T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF HISTOLOGY AND EMBRYOLOGY

HISTOPATHOLOGICAL EFFECT OF HIGH FAT DIET ON SUBSTANTIA NIGRA PARS COMPACTA IN ROTENONE-INDUCED PARKINSON'S TYPE MOUSE MODEL

MASTER OF HISTOLOGY AND EMBRYOLOGY THESIS

AYBÜKE ÖZER

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SUPERVISOR
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APPROVAL

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

15/06/2023

Signature

Aybüke Özer

DEDICATION

To my family…

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LIST OF SYMBOLS AND ABBREVIATIONS

6-OHDA: 6-Hidroksidopamin

AD: Alzheimer’s Disease

AAV2: Adeno-associated viral vector serotype 2

ALDH: Aldehyde dehydrogenase

BG : Basal ganglions

CAT: Catalase

COMT: Cathecol-o-methyl transferase

CNS: Central nervous system

H&E: Hematoxylen&Eosin

DA: Dopamine

DAergic: Dopaminergic

DAT: Dopamine transporter

DJ-1: Parkinson disease protein 7

DMSO : Dimethyl sulfoxide

DOPA : Decarboxylase, dopamine β-hydroxylase

DOPAC : 3,4-Dihydroxyphenylacetic Acid

DOPAL : 3,4-Dihydroxyphenyl Acetaldehyde

GABA: γ-aminobutyric acid

GSH : Glutatyon

GPi: Globus Pallidus Internal Segment

GPx: Glutathione peroxidase

GDNF: Glial cell line-derived neurotrophic factor

GSH: Reduced glutathione

HFD: High fat diet

IL : Interleukin

IFN-γ: Interferon gamma

iNOS: Inducible NO-synthase

IP.: Intraperitoneal

LB: Lewy body

L-DOPA: Levodopa

LRRK2: Leucine-rich repeat kinase 2

MAO: Monoamine oxidase

MPTP: 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine

MPP+: 1-methyl-4-phenylpyridinium

NO: Nitric Oxide

PBS: Phosphate buffered saline

PD: Parkinson's disease

PNS: Peripheral nervous system

ROS: Reactive oxygen species

SN: Substantia nigra

SNCA: Alpha-Synuclein

SNPc: Substantia nigra pars compacta

SNr: Substantia nigra pars reticulata

STR: Striatum

SOD: Superoxide dismutase

TH: Tyrosine Hydroxylase

TNF-a: Tumor necrose factor alfa

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end

Labeling

VMAT2: Vesicular monoamine transporter 2

VPS35: Vacuolar protein sorting ortholog 35

ABSTRACT

Özer, A. (2023). Histopathological Effect of High Fat Diet on Substantia Nigra Pars Compacta in Rotenone-induced Parkinson's Type Mouse Model. Yeditepe University, Institute of Health Science, Department of Histology and Embryology MSc Thesis, İstanbul.

Parkinson's disease (PD) is one of the common neurodegenerative diseases characterized by the loss of dopaminergic neurons located in the substantia nigra pars compacta (SNPc) area. Many studies have been conducted on mitochondrial dysfunction, oxidative stress and neuroinflammation to explain the neuronal death mechanisms involved in the pathogenesis of Parkinson's disease, but, although the processes are thought to cause Parkinson's disease, the reason is not fully understood. Since the cause is still unclear in most cases of Parkinson's disease, only symptomatic treatments such as pharmacotherapy, stereotactic neurosurgery and physiotherapy are available. Although these treatments reduce motor symptoms and improve the patient's quality of life, the clinical need for new treatments to prevent, stop or slow the progression of the disease is not fully understood. Furthermore, many studies now suggest an association between neurodegeneration and metabolic disorders. Long-term changes in diet have the potential to change the lipid composition of the brain. High fat diets (HFDs) are strongly associated with obesity and exert adverse effects on cognitive and behavioral functions. Epidemiological studies have shown that patients with obesity are at increased risk for neuropsychiatric disorders, including anxiety and depression. Agents used to induce neurodegeneration related to Parkinson's disease in rodents include rotenone. Rotenone is a pesticide that readily crosses the blood-brain barrier following systemic injection and inhibits complex I of the mitochondrial respiratory chain, inducing oxidative stress and subsequent pathology not limited to dopaminergic neurons. Rotenone is widely metabolized in vivo and the choice of route of exposure greatly influences the dose used. This model is based on the assumption that a high fat diet has an adverse effect. It was observed that rotenone substance caused loss of locomotor movement as in Parkinson's disease, and it was understood that the application of a high fat diet caused the exacerbation of these movements. Hematoxylin eosin, cresyl violet and silver staining were used to notice pathological findings, and as anticipated, intense pathological discoveries were observed in the PD+HFD group compared to the rotenone group. H-

Scores were calculated in iNOS immunohistochemistry staining, and there are notable distinctions between the results of the PD+HFD and control groups ($p<0.05$). Ultimately, the apoptotic index was calculated with the TUNEL experiment, the highest values were acquired in the rotenone group and PD+HFD group, the second most increased in the HFD group, and the lowest in the control ($p<0.05$). As a result, in this study, high fat diet administration and duration caused a decrease in dopaminergic neurons in the substantia nigra pars compacta and increased the number of apoptotic neurons in a rotenone-induced Parkinson type mouse model.

Key words: Parkinson’s Disease, Dopaminergic Neurons , Rotenone, Oxidative Stress, Neurodegenerative Diseases

ÖZET

Özer, A. (2023). Yüksek Yağlı Diyetin Rotenone ile Oluşturulan Parkinson Tipi Fare Modelinde Substantia Nigra Pars Compacta Üzerine Histopatolojik Etkisi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Histoloji ve Embriyoloji ABD, Master Tezi. İstanbul

Parkinson hastalığı (PH), substantia nigra pars compacta (SNPc) bölgesinde yer alan dopaminerjik nöronların kaybı ile karakterize edilen yaygın nörodejeneratif hastalıklardan biridir. Parkinson hastalığının patogenezinde yer alan nöronal ölüm mekanizmalarını açıklamak için mitokondriyal disfonksiyon, oksidatif stres ve nöroinflamasyon üzerine birçok çalışma yapılmıştır, ancak süreçlerin Parkinson hastalığına neden olduğu düşünülse de nedeni tam olarak anlaşılamamıştır. Çoğu Parkinson hastalığı vakasında neden hala belirsiz olduğundan, sadece farmakoterapi, stereotaktik beyin cerrahisi ve fizyoterapi gibi semptomatik tedaviler mevcuttur. Bu tedaviler motor semptomları azaltmasına ve hastanın yaşam kalitesini iyileştirmesine rağmen, hastalığın ilerlemesini önlemek, durdurmak veya yavaşlatmak için yeni tedavilere yönelik klinik ihtiyaç tam olarak anlaşılamamıştır. Ayrıca, birçok çalışma artık nörodejenerasyon ve metabolik bozukluklar arasında bir ilişki olduğunu düşündürmektedir. Diyetteki uzun vadeli değişiklikler, beynin lipid bileşimini değiştirme potansiyeline sahiptir. Yüksek yağlı diyetler (YYD'ler) obezite ile güçlü bir şekilde ilişkilidir ve bilişsel ve davranışsal işlevler üzerinde olumsuz etkiler gösterir. Epidemiyolojik çalışmalar, obezitesi olan hastaların anksiyete ve depresyon dahil olmak üzere nöropsikiyatrik bozukluklar için yüksek risk altında olduğunu göstermiştir. Kemirgenlerde Parkinson hastalığına bağlı nörodejenerasyonu indüklemek için kullanılan ajanlar arasında rotenon bulunur. Rotenone, sistemik enjeksiyonun ardından kan-beyin bariyerini kolayca geçen ve mitokondriyal solunum zincirinin kompleks I'ini inhibe ederek oksidatif strese ve ardından dopaminerjik nöronlarla sınırlı olmayan patolojiye neden olan bir pestisittir. Rotenon, in vivo olarak büyük ölçüde metabolize edilir ve maruz kalma yolunun seçimi, kullanılan dozu büyük ölçüde etkiler. Bu model, yüksek yağlı bir diyetin olumsuz bir etkiye sahip olduğu varsayımına dayanmaktadır. Rotenone maddesinin Parkinson hastalığında olduğu gibi lokomotor hareket kaybına neden olduğu görülmüş ve yüksek yağlı diyet uygulamasının bu hareketlerin şiddetlenmesine neden olduğu anlaşılmıştır. Hematoksilen eozin, kresil viyole ve gümüş

boyama ile patolojik bulgular fark edildi ve beklendiği gibi rotenon grubuna göre PH+YYD grubunda yoğun patolojik bulgular gözlendi. iNOS immünohistokimya boyamasında H-Skorları hesaplandı ve PH+YYD ile kontrol grubu sonuçları arasında belirgin farklar vardı ($p<0.05$). Son olarak TUNEL deneyi ile apoptotik indeks hesaplandı, en yüksek değerler rotenon grubu ve PH+YYD grubunda, ikinci en yüksek değerler YYD grubunda ve en düşük kontrol grubunda elde edildi ($p<0,05$). Sonuç olarak, bu çalışmada, yüksek yağlı diyet uygulaması ve süresi, rotenon ile indüklenen Parkinson tipi fare modelinde substantia nigra pars compacta'da dopaminerjik nöronlarda azalmaya ve apoptotik nöron sayısında artışa neden olmuştur.

Anahtar Kelimeler: Parkinson Hastalığı, Dopaminerjik Nöronlar, Rotenon, Oksidatif Stres, Nörodejeneratif Hastalıklar

1. INTRODUCTION AND PURPOSE

Parkinson's disease (PD) is the second most frequent neurodegenerative disease following Alzheimer's disease (1). PD is characterized neuropathologically by the presence of α-synuclein-containing Lewy bodies in the substantia nigra of the brain (2). Parkinson's disease in the clinic; is demonstrated by motor symptoms such as rigidity, resting tremor, postural instability, bradykinesia, and difficulties in initiating movement (3, 4). Movement dysfunction in Parkinson's disease occurs as a result of the progressive death of dopaminergic neurons in the substantia nigra pars compacta (SNPc), which is considered to be involved in the basal ganglia (BG) system in motor control (5). Dopaminergic neurons interact with neurons in the BG, which is designated as the junctional part of the movement, by releasing the neurotransmitter called dopamine, and this interaction is responsible for our motor movements (6). The reason for cell death in Parkinson's disease is not fully understood, nevertheless, biochemical processes such as free radical-mediated oxidative damage, damaged mitochondrial complex I activity, and neuroinflammation are among the mechanisms expressed as cell mortality in Parkinson's disease (7, 8, 9, 10).

Rotenone compound creates PD like pathology; therefore, it is one of the common neurotoxins to model PD (11). As a result of the pathological events caused by rotenone, it causes events such as triggering of astrocyte and microglial activation, accumulation of a-synuclein, widespread expression of Lewy Body in the brain, and decrease in TH neurons in SNPc (12, 13, 14, 15, 16). High fat diet consumption seems to be associated with an increased risk of neurocognitive impairment in the brain (17).

A high fat diet is one of the most repeatedly used human obesity models in laboratory animals for obesity research (18). Luscious high fat and high sugar nutriment are rewarding and their utilization is associated with modification in the brain's reward circuitry (19). Research demonstrates that a high fat diet consumed in adolescence and the juvenile period has negative effects on hippocampal memory and neuronal functions in the majority (20). Primarily, in cognitive disorders caused by a high fat diet; Decreased neurogenesis, neuroinflammation, altered synaptic plasticity and dopaminergic neuron loss are noteworthy (21, 22).

In the thesis study, it is hypothesized that a high fat diet can aggravate the pathological effects in neurons in the rotenone-induced PD mouse model. This study aims to conduct investigated the effect of a high fat diet on morphological changes in dopaminergic neurons located in the substantia nigra pars compacta in a rotenone-induced PD model.

2. LITERATURE REVIEW

2.1. Central Nervous System

Central Nervous System (CNS) implicates the brain and spinal cord. Besides that is made up of about 100 billion neurons and entitled glial cells. The neuron, soma or cell body consists of an individual axon and multiple dendrites (23). The central nervous system consists of the brain, cerebellum and spinal cord, and its essential assignment can be summarized as receiving, analyzing, evaluating and responding to instructions generated by mechanical and chemical changes comprising among and outside of the organism (24).

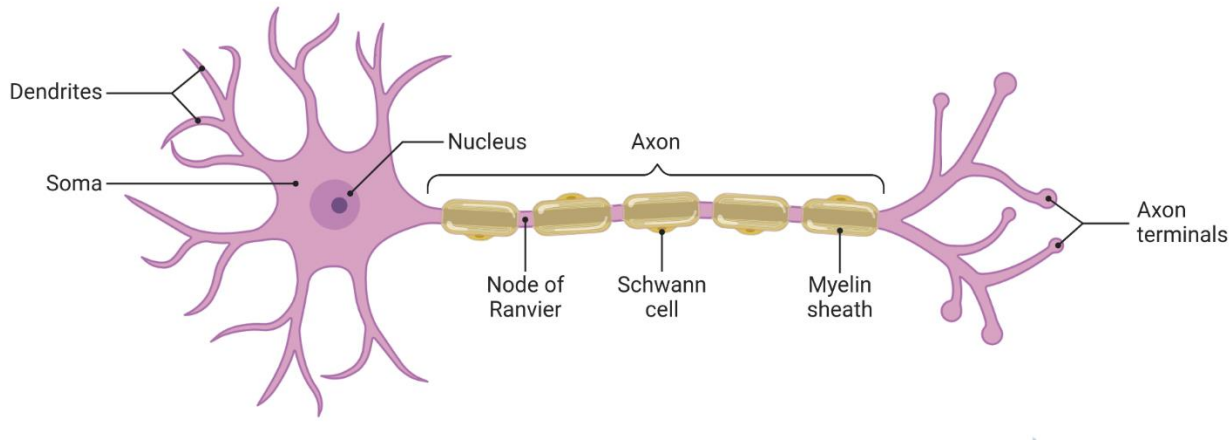

Figure 1. General structure of the neuron (23).

2.2. Basal Ganglia

Basal ganglia (BG) are a group of nuclei that have roles in the execution and preservation of learned movement patterns, as well as in cognitive functions such as ascertaining, planning, reward behaviour and response selection. BG dispatch afferent and efferent signals to the convenient regions to perform functions (23). Instructions coming from the cerebral cortex to the striatum, which is the principal afferent nucleus of the basal ganglia, are transferred to the Globus Pallidus internal segment (GPi) and substantia nigra pars reticularis (SNr). The knowledge coming here is transferred to these regions utilising direct and roundabout routes. While the direct pathway is related to gamma-aminobutyric acid (GABA), substance P, dynorphin and D1 receptors; The indirect pathway runs through GABA, enkephalin and D2 receptors (25, 26).

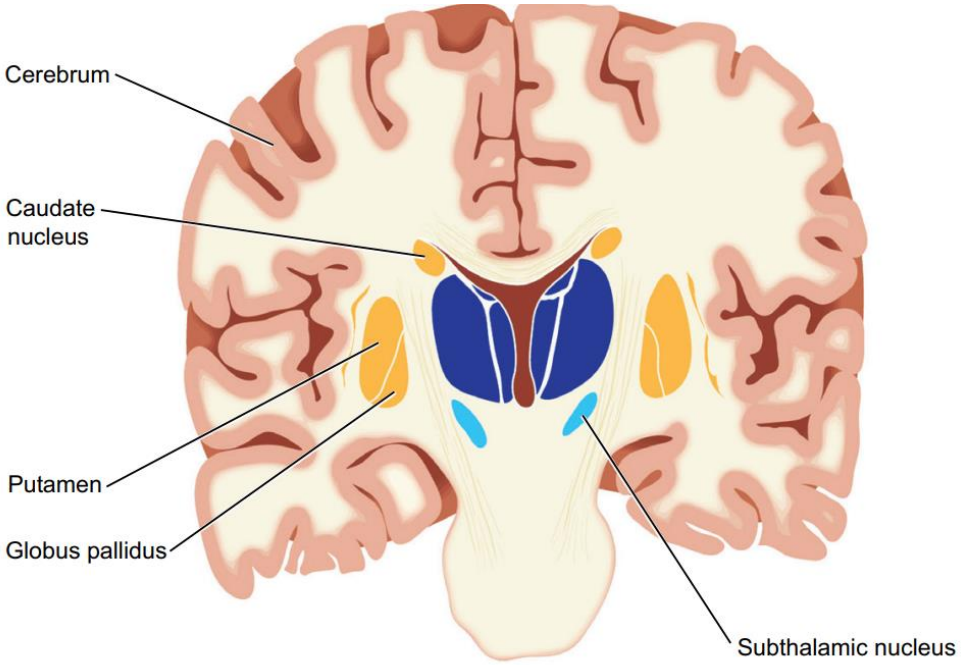

Figure 2. Structure of Basal ganglia (25)

2.3. Substantia Nigra

The substantia nigra is the basal ganglia construction in the midbrain and actins an important role in the movement. Due to high levels of neuromelanin dopaminergic neurons appear darker than neighbouring domains (25). Therefore it was named in Latin as 'dark substance' (27).

