THE EFFECT OF PHOTOBIOMODULATION AND VITAMIN D ON TNF-ALPHA INDUCED TOXICITY IN SH-SY5Y CELLS

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ABSTRACT

THE EFFECT OF PHOTOBIOMODULATION AND VITAMIN D ON TNF-ALPHA INDUCED TOXICITY IN SH-SY5Y CELLS

Almsalmeh, Abedullraziq

Master's Program in Neuroscience

Supervisor: Assist. Prof. Dr. Mehmet OZANSOY

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The central nervous system is considered as one of the most important systems in the human body, because it controls all body systems by sending nerve impulses with the necessary commands. Neurodegenerative diseases are described as progressive and age-related disorders affecting central nervous system. This study aimed to use the photobiomodulation in human neuroblastoma cell line (SH-SY5Y) after being applied with TNF-α as a toxic agent, and vitamin D was also used in combination with the low-level laser. The cells were cultured in specific conditions suitable for their growing and propagation. The results showed that photobiomodulation led to an increase in cell viability in the presence of TNF-α and Vitamin D3. In addition, the amount of lactate dehydrogenase enzyme was found to be reduced in these cells.

Photobiomodulation can be used as one of the promising tools for treating many diseases affecting the CNS, especially neurodegenerative diseases, without having serious side effects.

Key Words: SH-SY5Y, photobiomodulation, TNF-α, Vit. D3, Cell Viability.

ÖZ

THE EFFECT OF PHOTOBIOMODULATION AND VITAMIN D ON TNF-ALPHA INDUCED TOXICITY IN SH-SY5Y CELLS

Almsalmeh, Abedullraziq
Sinirbilim Yüksek Lisans Programı
Tez Danışmanı: Dr. Öğr. Üyesi Mehmet OZANSOY

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Merkezi Sinir Sistemi, nöral sinyaller yoluyla insan vücudundaki tüm organ sistemlerini control etmektedir. Nörodejeneratif hastalıklar, merkezi sinir sistemini etkileyen yaşa bağlı ve ilerleyici karakterde olan hastalıklardır. Bu çalışmanın amacı düşük-seviye lazer uygulamasının TNF-α ile insan nöroblastoma hücre hattında (SHSY-5Y) oluşturulan toksisite modelinde etkisinin araştırılmasıdır. Çalışmada Vitamin D3 de kullanılmıştır. Bulgular TNF-α ve Vitamin D3 varlığında fotobiyomodülasyonun hücre canlılığını arttırdığını göstermektedir. Öte yandan fotobiyomodülasyon ile hücrelerde bulunan laktat dehidrogenaz enzim miktarı azalmaktadır.

Fotobiyomodülasyon, nörodejeneratif hastalıklar gibi merkezi sinir sistemini etkileyen hastalıkların gelecekteki tedavilerinde ciddi yan etkileri olmaması açısından önemli bir seçenek oluşturabilir.

Anahtar Kelimeler: SH-SY5Y, Fotobiyomodülasyon,TNF-α, Vit. D3, Hücre canlılığı

DEDICATING

I would to express my wholehearted thanks to my beloved family, especially my mother lovely who endured all pain to make all my dreams come true by supporting me continuously in every way. I want to dedicate this success to my father's soul (God rest his soul).

I want to dedicate this success to who is love me and I love him.

I want to dedicate this success to who was and still supports me, to who fills my heart with the whole of love. Because of their unconditional love and prayers, I have the chance to complete this thesis.

Finally, I thank all those who did not stand by me, who stood in my way and disturbed my work, without their presence I did not feel the pleasure of the research and the sweetness of the positive competition.

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I want to thank my colleague Bervis Hemis who assists me during my research. I wish you a very beautiful future in medicine.

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ABBREVIATIONS

Ach E1	Acetyl cholinesterase inhibitor
AD	Alzheimer's disease
Aβ	Amyloid-beta
APP	Amyloid precusor protiens
ALS	Amyotrophic lateral sclerosis
APO E	Apolipo protein E
CNS	Central nervous system
CcO	Cytochrome – c – oxidase
EOAD	Early onset of Alzheimer's disease
EAE	Experimental autoimmune encephalomyelitis
FR	Far red
FADD	Fas-associated death domain protein
FLD	Frontotemporal labor dementia
Hrs	Hours
HD	Huntington's disease
INF-γ	Interferon- gamma
IL-1β	Interleukin-1-beta
IU	International unit
LOAD	Later onset of Alzheimer's disease
LED	Light emitting diode
LLLT	Low light laser therapy
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
MAP	Microtubule-associated protein
mW	Milli watt
MTS	Mitochondrial targeting sequence
MS	Multiple sclerosis
NIR	Near infra-red
MAO	Monoamine oxidase
MAO B	Monoamine oxidase B
nm	Nano meter

