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

HISTOPATHOLOGICAL EVALUATION OF HIGH-DOSE 25 HYDROXY CHOLECALCIFEROL IN THE HIPPOCAMPUS AND CEREBRAL CORTEX MEMBERS IN A SCOPOLAMİNE-INDUCED ALZHEIMER'S TYPE DEMENTIA MODEL IN MICE

THE MASTER OF HISTOLOGY AND EMBRYOLOGY THESIS

YELDA IŞIKBAY

İSTANBUL-2023

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

HISTOPATHOLOGICAL EVALUATION OF HIGH-DOSE 25 HYDROXY CHOLECALCIFEROL IN THE HIPPOCAMPUS AND CEREBRAL CORTEX MEMBERS IN A SCOPOLAMINE-INDUCED ALZHEIMER'S TYPE DEMENTIA MODEL IN MICE

THE MASTER OF HISTOLOGY AND EMBRYOLOGY THESIS

YELDA IŞIKBAY

SUPERVISOR
ASSOC. PROF. ALEV CUMBUL

İSTANBUL-2023

APPROVAL

Institute: Yeditepe University Institute of Health Sciences

Programme: Master Program in Histology and Embryology

Title of the Thesis: Histopathological evaluation of high-dose 25 hydroxy cholecalciferol in the Hippocampus and Cerebral Cortex members in a scopolamine-induced Alzheimer's Type Dementia Model in Mice.

Owner of the Thesis: Yelda Işıkbay

Examination Date: 15 / 06 /2023

The Jury judges that the candidate named above has satisfactorily completed his thesis.

Chair of the Jury:	
Supervisor:	Assoc. Prof. Alev Cumbul (Yeditepe University, Faculty of Medicine, Department of Histology and Embryology)
Member/Examiner:	Prof. Aylin Yaba Uçar (Yeditepe University, Faculty of Medicine, Department of Histology and Embryology)
Member/Examiner:	Assoc. Prof. Salime Pelin ERGÜVEN

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 .....

(Signature)

Prof. Dr. Bayram Yılmaz

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 acknowledgement has been made in the text.

15/06/2023

Signature

Yelda Işıkbay

DEDICATION

To my family…

ACKNOWLEDGEMENTS

I would like to thank my esteemed advisor, associate professor Dr Alev Cumbul, who has supported me in this thesis that I have carried out. Knowing that you are always by my side gave me strength. I am very lucky to work with Alev Cumbul.

In addition, I would like to thank Professor Dr Aylin Yaba Uçar, our head of histology and department, for their support and contribution to my view of life. It has been very instructive for me, both spiritual and scientific, thank you again.

I would like to express my respects and thanks to Prof. Dr Ece Genç, who values his student and is very valuable, who always opened his door and always helped in every way throughout the thesis.

I would like to thank the veterinarian Engin Sümer, who provided me with information and helped me in every subject in my animal experiments, and did not spare his moral support.

I would like to express my gratitude to YUDETAM, who works passionately to care for my animals.

I would like to thank my dear friend, department friend, and life partner Aybike Özer, who did not leave me alone in every aspect of my life. He always encouraged me in this process and made me strong.

I offer my most sincere love and thanks.

Finally, I would like to express my endless gratitude, love and respect to my beloved mother who brought me to this day, to my dear father whom I lost and whom I know watching over me, and to my dear sister Yağmur, who was always there for me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

7DHK: 7- dehydrocholesterol

AD : Alzheimer’s Disease

APLP1: Amyloid Beta Precursor Like Protein 1

APLP2: Amyloid Beta Precursor Like Protein 2

APOE: Apolipoprotein

APP Amyloid Precursor Protein

Aβ: β-Amyloid Peptide

BPD: Binding Vitamin D

IL1: The Interleukin-1 Family

İNOS: Inducible Nitric Oxide Synthase

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NFT: Neurofibrillary tangles

NGF: Nerve Growth Factor

PD: Parkinson Disease

PNES1: Presenilin 1

PNES2: Presenilin 2

PTH: Parathyroid Hormone

RCAN: Regulator Of Calcineurin 1

ROS: Proto-oncogene Tyrosine-protein Kinase

SOD2: Superoxide Dismutase 2

UVB: Ultraviolet B

VDR: vitamin D receptor

β42: Beta-Amyloid42

ABSTRACT

Isıkbay, Yelda. (2023). Histopathological evaluation of the effect of high-dose 25 hydroxy cholecalciferol on the Hippocampus and Cerebral Cortex region in a scopolamine-induced Alzheimer's Type Dementia Model in Mice, Yeditepe University, Institute of Health Sciences, Department of Histology and Embryology, MSc Thesis, İstanbul.

This study was designed to understand and investigate how 25 hydroxy calciferol (vitamin D) induces Alzheimer's-type dementia in mice, and how dementia has a positive or negative effect on the formula. Alzheimer's is a global health problem.

An irreversible and progressive neurodegenerative management of Alzheimer's disease. Cognitive changes are receptors with cerebral atrophy neurons veal loss and neuropathological effects involving misfolded and aggregated cells of β-amyloid peptides (Aβ) and tau proteins. Two main pathological structures are depending on the disease. These are amyloid plaques that accumulate outside the cell and neurofibrillary tangles that form inside the cell. These proteins, which accumulate inside and outside the cell, impair axonal transport, interneuron observers, and neurotrophic factor, envelop intracellular shields and increase oxidative stress. Twenty c57bl/6 male mice with a body weight of 20-25 g were used in this study. Vitamin D tests were done. Accordingly, investigate the underlying causes of AD and. The development of treatments that will change the course of the illness becomes even more important. Diagnostic criteria were established in the years when biomarkers for AD diagnosis, patient anamnesis, clinical examination, neuropsychological tests and differential diagnosis were not available. Neurotrophin production of some protective vitamin D, which has been made in recent years, stimulates neuronal shield shelter by affecting the spread of shield channels and prevents oxidative stress by regulating some detoxification steps. And in this way, it is observed that it can exert a protective effect on neurons. Vitamin D is formed as a group of stars that are soluble in fat and organic solvents but insoluble in water. Until now, it was not discussed whether it should be evaluated as a hormone or a vitamin. However, it is studied as a prohormone by researchers because it reaches organs and tissues through its archives and undertakes important points. We aimed to examine the morphological changes of vitamin d in the hippocampus and cerebral cortex in the Alzheimer's fee model by histological analysis, and our guess is that we aim to have a protected area of vitamin

d in this model. VDR is known to occur in large areas of the brain such as the cerebellum, hypothalamus, basal nodes and hippocampus. It has been suggested that vitamin D's effect on neuronal cell death and functionality may be shown by decreasing the expression of L-type calcium channels or increasing the level of VDR in order to facilitate the recovery of neurons or to facilitate the turn back of the brain to its previous function after neuron detriment. Premised on this, we think of that high-dose vitamin D increases the VDR level in the hippocampus and can act against neurodegenerative processes, and has a neuroprotective effect on the elimination of amyloid plaques, which is the most important pathology of Alzheimer's disease.The H-score was calculated on iNOS Amyloid-Beta immunohistochemistry staining, and there was a significant difference between the results of the scopolamine and control groups. Amyloid-Beta immunohistochemistry staining showed intense amyloid plaque formation of scopolamine cells and was rare in the test instruments.

Key words: Alzheimer’s Disease, Vitamin D, Scopolamine, Dementia, Amyloid plaques

ÖZET

Isıkbay, Yelda. (2023). Yüksek doz 25 hidroksi kolekalsiferolün Farelerde skopolamin ile oluşturulan Alzheimer Tipi Demans Modelinde Hipokampüs ve Serebral Korteks bölgesine etkisinin histopatholojik olarak değerlendirilmesi, Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Histoloji ve Embryoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Bu çalışma, 25 hidroksi kalsiferolün (D vitamini) farelerde Alzheimer tipi bunamayı nasıl tetiklediğini ve bunamanın formül üzerinde nasıl olumlu veya olumsuz bir etkiye sahip olduğunu anlamak ve araştırmak için tasarlanmıştır. Alzheimer küresel bir sağlık sorunudur. Alzheimer hastalığı geri dönüşümsüz ve ilerleyici nörodejeneratif hastalıktır.

Bilişsel değişiklikler, serebral atrofi nöronları kaybı olan reseptörler ve β-amiloid peptitlerinin (Aβ) ve tau proteinlerinin yanlış katlanmış ve kümelenmiş hücrelerini içeren nöropatolojik etkileridir. Hastalığa bağlı olarak 2 ana patolojik yapı vardır. Bunlar hücre dışında biriken amiloid plaklar ve hücre içinde oluşan nörofibriler yumaklardır. Hücre içinde ve dışında biriken bu proteinler aksonal taşımayı, nöronlar arası gözlemcileri ve nörotrofik faktörü bozarak hücre içi alanları sarar ve oksidatif stresi artırır. AD'nin altında yatan nedenlerin araştırılması ve hastalığın seyrini değiştirecek tedavilerin geliştirilmesi daha da önem kazanmaktadır. Tanı kriterleri AD tanısı için biyobelirteçlerin, hasta anamnezinin, klinik muayenenin, nöropsikolojik testlerin ve ayırıcı tanının olmadığı yıllarda oluşturulmuştur. Son yıllarda yapılan bazı koruyucu D vitaminlerinin nörotrofin üretimi, kalkan kanallarının yayılmasını etkileyerek nöronal kalkan barınağını uyarır ve bazı detoksifikasyon adımlarını düzenleyerek oksidatif stresi önler. Ve bu sayede nöronlar üzerinde koruyucu bir etki gösterebildiği gözleniyor. D vitamini, yağda ve organik çözücülerde çözünen ancak suda çözünmeyen bir yıldız grubu olarak oluşur. D vitamini, yağda ve organik çözücülerde çözünen ancak suda çözünmeyen grup olarak oluşur. Şimdiye kadar hormon olarak mı yoksa vitamin olarak mı değerlendirilmesi gerektiği tartışılmamıştı. Ancak arşivleri aracılığıyla organ ve dokulara ulaştığı ve önemli noktaları üstlendiği için araştırmacılar tarafından bir prohormon olarak incelenmektedir. Bu çalışmada vücut ağırlığı 20-25 g olan yirmi tane c57bl/6 erkek fare kullanıldı. D vitamini testleri yapıldı. Buna göre Alzheimer modelinde D vitamininin hipokampus ve serebral korteksteki morfolojik değişimlerini histolojik analizle incelemeyi amaçladık ve

tahminimiz bu modelde D vitamininin korunan bir alana sahip olmasını hedefliyoruz. VDR'nin beyincik, hipotalamus, bazal düğümler ve hipokampus gibi beynin geniş alanlarında meydana geldiği bilinmektedir. D vitamininin nöronal hücre ölümü ve işlevselliği üzerindeki etkisinin, nöronların iyileşmesini kolaylaştırmak veya nöron hasarından sonra beynin önceki işlevine geri dönüşünü kolaylaştırmak için L tipi kalsiyum kanallarının ekspresyonunu azaltarak veya VDR seviyesini artırarak gösterilebileceği öne sürülmüştür. Buna dayanarak, yüksek doz D vitamininin hipokampustaki VDR seviyesini arttırdığını ve nörodejeneratif süreçlere karşı hareket edebileceğini ve Alzheimer hastalığının en önemli patolojisi olan amiloid plakların ortadan kaldırılması üzerinde nöroprotektif bir etkiye sahip olduğunu düşünüyoruz. iNOS Amiloid-Beta immünohistokimya boyamasında H skoru hesaplandı ve skopolamin ve kontrol grubu sonuçları arasında anlamlı fark vardı. Amiloid-Beta immünohistokimya boyaması, skopolamin hücrelerinde yoğun amiloid plak oluşumu gösterdi.

Anahtar kelimeler: Alzheimer hastalığı , Vitamin D, Scopolamine, Demans, Amyloid plak.

1. INTRODUCTION AND PURPOSE

Alzheimer's disease is a rapidly spreading neurodegenerative disease today. Alzheimer's disease is known as memory, memory problems and loss of thinking and speech and daily life and results in irreversible neuronal losses. In most people with Alzheimer's, symptoms first appear at a later age. Although estimates vary, experts suggest that more than 6 million people, most of whom are 65 years of age or older, may have dementia caused by Alzheimer's It is known that this disease, which greatly adversely affects the quality of life of the person, occurs as a result of damage to one or more parts of the brain responsible for memory, learning, spatial memory and cognitive functions (1). It is known that this disease, which greatly adversely affects the quality of life of the person, occurs as a result of damage to one or more parts of the brain responsible for memory, learning, spatial memory and cognitive functions. There are two basic pathological structures in the basis of the disease. The first of these is amyloid plaques that accumulate outside the cell, and the second is neurofibrillary tangles that form inside the cell (1).

These proteins, accumulating inside and outside the cell, cause disruption of axonal transport, interneuron transmission, neurotrophic factor synthesis, intracellular calcium balance, and increase in oxidative stress (2).Although Alzheimer's is a very common public health problem, there is no definitive treatment for the disease and only drugs that delay its progression are available (2, 3). In AD, the disease begins with atrophy of the entorhinal cortex and hippocampus and spreads to most parts of the cortex except the occipital lobes as clinical symptoms worsen (3).Apart from being a vitamin, vitamin D is actually a hormone. It is an antiproliferative and immunomodulatory secosteroid hormone. Its main role in the human body is to provide calcium metabolism and participate in the regulation of bone structure. It also plays an important role in the growth of bone and the regulation of the balance of production and destruction. In the last decade, the effects of vitamin D on the brain have been investigated, arguing that vitamin D may have important effects on the brain and that it is classified as a neurosteroid (4).

Vitamin D receptor (VDR) has been shown to be found in more than 50 tissues. The presence of VDR has been detected in the cerebellum, thalamus, hypothalamus, basal ganglia, hippocampus, olfactory system, temporal and orbital regions (5, 6).It has been thought that the neuroprotective effect of vitamin D may be exerted by decreasing the expression of L-type calcium channels or increasing the level of VDR. Therefore, vitamin D deficiency can be considered as a serious factor that increases the risk of neurological diseases (7). In addition, it has been shown in various studies that age-related cognitive functions are also associated with VDR polymorphisms, and that vitamin D levels decrease in Alzheimer's patients and the elderly with cognitive impairment. Studies show that vitamin D-related mechanisms may play a role in Alzheimer's disease and neurodegeneration (8).We think that vitamin D may be an important key molecule that can regulate the mechanisms that control oxidative stress, control immune response and neurotrophic factor synthesis, alleviate the effects of neurodegeneration mechanisms, and regulate the changing expressions of various proteins in neurodegenerative diseases, especially Alzheimer's. In the scopolamine-induced AD mouse model, it is hypothesized that high-dose vitamin D can reduce pathological effects in neurons. This study aims to investigate the effect of high-dose vitamin d on morphological changes in hippocampus and cerebral cortex neurons in a scopolamine-induced Alzheimer's Disease model. We aimed to contribute to the knowledge on the molecular pathogenesis of AD and to lay the groundwork for new approaches for new treatment possibilities. (9)

2. LITERATURE REVIEW

2.1. Anatomies of the Hippocampus and Cerebral Cortex

The external shell of the brain is called the cortex. The forebrain region is separated into two hemispheres by a line of lines running through the middle. It covers almost all the structures of the brain. It is the most developed part of the human brain. Accountable for functions such as thinking, perception, and language Each hemisphere is separated into four lobes: occipital, temporal, parietal and frontal (10).The cerebral cortex is the typically 2- 3 mm thick, covering the gyri and sulci. The slit in the sagittal position, which separates the hemispheres from each other, is called the fissura longitudinalis. The hemispheres are attached by a bundle of nerve fibers called the corpus callosum. The corpus callosum allows your two hemispheres to communicate with each other.The cortex is grey because the nerves in this area lack the isolation (myelin) that makes most other parts of the white brain appear (11).Mostly the cerebral cortex is the neocortex. It contains 10 to 14 billion neurons. It is called “neo” because it is the “newer” part of the brain. All your perceptions, awareness, and motor skills are managed here. It is also the most developed and complex part of our nervous system (12).

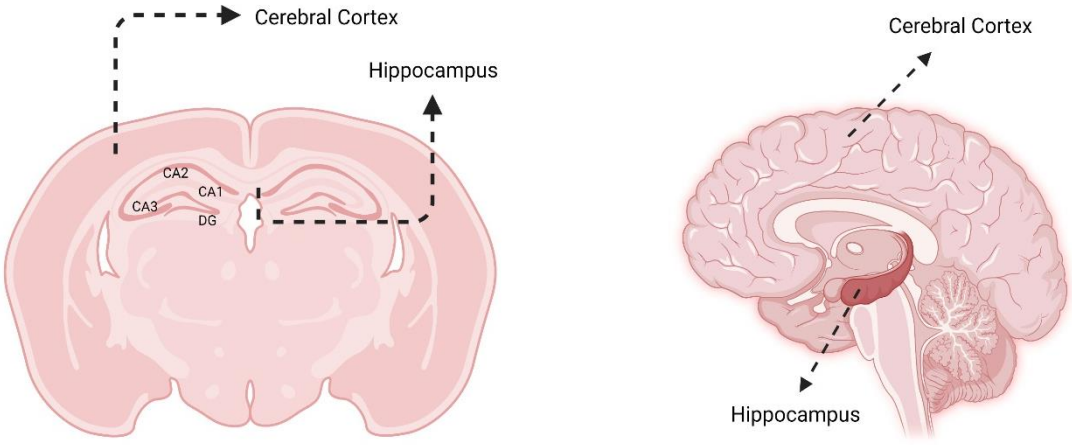


Figure 1: Location of the hippocampus and cerebral cortex.(Cornu Ammonis 1 (CA1), Cornu Ammonis 2 (CA2), Cornu Ammonis 3 (CA3), Dentat Girus DG) **(**13)

The hippocampus is considered to be C in the coronal sections of the brain. It is called the "hippocampus" because of its resemblance to a seahorse. It is located in the median portion of the temporal lobe and is closely related to the temporal horn of the lateral ventricle. The hippocampus is a layer of gray matter. It is about 5-8 cm long. The hippocampus region has an important role in memory and navigation. It is an extension to the neocortex through a large passage called the enthorinal cortex. It is where different neuronal structures are properly organized (14)

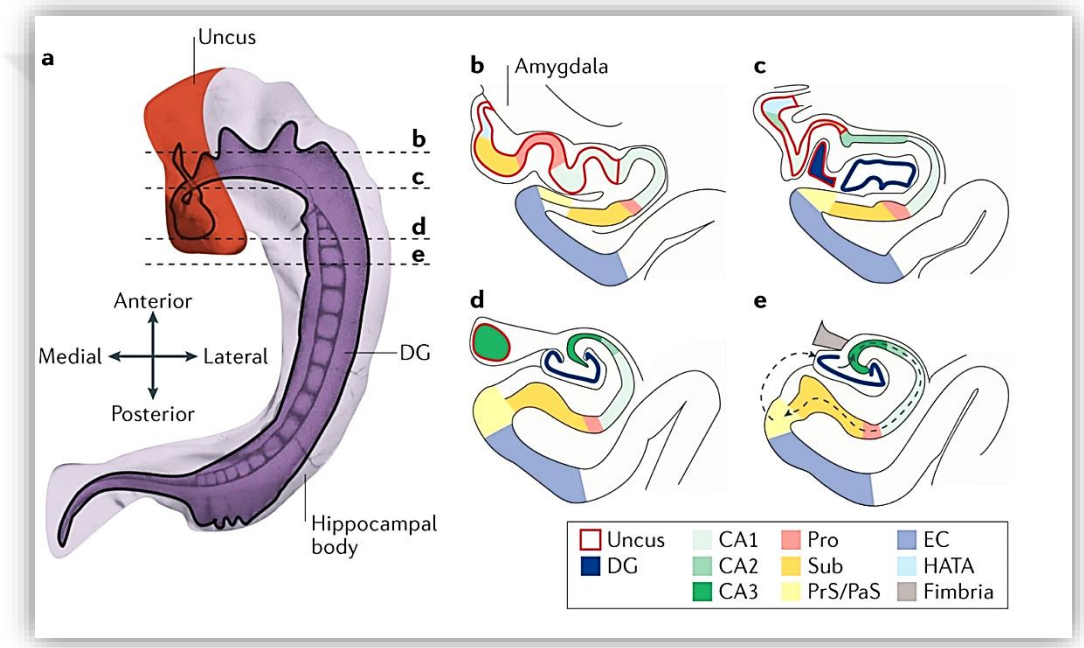


Figure 2. Anatomy of the anterior hippocampus **(**12**).**

2.2. The Histology of Hippocampus and Cerebral Cortex

The cerebral cortex is covered with meninges and is often referred to as pale matter. The cortex is pale because the nerves in this area are deficient in myelin (15). The cells of the cerebral cortex are arranged in diapers. They are 2-3mm in thickness. Gray matter contains a molecular layer, Purkinje cell layer and granular cell layer. While the molecular Layer contains Stellate cells and Basket cells; Purkinje cell Layer; contains Purkinje cells and Granule cell Layer; Contains Granule cells, Golgi cells, Lugaro cells and Unipolar Bushy cells (16).

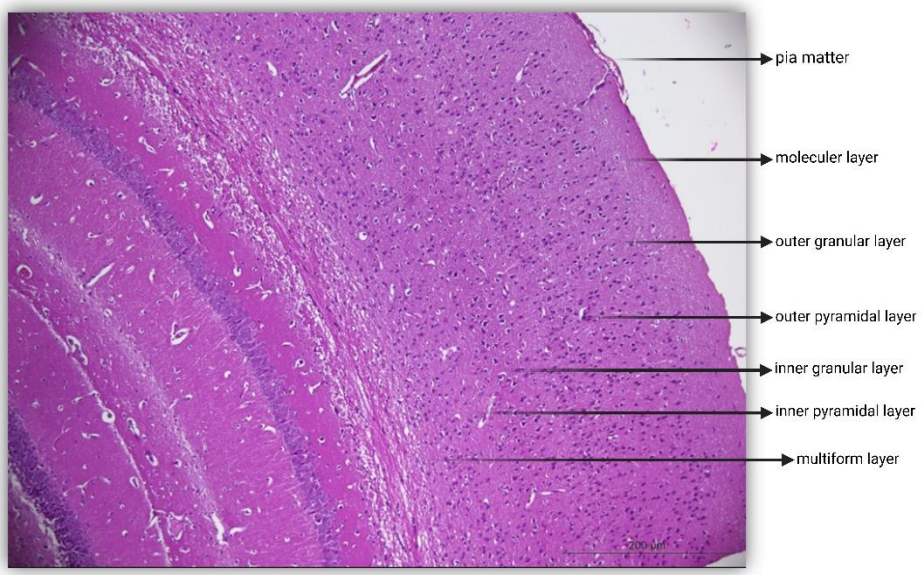


Figure 3. A photomicrograph of a section in the cerebral cortex of control adult rat showing the general histological structure of the cerebral cortex (H&E 200X) **(**17**).**

The hippocampus consists of two main regions; Dentate Gyrus (DG) and Cornu Ammonis (CA) The hippocampus is morphologically arched and sagittally located as a dilated anterior segment and a narrow posterior segment, about 4-5 cm in length and 1cm in width. Due to the constructional features of the different cell types it has, CA is reviewed by splitting into sub-areas termed cornu ammonias 1, 2, 3 and 4 (CA1, CA2, CA3 and CA4) (18).This difference is found in the basic structure of the morphology of cortical neural networks. It is known that the CA1 area is toward the understory and the CA4 area is toward the dental gyrus in terms of ease of orientation in the hippocampus. The hippocampus is histologically composed of many thin and interconnected layers. Although these layers vary from author to author in the literature, there are generally 6 layers. Histologically; Starting from the ventricular surface outward, the layers of the hippocampus are arranged as follows (19).

I. Alveus
II. Stratum oriens
III. Stratum pyramidale
IV. Stratum radiatum
V. Stratum lacunosum and Stratum moleculare.

The nerve extensions of the fornix overlying the ventricular surface of the hippocampus appear in the form of a thin white strip called elves. The hippocampus receives the major part of its contribution of excitation of the entorhinal cortex.

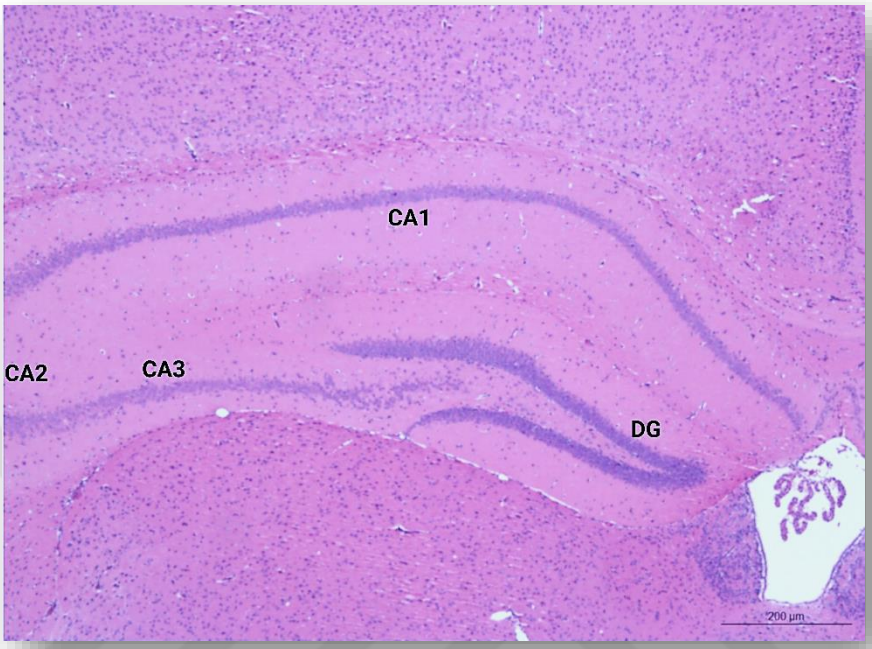


Figure 4. Hippocampus histology.H&E staining of coronal hippocampus sections. A section from control showing the different areas of the hippocampal formation where the hippocampus proper is formed of the Cornu Ammonis (CA) as CA1, CA2 & CA3 regions, and Dentate Gyrus (DG) is seen surrounding hilar region by its upper & lower limbs (20).