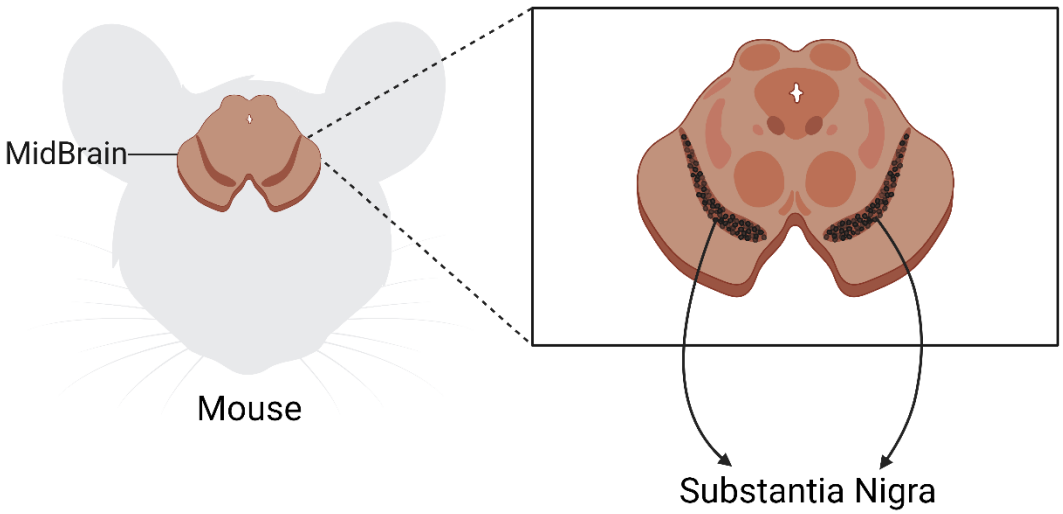

Figure 3. Substantia nigra in mouse brain

There is one substantia nigra on either side of the midline of the brain. Substantia nigra, consists of two parts, the substantia nigra pars compacta (SNpc) and the substantia nigra pars reticularis (SNpr) (27, 28). The SNpc is a densely packed nuclear region and for this reason, this domain glances darker (27). The SNpr is a restrictive GABAergic nucleus that functions in co-occurrence with the globus pallidus internus (GPi) as the final throughput of the basal ganglia's straight and indirect pathways (27).

2.4. History and Definition of Parkinson's Disease

Parkinson's disease (PD) was first aforementioned by the English neurologist James Parkinson in 1817 in a 66-page article comprising 5 portions and was defined as 'shaking palsy' (28, 29). Jean-Martin Charcot, as a result of his studies, added considerable details such as bradykinesia, muscle rigidity, and micrography to James Parkinson's observations and amplified the definition of the disease (30). He later named the disease 'Parkinson's disease', honouring James Parkinson (30). In 1912, Fritz Heinrich Lewy defined inclusion bodies, which are still considered pathological markers in PD, and in 1919 Konstantin Nikolaevich Tretiakoff and his group designated the loss of dopaminergic neurons (31). In the late 1950s, Arvid Carlsson and his workmates found that DOPA (a precursor to dopamine) notably alleviated Parkinsonism and propounded that dopamine is a neurotransmitter involved in motor control (32). Bertler and Rosengren reported that DA is extensively found in the basal ganglia of animals (33, 34). Hornykiewicz, who works in the UK, studied the work of Bertler and Rosengren and reported that DA levels fell in six cases of parkinsonism (35). It is characterized by diminished DA in the substantia nigra pars compacta (SNpc) region in PD and abnormal α-synuclein protein accumulation in the cytoplasm of neurons in dissimilar components of the brain (36). Today, the cause of these pathophysiological events responsible for the formation of PD is still not precisely elucidated (37).

2.5. Epidemiology of Parkinson's Disease

PD is the most widespread age-related neurodegenerative disorder after Alzheimer's disease (38). The incidence of Parkinson's disease differs according to ethnicity and sexuality. By ethnicity, the diffusiveness of Parkinson's disease is highest in white people such as Europeans and North Americans and least in Black people in

Africa. Furthermore, it is more likely to be seen in men than in women (38). Its extensiveness was reported as 173 cases per 100,000 people aged 55 to 64 years and 1903 cases per 100,000 people over 80 years of age. Its incidence was reported as 30 cases per 100,000 people in women aged 60-69, 103 cases per 100,000 people in women over 80 years of age, 58 cases per 100,000 people in men aged 60-69 and 258 cases per 100,000 people over 80 years old (39).

2.6. Etiology of Parkinson's Disease

The pathophysiological process of the disease is unknown in the etiology of PD, which is the second most widespread neurodegenerative disease. The excessive level of neuronal corruption seen in the pathology of the ailment is thought to arise as a result of the interaction of genetic and environmental factors (40, 41).

2.6.1. Environmental Factors and Genetic Factors

Environmental factors alone or in interplay with genetic factors are efficient in the formation and progression of PD. When an incident happened in 1983, after the injection of medications contamined with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to more than one person, it was noticed that PD symptoms occurred in people and it was reported that MPTP damaged dopaminergic cells (38). Polluted water use for PD was positively associated with long-term or high-dose exposure to heavy metals; It has been reported in numerous studies that vitamin E is negatively associated with smoking, alcohol and coffee consumption (42).

Looking at the genetic factors of PD, only 10% of cases appear to be associated with a clear Mendelian inheritance pattern (43). As a consequence of genetic studies, genes and loci called 'PARK' that cause monogenic forms of PD have been identified and two forms of PD are autosomal dominant and autosomal recessive (44). Of these, SNCA (PARK1 and 4), LRRK2 (PARK8) and VPS35 (PARK17) are involved in the autosomal dominant inheritance, while those responsible for autosomal recessive inheritance are Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7) (44).

Table 1. Conjectural risk factors straight or implicitly associated with PD (44).

Straight associated agents	**Implicitly associated agents**
Pesticide exposure	Smoking
Well water	Alcohol
Heavy metals	Non-steroidal anti-inflammatory use
Proteasome inhibitors	Vitamin E

2.7.Pathology of Parkinson's Disease

The significative properties of Parkinson's disease are neuronal loss in definite areas of the substantia nigra, diffuse intracellular protein (α-synuclein) backlog, and the presence of cytoplasmic inclusions is called Lewy Bodies (40, 45). The existence of alpha-synuclein characterizes Lewy Bodies (45). In humans, thereabout 450,000 dopaminergic neurons are found in the SNPc, and the loss of dopaminergic neurons must be between 60 and 80 per cent for clinical indications to transpire prominently (46, 47).

Neuronal loss in the SNPc region of the brain takes place in the ventrolateral, medial, ventral, and dorsal zones, respectively (46). Nonetheless, degeneration of dopaminergic neurons happens predominantly in the ventrolateral portion of the substantia nigra pars compacta (46). In the dorsal and intermediate parts of the putamen, striatal dopamine loss comprises, resulting in akinesia and rigidity (48). It has been determined that neuronal damage in the nigrostriatal pathway, which is one of the principal causes of PD, correlates with clinical pathology (49). Dissimilar from the disruption of dopaminergic neurons in the nigrostriatal pathway, catecholaminergic neurons, serotonergic neurons, and neurons in the vagus areas of the brain are most affected in PD (49, 50).

The existence of Lewy bodies in PD brains was initially defined by Friedrich Heinrich Lewy in 1912 as spherical inclusions in the brainstem of Parkinson's clients (51). Lewy bodies are round in shape, 5 to 25 μm in diameter, and importantly, ubiquitin, cytoskeletal elements, cell stress proteins, and synaptic vesicular proteins are the protein components of Lewy bodies (52).

Although the precise mechanism for the pathogenesis of PD is unknown, notice based on biochemical data, all obtained from the brains of Parkinson's patients, has helped elucidate the pathogenesis. Hence, pathological α-synuclein aggregation in the substantia nigra pars compacta region of the brain, excitotoxicity, protein aggregation, oxidative stress, and mitochondrial dysfunction form the basis of pathogenetic contraptions (53, 54).

2.7.1. Oxidative Stress

Cells continually manufacture free radicals during their metabolic activities. These radicals enclose unpaired electrons and are immensely reactive (55). For all biological systems to function properly, it is requisite to maintain a balance amid the formation and excretion of free radicals (56). Free radicals can also cause detriment to nucleic acids, lipids, and proteins if the balance is disturbed (57). The pathological process that befalls when this balance is disturbed is entitled oxidative stress.

Free radicals are separated into two forms ROS and Nitric oxide (NO) (56). ROS and NO are neutralized by the antioxidant defensive system and the organism is safeguarded from oxidative injury. These free radicals comprise enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and numerous non-enzymatic antioxidants, bearing vitamins A, E, glutathione, ubiquinone and flavonoids (58). Furthermore, since ROS fail to be detoxified in the SN in pathological conditions such as PD, it forms free iron ions and hydroxyl radicals, consequencing in the degeneration of dopaminergic neurons (56, 58, 59).

Improvements in lipid peroxidation enhancements in the SN region of the brain divisions of Parkinson's patients were caused by oxidative stress (60). Free radicals assault lipid membranes, bringing about lipid peroxidation (61).

Melanin is released into SN through the enzyme monoaminoxidase (MAO) in dopaminergic neurons. The release of melanin, which occurs as a result of oxidative deamination and autoxidation of DA, prohibits the accumulation of toxic metabolites by estranging free radicals and reactive metals from neurons (62, 63). Besides, it has been shown that melanin accumulates locally in neurons and ties up to free metals, causing cell demise (63).

Glutathione (GSH) is an antioxidant from the thiol tripeptide group that safeguards against oxidative stress-induced harm by cleansing free radicals (64). However, it is found at slighter levels in the SNPc relative to distinct regions of the brain (64, 65).

2.7.2. Mitochondrial Dysfunction

The essential function of mitochondria, called the 'powerhouse' of the cell, is to breed energy in the form of adenosine 5' triphosphate (ATP) (66). Besides, mitochondria carry out plenty of other tasks without energy production. Distinct tasks are cell death, arrangement of calcium metabolism and manufacture (66). It converts nutrients and oxygen into adenosine triphosphate (ATP) through oxidative phosphorylation, scavenges free radicals and controls programmed cell death (66).

Mutations in specific genes cause mitochondrial dysfunction and are known to act on forms of familial illness (67). Parkin, α-synuclein, DJ-1, UCHL-1, LRRK2, PINK1, NURR1, VPS35, and HtrA2 have pathogenic mutations supporting mitochondrial dysfunction to be pertinent in familial PD (68, 69).

The initial connection between mitochondria and PD came from the exploration that a neurotoxin that causes PD impedes mitochondrial respiration (67, 70). In the 1980s, it was defined that the synthetic heroin substance, which is utilized illegally by drug addicts and sources PD symptoms in these people, encloses MPTP (71). Additionally, toxins such as rotenone, pyridaben, trichloroethylene and fenpyroximate are other complex I inhibitors that bring about neurodegeneration and augment many pathological properties of PD (67, 72). It was defined that there was a selective degradation of 30-40% in the complex I activity of the mitochondrial respiratory chain in the SNPc area of Parkinson's clients (73). Hence, mitochondrial dysfunction, which is still the subject of intense research, has significant effects on the degeneration of dopaminergic neurons.

Abnormal metabolic function, abnormal morphology, and impaired fission-fusion balance in mitochondria have all been observed in forms of PD. Impaired mitochondrial function leads to increased oxidative stress and affects cellular pathways, leading to damage to intracellular components and cell death (Figure 5) (66).

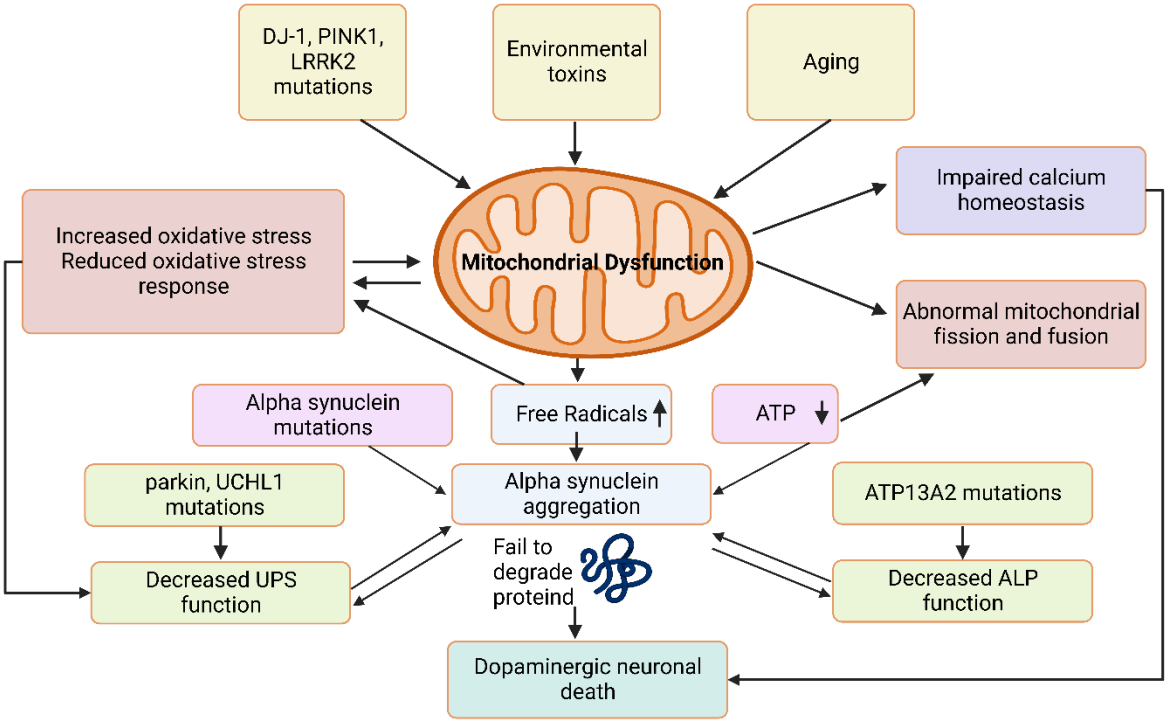

Figure 4. Mitochondria dysfunction and dopaminergic cell death in PD pathogenesis. All of these may directly or indirectly affect the mitochondrial function of protein degradation systems, including UPS and ALP, thereby causing the death of dopamine neurons (66).

2.7.3. Excitotoxicity

Glutamate is one of the body's most considerable neurotransmitters, found in more than 50 per cent of nerve tissue and plays a significant role in neuronal excitation (74). Glutamate receptors; when considered into two principal groups ionotropic receptors and metabotropic receptors, the process that detriments neuronal cells and leads to death as a result of excessive stimulation of ionotropic receptors by glutamate and similar substances are expressed as excitotoxicity (74).

It has been reported that there is an increase in glutamate or other endogenous glutamatergic agonists in neurodegenerative disorders, and the excitotoxic response resulting from this enhancement may be significant in determining the degree of tissue (75).

The overactivation of glutamate receptors on dopaminergic neurons in the substantia nigra pars compacta region may be involved in a role in the pathophysiology of PD (76, 77).

2.7.4. Neuroinflammation

One of the most obvious causes of PD is neuroinflammation (78). Labours show that inflammation originates from cells called microglia (79). Microglia are neuroimmune and neuroinflammatory cells that play a role in the advancement, regular functioning, ageing and injury of the central nervous system (80). Microglia exhibit a resting phenotype in the healthy brain. Nevertheless, conditions such as pathogen invasion, injury, and toxic protein aggregation are activated below stress (80). When microglia become overactive, the tissue deteriorates, causing an inflammatory reply in neurons along with neighbouring glial cells in the central nervous system (81). In PD brains, particularly in the substantia nigra and the striatum, the microglia levels are appreciably upraised (82).

The excessive activation of microglia leads to the formation of proinflammatory and cytotoxic factors such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) (83). This increments iNOS manufacture and exacerbates NO-induced DA neuronal damage. Nevertheless, the causes of excessive activation of microglia include pathological proteins such as misfolded α-synuclein, LRRK2, parkin, and DJ-1 (84).

2.7.5. Protein Accumulation and Misfolding

Accumulation and misfolding of α-synuclein are highly implicated in the pathogenesis of both sporadic and heredity PD (2). α-synuclein is a crucial protein predominantly stated in the substantia nigra, which is unfolded naturally and regulates the stability of dopaminergic neurons (2). While the role of α-synuclein is not fully understood, animal research has frankly shown that it plays a significant role in the arrangement of synaptic plasticity and neurotransmission of dopamine (85). On the other hand, some researchers propose that α-synuclein controls the presynaptic release of dopamine (86). Even though it is in terms of physiological status remains undefined, α-Syn has been involved in the etiology of two extensive neurodegenerative diseases, Alzheimer's disease and PD (86).

As a result of research conducted in 1950, the principal cause of motor symptoms was the loss of striatal DA; It has been reported that non-motor symptoms are related to glutamatergic, cholinergic, serotonergic and adrenergic systems (87). Bradykinesia,

rigidity, rest tremor, and postural instability are features of significant motor symptoms. Depression and sleep disorders are included as non-motor clinical features (88). As there is no precise diagnostic test for PD, the diagnosis is based on the clinical signs of PD (89).

Table 2. An overview of the clinical symptoms of PD (3)

Motor symptoms	Non-motor symptoms
Bradykinesia	Depression
Hypomimia	Insomnia
Tremor	Restless legs syndrome
Rigidity	Cognitive impairment
Postural instability	Sexual dysfunction
Dystonia	Sensory disorders

2.8. Current Treatment Approaches for Parkinson's Disease

It is purposed at reducing the advancement of the disorder, diminishing the loss of functional processes and enhancing the quality of life in PD patients. Regardless, since PD is a multifactorial disease group, there is no thoroughly preventive treatment method for the disease, since its etiology and pathogenesis are not fully understood (90, 91).

2.8.1. Medical Treatment of Parkinson's Disease

Pharmaceuticals are the widely used treatment for PD (92, 93). As disputed, diminished amounts of dopamine in the striatum are hallmarks of PD, and the primary approach in the medical treatment of PD is to correct the deficiency of DA due to dopaminergic neuron degeneration causing symptoms (92). Levodopa is the most efficient and received the gold-standard drug in the treatment of PD today (94). It cannot cross the blood-brain barrier of DA, thence its precursor L-Dopa is used in the treatment (95). In clinical practice, DA decarboxylase peripheral inhibitors are combined with carbidopa or benserazide (96). Subsequently, levodopa is transformed into dopamine. Thus, dopamine can be broken down by the enzymes such as catechol-o-methyl

transferase (COMT) (97). Tolcapone or entacapone, which hinders the COMT enzyme, is used as adjunctive treatment with Levodopa to improve its bioavailability (97).

Levodopa cannot restore the death of dopaminergic neurons. Therefore, motor complications occur when levodopa is used for a long time (98). With motor fluctuations in the later stages of treatment, concomitant dyskinesias constitute a significant problem (98, 99). To overcome the motor complications and dyskinesias, dopamine agonists such as apomorphine, pergolide, and pramipexole are used (100). Dopamine agonists are preferred because they are the most effective drug after L-Dopa in the therapy of Parkinson's, and they develop fewer motor complications (100). On the other hand, the negative part is, dopamine agonist remedies can lead to urge control disorders (101). In addition, selegiline, rasagiline and safinamide, MAO-B inhibitors, inhibit MAO-B, which is liable for DA catabolism in the brain and improves levodopa effectiveness (102). MAO-B inhibitors have a restricted therapeutic effect (103).