NDs	Neurodegenerative disease
NFκ B	Neuclear factor kappa B
NMDA	N-methyl-D-aspartate receptor
PHFs	Paired helical filaments
PA	Parkinson's disease
PSEN 1	Presenilin 1
PSEN 2	Presenilin gene 2
PBM	Photobiomodulation
RIP1	Receptor interacting protein 1
ROS	Reactive oxygen species
SOD1	Superoxide dismutase gene 1
TRADD	Tumor receptor associated death protein
TNF-α	Tumor necrosis factor-α
TNFR-1	Tumor necrosis factor 1
TNR-2	Tumor necrosis factor 2
TRAF-2	Tumor receptor associated factor 2
Vit. D	Vitamin D

SYMBOLS

2-ccPA	2-carba-cyclic phosphatidic acid
CO_2	Carbon dioxide
$CoCl_2$	Cobalt chloride
DMEM	Dulbecco′s modified Eagle′s medium
FBS	Fetal bovine serum
LDH	Lactate dehydrogenase
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO HCl	Nitric oxide
PBS	Phosphate buffered saline
TBZ	Tetrabenazine

Chapter 1

Introduction

The nervous system is considered one of the most important systems of the body because of its important role related to all body functions. And just like other systems of the human body, it is susceptible to diseases, as the number of diseases that may affect the nervous system exceeds 600, and neurodegenerative diseases often emerge in this context. They are recognized in the late stages in adults, which include losing specific neurons which then causes a loss in their properties in the central nervous system. (Matilla-Dueñas et al., 2017)

Neurodegenerative diseases (NDDs) are defined as chronic degenerative illnesses of the brain and spinal cord that affect tens of millions of people worldwide. Symptoms of these diseases begin to appear with age, and they are described as the fourth leading reason of death in the area of developed countries, and they are more widespread in developing countries. Besides plenty of research, the underlying mechanisms have still been inconspicuous so far, and the pathogenic etiology accurate identification in traditional neurodegenerative diseases has not been clearly identified yet. (Zhou et al., 2013)

The classical neurodegenerative diseases shared several intracellular mechanisms such as mitochondrial dysfunctions, axonal transport, apoptosis, and protein degradation. Probable pathogen participation and new evidence from genomic sides appearing similarities between classical NDDs and virally-induced NDDs in humans, wherein some of the recent studies display a boost for possible connections of viruses with conventional neurodegenerative illnesses in humans. (Zhou et al., 2013)

The activity of immunity in the central nervous system can be classically seen in various immune-mediated disorders, human neurodegenerative diseases also in viral infections. Despite predominantly immune responses that may participate directly or indirectly in neuronal damage or loss, immune reactions are not fully destroyed because of their benefits in restoration and regeneration. Rising responses of the immune system may be a symptom of the pathogenic process, which might affect the precise balance between advantageous and injurious reflexes. (Zhou et al., 2013)

The process of neurodegeneration in the brain occurs in the form of sequential stages, as these diseases affect specific areas of the brain and then develop to affect other areas. (Ransohoff, 2016)

Research has also shown that the genetic factors causing neurodegenerative diseases constitute a small percentage of the factors, while the majority of these diseases occur as a result of the interactivity occurring between two different factors, the first of these factors is the genetic factor and the other is the environmental factor. (Coppedè et al., 2006)

There are many pathological conditions classified as neurodegenerative diseases, and each of these diseases has its symptoms and diagnostic methods, which are recognized in many disorders, like Parkinson's (PD), Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and many other neurological conditions. (Ransohoff, 2016)

Among the signs and indicators related to neurodegenerative diseases is the chronic increase of tumor necrosis factor. These markers associated with cytokines have been set among the targets that may contribute to the recovery process from these diseases, particularly in the early phase of the illness. (Clark and Vissel, 2018)

New research studies indicate the possibility of using low-level lasers in the treatment of many diseases, including neurodegenerative disorders affecting the brain tissue, and lead to the emergence of various symptoms such as accumulation and increase in amyloid-beta and the process of excessive phosphorylation of tau and other abnormal activities. (de la Torre, 2017)

Chapter 2

Literature Review

2.1. What Is Meant by Neurodegenerative Diseases?

2.1.1. Definition of neurodegenerative diseases. Neurodegenerative diseases are considered as chronic degenerative disorders of the central nervous system (CNS) (brain, optic nerve, and spinal cord) occurring from neurodegenerative processes. (Zhou et al., 2013) It is a disease caused by an imbalance in the association of neurons with each other in an area of the brain and this leads to disruption of other brain activities, and this may lead to the occurrence of many problems in the mechanism of brain functioning on a large scale. (Berman, 2018). Neurodegenerative diseases cause neuron death. We can divide the "neurodegenerative" word into two parts neuro: which means brain, and degenerative: which means breaking down or, dying. Neurodegenerative illnesses are considered a large model of the damaging effects of lost communications between the brain cells. These illnesses can affect the human's movement, intelligence, recollection, and speech. Neurodegenerative diseases are so sophisticated, and the reason for many of these disorders is still a secret. (Berman, 2018)

