A and B) Photomicrograph of H&E and Congo red Magnification Power 100x, 400x: Represented normal histological sections. in control male rats of cortex layers with medulla in cerebrum (A) cerebellum (B): Cerebral-cortex is consist from six layers there are Molecular (I-Mol.), external granular (II- Eg), external pyramidal (III-Pym.), internal granular (IV-Ig.), ganglionic (V-Gan) and multiform (VI- multi) cells layers. Furthermore, cerebellar-cortex which is formed three strata; Molecular (I-Mol.), Purkinje and granular (Gr.) cells layers and medulla. represented neuroglia, neuron and neuropil respectively.

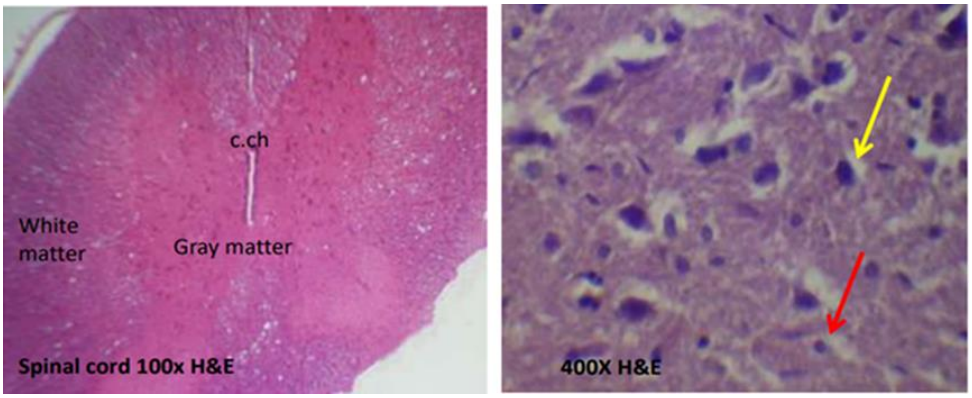

Figure (4-17) : Photomicrograph in control male rats group in hematoxylin and eosin stain and Magnification Power 100x, 400x : reveal the normal architecture of spinal cord tissues which is consist from the white and gray matter with central canal (c.ch) mediate it, the (Red and Yellow arrows) represented neuroglia and neuron respectively.

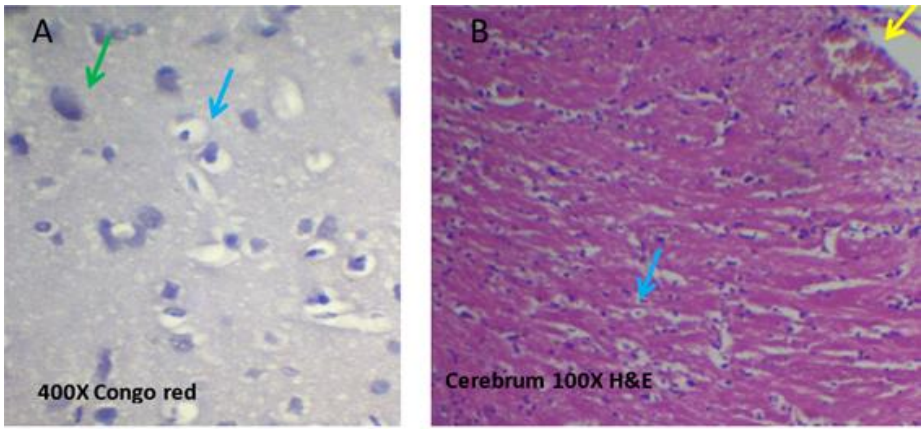

Figure (4.18) :(A&B): Photomicrograph represented cerebral hypercholesterolemic male rats in CR and H&E stain and Magnification Power 100x, 400x: Showing changes occur disarrangement of neuro-cortical cells with significant increase of neuroglia cells and shrunken & degeneration (blue arrows) with congestion of blood vessel (yellow arrow). Furthermore pyknotic nuclei (green arrow)

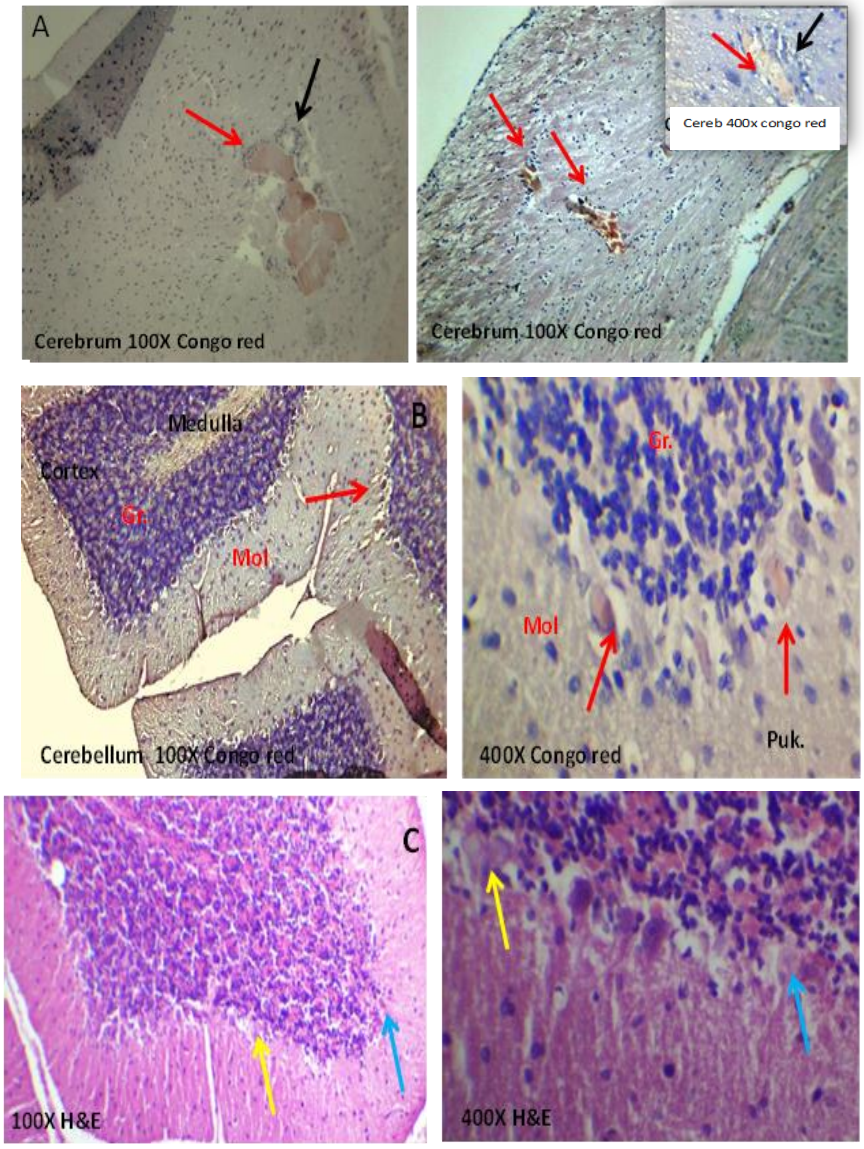

Figure (4-19): (A,B and C):Photomicrograph represented brain (cerebral and cerebellum) section of hypercholesterolemic male rats in CR and H& E stain and Magnification Power 100x, 400x : Reveal disorder pattern of neocortex and nero necrosis by significant deposits a beta-amyloid plaque in the neural tissues remarkable by highlight red color (Red arrow) protruding in the cerebral cortex strata but, mild in cerebellar cortex. In Addison Inflammatory cells infiltrations (Black arrow). Furthermore, nicroptic neuronal karyorrhexis of degenerated purkinje cells (yellow arrow) with deformity of structure (Blue arrow).

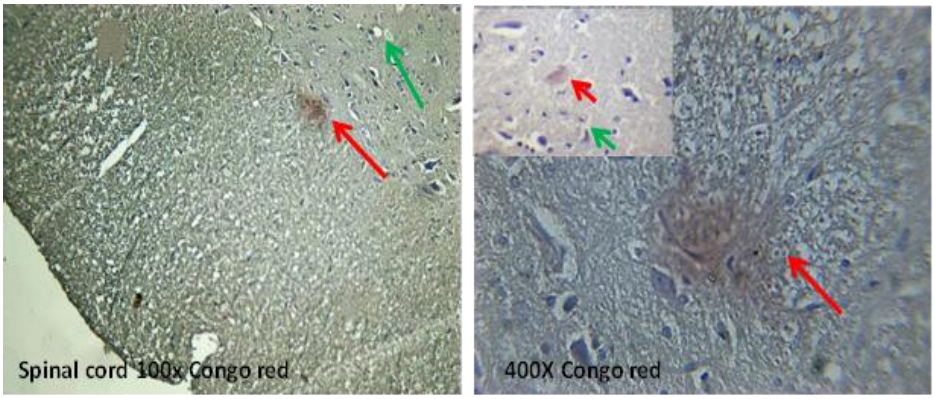

Figure (4-20): Photomicrograph represented spinal cord section of hypercholesterolemic male rats in CR stain and Magnification Power 100x, 400x : Reveal mild deposition of beta –amyloid in highlight red color of the gray matter(red arrows) with significant glia cells and neuronal karyorrhexis (green arrow).