2.8.2. Surgical Treatment of Parkinson's Disease

Although there are effective pharmacological agents for the treatment of PD, surgical treatment options can be used in cases where medical therapy is insufficient and the patient standard is negatively impacted.

In deep brain stimulation execution, it has become the most crucial option preferred in surgical treatment since it enhancements the quality of life in patients due to symptomatic control in cardinal findings (104). In deep brain stimulation, a medical apparatus that sends electrical signals to specific parts of the brain resides in the subthalamic nucleus, the globus pallidus interna or thalamus (94). The method of treatment of neurostimulation is not preferred in patients with moderate or severe dementia, as only the first 3-4 years of PD respond well to medical treatment (104, 105). Deep brain stimulation has significant vantages; it permits the physician to diminish the dose of medication, hence reducing its undesirable side effects such as tremors, rigidity and bradykinesia are reduced (106) .

2.8.3. Physiotherapy and Rehabilitation of Parkinson' Disease

Physiotherapy and rehabilitation approaches play a crucial role in the management of PD by supplying methods to enhance physical capacity and overcome drug-resistant motor symptoms such as gait, balance and posture disorders (107). The physical treatment delivers clinically expressive advantages for PD clients (107).

Nowadays, any form of treatment method used in PD is not aimed at stopping the progression of the disease. It aims to improve individuals' quality of life by controlling the symptoms of illness (108).

2.8.4. New Treatment Approaches in Parkinson's Disease

Many clinical experiments include gene treatment approximations for the cure of PD. Gene therapies involving viral vector-mediated delivery of therapeutic genes encoding DA synthesis to the putamen and SN regions of the brain in the therapy of PD play an influential role (109). The aromatic amino acid decarboxylase is liable for the transformation of levodopa to dopamine and is present in low amounts in the PD brain (110). Targeted genes encoding enzymes such as aromatic L-amino acid decarboxylase (AAV2-AADC), neurturin (AAV2-NRTN), glia-derived neurotrophic factor (GDNF), glutamic acid decarboxylase (GAD) are injected into the putamen and SN bilaterally via viral vector (111). The clinical application of gene therapy depends on overcoming biosafety problems (110, 112).

Besides conventional medicine, complementary and alternative medicine is widely used to prevent the progression of PD today (113). Biologically based practices such as homoeopathy, acupuncture, hypnosis, meditation and herbal products, the most prevalent one today is phytotherapy based on herbal products (113, 114). Herbal products or compounds derived from plants can be used independently or in combination with conventional medicine approaches (114). It has been shown that compounds obtained from plants inhibit striatal dopaminergic neuron degeneration by preventing processes such as mitochondrial dysfunction and oxidative stress seen in the pathogenesis of the disease (115).

As a result of this information, there is presently no known efficient neuroprotective or neurorestorative treatment for PD (116).

2.9. High-Fat Diet and Its Metabolic Effects

Many a high fat diet is one of several models of human obesity used in experimental animals for obesity research (18). Metabolic events caused by excessive consumption of foods are obesity, insulin resistance, type-2 diabetes, non-alcoholic steatohepatitis and dyslipidemia (117, 118). Furthermore, these metabolic conditions are risk factors that improve the incidence of cardiovascular disease and type 2 diabetes (118).

In a high fat diet, adipose tissue mass gains and inflammation develops in peripheral tissues (119). As a result of the changes in the adipose tissue, proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), free fatty acids and leptin are released from the adipose tissue (120). An increment in pro-inflammatory cytokines has been shown to stimulate lipolysis of TNF-α in adipocytes (120). A chronic free fatty acid flow occurs in the liver, brain, muscle, and pancreas, resulting in the disorder of fatty acid oxidation (121). This condition is characterized by insulin resistance (121).In addition, it is stated that consumption of a high fat diet causes an increase in serum total cholesterol, serum LDL levels and triglyceride levels, and a reduction in HDL levels (122, 123).

Calorie intake, peripheral glucose, and fat metabolism are neuronally regulated in the central nervous system (124). Insulin and leptin hormones secreted from adipose tissue attend to the regulation of energy homeostasis in the arcuate nucleus in the hypothalamus (125). Resistance to insulin and leptin signals leads to increased weight gain (126).

2.10. Leptin

Leptin is a protein hormone that is structurally related to the cytokine family and is named the 'Obese' gene (127). It is effective in the central regulation of food intake and body weight homeostasis in humans (128, 129). Mutation of the human leptin gene has been shown to cause early-onset morbid obesity. Known to contribute to endocrine functions, leptin affects the hypothalamus with negative feedback and plays a critical role in the pathogenesis of obesity by regulating energy intake (130). This hormone is mainly expressed in white adipose tissue, as well as in the stomach, hypothalamus, pituitary, skeletal muscle, placenta, and mammary glands (131).

Obesity is strongly associated with hyperleptinemia in rats and humans fed a high fat diet (132). As a result of the observations, similar to insulin resistance in diet-induced obesity, there is a disruption in the hypothalamic pathways (132). Furthermore, the cellular mechanisms underlying obesity-induced insulin resistance may also impair leptin signalling (132, 133).

Leptin crosses the blood-brain barrier, reducing appetite and food intake and increasing energy expenditure in the hypothalamic nucleus (134). It provides energy expenditure by increasing sympathetic nervous system stimulation in peripheral tissues (135). Through its receptors in the brain, it supplies neuronal regulation of energy intake and feeding behaviour (134).

2.11. Neuronal and Behavioral Effects of High-Fat Diet

Obesity is related to inflammation, oxidative stress and mitochondrial dysfunction, resulting in the progress of neurodegenerative disorders (136). Additionally, diet-induced obesity has been linked to behavioral disorders such as depression (17, 19). According to research, high fat diet consumption in the juvenile period causes unfavorable effects on hippocampal memory and neuronal functions in adulthood (20). A HFDs in the juvenile period has been demonstrated to boost proinflammatory cytokines and cause impaired learning and memory (137). Furthermore, it is thought that middle-aged obese individuals may develop brain atrophy and potentially have a higher risk of cognitive decline than healthy individuals (138).

The enlarge in unsaturated fatty acids in obesity contributes to insulin resistance (139). Since changes in insulin signalling impair the control of neuronal cell metabolism and cell survival, it may cause cognitive disorders as well as affect progressive neuronal loss (140).

The expansion in proinflammatory cytokines and leukocyte infiltration in obesity cause oxidative damage by increasing the production of free radicals (141). Oxidative stress is associated with neurodegenerative disorder as it can initiate the apoptosis process and cellular necrosis (141, 142).

Generally, a HFDs causes morphological changes in the brain (143). Elevated adiposity is considered a risk factor for diseases that cause cognitive impairment, such as Alzheimer's disorder and PD (143, 144, 145).

2.12. Animal Models of Parkinson's Disease

Animal models are exceptionally beneficial in understanding the pathology and origin of the disease and enabling the development of possible new treatments. Studies on humans are limited since it is often not possible in terms of ethical rules. Animal models, on the other hand, contribute to the understanding of the mechanisms underlying the pathogenesis of the disease and help to develop new methods of therapy by concentrating on these mechanisms (146). Neurotoxin-induced, genetically engineered and pharmacological models are used as animal models of PD (147). In addition, multifarious neurotoxins such as 6-hydroxydopamine, MPTP, rotenone and paraquat are used to mimic PD in animals. These models are effective at inducing nigrostriatal damage and can mimic the PD model (148).

2.12.1. Rotenone

Known as a herbicide, rotenone is highly lipophilic and can cross the blood-brain barrier easily. Systemically administered rotenone to animals leads to the degeneration of dopaminergic neurons and PD (16). It principally impedes complex I of mitochondria.

Unlike other models, rotenone formation provides a Lewy Body, a key indicator of PD (149, 150). Rotenone leads to the degeneration not only of dopaminergic neurons but also serotonin and cholinergic neurons (149, 151).

Individuals exposed to rotenone were found to be approximately three times more likely to develop the disease than those not exposed (152). Based on these findings, rotenone has been considered suitable for the use of neurotoxic compounds in neurodegenerative diseases (153, 154, 155, 156).

Rotenone readily passes cell membranes and inhibits mitochondrial complex I in all cells, it causes specific nigrostriatal damage. Cytoplasmic DA in neurons undergoes oxidative deamination catalyzed by monoamine oxidase-A to form catecholaldehyde, 3,4-dihydroxyphenylacetaldehyde. DOPAL is cytotoxic both when administered exogenously and when produced intracellularly. DOPAL also potentially oligomerizes alpha-synuclein and alpha-synuclein oligomers are thought to be pathogenic in PD (Figure 6) (157).

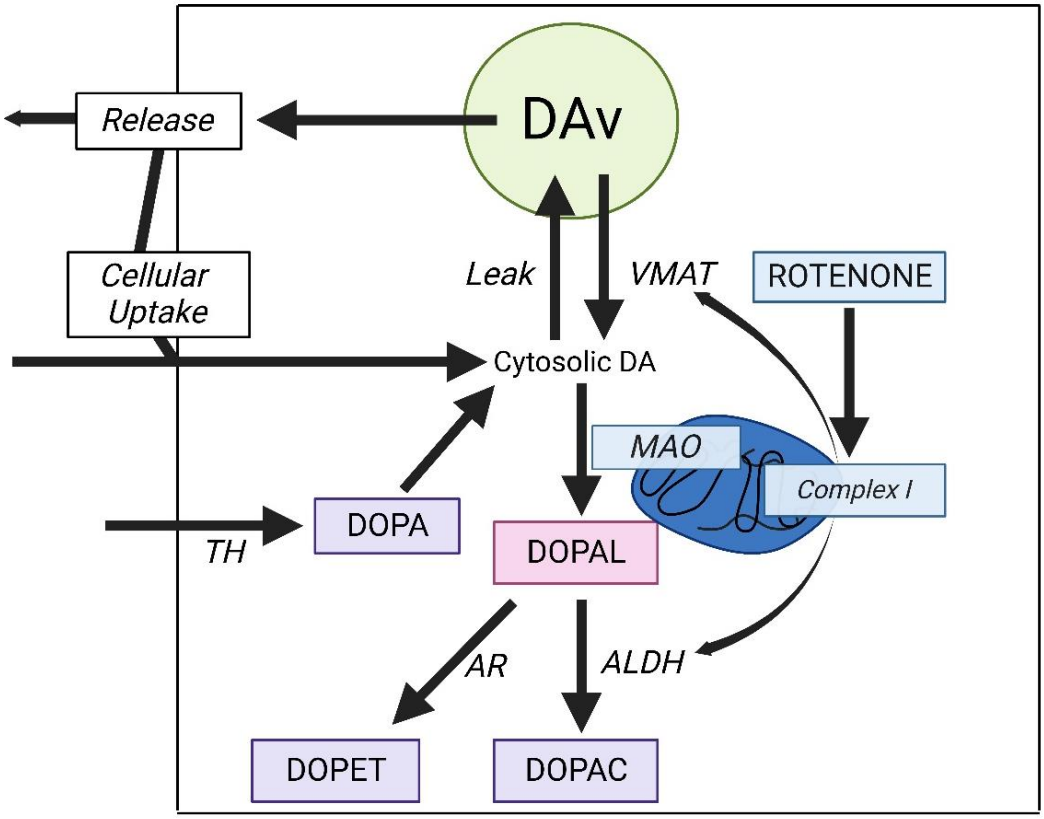

Figure 5. Schematic diagram of the toxicity of the rotenone metabolism. ALDH, aldehyde dehydrogenase; AR, aldehyde/aldose reductase; DA, dopamine; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; MAO-A, monoamine oxidase-A; PD, Parkinson's disease; VMAT2, type 2 vesicular monoamine transporter (157).

2.12.2. 6-hydroxydopamine (6-OHDA) Model

6-OHDA is a hydroxylated analogue of DA and is a neurotoxin that causes the degeneration of dopaminergic neurons in the nigrostriatal pathway (151). It cannot cross the blood-brain barrier (151). Therefore, it is performed by stereotaxic injection directly into the substantia nigra, medial forebrain bundle, or striatum only (146). After the lesion is created by injection of 6-OHDA, the damage of catecholamines by the manufacture of free radicals by oxygen leads to the loss of dopaminergic neurons in the nigrostriatal pathway (158). 6-OHDA is the most widely used neurotoxin when creating a PD phenomenon (159).

2.12.3. MPTP Model

A synthetic drug MPTP causes PD in humans (160). Due to its lipophilic nature, it can easily cross the blood-brain barrier (151, 161). It is selectively absorbed into the dopaminergic neurons via DAT and converted to its active form MPP+ by the activity of MAO (162) MPP+ inhibits mitochondrial complex I and increases oxidative stress (163). Although α-aggregations are seen in MPTP animal models, they are not similar to Lewy bodies seen in PD patients. It is most frequently used in mice and non-human primates and is given in a systemic manner (146). Nowadays, the MPTP model is considered the gold standard for evaluating new strategies for the therapy of PD symptoms (164, 165, 166).

2.12.4. Paraquat Model

The paraquat (1,1'-dimethyl-4,4'-bipyridinium) is structurally MPP+ it resembles (168). It is a herbicide (169). Unlike MPP+, it causes oxidative stress by producing reactive oxygen species for its toxic effect (158). Paraquat can cross the blood-brain barrier even though it is restricted and leisurely (150, 169). LB-like pathology and decreased locomotor activity are seen in the group exposed to Paraquat (170). In the paraquat model, its use is not preferred since there are contradictory and variable effects in conditions of DAergic cell death, striatal DA loss and behavioral results (171).

2.12.5. Genetic Models

PD is a sporadic illness, while a small number of cases have earlier-onset familial PD. Molecularly engineered animals are an attractive area of investigation in familial PD research. For this purpose, in order to comprehend why mutations in typical genes cause PD, mutations of genes implicated in the etiology of PD have been especially expressed in animal models (172). Contentious consequences reside among transgenic animal models that overexpress α-synuclein. A transgenic mouse form overexpressing α-synuclein was unsuccessful in demonstrating dopaminergic degeneration (173). Conversely, it evidently demonstrates the attendance of LB similar inclusions (173).

Furthermore, research is needed to gain a significant understanding of the genetic foundation of PD and to uncover new therapeutic approaches.

3. MATERIAL AND METHODS

3.1. Material

The chemicals, reagents, antibodies, commercial kits, and technical equipment are listed beneath in this research.

3.1.1. Chemicals and Reagents

- Neutral formalin solution (Formalin, Buffered, Fisher Scientific, SF100-4)
- DAB Substrate (UltraVision Detection System Large Volume DAB Substrate System (RTU))
- Normal horse serum (Vector Laboratories, 30022)
- Entellan mounting medium (Bio Mount HM, Bio-Optica, 05-BMHM500)
- Hydrogen peroxide solution 30 % (Sigma Aldrich, H1009, Germany)
- Gill's Hematoxylin (Gill's hematoxylin No.3, Bio-Optica, 05-06015/L)
- Eosin (Eosin Y Alcoholic Solution, Bio-Optica, 05-10003/L)
- Phosphate buffered saline tablets (Sigma)
- Sodium chloride (Sigma Aldrich, S6546, Germany)
- Sodium dihydrogen phosphate (Sigma Aldrich, 106370, Germany)
- Sodium citrate tribasic dihydrate (Sodium Citrate Tribasic Dihydrate, Sigma Aldrich, 6132-04-3)
- Triton X-100 (Roche, Cat. No. 11332481001)
- Xylene (Sigma Aldrich, No:1.08633, Germany)

3.1.2. Antibodies

- iNOS (1:200, PA5-16524, Thermo Fisher Scientific , San Jose , CA , ABD)

3.1.3. Stain Kits

- In situ cell death detection kit, POD, Sigma Aldrich (Terminal deoxynucleotidyl transferase enzyme solution, Nucleotide mixture in reaction buffer, Anti-fluorescein antibody conjugated with horse-radish peroxidase)
- Vectastin Universal Quick Kit, Vectastin (Normal Horse Serum, Biotinylated Universal Secondary antibody, Streptavidin/Peroxidase preformed complex)
- Bielschowsky Silver Stain Kit ab245877 (Formalin Solution (20%), Silver Nitrate Solution (20%), Citric Acid Solution , Nitric Acid Solution)

3.1.4. Laboratory Equipments

- M-polylysin slides (Poly-Lysine, Thermo Fisher Scientific, 165014)
- Propylen centrifuge tubes; 50 mL, 15 mL, 2 mL,0.5 mL (Isolab)
- Micropipettes 10 µL, 100 µL, 200 µL, 1000 µL (Eppendorf)

3.1.5. Laboratory Technical Instruments

- Light microscope (Leica CTR 6000)
- Microtome (Leica, RM 2245)
- Incubator (Memmert)
- Locomotor activity cage (MAY - Activity Monitoring System - Commat Ltd., TR)
- pH meter (Hanna instruments PH211, Germany)
- Tissue embedding automat (Leica, EG 1160)
- Staining Automat (Leica, Autostainer XL ST5010)
- Tissue processing automat (Leica, TP 1020)

3.2. Methods

3.2.1. Experimental Setup and Groups

Adult male C57BL/6 mouse (18-21g) were obtained from Yeditepe University Experimental Research Center (YUDETAM). All of the experiments were authorized by the Ethical Committee of Yeditepe University Experimental Research Center and the use of animals complied with the US National Institutes of Health Guide for Care and Use of Laboratory Animals. Animals were maintained in standard housing terms with standard temperature, humidity, 12-h light/dark cycles, and free access to food and water.

24 mouse were randomly divided into 4 experimental groups as follows: intraperitoneal (IP) DMSO-injected control group (C), IP 2,5 mg/kg/day rotenone injected group as control to Parkinson's disease model (PD), high fat diet fed group (HFD), IP 2,5 mg/kg/day rotenone injected and high fat diet fed (PD+HFD) group. Each group has 6 animals [Table 3].