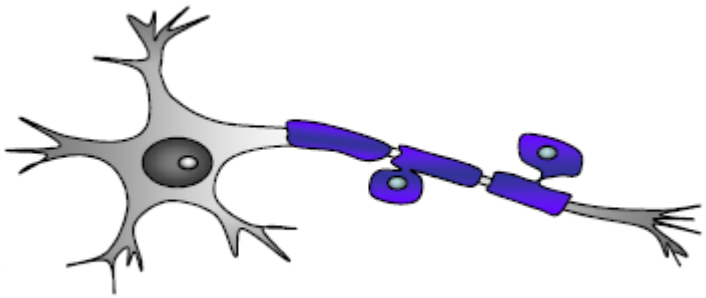

***Figure 1**: The nerve in normal case **(**Star et al, 2012).*

Neurodegenerative diseases are considered one of the most common diseases that affect the nervous system, and the rates of injury with these diseases vary from

one type to another, and from one sex to the other, as some diseases have a higher prevalence in males than females and vice versa. In addition to the fact that the prevalence of these diseases depends on age, the risk for this disease also connects with age, when age increases the risk to get the diseases will increase, and what is confirmed is that the most prevalent of these is Alzheimer's disease, followed by Parkinson's disease, while the rest of the diseases spread at lower and different rates. (Erkkinen et al., 2018)

As the number of people diagnosed with neurodegenerative diseases is estimated at tens of millions around the world, a number that is likely to increase with the increase in age, and the cases of people with Alzheimer's disease come as the highest percentage of patients with neurodegenerative diseases, as the number of people infected with it exceeds 50 million, while those with Parkinson's disease Their number exceeds 10 million patients all over the world, and the rest of the neurodegenerative diseases spread in large numbers, but they are less than these two diseases, and the percentage increases with age. (Han and Lu, 2020)

2.1.2. The effect of TNF-α in induceing injuries in cells. The scientist defines the tumor necrosis factor (TNF-α) as a cytokine produced from inflammation that's produced through macrophages/monocytes which happen during a severe inflammation and it's responsible for several interactions happening within the cells. (Idriss and Naismith, 2000)

TNF-alpha was first discovered in 1975 by Carswell and his associates, as it appeared as a protein in the serum of mice infected with bacteria. (Al-Gayyar and Elsherbiny, 2013)

The researches were confirmed that the TNF-α leads to the killing of the cancer cell line in vivo and in vitro models also, there is a connection between the TNF-α and several activities in the cell; like cell stay live, propagation, apoptosis, and cell differentiation have appeared. And it's seeming so important in the inflammatory immune response to induce and maintain it. (Al-Gayyar and Elsherbiny, 2013)

Through the studies appeared that the TNF-α interacts with two similar receptors: the first one is called p55 (TNFR1) and the other was p75 (TNFR2) and these interactions show on the neuron cells, microglia, and astrocytes in the central

nervous system (CNS). The TNFR1 is considered only for apoptosis while TNFR2 supports cell survival. (Al-Gayyar and Elsherbiny, 2013)