In the pathway of the central nervous system, the cerebrum, cerebellum and spinal cord are the essential components of central system and relay station. We studied the histological structures these organs, according to the outcomes of our research, surveys were done to the structure of each of them. The histological characteristics features of central nervous tissues constituent with the prior studies by **(Garman, 2011; Mahdi et al., 2021).**

The examination microscopic section of the male rats, were fed normal diet showing normal pattern histological architecture of neural tissues consequences of similar of line with other studies in which they mentioned there are basic structure (neuron and neuroglia) of central nervous system this agrees with **(Garman *et al*., 2011; de Oliveira *et al*., 2020; Mahdi et al., 2021)**

Hypercholesterolemia caused by diet accelerates rats with Aβ accumulation in their brain tissues.This result agreement with **(Swanson, 2004; Rajamohamedsait & Aut, 2012; Hussain *et al*., 2019 Hussain, A *et al*., *2023*; Raheem *et al*., 2023)**. These were resemble the study results deposition of beta amyloid on the nervous tissue clearly in cerebral cortex and mild in cerebellum and spinal cord furthermore, amyloid fibrils in several proteins, react positively with Congo red amyloid dye ultrastructure of biofilms found inside cells (purkinje cell) and excreta cellular in cerebrum and spinal cord tissues which as represented a test for confirmation of the amyloid nature of protein aggregates, when bound to it will show a red color **(Iqbal *et al*., 2005; Greenwald & Riek., 2010; Allen *et al*., 2018).**

Cholesterol is needed for active axonal development, synapse formation, and remodeling but cannot be supplied by the neuron's distant cell body (**Raheem *et al*., 2023).** Microglia are crucial for maintaining brain balance and could be used as a therapeutic focus for neuronal injury (**Wang *et al*., 2022).** Damaged microglia with high cholesterol buildup may result from downregulated cholesterol production pathways **(Raheem *et al*., 2023).**

The glia cell are important roles in brain such as metabolism modifications, glucose transmission and blood-brain barrier maintain physiological function regulation and distraction, low numbering of microglia is one of the indications of neuropathology due to the stress factors (**Hussain, A *et al*., *2023*)**. The match opinion of our results examination of decrease average number and disruptions glia in nervous tissues showed in HCD group.

As well, one of the main causes of cell damage is oxidative stress, which is brought on by an excess of ROS and poor metabolism., the brain is

represented to be especially vulnerable to oxidative damage, united in opinion of our research results of the neuro- cortex necrosis with deeply stained pyknotic nuclei, necrotic karyorrhexis of degenerated neurons and purkinje cells and inflammatory markers infiltration cells **(Zhou *et al*., 2008)**.

Disorder of cholesterol homeostasis in the brain affect several neurodegenerative diseases, so as proliferation and accumulation of fats in glial cells **(Singh *et al*., 2019).** who contributed to our study of the increased number of the neuroglia cell (gliosis'), distortion neurocyte of the nervous tissues lead to the neuroglia divergence to provide a higher proportion of the cholesterol needed to form synapses.

Chapter Five

Conclusions
&
Recommendations

5.Conclusions & Recommendations

5.1. Conclusions:

From the results of the current study obtained the following: -

1- TC, TG and LDL levels rise along with a decrease in HDL, causing the body to become more dyslipidemia. Hypercholesteremia has also been shown to lower the body's levels of antioxidant capacity

2- Hypercholesteremic diet causes increase of oxidative stress (increase MDA), and decrease antioxidant stress (decrease GSH), which in turn depletes BDNF and leading to a decrease in glutamate and other master neurotransmitters in the brain.

3- Hypercholesterolemic diet no significant on synapsin-1.

4- Hypercholesterolemic diet causes decreased nitric oxide level.

5- Hypercholesterolemic diet causes down-regulation in the ARC gene.

6- Histologically, inflammation and beta amyloid buildup are visible in the brain and spinal cord tissue.

5.2. Recommendations:

1-Further studies on the biomarkers related with BBB and hypercholesteremia.

2-Studying effect of hypercholesteremia on the brain by electroencephalogram (EEG) in of different environments

3-The role of medications in preventing the neurological effects that hypercholesterolemia causes.

4-Studying the effect of interactions between hypercholesterolemic diet and Apo-E protein in the context of cerebral dysfunction

Chapter Six
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Appendixes

Appendixes I

Determination of Serum Total Cholesterol (TC) Concentration:

Total Cholesterol (TC) was detected by use Enzymatic method described by Allain and al, (Allain C. C. *et al*., Clin. Chem. (1974), 20/4, p.470-475). which reaction scheme is as follows:

ce

Cholesterol esters ⟶ cholesterol +free fatty acids

Cholesterol +O2 ⟶ cholesten4 one3 +H2O2

2H2O2+phenol+PAP⟶ quinoneimine (pink) +4H2O2

Procedure:

At room temperature Add 1000 µL of reagent and after that add 10 µL of Blank, Standard, Control or specimen Let them stand at 37 C for 5 min record absorbance at 500nm against reagent blank.

Calculation

The result was calculated according to this equation

$$\textbf{Total Cholesterol (TC)} = \frac{abs(assay)}{abs(standerd)} \times \textbf{standerd concentration}$$

$abs(assay) = absorbance\ of\ sample$

$abs(standerd) = absorbance\ of\ stander$

Appendixes II

Determination tri glycerides (TG) concentrations

Total Serum Triglyceride was detected by use Fossati and Prencipe method associated with Trinder reaction. Reaction scheme (Fossati P., Prencipe L., Clin. Chem. (1982), 28, p.2077-2080.), (Trinder P. Ann. Clin. Biochem. (1969), 6, p.27-

29.).it is as follows:

Triglycerides lipase ⟶ Glycerol + free fatty acids

GK

Glycerol + ATP ⟶ glycerol kinase + ADPGlycerol 3

GPO

Phosphate + O2 ⟶ Dihydroxyacetone Phosphate + H2O2 POD

H2O2 + 4 Chlorophenol + PAP ⟶ Quinonimine (pink) + H2O

The absorbance of the coloured complex (quinoneimine), proportional to the amount of triglycerides in the specimen, is measured at 500 nm.

REAGENTS PREPARATION

Add promptly the contents of vial R2 (Enzymes), into vial R1 (Buffer). Mix gently and wait for complete dissolution before using reagent (approximately 2 minutes).

Procedure:

The procedure was done as shown in the table below:

Pipette into well identified test tubes	blank	Standard	assay
Regent	1ml	1ml	1ml
Demineralized water	10ul		
Standard		10ul	
Specimen			10ul

Calculation

The result was calculated according to this equation

$$\text{Serum Triglyceride (TAG)} = \frac{abs(assay)}{abs(standerd)} \times \text{standerd concentration}$$

Appendixes III

Determination of Serum High-Density lipoprotein-cholesterol

PRINCIPLE

Accelerator selective detergent methodology. Direct method, without specimen pre-treatment. During the first phase, LDL, VLDL particles and Chylomicrons generate free Cholesterol, which through an enzymatic reaction, produce Hydrogen peroxide. The generated peroxide is consumed by a peroxidase reaction with DSBmT yielding a colourless product. During the second phase, specific detergent solubilizes HDL-Cholesterol. In conjunction with CO and CE action, POD + 4-AAP develop a colored reaction

which is proportional to HDL-Cholesterol concentration. The absorbance is measured at 600 nm.

PROCEDURE

The procedure was done as shown in the table below:

Tubes	**Blank**	**Calibrator**	**assay**
Regent 1 300 µL	**300 µL**	**300 µL**	**300 µL**
Calibrator		**3 µL**	
Specimen			**3 µL**

Let stand in 37°C for 5 min and Record absorbance of them at 600 nm against reagent blank. Add 100 µL of regent 2 Let stand in 37°C for 5 min and Record absorbance of them at 600 nm against reagent blank.

Calculation

The result was calculated according to this equation

$$\textbf{HDL-C-} = \frac{\Delta\, \boldsymbol{abs.assay}}{\Delta \boldsymbol{abs.calibrator}} \times \textbf{calibrator concentration}$$

Δ abs.assay = the different between the two record for the assay

Δabs.calibrator= the different between the two record for the calibrator

Appendixes IV

Determination of Serum Low Density lipoprotein-cholesterol

PRINCIPLE

Direct method using selective detergents without specimen pretreatment. During the first phase, only non-LDL lipoproteins are solubilised by detergent 1. Such generated Cholesterol, subjected to Cholesterol oxidase (CO) and Cholesterol esterase (CE) actions, produces a colourless compound. During the second phase, detergent 2 solubilises LDL-Cholesterol. The chromogenic coupler allows for colour formation that is proportional to the concentration of LDLCholesterol. The absorbance is measured at 546 nm (520-580).

PROCEDURE

The procedure was done as shown in the table below:

Tubes	Blank	Calibrator	assay
Regent 1	300 µL	300 µL	300 µL
Calibrator		3 µL	
specimen			3 µL

Let stand in 37°C for 5 min and Record absorbance of them at 546 nm against reagent blank. Add 100 µL of regent 2 Let stand in 37°C for 5 min and Record absorbance of them at 546 nm against reagent blank.