2.3. Description and history of Alzheimer's Disease

Alois Alzheimer was born on 14 June 1864 in the small neighbourhood of Marktbreit in Bavaria. Tübingen studied at the University of Aschaffenburg, Berlin, where he obtained his doctorate in 1887. He and renowned neurologist Franz Nissl have studied the normal and pathological anatomy of the cerebral cortex. As a meticulous laboratory worker, Alzheimer provided detailed information about the microscopic structure of tissue. In 1903, he started working with the famous psychiatrist Emil Kraeplin at the Psychiatric Clinic of the University of Munich (21). When he was admitted to the hospital, his examination revealed disorientation and memory impairment, as well as

difficulty writing and reading. Symptoms progressed over time, hallucinations and other cognitive dysfunctions were added to the table. In 1906, Agust D. died. Alzheimer's demanded the brain from his old clinic in Frankfurt to autopsy his clinical records. In the microscopic investigation of the patients' brains, the cortex was thinner than ordinary and there were two abnormal findings in the brain (22). One of other was the senile plaques, formerly detected in the brains of the elderly, and neurofibrillary tangles stained with silver paint, which was used for the first time at that time. Neurofibrillary tangles had never been described before, and the finding made new sense (21).

Alzheimer's presented this phenomenon in 1906 at the "South-West German Congress of Mental Illnesses" as "a peculiar disease of the cerebral cortex". The person who named the disease, Alzheimer's, Dr. Alzheimer's clinical chief is Dr. Emil Kraepelin. Page 627 of the 8th edition of Kraepelin's 1910 book Clinical Psychiatry uses the term "Alzheimer's Disease" after the title "Senile Brain Injury." (23). AD can be categorized as a function of family and age. It is divided into two according to whether there is a first-degree relative with dementia in the family or not, that is, hereditary or sporadic Alzheimer's, whichever is the most common form of Alzheimer's disease and occurs after the age of 65. At the same time, according to age, it can be divided into under 65 years old, which we call the early stage, and 65 years and over, whichever we call the late stage (24).

2.4. Alzheimer's Epidemiology

Alzheimer's disease is the most common cause of neuron-degenerative dementia. It is responsible for 2/3 of all dementias. The prevalence of AD in persons over the age of 65 is approximately 10%. The prevalence increases with rising age and reaches 45% in those over 85 years of age (25). In the study of the pervasiveness of Alzheimer's disease in Turkey conducted in Kadıköy, Istanbul, the pervasiveness of AD was found to be 11% among people over 70 years of age. In line to this prevalence value, it is assumed that there are 250-300 thousand patients with AD in Turkey (26). It is anticipated that the number of AD patients will gradually increase with increasing average life expectancy. It is predicted that 50 million AD patients will be in the USA in 2050. It is thought that approximately 35 million people in the world have Alzheimer's disease in 2010. One case emerges every 7 seconds, and if a new treatment approach is not found in the AD, it is expected to double in 20 years (27). The prevalence of Alzheimer's disease in Turkey is

nearly similar to that of western countries. The prevalence of Alzheimer's disease is 11% among people older than 70 years. According to the 2008 census of the population over 65 years of age in our country, it is estimated that there are over 450,000 Alzheimer's patients in Turkey (28).

2.5. Alzheimer's Disease Risk Factors

2.5.1. The Age Factor

Age is a major risk factor in Alzheimer's disease.As a result, in the normal brain, there is an age-related decrease in brain volume and weight, enlarged ventricles, and loss of synapses and dendrites in the selected areas (29). According to the global Alzheimer's statistics from 2016, there are currently approximately 46.8 million Alzheimer's patients worldwide.

The aging of the world's population is estimated to aggravate this problem .The number of AD patients is estimated to double almost every 20 years. Thus, the population of AD should reach 74.7 million in 2030 and 131.5 million in 2050 (30).

2.5.2. Gene Factor

Following age, the most important risk component is genetic. The genes you inherit from our forefathers can contribute to your risk of AD development. Mutations in these genes Presenilin 1 (chromosome 14), presenilin 2 (chromosome 1) and amyloid precursor protein (chromosome 21) genes are responsible for the predominant transmission of the disease. The common feature of mutations in these genes is a significant increase in the level of amyloid beta (Aβ), the main ingredient of senile plaques formed by the peptide secretes enzyme of Amyloid-beta precursor protein (APP). The functions of these three proteins are little known and entirely unknown (31). It was resolved that the ε4 allele of apolipoprotein E, a protein involved in cholesterol transportation, develops more frequently in patients with AD. The common allele of APOE ε4 is responsible for an important proportion of Alzheimer's disease, but how it affects its heritability is unclear (32).

2.5.3. Down Syndrome Factor

People with Down syndrome have a top risk of developing Alzheimer's disease. This is forasmuch the genetic customization that cause Down syndrome can cause

amyloid plaques to accumulate in the brain over time. According to the 2002 report from Kanad, a public health agency, 1 in 800 newborns have Down syndrome. The average lifespan of people with Down syndrome is 49 years. These people have an extra copy of the gene that produces the Regulator Of Calcineurin 1 (RCAN) protein; this leads to the excessive making of RCAN1 protein. As a result of overproduction, neuronal death occurs and the life expectancy of patients is abbreviated (33).

Also found that some people with Alzheimer's disease also had high levels of RCAN1 protein. Down-regulation of the gene is thought to be resulted of stroke, hypertension, or the presence of beta-amyloid protein. A 2011 analysis suggested that RCAN1 protein functions as a mediator of stress and Aβ-induced neuronal death, and an extra copy of the RCAN1 protein on chromosome 21 and they said that overexpression of RCAN1 interacts with down syndrome and the pathogenesis of AD (34).

In another study, beta-amyloid plaques and tau tangles, which are regarded to be the differentiating features of Alzheimer's, were observed in the brains of almost all individuals as a result of the autopsy performed on individuals with Down syndrome at the age of 40. But we can't say that every person with Down syndrome has Alzheimer's disease (35, 36).

2.5.4. Head Trauma

We do not know the biological mechanism through which head trauma causes AD. It has been shown in animal and human studies that trauma causes neuronal damage, which increases the accumulation of amyloid Aβ and becomes amyloid plaques. According to the study published in the German journal Bild der Wissenschaft, GGA1 and GGA3 bearer proteins in brain nerve cells decline in brain or skull traumas. In Alzheimer's disease, plaques developed because of this accumulation to the detriment nerve cells (37).

2.6. Alzheimer's Disease Pathophysiolog

AD is a cognitively impaired synaptic dysfunction. AD is a very complex procedure involving numerous mechanisms and associated with neuronal personalization. Extracellular Aβ plaques and intracellular agglomerated neurofibrillary tangles (NFT) were the first pathological structures that drew attention when the brain was examined in AD cases (38).NFTs are double filament helices formed by

hyperphosphorylation of tau protein. There is a pathology of AD, pathological findings that can be observed through microscopy, one of which is neurofibrillar entanglements containing tau and the other is plaques containing Aβ (39). Additionally, anatomically, atrophy of the hippocampus and cerebral cortex, enlargement of the sulci and ventricles, and shrinkage of the gyri is among the macroscopic pathological findings of AD (40).

2.6.1. Genetic Mechanisms

One of the most usual causes of Alzheimer's disease seen in the ageing period is the genetic factor.

Genetic risk factors involved in AD are mutations in genes encoding the APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2). AD can be classified into two groups early-onset and late-onset Alzheimer's disease (41).

Mutations in the APP, PSEN1 and PSEN2 genes cause early-onset Alzheimer's disease. Advancements in the early onset of Alzheimer's disease are spreading over long years. The genetic risk factor responsible for late-onset Alzheimer's disease is the ε-4 allele of the gene encoding apolipoprotein E (APOE). The number of cases is higher than in early-onset AD. The average life expectancy for Alzheimer's disease is 8 to 10 years (42).

The ε-4 allele of ApoE is an important serum protein involved in the transport of cholesterol and its metabolism and is an important factor for AD since it is present in approximately 20% of the population (43). APP is degraded by secretases, pathogenic mutations in these genes cause APP methanolysis to be disturbed, and therefore, APP secretases cannot perform the degradation process. Too much beta 42 peptide is produced, resulting in the accumulation of neurotoxic Aβ in the brain of patients with Alzheimer's disease (44). ApoE exhibits an affinity Aβ peptide and its ratio in amyloid plaques is very high.

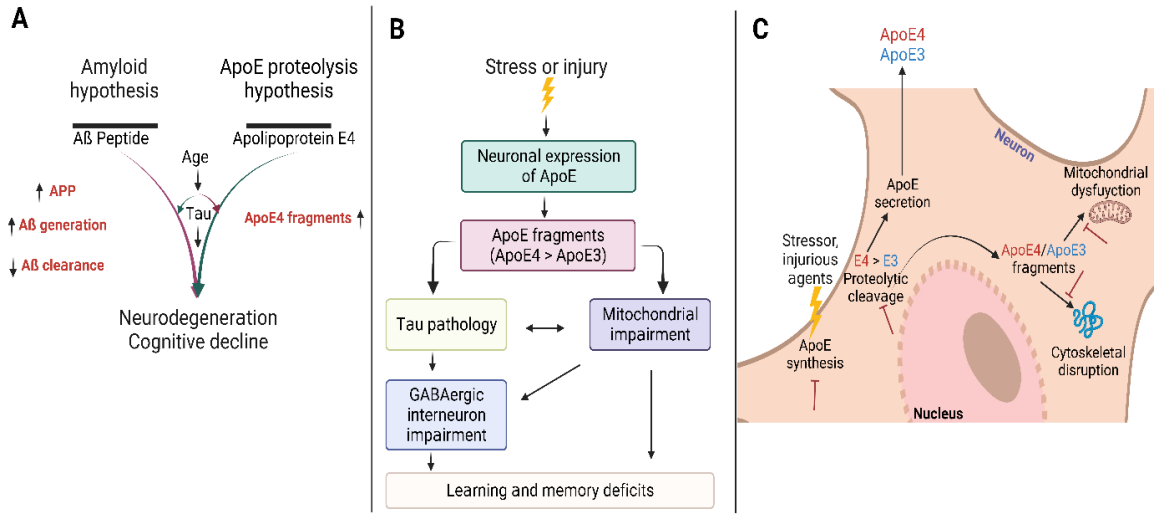


Figure 5. ApoE4 in AD Pathogenesis and Related Therapeutic Strategies.
(A)ApoE4 probably has both Aβ-dependent and Aβ-independent roles in AD pathogenesis. ApoE4 impairs Aβ clearance and promotes Aβ deposition (left). (B) The apoE proteolysis hypothesis suggests that, in response to stresses or injuries, neuronal apoE expression is triggered to facilitate neuronal repair**.** (C) Neuronal expression of apoE and apoE proteolysis-related neurotoxicity represent targets for the development of novel therapeutics. Inhibiting apoE4 expression in neurons should reduce the level of toxic apoE4 fragments and their downstream detrimental effects (45).

2.6.2. Processing of Amyloid Precursor Protein

The fundamental structure of AP is the Aβ peptide. While Aβ is produced from its precursor protein throughout life, it is best known as the major component of amyloid plates, the neuropathological characteristic of AD (46). Members of the membrane-related APP family interact in cis or trans, allowing them to function as cell adhesion molecules. Many extracellular and intracellular attachment partners have been identified (47).APP is transmitted to axon following dissociation in the endoplasmic reticulum and Golgi, where it is transported to synaptic terminals by fast axonal transport (48) APP belongs to a family of proteins that includes amyloid beta precursors like protein 1 (APLP1) and amyloid beta precursors like protein 2 (APLP2) in mammals (48).

While APP has been studied extensively since its definition, its physiological function has not yet been fully resolved. APP is thought to affect neurite growth and

synaptogenesis, transmembrane signal transduction, cellular adhesion and calcium metabolism, but further studies are needed for these (48, 49).

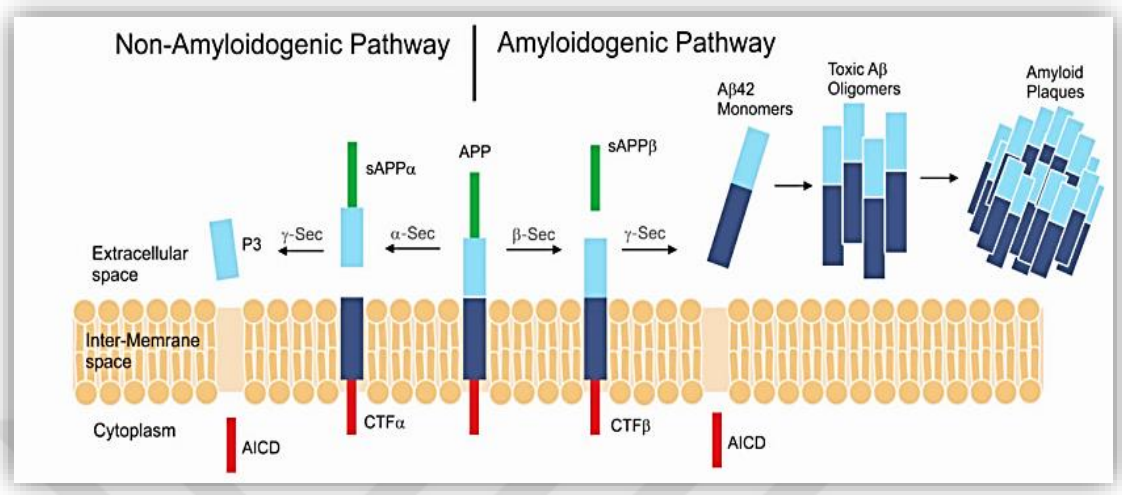


Figure 6. A schematic representation of beta-amyloid pathway that leads to AD pathology. The cleavage of APP β-secretase and subsequently by γ -secretase results in the production of an amyloid plaque. The nonpathogenic (nonamyloidogenic pathway) is initiated by α-secretase releasing sAPPα into the intracellular space. The resulting CTF-α fragment is cleaved by γ -secretase in the intermembrane space resulting in both the AICD and p3 fragments that are both non-plaque forming elements (50).

2.6.3. Neurofibrillary Tangles

NFT was first described as "neurofibrils" that form thick bundles near the cell surface of affected neurons. The major component of Lewy's bodies, neurofibrillar deposits, is α-synuclein, a presynaptic protein. The main component of neurofibrillar entanglements is the tau protein, which is involved in the corporation and stabilisation of microtubules, and the tau protein builds up within neurons, resulting in abnormal aggregation (51).

Healthy neurons are supported by structures called microtubules that help guide molecules in the cellular body toward the axon and dendrites (52).

In healthy neurons, tau typically binds to microtubules and stabilizes them, but AD chemical changes consequence in abnormal accumulation of tau protein in neurons. By causing tau to separate from microtubules and attach to other tau molecules, thread-like structures are formed that eventually join together to form knots inside neurons (53).

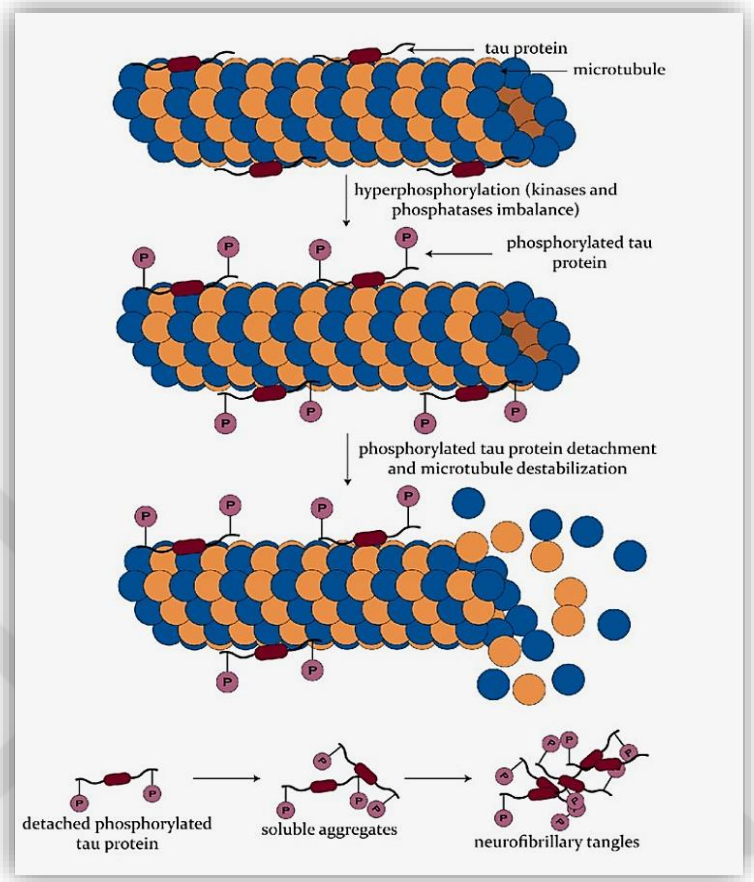


Figure 7. The formation of neurofibrillary tangles through the process of tau protein Hyperphosphorylation (54).

2.6.4. Mitochondrial Dysfunction in Alzheimer's Disease

Genetic, oxidative stress, inflammation, Aβ, mitochondrial malfunction is effective in the formation and progression of AD. The most common form of neurodegenerative cell death is the intrinsic mitochondrial apoptotic pathway (55). This pathway controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial inter-membrane space. Mitochondrial dysfunction falls into two categories; The first type (primary dysfunction) refers to the dysfunction caused by a mutation in a gene associated with the ATP-producing pathway (56).The second type (secondary dysfunction) refers to dysfunction caused by other genetic or metabolic abnormalities and deficiencies that may interfere with mitochondrial synthesis of ATP(57). Reactive oxygen species (ROS) are a by-product of mitochondrial respiratory

chain activity. ROS focusing is mediated by mitochondrial antioxidants such as manganese superoxide dismutase (SOD2) and glutathione peroxides.

Excessive production of ROS is a central feature of all neurodegenerative disorders. In addition to the specified neuropathological symptoms of AD, disturbance of Ca homeostasis, accumulation of Aβ in secretory pathways, presence of Aβ in mitochondria, which cause for mitochondrial dysfunction and is thought to be associated with intracellular lesions such as increased reactive oxygen species (58).

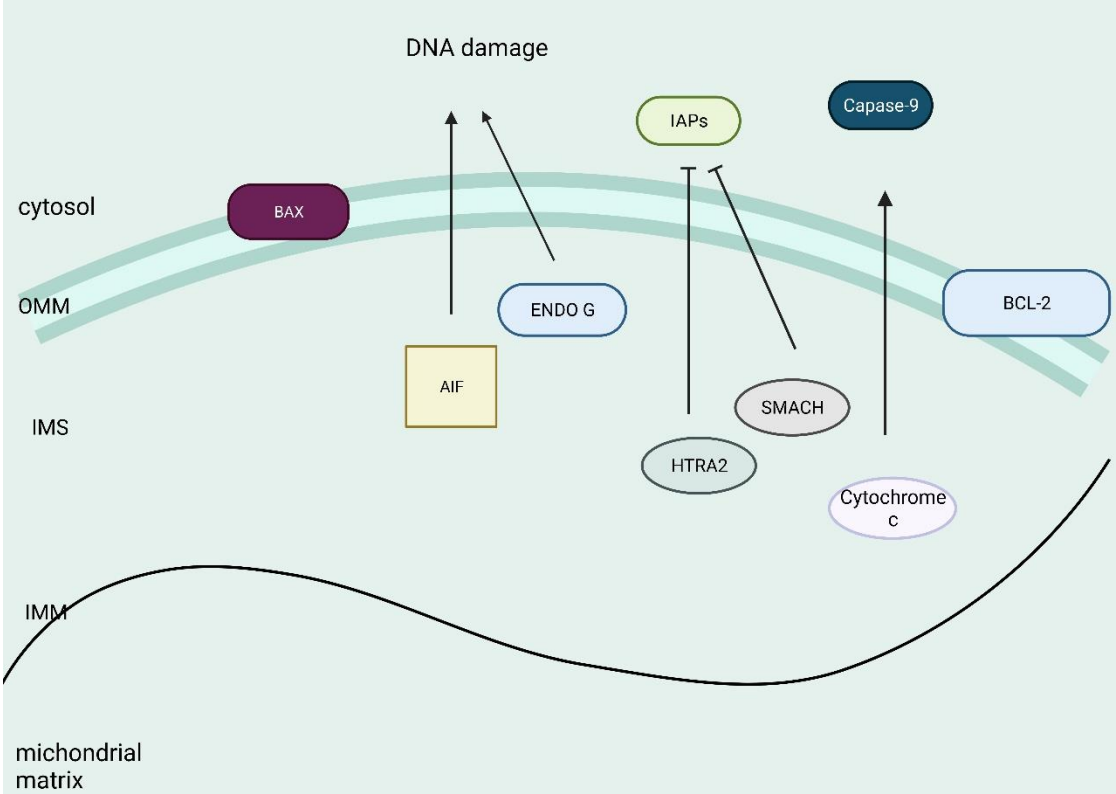


Figure 8: Role of mitochondria in apoptosis. Several intermembrane space (IMS) proteins are pro-apoptotic if released into the cytosol. Cytochrome c (C) activates caspase-9. SMAC (second mitochondrial activator of caspases) and HTRA2 inhibit cytosolic inhibitor of apoptosis proteins (IAPs) (59).

Neurons communicate with their target tissues through synapses, where they release chemicals known as neurotransmitters. The brain needs a lot of energy for neurotransmission. This is why mitochondria are abundant with synapses. Decreased

mitochondrial numbers and decreased energy metabolism are also the first detected impairments in the AD brain (59).

2.6.5. Oxidative Stress

Oxidative stress (OS) plays an important role in the pathogenesis of AD, which is currently the most devastating (60).It is a lipophilic gas that is synthesized endogenously by various cell types and is involved in multiple bioregulatory roles in a explicit area of inflammation, the nervous and cardiovascular systems, and bone resorption. In physiological structures, ROS produced from mitochondria, NADPH-oxidase (Nox) and xanthin oxidase (XO) are maintained at low levels of endogenous antioxidants. The pathogenesis of various neurodegenerative disorders such as AD and Parkinson's disease (PD) is associated with the accumulation of misfolded proteins. And the aggregation of engineered proteins can trigger the inflammatory response in the brain, triggering the significant release of ROS followed by the OS (61).There is an equilibrium between the production of ROS and the antioxidant defense mechanism. However, in AD, the activity of antioxidant enzymes changes, resulting in the accumulation of oxidative damage (62). In addition, when the brains of individuals with AD were examined at autopsy, high levels of oxidized proteins, glycosylated products, aldehydes, ketones, cholesterol, and oxidative modifications in RNA and nuclear and mitochondrial DNA were build in brain tissue (63). For this reason, an increase in ROS manufacture may be sufficient to spread ROS damage to other mitochondria eventually touching the whole cell, and ROS levels may affect multiple cellular functions, including loss of synaptic activity. As a result, impaired mitochondria can trigger the discharge of cytochrome c, which activates apoptosis (64)(65).