Table 3. The experimental groups and number of animals

Groups	Numbers
IP DMSO injected control group (C)	n=6
IP rotenone injected group (PD)	n=6
High fat diet fed group (HFD)	n=6
IP rotenone injected and high fat diet fed group (PD+HFD)	n=6

To develop obesity in groups fed with high fat diet feed, an 8-week ad libitum access with 60% fat-containing feed was planned, and the development of obesity in mice was followed (174). Animals were allowed free access to water and food during the entire experiment.

During the experiment, the general conditions of the animals were checked daily. In addition, parameters such as the animals' mobility, posture and the state of their feathers were also followed. Weight tracking once a week, on Sundays; Feed consumption was monitored and recorded three times a week, on Mondays, Thursdays and Sundays.

Table 4. The nutrient ingredients of a high fat diet feed

Metabolizable energy		%60 fat %20 protein 5,150 kcal/kg
Food items (%)	Protein(N x 6,25)	24,4
	Fat	34,6
	Fiber	6,0
	Ash	5,3
	Starch	0,1
	Sugar	9,4

	Non-nitrogenous components	26,3
Vitamins(kg)	A vitamin	15000IU
	D3 vitamin	1500IU
	E vitamin	150mg
	K vitamin	20mg
	B1vitamin	25mg
	B2 vitamin	16mg
	B6 vitamin	16mg
	B12 vitamin	30 µg
	Folic acid	16mg
	Biotin	300 µg
Trace Elements (mg/kg)	Iron	168
	Magnesium	95
	Zinc	65
	Copper	13
	Iodine	1,2
	Selenium	0,2

Table 5. Experiment animal group charts

Control group (n=6)

The group fed with standard pellets and 2,5 mg/kg/day DMSO

Parkinson's Disease group (n=6)

The group fed with standard pellets and 2,5mg/kg/day rotenone

High fat diet group (n=6)

High fat diet fed group

High fat diet group +Parkinson's Disease group (n=6)

High fat diet fed group and 2,5mg/kg/day rotenone

In the last ten days of the experiment, rotenone was injected into the rotenone group(PD) and the rotenone+high fat diet feed group (PD+HFD). All mice were tested for locomotor activity twice on the last day of the injections. All mice were pre-trained on locomotor activity. At the end of 8 weeks, all animals were dissected and the brain, liver and kidney tissues were dissected.

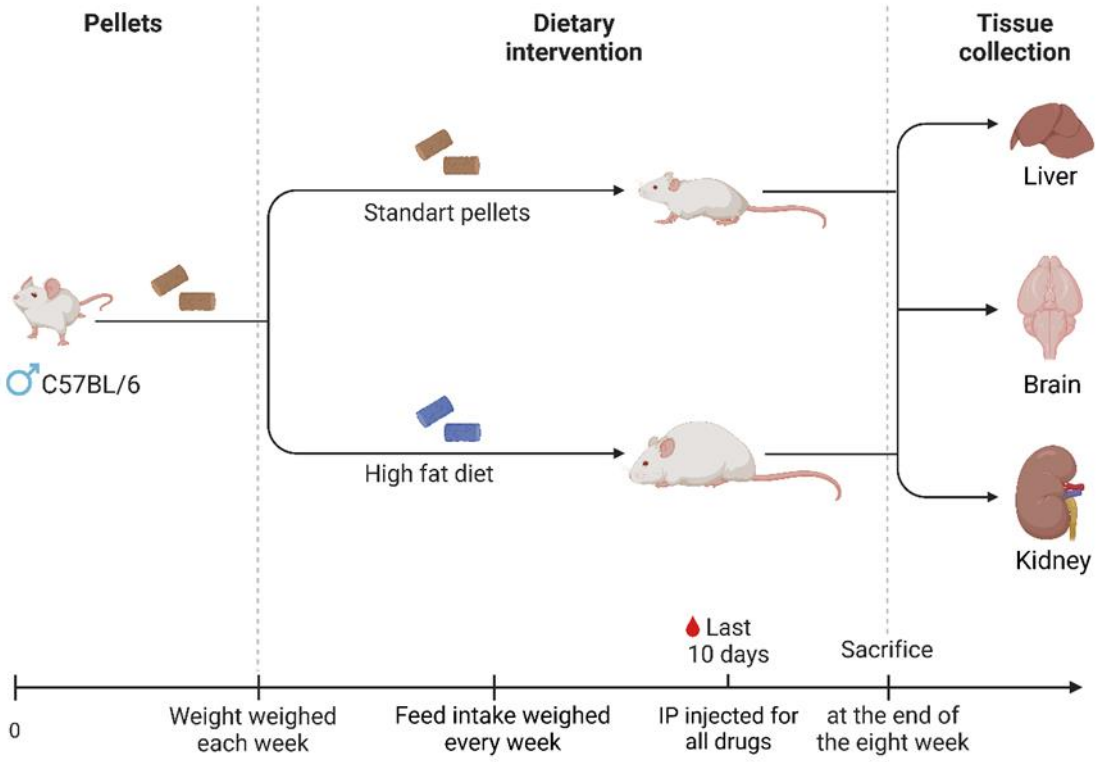

Figure 6. Experiment flow diagram

3.2.2. Drug Administration and High-Fat Diet

Drug administration of groups are followed as; C group of the mice were intraperitoneally injected with DMSO, PD group of the mice were intraperitoneally injected with 2,5 mg/kg/day rotenone (175), PD+HFD group of the mice were intraperitoneally injected with 2,5 mg/kg/day rotenone (175).

PD group and PD+HFD group of the mice were intraperitoneally injected with rotenone 2,5 mg/kg/day for ten days. DMSO was dissolved in olive oil. Also, rotenone was dissolved in DMSO (175).

3.2.3. Sacrification of the Animals

Afterwards the locomotor activity test, animals were decapitated, and their brains were dissected out and their substantia nigra regions were reserved and placed in %10 neutral formaldehyde solution in order to use in histological analysis. Furthermore, the kidney and liver tissues of the animals were also removed.

3.2.4. Spontaneous Locomotor Activity

At the beginning of the experiment and before the decapitation of animals, an animal activity monitoring system (MAY - Activity Monitoring System - Commat Ltd., TR) was used to record motor activity measurements [Figure 8]. The locomotor activity cage is a rectangular system (45x45x30 cm) including infrared light sources on each side. An electromechanical counter was placed on the floor of the cage. Moves of animals disrupt the infrared beams and the electromechanical counter enumerates them as locomotor activity. Each mouse was separately placed in the cage and spontaneous motor activity was registered at 5 min intervals throughout a 10 min duration. All records were performed between 9 a.m. and 3 p.m. The cage was sterilized with a wet towel prior to each recording. Stereotypes, ambulatory, vertical, and horizontal motor activities and distance travelled by the animals were calculated.

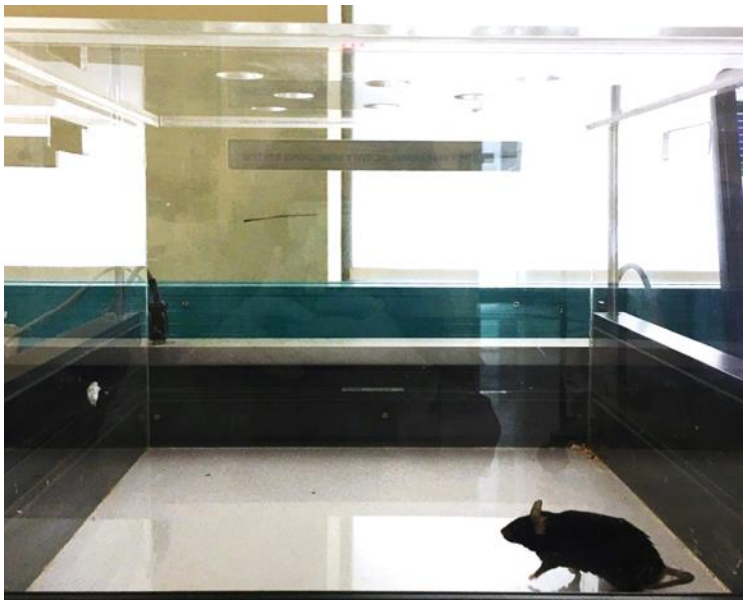

Figure 7. Spontaneous locomotor activity

3.2.5. Histological Techniques

3.2.5.1. Tissue Processing

Brain, liver and kidney tissues were put in cassettes and stationed in the tissue processor. The tissues were fixed for 4 hours in a neutral formaldehyde solution. After the tissues were fixed, dehydration was performed in ethanol series (70%, 80%, 90%, 96%, 100%) with the follow-up device. After dehydration, the tissues were cleared in xylene and embedded in paraffin blocks. All paraffin blocks were positioned in a microtome, and 5 micrometer sections were acquired on the slides.

Tissue tracking was performed according to the following steps [Table 6].

Table 6. Stages in tissue processing

Fixation	Neutral formaldehyde solution	2 Hours
	Neutral formaldehyde solution	2 Hours
Dehydration	70% Alcohol	2 Hours
	80% Alcohol	2 Hours
	90% Alcohol	2 Hours
	96% Alcohol	2 Hours
	96% Alcohol	2 Hours
	100% Alcohol	2 Hours
Clearing	Xylene I	1.5 Hours
	Xylene II	1.5 Hours
Paraffin Impregnation	Paraffin	3 Hours
	Paraffin	3 Hours

3.2.5.2. Hematoxylin Eosin Staining

Hematoxylin Eosin staining is standard staining in histology laboratories and is typically used to explore the pathology in tissue sections. Hematoxylin is a positively charged basic dye. Thus, stains negatively charged nucleic acid parts in the nucleus blue colour. Contrarily, eosin dye is a negatively charged acidic dye, for this reason, it stains positively charged constructions like proteins in the cytoplasm pink colour (176).

All the solutions were prepared in advance and positioned into copulin jars which were found in the staining automat and the protocol described down was followed:

- The sections were dried in an incubator at 75°C for 30 minutes.
- Then the sections were cleared in xylene I and II for 1 minute each.
- The slides were hydrated in decreasing alcohol series (100%, 96%, 96% ,90%) for 3 minutes each.
- Every section was washed in distilled water.
- The sections were stained with Mayer's hematoxylin solution for 6 minutes and rinsed inwater for 2 minutes.
- The slides were decolorized in acid alcohol for 3 minute.
- The slides were washed in tap water for 3 minute.
- The sections were subjected to 80% ethanol for 3 minutes.
- Then the sections were counterstained with Eosin for 5 minutes.
- The sections were dehydrated in increasing alcohol series (90%, 96%, 96%, 96%, and 100%) for 3 minutes each.
- Ultimately, the slides were cleared in xylene I and II for 5 minutes each.
- The slides were coverslipped with entellan using an automated glass coverslipper.

3.2.5.3. Cresyl Violet Staining

Cresyl violet is a basic dye and stains Nissl substance in the cytoplasm of neurons dark blue. Cresyl violet staining is usually used to indicate the constructional features of the neurons (177). Two solutions, A and B solution, were prepared in advance. To make ready solution A, sodium acetate was dissolved in distilled water. For solution B, glacial acetic acid was diluted with distilled water. Afterwards, A and B solutions were mixed, and the pH was modified to 3.8-4.0 with acetic acid. Cresyl violet acetate was dissolved in this solution and left on the magnetic stirrer overnight in the dark. The staining solution

was filtered with filter paper earlier staining the sections (178). All solutions were set into copulin jars, and the protocol clarified beneath was followed:

1. The sections were dried in an incubator at 75°C for 30 minutes.
2. The sections were placed in xylene solution for 25 minutes at room temperature.
3. Then the slides were washed with distilled water for 3 minute.
4. The sections were stained with Cresyl Violet for 25 minutes.
5. The slides were washed with distilled water.
6. The sections were then dehydrated in ascending alcohol series (70%, 80%, 90%, 96%,96%, and 100%) for 5 minute each.
7. Lastly, the slides were cleared in xylene I and II for 3 minutes each.
8. The slides were coverslipped with entellan using an automated glass coverslipper.

3.2.5.4. Silver Staining

Bielschowsky's silver stain is a very useful tool to detect nerve fibers. It can be used to stain axons, neurofibrils and senile plaques in the central nervous system (179). Firstly, three stock solutions were prepared. These; Ammonia silver solution, Prepare Developer Solution and ammonia water. Initially, silver nitrate solution and ammonium hydroxide were mixed to prepare an ammonia silver solution. If there is a precipitate in the resulting solution, it was filtered with filter paper. Then, distilled water, formalin solution, citric acid solution and nitric acid solution were mixed to prepare Prepare Developer Solution. Finally, concentrated ammonium hydroxide was mixed with distilled water to prepare ammonia water. All of the solutions were put into copulin jars and the protocol explained below was followed:

1. The sections were dried in an incubator at 75°C for 30 minutes.
2. The sections were placed in xylene solution for 20 minutes at room temperature.
3. Then the slides were washed with distilled water for 3 minutes.
4. Place a chemically cleaned staining jar containing 25 ml of Silver Nitrate Solution (20%) was placed in the waterbath.
5. Then, the slide was placed in warmed Silver Nitrate Solution (20%) and incubated at 40°C for 15 minutes.

6. During the incubation, Ammonia Silver Solution was placed in the waterbath to stabilize the temperature.
7. The slide was removed from Silver Nitrate Solution (20%) and rinse in 4 changes of distilled water.
8. The slide was placed in warmed Ammonia Silver Solution and incubated at 40°C for 10 minutes.
9. The slide was placed directly into Developer Solution. It waited until the tissue section turned yellow/brown (5-20 seconds).
10. Then, the slide was removed from the Developer Solution and placed directly in Ammonia Water for 30 seconds.
11. Then the slides were washed with distilled water, applied adequate Sodium Thiosulfate Solution (5%) to completely cover tissue section and incubate for two minutes.
12. Then the slides were washed with distilled water.
13. Sections were dehydrated in 100% for 3 min and cleared in xylene for 3 min.
14. The slides were coverslipped with entellan using an automated glass coverslipper.

3.2.5.5. Immunohistochemistry for iNOS

Immunohistochemistry is a greatly sensitive procedure to detect the existence and location of the related proteins in tissues. Nitric oxide is an inorganic compound that plays a significant role in neurotransmission and cytotoxicity. NO production is continued by the nitric oxide synthase (NOS) family (180). Inducible nitric oxide synthase (iNOS) is one of these synthases although the aberrant amount of its induction can reason damage in cells and the progression of the pathology of diseases such as Parkinson's Disease (181). Five solutions were prepared in advance. Firstly, 3% hydrogen peroxide solution was prepared with 1 ml 30% Hydrogen peroxide stock solution and 99 ml PBS. Secondly, 0,1 g tri–Na Citrate x2H2O and 0,1 ml Triton X-100 were dissolved in 100 ml distilled water to prepare 100 ml 0.5 M permeabilization solution. After that, 5.88 g tri–Na Citrate x 2H2O was dissolved in 200 ml distilled water, and pH was adjusted to 6 with HCL titration resulting in 200 ml 0,1 M citrate buffer (antigen retrieval solution). Additionally, to have a washing solution, one phosphate-buffered saline (PBS) tablet (Sigma) was

dissolved in 100 ml distilled water. In conclusion, 10 µl DAB was added to the 200 µl substrate and kept on ice.

The solutions were set into copulin jars, and the protocol described under was followed:

1. The sections were dried in an incubator at 75°C for 30 minutes.
2. The sections were placed in xylene solution for 30 minutes at room temperature.
3. The slides were then rehydrated in decreasing alcohol series (100%, 96%, 90%, 80%, and 70%) for 3 minutes each.
4. Then the slides were washed with PBS for 5 minutes at room temperature.
5. The slides were incubated in permeabilization solution for 8-10 mins in ice.
6. Then the slides were washed with PBS for 3 minutes at room temperature.
7. The slides were placed in citrate buffer solution (pH 6.0) in a 750-watt microwave for 2min for antigen unmasking.
8. Then the slides were washed with cold PBS for 6 minutes.
9. Then the slides were washed with PBS for 6 minutes at room temperature.
10. The slides were placed in 3% hydrogen peroxide solution for 30 minutes in order to quench endogenous peroxidase activity.
11. Then the slides were washed with PBS for 6 minutes.
12. All specimens were incubated in blocking solution (2.5% RTU Normal Horse Serum VECTASTAIN KIT) for 30-40 min in a humidified chamber.
13. Blocking solution was removed from slides and slides washed with PBS for 5 min.
14. Primary diluted (1:200) antibody (iNOS) was dropped on all sections and incubated at 4°C overnight in a humidified chamber.
15. Then the slides were washed with PBS for 6 minutes.
16. Secondary antibody (RTU Biotynlated Universal Antibody Anti-Rabbit/mouse IgG) was dropped on all sections and incubated at room temperature for 30-40 min in a humidified chamber.

17. Then the slides were washed with PBS for 6 minutes.
18. Vectastain RTU ABC Reagent was dropped on all sections and incubated at room temperature for 30-40 min in a humidified chamber.
19. Then the slides were washed with PBS for 6 minutes.
20. All slides were incubated in DAB chromogen & peroxidase substrate mixture for 2-3 min.
21. Then the slides were washed with PBS for 5 minutes.
22. Each specimen was counterstained with Gill's hematoxylin for 5 min, then washed in tap water.
23. Sections were dehydrated at in 100 % for 3 min and cleared in xylene for 3 min.
24. The slides were coverslipped with entellan using an automated glass coverslipper.

3.2.5.6. TUNEL Assay: (In Situ Cell Detection Kit, Pod) Roshe*

TUNEL assay is a dependable procedure to detect apoptotic neuron death. DNA double-strand breaks, which appear during apoptosis are specifically labelled and visualized with TUNEL assay. In this technique, the terminal deoxynucleotidyl transferase (TdT) enzyme adds fluorescein-labeled nucleotides to the free 3'-OH DNA ends which are then visualized with an anti-fluorescein antibody labeled with converter-POD and substrate reaction (182). Furthermore, TUNEL POD is also used for the conversion of fluorescence-based TUNEL detection into a colorimetric labeling suited for transmission light microscopy. The protocol explained below was followed:

1. The sections were dried in an incubator at 75°C for 30 minutes.
2. The sections were placed in xylene solution for 30 minutes at room temperature.
3. The slides were then rehydrated in decreasing alcohol series (100%, 96%, 90%, 80%, and 70%) for 5 minutes each.
4. Then the slides were washed with PBS for 5 minutes at room temperature.
5. The slides were kept in Proteinase K (20-37 ºC) for 30 minutes.