Calculation

The result was calculated according to this equation

$$\text{LDL-C-} = \Delta \frac{\boldsymbol{abs.assay}}{\boldsymbol{\Delta abs.calibrator}} \times \text{calibrator concentration}$$

Δ abs.assay = the different between the two record for the assay

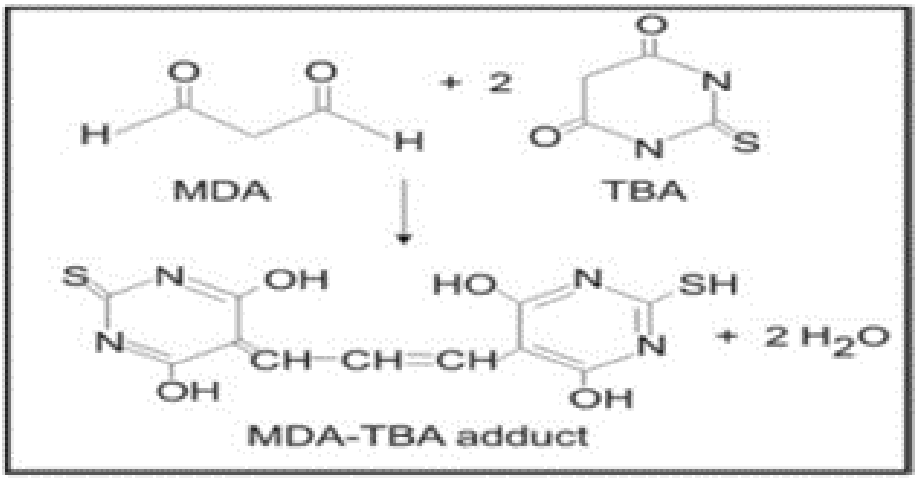
Δabs.calibrator= the different between the two record for the calibrator

Appendixes V

Determination of Serum Malondialdehyde (MDA) and homogenised brain tissue concentration (µ mol /L):

Principle:

This method quantifies lipid peroxides by measuring aldehyde breakdown products of lipid peroxidation. Basic principle of the method is the reaction of one molecule of malondialdehyde and two molecules of thiobarbituric acid to form a red MDA-TBA complex which can be measure at 535 nm.



Stock TCA – TBA – HCl Reagent:

It was prepared by dissolving 15% W/V trichloroacetic acid and 0.375% W/V thiobarbituric acid and 0.25N HCl to make 100 ml (2.1 ml of concentrated HCl in 100 ml). This solution was mildly heated to assist in the dissolution of TBA. Dissolved 15 gm TCA and 0.375 mg thiobarbituric acid in 0.25 N HCl and volume was made up to 100 ml with 0.25 N HCl.

Procedure:

To 0.4 ml of serum, 0.6 ml TCA-TBA-HCl reagents were added. It was mixed well and kept in boiling water bath for 10 minutes. After cooling 1.0 ml freshly prepared 1N NaOH solution was added to eliminate centrifugation. This absorbance of pink colour was measured at 535 nm against blank which contained distilled water in place of serum. In blank 0.4 ml distilled water and 0.6 ml TCA-TBA-HCl reagent was mixed and boiled. Blank was always taken.

Calculation:

extinction coefficient of MDA at 535 nm is = 1.56 × 105

MDA concentration = χ / 0.0624 nmol / ml

Appendixes VI

Determination of serum Reduced Glutathione (GSH) and homogenised brain tissue concentration:

First, 3.0 mL precipitating solution containing metaphosphoric acid, Na2EDTA and NaCl was added to 2.0 mL of the sample. The mixture was centrifuged at 4500 × g for 10 min. 1.0 mL of supernatant was added to 4.0 mL of 0.3 M Na2HPO4 solution and 0.5 mM DTNB (5,5 -dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as nmol GSH/mg protein in the tissues and mmol GSH/g Hb in whole blood using the reduced glutathione as a reference (hemoglobin levels were estimated in whole blood using the Drabkins' solution).

Appendixes VII

Determination of the brain tissue nitric oxide (NO) concentrations:

SPECIFICITY

When it comes to detecting rat nNOS/NOS1, this assay offers great specificity and good sensitivity. There was no discernible cross-reactivity or interference between rat nNOS/NOS1 and its equivalents.

DETECTION RANGE

31.25 mlU/ml-2000 mlU/ml.

PRINCIPLE OF THE ASSAY

The quantitative sandwich enzyme immunoassay method is used in this assay. An anti-nNOS/NOS1 antibody has been pre-coated on a microplate. After pipetting standards and samples into the wells, the immobilized antibody binds any nNOS/NOS1 that is present. A biotin-conjugated antibody that is specific for nNOS/NOS1 is added to the wells after any unbound materials have been removed. Horseradish peroxidase (HRP) coupled with avidin is added to the wells after washing. A substrate solution is added to the wells after a wash to remove any unbound avidin-enzyme reagent, and color develops in proportion to the amount of nNOS/NOS1 bound in the first phase. The color development is halted, and the color's intensity is gauged.

SAMPLE COLLECTION AND STORAGE

 Serum Before centrifuging samples for 15 minutes at 1000 ×g, let them clot for two hours at room temperature or overnight at 4°C in a serum

separator tube (SST). Assay and remove serum right away, or aliquot and keep samples at -20°C or -80°C. Do not freeze-thaw repeatedly.

 Plasma Use EDTA or heparin as an anticoagulant to gather plasma. Within 30 minutes of collection, centrifuge at 1000 x g for 15 minutes at 2 to 8°C. Samples should be stored at -20°C or -80°C and assay right away or in an aliquot. Do not freeze-thaw repeatedly. Before the assay, centrifuge the material once more once it has thawed.

 Supernates from Cell Culture Particulates can be removed by centrifugation for 15 minutes at 1000 x g, 2 - 8°C. Samples can be stored at -20°C or -80°C, or they can be aliquoted and assayed right away. Do not freeze-thaw repeatedly. 100 mg of tissue was homogenized in 1 ml of 1X PBS after being washed with 1X PBS and kept at -20°C for the night. Following two rounds of freezing and thawing to rupture the cell membranes, the homogenates were centrifuged at 5000 x g for 5 minutes at a temperature between 2 and 8°C. The supernate was taken out and tested right away. Samples can also be aliquoted and kept at -20°C or -80°C. Before the assay, centrifuge the material once more once it has thawed. Do not freeze-thaw repeatedly.

 Adherent Cell Lysates

(**1) Cell**: After removing the media, give the cells one ice-cold PBS (pH 7.2–7.4) rinse. After removing the cells with a scraper, move them to the proper tube. Use 1xPBS (pH 7.2–7.4) to dilute the cell suspension until the concentration of cells reaches 100 million/ml. After that, store at -20°C for the night. Following a pair of freeze-thaw cycles aimed at disrupting the cell membranes, the cell lysates underwent a 5-minute centrifugation at 5000 x g,

2 - 8°C. Get the supernatant together. Cell lysates must be assayed right away or separated and kept at -20°C. Before the assay, centrifuge the material once more once it has thawed. Don't do freeze-thaw cycles too often..

(2) Suspension Cell: Gather cells in the proper tube, then centrifuge at 1000x g, 2–8°C, for 5 minutes. After extracting the supernatant, resuspend the cells in 1xPBS (pH 7.2–7.4). 7. Centrifuge at 1000 x g, 2 - 8°C for 5 minutes. Take the supernatant out. Use 1xPBS (pH 7.2–7.4) to dilute the cell until the concentration reaches 100 million/ml. After that, store at -20°C for the night. Following a pair of freeze-thaw cycles aimed at disrupting the cell membranes, the cell lysates underwent a 5-minute centrifugation at 5000 x g, 2 - 8°C. Get the supernatant together. Cell lysates must be assayed right away or separated and kept at -20°C. Before the assay, centrifuge the material once more once it has thawed. Don't do the freeze-thaw cycle again.**REAGENT PREPARATION**

Note:

λ Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.

λ Bring all reagents to room temperature (18-25°C) before use for 30min.

λ Prepare fresh standard for each assay. Use within 4 hours and discard after use.

λ Making serial dilution in the wells directly is not permitted. λ Please carefully reconstitute Standards according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that

pipettors are calibrated. It is recommended to suck more than 10μl for once pipetting.

λ Distilled water is recommended to be used to make the preparation for reagents. Contaminated water or container for reagent preparation will influence the detection result.

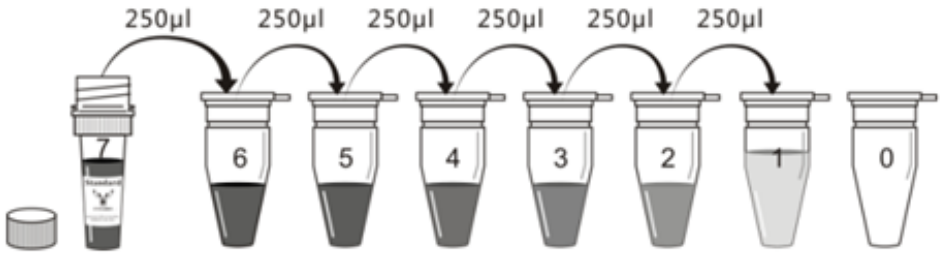
1. Centrifuge the vial before opening the biotin-antibody (1x) container. The biotin-antibody needs to be diluted 100 times. 10 μl of biotin-antibody plus 990 μl of biotin-antibody diluent is a recommended 100-fold dilution.

2. HRP-avidin (1x): Before opening, centrifuge the vial. HRP-avidin needs to be diluted 100 times. 10 μl of HRP-avidin plus 990 μl of HRP-avidin Diluent is a recommended 100-fold dilution.