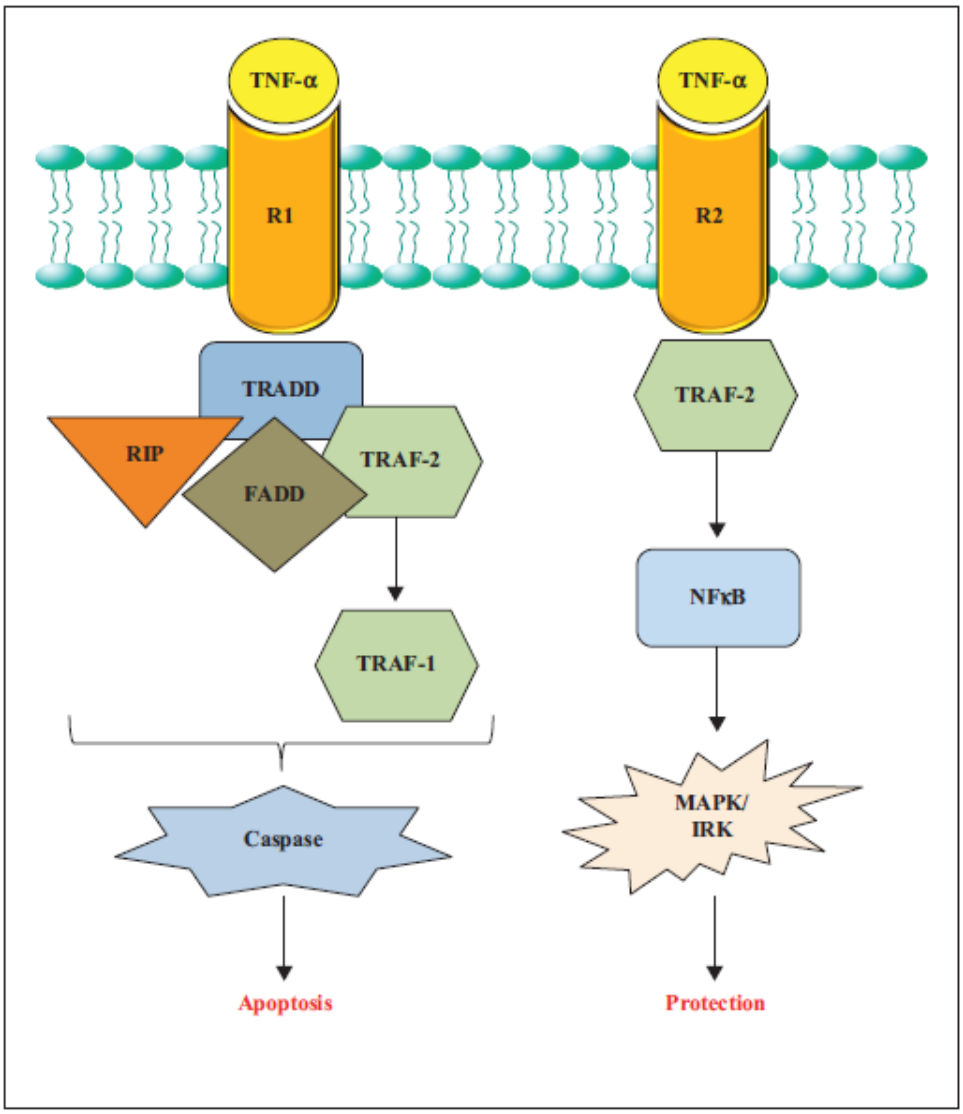

***Figure 2**: Diagram illustrate the mechanism of activity TNF-α.(Al-Gayyar & Elsherbiny, 2013)*

Figure (2) illustrates the binding between the TNF-α and the receptor 1 (R1) leading to stimulate (TNF-R1) associated death domain protein (TRADD) and this work as a base to induce another three mediators at least, the first is Fas-associated death domain protein (FADD), the second is TNF-receptor-associated factor 2 (TRAF2) and the third one is receptor-interacting protein 1 (RIP1), which activate the caspases and apoptosis. While the TNF-R1 activates apoptosis, the TRAF2 quickly

stimulates the nuclear factor kappa B (NFκB) that's inducing cell survival. (Al-Gayyar and Elsherbiny, 2013)

TNF-α is considered as the main moderator in causing many pathological pathways in a lot of neurological diseases like Parkinson's, ischemia, Alzheimer's, multiple sclerosis, amyotrophic lateral sclerosis, and peripheral autoimmune disorders such as juvenile arthritis and rheumatoid, Crohn's disease, and ankylosing spondylitis. (Olmos and Lladó, 2014)

Through the studies which conducted by Olmos and Lladó, it was found that the TNF-α is not the main reason for motor neurodegeneration, but TNF-alpha can work with a complementary rhythm with the other cytokines like interferon-γ (IFN-γ) and interleukin-1β (IL-1β) to induce cell toxicity and neuronal cells apoptosis. (Olmos and Lladó, 2014)

The studies indicate that there is an effect of high TNF- α secretion inside the brain, and it seems obvious through different disorders at the beginning of the non-infectious neurodegenerative disorders. The TNF-α regulates the changes in the level of α-synuclein, degree of insulin resistance, β-amyloid, and tau level in the majority of neurodegenerative disorders. (Clark and Vissel, 2018)

It has been proven through previous studies that tumor necrosis factor levels should be reduced to help reduce the appearance of both Parkinson's and Alzheimer's and that the chronic increase in levels of cytokines affects the development of such diseases, and this prompted scientists to say that factors that reduce levels of cytokines can reduce the incidence of Parkinson's and Alzheimer's diseases. (Clark and Vissel, 2018)

Through the studies carried out by Liu and colleagues, it was shown that there is an effect of TNF-α on SH-SY5Y cell line, as the higher the concentration of TNF-α, the effect will be increasing on the cells in terms of reducing cell viability and increase the cell apoptosis. This was confirmed by the use of several cell viability assay methods such as [3–(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (MTT), Caspases 3,9, and reactive oxygen species (ROS) assay. (Liu et al., 2019)

Scientists have used the SH-SY5Y neuroblastoma cell line in many studies and research, as it was not limited to a specific disease type, but was used in the study of many neurodegenerative diseases such as Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, and others. (Xicoy et al., 2017)

Through the research done by Pregi and his colleagues, it was observed that there is an effect of the TNF-alpha on the SH-SY5Y cells and this effect leads to toxicity to cells through following two pathways, one of them leading to cell death. (Pregi et al., 2009)