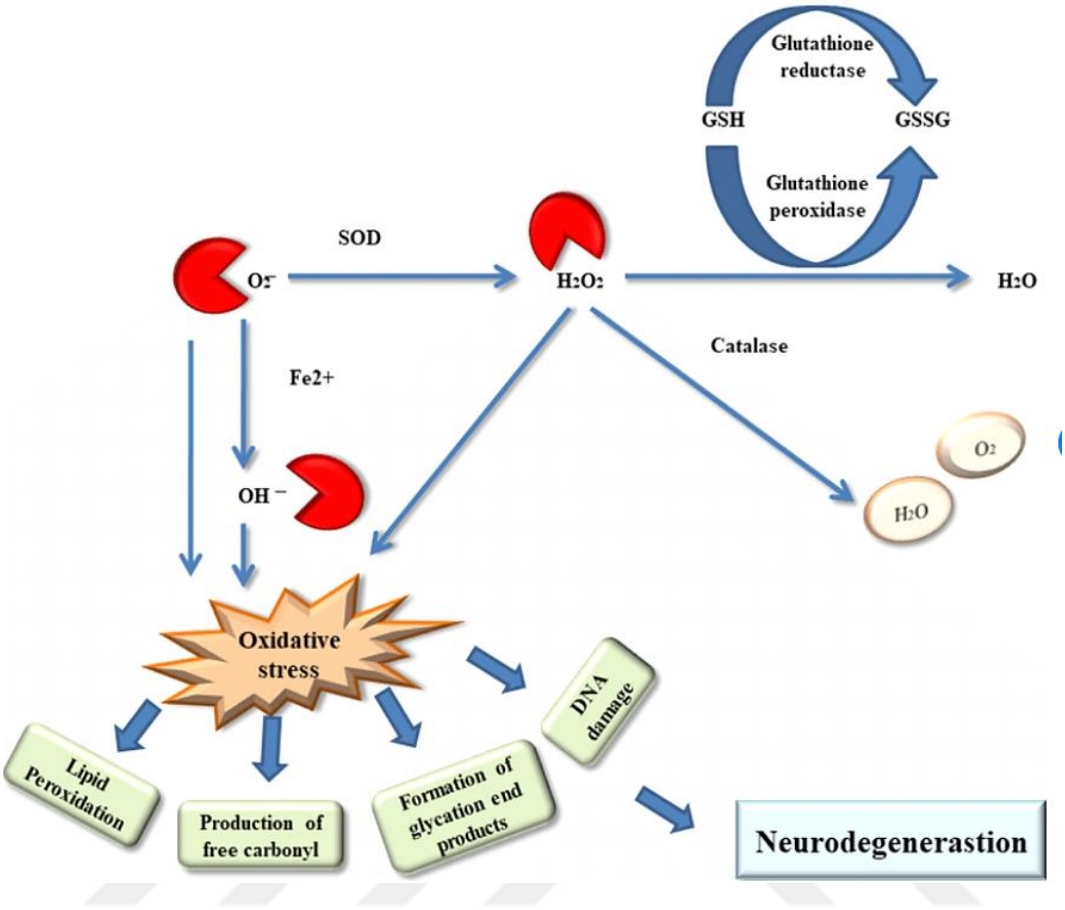


Figure 9. Oxidative stress and mitochondrial dysfunction in Alzheimer's diseases (66)**.**

2.6.6. İnflammation and Alzheimer's

Inflammation occurs in pathologically vulnerable areas of the AD brain. The accumulating of degenerating tissue and highly accumulating abnormal materials in the circumference is classic manifestations of inflammation (67).Likewise, neurons and neuritis and highly insoluble amyloid β-damaged peptide deposits and neurofibrillary tangles in the AD brain are strictly stimuli for inflammation. Many studies now suggest that neuroinflammation plays a fundamental role in the neuropathologic changes observed in AD (68).We know that non-steroidal anti-inflammatory drugs selectively reduce the amyloid β42. Although some cross-sectional and retrospective studies have shown that nonsteroidal anti-inflammatory drugs reduce the risk of Alzheimer's and slow the progression of the disease, prospective studies could not support these findings. Studies of amyloid beta-immunization, TNF-α and complement factor inhibitors are under way (69).Brain inflammation appears to have a dual role that plays a significant

role during the acute phase response, but becomes harmful when a persistent response occurs. Microglia are chronically active and releases a variety of proinflammatory and toxic products, including ROS, NO, and cytokines. In patients with recent head injury and death, there is an increase in cerebral Aβ deposits 1-3 weeks after injury, and high levels of interleukin 1 (IL-1) have been shown to be responsible for APP production and Aβ load (70).

Neuroinflammation observed in AD comes first in triggering Aβ burden and tau hyperphosphorylation; This suggests that there may be a leading link between AD pathologies. Microglia have a major place in AD research.

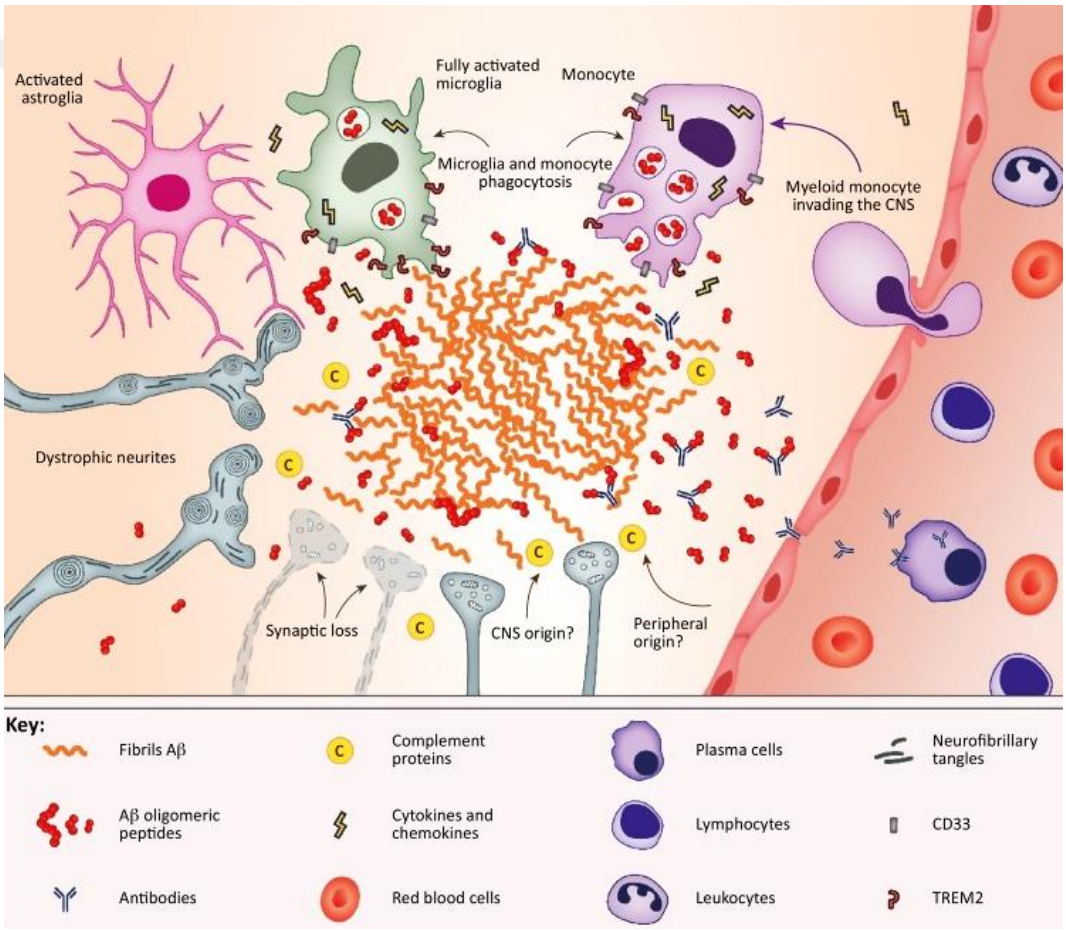


Figure 10. Schematic Representation of Late Central Nervous System (CNS) Inflammation in Alzheimer Disease (AD) Pathology, Representing an Active Inflammatory/Immune Response Centered Around Amyloid Plaques(70).

3. Alzheimer's Animal Models

Conclusive diagnosis of the disease is undertaken by histopathological diagnosis of the presence of amyloid plaque, the intraneuronal neurofibrillary tangle formation around neurons in brain tissues and corresponding amyloid angiopathy, granulovacuolar degeneration and Hirano authorities in an autopsy of patients with typical clinical features. Animal models are central to the study of disease pathology and the evaluation of the potential of new therapeutic approaches.

3.1. Transgenic Alzheimer's Models

Transgenic animals are recombinant animals that carry a gene called recombinant their own genome, which is not potential to occur spontaneously in nature from some other organism, mostly formed by cutting DNA molecules obtained from different biological species and combining different DNA fragments obtained. The first genetically engineered mouse produced by microinjection and developed mainly for disease modelling was published in 1980. Experimental designs involving transgenic animals are also referred to as transgenic designs. Among transgenic animals, mice are the most commonly used species since 90% of their genomes are known. Suited to the desired pathological process to create. Animals carrying the transgene are used (71).

3.1.1. Aβ Models

Aβ directly stimulates neuron-toxicity, leading to basal prophylaxis. This is the factor responsible for the cholinergic destruction of the brain. AD has a very complicated structure and bears all the pathological characteristics of AD. It is very difficult to construct a transgenic model. As such, most models targeted the most important pathological features of AD, not all features. While the first models concentrated on amyloid deposition, after the discovery of the APP, AD family. Models for the overproduction of transgenes carrying mutations have come into use (72). At the extremity of the Aβ gene, the mutation is directly related to the formation of plaques in the models. Mutations in terminal N increase production of Aβ40 and Aβ42, whereas mutations in terminal C increase production of Aβ1-42. Many of these models are genetically engineered.

It has been reported that APP is produced much more than endogenous APP (73).

3.1.2. Tau Models

IT is known that the overproduction of the human Tau protein causes both the formation of hyperphosphoryl Tau and neurofibrillar degradation. Dystrophies neuritis caused by pathological hyperphosphorylated Tau deposition surrounding aged plaques have been demonstrated in models with high plaque burden.

- JNPL3: It is the first model to show NFT formation and related cell loss, it is created by the P301L mutation.
- TAPP: It is a hybrid model. It arose by crossing Tg2576 and JNPL3. This model reveals MAPT pathology in the forebrain more clearly than JNPL3
- Mutant APP/PS1: The first mice used in this model were reported to express mutant APP/PS1 but not overproduce Tau.

In these mice, excessive production of Aβ stimulates hyperphosphorylation in the endogenous mouse Tau but does not induce NFT formation. The reason for this is the low NFT ability of the endogenous mouse Tau (74).

3.2.Non-Transgenic Mouse Model

Non-transgenic models were used to test the 'cholinergic damage' hypothesis, which argues that cognitive impairment in elderly dementia patients is due to cholinergic deficiency. Chemical applications (scopolamine, colchicine, aluminum and p75NTR) or the formation of iatrogenic lesions are used to stimulate cortical cholinergic neuron loss observed in AD. Of the stimulant compounds used, scopolamine, colchicine and although aluminium is non-specific, p75NTR is cholinergic specifically for nerves.

4. Vitamin D

First identified as a vitamin at the beginning of the 20th century, vitamin D is now considered a prohormone. It has been reported that humankind was aware of the substance defined as vitamin D, even in antiquity. A unique characteristic of vitamin D is that besides dietary intake, it can also be synthesized by the human body under the influence of sunlight. In the time since the uncovering of vitamin D, it has been known that vitamin D is not only in bone, but also has its own explicit mechanism composed of transport proteins, metabolic enzymes and the vitamin D receptor (VDR) to arbitrate the actions of vitamin D (75).The presence of vitamins was first

demonstrated by McCollum and Davis (1913) work at the University of Wisconsin (1913) that developed an eye disease, xerophthalmia, which can be treated with butter, oil or cod liver oil, in mice fed a highly purified diet. He observed that it prevents neuritis, called inflammation of the optic nerve, the nerve that provides vision. He described it as "vitamin B". Sir Edward Mellanby, without knowing it, kept the dogs in closed kennels and deprived them of the light of the sun. Mellanby was aware of McCollum, Osborne, and Mendel's experiments and, as a result, he used cod liver oil to process his animals. However, unbeknownst to Mellanby, cod liver oil not only contained vitamin A. It was discovered by McCollum but also contained vitamin D. Mellanby speculated that this was another property of vitamin A, namely the prevention of skeletal rachitic symptoms (76). This revelation ended the quandary that cod liver oil equates to ultraviolet light in curing rickets; sunshine resulted a alteration in the unsaponifiable fraction of the lipid component of the diet to become the antirachitic factor.

4.1.Vitamin D Uncovering

Vitamin D was first discovered during the industrial revolution of the late 1800s, when Britain was confronted with an unprecedented epidemic of rickets. In 1918, Sir Edward Mellanby demonstrated that the disease was caused by nutritional deficiency, and soon young, lean patients were successfully treated with cod liver oil (76).Vitamin D; is one of the fat-soluble vitamins that can be stored in the body for an extended period of time. It is also regarded as a hormone because it is a steroid of endogenous synthesis (77).

Vitamin D, which has been produced since the beginning of life on earth, is called the sun vitamin because most of it (90-95%) is synthesized under the influence of the sun (Akkoyun et al., 2014). 7-dehydrocholesterol (7-DHK), which is the provitamin D3 in the skin, absorbs Ultraviolet B (UVB) rays when exposed to sunlight and converts to previtamin D3 and to vitamin D3 as a result of its thermal photo-isomerization (≥25) °C) (78). There is a great deal of research demonstrating that vitamin D has important health benefits. Vitamin D lowers the risk and severity of autoimmune diseases, cardiovascular diseases, cancer, dementia, type 1 and 2 diabetes, and respiratory tract infections (78). More recently, experimental and preclinical evidence suggests an association between vitamin D status and cognitive function. Human studies strongly support an association

between low circulating levels of 25-hydroxyvitamin D (25 (OH) D) and cognitive disorders or dementia among aging populations.

At the same time, animal studies suggest that vitamin D supplementation protects against biological processes associated with AD and enhances learning and memory in various animal models of aging and AD (79). Regarding vitamin D metabolism in the brain, it has been shown that the hydroxylation active metabolite 1,25 (OH) 2 D3 is synthesized in glial cells by CY P24A1, a mitochondrial monooxygenase that catalyzes reactions including the hydroxylation of vitamin D3, one of the cytochrome P450 enzyme systems.

4.2. Vitamin D Metabolism

Vitamin D is a secosteroid hormone that exists in two main forms according to the source, vitamin D2 (ergocalciferol) of plant origin and vitamin D3 of animal origin (cholecalciferol) are known in two forms. Vitamin D3 can be absorbed photochemically or produced in the epidermis through the effect of UVB on 7-dehydrocholesterol (80). For its activation, it needs to first be processed to 25hydroxycholecalciferol [25 (OH) D] in the liver by the enzyme 25-hydroxylase. The second stage is the conversion of the enzyme 1,25 1α-hydroxylase into dihydroxycholecalciferol [1,25 (OH) 2D] through the kidneys. It's the active part of vitamin D (81).

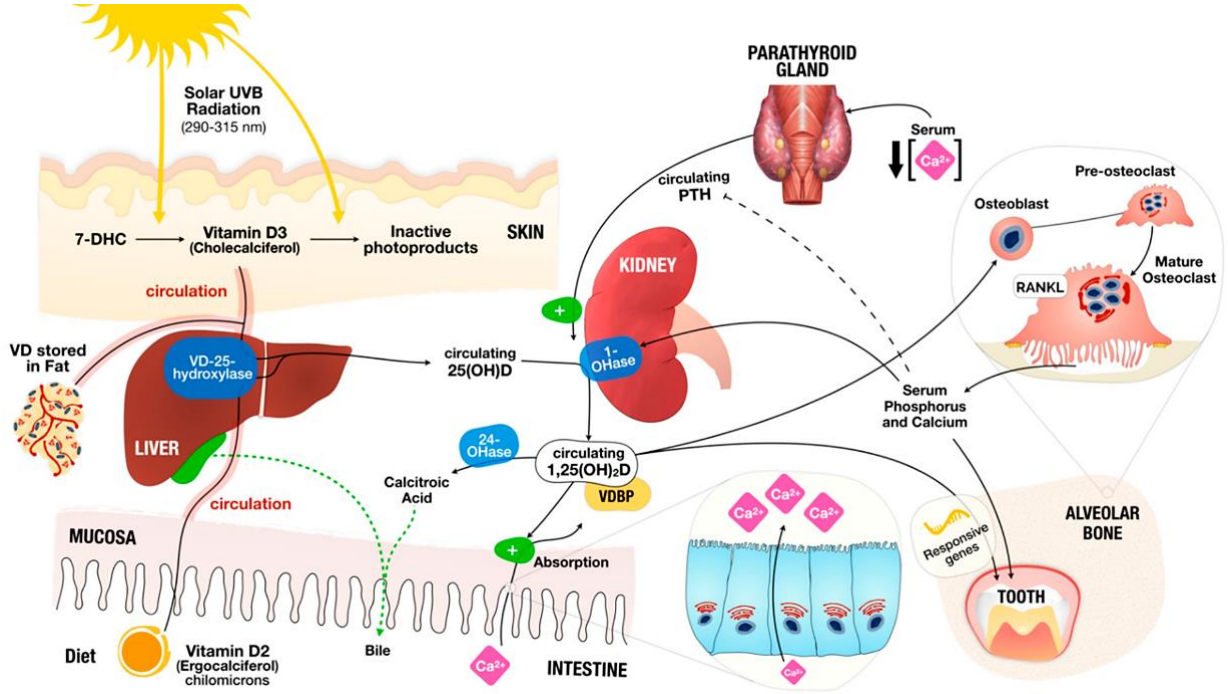


Figure 11. Vitamin D and Calcium, Phosphorus and Bone Metabolism (82).

Vitamin D acts like a hormone and subsidizing serum calcium and phosphate metabolic process through endocrine activity, regulation of internecine assimilation Vitamin D also acts as an autocrine and paracrine agent regulating cell distinction, cell maturation and the innate immune system (83). In target tissues, calcitriol binds to VDR, a member of the nuclear receptor family of ligand-activated transcription factors, inducing both genomic and non-genomic regulation of downstream targets involving a variety of biological functions.

Recently, an alternative pathway for vitamin D metabolism has been reported through CYP11A1, also known as cytochrome side-chain cleavage enzyme P450 (84).

The occupation of the hydroxylase enzyme, which is synthesized from the distal tubules in the kidney, is responsible for the regulation of the balance of parathyroid hormone (PTH), calcium and phosphorus in the blood and body fluids. This also enables calcium to move from bone to blood. PTH induces a decrease in the reabsorption of phosphate in the kidneys, resulting in a decrease in the level of phosphorus in the blood. This. the effect causes an insufficient calcium-phosphor problem in the extracellular space, causing a mineralizing disorder in the newly formed collagen matrix (85).Vitamin D and its metabolites are transported from where they are produced to the target tissues.

The protein binding vitamin D (BPD), which binds to ~85% of the circulating 25OHD and 1.25 (OH) 2D, is mainly responsible for this type of transport (86).

4.2.Vitamin D Receptor

VDR, which is a vitamin D receptor, is within the nuclear receptor family of intracellular steroid receptors. Consequently, only free vitamin D which can enter the cell can bind to the receptor and demonstrate biological activity. VDR regulates the expression of many genes implicated in calcium/phosphate homeostasis, cell proliferation and differentiation, and immune response (87). Accurate identification of VDR in tissues is of swell significance to understand the physiopathological significance of vitamin D. It is known that calcitriol triggers numerous metabolic belongings in the body with intracellular VDR. Studies have demonstrated that the VDR is present in many target cells of the body and that it performs its functions with the enzyme 1α-hydroxylase (88). Calcitriol produces metabolic effects in the organism through intracellular VDR. Studies have demonstrated that the VDR is present in many target cells of t he organism and performs its functions with the enzyme 1α-hydroxylase. VDR is a transcription factor that has a strong affinity with calcitriol and belongs to the family of nuclear receptors of intracellular steroid receptors (89).It is also a proseous nuclear receptor found in the prostate, ovaries, breast and skin, as well as in the brain, heart, pancreas, kidney, intestine and colon. VDR mediates the biological effects of its ligand by regulating the transcription of target genes (90). Since the expression levels of VDR in various cells and tissues are greatly variable in the research transported out, the idea that it can change with the differentiation of the cell is still being investigated.

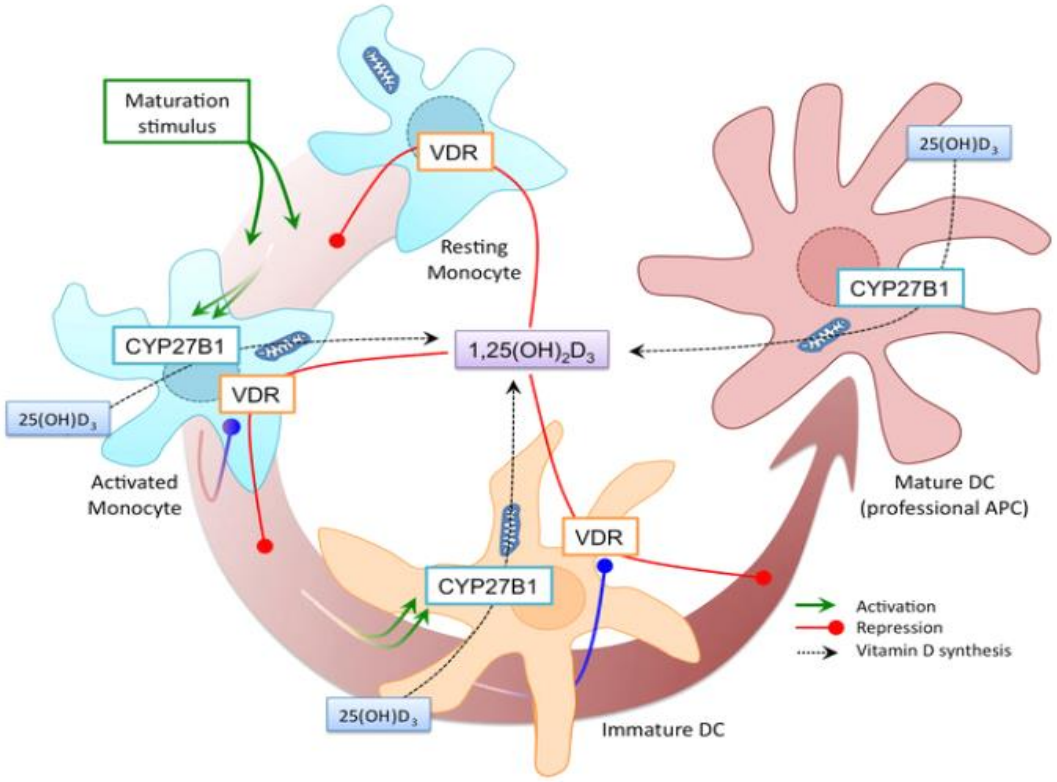


Figure 12 . Regulation of vitamin D receptor (VDR) and 25-hydroxyvitamin D3 1-α-hydroxylase (CYP27B1) synthesis during maturation of antigen presenting cells (APC) (91).

4.3.D vitamin and Alzheimer

The evidence demonstrates the role of vitamin D in regulating the development and function of nerve cells and, in this regard, the potential impacts of vitamin D deficiency. The participation of vitamin D in central nervous system reception is supported by the presence of the vitamin as well as the enzyme 25 (OH) D3-1a-hydroxylase, which is responsible for the formation of the active form of vitamin D. VDR receptors in the brain appear mainly in the hypothalamus and dopaminergic neurons of the substantia nigra. Vitamin D has been described as having varying roles in cell proliferation, differentiation, neurotransmission and neuroplasticity in the neurological system. In the last decade, it has been argued that vitamin D is classified as a neurosteroid termed steroid hormones synthesized in the brain or nervous system, and the effects of its different functions in the brain have been started to be investigated(92). Concerning vitamin D metabolism in the brain, it has been shown that the active metabolite 1,25 (OH) 2 D3 is synthesized in glial

cells by the hydroxylation process, which is one of the cytochrome P450 enzyme systems, CY P24A1. Studies have shown that the expression of VDR is high in pyramidal cells located in the CA1 and CA2 regions of the hippocampus, which are important in cognitive functions(93). 25-hydroxyvitamin D (25OHD), a metabolite of vitamin D, is a serum biomarker of vitamin D. In particular, this predisposition to lower 25OHD levels in the elderly has been associated with a higher risk for numerous age-related disorders, including cancer and metabolic and vascular diseases(94).Vitamin D deficiency has been inferred as a higher risk of cognitive decline in older people, and a normal level of vitamin D may support healthy brain aging. The protective action of vitamin D on the nervous system catches the eye. Specifically, the synthesis of nerve growth factor (NFG), a neuropeptide that is primarily involved in the regulating of growth, advancement, proliferation, and survival of explicit target neurons, is regulated by Vitamin D. In addition to all these, Vitamin D also plays a role in the regulation of the synthesis of some other neurotrophins (95).

Vitamin D also interferes with the synthesis of inducible nitric oxide synthase (iNOS). When iNOS is expressed at elevated levels, nitric oxide is generated, damaging neurons and oligodendrocytes. It is worth noting that the increase in the level of iNOS in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and multiple sclerosis is an important factor in this context(96). A shortage of vitamin D can be a risk factor for AD. A meta-analysis from 2019 found that vitamin D deficiency (<10 ng/mL) was positively correlated with AD risk, and each 10 ng/mL vitamin D supplementation could reduce AD risk by 17% (97).