3. Wash Buffer (1x) - Bring the concentrate to room temperature and stir slowly until the crystals are totally dissolved if any have developed in the concentrate. To make 500 ml of Wash Buffer (1 x), dilute 20 ml of Wash Buffer Concentrate (25 x) with deionized or distilled water. 10

4. Customary Centrifuge the standard vial for thirty seconds at 6000–10,000 rpm. Use 1.0 milliliter of Sample Diluent to reconstitute the Standard. Never use another diluent in its place. The stock solution that results from this reconstitution is 2000 mlU/ml. Before creating dilutions, mix the standard to guarantee full reconstitution and let it sit with mild stirring for at least 15 minutes. Fill each tube (S0-S6) with 250 μl of Sample Diluent using a pipette. To create the two-fold dilution series (below), use the stock solution. Before the next transfer, properly combine each tube. The high standard is the undiluted Standard (2000 mlU/ml). The zero standard is represented by

Sample Diluent (0 mlU/ml).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
mlU/ml	2000	1000	500	250	125	62.5	31.25	0

ASSAY PROCEDURE

Before using, bring all samples and reagents to room temperature. Before the assay, centrifuge the material once more once it has thawed. It is advised that duplicate assays be performed on all samples and standards.

1. Follow the instructions in the preceding sections to prepare all reagents, working standards, and samples.

2. To determine how many wells to use, consult the Assay Layout Sheet. Place any extra wells and the desiccant back into the pouch, close the ziploc bag, and store the unused wells at 4°C.

3. Fill each well with 100µl of the standard and sample. Use the included adhesive strip to cover. At 37°C, incubate for two hours. To keep track of the standards and samples tested, a plate arrangement is offered.

4. Empty each well's contents; do not wash.

5. Add 100µl of Biotin-antibody (1x) to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)

6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

7. Add 100µl of HRP-avidin (1x) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.

8. Repeat the aspiration/wash process for five times as in step 6.

9. Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.

10. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing

11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate.Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

It is recommended to use the expert soft "Curve Expert," which can be downloaded from our website, to design a standard curve.

Take the average of the duplicate readings for each standard and sample, and subtract it from the average of the zero standard optical density.

Reduce the data to a standard curve using tools that can generate a four parameter logistic (4-PL) curve-fit. Alternatively, graph the mean absorbance of each standard on the x-axis against the concentration on the y-axis to generate a standard curve. Next, make the best fit curve using the points on the graph. The data can be linearized by plotting the log of the nNOS/NOS1 concentrations against the log of the O.D. and regression analysis can be used to find the line that best fits the data. This process will yield a data fit that is sufficient but not as exact. The concentration read from the standard curve must be multiplied by the dilution factor if samples have been diluted.

Appendixes VIII

Estimated of serum Brain Derived Neurotropic Factor (BDNF) concentration

This ELISA kit applies to the in vitro quantitative determination of Rat Aβ1-42 concentrations in serum, plasma and other biological fluids.

Specification

- Sensitivity: 3.5 pg/mL
- Detection Range: 7.82-500 pg/mL
- Specificity: This assay has high sensitivity and excellent specificity for detection of Rat BDNF. No significant cross-reactivity or interference between Rat BDNF and analogues was observed.

Test Principle

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody

specific to Brain Derived Neurotrophic Factor (BDNF). Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to Brain Derived Neurotrophic Factor (BDNF). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Brain Derived Neurotrophic Factor (BDNF), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Brain Derived Neurotrophic Factor(BDNF) in the samples is then determined by comparing the OD of the samples to the standard curve.

KIT Components & Storage

Reagents	Quantity		Storage Condition
	48T	96T	
Pre-Coated Microplate	6 strips x 8 wells	12 strips x 8 wells	-20°C (6 months)
Standard (Lyophilized)	1 vial	2 vials	-20°C (6 months)
Biotinylated Antibody (100×)	60 µL	120 µL	-20°C (6 months)
Streptavidin-HRP (100×)	60 µL	120 µL	-20°C (6 months)
Standard/Sample Diluent Buffer	10 mL	20 mL	4°C
Biotinylated Antibody Diluent	6 mL	12 mL	4°C
HRP Diluent	6 mL	12 mL	4°C
Wash Buffer (25×)	10 mL	20 mL	4°C
TMB Substrate Solution	6 mL	10 mL	4°C (store in dark)
Stop Reagent	3 mL	6 mL	4°C
Plate Covers	1 piece	2 pieces	4°C

For a week, keep the kit stored at 4°C. After the kit is received, store the items individually under the following circumstances if they are not used up in a week.

Note

All kit components have been formulated and quality control tested to function successfully. Do not mix or substitute reagents or materials from other kits, detection effect of the kit will not be guaranteed if utilized separately or substituted.

Materials Required, Not Supplied

1. Microplate reader capable of measuring absorbance at 450 ± 10 nm.

2. High-speed centrifuge.

3. Electro-heating standing-temperature cultivator.

4. Absorbent paper.

5. Double distilled water or deionized water.

6. Single or multi-channel pipettes with high precision and disposable tips. 7. Precision pipettes to deliver 2 μL to 1 mL volumes.

Sample Collection and Storage

Samples for serum: should be gathered and placed in a serum separator tube. Following clotting for two hours at room temperature or overnight at 4°C, centrifuge for 20 minutes at 1000 × g. Assay recently made serum right away, or save samples in an aliquot for later use at -20°C or -80°C. Do not freeze-thaw repeatedly.

Plasma: Gather plasma while using heparin or EDTA as an anticoagulant. Within 30 minutes of collection, centrifuge samples at 1000 × g and 2–8°C for 15 minutes. Assay the plasma right away, or save the samples in an aliquot and store them at -20°C or -80°C for later use. Do not freeze-thaw repeatedly.

Tissue homogenates: There are numerous techniques for creating tissue homogenates, depending on the kind of tissue. 1. Weigh the tissues before to homogenization and rinse them with PBS that has been chilled beforehand to get rid of all extra blood.

2. Using a glass homogenizer on ice (micro tissue grinders, too), mince the tissues into small pieces and homogenize them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular location of the target protein; PBS can be used as the lysis buffer of most tissues) (w:v = 1:9, e.g. 900 μL lysis buffer is added in 100 mg tissue sample).

3. Use an ultrasonic cell disrupter to pound the resulting suspension until the solution becomes transparent.

4. Next, centrifuge the homogenates at 10,000 × g for 5 minutes. Gather the supernatant and analyze right away, or keep the cell lysates. Before performing an assay, the cells must be lysed in accordance with the guidelines below.

1. Adherent cells should be gently rinsed with pre-cooled PBS, separated using trypsin, and then collected by centrifugation at 1000 × g for five minutes (direct centrifugation can be used to collect suspension cells).

2. Use pre-cooled PBS to wash cells three times.

3. Next, resuspend the cells in 10 cells/mL of newly prepared lysis buffer. The cells could undergo ultrasonication until the solution is clear if that is required.

4. Centrifuge at 1500 × g for 10 minutes at 2-8°C to remove cellular debris. Assay immediately or store aliquots at ≤ -20°C

Cell culture supernatants and other biological fluids - Centrifuge samples at 1000 × g for 20 minutes. Collect the supernatant and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated

Sample notes

1. To prevent loss of bioactivity and contamination, samples must be stored at -20°C (≤ 1 month) or -80°C (≤ 2 months). Samples that will be utilized within 5 days may be stored at 4°C. Do not freeze-thaw repeatedly.

2. Sample hemolysis should not be used because it will affect the outcome.

3. Bring samples to room temperature prior to doing the experiment.

4. In the event that the test material concentration in your sample is higher than that of the Standard product, kindly make the necessary multiple dilutions in accordance with the real circumstances (a preliminary experiment is advised to ascertain the dilution ratio).

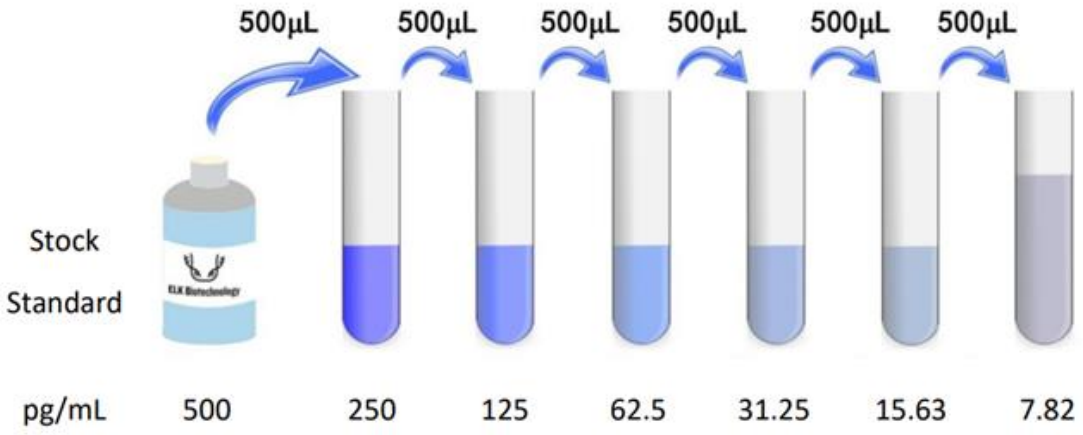
Reagent Preparation

1. Before using, bring all kit parts and samples to room temperature (18–25°C).

2. Please only remove the strips and reagents needed for the current experiment and save the remaining strips and reagents as directed if the kit won't be used up in one sitting.