The process of neuroinflammation is defined as the response of brain cells to infection and other factors that may lead to the process of cell death, as the process of chronic inflammatory response in the central nervous system conduct to an increase in the production and accumulation of cytokines and other neurotoxic factors and the stimulation of glial cells. (de Araújo Boleti et al., 2020)

Several studies have shown that microglia, which are innate in the brain, play a role in the development of neurodegenerative diseases and neuroinflammatory processes. (de Araújo Boleti et al., 2020)

It should be noted that research has shown the process of nervous inflammation contributes to protection and prevention in the body, but the process of turning this inflammation into a chronic state of nerve inflammation leads to an excessive increase in the secretion of cytokines and neurotoxic factors in the brain, and thus the death of neurons. (de Araújo Boleti et al., 2020)

Among the factors that may lead to the occurrence of neuroinflammation are pathological conditions of aging or brain injuries, in addition to diseases that occur as a result of an imbalance in metabolic processes such as diabetes, obesity, depression, and high blood pressure. (de Araújo Boleti et al., 2020)

The process of deposition of beta-amyloid (it's the peptides that are considered the main components of senile plaque in the people who are suffered from Alzheimer's disease), as it leads to the stimulation of inflammation in microglia, and these plaques are not degraded by enzymes, and therefore this leads to increase neuroinflammation, in addition to the control of the pro-amyloid protein by other cytokines. This process controls the production of Aβ peptides, which in turn secrete additional neurotoxic agents. (de Araújo Boleti et al., 2020)
Parkinson’s is linked to Neuroinflammation and microglia stimulation, which leads to the accumulation and deposition of α-synuclein and ubiquitin in Lewy neuritis and Lewy body. Studies indicate that microglia also contribute to the development and emergence of multiple sclerosis. (de Araújo Boleti et al., 2020)

Through the studies carried out by Xiaoxu Chen and his colleagues, it was shown that the process of stimulation of astrocytes, which leads to the secretion of cytokines that contribute to the occurrence of inflammation, protects the nervous system, but the continuous stimulation of these cells leads to an increase in the secretion of cytokines, including TNF- α and thus an increase in factors Inflammatory nervous system. (Chen et al., 2018)

Glial cells may secrete TNF-alpha in order to stimulate the neurodegeneration process in the hippocampus. Also, research has shown that neurotoxins contribute to the overproduction of TNF-α by astrocytes, which in turn leads to neuronal damage. (Chen et al., 2018)

Microglia play the function of the immune system in the brain, where they work constantly to check the brain and produce stimulating factors for astrocytes, a type of glial cells that carry out the support process as the microglial cells secrete anti-inflammatory factors. The recurrent inflammation may be caused by environmental factors or internal causes, and as a result of this recurrence may increase the inflammatory response, which leads to an increase in the production of neurotoxic agents, which in turn increases the underlying disease cases. Thus, Christopher Glass and his colleagues concluded that trying to reduce neuroinflammation could reduce the production of neurotoxic agents, while potentially not affecting the main cause of the disease. (Glass et al., 2010)

2.1.3. Etiology of neurodegeneration. Studies have shown that the happening of neurodegenerative diseases is partly due to the state of aging, maybe also, according to the infection caused by viruses that attack nerves, besides genetic diseases and mutations, these diseases evolved, furthermore proteins accumulation in particular brain regions or chronic activation of the immune response innate in the nervous system (Stephenson et al., 2018). Neurodegenerative diseases causative factors had been studied and mentioned in many researches but aging is the most critical one. Studies have shown that the elderly are more susceptible to infection, and the higher the age, the greater the likelihood of developing such diseases. (Hou et al., 2019)

The inflammation is considered a sword with double edges; to maintain physiological homeostasis the inflammation should be controlled which is conclusive in the natural recovery operation. (Lyons, 2015)