6. Then the slides were washed with PBS for 5 minutes.
7. Then blocking buffer is added at room temperature and incubated for 10 minutes.
8. The slides were washed with PBS for 5 minutes.
9. Then TdT enzyme is added and kept at 37 ºC for 1 hour.
10. Then the slides were washed with PBS for 5 minutes.
11. Add 50 ml of streptavidin-HRP and incubate at 37 ºC for 30 minutes.
12. Then the slides were washed with PBS for 5 minutes.
13. DAB solution was added and waited between 30 seconds and 5 minutes.
14. Then the slides were washed with PBS for 5 minutes.
15. Each specimen was counterstained with Gill's hematoxylin for 5 min, then washed in PBS for 5 minutes.
16. Sections were dehydrated in ascending alcohol concentrations, cleared in xylene.
17. The slides were coverslipped with entellan using an automated glass coverslipper.

3.2.6. Analysis by Light Microscopy

Every section was examined beneath a light microscope (Leica DM 4000B, Wetzlar, Germany) and photographed by CCD digital camera (Optronics Microfire 1600x1200P, Goleta, CA, USA) with the assistance of LAS Version 4.1.0 program. Hematoxylin and Eosin, Silver and Cresyl violet stained sections were assessed histopathologically. H-score analysis was performed on iNOS immunohistochemical stained sections. Ultimately, the apoptotic index was calculated by the Stereo Investigator method for the analysis of the sections.

3.2.7. H-score Analysis for iNOS Immunohistochemistry

The H score ("histo" score) is calculated by multiplying the staining ratio with an ordinal value identical to the intensity. The final score varied from 0 (no staining in the tumor) to 300 (no staining in the tumor) (diffuse intense staining of the tumor). To calculate the H-score, three sections of each animal were resulted with their percentages as their staining density from 0 to 3 degrees (0=none, 1=mild, 2=moderate, 3=severe).

The percentage of cells at each staining intensity level is calculated. Percentages and degrees were calculated using the formula presented beneath.

$$[1 \times (\%\ \text{cells}\ 1+) + 2 \times (\%\ \text{cells}\ 2+) + 3 \times (\%\ \text{cells}\ 3+)]$$

The final score, ranging between 0 to 300, gives more relative numbers to demonstrate staining density in a sample tissue. Afterwards, the mean of all sections was calculated (183).

3.2.8. Apoptotic Index Calculation for TUNEL Assay

To calculate the apoptotic index, per section of all animals was divided into five zones. Afterwards, both apoptotic cells and normal cells were counted in each area. Eventually, apoptotic cells were divided into apoptotic + normal cells to obtain the apoptotic index (182).

3.2.9. Statistics

GraphPad Prism®8 was used as the statistical analysis program. All data were analyzed as mean ± SEM. The Shapiro-Wilk normality test was used to determine the normal distribution of data. Data were considered significant if P values were more down than 0.05. For the locomotor activity parameters (distance travelled, stereotypic activity, ambulatory activity, horizontal activity, vertical activity) the distinctions among groups were analyzed with one-way Anova. Feed consumption data were analyzed using one-way Anova. Weight data were analyzed with 2-way Anova. Tukey's multiple comparison tests were used to compare the data of all groups.

4. RESULTS

4.1. Spontaneous Locomotor Activity

To investigate the impacts of a high fat diet on the motor activity of experimental animals, the locomotor activities of the animals were measured with a motor activity monitoring technique. Stereotypic, ambulatory, vertical, and horizontal motor activities and distance travelled by the animal were measured.

4.1.1 Distance Travelled

In the investigation before the decapitation of the animals, as anticipated, the distance travelled by animals in the PD group has extensively reduced compared to the Control group ($p<0.0001$). Likewise, animals in HFD and PD+HFD groups have also experienced a decline in their locomotor activity i.e., when contrasted to the Control group, and substantial statistical importance was computed for both groups. There is a substantial distinction in the distance travelled by the animals in the HFD and PD+HFD groups, the HFD is slightly higher, so considerable statistical significance was observed between the HFD and PD+HFD groups. Considering the last measures of the animals before decapitation, it is seen that the distance travelled by the animals is the lowest in the PD+HFD group compared to the HFD, PD and C groups.

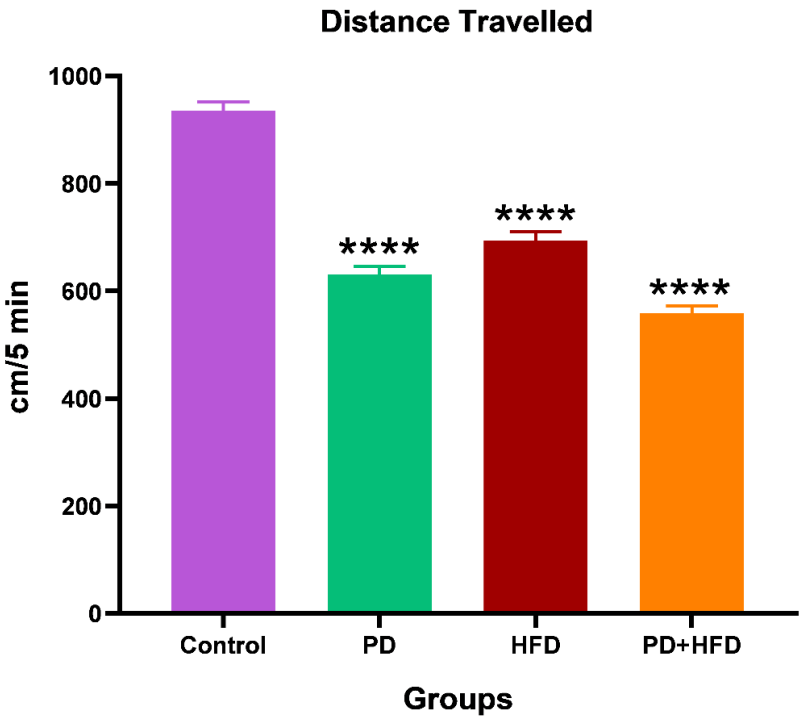

Figure 8. Graphs comparing distance travelled by the animals. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are offered as centimeters of the distance travelled by the animal in 5 minutes. Data are expressed as mean ± SEM (****p<0.0001, compared with C group).

4.1.2 Stereotypic Activity

The animals in the PD+HFD group have the lowest stereotypic activity compared to C, PD and HFD groups (p<0.0001). Nevertheless, the PD+HFD group had a significant reduction in their stereotypic activity compared to the animals in the HFD group fed the high fat diet (p<0.0001). The stereotypic activity of the animals in the PD+HFD group is lower than the C group. Furthermore, animals fed only a high fat diet showed a statistically significant decrease compared to group C (p<0.0001). Likewise, the stereotypic activity by animals in the PD group has considerably reduced compared to the C group (p<0.0001).

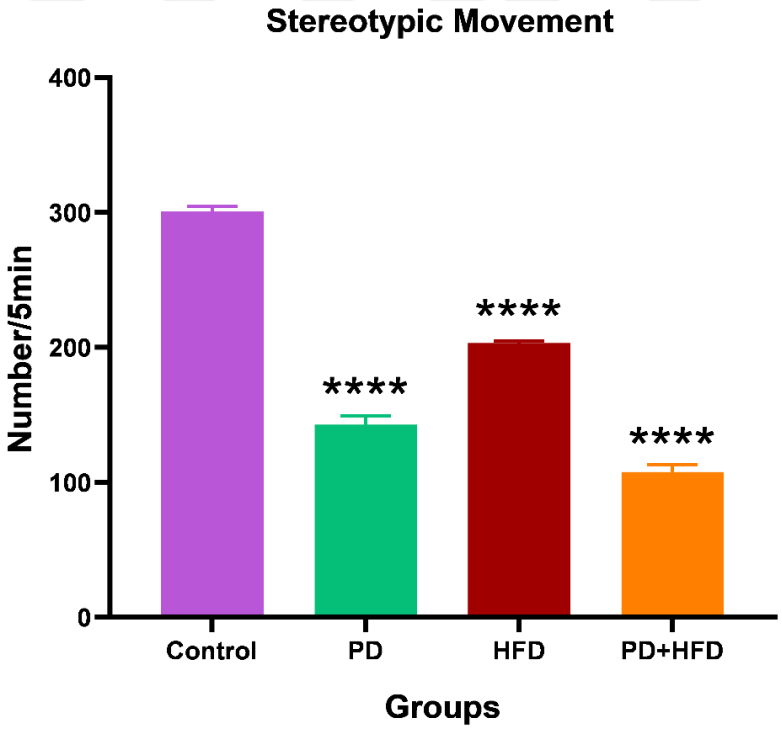

Figure 9. Graphs comparing stereotypic movement of animals. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are presented as number of stereotypic

movement in 5 minutes. Data are expressed as mean ± SEM (****p<0.0001, compared with C group).

4.1.3 Ambulatory Activity

The animals in the PD+HFD group have the lowest ambulatory activity compared to C, PD and HFD groups (p<0.0001), and when the HFD group is compared with the PD+HFD, there is a statistically significant difference. Animals in the PD group had lower ambulatory activity rates compared to the C groups (p<0.0001). The ambulatory activity of the animals in the HFD group is less than the C group.

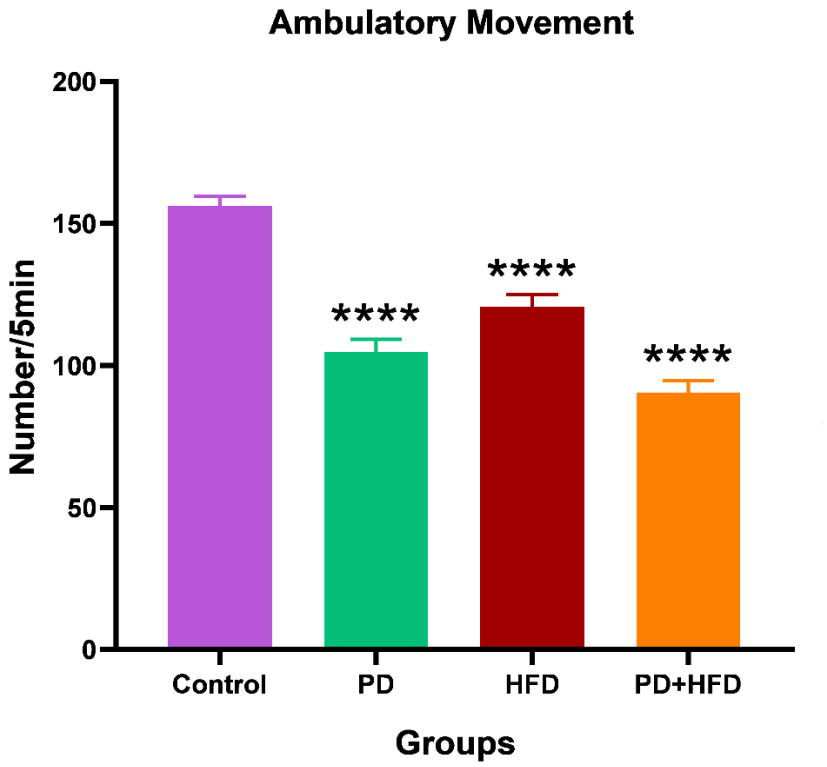

Figure 10. Graphs comparing the ambulatory movement of animals. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are presented as number of ambulatory movement in 5 minutes. Data are expressed as mean ± SEM (****p<0.0001, compared with C group).

4.1.4 Horizontal Activity

In line with the measurements before the decapitation of the animals, the horizontal activity of the animals in the PD+HFD group has the lowest horizontal activity compared to C, PD and HFD groups ($p<0.0001$). Nonetheless, the PD+HFD group had a significant decrease in their horizontal activity compared to the animals in the HFD group fed the high fat diet ($p<0.0001$). The horizontal activity of the animals in the PD+HFD group is lower than the C group. Similarly, animals fed only a high fat diet showed a statistically significant decrease compared to group C ($p<0.0001$). In addition, the horizontal activity by animals in the PD group has considerably reduced compared to the C group ($p<0.0001$).

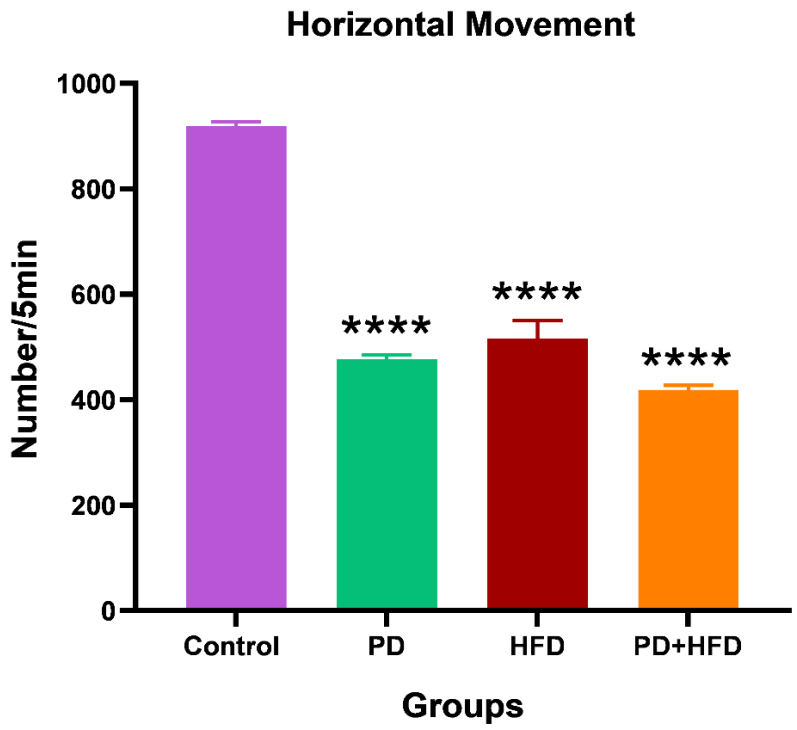

Figure 11. Graphs comparing the horizontal movement of animals. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+YYD) groups. Data are presented as number of horizontal movement in 5 minutes. Data are expressed as mean ± SEM (****$p<0.0001$, compared with C group).

4.1.5 Vertical Activity

The vertical activity of the animals in the PD+HFD group has the lowest vertical activity compared to C, PD and HFD groups ($p<0.0001$). Nevertheless, the PD+HFD group had a significant decrease in their vertical activity compared to the animals in the HFD group fed the high fat diet ($p<0.0001$). The vertical activity of the animals in the PD+HFD group is lower than the C group. Likewise, the HFD group fed only a high fat diet showed a statistically significant decrease compared to group C ($p<0.001$). Moreover, the vertical activity by animals in the PD group has considerably reduced compared to the C group ($p<0.001$).

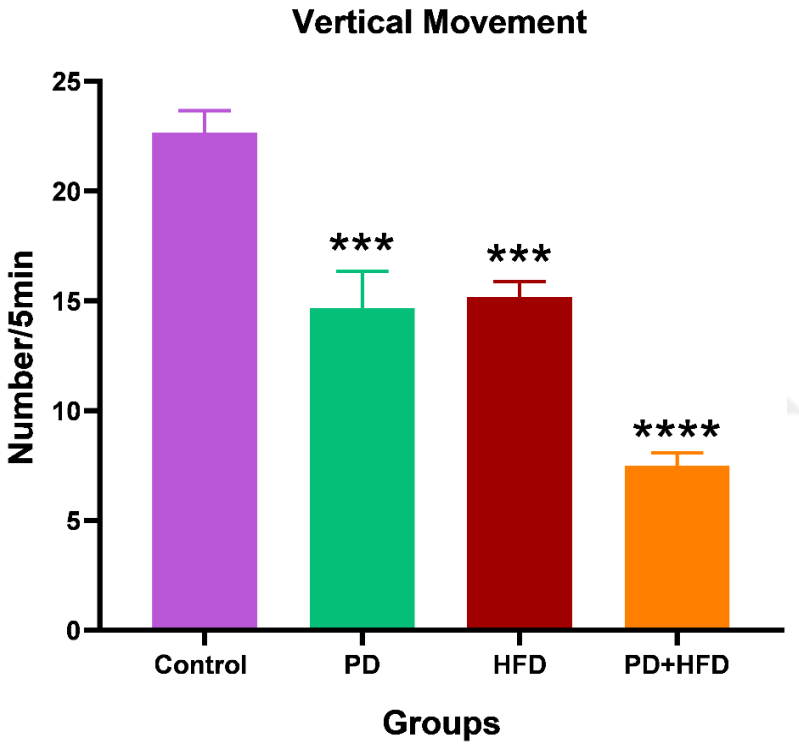

Figure 12. Graphs comparing the vertical movement of animals. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are presented as number of vertical movement in 5 minutes. Data are expressed as mean ± SEM (****$p<0.0001$, ***$p<0.001$ compared with the C group).

4.2. Histology

TUNEL positive neurons were counted in both left and right substantia nigra pars compacta as well. Furthermore, iNOS positive neurons were demonstrated in 40x objective. Ultimately, hematoxylin and eosin, silver and cresyl violet stainings were conducted in order to view general histopathological changes.