3. Use double-distilled water to dilute the 25×Wash Buffer into a 1×Wash Buffer.

4. Standard Operating Solution: Centrifuge the Standard for one minute at 1000 × g. Shake gently (not to froth) and let the Standard sit at room temperature for 10 minutes before reconstituting it with 1.0 mL of Standard Diluent Buffer. 500 pg/mL is the Standard's concentration in the stock solution. As indicated in the photo below, please set up 7 tubes with 0.5 mL of Standard Diluent Buffer and use the Diluted Standard to create a double dilution series. Pipette the solution up and down multiple times to ensure that each tube is properly mixed before the next transfer. The last EP tubes with the standard diluent are the Blank, which have a 0 pg/mL. Set up 7 points of Diluted Standard, such as 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.63 pg/mL, and 7.82 pg/mL. Please utilize the new Standard Solution for every experiment to ensure the authenticity of the results. As the Standard is diluted from a high concentration to a low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as a Blank and do not pipette solution into it from the former tube.



5. 1×Biotinylated Antibody and 1×Streptavidin-HRP - Briefly spin or centrifuge the stock Biotinylated Antibody and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated Antibody Diluent and HRP Diluent, respectively.

6. TMB Substrate Solution - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial

Assay Procedure

1. Ascertain the wells for the sample, blank, and diluted standard. Set up one blank well and seven standard wells. Fill the relevant wells with 100 µL of either Standard Working Solution (please see Reagent Preparation) or samples. Put the plate cover on top. At 37°C, incubate for 80 minutes.

It is important to ensure that solutions are introduced to the bottom of the micro-ELISA plate, avoiding contact with the inner wall and creating as much foaming as possible.

2. Transfer the contents of every well out. After aspirating the solution, fill each well with 200 μL of 1×Wash Solution, and let it settle for one to two minutes. Snap the plate onto absorbent paper to completely remove any residual liquid from each well. Wash everything three times. Aspirate or decant any leftover Wash Buffer after the last wash. Turn the plate over and dab it on absorbent paper. Notes: (a) The pipette tip should not come into contact with the well walls when adding Washing Solution to prevent contamination. (a) To prevent the washing liquid from contaminating neighboring wells, be careful to pour it directly into the well.

3. Fill each well with 100 μL of the Biotinylated Antibody Working Solution. Place the Plate Cover on top of the wells and incubate for 50 minutes at 37°C.

4. As in step 2, repeat the aspiration and washing process three times in total.
5. Fill each well with 100 μL of the Streptavidin-HRP Working Solution, place the plate sealer over the wells, and incubate at 37°C for 50 minutes.

6. Carry out the aspiration and washing procedure five times in total, just like in step 2.

7. Fill each well with 90 μL of TMB Substrate Solution. Replace the plate cover. Incubate in the dark for 20 minutes at 37°C (do not exceed 30 minutes). The addition of TMB Substrate Solution will cause the liquid to turn blue. Before measuring the OD, preheat the microplate reader for approximately fifteen minutes.

8. Fill each well with 50 μL of Stop Reagent. The addition of Stop Reagent will cause the liquid to turn yellow. Tap the side of the plate to combine the liquid. To make sure there is adequate mixing, lightly tap the plate if the color

change is not uniform. The TMB Substrate Solution and the Stop Reagent should be inserted in the same order.

9. Remove any fingerprints and water droplets from the plate's bottom and make sure the liquid's surface is bubble-free. After that, start the microplate reader and start measuring at 450 nm right away.

Calculation of Results

Subtract the average zero Standard optical density from the average of the duplicate readings for each Standard, Control, and Sample. Draw a best fit curve through the graph's points after creating a standard curve with the Rat BDNF concentration on the y-axis and absorbance on the x-axis. The concentration read from the Standard curve must be multiplied by the dilution factor if samples have been diluted. using a plotting program like curve expert, for example.

Declaration

1. The kit might not be appropriate for unique experimental material, including gene knockout research, where the experiment's validity is questionable.

2. Because they don't match the detection and capture antibodies employed in this product, some natural or recombinant proteins, such as bacterial and eukaryotic recombinant proteins, might not be detected.

3. Inconsistent test findings cannot be ruled out because this kit is not compared with comparable kits from other manufacturers or goods that use different methods to identify the same substance.

Appendixes IX

Estimated of serum Synapsin-1 concentration

All kit components have been formulated and quality control tested to function successfully. Do not mie or substitute reagents or materials from other kits, detection effect of the kit will not be guaranteed if utilized separately or substituted.

Specification

Sensitivity: 0.061 ng/mL

Detection Range: 0.16-10 ng/ml

Specificity: This assay has high sensitivity and excellent specificity for detection of Rat SYNI. No significant cross-reactivity or interference between Rat SYNI and analogues was observed.

Materials Needed; Not Provided

1. Microplate reader with 450 ± 10 nm absorbance measurement capability.

2. A centrifuge that runs fast.

3. A cultivator with an electro-heating standing temperature.

4. Absorbent paper.

5. Deionized or double-distilled water.

6. Disposable tip single or multichannel pipettes with excellent precision.

7. Accurate pipettes that can deliver volumes from 2 μl to 1 ml

Notes on Safety

1. Human use of this kit is not recommended; it is exclusively intended for laboratory research and development.

2. Reagents ought to be handled carefully and appropriately since they are potentially dangerous materials.

3. To prevent skin and tissues from coming into touch with Stop Solution and TMB, gloves, lab coats, and goggles should be used at all times. If contact occurs, thoroughly wash with water.

Test Principle

Sandwich enzyme immunoassay is the test principle used in this kit. An antibody specific to Rat SYN1 has been pre-coated on the microtiter plate included in this kit. After adding standards or samples to the proper microtiter plate wells, a biotin-conjugated antibody that is specific to Rat SYN1 is applied. Each microplate well is then filled with horseradish peroxidase (HRP)-conjugated avidin, and the mixture is incubated. Only the wells containing Rat SYN1, biotin-conjugated antibody, and enzyme-conjugated Avidin will change color when the TMB substrate solution is introduced. A sulphuric acid solution is added to stop the enzyme-substrate reaction, and the color change is determined spectrophotometrically at 450 nm ± 10 nm. The The OD of the samples is then compared to the standard curve to determine the concentration of Rat SYN1 in the samples.

Sample Collection and Storage

Samples for serum: should be gathered and placed in a serum separator tube. Following clotting for two hours at room temperature or overnight at 4°C, centrifuge for 20 minutes at 1000 x g. Assay recently made serum right away, or save samples in an aliquot for later use at -20°C or -80°C. Do not freeze-thaw repeatedly.

Plasma: Gather plasma while using heparin or EDTA as an anticoagulant. Centrifuge samples within 30 minutes of collection at 1000 x g and 2-8°C for 15 minutes. Assay the plasma right away, or save the samples in an aliquot and store them at -20°C or -80°C for later use. Do not freeze-thaw repeatedly.

Tissue homogenates: Depending on the kind of tissue, different procedures will be used to prepare the homogenates.

1. Weigh the tissues before to homogenization and rinse them with PBS that has been chilled beforehand to get rid of all extra blood.

2. Using a glass homogenizer on ice (micro tissue grinders, too), mince the tissues into small pieces and homogenize them in fresh lysis buffer (different lysis buffer needs to be chosen based on subcellular location of the target protein; PBS can be used as the lysis buffer of most tissues) (w:v = 1:9, e.g. 900 µl lysis buffer is added in 100 mg tissue sample).

3. Use an ultrasonic cell disrupter to pound the resulting suspension until the solution becomes transparent.

4. Next, collect the supernatant and perform an assay after centrifuging the homogenates for five minutes at 10,000 x g. Store in aliquots at ≤ -20°C or use right away.

*Note: To acquire a more accurate concentration of the test material per mg of protein, it is advised to test tissue homogenates for protein concentration concurrently with the tests for protein concentration. You can buy our product, the BC016, BCA Protein Concentration Determination Kit, for protein detection.

Lysates of cells - Before performing an assay, the cells must be lysed in accordance with the guidelines below.

1. Adherent cells should be gently rinsed with pre-cooled PBS, separated using trypsin, and then collected by centrifuged at 1000 × & for 5 minutes (direct centrifugation can be used to collect suspension cells).

2. Use pre-cooled PBS to wash cells three times. 3. If required, then resuspend the cells in new lysis buffer at a concentration of 10 cu/ml.

4. To eliminate cellular derib, centrifuge at 1500 for 10 minutes at 2–8°C.

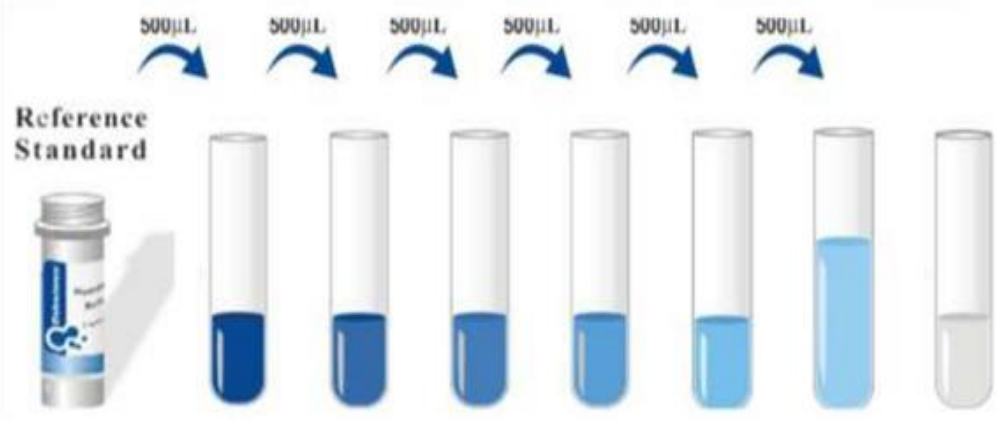
Preparing Reagents

Before using, bring all kit parts and samples to room temperature (18–25°C).