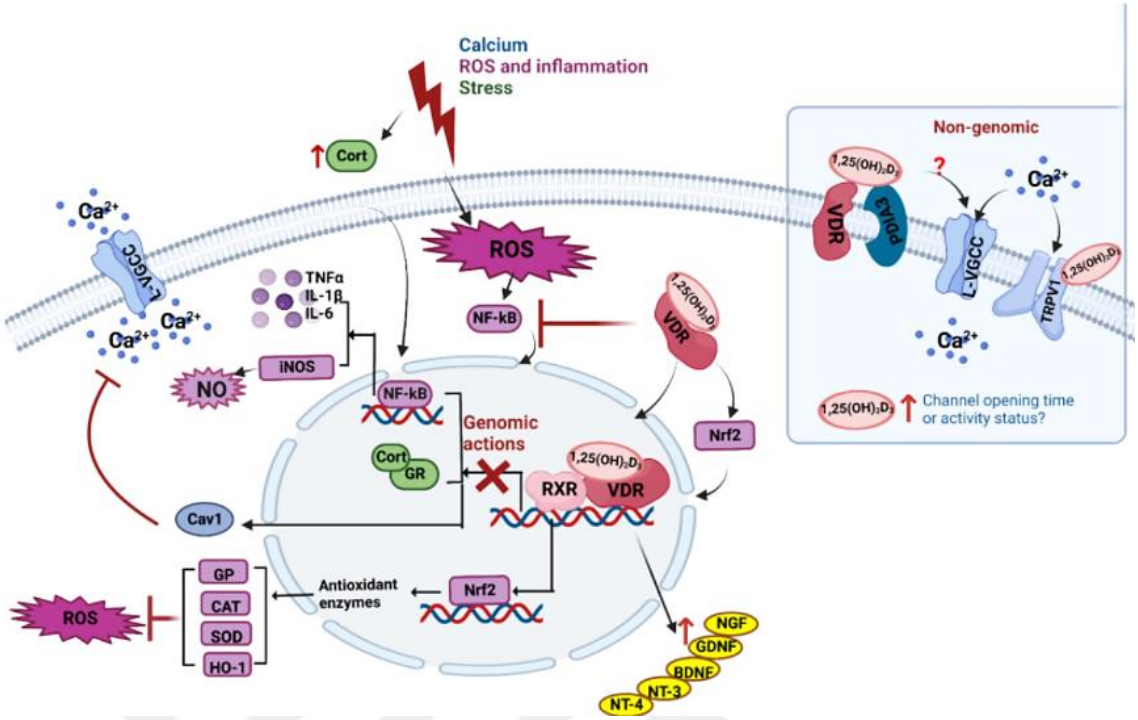


Figure 13. Neuroprotective actions of vitamin D in brain**. (**97)

The presence of 25 (OH) D3 and 1.25 (OH) 2D3, which are metabolites of vitamin D, has been detected in adult spinal fluid (98). In a consideration conducted on rats, an increase in the number of VDRs in the subventricular region was observed in vitamin D intake and it was found that vitamin D has a regulatory function in cell proliferation. In a study conducted on rats, an increase in the number of VDRs in the subventricular region was ascertained in vitamin D intake and it was determined that vitamin D has a regulatory role in cell proliferation. In some other study in rats, vitamin D intake augmented the diameter of the axon and enhanced neural sensor responses. It was pointed out that vitamin D in particular can be used as a therapeutic agent in nerve lesions, as it has a potential effect on axonal regeneration (99, 100). Our study is based on the fact that vitamin D may have a protective effect in the Alzheimer-like dementia model and reduce pathological symptoms.

5. MATERIAL and METHODS

5.1. Materials

5.1.1. Chemicals and Reagents

- Solution (37 %) (Merck)
- Cresyl violet acetate (Santa Cruz Biotechnology)
- DAB Chromogen (Thermo Fisher Scientific)
- DAB Substrate Buffer (Thermo Fisher Scientific)
- Di-sodium hydrogen phosphate (Merck)
- Entellan mounting medium (BioMount)
- Hydrogen peroxide solution 30 % (Sigma)
- Mayer's Hematoxylin (Thermo Fisher Scientific)
- Phosphate buffered saline tablets (Sigma)
- Saline (Isotonic 9 % NaCl) (Polifarma)
- Sodium chloride (Sigma)
- Sodium dihydrogen phosphate (Sigma)
- Congo Red (Merck)
- Tri-sodium citrate dihydrate (Merck)
- Triton X-100 (Roche)
- Xylene (Sigma)

5.1.2. Antibodies

- iNOS (Abcam iNOS,2977S)

5.1.3. Commercial Kits

- In situ cell death detection kit, POD, Roche (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)

5.1.4. Laboratory Equipments

- M-polylysin slides (25*75*1,0 mm) (Thermo Scientific)
- Propylen centrifuge tubes; 50 mL, 15 mL, 2 mL,0.5 mL (Isolab)

5.1.5. Laboratory Technical Instruments

- Automatic Glass Cover slipper (Leica)
- Stereological workstation and light microscope (Leica)
- Incubator (Memmert)
- Locomotor activity cage (Commat, MAY)
- Microscope (Leica)
- pH meter (Hanna instruments PH21)
- Staining Automat (Leica)
- Microtome
- Tissue embedding automat
- Tissue Processing automat

5.2.Methods

5.2.1. Experimental Setup and Groups

10 week old male C57BL/6 albino rats (17-20g) were obtained from Yeditepe University. Experimental Research Center (YUDETAM). All of the experiments were approved by the. Ethics Committee of Yeditepe University Experimental Research Center and the use of animals was in observance with the US National Institutes of Health Guide for Care and Use of Laboratory Animals. The animals were kept in standard accommodation weather conditions with constant temperature, humidity, 12-hour light/dark cycles and free access to food and water. 220 mice, 5 in each group, were randomly divided into 4 experimental groups as follows (101);

1. Control group (K) (n=5) IP 1.5 mg/kg/day, injected with saline intraperitoneally (IP),
2. IP as scopolamine injected group (SC) to Alzheimer's dementia model (n=5), IP 1 mg/kg/day,
3. Vitamin D injected group (Dvit) IP as 10,000IU/day and

Vitamin D and scopolamine injected (n=5), 10,000IU/day scopolamine and 1 mg/kg/day scopolamine injected IP as the

Table 1. The experimental groups and number of animals.

Groups	Numbers
IP saline injected control group (C)	n=5
IP scopolamine injected group (AD)	n=5
IP vitamin D injected group (DVİT)	n=5
IP scopolamine and vitamin D injected group (AD+DVİT)	n=5

All substances were injected for 21 days and on the last day of injections locomotor activity test is conducted for all rats. At the end of 21 days of injections all animals were dissected, and brain tissues were dissected.

5.2.2. Drug Treatment

Drug administration groups; Intraperitoneal saline in group C, scopolamine 1.5 mg/kg/day intraperitoneally for mice in group AD, 10,000 IU/day vitamin D intraperitoneally for the vitamin D group, 1.5 mg/kg/day intraperitoneal Scopolamine and 10,000 IU/day for AD + DVIT group Vitamin D were injected. This drug administration was injected for 21 days (101). Scopolamine was dissolved in saline. Vitamin D was delivered directly via the intraperitoneal route.

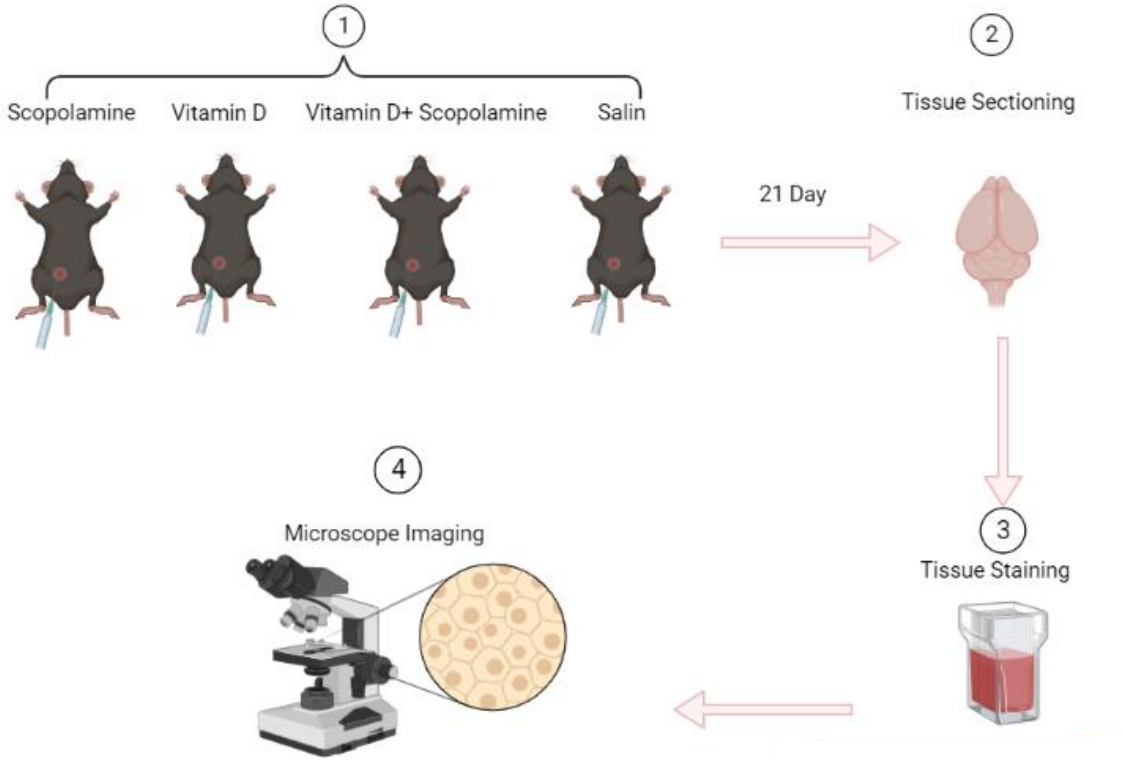


Figure 14. Experiment Plan

5.2.3. Sacrification of the Animals

Subsequently the locomotor activity test, the animals were decapitated, their brains were dissected, the hippocampus and cerebral cortex regions were separated and placed in 10% neutral formaldehyde solution for histological analysis (102).

5.2.4. Spontaneous Locomotor Activity

Before the animals were beheaded, the animal activity monitoring system (MAY - Activity Monitoring System - Commat Ltd., TR) was used to record measurements of motor activity (Figure 15). Infra-red light sources found on each side of the rectangular

frame (45x45x30 cm) in the locomotor activity cage. Electric mechanical meter placed on the floor of the cage. Moves of animals disrupt the infrared beams and electromechanical counters count them as locomotor activity. Each mouse was individually placed in the cage and locomotor activity was recorded for 5 minutes. All records were performed between 10 a.m. and 4 p.m. The cage was cleaned using a wet towel prior to each registration. Stereotypical, ambulatory, vertical, horizontal motor activities and distance traveled by the animals were measured (103).

Figure 15. Spontaneous locomotor activity

5.3. Histological Techniques

5.3.1. Tissue processing

All brain tissue was put into cassettes and placed in the tissue processor and followed the steps below which are outlined in(Table 2);

Table 2. Steps of Tissue Processing

Tissue Processing	Tissue Processing	Time
Fixation	-Neutral formaldehyde solution I -Neutral formaldehyde solution II	-2 Hours -2Hours
Dehydration ↓	1. 70% Alcohol 2. 80% Alcohol 3. 90% Alcohol 4. 96% Alcohol 5. 96% Alcohol 6. 100% Alcohol	-2 Hours -2 Hours -2 Hours -2 Hours -2 Hours -2 Hours
Clearing	-Xylene I -Xylene II	-1.5 Hours -1.5 Hours
Paraffin Impregnation	-Paraffin I -Paraffin II	-3 Hours -3 Hours

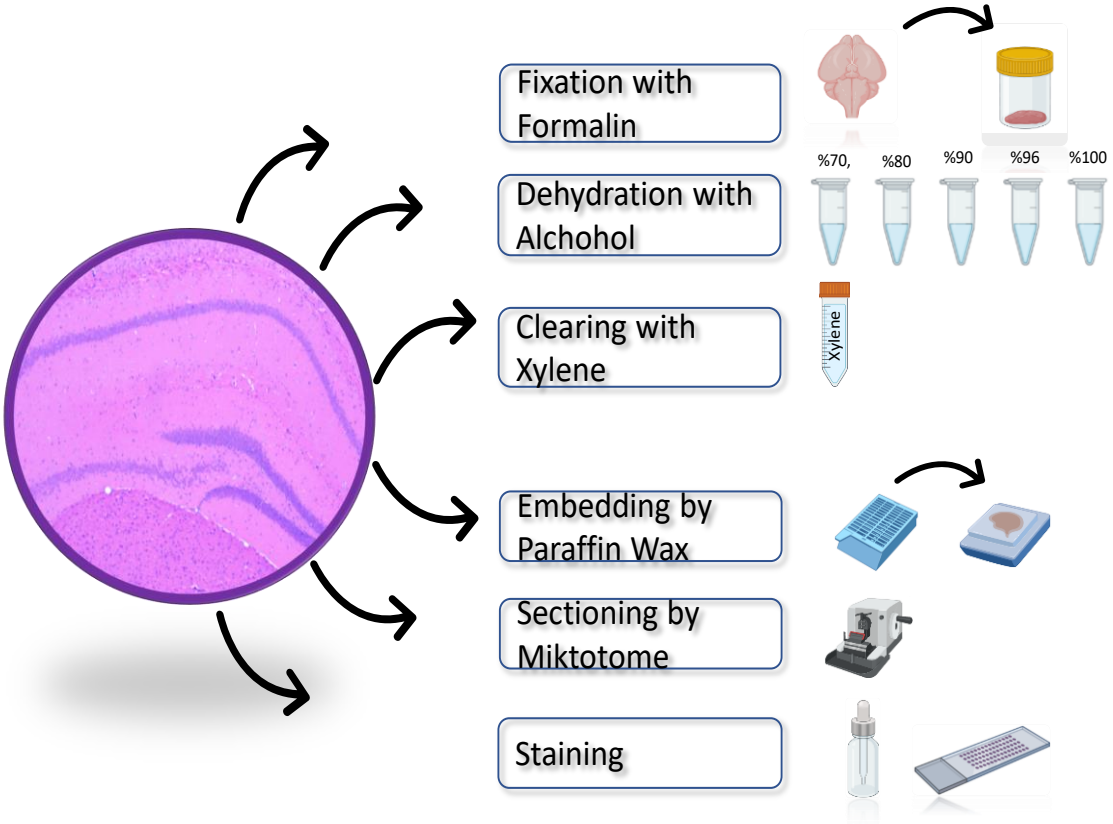


Figure 16 . Tissue Processing.

5.3.1.1.A. Fixation

The fixative solution (10% neutral buffered formalin (pH 7.4) was preprepared. 4g monobasic sodium phosphate (NaH2PO4) and 6.5g dibasic sodium phosphate (Na2HPO4) were weighed and dissolved in 900 ml of distilled water. 100 mL formaldehyde (37%) was added to the solution to have a final volume of 1000 mL. The tissues were secured for 4 hours in a neutral formaldehyde solution (104).

5.3.1.1.B. Dehydration

The tissues were dehydrated in ascending alcohol series (70%, 80%, 90%, 96%, 96%, and 100%) for 2 hours each.

5.3.1.1.C. Clearing

The tissues were cleared in xylene I and II for 1.5 hours each.

5.3.1.1..D. Paraffin Impregnation

All tissues were placed in paraffin for 3 hours. In the end, they are embedded in paraffin blocks with the aid of a tissue embedding device.

5.3.1.1.E. Sectioning

All paraffin blocks were placed in a microtome and 5 micrometer sections were acquired on the slides.

5.3.2. Hematoxylin Eosin Staining

Hematoxylin and eosin staining is one of the most elemental types of tissue staining used in histology. Hematoxylin is dark blue-purple and stains nucleic acids in a poorly understood reaction. Eosin is pink, staining the proteins in a non-specific way. In a typical tissue, the nuclei are blue-black, and the cytoplasm and extracellular substance are coloured in various shades of pink (105). Hematoxylin is a positively charged basic dye, in contrast, eosin dye is negatively charged. The staining machine for Hematoxylin Eosin Staining was followed by the protocol described below;

1) The sections were dried in an incubator at 75oC for 20 minutes.
2) Then the sections were cleared in xylene I and II for 1 minute each.
3) The slides were hydrated in descending alcohol series (100%, 96%, 96% ,90%) for 2 minutes each.
4) All sections were washed with distilled water.
5) The sections were stained with Mayer's hematoxylin solution for 5 minutes and rinsed in water for 2 minutes.
6) The slides were decolorized in acid alcohol (396 mL 70% ethanol was mixed with 4 mL glacial acetic acid) for 1 minute.
7) The slides were washed in tap water for 1 minute.
8) The sections were subjected to 80% ethanol for 2 minutes.
9) Then the sections were counterstained with Eosin for 3 minutes.
10) The sections were dehydrated in ascending alcohol series (90%, 96%, 96%, 96%, and 100%) for 1 minute each.
11) Finally, the slides were cleared in xylene I and II for 2 minutes each.
12) The slides were coverslipped in entellan mounting medium using an automatic glass coverslipper.

5.3.3. Cresyl Violet Staining

Cresyl violet is commonly used to show the Nissl substance in neurons and cellular nuclei. It shows the specific structure of neurons. Due to its RNA content, Nissl substance is very basophilic and stains very strongly with basic aniline dyes. Cresyl violet is a base dye and colours the Nissl substance in the cytoplasm of dark blue neurons. Preparation of solution A with sodium acetate dissolved in distilled water. For solution B, glacial acetic acid dissolved in distilled water. The two solutions were then mixed and the pH adjusted to 3.8-4.0 with acetic acid. Cresyl violet acetate was dissolved in this solution and left overnight on a magnetic stirrer in the dark. Before staining the sections, the staining solution was filtered with filter paper. All solutions were placed in copulin jars, and the protocol described below was followed (106);

1) The sections were dried in an incubator at 75°C for 20 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 1 minute.

4) The sections were stained with Cresyl Violet for 20 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 1 minute each.

7) Finally, the slides were cleared in xylene I and II for 2 minutes each.

8) The slides were coverslipped in entellan mounting medium using an automatic glass coverslipper.

5.3.4. Congo Red Staining

Congo red is an organic component which is the sodium salt of 3,3'-bis. It's an azoic stain. Congo red can be dissolved in water. Staining with Congo Red (CR) is a qualitative method used for the identification of amyloids in vitro and in tissue sections. It is a highly selective dye of Congo red dye for amyloid (107). Due to their small molecular size and penetration through the blood-brain barrier, Congo red may be used for ante-mortem and in vivo visualization and quantification of amyloid in the brain. It is the result of the binding affinity of Congo red with enriched fibril proteins in the β-sheet conformation.

In our study, we used Congo Red stain, which has higher affinity for amyloid (108). All solutions were placed in copulin jars, and the protocol described below was followed ;

1. Congo Red solution;
    i. Sodium chloride 2 g
    ii. Dissolved in deionized water 32 ml
2. Congo Red powder 0.5 g ;
    i. Suspended in 100 % absolute alcohol 68 ml
3. Combine the two solutions (Store at room temperature for months)
4. II. Alcohol 50 %
    i. reagent alcohol ~50ml
    ii. deionized water ~50ml
5. III. Alcohol 70 %
    i. reagent alcohol ~70ml
    ii. deionized water ~30ml
6. IV. Alcohol 80 %
    i. reagent alcohol ~80ml
    ii. deionized water ~20ml
7. Alcohol 95 %
    i. reagent alcohol ~95ml
    ii. deionized water ~ 5ml

Staining Procedure ;

1. Place the coverslip with section in a ceramic staining rack .

2. Immerse sections in Harris Hematoxylin for 3 – 4 dips.

3. Wash with tap water until the water is clear.

4. Filter the Congo Red solution into a Columbia staining dish

5. Place coverslips with sections in the Columbia staining dish with the filtered Congo Red solution for 3 minutes at room temperature.

6. Wash with several exchanges of deionized H_2O.

5.3.5. Immunohistochemistry for iNOS

Nitrous oxide is an inorganic compound that plays a major role in neurotransmission and cytotoxicity. Nitric oxide syntheses constitute a family of enzymes that catalyze the production of nitric oxide from L-arginine. Overproduction of reactive species, particularly reactive oxygen (ROS) and nitrogen (RNS) species, and the imbalance of the body's antioxidant enzyme systems cause the destruction of cellular structures, lipids, proteins, and genetic material such as DNA and RNA. Moreover, the effects of reactive species on mitochondria and their metabolic processes lead to the oxidation of mitochondrial proteins, lipids and DNA, ultimately leading to an increase in ROS/RNS levels (109). NO is a significant cell signaling molecule. Inducible nitric oxide synthase (iNOS) is one of these syntheses, but the abnormal amount of its induction can cause damage to cells and advance the pathology of diseases such as Alzheimer's Disease. Five solutions were prepared in advance. At first, 3% hydrogen peroxide solution was prepared with 1ml 30% Hydrogen peroxide stock solution and 99 ml PBS. After that, 0,1 g tri–Na Citrate x 2H2O and 0,1 ml Triton X-100 were dissolved in 100 ml distilled water in order to prepare 100 ml 0.5 M permeabilization solution. Next, 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 (retrieval solution). Furthermore, to have a washing solution, one phosphate-buffered saline (PBS) tablet (Sigma) was dissolved in 100 mL distilled water. Finally, 10 µl DAB was added to the 200 µl substrate and kept on ice.

All 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 20 minutes.

2. The sections were placed in xylene solution for 20 minutes at room temperature.

3. The slides were then rehydrated in descending alcohol series (100%, 96%, 90%, 80%, and 70%) for 3 minutes each.

4. Then the slides were washed with PBS for 3 minutes at room temperature.

5. The slides were incubated in permeabilization solution for 8 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 1 min for antigen unmasking.

8. Then the slides were washed with cold PBS for 5 minutes.

9. Then the slides were washed with PBS for 5 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 5 minutes.

12. All specimens were incubated in blocking solution (2.5% RTU Normal Horse Serum VECTASTAIN KIT) for 30 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 5 minutes.

16. Secondary antibody (RTU Biotynlated Universal Antibody Anti-Rabbit/mouse IgG) was dropped on all sections and incubated at room temperature for 30 min in a humidified chamber.

17. Then the slides were washed with PBS for 5 minutes.

18. Vectastain RTU ABC Reagent was dropped on all sections and incubated at room temperature for 30 min in a humidified chamber.

19. Then the slides were washed with PBS for 5 minutes.

20. All slides were incubated in DAB chromogen & peroxidase substrate mixture for 3 min.

21. Then the slides were washed with PBS for 5 minutes.

22. Each specimen was counterstained with Gill's hematoxylin for 4 min, then rinsed in tap water.

23. Sections were dehydrated at in 100 % for 2 min and cleared in xylene for 2 min.

24. The slides were coverslipped in entellan mounting medium with the aid of an automatic glass cover slipper.

5.3.6. TUNEL Assay: (In situ Cell Detection Kit, Pod) Roche

TUNEL assay is a reliable method to detect apoptotic neuron death. DNA double-strand breaks which occur during apoptosis are precisely labeled 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 POD and substrate reaction . The TUNEL test makes it possible to detect apoptosis in tissue sections in situ(110). TUNEL is the abbreviation for the final labelling of deoxynucleotidyl transferase biotin-dUTP. The protocol explained below was followed ;

1) The sections were dried in an incubator at 75°C for 20 minutes.

2) The sections were placed in xylene solution for 20 minutes at room temperature.

3) The slides were then rehydrated in descending alcohol series (100%, 96%, 90%, 80%, and 70%) for 3 minutes each.

4) Then the slides were washed with PBS for 1 minute at room temperature.

5) The slides were placed in a citrate buffer solution in a 750-watt microwave for 1 min.

6) Then the slides were washed with cold PBS for 1 minute.

7) Then the slides were washed with PBS for 1 minute at room temperature.

8) A hydrophobic barrier was made around the tissue with a barrier pen. The slides were incubated in a blocking solution (normal horse serum) in a humidified chamber at room temperature for 30 minutes.

9) Then the slides were washed with PBS for 1 minute at room temperature.

10) TUNEL reaction mixture (45 µl label solution was mixed with 5 µl enzyme solution for one section) was prepared. The sections were incubated in a TUNEL reaction mixture in dark, 37°C humidified chambers for 60 minutes.

11) Then the slides were washed with 1X PBS three times for 5 minutes each.

12) Converter-POD was added to the sections and incubated in dark, 37°C humidified chambers for 30 minutes.

13) Then the slides were washed with 1X PBS three times for 5 minutes each.

14) The reaction was visualized by using DAB (1000 µl DAB substrate buffer was mixed with 50 µl DAB chromogen) solution as a chromogen.

15) Then the slides were washed with 1X PBS three times for 5 minutes each.

16) The sections were counterstained with Mayer's hematoxylin and rinsed in tap water.

17) Sections were dehydrated in ascending alcohol concentrations, cleared in xylene.

18) The slides were coverslipped in entellan mounting medium

5.3.Analysis by Light Microscopy

Each section was examined under a light microscope (Leica DM 4000B, Wetzlar, Germany) and photographed with a CCD digital camera (Optronics Microfire 1600x1200P, Goleta, CA, USA) with the help of LAS Version 4.1.0 program. Sections stained with hematoxylin and eosin, silver and cresyl purple were evaluated histopathologically. H-score analysis was performed on iNOS stained sections. Finally, the apoptotic index was calculated based on the analysis of the Tunnel assayed sections.

5.4.H-score Analysis for iNOS,

The H score ("histo" score) is obtained by multiplying the coloration ratio by an ordinal value corresponding to the intensity level. Three sections of each animal were noted as percentages of coloration intensity from 0 to 3 grades (0 = absent, 1 = mild, 2 = moderate, 3 = severe) to calculate the H score. The percentage of cells at each dye strength level is calculated. Percentages and degrees are multiplied by the following formula;

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

A final score between 0 and 200 gives more relative numbers to indicate the intensity of coloration in a sample tissue. Then, the average of all partitions is taken (111).

5.5.Apoptotic Index Calculation for TUNEL Assay

To calculate the apoptotic index, each part of each animal was subdivided into five fields and apoptotic cells and normal cells were enumerated in each field. Subsequently, apoptotic cells are divided into apoptotic normal cells to obtain the apoptotic index (112).

5.6.Statistics

GraphPad Prism®6 was used as a statistical analysis software. All data were analyzed as mean ± SEM. The Shapiro-Wilk normalcy test was used to determine the normal distribution of the data. The data were considered meaningful if the P values were less than 0.05. The Tukey Multiple Compare test was used to compare all data in the group.

6. RESULT

6.1. Spontaneous Locomotor Activity

To examine the effects of vitamin D on animal motor activity, animal locomotor activities were measured using a motor activity surveillance system. Stereotyped, ambulatory, vertical and horizontal motor activities and distance traveled by the animal were measured.