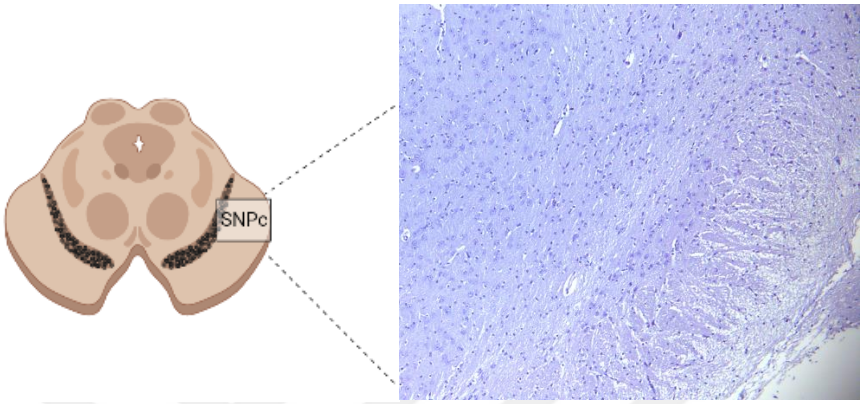

Figure 13. Section of the brain region used in all histological stages in the experiment, The magnification is x10

4.2.1. Hematoxylin Eosin Staining

Hematoxylin Eosin staining was performed in order to histopathologically examine the substantia nigra pars compacta. When the sections of the control group were examined histopathologically, it was observed that they had a normal histological appearance. Severe neuronal degeneration and severe hyperemia in the vessels were observed in the PD group. Moderate degeneration of neurons and hyperemia in vessels were observed in the HFD group fed a high fat diet. When the sections of the PD+HFD group were examined histopathologically, severe degeneration was observed in the neurons (Figure 15).

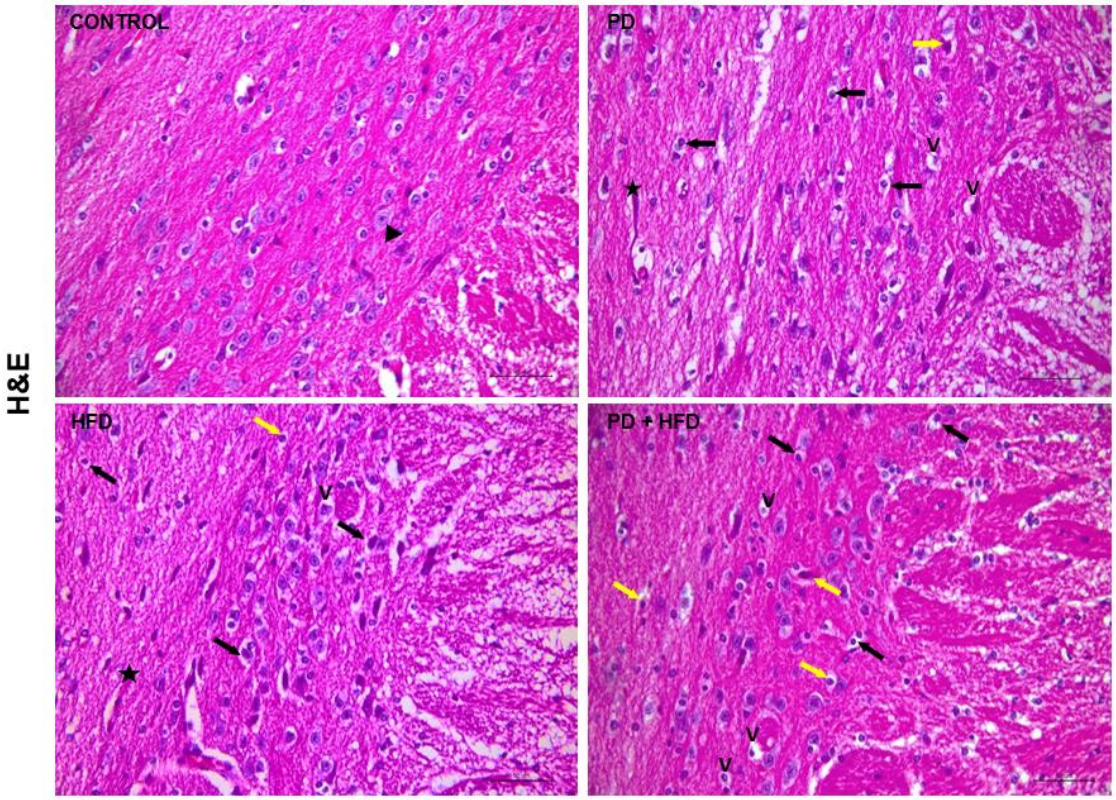

Figure 14. Photomicrographs demonstrate Hematoxylin Eosin staining in the SNPc. Control group; normal histological structure, normal neuron (arrowhead), PD group; severe neuronal degeneration (black arrows), haemorrhage (★), cytoplasmic vacuolations (V), pycnotic dark stained nuclei (yellow arrows), HFD group; moderately severe neuronal degeneration (black arrows), haemorrhage (★), cytoplasmic vacuolations (V), pycnotic dark stained nuclei (yellow arrows), PD+HFD group; heavy neuronal degeneration (black arrows), haemorrhage (★), cytoplasmic vacuolations (V), pycnotic dark stained nuclei (yellow arrows), DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, H&E, Bar: 50 µm

4.2.1.1.Histopathological Assessment of Liver Tissues

In light microscopic examinations, standard parenchyma structure was observed in the general sections of the H&E stained control group. In each lobule, hepatocytes were lying radially from the central vein in the vicinity of the sinusoids, and the nuclei of hepatocytes were observed in the centre of the cell and the round shape structure. In the liver sections of the PD group, a slight increase in vacuolization was observed, reflecting the degeneration of hepatocytes in the parenchyma. Mild vasocongestion in enlarged

sinusoids and leukocyte infiltration around the central vein was observed. In the HFD group, it was observed that vacuolization increased severely, reflecting the degeneration of hepatocytes. Slightly enlarged sinusoids were observed. In addition, large lipid droplets in hepatocytes; Macrovesicular steatosis was observed. Also, minor lipid droplets in hepatocytes; Microvesicular steatosis was observed. In the PD+HFD group, moderate vacuolization was observed, reflecting the degeneration of hepatocytes in the parenchyma. Large lipid droplets in hepatocytes; Mild macrovesicular steatosis was observed. In addition, minor lipid droplets in hepatocytes; Mild microvesicular steatosis was observed. Besides, leukocyte infiltration was observed in the liver parenchyma and periportal area compared to the HFD group. Also, an altered tissue architecture was observed (Figure 16).

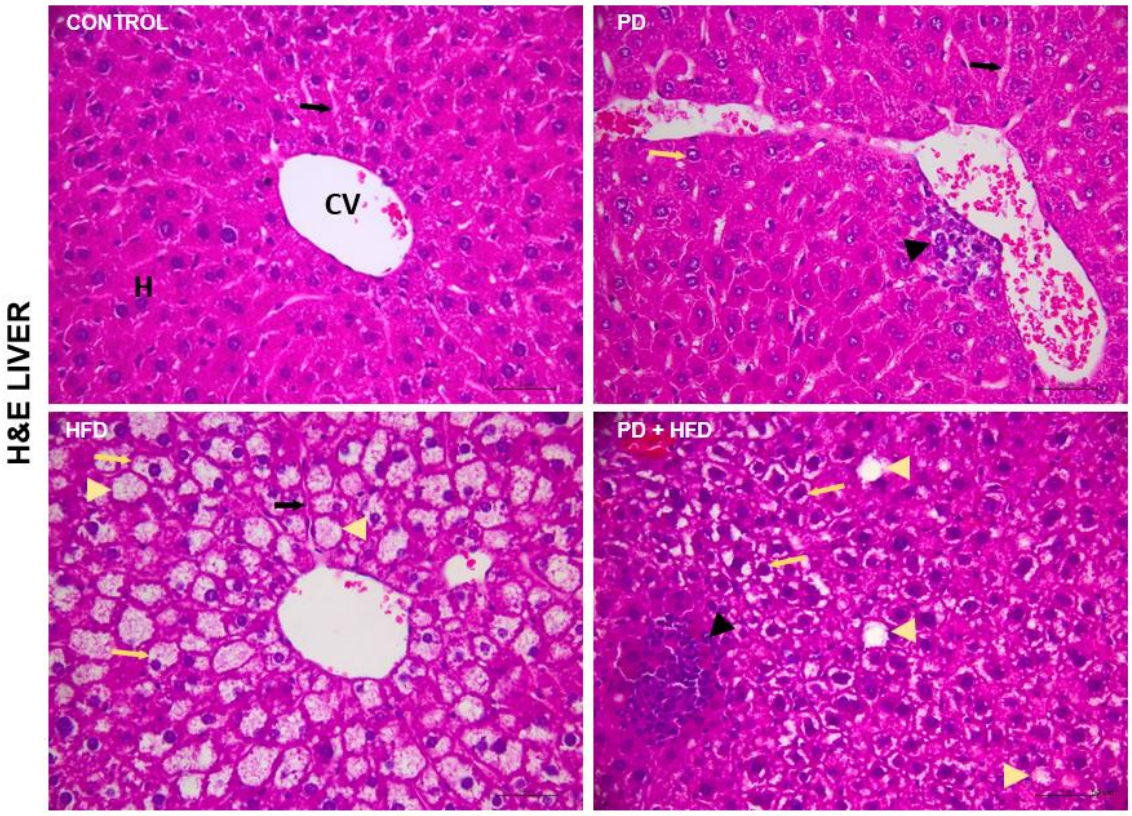

Figure 15. Photomicrographs demonstrate Hematoxylin Eosin staining in the liver. Control group; normal liver lobule structure, preserved hepatic tissue structure (H), hepatocytes lined up as radial plaques from the central vein and sinusoids between them (black arrows), PD group; mild vacuolization reflecting degeneration in hepatocytes (yellow arrows), enlargement of sinusoids (black arrows), leukocyte infiltration around the central vein (black arrowhead), HFD group; highly severe vacuolization reflecting

degeneration in hepatocytes (yellow arrows), enlargement of sinusoids (black arrows), high hepatic steatosis (yellow arrowheads), PD+HFD group; Moderate vacuolization reflecting degeneration in hepatocytes (yellow arrows), leukocyte infiltration in liver parenchyma and periportal area (black arrowhead), moderate hepatic steatosis (yellow arrowhead), DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, H&E, Bar: 50 μm

4.2.1.2.Histopathological Assessment of Kidney Tissues

In light microscopic examinations, renal parenchyma of a normal histological shape was observed in the general sections of the H&E-stained control group. Glomerular structure and tubules had a normal appearance. In the kidney sections of the PD group, slight congestion of glomerular tufts was observed. In addition, interstitial mononuclear cell infiltration was observed. In the HFD group, mild congestion of the renal blood vessels was observed. Besides, intense congestion of glomerular tufts was observed. Tubular necrosis was widespread. In the PD+HFD group, severe congestion of the renal blood vessels was observed. Besides, heavy congestion of glomerular tufts was observed. Tubular necrosis was widespread in this group as in the HFD group. In addition, interstitial mononuclear cell infiltration was observed compared to the HFD group (Figure 16).

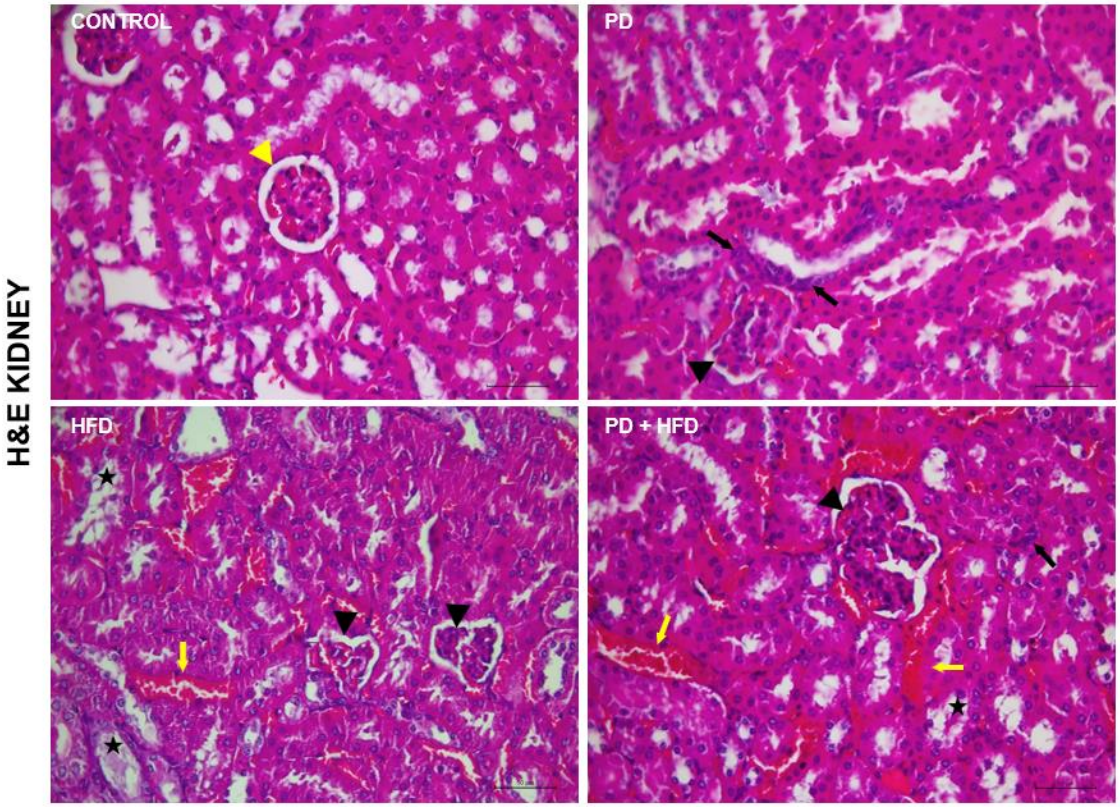

Figure 16. Photomicrographs demonstrate Hematoxylin Eosin staining in the kidney. Control group; normal renal parenchyma structure, preserved glomerular structure (yellow arrowhead), PD group; slight congestion of glomerular tufts (black arrowhead), moderate intensity interstitial mononuclear cell infiltration (black arrows) HFD group; mild congestion of the renal blood vessels (yellow arrows), intense congestion of glomerular tufts (black arrowhead), tubular necrosis (★), PD+HFD group; severe congestion of the renal blood vessels (yellow arrows), heavy congestion of glomerular tufts (black arrowheads), tubular necrosis (★), slight interstitial mononuclear cell infiltration (black arrow), DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, H&E, Bar: 50 µm

4.2.2. Cresyl Violet Staining

Cresyl violet staining was performed in order to histopathologically examine the tissues. According to the evaluation of Cresyl violet stained sections, intact neuronal cells and degenerated neuronal cells were determined in all groups. While these abnormalities were most intensely observed in the PD+HFD group, they were observed less frequently in the HFD and PD groups (Figure 17).

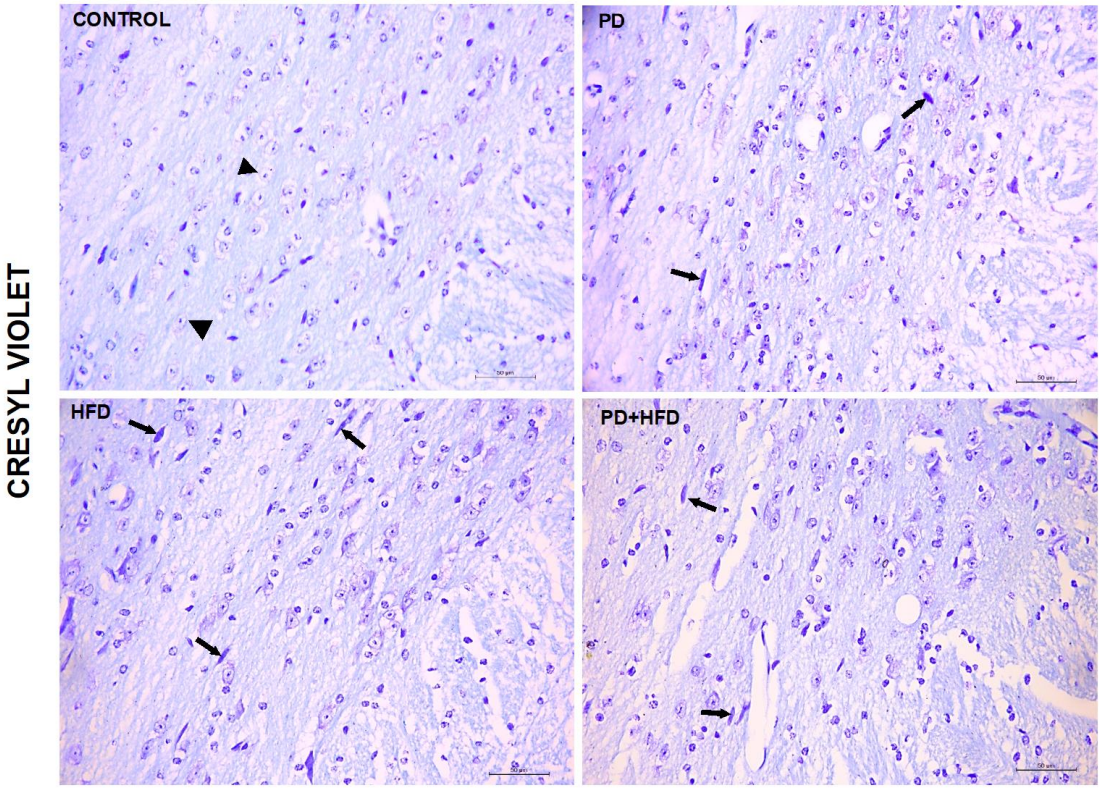

Figure 17. Photomicrographs demonstrate Cresyl Violet staining in the SNPc. Cresyl violet staining of different groups the intact neuronal cell (black arrowheads), the degenerated neuronal cell (black arrows). DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, Bar: 50 µm

4.2.3. Silver Staining

Silver staining was performed to examine the tissues histopathologically. According to evaluation of silver stained sections, normal neurons, degenerated neurons and neurofibrillary tangles were determined in all groups. While these abnormalities were most intensely observed in the PD+HFD group, they were observed less frequently in the PD and HFD groups. No abnormality was observed in group C (Figure 18).