2. Please only remove the strips and reagents needed for the current experiment and save the remaining strips and reagents as directed if the kit won't be used up in one sitting.3. Dilute the 25x Wash Buffer into 1x Wash Buffer with double-distilled Water.

4. Centrifuge the Standard Working Solution for one minute at 1000 g. Shake gently (not to froth) and let the Standard sit at room temperature for 10 minutes before reconstituting it with 1.0 ml of Standard Diluent Buffer. In the stock solution, the Standard has a concentration of 10 ng/ml. As indicated in the photo below, please set up 7 tubes with 0.5 mL of Standard Diluent Buffer and use the Diluted Standard to create a double dilution series. Pipette the solution up and down multiple times to ensure that each tube is properly mixed before the next transfer. Establish seven diluted standard points, such as 10ng / m * L 5ng / m * L 2.5ng / m * L 1.25ng / m * L 0.63ng / m * L 0.32 ng/ml,

0.16ng / m * L and the Blank, at 0 ng/ml, is the final EP tube with Standard Diluent. Please utilize the new Standard Solution for every experiment to ensure the authenticity of the results. Replace the pipette tip after each dilution while lowering the concentration of the Standard from a high concentration to a low concentration. Remember that the final tube is a blank, so don't pipette solution from the earlier tube into it.



5. One biotinylated antibody and one streptavidin-HRP – Before using, briefly spin or centrifuge the stock amounts of these two substances. Use Biotinylated Antibody Diluent and HRP Diluent, respectively, to dilute them to the working concentration 100 times.

6. TMB Substrate Solution: Use sterile tips to aspirate the required dosage of the solution; do not re-pour the residual into the vial.

Notes 1. Please keep the reagents in accordance with the instructions after receiving the kit. The plates are able to be separated into individual strips. Kindly use it in batches as needed. After the initial test, it is advised that the remaining reagents be used within a month.

2. There is an absolute ban on reusing the test tubes, pipette tips, or reagents used in this experiment; doing so will compromise the experiment's outcomes. With the exception of TMB, Washing Buffer, and Stop Reagent, kit reagents from different batches cannot be combined.

3. Due to their modest volumes, streptavidin-HRP, biotinylated antibody, and lyophilized standards may disperse throughout the tube while being transported. Before using, please centrifuge at 1000 for one minute. After that, carefully pipette the solution four or five times. Please use the appropriate Dilution Solution and configure the Standard, Biotinylated Antibody and Streptavidin-HRP Working Solution in accordance with the needed amount; these solutions cannot be combined.

4. Before using, bring all of the reagents to room temperature (18–25°C). It is common for crystals to form in the concentrate (25x). Gently bring it to room temperature (heating should not go above 40°C). Stir until all of the crystals have dissolved.

5. Aim to dissolve the standard fifteen minutes prior to the exam. You may only use this Standard Working Solution once. Please dispose of the dissolved Standard if it hasn't been used entirely. The insertion of the sample must happen quickly. Ideally, every sample addition should be under control in less than ten minutes. It is advised to test duplicate wells to verify experimental accuracy. Additionally, when pipetting chemicals into wells, maintain a consistent order of additions to ensure that each well receives the same amount of incubation time.

6. After the washing procedure, pat dry with absorbent paper any leftover washing liquid in the reaction well. When using paper to absorb water, do not

place it straight into the reaction well. In order to avoid affecting the microplate reader reading, be sure to remove any remaining liquid and fingerprints from the bottom of the plate.

7. TMB Substrate Solution is light sensitive, avoid prolonged exposure to light. Dispense the TMR Substrate Solution within 15 minutes followind the wasting of the micrositer plate in adultion, avoid contact between TMB Substrate Solution and metal to prevent color development. TMB is contaminated if it turns blue color before use and should be discarded. TMB is toxic, avoid direct contact with hands.

8. Bacterial or fungal contamination of either samples or reagents or cross-contamination, between reagents may cause erroneous results.

Assay Procedure

Identify the wells for the sample, blank, and diluted standard 1. Set up one blank well and seven standard wells. Fill the appropriate wells with 100 µl of either the Standard Working Solution (please see Reagent Preparation) or the samples. Put the plate cover on top. At 37°C, incubate for 80 minutes. It is important to ensure that solutions are introduced to the bottom of the micro ELISA plate, avoiding contact with the inner wall and creating as much foaming as possible.

2. Transfer the contents of every well out. After aspirating the solution, fill each well with 200 µl of 1x Wash Solution, and let it sit for one to two minutes. Snap the plate onto absorbent paper to completely remove any residual liquid from any weils. Wash everything three times. Aspirate or decant any leftover Wash Buffer after the last wash. Turn the plate over and dab it on absorbent paper.

Notes: (a) The pipette tip should not come into contact with the weils wall when adding Washing Solution to prevent contamination.

(a) To prevent the washing liquid from contaminating neighboring wells, be careful to pour it directly into the well.

3. Fill each well with 100 microliters of the biotinylated antibody working solution, then cover the wells with the plate.Cover and incubate for 50 minutes at 37°C.

4. Carry out the aspiration and washing procedure three times in total, just like in step 2.

5. Fill each well with 100 µL of the Streptavidin-HRP Working Solution, place the plate sealer over the wells, and incubate at 37°C for 50 minutes.

6. Carry out the aspiration and washing procedure five times in total, just like in step 2.

7. Fill each well with 90 µl of TMB Substrate Solution. Replace the plate cover. Incubate in the dark for 20 minutes at 37°C (do not exceed 30 minutes). The addition of TMB Substrate Solution will cause the liquid to turn blue. Before measuring the OD, preheat the microplate reader for approximately fifteen minutes.

8. Fill each well with 50 µl of Stop Reagent. The addition of Stop Reagent will cause the liquid to turn yellow. Tap the side of the plate to combine the liquid. To make sure there is adequate mixing, lightly tap the plate if the color change is not uniform. The TMB Substrate Solution and the Stop Reagent should be inserted in the same order.

9. Remove any fingerprints and water droplets from the plate's bottom and make sure the liquid's surface is bubble-free. After that, start the microplate reader and start measuring at 450 nm right away.

Declaration

1. The kit might not be appropriate for unique experimental material, including gene knockout research, where the experiment's validity is questionable.

2. Because they don't match the detection and capture antibodies employed in this product, some natural or recombinant proteins, such as bacterial and eukaryotic recombinant proteins, might not be detected.

3. Inconsistent test findings cannot be ruled out because this kit is not compared with comparable kits from other manufacturers or goods that use different methods to identify the same substance.

Calculation of Results

Subtract the average zero Standard optical density from the average of the duplicate readings for each Standard, Control, and Sample. Draw a best fit curve through the graph's points after creating a standard curve with the Rat SYN1 concentration on the y-axis and absorbance on the x-axis. The concentration read from the Standard curve must be multiplied by the dilution factor if samples have been diluted. using certain plotting programs, such as curve expert.

Appendixes X

Measurement of Glutamate in Tissue.

Sample gathering 1. Pyrogen and endotoxins should not be present in the blood collection tube.

2. Serum and plasma cannot be collected from specimens exhibiting hemolysis or hyperlipidemia.

3. The samples ought to have a transparent, clear appearance. Centrifugation should be used to eliminate all of the suspension.

4. To prevent recurrent freeze-thaw cycles, collected samples that are not promptly recognized should be separated into single-use portions and frozen in a refrigerator between -20°C and 80°C.

5. Make appropriate multiple dilutions based on the actual conditions of the samples (pre-experiment is strongly advised in order to confirm the dilution ratio).

6. Gather specimens and make an effort to obtain two doses in order to prevent a scarcity of specimens for additional tests in the event that an assay's failure postpones the experimental procedure.

7. Take precautions when gathering specimens (using gloves, a respirator, etc.), keeping in mind that each specimen may pose a risk.

8. The biological safety cabinet should be used for specimen processing. Make sure the biological safety cabinet is used correctly. Procedures for the samples 1. Serum: Overnight, refrigerate the collected whole blood at 4°C. Next, centrifuge it at 1000–3000 rpm for 10 minutes. Test the supernatant right away, or store samples at -20°C or -80°C for three to six months.

2. Plasma: As an anticoagulant, take heparin, sodium citrate, and EDTA. Stir thoroughly after adding the plasma. Centrifuge mixture at 1000–3000 rpm for

10 minutes. Test the supernatant right away, or store samples at -20°C or -80°C for three to six months.

3. Tissue homogenate: After washing tissue slices with 0.01 MPBS, add tissue protein extraction reagent in a volume of 1G (5–10 ml) and combine with ice water. The mixture must be mixed and centrifuged for 10 minutes at 5000–10,000 rpm.Take the supernatant and test it right away, or store it at -20°C or -80°C for three to six months.

4. Cell culture: Centrifuge at 1000–3000 rpm for 10 minutes.Test the supernatant right away, or store samples at -20°C or -80°C for three to six months.

5. Perform centrifugation for 10 minutes at 1000–3000 rpm for fluids such as urine, ascites, and cerebrospinal fluid. Test the supernatant right away, or store samples at -20°C or -80°C for three to six months. Note: The fundamental ideas behind sample dilution Before deciding to dilute the samples, the user should consult the references to determine the likely content of the samples. The diluted sample content must fall within the best detection range of the specified ELISA Kits. A thorough record of the sample's dilution should be kept.