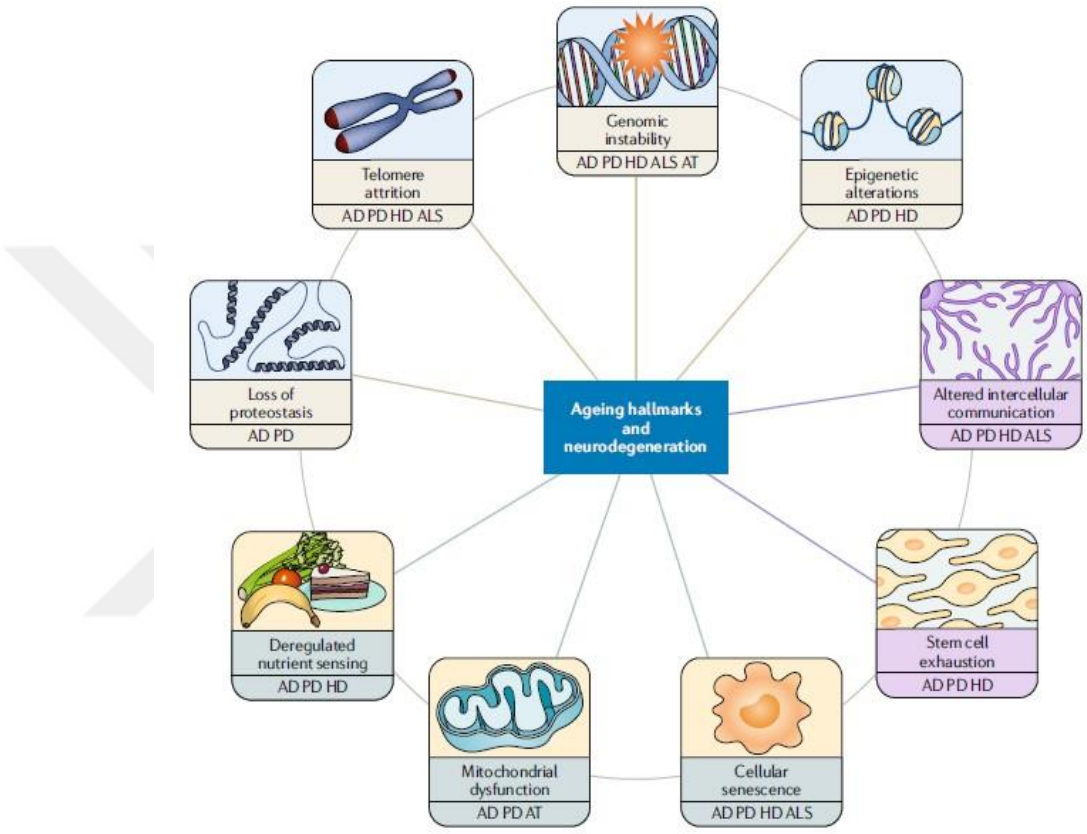

Figure 3: *Figure lists age-related factors that are associated with the onset of neurodegenerative diseases (Hou et al, 2019).*

α-Synuclein is a protein that is abundantly found in nerve endings and presynaptic regions. Studies have shown its association with the development of many degenerative diseases, but its natural function is still being studied. Synuclein is produced in its three forms (alpha, beta, and gamma) in the brain areas like of the hippocampus, cerebellum, neocortex, thalamus, and striatum. (Burré et al., 2018)

Research studies conducted by researchers indicate that the process of phosphorylation of α-synuclein protein contributes to the occurrence of neurodegenerative diseases, especially Parkinson's, as the phosphorylation process

that occurs on the amino acid serine-129 is the most prevalent form, which may lead to the accumulation of α-synuclein and the formation of inclusions. Some studies also indicate that the increase in phosphorylation of α-synuclein may lead to affecting its solubility, ability to membranes binding, and cellular distribution, which thus encourages the pathological condition. (Rocha et al., 2018)

Research also showed that α-synuclein contributes to the obstruction of mitochondrial protein import processes in Parkinson's disease. This is done through the sequence at the end of the α-synuclein, which is the mitochondrial targeting sequence (MTS), as this peptide sequence contributes to the transfer of mitochondrial proteins that are nuclear encoded to the mitochondria. (Rocha et al., 2018)

Tau is a protein present in the central nervous system in a high percentage. It is a type of protein associated with microtubules (MAP) and contributes to the process of stabilizing these neuronal microtubules. Microtubules have very important functions in maintaining cells, their development, and the processes they carry out, but the most important function is to maintain the stability of microtubules and by maintaining this stability, the various functions of the cell are regulated. (Muralidar et al., 2020)

Increased phosphorylation at the C-terminus of tau results in the protein's self-assembly and this leads to the formation of paired helical filaments (PHFs) [which are defined as abnormal proteins filaments for tau protein that are hyperphosphorylated and at some stages can be twisted around each other]. This aggregated protein leads to impaired cell functions and axonal transport processes as well as instability of neuronal microtubules and phosphorylated tau leads to the stimulation of proteins that cut neuronal microtubules such as katanin, in addition to many disorders that occur as a result of excessive phosphorylation of tau, the most important of which is the loss of ability to maintain on the stability of neuronal microtubules. (Muralidar et al., 2020)

2.1.4. The genetic connections with neurodegenerative diseases. Studies have shown that the emergence of neurodegenerative diseases is linked to genetic factors, especially Alzheimer’s disease, which is a disease that develops very slowly long before the appearance of its symptoms. (Matilla-Dueñas et al., 2017)

Presenilin genes (PSEN1, PSEN2) and the amyloid precursor protein gene (APP) have been considered as the main genes which could have mutations that cause

Alzheimer's, while a mutation in these genes develop the rate of early-onset of it (EOAD), while the apolipoprotein E (Apo E) gene located on chromosome number 19 increases the rate of late-onset of it (LOAD). (Matilla-Dueñas et al., 2017)