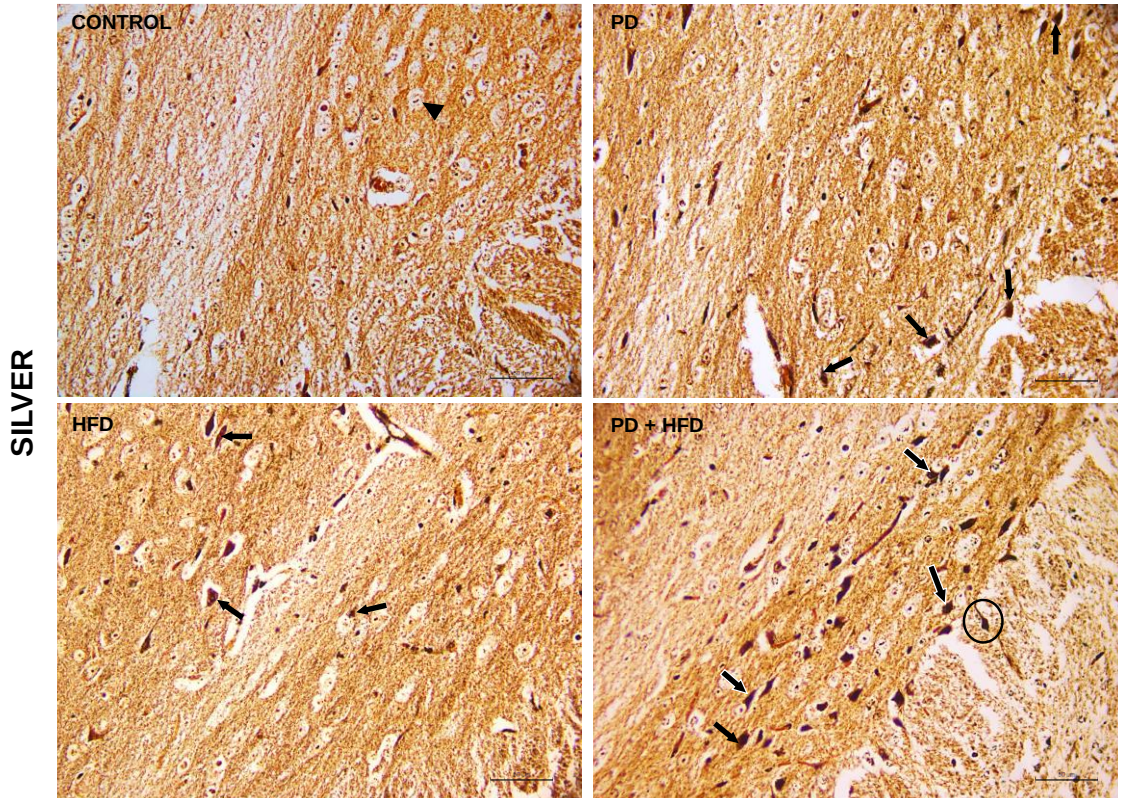

Figure 18. Photomicrographs demonstrate Silver staining in the SNPc. Silver staining of different groups the intact neuronal cell (black arrowhead), the degenerated neuronal cell (black arrows), neurofibrillary tangles (circle shape), DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, Bar: 50 µm

4.2.4. TUNEL Assay

The sections from all the groups were investigated for TUNEL positive neurons. TUNEL assay gives reliable data on the number of apoptotic neurons. Photomicrographs demonstrated sections taken from the SNPc (Figure 19).

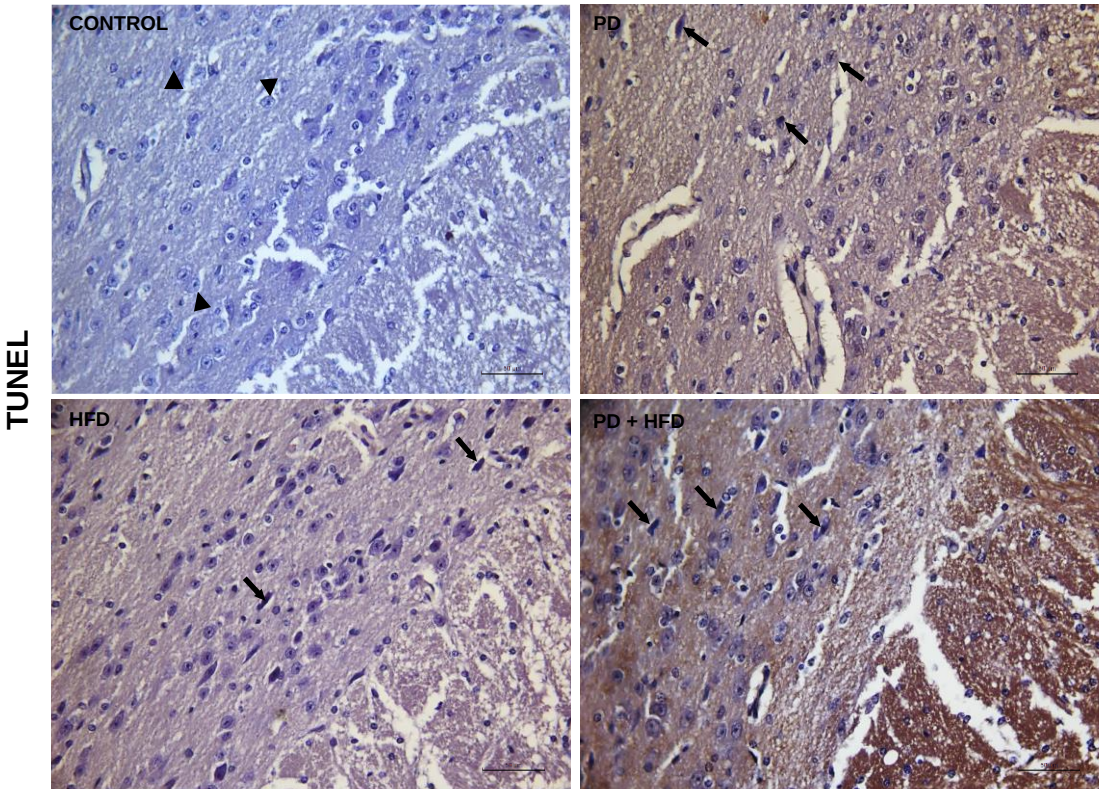

Figure 19. Photomicrographs demonstrate TUNEL positive neurons in the SNPc. The intact neuron is demonstrated with black arrowheads. Apoptotic neuron (TUNEL positive neuron) is demonstrated with black arrows. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, Bar: 50 μm

Rotenone injection induced apoptosis in PD and PD+HFD groups as compared to all other groups ($p<0.001$). The PD+HFD group fed a high fat diet had a higher number of TUNEL positive neurons compared to the HFD group ($p<0.001$). In addition, in the HFD group, the number of apoptotic neurons was very high compared to the control group ($p<0.001$). However, the number of TUNEL positive neurons was still higher in the PD+HFD group as compared to all groups ($p<0.001$) (Figure 20).

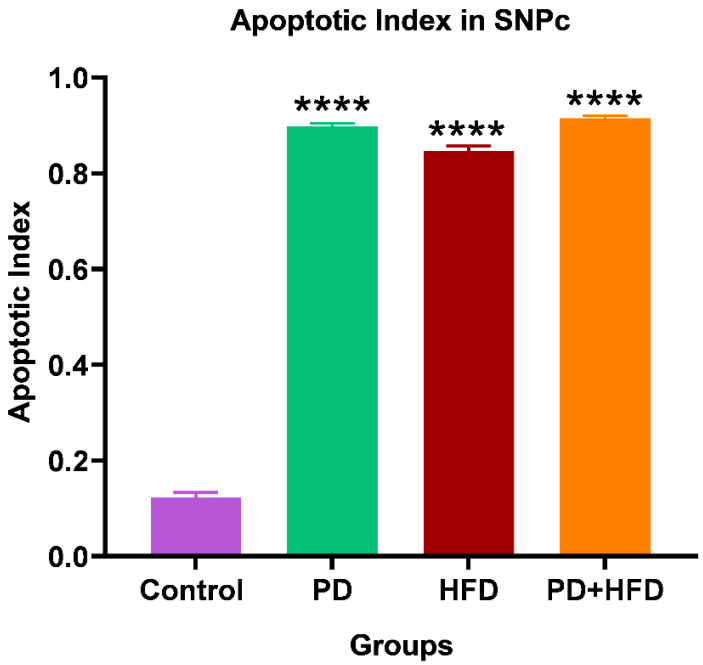

Figure 20. Graphs comparing TUNEL positive neurons in SNPc. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are expressed as mean ± SEM (****$p<0.0001$, compared with the C group).

4.2.5. Immunohistochemistry for iNOS

The sections from all of the groups were examined for iNOS immunoreactive neurons. Photomicrographs demonstrate sections taken from the SNPc stained with iNOS immunohistochemistry (Figure 21). H-scores were calculated for all groups (Figure 22).

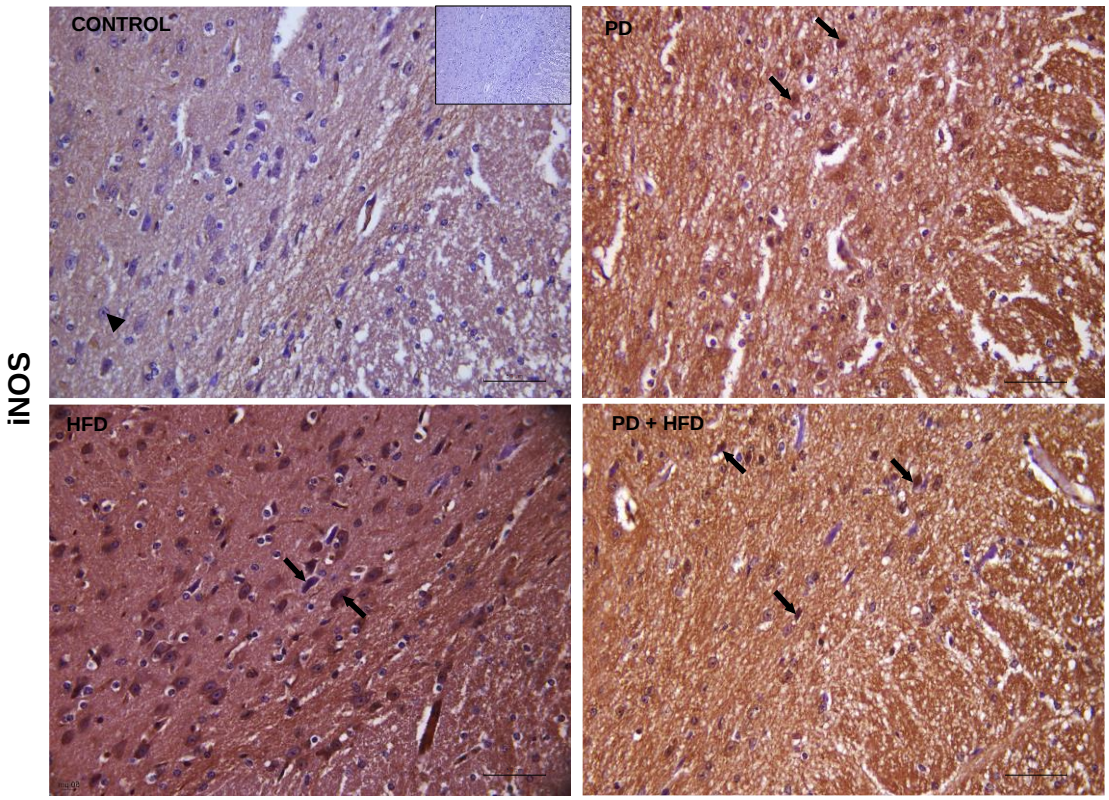

Figure 21. Photomicrographs demonstrate iNOS immunoreactivity neurons in the SNPc. The intact neuron is demonstrated with a black arrowhead. The degenerated neuronal cell (black arrows), Inset; iNOS negative control, Control group; negative iNOS expression, PD group; medium expression of iNOS, HFD group; mild iNOS expression, PD+HFD group; severe iNOS expression, DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups, The magnification is x40, Bar: 50 µm

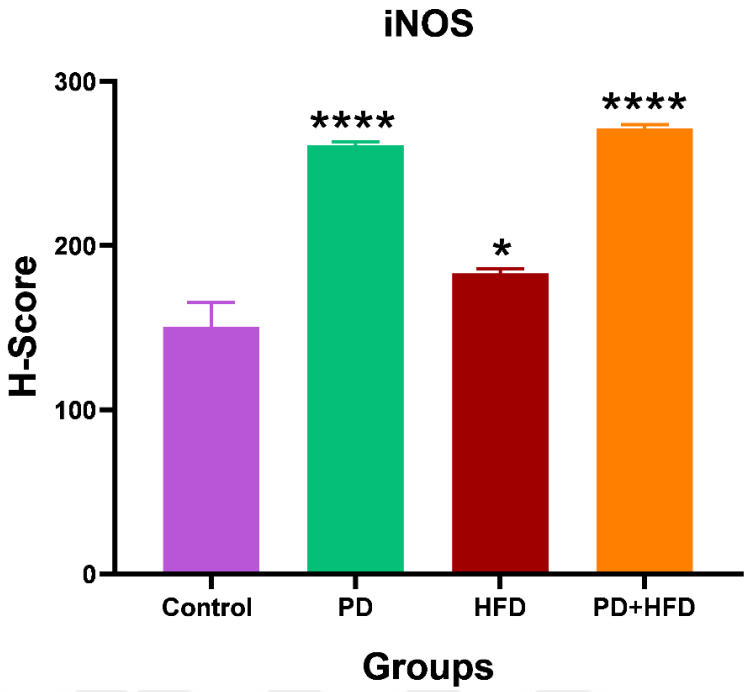

Figure 22. Graphs demonstrate iNOS H-Score in the SNPc. When comparing the H-Scores of iNOS, it can be seen that the PD group and PD+HFD group have a higher H-Score for iNOS than group Control ($p<0.0001$), whereas the HFD group has a slightly lower H-Score for iNOS ($p<0.1$). Moreover, the PD group has a higher H-Score for iNOS than the HFD group ($p<0.0001$). DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are expressed as mean ± SEM (****$p<0.0001$, *$p<0.1$ compared with the C group).

4.3. Weight Changes In Experimental Animals

The changing weight averages of mice fed a high fat diet and standard mice feed for 8 weeks from the start of the experiment are shown in Figure 23.

The difference between measurements is statistically significant ($p<0.001$). The HFD group, which had an average weight of 33 g at the beginning, increased to an average of 54.5 grams after 8 weeks of a high fat diet. Considering the PD+HFD group, the mice's weight, which was 32.1 g at the beginning, increased to an average of 37 g. However, in the PD+HFD group, there was a decrease in weight average with the injection of rotenone in the last 10 days of the experiment. While the PD group had an average weight of 29.5 g at the beginning, it decreased to 23.5 g at the end of 8 weeks. In the control group, the mice's weight, which was 27 g at the beginning, increased to an average of 29.7 g.

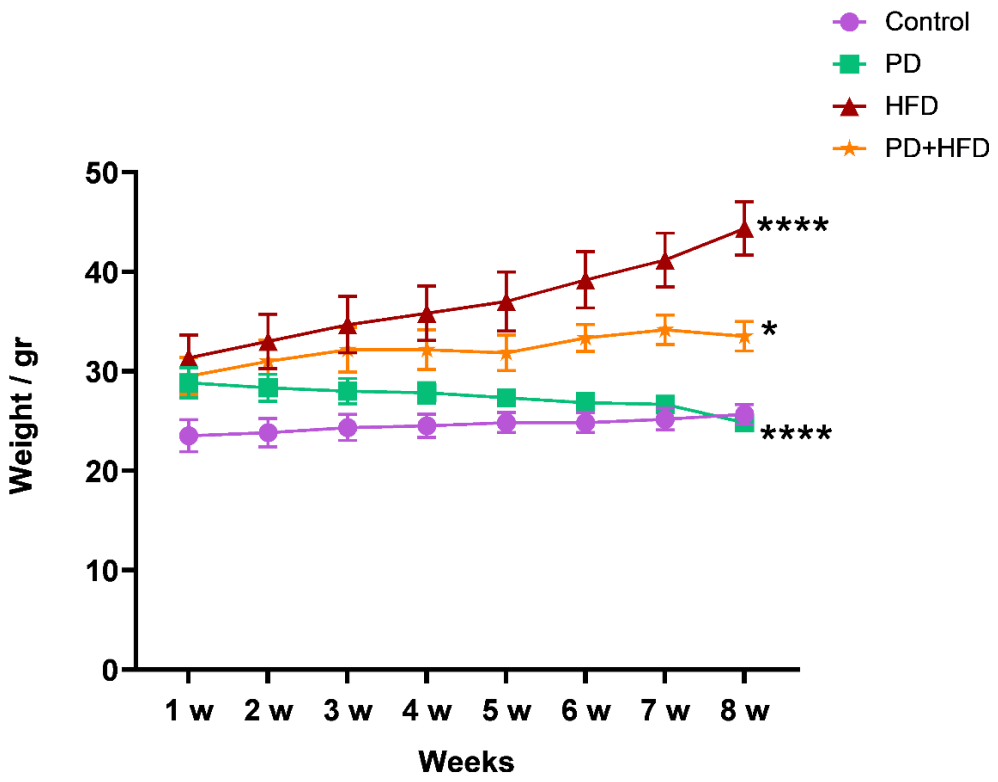

Figure 23. Weekly average weight values of mice during the experimental period. When comparing the weights, it can be seen that the HFD group has a higher weight than the group Control ($p<0.0001$), whereas the PD+HFD group has a slightly lower weight ($p<0.1$). Furthermore, the PD group has a lower weight than all groups. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are expressed as mean ± SEM (****$p<0.0001$, *$p<0.1$ compared with the C group).

4.4. Feed Consumption of Experimental Animals

The average feed consumption of mice fed a high fat diet and standard mice feed for 8 weeks from the start of the experiment is shown in Figure 24. Although the Control group had the highest total average feed consumption at the end of the 8 weeks, the average feed consumption decreased in the last weeks. In the HFD group, although the total average feed consumption was lower than in the Control group, the average feed consumption continued to increase steadily for 8 weeks ($p<0.1$). In the PD group, the total average feed consumption was close to the Control group, but the average feed consumption of the PD group decreased a lot in the last weeks. The PD+HFD group had

the lowest total average feed consumption($p<0.001$). Likewise, the average feed consumption rate of the PD+HFD group decreased considerably in the last week.