6.1.1. Distance Travelled

In this test, which was performed to measure the distance covered, the distance traveled by the animals in the AD group was considerably reduced compared to the C group, as expected in the examinations of the animals before decapitation ($p<0.01$). Similarly, animals in the AD + DVIT groups also experienced a decrease in locomotor activity, ie compared to group C. The distance traveled by the animals in the DVIT and C groups was significantly closer, in fact, the distance traveled by the animals in the DVIT group was higher. Considering the last measurements of the animals before decapitation, it is seen that the distance traveled by the animals is the lowest in the AD group compared to the DVIT, AD+DVIT and C groups. In other words, animals in the AD DVIT group experienced an increase in locomotor activity when they received DVIT supplementation over the AD group, though statistically non-significant. Based on the control group, there is significance in the AD and AD + DVIT groups.

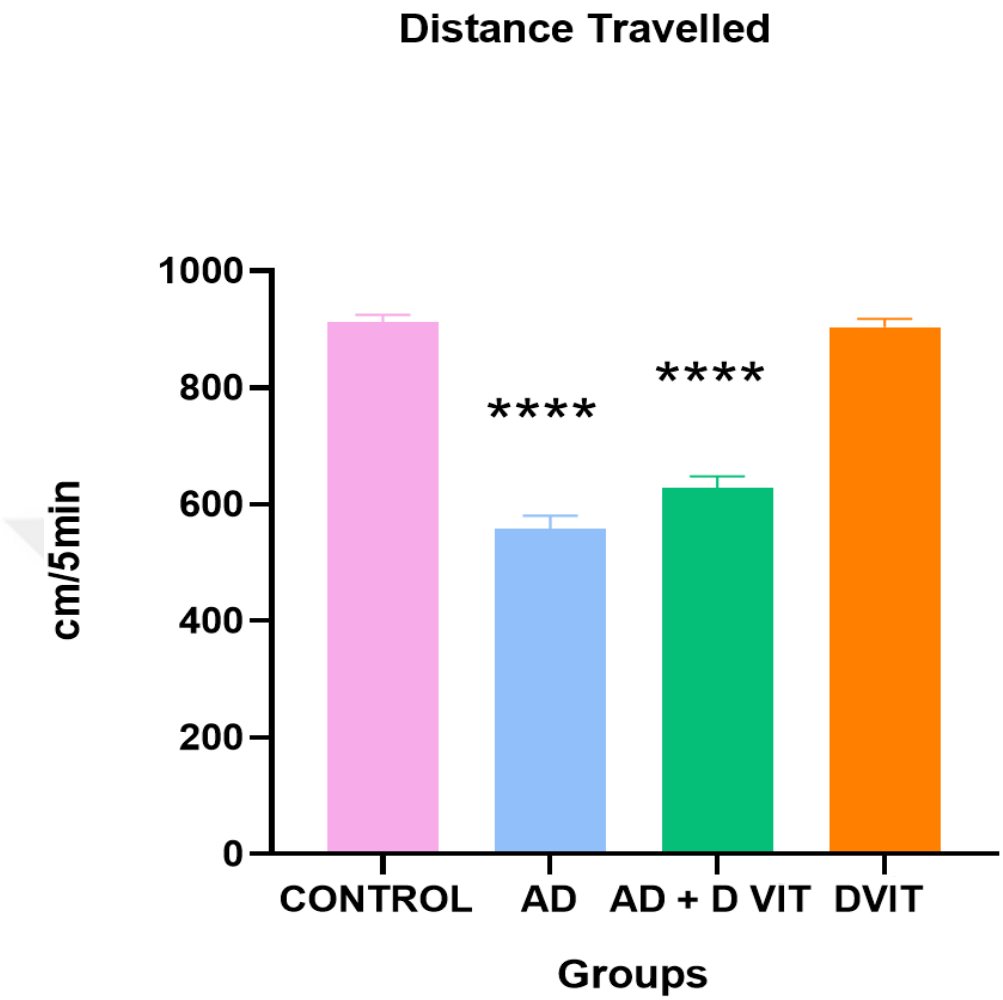


Figure 17. Charts comparing distance traveled by animals**.**
Saline administered control group (C), vitamin D injected (DVIT), scopolamine injected (AD), scopolamine and vitamin D injected (DVIT+AD) groups (****p<0.0001 Compared with the control group.)

6.1.2. Stereotypic Activity

As expected, animals in Group AD had the lowest stereotypical activity compared to Groups C, DVIT and AD+DVIT ($p<0.001$, $p<0.0001$ and $p<0.05$, respectively). However, after DVIT supplementation, it was observed that the stereotypical activities of the animals in the AD+DVIT group increased compared to the AD group ($p<0.05$). Although not statistically significant, the stereotypical activity of animals in the

AD+DVIT group was lower than that of group C. In addition, after DVIT supplementation alone, stereotypical activity was observed in animals compared with Group C, without any statistical significance.

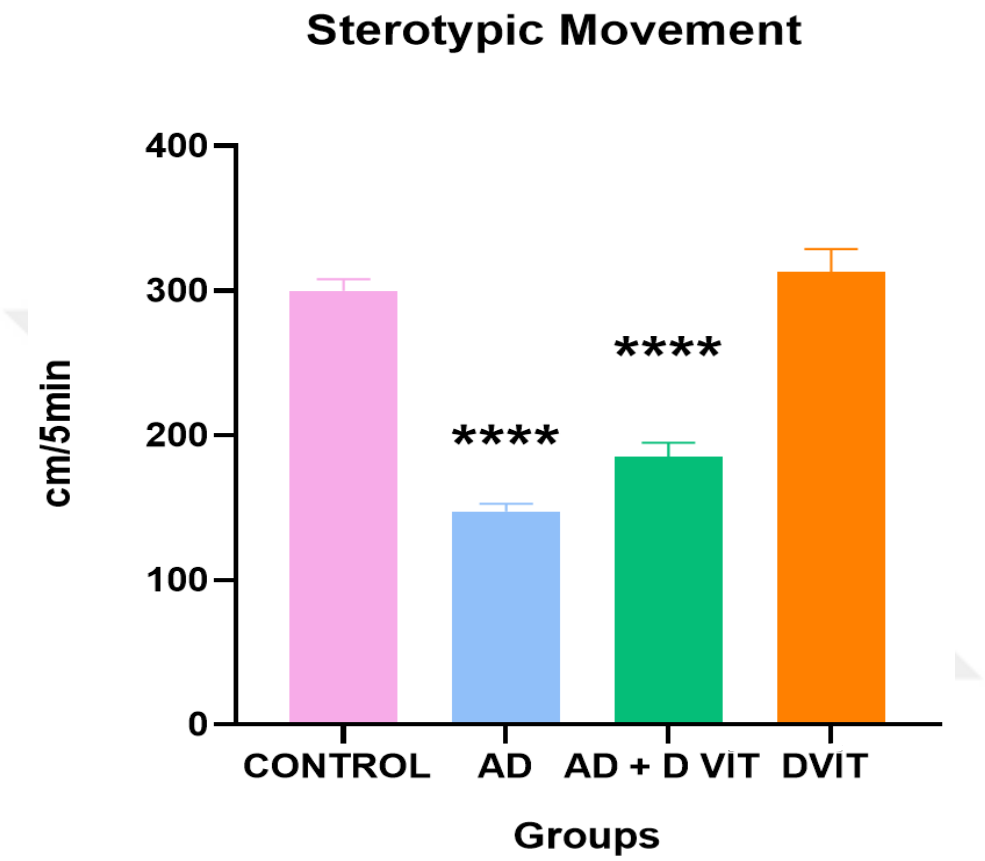


Figure 18 : Charts comparing stereotypical movements of animals**.**
Saline administered control group (C), vitamin D injected (DVIT), scopolamine injected (SC), scopolamine and vitamin D injected (AD + DVIT) groups. Data are presented as the number of stereotypical movements in 5 minutes. (****p<0.0001 Compared with the control group.)

6.1.3. Ambulatory Activity

Ambulatory activity in the AD group decreased significantly relative to Group C ($p<0.01$). Ambulatory activities of DVIT and AD+DVIT animals also declined slightly compared to Group C, but statistical significance was not calculated for both groups. Animals in the AD group had lower rates of outpatient activity compared to the DVIT

and AD DVIT groups ($p<0.01$, $p<0.05$, respectively). However, following DVIT supplementation, outpatient activity in the AD DVIT group increased significantly relative to the AD group ($p<0.05$).

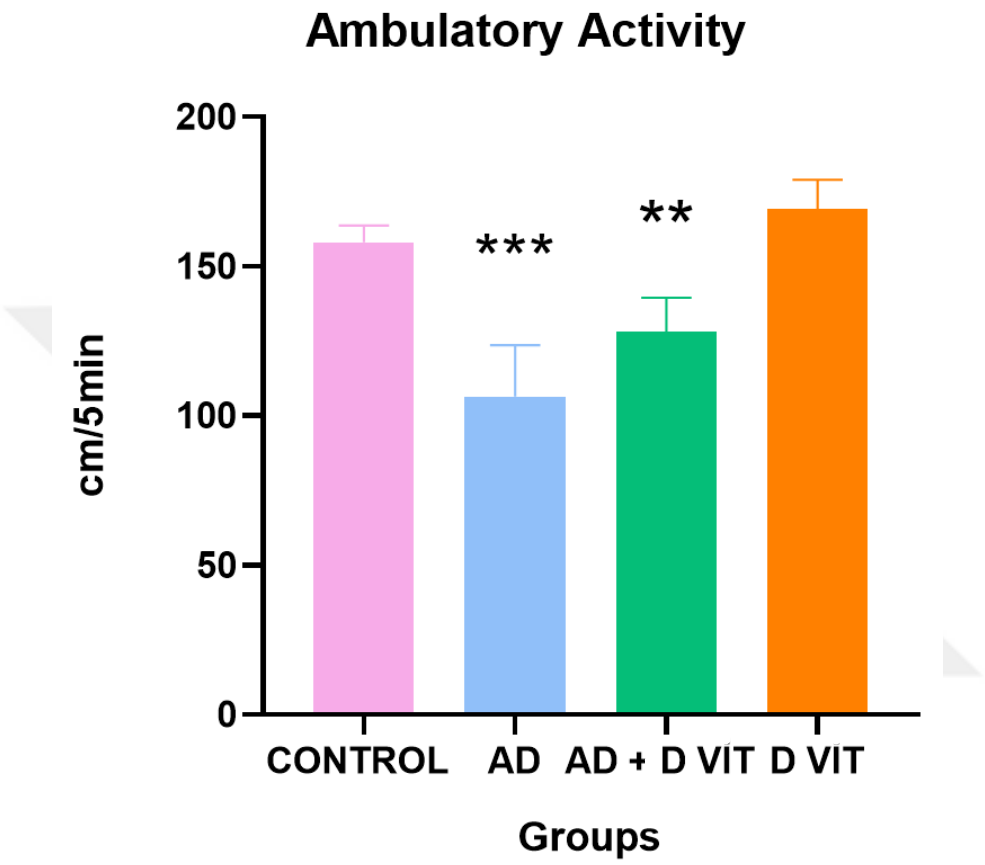


Figure 19. Charts comparing Ambulatory movements of animals**.**
Saline administered control group (C), vitamin D injected (DVIT), scopolamine injected (AD), scopolamine and vitamin D injected (AD + DVIT) groups.(***p<0.001, **p<0,01 Compared with the control group.)

6.1.4. Horizontal Activity

Based on pre-decapitation measurements, horizontal activity in the AD group decreased as needed compared to group C ($p<0.0001$). Similarly, there was a slight decrease in horizontal activities of the DVIT and AD DVIT animals relative to Group C (NS, $p<0.01$, respectively). The AD DVIT group was less horizontal than the DVIT group ($p<0.05$). On the other hand, treatment after DVIT supplementation increased horizontal

activity in the AD+DVIT group, resulting in a higher horizontal activity rate than the AD group, but statistical significance was not calculated.

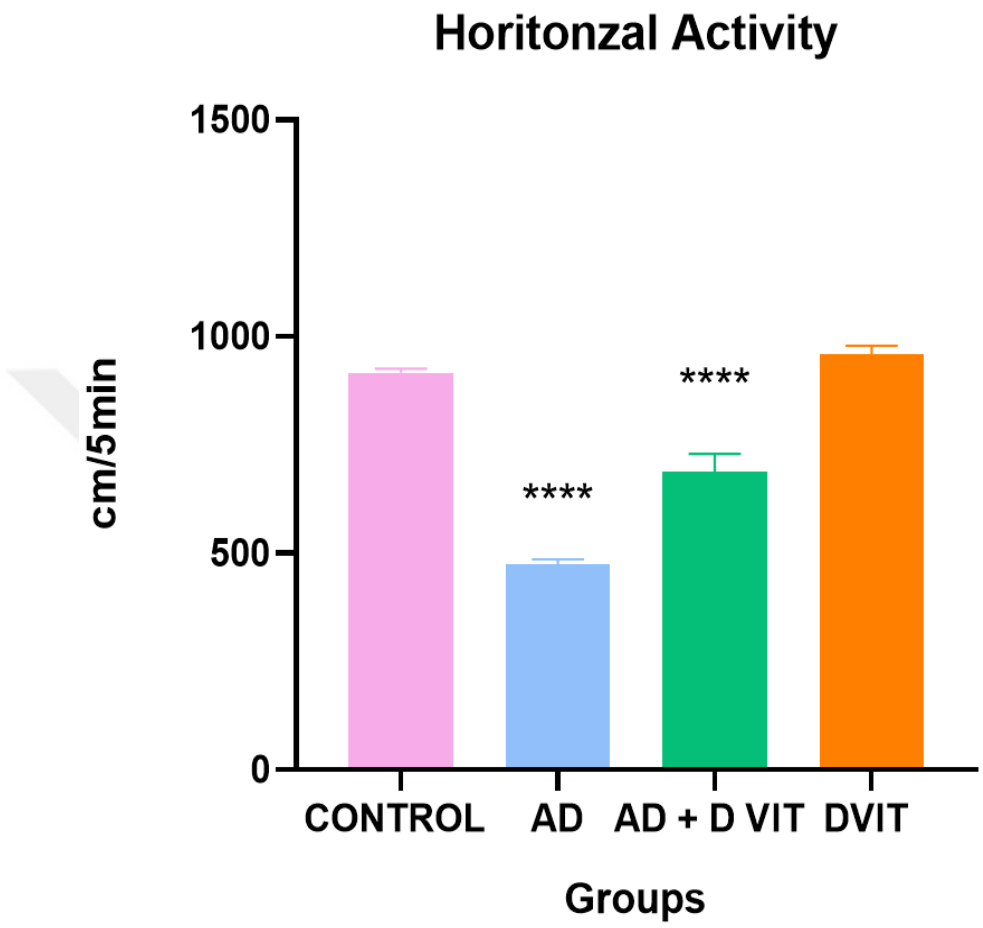


Figure 20. Graphs comparing horizontal movement of animals**.**
Saline administered control group (C), vitamin D injected (DVIT), scopolamine injected (AD), scopolamine and vitamin D injected (AD + DVIT) groups (****p<0.0001 Compared with the control group.)

6.1.5. Vertical Activity

As expected, the AD group had the lowest level of vertical activity relative to Group C, DVIT and AD DVIT . No significant differences were found between Group C and DVIT, and statistical significance was not calculated. However, when the DVIT group and the AD+DVIT group are compared, it is seen that the animals in the AD+DVIT group have lower vertical activity than the animals in the DVIT group ($p<0.05$). While adding

DVIT slightly increased the vertical activity ratio of AD group DVIT comparisons, the calculated statistical significance was not significant.

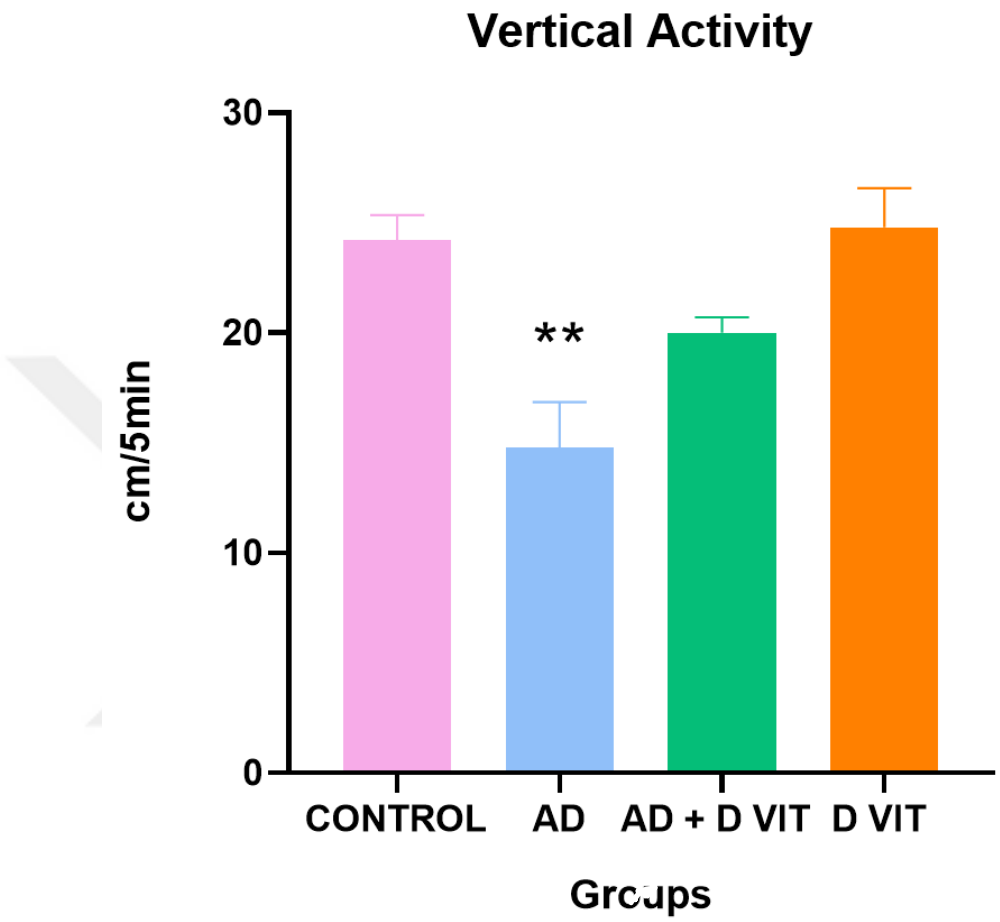


Figure 21. Graphs comparing Vertical movement of animals**.**

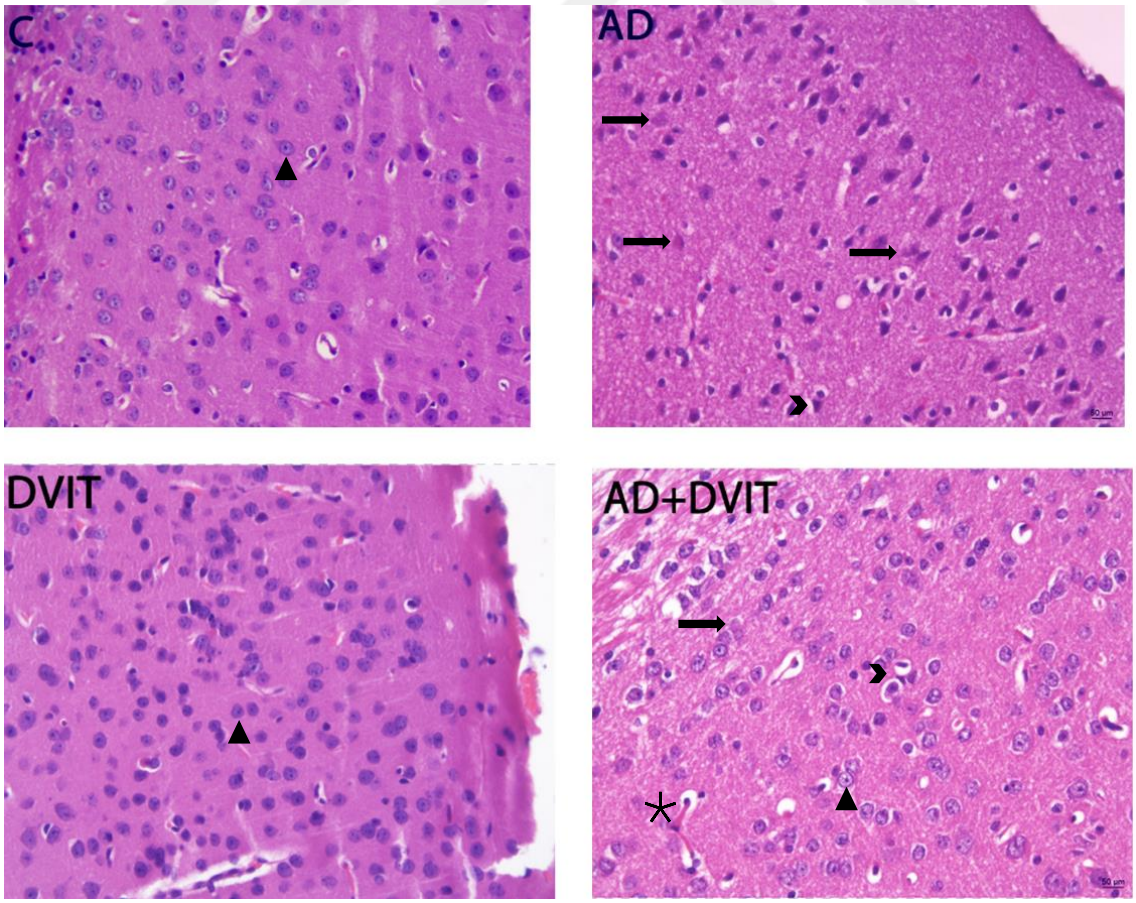
Saline administered control group (C), vitamin D injected (DVIT), scopolamine injected (AD), scopolamine and vitamin D injected (AD + DVIT) groups.(**p<0.01 Compared with the control group)

6.2. Histology

Amyloid beta and TUNEL positive neurons were counted in the cerebral cortex and hippocampus. Additionally, iNOS positive neurons are shown in the 10x, 20x, 40x lens. Finally, stains of haematoxylin and eosin, Congo red and cresyl violet were carried out to observe general histopathological changes.

6.2.1. Hematoxylin Eosin Staining

The Hematoxylin&Eosin staining was performed for histopathologic examination of the tissues. According to the evaluation of the sections stained with H&E; As expected, amyloid plaques and neurofibrillary tangles were determined in the sections of the AD and AD+ DVIT groups according to the C and DVIT groups. Although these anomalies were observed most intensely in the AD group, they were observed less frequently in the AD+DVIT group. Anomalies were not observed in groups C and DVIT.