As for Parkinson's disease, which is the second most widespread neurodegenerative disorders after Alzheimer's disease, studies have shown that there is a link between many mutations that occur on the genes and the occurrence of the disease. The emergence of patterns linked to the X chromosome and the presence of autosomal dominant inheritance patterns. (Matilla-Dueñas et al., 2017)

Among the gene loci in which studies have shown mutations that lead to the emergence of Parkinson's disease are PARK3, PARK10, and PARK11, in addition to two SNCAIP genes, which encode a protein that interacts with α-synuclein. (Matilla-Dueñas et al., 2017)

As for amyotrophic lateral sclerosis (ALS), research has found that about 10% of the disease is of family origin, and studies have shown the association of six genes and eight different regions on the chromosomes with the disease. The most prominent of these genes that have been identified is the cytosolic copper-zinc superoxide dismutase gene (SOD1), which was the first gene to be identified as being associated with ALS, and the relationship of the alsin gene was also discovered, which contributes to encoding a protein that has a role in the vesicle trafficking process and organization of the cytoskeleton. (Coppedè et al., 2006)

Studies also have shown the presence of a genetic factor that poses a risk of multiple sclerosis in the population of northern regions of Europe, which is the HLA-DRB1 * 1501 risk haplotype, as it rises the incidence of the disease by about three times, but this hypothesis still needs to be proven by epidemiological studies. (Ascherio et al., 2010)

According to several studies and reviews conducted by Olsson and his partners, the focus has been on HLA class I and II genes, which have the greatest risk related to the occurrence of multiple sclerosis. (HLA is defined as a region that contains about 200 genes located on human chromosome number 6, as its functions are concentrated in the immune system. Some of these genes, category 1 genes, encode molecules that present peptide antigens to CD8+ cytotoxic T cells, while Class 2 genes provide molecules that help to bind to the peptide antigens, they encode to CD4+ TH cells). (Olsson et al., 2017)

Huntington's disease is a disease that can affect the nervous system which leads to the emergence of motor disorders and a state of tension in addition to cognitive and behavioral problems. These diseases can appear and affect humans at the age between 1-80 years. The disease is due to a dominant genetic factor with a special phenotype, in which the huntingtin protein is produced by greatly repeating the CAG nucleotide sequence on chromosome 4 and thus forming a long chain of Glutamine at the N-terminus, which plays a toxic role in this tail. (Walker, 2007)

2.1.5. Molecular neuropathology of neurodegenerative diseases. Research has shown that the apolipoprotein E (APOE) gene is one of the most important factors participating in the development of neurodegenerative diseases, specifically in relation to Alzheimer's disease, as APO E is associated with several major alleles, namely *APOE-Ɛ2*, *APOE-Ɛ3*, and *APOE-Ɛ4*. APOE4 was considered as the most dangerous alleles were associated with the emergence of neurodegeneration, and the increased risk in the presence of this allele may reach 12 times if compared to APOE3, also the studies have found that it is possible to treat the damaged hippocampus neurons in APOE2 whereas it is not possible in APOE4. (Clark and Vissel, 2018)

ApoE is defined as a glycoprotein that is predominantly present in the liver and brain and contributes to endocytosis through receptors for lipoprotein molecules such as cholesterol, which are essential for common brain tasks and myelin synthesis. (Breijyeh and Karaman, 2020)

The review by Breijyeh and Kraman confirmed the association of amyloid-beta in the development of degenerative diseases, particularly Alzheimer's disease since Aβ protein is deposited outside the cell in various forms; α-secretase and γ-secretase enzymes, which act as cleavage enzymes, are responsible for the formation of Aβ by degrading the amyloid precursor protein (APP). Where these enzymes break down the APP into parts that vary in size for each part, as some of them consist of 43, 45, 46, 48 amino acids, 49 or 51 amino acids until they reach the final forms, which are 40 and 42 Aβ. As a result, the accumulation of 40 and 42 amyloid-beta contributes to increased neurotoxicity in the brain. The process of aggregation of amyloid plaques in the cerebral cortex, hippocampus, and amygdala leads to activation of microglia and astrocytes, loss of synaptic areas, and the emergence of motor impairment. (Breijyeh and Karaman, 2020)