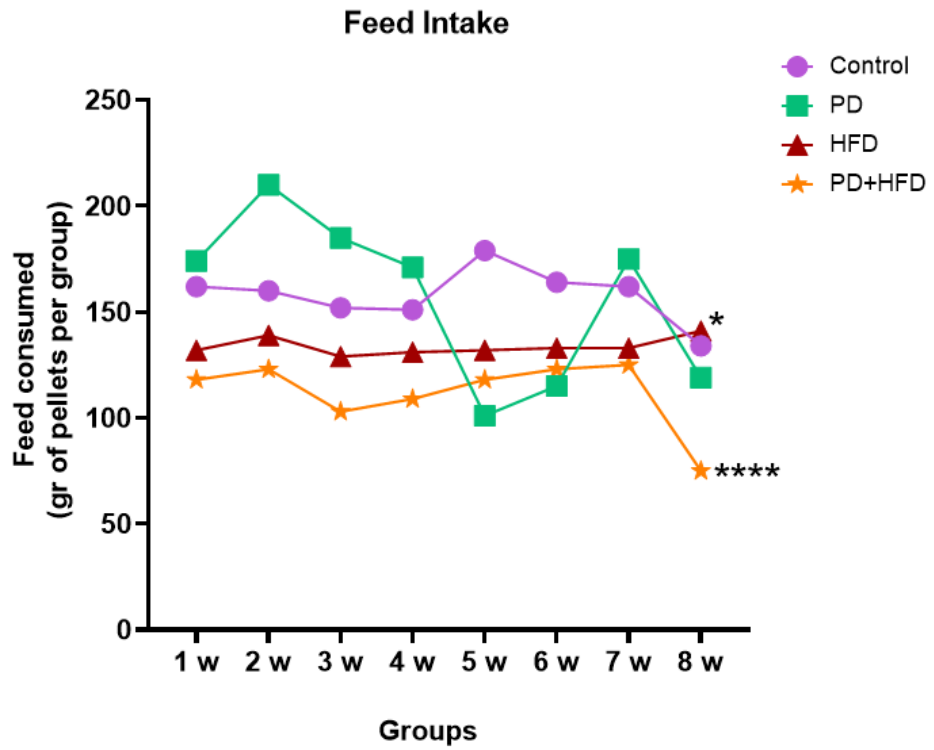

Figure 24. Weekly average feed consumption values of mice during the experimental period. When comparing the feed consumption, it can be seen that the PD+HFD group has a lower average feed consumption than the group Control ($p<0.0001$), whereas the HFD group has a slightly lower average feed consumption ($p<0.1$). Furthermore, In the PD group, the total average feed consumption was close to the Control group. DMSO treated control group (C), fed a high fat diet (HFD), rotenone injected (PD), fed a high fat diet and rotenone injected (PD+HFD) groups. Data are expressed as mean ± SEM (****$p<0.0001$, *$p<0.1$ compared with the C group).

5. DISCUSSION AND CONCLUSION

Parkinson's Disease is one of the most common neurodegenerative diseases, and individuals have difficulty in performing their daily activities over time and their quality of life is adversely affected. Important clinical appearances are resting tremors, postural instability, bradykinesia, and rigidity, all of which negatively affect patients' quality of life (184, 185). Studies on the treatment of this disease, which causes socioeconomic and physical losses, are gaining importance day by day. Although the pathogenesis of PD is not fully understood, it is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta region of the brain (38). Degeneration of dopaminergic neurons reduces the amount of dopamine in the striatum. From this point of view, experimental Parkinson's models have made great contributions to elucidating the pathogenesis of PD and identifying potential neuroprotective agents. Many methods are used to create a Parkinson's model. The most commonly used methods are 6-OHDA, MPTP, Paraquat, Rotenone (146, 147). Rotenone causes pathological events such as dopaminergic neuron degeneration, increased microglial activation, α-synuclein accumulation by causing oxidative stress, mitochondrial dysfunction, increased ROS production in mice and rats (14, 15, 16, 186, 187, 188, 189). In the light of this information, Rotenone was used to create a PD model in our study.

Nowadays, obesity is increasing due to inadequate and unbalanced nutrition, decreased physical activity, and the effects of genetic and environmental factors. One of the main causes of obesity is excessive consumption of unhealthy foods with high fat and sugar content (190). There are several models of obesity in animals. Although there are many dietary sources, high fat diets are frequently used and the diets that are frequently used are 45%-60% fat diets (18). In the literature, there are studies in which significant weight gain was observed with a high fat diet in rodents (191). Obesity is associated with decreased cognitive functions, according to human and animal studies (192, 193, 194, 195). As a result of obesity and metabolic disorders caused by a high fat diet, psychopathologies such as depression and cognitive dysfunction may occur.

The main clinical symptom of PD is motor abnormalities due to the disturbed balance of the direct and indirect pathways of the basal ganglia. In experimental Parkinson's studies, negative effects of rotenone on many motor performance tests have

been reported (196), and investigations are demonstrating that HFD consumption may also affect locomotor activity behaviors (197). In this study, the locomotor activity parameters were examined with an activity monitoring system (198). The distance travelled is the measurement in centimeters of the area covered by the animal in the activity cage. The stereotypical activity is the number of times the mouse licks its forelimbs, moves it over its head, and bites its own fur. The ambulatory activity corresponds to the number of squares crossed in the activity cage. Vertical activity refers to the numeral of times the rat stands on its hind feet. In this study, all locomotor activity parameters were decreased in animals with rotenone lesions in Parkinson's disease. Rotenone-treated high fat diet-fed mice group (PD+HFD), rotenone-administered (PD), high fat diet-fed (HFD) and control group were still the group with the lowest locomotor activity parameters when compared to animals. The reason for this is that according to the studies, a high fat diet causes low locomotor activity and, rotenone application causes locomotor activity deficiencies (196, 199, 200, 201) . There was a decrease in locomotor activity parameters in the high fat diet group (HFD) compared to the control. Similarly, locomotor activity parameters were lower in the rotenone-administered (PD) group compared to the control group and the group fed a high fat diet. As an explanation, it has been proven that feeding a high fat diet has a negative effect on the locomotor activity of animals. Bittencourt, A. et al fed rats with a high fat diet for 25 weeks in their study (202). They showed that there were impairments in locomotor activities (202). Chi Kin Wong et al., on the other hand, fed rats with a high fat diet for 8 weeks and observed a decrease in locomotor activities in rats (203). As a result, the PD+HFD group had the lowest locomotor activity parameters.

The liver is the main site of lipid metabolism. On the one hand, it takes free fatty acids from the blood by synthesizing triglycerides, on the other hand, it secretes the synthesized endogenous triglycerides into the blood and thus transports them to the extrahepatic tissues. Both situations are in a dynamic balance. It is reported that a high fat diet has a significant effect on lipid metabolism in rat liver by disrupting this balance (204). In groups fed a high fat diet, liver tissue degeneration increased as expected. In studies in the literature, cell damage and lobular inflammation in the liver tissue have been shown in experimental animals fed a high fat diet (205). The HFD group had a high effect on liver tissue damage compared to the Control group. A severe effect on liver tissue damage was observed in the PD+HFD group compared to all groups. In one study,

experimental obesity was developed in rats on a high fat diet, resulting in fatty liver status (206). In addition, severe steatosis and ballooning have been observed in obese rats in the literature (206). Other studies have shown that fatty liver, the first stage of nonalcoholic fatty liver disease, is strongly associated with obesity and that fatty liver can result in severe liver damage, eventually including cirrhosis (207, 208, 209). Likewise, within our study, H&E staining of liver sections of the HFD and PD+HFD groups showed steatosis, hepatocyte ballooning morphology and pathology associated with inflammation. In addition, it was observed that the PD group was worse than the control group's liver. We can say that this is due to the toxic effect of rotenone in the liver of mice (210, 211). In conclusion, the PD+HFD group had the most damaged liver morphology.

The kidney is one of the earliest organs to respond to a high fat diet and exhibits a programmed inflammatory response to a high fat diet. Clinical and experimental studies have shown that the characteristic features of obesity-related kidney injury are glomerular hypertrophy, thickening of the glomerular basement membrane, mesangial matrix expansion, and increased kidney inflammation (212). High fat diet (HFD) group and PD+HFD group showed the worst pathology in our study, which is consistent with the literature. In a study, it was shown that a high fat diet significantly increased inflammatory cells compared to the control group in histological kidney analysis (213). In our study, the most increased damage in kidney sections stained with H&E was in PD+HFD group mice and was recorded in HFD, PD and Control groups, respectively. Xiao-Wen Jiang et al, reported that rotenone plays a significant role in the apoptosis of kidney cells and showed that rotenone causes nephrotoxicity in rats (214). Consistent with the literature in our study, the PD group had more kidney damage compared to the Control group. The most remarkable feature seen in the HFD group and the PD+HFD group were tubular vacuolation and tubular dilatation, which had moderate effects on the glomerular structure. This observation of tubular vacuolization has been previously reported in HFD-induced obese mouse models and is associated with chronic kidney disease (215, 216, 217). As a result, mice in the PD+HFD group are associated with renal damage likely to lead to progressive renal disease from a high fat diet.

The association between obesity and PD has appeared to play an essential role in both obesity and PD, with the loss of dopaminergic neurons in common and lower dopamine levels in the hypothalamus and striatum. Mechanisms associated with obesity

and PD are multifactorial (218, 219, 220). In this study, hematoxylin eosin, cresyl violet and silver stains were also performed to examine the tissues morphologically. In our study, severe neuronal degeneration was observed in the PD group. In the PD+HFD group, when compared to the Control and HFD groups, severe degeneration was observed in the neurons when examined histopathologically. Interestingly, in silver staining, a rare number of neurofibrillary tangles were seen in the rotenone treated high fat diet group (PD+HFD) (221). In the literature, Julie A Schneider et al (222) examined the brains of 86 deceased elderly Catholic clergymen who did not have Parkinson's disease. They showed that neurofibrillary tangles in the substantia nigra were present in 67 of 86 people and that neurofibrillary tangles in the substantia nigra were associated with gait disturbance in elderly people with or without dementia. Dementia is increasingly recognized in cases of Parkinson's disease (PD); such cases are called PD dementia (PDD) (223). In addition, studies have shown that the pathologies of Aβ plaques, neurofibrillary tangles, and α-synuclein in the brains of Alzheimer's disease and Parkinson's disease overlap in the brains of dementia (PDD) patients (224). Based on all these, our study makes us think that in the rotenone treated group with a high fat diet (PD+HFD), the high fat diet exacerbates the disease and it is possible to develop dementia(PDD) in the coming years.

Inducible Nitric Oxide Synthase iNOS can be found in the ageing brain due to its effects on the balance between brain injury and repair (180, 225, 226, 227). Among the proinflammatory factors, reactive nitrogen species such as nitric oxide and peroxynitrite play an important role in the toxicity of dopaminergic neurons (228). Studies have shown that iNOS inhibitors have neuroprotective effects (229). In addition, Rotenone causes pathological events such as oxidative stress, mitochondrial dysfunction, increased ROS production, and dopaminergic neuron degeneration in mice and rats (14, 15, 188, 189, 230, 231, 232) . When iNOS expression is evaluated, it is seen that the iNOS expression of rotenone-induced PD group, high fat diet fed and rotenone-induced PD+HFD group mice is significantly increased compared to the Control and HFD groups. Studies have shown that one of the mechanisms by which HFD-induced obesity can lead to neurological disorders is increased neuroinflammation and ROS production, which causes increased oxidative damage in the CNS (233, 234, 235, 236, 237). In short, obesity caused by HFD is characterized by oxidative stress (238). In our study, the iNOS expression of the HFD group was higher than that of the Control group. Morrison et al

(239), demonstrated that 20-month-old male mice fed 60% fat HFD had increased oxidative stress and cognitive decline after 4 months compared to controls. The PD+HFD group had the highest expression of iNOS. The reason why the highest iNOS expression was found in the PD+HFD group in our study is; It can be interpreted that a high fat diet causes iNOS expression (202, 238, 240) and the inflammation that develops after rotenone injection leads to iNOS expression with activation of microglia (189, 230, 241, 242, 243). In direct ratio to the literature, the highest iNOS expression was observed in the PD+HFD group.

Although contentious studies exist regarding apoptosis in nigral degeneration, various studies suggest that apoptosis plays a role in neuron degeneration in humans, in vivo and in vitro models of PD (244). The TUNEL method is used extensively in detecting apoptotic cells. It is an extremely reliable method for detecting apoptosis, as it tags DNA double-strand breaks. Several studies show TUNEL positive neurons in rotenone animal models (245, 246, 247). Many studies show that mice or rats fed a high fat diet may play a role in neuronal cell death associated with obesity (248, 249). In this study, consistent with the literature, the PD and PD+HFD groups caused a significant increase of TUNEL positive neurons in the substantia nigra pars compacta compared to the Control group. Compared with the PD and PD+HFD groups, the HFD group had lower apoptotic neuron counts in the substantia nigra pars compacta but was very high compared to the Control group. The PD+ HFD group had the highest number of apoptotic neurons compared with all groups. It suggests that rotenone may cause apoptosis due to neuroinflammation and that a high fat diet may negatively affect the apoptosis of dopaminergic neurons.

Although there are various models of obesity in animals, high fat diets are frequently used. Commonly used diets are diets with 45%-60% fat (18, 174). Studies have shown that hypercaloric diets, such as high fat diets, are easier to develop obesity in animals and the closest model for obesity in humans(174, 250). In this 8 week study conducted with the HFD and PD+HFD groups fed with a high fat diet, 60% of the energy value of which consists of fats, the weight of the mice increased significantly compared to the control group. PD and Control groups were fed with standard mouse feed. The PD group mice had the lowest overall weight, with an extra fall in weight in the final week. Similarly, the weight of the PD+HFD group mice also decreased in the final week. This

is thought to be related to rotenone (210). In the HFD group, it was noted that there was a continuous weight increase. In a study examining whether a high fat diet causes PD like symptoms after the development of experimental obesity in C57BL/6 mice, the mice were followed for 20 weeks, and the mice were shown to weigh an average of 45.8 ± 2.6 g at the end of the experiment (251). In our study, it was saved that the HFD group mice weighed 54.5 g on average at the end of 8 weeks.

In this study, although the total average feed consumption in the HFD group was lower than in the Control group, the average feed consumption continued steadily throughout the total experiment time. In a similar study, researchers showed that the group fed with a high fat diet consumed less feed, on average compared to the control group, but the weight increase continued regularly (251). At the end of the experiment, the total average feed consumption was the lowest in the PD+HFD group compared to the Control group, while the total average feed consumption in the PD group was close to the Control group.

Millions of patients worldwide suffer from Parkinson's disease and its incidence is increasing with the aging population (252). High fat diets (HFDs) are powerfully related with obesity and exert harmful impacts on cognitive and behavioural functions (253). Epidemiological studies and animal studies have also shown associations between obesity and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (236, 254). Current studies show that a high fat diet may have adverse effects in terms of Parkinson's disease (251, 255, 256). Many studies have also shown that a high fat diet makes substantia nigra neurons more vulnerable to Parkinson's disease neurotoxins.

In our study, high fat diet administration and duration caused a decrease in dopaminergic neurons in the substantia nigra pars compacta and increased the number of apoptotic neurons. On the other hand, impaired locomotor activity parameters were observed in groups fed a high fat diet. Motor and behavioural symptoms were similar to those seen in Parkinson's patients. Rotenone may be an alternative to a Parkinson's model to investigate the effects of a high fat diet. In addition, the result of this study shows that a high fat diet negatively affects rotenone Parkinson model pathology morphologically. Exposure to a high fat diet and consequent obesity may ensure greater susceptibility to environmental toxins and exacerbate the pathogenesis of Parkinson's disease. Nevertheless, because Parkinson's disease is a highly complex disease involving many

pathological mechanisms simultaneously, further research is needed to determine whether a high fat diet is a replaceable risk factor for Parkinson's disease.

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7. APPENDICES

7.1 EHICAL APPROVAL

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2021-058	14.10.2021	2021/10	2021/10-9	Dr. Öğr. Üyesi Alev Cumbul
'Yüksek Yağlı Diyetin Rotenone ile Oluşturulan Parkinson Tipi Fare Modelinde Substantia Nigra Pars Compacta Üzerine Histopatolojik Etkisi ' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Fare / C57BL/6	24	Erkek

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Dr. Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Prof. Dr. Özlem MALKONDU	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Atakan Mücahit YAVUZ	
Üye	Ahmet ŞENKARDEŞLER	

7.2 CURRICULUM VITAE

Personal Information

Name	Aybüke	**Surname**	Özer

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Master	Institute of Health Sciences/Department of Histology and Embryology	Yeditepe Univesity	2023
University	Faculty of Science / Molecular Biology and Genetics	İstanbul Yeni Yuzyil Univesity	2018
High school	Science Branch	Orhan Cemal Fersoy High School	2012

[#] All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Languages	Grades ([#])
-	-

Work Experience (Sort from present to past)

Position	Institute	Duration (Year - Year)
-	-	-

Computer Skills

Program	Level
Microsoft Office (Excel, Word, Powerpoint)	Good
GraphPad	Good
EndNote	Good
Image Lab	Good

****Excellent, good, average or basic**

Scientific works

Articles published in other journals

Proceedings presented in international scientific meetings and published in proceedings book.

Journals in the proceedings book of the refereed conference / symposium

Others (Projects / Certificates / Rewards)

Frances King School of English Certificate - 2019
Certificate of Animal Use in Experimental research by Animal Research Ethics Committee of Yeditepe University,2020
4th East Mediterranean Congress of Laboratory Animal Science Oral Presentation 2nd Award**.** Ezgi TALO[1], Muhammed HAMİTOĞLU[1], Alev CUMBUL[2], Engin SÜMER[3], Aybüke ÖZER[2], Rim ALOMAR[1], Yelda IŞIKBAY[2], Ahmet AYDIN[1] Investigation of Effects of Oral Anti-diabetics on Oxidative Stress Parameters and Comparison with Healthy Cells 9-11 Dec 2021
15. YÜTBAT National Medical Student Congress 'Innovations in Neuroscience' 2022