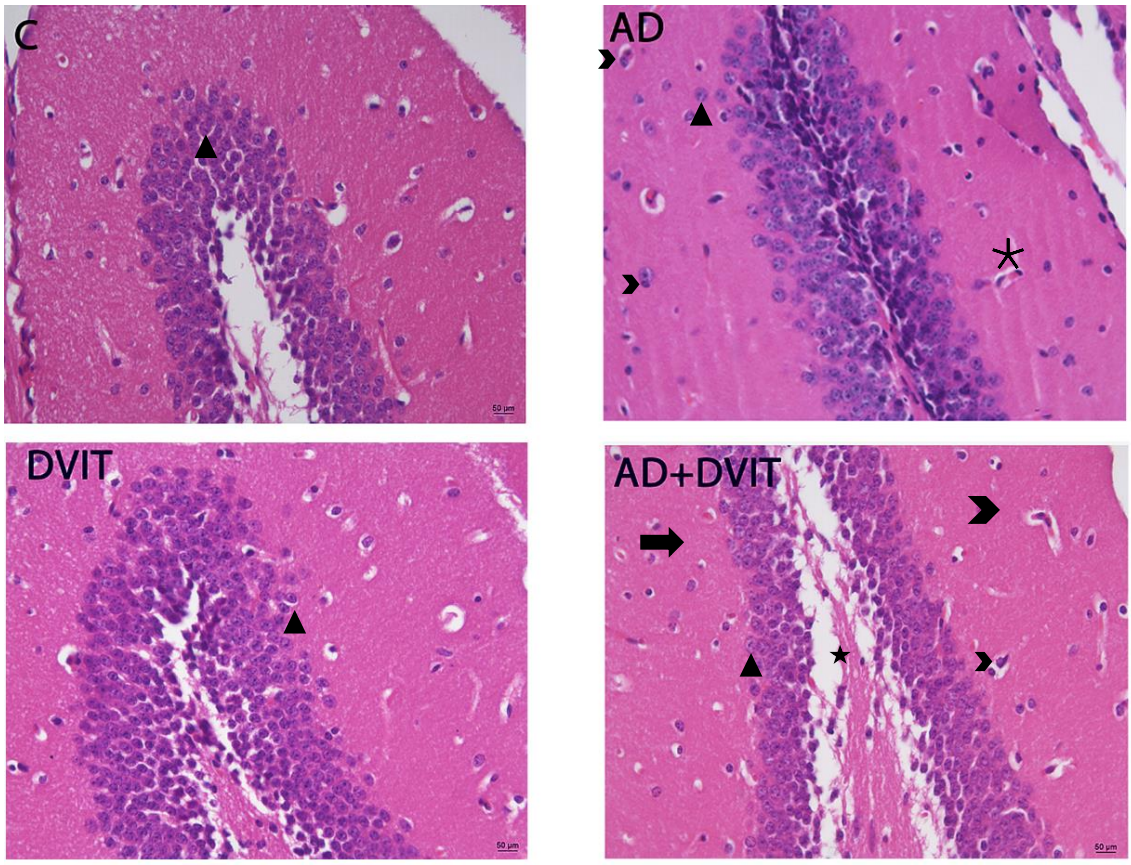
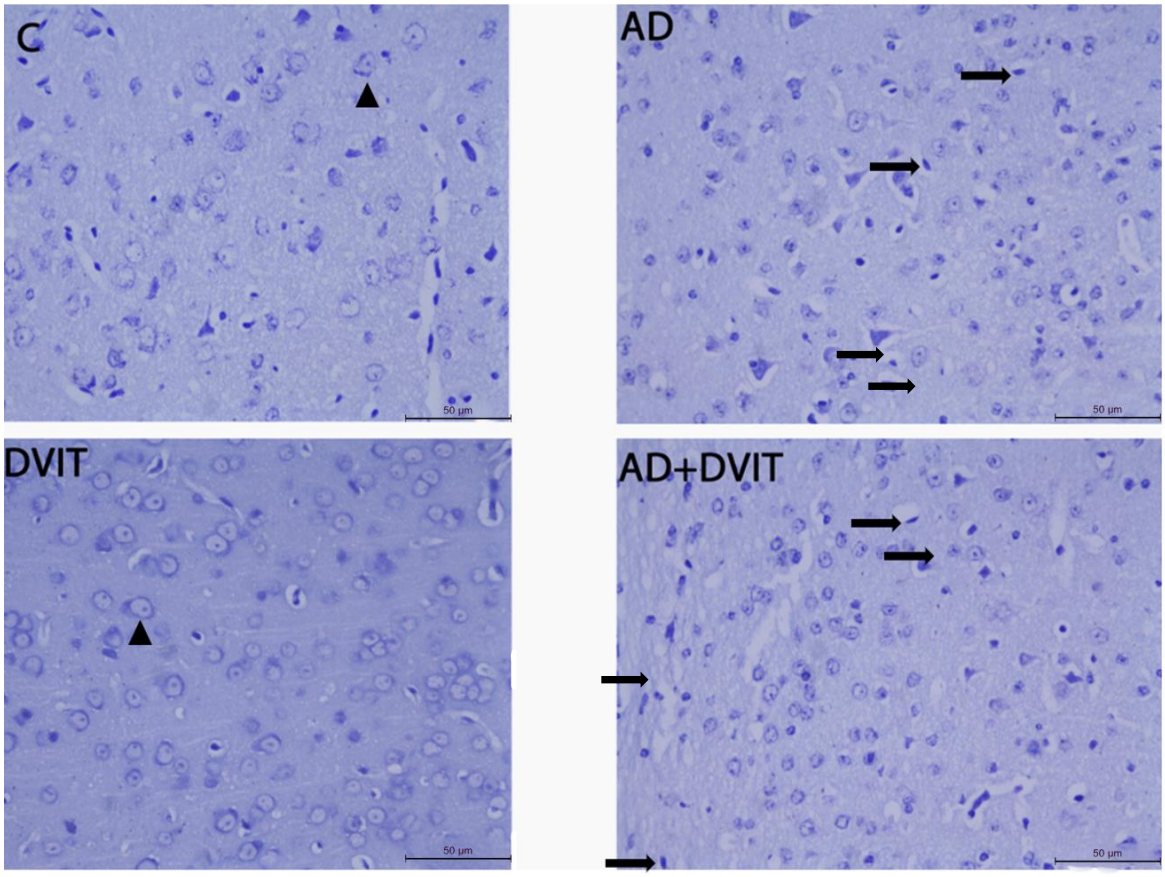


Figure 22. Photomicrographs demonstrate Hematoxylin&Eosin staining in the cerebral cortex and hippocampus. Scale bar represents, 50 μm in x40.Arrowheads indicates neurofibrillary tangles while arrows indicate amyloid plaques, The triangle represents the normal neuron and the star represents the capillary vessel.

6.2.2. Cresyl Violet Staining

Cresyl Violet was performed to examine the tissues histopathologically. In the evaluation of the sections stained with Cresyl Violet, amyloid plaques and neurofibrillary tangles were determined in the sections of the AD and AD+ DVIT groups according to the C and DVIT groups. While these abnormalities were most intensely observed in the AD group, they were observed less frequently in the AD+ DVIT group. No abnormality was observed in C and DVIT groups.



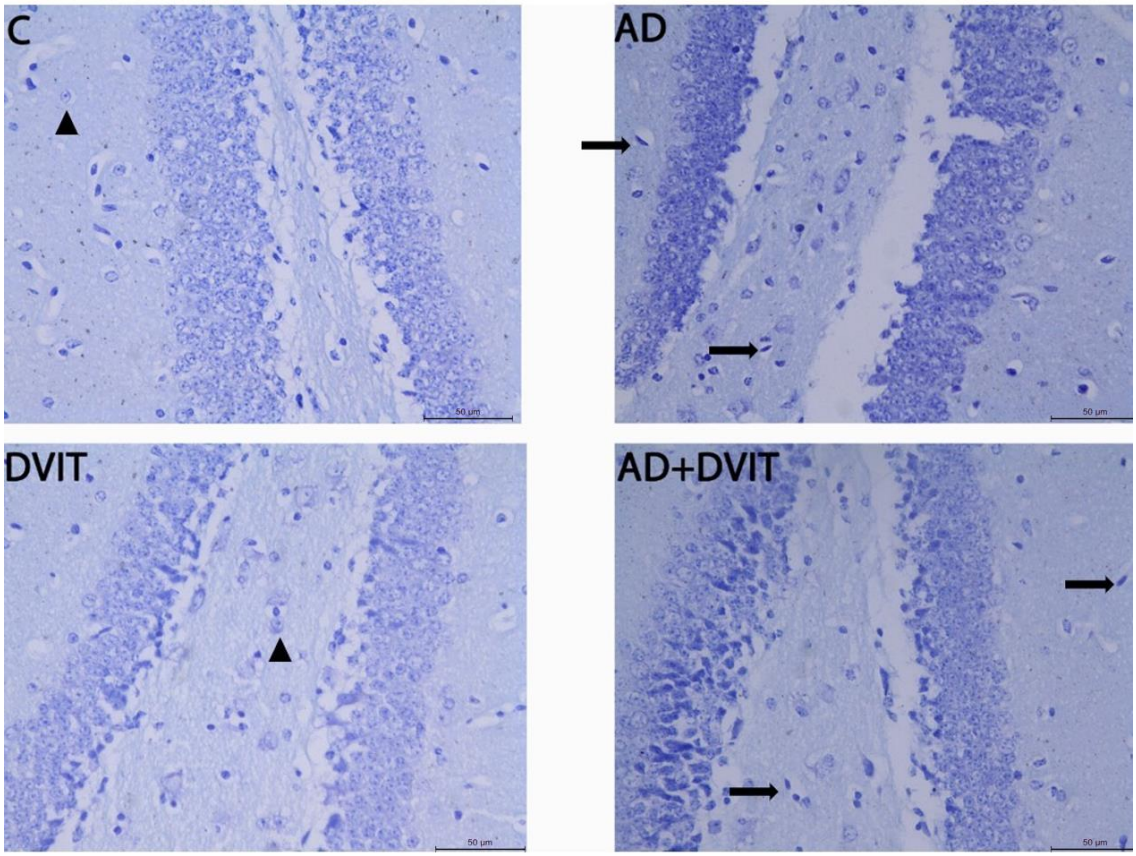
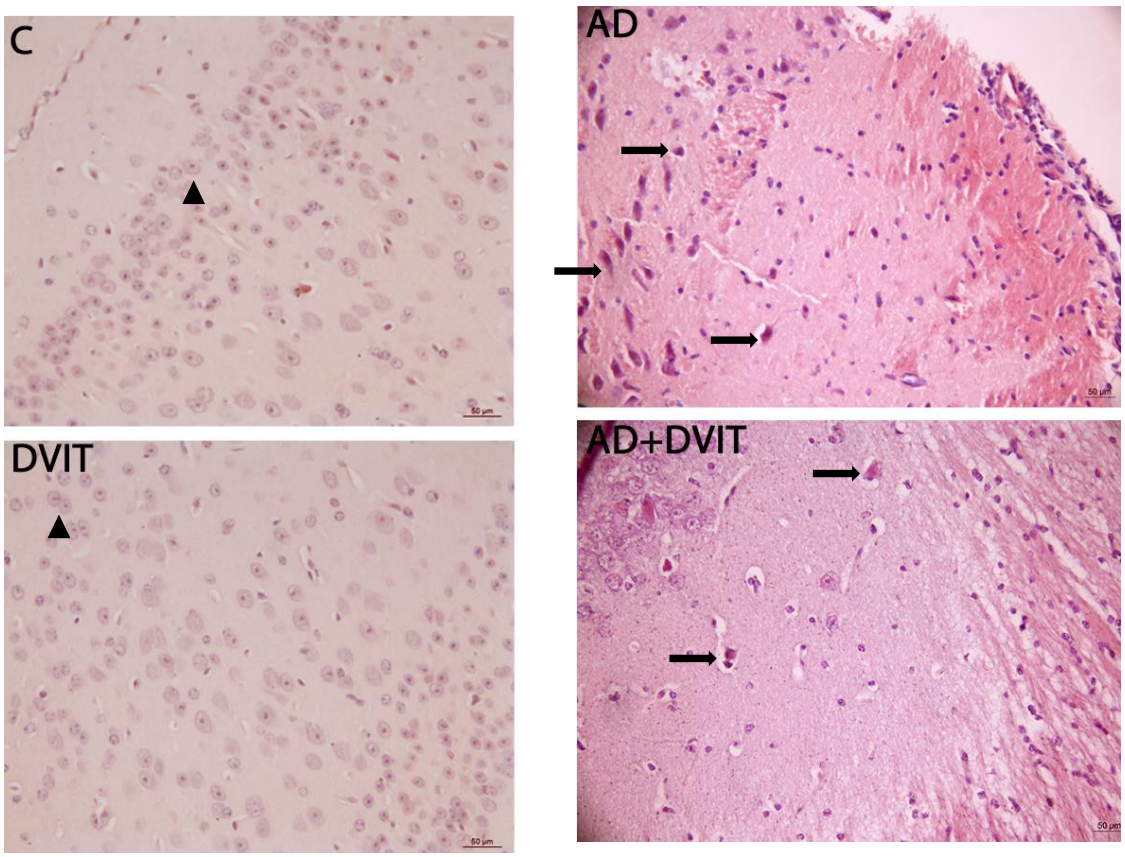


Figure 23: Photomicrographs demonstrate Cresyl Violet staining in the cerebralcortex and hippocampus**.** Scale bar represents, 50 µm in x40 Arrowheads indicates neurofibrillary tangles, The triangle represents the normal neuron and the arrow represents the apoptotic neuron.

6.2.4. Congo red

Congo Red was performed to examine the tissues histopathologically. In the evaluation of the sections stained with Congo Red, amyloid plaques were determined in the sections in the AD and AD+DVIT groups according to the C and DVIT groups. While these abnormalities were most intensely observed in the AD group, they were observed less frequently in the AD+DVIT group. No abnormality was observed in the C and DVIT groups.



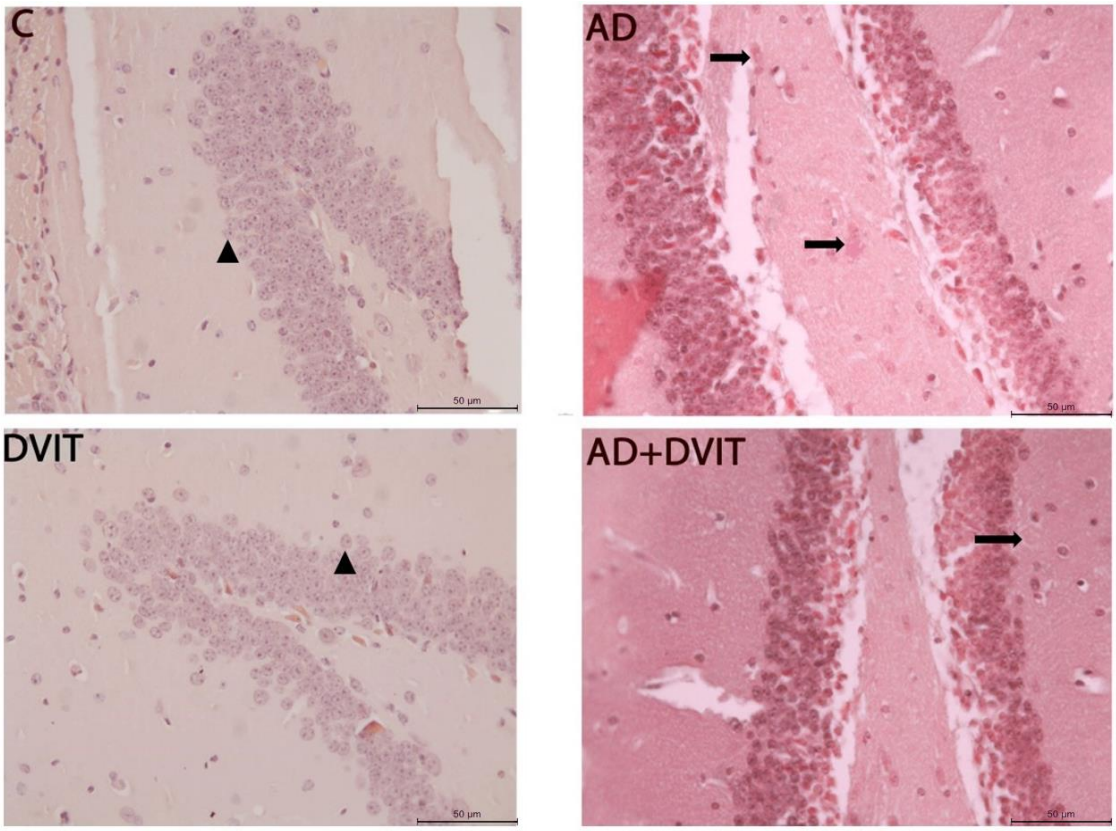
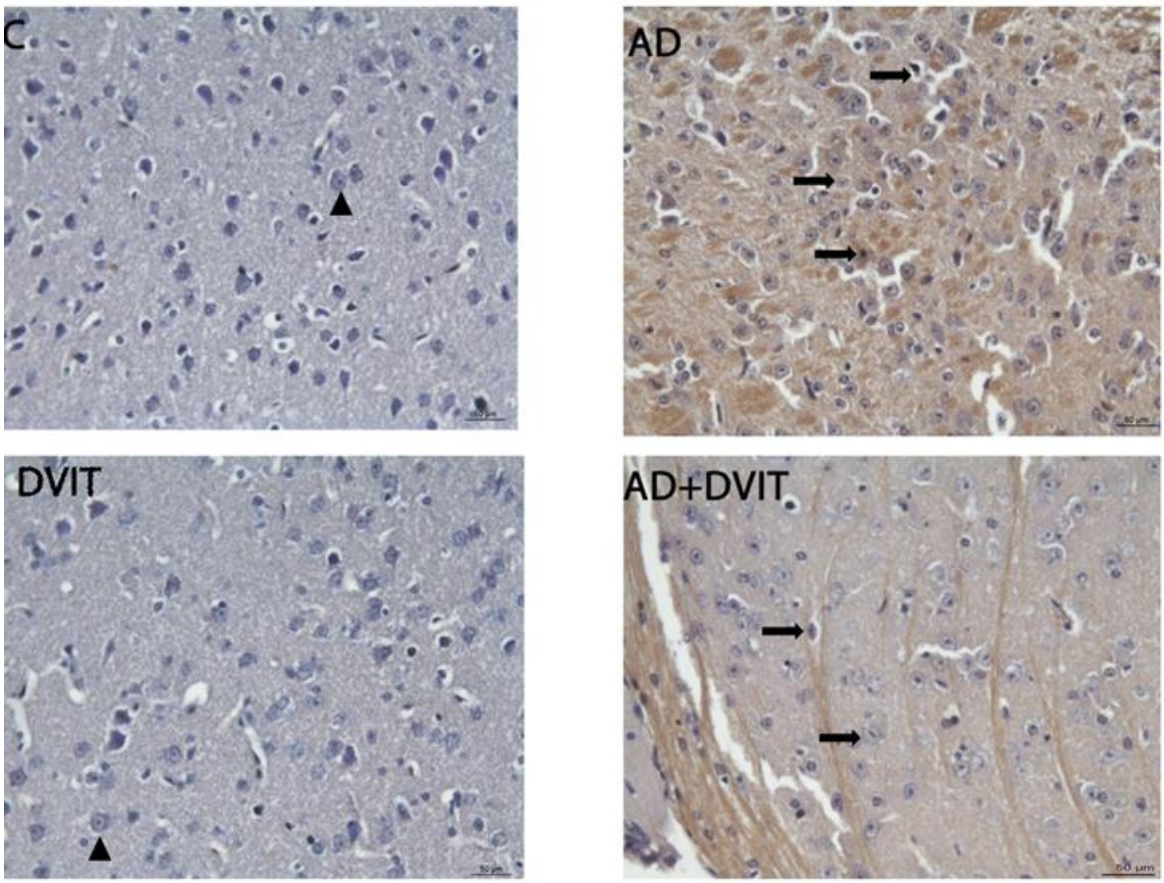


Figure 24. Photomicrographs demonstrate Congo red staining in the cerebral cortex and hippocampus. Scale bar represents, 50 µm in x40 triangle represents normal neuron and arrow represents amyloid plaque.

6.2.5. TUNEL Assay

Coronal sections from all groups were analyzed for TUNEL positive neurons. The TUNEL test gives reliable data on the number of apoptotic neurons. Photomicrographs show sections from the cerebral cortex and hippocampus. Apoptotic Index ($P<0.05$) was calculated for all groups.



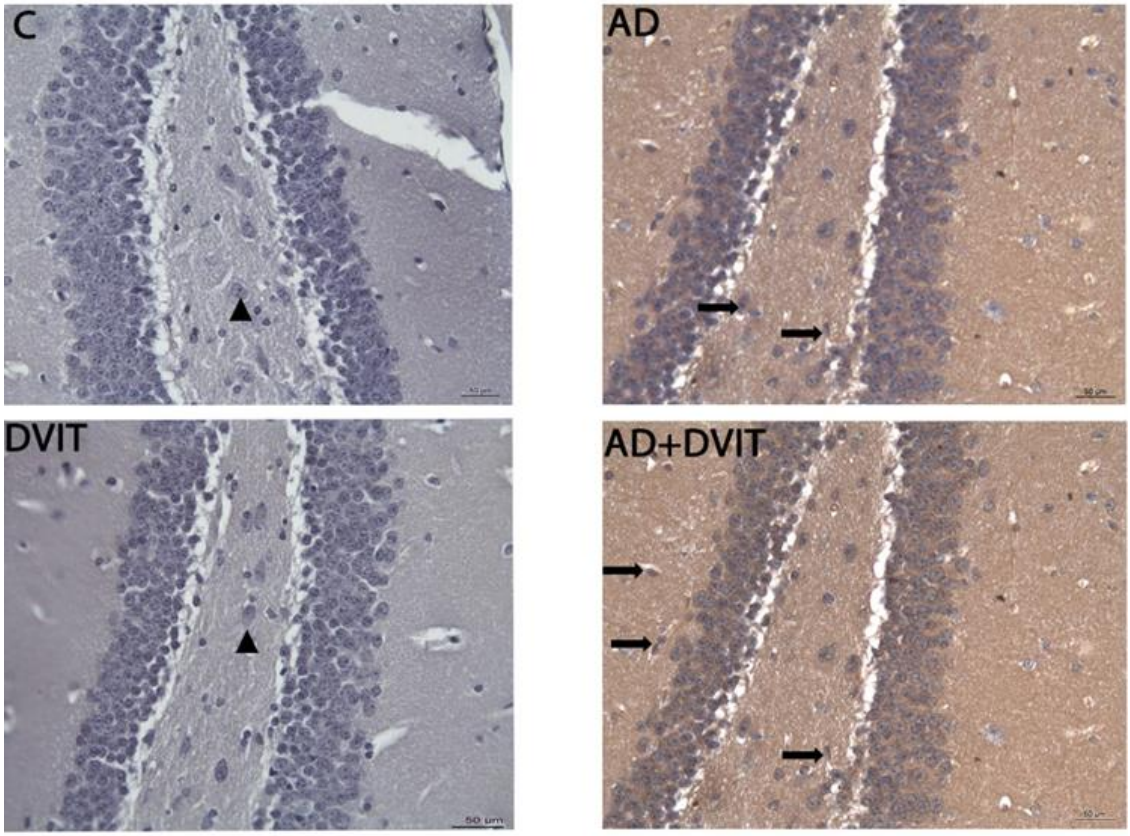


Figure 25. Photomicrographs demonstrate TUNEL positive cells in the cerebral cortex and hippocampus Scale bar represents, 50 μm in triangle represents normal neuron and arrow represents apoptotic neuron.

6.2.5.1. Tunel Cortex AP Index

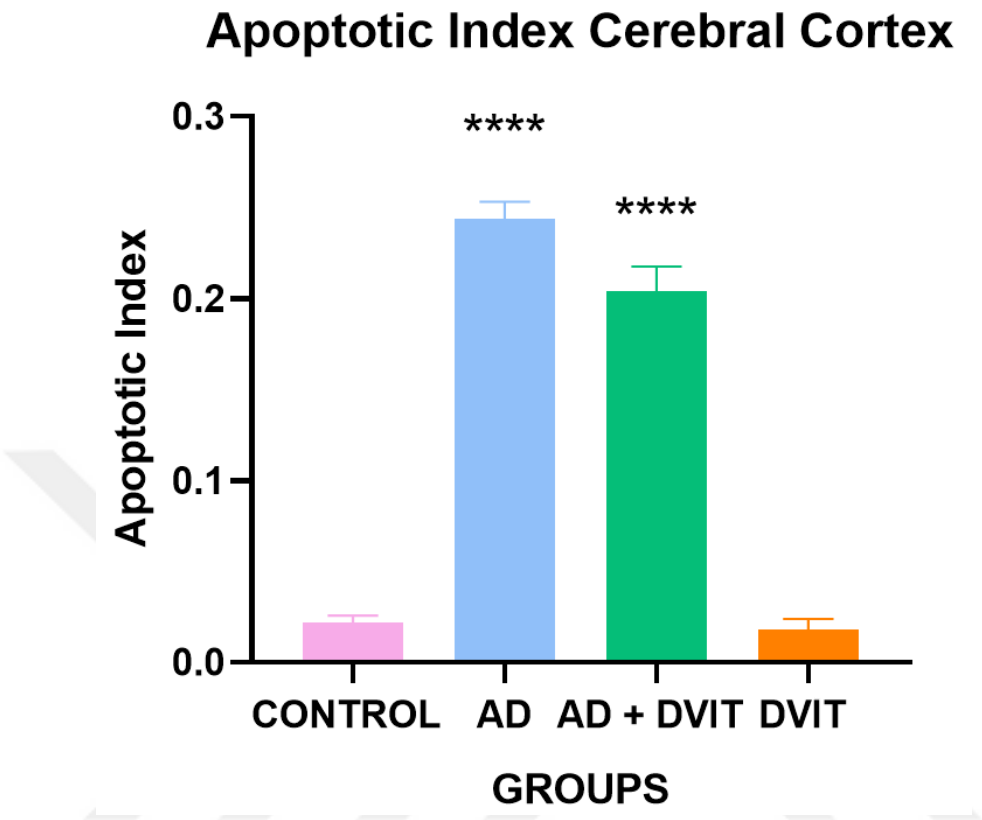


Figure 26. Graph of Apoptotic Index for Cerebral Cortex (P<0.05) When the apoptotic index of the cerebral cortex is compared, it can be said that the AD Group and AD+DVIT Group have a higher apoptotic index than Group C (p<0.0001, p<0.0001, respectively). For cerebral cortex, the AD+DVIT Group has a lower apoptotic index than the AD Group (p<0.0001). According to the Tukey Multiple Comparison Test, the AD Group in the cerebralcortex region has a higher apoptotic index than the DVIT Group of the hippocampus (****p<0.0001).