Before used, the kit should be stored between 2 and 8 degrees Celsius. All other ingredients must not be frozen, with the exception of the redissolved standard samples.

2. The concentrated enzyme-conjugates for the concentrated biotinylated Rat Glu antibody are modest in size. During transit, the tubes may bump or maybe invert, which could result in the liquid adhering to the wall or cap. In order to

shake off the adhering liquid that has settled to the tube bottom, the tubes should be manually shaken or centrifuged for one minute at 1000 rpm.

3. There's a chance the concentrated washing buffer will crystallize. When diluting, use a water bath to aid in the dissolution. It is necessary to completely dissolve the crystals while making the washing buffer.

4. The Rat Glu lyophilized standard sample must be used only once and cannot be divided throughout the testing procedure. The sample's lower concentration means that it will fast become inactive after dissolution.

5. The instructions should be strictly followed when operating. It is not permitted to use various batch numbers of components in combination with this reagent.

6. Verify that the spiral hybrid instrument thoroughly mixed the reagent. Enough mixing is especially crucial for the reagent in the microplate to yield the desired test result. Therefore, using the micro-oscillator (at the lowest frequency) is preferable. To ensure that the reaction liquid in the microplate is thoroughly mixed, manually shake it for one minute in a circular motion if there is no micro-oscillator.

7. The ELISA for the experiment needs to be properly preheated previously and operated strictly in accordance with the handbook standard.

8. There should be several pores when evaluating Rat Glu standard samples using enzyme immunoassay.

9. Store the unused microplates in a raw foil bag between 2 and 8 degrees Celsius.

10. The chromogen reagent is light-sensitive. As a result, it must not be in direct sunlight.

11. It is not recommended to utilize expired kits in experiments.

12. The ELISA values must be taken into consideration while determining the test results. The wavelengths for dual-wavelength tests should be adjusted to 450 nm and 630 nm, respectively.

13. All wastes, cleaning solutions, and samples ought to be handled as biowaste. Sulfuric acid (1M) should be used as Color Reagent C, and handle it with caution.

14. Every step should involve adding an instrument to the sample. Adjust the adding device's precision to prevent experimentation errors. Within five minutes, the single sample addition time should be under control. The salvo for sample-adding is suggested just in case the samples surpass the limits.
15. According to the experiments, adhesive closures must be cropped or they cannot be reused. Apply an adhesive strip to the compacted
16. Multiple pores are better since test determination and standard curve should be made simultaneously in every experiment. In the event that the test sample's content exceeded the maximum allowable concentration (measured by the sample's OD value), dilute to a specific multiple by sample diluents (n times), test the outcome, and then multiply the result by dilution ratios for computation.

17. Because NaN3 inhibits the activities of horseradish peroxidase, the sample containing NaN3 cannot be evaluated (HRP).

18. When using a plate washer to wash a board, each well should have a volume of liquid injection that is greater than 350μl. Verify whether the

sampling head is stuck. However, while hand-washing a board, the water-absorbing substance including paper scraps should be utilized carefully to avoid chromogen reagent and exogenous peroxidase analogue reactions.

19. Read the OD within 10 minutes of Color Reagent C stopping the reaction.

20. The mean value will be the computation result for the multiple pores experiment.

21. Sample hemolysis is not a good candidate for this test since it could lead to a false positive result.

22. After adding samples, the test strips should be placed in a closed box, and the surrounding humidity should be maintained at roughly 60%.

23. Maintaining the thermostat's internal temperature at 37°C requires routine calibration checks. Make sure the temperature in the experiment is constant. 24. Every component for the 48T Elisa Kit is 50% of the 96T total. 25. The English instruction will take precedence in the event of any discrepancy.

Getting ready for tests

1. Please remove the Elisa Kit from the refrigerator twenty minutes before using it, and wait until it reaches room temperature before taking a test.

2. Use double-distilled water to dilute the concentrated washing solution (1:25). Return the unused item.

3. Standard sample of Rat Glu: After adding 1.0 ml of diluent to the Rat Glu lyophilized standard sample, let it sit for 30 minutes. Once the sample has fully dissolved, give it a little stir, label the tube, and take dilutions as necessary.(Using the following concentration values to create the standard

curve is advised: 20, 10, 5, 2.5, 1.25, 0.625, 0.312 nmol/ml). Note: Ensure that the lyophilized standard is thoroughly combined and dissolved. 4. The standard sample dilution method's legend: Label seven clean tubes with the following: ②,③,⑿,⑤,⑥,⑦, and ⑧, accordingly. Fill each tube with 300μl of the standard sample diluent. Pour 300μl of the diluent from tube ① into tube ② and thoroughly stir. Additionally Pour 300μl of the diluent from tube ② into tube ③, then thoroughly mix. Follow the previous instructions up to the tube. **. Negative control is the standard sample dilution in tube ⑧.

Redissolved standard liquid (20 nmol/ml) should be disposed of, not reused.

It is not possible to reuse the reconstituted standard stock solution.

5Biotinylated Rat Glu Antibody Liquid: Use an antibody diluent to dilute the concentrated biotinylated antibody (1:100) to create biotinylated antibody liquid, according to the required amount. It is best to prepare thirty minutes in advance. Plus, it's just valid that one day.

6. Enzyme-conjugate liquid: Dilute the concentrated enzyme-conjugate with enzyme-conjugate diluent (1:100) according to the required amount to create enzyme-conjugate liquid. It is best to prepare thirty minutes in advance. That day is the only time it can be used.

7. Color Reagent Liquid: Mix 9:1 Color Reagent A and Color Reagent B 30 minutes in advance to make Color Reagent Liquid. Cleaning technique 1. Automatic plate-washing machine: 350μl of lotion is needed, and the time between injection and extraction should be between 20 and 30 seconds. Before using the machine, make sure you understand its operating instructions well. 2. A manual plate-washing machine: fill each well with 350μl with lotion

and let it sit for 30 seconds. As soon as possible, shake each well dry, then use absorbent paper to clean it. To prevent contamination and well-jumping, be mindful of the lotion-adding step when washing the plates...

Steps:

1. Remove the necessary strips from the zip-lock bag and let them come to room temperature. Repackage the unused strips and desiccant into the sealed aluminum foil bag and store it between 2 and 8 degrees Celsius.

2. Set aside the blank wells (you might overlook them if you're using a dual-wavelength reading plate).

3. Fill 0 nmol/ml wells with standard diluent and add samples or varying concentrations of Rat Glu standard samples to matching wells (100µl for each well). Adhesive tapes are used to seal the reaction wells, and they are then incubated for 90 minutes at 37°C.

4. 30 minutes in advance, prepare the liquid biotinylated Rat Glu antibody.

5. Repeatedly wash the Elisa plate

6. Fill each well with 100µl of the biotinylated Rat Glu antibody liquid. Reaction wells are sealed using adhesive tapes, and they are incubated for 60 minutes at 37°C.

7. 30 minutes in advance, prepare the enzyme-conjugate liquid.

8. Three times, wash the Elisa plate.

9. Pour 100µl of the enzyme-conjugate liquid into each well, excluding the blank wells. Using adhesive tape, seal the reaction wells, and then hatch them in an incubator for 30 minutes at 37°C.

10. Five times, wash the Elisa plate.

11. Fill each well with 100µl of Color Reagent liquid (as well as the blank well), and hatch the eggs at 37°C in a dark incubator. Hatching can be halted when the color for a high concentration of the standard curve becomes darker and a color gradient forms. Within thirty minutes, the chromogenic process needs to be under control.

12. Fill each individual well (as well as the blank well) with 100µl of Color Reagent C. Blend thoroughly. In ten minutes, read the OD at 450 nm.

Note: Color reagent solution and Color reagent C are the only reagents that need to be added to the blank well.

Appendixes XI

Determination Gene expression activity regulatory cytoskeleton associated protein (ARC):

This approach was carried out according to the comparative Ct approach (ΔΔCt) with normalization to the level of the control group in the presence of the transcript levels to those of GAPDH mRNA. This was achieved according to the recommendation of (Schmittgen and Livak, 2008).

1- For this purpose, amplifying of the ARC gene was carried out using the following primers:

Primer name	Sequence (5'->3')	Target

ARC-Forward	GACAAGGCAGAGGAGAGTGTC	Gene of Interest
ARC-Reverse	CCGGAGTGACTAATGTGCTCT	
B-actin-F	GTGGCACCACCATGTACCCAGGCAT	Housekeeping gene
B-actin-R	ACTACAGGGCTGACCACACCCCACT	

1. Quantitative Reverse Transcription Real-Time PCR (RT-qPCR):

A. RNA extraction:

After 4 weeks, the rats were being sacrificed meanwhile brain tissue was collected and added with Triazol for RNA extraction as follow:

1-200 µg of brain tissue from each sample was lysed by adding 600 µl of Triazol in a 1.5 ml micro-centrifuge tube and left for 5 minutes at room temperature with physical sonication to release the RNA.

2-This followed by centrifugation at 13000 rpm for 2 minutes meanwhile the supernatant was transferred into new tube and 600 µl of ethanol was added and vortexed.

3-The lysate was carefully transferred into the upper reservoir of the spin column for RNA binding which then centrifuged at 13,000 rpm for 1 minute meanwhile the flow-through was discarded.

4-The spin column was washed with washing buffer then exposed to DNase by adding 45 µl of DNase buffer and 5 µl of DNase which then left for 15 minutes.