6.2.5.2. Tunel Hippocampus AP Index

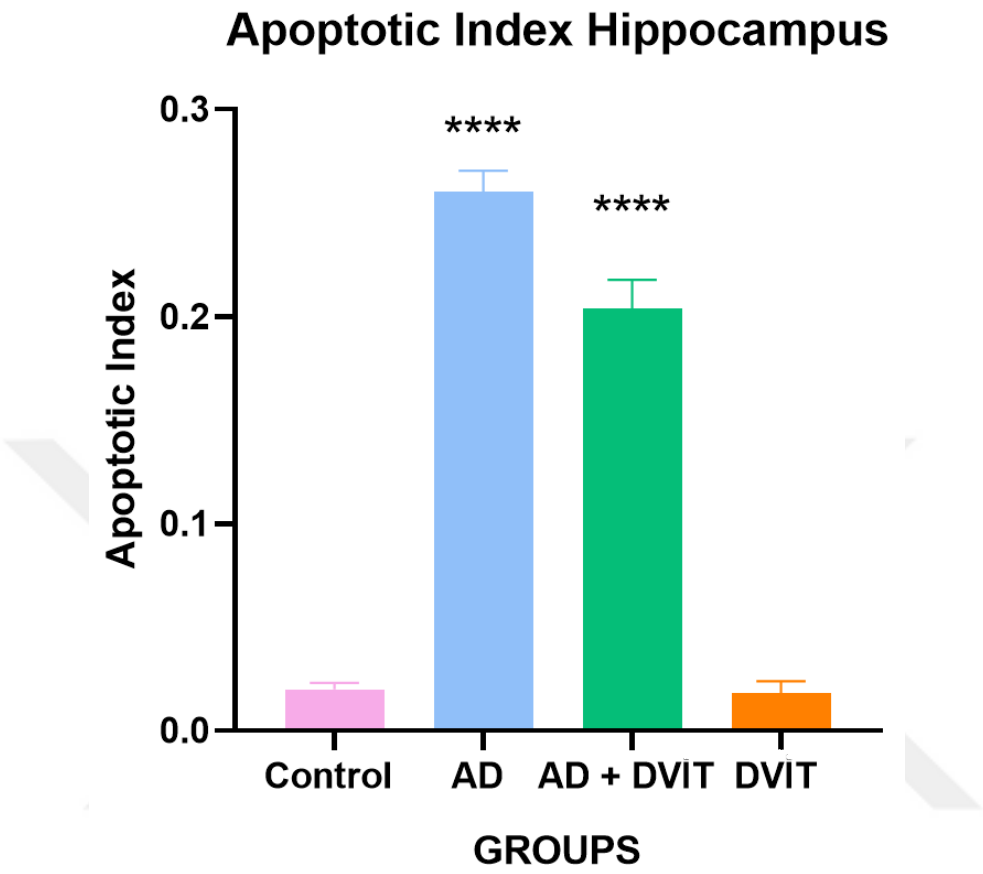
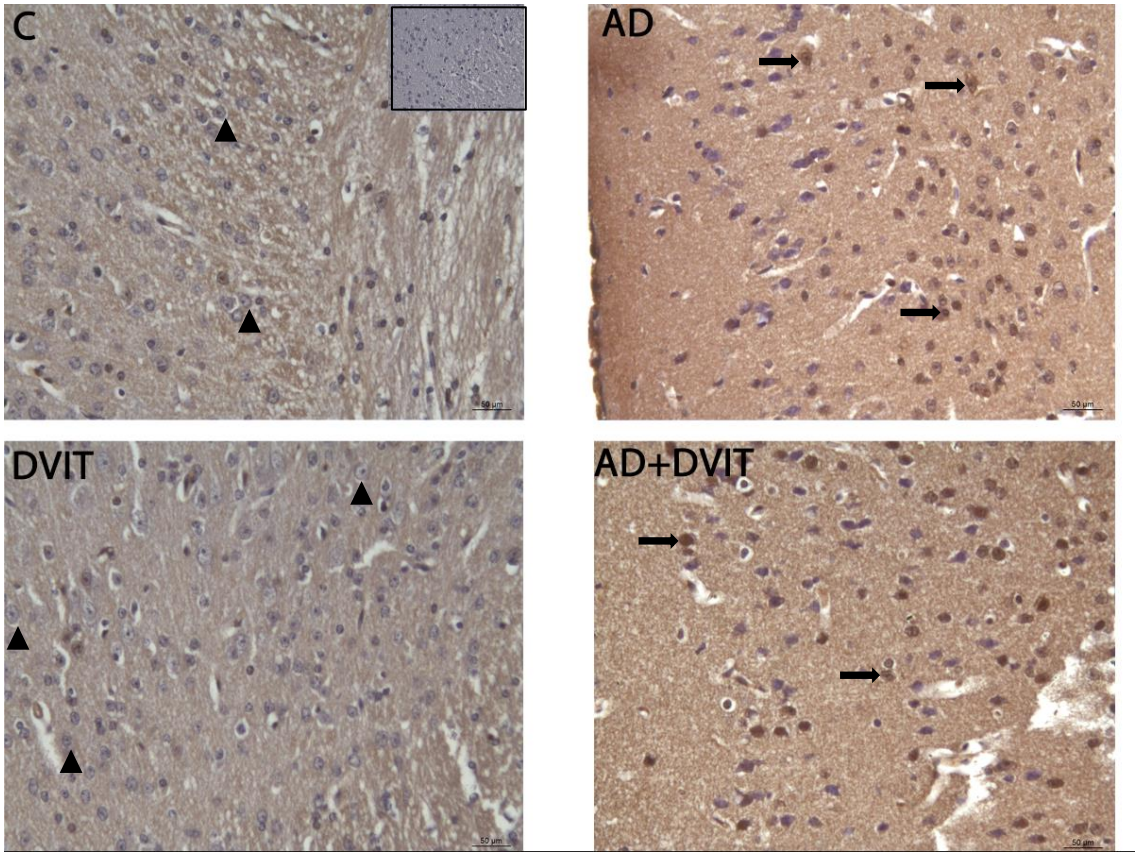


Figure 27. Graph of Apoptotic Index for Hippocampus(P<0.05).Considering the apoptotic index of the hippocampus, it can be said that AD Group and AD+DVIT Group have higher apoptotic index than Group C (p<0.0001, p<0.0001, respectively). The AD+DVIT Group has a lower apoptotic index for the hippocampus than the AD Group (p<0.0001). According to the Tukey Multiple Comparison Test, the AD Group in the hippocampus region has a higher apoptotic index than the DVIT Group of the hippocampus (****p<0.0001).

6.2.7. Immunohistochemistry for iNOS;



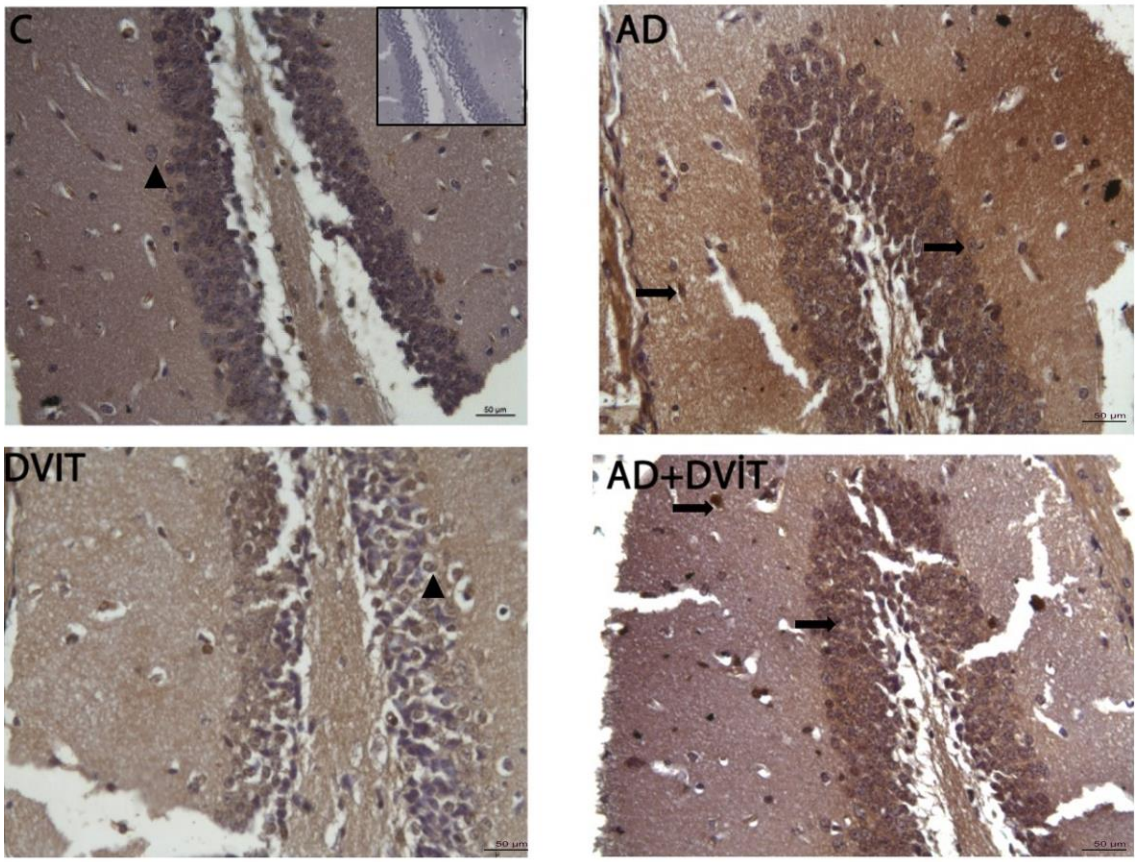
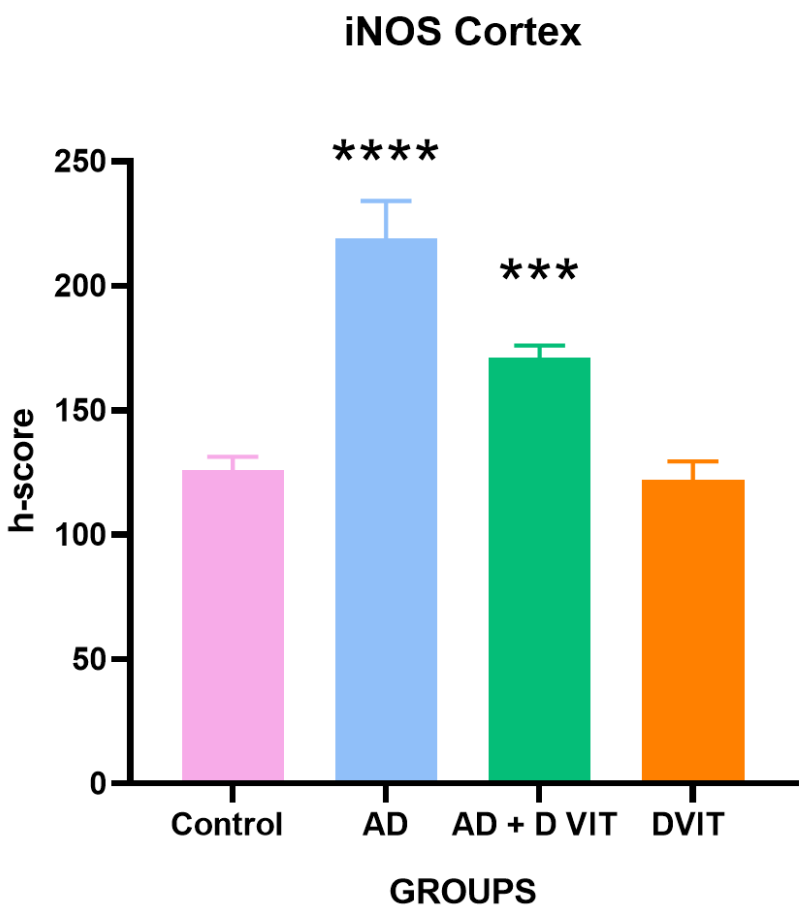


Figure 28. Photomicrographs demonstrate iNOS immunoreactivity in the cerebral cortex and Scale bar represents, 50 μm in x40 Photomicrographs demonstrate iNOS immunoreactivity in the cerebral cortex. triangle represents normal neuron and arrow represents apoptotic neuron.

6.2.7.1.Cerebral Cortex iNOS H-Score



Figüre 29. Graph of iNOS H-score Cerebral Cortex (P<0.05) When the cerebral cortex iNOS H-Scores are compared, it is seen that the AD Group and AD+DVIT Group have a higher H-Score for iNOS than Group C, while the DVIT Group has a slightly lower H-Score for iNOS (p<0.0001, p, respectively). The AD Group has a higher H-Score for iNOS than the DVIT Group (p<0.0001). The AD+DVIT Group has a lower H-Score forthe cerebral cortex for iNOS immunochemistry than the AD Group (p=0.0248).

6.2.7.2.Hippocampus iNOS H-Score

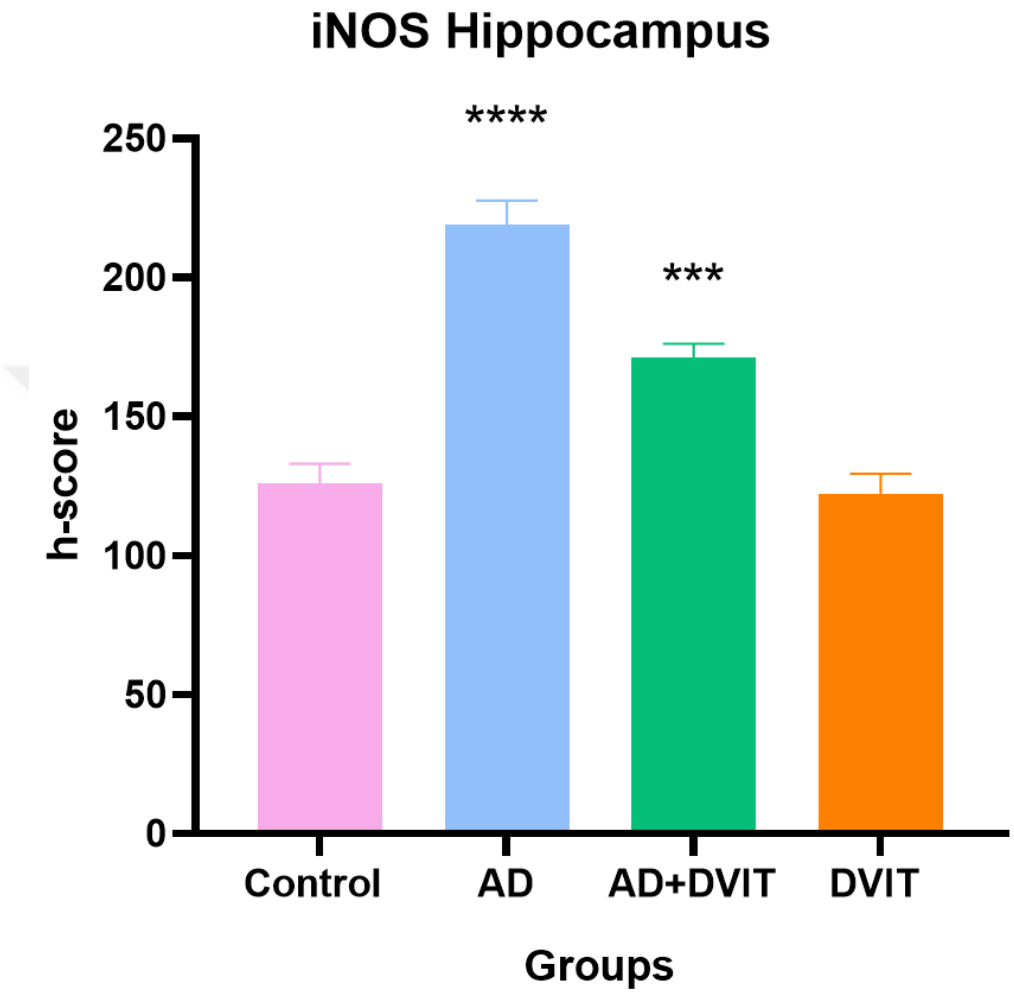


Figure 30. Graph of iNOS H-score Hippocampus(P<0.05) Considering the hippocampus iNOS H-Scores, it is seen that the iNOS H-Score of the AD Group and AD+DVIT Group is higher than that of Group C (p<0.0001, p, <0.001 respectively). Group AD has a higher H-Score for iNOS than Group DVIT (p<0.0001). Group AD+DVIT has a lower H-Score for iNOS immunochemistry than Group AD for hippocampus (p=0.0099). The AD Group in the hippocampus region has a higher H-Score for iNOS immunochemistry than the AD Group of the hippocampus (p<0.0001, p<0.0001).

6.2.7.3. D vitamin serum

For vitamin D level, all blood was drawn from the heart before all animals died. Vitamin D serum levels were measured and the resulting statistics are;

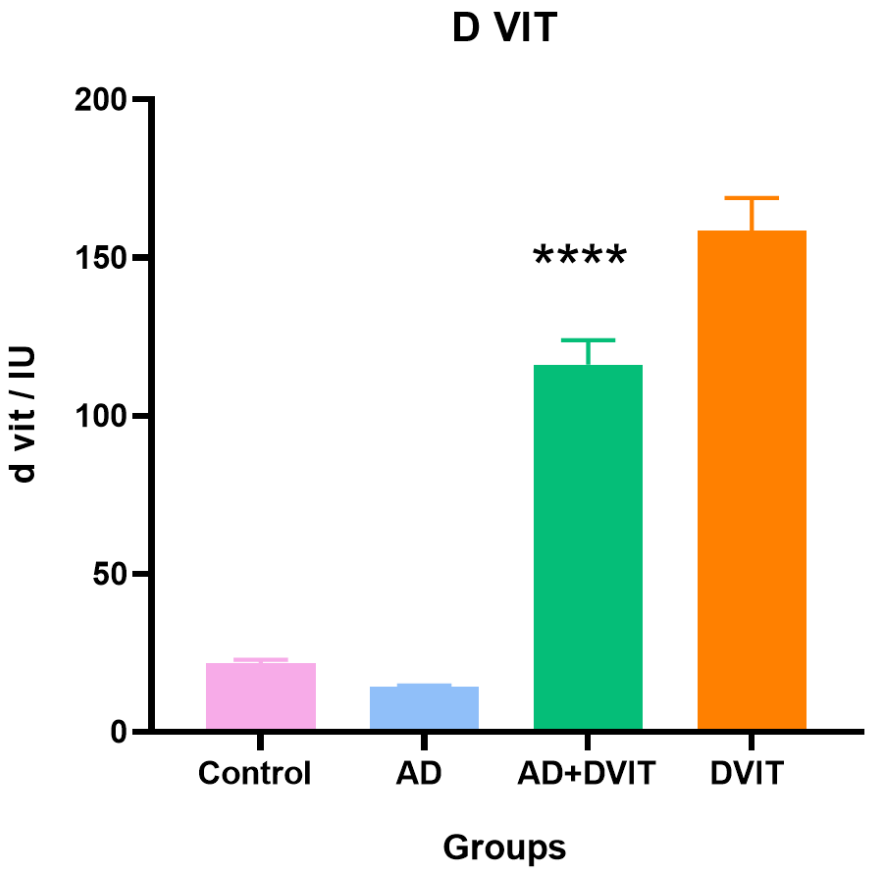


Figure 31. D vitamin serum (****p<0.0001). When the vitamin D levels were examined, it was determined that the DVIT Group and AD+DVIT group were found to be higher than the AD and C groups (p<0.0001). It was also found that the vitamin D levels were lower in the AD and C groups as an estimate (p=0.8114).

7. DISCUSSION

AD is the most common and progressive illness nowadays. Losing the ability to think over time with continuous progress creates severe consequences such as an individual's inability to do their daily work. The most remarkable pathological features of AD are tau and neurofibrillary tangle. Scopolamine is a muscarinic cholinergic receptor antagonist. The effect of emotional memory and its forgetting has not been fully elucidated yet (9). Scopolamine is a neurotoxin that reproduces the effects of Alzheimer's disease when administered appropriately. This is why it is one of the most commonly used neurotoxins for modeling Alzheimer's disease. So when we look at current disease studies, we focus on early diagnosis, preventative treatment, and forms of treatment that will reduce the time the patient needs care. The impact of scopolamine on memory and forgetfulness has yet to be fully understood. Scopolamine is a neurotoxin that reproduces the effects of Alzheimer's disease when administered correctly, making it one of the most commonly used neurotoxins for modeling Alzheimer's disease. Scopolamine temporarily impairs attention retention, treatment, and information acquisition in rodents and humans. Because of this effect, scopolamine was an appropriate pharmacological tool in the modelling of learning and memory disabilities (113). Vitamin D promotes intestinal absorption of calcium and maintains appropriate serum calcium and phosphate levels to restore normal bone mineralization and prevent tetanus hypocalcemia (113).

In recent years, attempts have been made to understand the relationship between vitamin D and brain health, as well as several studies showing the effect of vitamin D deficiency on the brain. Not enough vitamin D is recognized as a serious risk factor for dementia and AD. They found that vitamin D is found in brain tissue, and those who have higher levels of vitamin D in their brain also reported higher cognitive function before dying (114).

A 2017 meta-analysis assumes that 25 (OH) D concentrations lower than 25 nmol/L increase the risk of dementia, particularly in adults and patients over the age of 65. He conducted a study that showed that vitamin D regulates the release of NGF, the small protein-like structure responsible for cortical as well as hippocampal neurons,

neurogenesis, or the development of new neurons, and the health and protection of mature ones (92).

In this study, scopolamine, a nerve agent, was used to stimulate behavioural and cognitive malfunctions in Alzheimer's disease. Effect of vitamin D in model created; behavioural and cognitive disorders, as well as motor activity, groups enriched with vitamin D were formed and compared (115). Thus, the distance covered by the animals, the As a result, the distance traveled by the animals, stereotypical activity ($p<0.001$), the ambulatory activity ($p<0.001$), the horizontal activity ($p<0.0001$), the horizontal activity ($p<0.0001$), the vertical activity ($p<0.001$). It was the lowest in the AD group. The AD group was the group where scopolamine and expected cognitive loss were applied. As we have observed, we show that the data causes cognitive data loss. Since the cerebral cortex is the area where the feedback and connections between the sensory and motor parts take place, and therefore contains the region responsible for motor activities, we think that these results may be due to intense neuronal loss and thus trigger cognitive disorders (116). Animals in the AD+ DVIT group showed an increase in stereotypic activity ($p<0.05$) and ambulatory activity ($p<0.05$) when supplemented with Dvit compared to the AD group. Also, horizontal activity, distance traveled by the animals, and vertical activity were also increased in the AD+ DVIT group compared to the AD group. Increased but not statistically significant. We believe Dvit supplementation has proven to be successful. In summary, the administration of DVIT in the scopolamine model has the potential to improve cognitive states. However, further studies must be carried out to determine the specific effects of DVIT on cognitive impairment (117).

Afterwards, amyloid plaques and neurofibrillary tangles were observed in AD and AD+DVIT group sections in the sections of Hematoxylin Eosin, Cresyl Violet, Congo red stainings we performed to examine the morphology of neurons. While these abnormalities were most intensely observed in the AD group as we expected, they were observed in the AD + DVIT group, although not as intensely as in the AD group. As expected, no pathology of AD was found in Groups C and DVIT. Therefore, in the scopolamine model, amyloid plaque and neurofibrillary tangle with AD pathology can be observed with individual histological stainings in the scopolamine model, and that Dvit supplementation in the AD + DVIT group has a protective effect on the cerebral cortex and hippocampus with Dvit, and we characteristic it to this circumstances (118). It was

then analyzed by iNOS staining and calculating the H-score. iNOS is known to be involved in the development of nitric oxide, a molecule with multiple biological activities (119). INOS may be found in brains affected by dementia because it plays a role in balancing brain damage and repair. Comparing iNOS H-Scores for cerebral cortex, AD Group and AD + DVIT Group seem to have higher H-Scores for iNOS than Group C ($p<0.000$). For the hippocampus, the situation with the H-Scores of iNOS was similar as we expected. The H scores of iNOS immunochemistry in the hippocampus region of the AD group and the AD + DVIT group were even higher than those of the C group ($p<0.0001$) (120). In one study, mice administered scopolomine showed characteristic delusional behavioural models, biochemical results consistent with neuroinflammatory changes and inflammation activation.

As expected, the AD + DVIT Group had a lower H-Score for iNOS immunochemistry for both cerebral cortex and hippocampus than the AD Group, indicating a positive result for Dvit supplementation (121) .The H-Score for Group AD iNOS in the cerebral cortex region is comparatively higher than the H-Score for Group DVIT for iNOS, meaning that dvit complementarity and scopolamine injections work as expected ($p<0.0001$) (122). Vitamin D while oxidative stress brings about the formation of AD.

Low levels of antioxidants and an increased risk of illness were also reported. Vitamin D whereas oxidative stress leads to the formation of AD.Low levels of anti-oxidant capacity and increased risk of disease have been reported (122). It is known that vitamin 1.25 (OH) 2 D3 is a free radical and inhibits the synthesis of Inos. Vitamin D while oxidative stress brings about the formation of AD. Low levels of anti-oxidant capacity and increased risk of disease have been reported.

It is known that vitamin 1.25 (OH) 2 D3 is a free radical and inhibits the synthesis of Inos. As a result, it is suggested that 1.25 (OH) 2 D3 can control the process of brain detoxification because it is effective in this cycle. We believe that vitamin D reduces oxidative stress and thus regulates inflammation, which is why we attribute the decline of the experimental group to this situation (123).In conclusion, there was a significant difference in iNOS immunohistochemical staining between the scopolamine and monitoring groups. Scopolamine can cause neurons to die in brain tissue, and we believe that vitamin D can help reduce neuroinflammation in the cerebral cortex and

hippocampus (124). The apoptotic index was calculated by the TUNEL experiment, and the highest values were obtained in the AD group, the second highest values in the AD + DVIT group, and the lowest values in the control and DVİT groups (125). Cerebral cortex was found to have a higher apoptotic index for AD Group and AD + DVIT Group than Group C. The situation is similar in terms of the apoptotic index of the hippocampus. The apoptotic index of the hippocampal region of the AD group and the AD + DVIT group was even higher than that of the C group (126) . AD + DVIT group had a relatively lower apoptotic score compared to the AD group, indicating a positive result for DVIT supplementation. In conclusion, it is promising to see the protective effect of vitamin D supplements. We think scopolamine could be a good substitute for Alzheimer's disease. In addition, the findings of this study show that vitamin D has morphologically promising effects on the modelled pathology of scopolamine-induced Alzheimer's disease. We hope that in this way it can at least have a protective effect for irreversible AD and therefore slow down this progressive disease. Consequently, this study can serve as a guide for the development of studies to prevent Alzheimer's disease with vitamin D supplementation in the future.

9. REFERENCES

1. Kuningas M, Mooijaart SP, Jolles J, Slagboom PE, Westendorp RG, van Heemst D. VDR gene variants associate with cognitive function and depressive symptoms in old age. Neurobiol Aging. 2009;30(3):466-73.

2. Thibault O, Gant JC, Landfield PW. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. Aging Cell. 2007;6(3):307-17.

3. Lane CA, Hardy J, Schott JM. Alzheimer's disease. Eur J Neurol. 2018;25(1):59-70.

4. Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. Front Neuroendocrinol. 2013;34(1):47-64.

5. Walbert T, Jirikowski GF, Prüfer K. Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the limbic system of the rat. Horm Metab Res. 2001;33(9):525-31.

6. Holick MF. Noncalcemic actions of 1,25-dihydroxyvitamin D3 and clinical applications. Bone. 1995;17(2 Suppl):107s-11s.

7. Taniura H, Ito M, Sanada N, Kuramoto N, Ohno Y, Nakamichi N, et al. Chronic vitamin D3 treatment protects against neurotoxicity by glutamate in association with upregulation of vitamin D receptor mRNA expression in cultured rat cortical neurons. J Neurosci Res. 2006;83(7):1179-89.

8. Bondi MW, Edmonds EC, Salmon DP. Alzheimer's Disease: Past, Present, and Future. J Int Neuropsychol Soc. 2017;23(9-10):818-31.

9. Chen WN, Yeong KY. Scopolamine, a Toxin-Induced Experimental Model, Used for Research in Alzheimer's Disease. CNS Neurol Disord Drug Targets. 2020;19(2):85-93.

10. Donovan AP, Basson MA. The neuroanatomy of autism - a developmental perspective. J Anat. 2017;230(1):4-15.

11. Ji JL, Spronk M, Kulkarni K, Repovš G, Anticevic A, Cole MW. Mapping the human brain's cortical-subcortical functional network organization. Neuroimage. 2019;185:35-57.

12. Martino J, De Witt Hamer PC, Vergani F, Brogna C, de Lucas EM, Vázquez-Barquero A, et al. Cortex-sparing fiber dissection: an improved method for the study of white matter anatomy in the human brain. J Anat. 2011;219(4):531-41.

13. Turk-Browne NB. The hippocampus as a visual area organized by space and time: A spatiotemporal similarity hypothesis. Vision Res. 2019;165:123-30.

14. Zeidman P, Maguire EA. Anterior hippocampus: the anatomy of perception, imagination and episodic memory. Nat Rev Neurosci. 2016;17(3):173-82.

15. Ward NS, Frackowiak RS. Towards a new mapping of brain cortex function. Cerebrovasc Dis. 2004;17 Suppl 3:35-8.

16. DeMaster D, Pathman T, Lee JK, Ghetti S. Structural development of the hippocampus and episodic memory: developmental differences along the anterior/posterior axis. Cereb Cortex. 2014;24(11):3036-45.

17. Afifi OK, Embaby AS. Histological Study on the Protective Role of Ascorbic Acid on Cadmium Induced Cerebral Cortical Neurotoxicity in Adult Male Albino Rats. J Microsc Ultrastruct. 2016;4(1):36-45.

18. Eckermann M, Schmitzer B, van der Meer F, Franz J, Hansen O, Stadelmann C, et al. Three-dimensional virtual histology of the human hippocampus based on phase-contrast computed tomography. Proceedings of the National Academy of Sciences. 2021;118(48):e2113835118.

19. Yassa MA, Stark CE. Pattern separation in the hippocampus. Trends Neurosci. 2011;34(10):515-25.

20. Seghatoleslam M, Alipour F, Shafieian R, Hassanzadeh Z, Edalatmanesh MA, Sadeghnia HR, et al. The effects of Nigella sativa on neural damage after pentylenetetrazole induced seizures in rats. J Tradit Complement Med. 2016;6(3):262-8.

21. O'Brien C. Auguste D. and Alzheimer's disease. Science. 1996;273(5271):28.

22. Foley P. Lipids in Alzheimer's disease: A century-old story. Biochim Biophys Acta. 2010;1801(8):750-3.

23. Maurer K, Volk S, Gerbaldo H. Auguste D and Alzheimer's disease. Lancet. 1997;349(9064):1546-9.

24. Rao KJ, Rao R, Shanmugavelu P, Menon R. Trace elements in Alzheimer's disease brain: a new hypothesis. Alzheimers Rep. 1999;2(4):241-46.

25. Gürvit İH. Demans sendromu, Alzheimer hastalığı ve Alzheimer dışı demanslar. by Öge AE İstanbul, Nobel Tıp Kitap Evleri Ltd Şti. 2004:367-415.

26. YAZICI TG, ŞAHİN HA. Alzheimer Hastalığı. Klinik Gelişim. 2010;23(1):48-52.

27. Hebert LE, Weuve J, Scherr PA, Evans DA. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. Neurology. 2013;80(19):1778-83.

28. Scheyer O, Rahman A, Hristov H, Berkowitz C, Isaacson RS, Diaz Brinton R, et al. Female Sex and Alzheimer's Risk: The Menopause Connection. J Prev Alzheimers Dis. 2018;5(4):225-30.

29. GAZİOĞLU H, KONUK M. ALZHEİMER HASTALIĞI İLE APOLİPOPROTEİN E GENOTİPLEMESİ ÜZERİNE BİR ÇALIŞMA.

30. Armstrong RA. What causes alzheimer's disease? Folia Neuropathol. 2013;51(3):169-88.

31. Khan S, Barve KH, Kumar MS. Recent Advancements in Pathogenesis, Diagnostics and Treatment of Alzheimer's Disease. Curr Neuropharmacol. 2020;18(11):1106-25.

32. Bellenguez C, Charbonnier C, Grenier-Boley B, Quenez O, Le Guennec K, Nicolas G, et al. Contribution to Alzheimer's disease risk of rare variants in TREM2, SORL1, and ABCA7 in 1779 cases and 1273 controls. Neurobiol Aging. 2017;59:220.e1-.e9.

33. Cano E, Canellada A, Minami T, Iglesias T, Redondo JM. Depolarization of neural cells induces transcription of the Down syndrome critical region 1 isoform 4 via a

calcineurin/nuclear factor of activated T cells-dependent pathway. J Biol Chem. 2005;280(33):29435-43.

34. Sun X, Wu Y, Chen B, Zhang Z, Zhou W, Tong Y, et al. Regulator of calcineurin 1 (RCAN1) facilitates neuronal apoptosis through caspase-3 activation. J Biol Chem. 2011;286(11):9049-62.

35. Fortea J, Vilaplana E, Carmona-Iragui M, Benejam B, Videla L, Barroeta I, et al. Clinical and biomarker changes of Alzheimer's disease in adults with Down syndrome: a cross-sectional study. Lancet. 2020;395(10242):1988-97.

36. DeTure MA, Dickson DW. The neuropathological diagnosis of Alzheimer's disease. Mol Neurodegener. 2019;14(1):32.

37. Li W, Risacher SL, McAllister TW, Saykin AJ. Traumatic brain injury and age at onset of cognitive impairment in older adults. Journal of Neurology. 2016;263(7):1280-5.

38. Mahoney SO, Chowdhury NF, Ngo V, Imms P, Irimia A. Mild Traumatic Brain Injury Results in Significant and Lasting Cortical Demyelination. Front Neurol. 2022;13:854396.

39. Rostowsky KA, Irimia A, for the Alzheimer’s Disease Neuroimaging I. Acute cognitive impairment after traumatic brain injury predicts the occurrence of brain atrophy patterns similar to those observed in Alzheimer’s disease. GeroScience. 2021;43(4):2015-39.

40. Nelson PT, Braak H, Markesbery WR. Neuropathology and cognitive impairment in Alzheimer disease: a complex but coherent relationship. J Neuropathol Exp Neurol. 2009;68(1):1-14.

41. Castellani RJ, Lee HG, Zhu X, Perry G, Smith MA. Alzheimer disease pathology as a host response. J Neuropathol Exp Neurol. 2008;67(6):523-31.

42. Sertkaya ME, Ergen B. Alzheimer Hastalığının Erken Teşhisinin Çoklu Değişken Kullanarak Tespiti. Avrupa Bilim ve Teknoloji Dergisi. 2022(35):306-14.

43. Bloom GS. Amyloid-β and tau: the trigger and bullet in Alzheimer disease pathogenesis. JAMA Neurol. 2014;71(4):505-8.

44. Wang X, Zhou X, Li G, Zhang Y, Wu Y, Song W. Modifications and Trafficking of APP in the Pathogenesis of Alzheimer's Disease. Front Mol Neurosci. 2017;10:294.

45. Huang Y, Mucke L. Alzheimer mechanisms and therapeutic strategies. Cell. 2012;148(6):1204-22.

46. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci. 2011;34:185-204.

47. Müller UC, Deller T, Korte M. Not just amyloid: physiological functions of the amyloid precursor protein family. Nature Reviews Neuroscience. 2017;18(5):281-98.

48. Schauenburg L, Liebsch F, Eravci M, Mayer MC, Weise C, Multhaup G. APLP1 is endoproteolytically cleaved by γ-secretase without previous ectodomain shedding. Sci Rep. 2018;8(1):1916.

49. Yanagida K, Maruyama R, Tagami S, Kudo T, Okochi M, Fukumori A. APLP2 is predominantly cleaved by β-secretase and γ-secretase in the human brain. Psychogeriatrics. 2023;23(2):311-8.

50. Bachurin SO, Bovina EV, Ustyugov AA. Drugs in Clinical Trials for Alzheimer's Disease: The Major Trends. Med Res Rev. 2017;37(5):1186-225.

51. Mandelkow EM, Mandelkow E. Biochemistry and cell biology of tau protein in neurofibrillary degeneration. Cold Spring Harb Perspect Med. 2012;2(7):a006247.

52. Aamodt EJ, Williams RC, Jr. Microtubule-associated proteins connect microtubules and neurofilaments in vitro. Biochemistry. 1984;23(25):6023-31.

53. Zeng K, Yu X, Mahaman YAR, Wang JZ, Liu R, Li Y, et al. Defective mitophagy and the etiopathogenesis of Alzheimer's disease. Transl Neurodegener. 2022;11(1):32.

54. Bălașa AF, Chircov C, Grumezescu AM. Body Fluid Biomarkers for Alzheimer's Disease-An Up-To-Date Overview. Biomedicines. 2020;8(10).

55. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006;443(7113):787-95.

56. Mandemakers W, Morais VA, De Strooper B. A cell biological perspective on mitochondrial dysfunction in Parkinson disease and other neurodegenerative diseases. J Cell Sci. 2007;120(Pt 10):1707-16.

57. Sas K, Robotka H, Toldi J, Vécsei L. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. J Neurol Sci. 2007;257(1-2):221-39.

58. Reddy PH, Beal MF. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. Trends Mol Med. 2008;14(2):45-53.

59. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006;443(7113):787-95.

60. Asiimwe N, Yeo SG, Kim MS, Jung J, Jeong NY. Nitric Oxide: Exploring the Contextual Link with Alzheimer's Disease. Oxid Med Cell Longev. 2016;2016:7205747.

61. Zuo L, Zhou T, Pannell BK, Ziegler AC, Best TM. Biological and physiological role of reactive oxygen species--the good, the bad and the ugly. Acta Physiol (Oxf). 2015;214(3):329-48.

62. Tönnies E, Trushina E. Oxidative Stress, Synaptic Dysfunction, and Alzheimer's Disease. J Alzheimers Dis. 2017;57(4):1105-21.

63. Sultana R, Mecocci P, Mangialasche F, Cecchetti R, Baglioni M, Butterfield DA. Increased protein and lipid oxidative damage in mitochondria isolated from lymphocytes from patients with Alzheimer's disease: insights into the role of oxidative stress in Alzheimer's disease and initial investigations into a potential biomarker for this dementing disorder. J Alzheimers Dis. 2011;24(1):77-84.

64. Torres AK, Jara C, Park-Kang HS, Polanco CM, Tapia D, Alarcón F, et al. Synaptic Mitochondria: An Early Target of Amyloid-β and Tau in Alzheimer's Disease. J Alzheimers Dis. 2021;84(4):1391-414.

65. Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS Sources in Physiological and Pathological Conditions. Oxid Med Cell Longev. 2016;2016:1245049.

66. Kumar K, Kumar A, Keegan RM, Deshmukh R. Recent advances in the neurobiology and neuropharmacology of Alzheimer's disease. Biomed Pharmacother. 2018;98:297-307.

67. Gasparini L, Ongini E, Wenk G. Non-steroidal anti-inflammatory drugs (NSAIDs) in Alzheimer's disease: old and new mechanisms of action. J Neurochem. 2004;91(3):521-36.

68. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, et al. Inflammation and Alzheimer's disease. Neurobiol Aging. 2000;21(3):383-421.

69. Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, et al. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc Natl Acad Sci U S A. 1989;86(19):7611-5.

70. Plassman BL, Havlik RJ, Steffens DC, Helms MJ, Newman TN, Drosdick D, et al. Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. Neurology. 2000;55(8):1158-66.

71. Maksimenko OG, Deykin AV, Khodarovich YM, Georgiev PG. Use of transgenic animals in biotechnology: prospects and problems. Acta Naturae. 2013;5(1):33-46.

72. Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. Med Princ Pract. 2015;24(1):1-10.

73. Chen ZY, Zhang Y. Animal models of Alzheimer's disease: Applications, evaluation, and perspectives. Zool Res. 2022;43(6):1026-40.

74. Krüger L, Mandelkow EM. Tau neurotoxicity and rescue in animal models of human Tauopathies. Curr Opin Neurobiol. 2016;36:52-8.

75. Jones G. 100 YEARS OF VITAMIN D: Historical aspects of vitamin D. Endocr Connect. 2022;11(4).

76. DeLuca HF. Vitamin D: Historical Overview. Vitam Horm. 2016;100:1-20.

77. Kulda V. [Vitamin D metabolism]. Vnitr Lek. 2012;58(5):400-4.

78. Sizar O, Khare S, Goyal A, Givler A. Vitamin D Deficiency. StatPearls. Treasure Island (FL): StatPearls Publishing

79. Landel V, Annweiler C, Millet P, Morello M, Féron F. Vitamin D, Cognition and Alzheimer's Disease: The Therapeutic Benefit is in the D-Tails. J Alzheimers Dis. 2016;53(2):419-44.

80. Chau YY, Kumar J. Vitamin D in chronic kidney disease. Indian J Pediatr. 2012;79(8):1062-8.

81. Cronin SC. The dual vitamin D pathways: considerations for adequate supplementation. Nephrol Nurs J. 2010;37(1):19-26, 36; quiz 27-8.

82. Botelho J, Machado V, Proença L, Delgado AS, Mendes JJ. Vitamin D Deficiency and Oral Health: A Comprehensive Review. Nutrients. 2020;12(5):1471.

83. Khojah HMJ, Ahmed SA, Al-Thagfan SS, Alahmadi YM, Abdou YA. The Impact of Serum Levels of Vitamin D3 and Its Metabolites on the Prognosis and Disease Severity of COVID-19. Nutrients. 2022;14(24).

84. Jeon SM, Shin EA. Exploring vitamin D metabolism and function in cancer. Exp Mol Med. 2018;50(4):1-14.

85. Jean G, Souberbielle JC, Chazot C. Vitamin D in Chronic Kidney Disease and Dialysis Patients. Nutrients. 2017;9(4).

86. Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzen E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. J Clin Endocrinol Metab. 1986;63(4):954-9.

87. Fathi N, Ahmadian E, Shahi S, Roshangar L, Khan H, Kouhsoltani M, et al. Role of vitamin D and vitamin D receptor (VDR) in oral cancer. Biomed Pharmacother. 2019;109:391-401.

88. Belorusova AY, Rochel N. Structural Studies of Vitamin D Nuclear Receptor Ligand-Binding Properties. Vitam Horm. 2016;100:83-116.

89. Dusilová-Sulková S, Šafránek R, Vávrová J, Horáček J, Pavlíková L, Palička V. Low-dose cholecalciferol supplementation and dual vitamin D therapy in haemodialysis patients. Int Urol Nephrol. 2015;47(1):169-76.

90. Wang Y, Zhu J, DeLuca HF. Where is the vitamin D receptor? Arch Biochem Biophys. 2012;523(1):123-33.

91. Fibla J, Caruz A. Vitamin D and HIV infection. 2010. p. 111-44.

92. Anjum I, Jaffery SS, Fayyaz M, Samoo Z, Anjum S. The Role of Vitamin D in Brain Health: A Mini Literature Review. Cureus. 2018;10(7):e2960.

93. Latimer CS, Brewer LD, Searcy JL, Chen KC, Popović J, Kraner SD, et al. Vitamin D prevents cognitive decline and enhances hippocampal synaptic function in aging rats. Proc Natl Acad Sci U S A. 2014;111(41):E4359-66.

94. Buell JS, Dawson-Hughes B, Scott TM, Weiner DE, Dallal GE, Qui WQ, et al. 25-Hydroxyvitamin D, dementia, and cerebrovascular pathology in elders receiving home services. Neurology. 2010;74(1):18-26.

95. Littlejohns TJ, Henley WE, Lang IA, Annweiler C, Beauchet O, Chaves PH, et al. Vitamin D and the risk of dementia and Alzheimer disease. Neurology. 2014;83(10):920-8.

96. Afzal S, Bojesen SE, Nordestgaard BG. Reduced 25-hydroxyvitamin D and risk of Alzheimer's disease and vascular dementia. Alzheimers Dement. 2014;10(3):296-302.

97. Chai B, Gao F, Wu R, Dong T, Gu C, Lin Q, et al. Vitamin D deficiency as a risk factor for dementia and Alzheimer's disease: an updated meta-analysis. BMC Neurol. 2019;19(1):284.

98. Balabanova S, Richter HP, Antoniadis G, Homoki J, Kremmer N, Hanle J, et al. 25-Hydroxyvitamin D, 24, 25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D in human cerebrospinal fluid. Klin Wochenschr. 1984;62(22):1086-90.

99. Cui X, McGrath JJ, Burne TH, Mackay-Sim A, Eyles DW. Maternal vitamin D depletion alters neurogenesis in the developing rat brain. Int J Dev Neurosci. 2007;25(4):227-32.

100. Chabas JF, Alluin O, Rao G, Garcia S, Lavaut MN, Risso JJ, et al. Vitamin D2 potentiates axon regeneration. J Neurotrauma. 2008;25(10):1247-56.

101. Patel P, Shah J. Vitamin D(3) supplementation ameliorates cognitive impairment and alters neurodegenerative and inflammatory markers in scopolamine induced rat model. Metab Brain Dis. 2022;37(8):2653-67.

102. Honório JE, Jr., Vasconcelos GS, Rodrigues FT, Sena Filho JG, Barbosa-Filho JM, Aguiar CC, et al. Monocrotaline: histological damage and oxidant activity in brain areas of mice. Oxid Med Cell Longev. 2012;2012:697541.

103. Satlin A, Volicer L, Stopa EG, Harper D. Circadian locomotor activity and core-body temperature rhythms in Alzheimer's disease. Neurobiol Aging. 1995;16(5):765-71.

104. Aziz SJ, Zeman-Pocrnich CE. Tissue Processing. Methods Mol Biol. 2022;2422:47-63.

105. Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. 2008;2008:pdb.prot4986.

106. Alvarez-Buylla A, Ling CY, Kirn JR. Cresyl violet: a red fluorescent Nissl stain. J Neurosci Methods. 1990;33(2-3):129-33.

107. Yakupova EI, Bobyleva LG, Vikhlyantsev IM, Bobylev AG. Congo Red and amyloids: history and relationship. Biosci Rep. 2019;39(1).

108. Frid P, Anisimov SV, Popovic N. Congo red and protein aggregation in neurodegenerative diseases. Brain Res Rev. 2007;53(1):135-60.

109. Islam MT. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. Neurol Res. 2017;39(1):73-82.

110. Pan J, Lei X, Wang J, Huang S, Wang Y, Zhang Y, et al. Effects of Kaixinjieyu, a Chinese herbal medicine preparation, on neurovascular unit dysfunction in rats with vascular depression. BMC Complement Altern Med. 2015;15:291.

111. Maae E, Nielsen M, Steffensen KD, Jakobsen EH, Jakobsen A, Sørensen FB. Estimation of immunohistochemical expression of VEGF in ductal carcinomas of the breast. J Histochem Cytochem. 2011;59(8):750-60.

112. Fabbri F, Carloni S, Brigliadori G, Zoli W, Lapalombella R, Marini M. Sequential events of apoptosis involving docetaxel, a microtubule-interfering agent: a cytometric study. BMC Cell Biol. 2006;7:6.

113. Rosen CJ, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, et al. IOM committee members respond to Endocrine Society vitamin D guideline. J Clin Endocrinol Metab. 2012;97(4):1146-52.

114. Navale SS, Mulugeta A, Zhou A, Llewellyn DJ, Hyppönen E. Vitamin D and brain health: an observational and Mendelian randomization study. Am J Clin Nutr. 2022;116(2):531-40.

115. Liu H, He Y, Beck J, da Silva Teixeira S, Harrison K, Xu Y, et al. Defining vitamin D receptor expression in the brain using a novel VDR(Cre) mouse. J Comp Neurol. 2021;529(9):2362-75.

116. Shen H, Wang Y. Correlation of locomotor activity and brain infarction in rats with transient focal ischemia. J Neurosci Methods. 2010;186(2):150-4.

117. Sultan S, Taimuri U, Basnan SA, Ai-Orabi WK, Awadallah A, Almowald F, et al. Low Vitamin D and Its Association with Cognitive Impairment and Dementia. J Aging Res. 2020;2020:6097820.

118. Gong Z, Huang J, Xu B, Ou Z, Zhang L, Lin X, Ye X, Kong X, Long D, Sun X, He X, Xu L, Li Q, Xuan A. Urolithin A attenuates memory impairment and neuroinflammation in APP/PS1 mice. J Neuroinflammation. 2019 Mar 14;16(1):62.

119. Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol. 2004 Oct 1;500(1-3):255-66.

120. Dursun E, Gezen-Ak D, Yilmazer S. A new mechanism for amyloid-β induction of iNOS: vitamin D-VDR pathway disruption. J Alzheimers Dis. 2013;36(3):459-74

121. Cheon SY, Koo BN, Kim SY, Kam EH, Nam J, Kim EJ. Scopolamine promotes neuroinflammation and delirium-like neuropsychiatric disorder in mice. Sci Rep. 2021 Apr 16;11(1):8376.

122. Hernández-Rodríguez M, Arciniega-Martínez IM, García-Marín ID, Correa-Basurto J, Rosales-Hernández MC. Chronic Administration of Scopolamine Increased GSK3βP9,

Beta Secretase, Amyloid Beta, and Oxidative Stress in the Hippocampus of Wistar Rats. Mol Neurobiol. 2020 Sep;57(9):3979-3988.

123. Dursun E, Gezen-Ak D, Yilmazer S. The Influence of Vitamin D Treatment on the Inducible Nitric Oxide Synthase (INOS) Expression in Primary Hippocampal Neurons. Noro Psikiyatr Ars. 2014 Jun;51(2):163-168.

124. Gezen-Ak D, Dursun E, Yilmazer S. The Effect of Vitamin D Treatment On Nerve Growth Factor (NGF) Release From Hippocampal Neurons. Noro Psikiyatr Ars. 2014 Jun;51(2):157-162.

125. Balaban H, Nazıroğlu M, Demirci K, Övey İS. The Protective Role of Selenium on Scopolamine-Induced Memory Impairment, Oxidative Stress, and Apoptosis in Aged Rats: The Involvement of TRPM2 and TRPV1 Channels. Mol Neurobiol. 2017 May;54(4):2852-2868

126. Gong Z, Huang J, Xu B, Ou Z, Zhang L, Lin X, Ye X, Kong X, Long D, Sun X, He X, Xu L, Li Q, Xuan A. Urolithin A attenuates memory impairment and neuroinflammation in APP/PS1 mice. J Neuroinflammation. 2019 Mar 14;16(1):62.

10. APPENDICES

10.1. 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
2022-43	11.10.2022	2022/10	2022/10-2	Dr.Öğr.Üyesi.Alev Cumbul

'Yüksek Doz 25hidroksi Kolekalsiferolün Farelerde Skopolamin İle Oluşturulan Alzheimer Tipi Demans Modelinde Hipokampüs ve Serebral Korteks Bölgesine Etkisinin Histopatholojik Olarak Değerlendirilmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Fare / BALB/c	20	Erkek

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Dr. 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	

10.2. CURRICULUM VITAE

Personal Information

Name	Yelda	**Surname**	Işıkbay

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Master	Institute of Health Sciences/Department of Histology and Embryology	İstanbul Yeditepe Univesity	2023
University	Faculty of Science and Letters/molecıler biology ve genetic	İstanbul yeni yüzyıl Univesity	2018
High school	Science Branch	Bahçelievler Cumhuriyet Anadolu 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
Photoshop	Good

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

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Others (Projects / Certificates / Rewards)

Francing king school of sertificate 2019
Certificate of Animal Use in Experimental research by Animal Research Ethichs Committee of Yeditepe University, 2020
4th East Mediterranean Congress of Laboratory Animal Science Oral Presentation 2st 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 PC05. 9-11 Dec 2021.
15. YÜTBAT National Medical Student Congress 'Innovations in Neuroscience' 2022