5-The spin column was then washed three times with washing buffer by adding 500 µl of washing buffer and centrifugation at 13000 rpm for 1 minute.

6- For RNA elution, the column was centrifuged empty to dry out the silica gel followed by adding 50 µl of elution buffer and centrifugation at 13000 rpm for 2 minutes.

RNA concentration measurement by Quantus™ Fluorometer (Promega, USA):

1-1X of TE buffer was prepared by diluting 20X TE Buffer (pH 7.5) with nuclease-free water.

2-The QuantiFluor® Dye working solution was prepared with 1X TE buffer to make a 1:400 dilution by combining 10µl of QuantiFluor® Dye with 3,990µl of 1X TE buffer with mixing.

3-The blank sample was prepared for the QuantiFluor® ONE RNA System by adding 200 µl of QuantiFluor® RNA Dye to a 0.5ml PCR tube.

4-The nucleic acid standard (RNA) was added in a 0.5ml PCR tube as recommended by the manufacturer (2 µl of standard to 200 µl of working solution).

5-Set up standard measurement by the Quantus Fluorometer reading then measure the RNA samples according to the recommended procedures by the Quantus machine.

2-cDNA synthesis

A- A total of RNA was reversed transcribed to cDNA using the kit from ADDBio (Korea) as following:

Substance	Amount
H2O	3 µl
Reverse transcriptase (RT) 2X add script cDNA	10 µl
DNTPs	2 µl
random oligos hexamer	1 µl
RNA	4 µl
Total volume	20 µl

B- The thermal conditions were as following:

Temperature	Time	Purpose
25 C	10 min	Priming
50 C	60 min	Reverse transcriptase (RT)
80 C	5 min	RT inactivation
4 C	On hold	Store cDNA

2. Quantitative Reverse transcriptase PCR (RT-qPCR) Preparation

2.1. RT-qPCR amplification:

Initially, the amplification was achieved using AddScript RT-qPCR Syber master (AddBio, Korea) and

A/ the reaction was including:

Substance	Amount
H2O	3 µl
AddScript RT-qPCR	10 µl
Forward primer (0.05 pmol/20 µl)	2 µl
Reverse primer (0.05 pmol/20 µl)	2 µl
Cdna	3 µl
Total	20 µl

Note: This was carried out for the internal reference gene in same components.

B/ The thermal conditions were carried out using BioRAD (USA) as following:

Temperature		Time	Repeat
Initial denaturation	95 C	5 min	1
Denaturation	95 C	20 sec	40x
Annealing	60 C	30 sec	40x with machine read
Extension	72 C	30 sec	40x
Melting analysis	95 C	15 sec	1
Melting analysis	60 C	60 sec	1
Melting analysis	+0.3 C of 95C	15 sec	With machine read

2.2. RT-qPCR data normalisation:

The delta-delta Ct method was used to normalize transcript levels to those of GAPDH mRNA as mentioned by **(Schmittgen &Livak, 2008);** in which the following formula was employed:

2-ΔΔCT = [(CT gene of interest- CT internal control) sample A- (CT gene of interest- CT internal control) sample B)].

Note:

Sample A means one certain group.

Sample B means another certain group.

Appendixes XII

Amyloid Staining Protocol for Congo Red Staining:

This is an altered version of the Highman's Congo Red stain. It can be applied to frozen sections as well as formalin-fixed, paraffin-embedded tissue sections containing amyloidosis in order to detect amyloid.

The nuclei will be stained blue, while the deposits of amyloid will be red.

Sections are typically 5 um thick. On the other hand, 10um thick sections will be more satisfactory in the event of insufficient amyloid deposits. In tissue sections, amyloid is stained with Congo red. Fixation: 10% Neutral Buffered formalin.

Section: 5–10 um paraffin sections (use 10 um thick sections if there are insufficient amyloid deposits).

Solutions and Reagents:

0.5% Congo red in 50% alcohol:

Congo red (Sigma, Cat# C-6277) ----- 0.5 g

50% Alcohol ---------- 100 ml

1% Sodium Hydroxide:

Sodium hydroxide ---------- 1 g

Distilled water ---------- 100 ml

Alkaline Alcohol Solution:

1% Sodium hydroxide ---------- 1 ml

50% alcohol ---------- 100 ml

Procedure:

1. Xylene Dewax - 2 minutes

2. Xylene Dewax - 2 minutes

3. Xylene Dewax - 2 minutes

4. Rinse in 100% alcohol for two minutes.5. Wash in Absolute Alcohol – 2 mins

6. Soak for two minutes in 90% alcohol

7. Soak for two minutes in 70% alcohol

8. Take a 2-minute shower with running water

9. For 15 to 20 minutes, stain in the Congo Red solution.

10. Use distilled water to rinse.

11. Quickly differentiate in an alkaline alcohol solution (5–10 dips).

12. Give it a minute of tap water rinsing.

13. Use Gill's hematoxylin as a counterstain for 30 seconds.

14. Give it a two-minute rinse with tap water.

15. Use 95% alcohol, switch to 100% alcohol twice for three minutes each to dehydrate.

16. Clear in two changes of three minutes each, using xylene or a replacement.

17. Use a resinous mounting medium to mount.

Findings:

Amyloid, eosinophil granules, and elastic fibers ——— red

Nuclei ---------- blue

الملخص

كان الهدف من هذه الدراسة هو التحقق من التغيرات الفسيولوجية للجهاز العصبي المركزي التي تحدث نتيجة اتباع نظام غذائي مرتفع الكولسترول. تم تقسيم الجرذان ال 20 عشوائيًا إلى مجموعتين (10/مجموعة): مجموعة السيطرة، التي تلقت نظامًا غذائيًا عاديًا ومجموعة النظام الغذائي الخاص بارتفاع نسبة الكولسترول في الدم(HCD) ، والتي تلقت مكملاً بنسبة 1٪ من الكوليسترول في طعامها لمدة 4 أسابيع. بعد انتهاء التجربة، تم جمع عينات من مصل الدم لقياس مستوى الدهون، Malnodialdehyde (MDA)، الجلوتاثيون(GSH) ، مستوى BDNF و synapsin-1. وتم تشريح الجرذان واخذ عينات من المخ لقياس التعبير الجيني للهيكل الخلوي المنظم للنشاط(ARC) ، ومستوى الغلوتامات، والمالنودياناالدهيد(MDA) ، والجلوتاثيون(GSH) ، وأكسيد النيتريك والفحص النسيجي.

أظهرت الدراسة الحالية زيادة معنوية عالية في الكوليسترول الكلي(TC) ، الدهون الثلاثية(TG) ، البروتين الدهني منخفض الكثافة (LDL) في مجموعة النظام الغذائي المرتفع الكوليسترول مقارنة بمجموعة السيطرة وفي المقابل حدث انخفاض معنوي في مستوى البروتين الدهني المنخفض الكثافة (HDL-C) لدى مجموعة النظام الغذائي المرتفع الكوليسترول مقارنة بمجموعة السيطرة.

أشارت النتائج إلى أن فرط كوليستيرول الدم كان له تأثير على مستويات عامل التغذية المشتق من الدماغ حيث انخفضت مستوياته بسبب الإجهاد التأكسدي، في حين ظلت مستويات السينابسين -1 في أنسجة المخ دون تغيير.وارتفاع في مستويات الغلوتاميت بسب فرط الكوليسترول و كانت هناك أيضًا انخفاضات ملحوظة في تركيزات أنسجة المخ والمصل المتجانسة من NO وGSH، إلى جانب الزيادات فيMDA ، كما أظهرت انخفاض التنظيم في التعبير الجيني للهيكل الخلوي (ARC) المنظم للنشاط في مجموعة النظام الغذائي المرتفع الكوليسترول مقارنة بمجموعة السيطرة.

علاوة على ذلك، يُظهر الفحص النسيجي المرضي لقسم الأنسجة نمط تمزق خلايا الأنسجة العصبية بدرجات متفاوتة من ترسيب التشابك الليفي العصبي على الخلايا العصبية، ويتجلى ذلك من خلال ترسب لويحات بيتا أميلويد داخل وخارج الخلية في الجهاز العصبي المركزي، والتي تتخلل طبقات المخ وأنسجة المخ. أقل مما كانت عليه في المخيخ، وأجزاء الحبل الشوكي. وكما اتضح، فإن نوى

ضمور الخلايا العصبية مع تكاثر الخلايا الدبقية العصبية وخلايا التسلل الالتهابية للأنسجة العصبية في مجموعة النظام الغذائي المرتفع الكوليسترول مقارنة بمجموعة السيطرة.

يمكن أن نستنتج أن النظام الغذائي الخاص بارتفاع نسبة الكولسترول في الدم يعزز مستويات مرتفعة من الإجهاد التأكسدي وقد ثبت أنه يرتبط ارتباطًا وثيقًا بأضرار الإجهاد التأكسدي والسلامة الهيكلية البيوكيميائية للخلايا العصبية والخلايا الدبقية في القشرة الدماغية بطريقة مهمة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب البيطري

قسم الفسلجة والادوية والكيمياء الحياتية

تأثير النظام الغذائي عالي الكوليسترول على مستوى الغلوتاميت والعامل العصبي المشتق من الدماغ والمؤشرات الحيوية الأخرى في الجهاز العصبي المركزي عند ذكور الجرذان

رسالة مقدمة إلى

مجلس كلية الطب البيطري في جامعة كربلاء في استيفاء جزء من متطلبات درجة ماجستير في الطب البيطري/الفسلجة

بواسطة

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