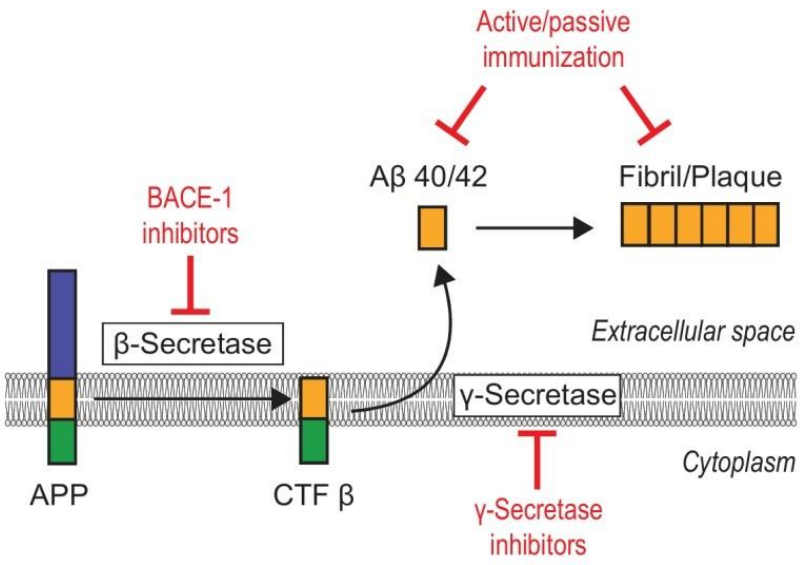

***Figure 4**: Degrading of the amyloid precursor protein to forming of amyloid-beta. (Zhou et al, 2018)*

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Research also showed that α-synuclein contributes to the obstruction of mitochondrial protein import processes in Parkinson's disease. This is done through the sequence at the end of the α-synuclein, which is the mitochondrial targeting

sequence (MTS), as this peptide sequence contributes to the transfer of mitochondrial proteins that are nuclear encoded to the mitochondria. (Rocha et al., 2018)

Tau is a protein present in the central nervous system in a high percentage. It is a type of protein associated with microtubules (MAP) and contributes to the process of stabilizing these neuronal microtubules. Microtubules have very important functions in maintaining cells, their development, and the processes they carry out, but the most important function is to maintain the stability of microtubules and by maintaining this stability, the various functions of the cell are regulated. (Muralidar et al., 2020)

Increased phosphorylation at the C-terminus of tau results in the protein's self-assembly and this leads to the formation of paired helical filaments (PHFs) [which are defined as abnormal proteins filaments for tau protein that are hyperphosphorylated and at some stages can be twisted around each other]. This aggregated protein leads to impaired cell functions and axonal transport processes as well as instability of neuronal microtubules and phosphorylated tau leads to the stimulation of proteins that cut neuronal microtubules such as katanin, in addition to many disorders that occur as a result of excessive phosphorylation of tau, the most important of which is the loss of ability to maintain on the stability of neuronal microtubules. (Muralidar et al., 2020)

2.1.6. The symptomatic treatment of NDDs. Research indicates that there are no effective treatments for neurodegenerative diseases, as the currently available treatments are used to treat symptoms only, while these treatments do not prevent the development of neurodegenerative diseases and do not treat the underlying causes of diseases. (Budd Haeberlein and Harris, 2015)

Scientists are seeking, through research and laboratory experiments, to find ways to treat neurodegenerative diseases, and one of the most important ways researchers are working on is to study the effect of anti-TNF, and they used infliximab and adalimumab, which are considered a monoclonal anti-TNF-α antibody. (Clark and Vissel, 2016)

Research by Clark and Vissel showed that there is an effect of tumor necrosis factor on changing the levels of glutamate in the brain, which is the main and most important neurotransmitter in the brain, as it participates in the majority of functions performed by the nervous system. (Clark and Vissel, 2016)

Scientists have thought about a mechanism for treating neurodegenerative diseases and found it logical that the treatment process should include a method to reduce the overproduction and accumulation of TNF, as it was evident that brain disorders, most of which are involved in TNF hyperactivity, are also associated with many of pathophysiological conditions. Studies have shown that the tumor necrosis factor significantly increases the level of glutamate production by activating glutaminase. (Clark and Vissel, 2016)

The importance of glutamate in the synaptic cleft lies in the fact that it is the main cause of rapid response processes in the brain caused by excitatory neurotransmitters. (Clark and Vissel, 2016)

As a result of studies carried out by Cummings and his colleagues, it was confirmed that the use of a group of medications that are taken in the treatment of symptoms of Alzheimer's disease. From these drugs donepezil, galantamine, and rivastigmine which are used as acetylcholinesterase inhibitors (AChEI), also use Memantine as N-methyl-D-aspartate (NMDA) receptor antagonist. (Cummings et al., 2019)

Another drug that has a double work, is Rasagiline which is working as a monoamine oxidase B (MAO B) inhibitor used in Parkinson's treatment and has a neuroprotective action with also, effect on amyloid processing. (Cummings et al., 2019)

Until now the USA food and drug administration did not approve special psychiatric drugs for the psychological symptoms of AD. (Cummings et al., 2019)

Hayes and colleagues have reviewed the use of several drugs in the treatment of symptoms resulting from Parkinson's disease. Including the use of Levodopa, where studies showed a significant effect on the disease. Also, drugs which are used in the treatment of Dopamine agonists (Pramipexole, Ropinirole, Rotigotine However, despite the benefits of dopamine agonists, they have some side effects such as affecting movement or excessive sleepiness. (Hayes et al., 2019)

Rasagiline and Selegiline are used as monoamine oxidase (MNO) inhibitors which decreases the metabolism of dopamine, thus relieving the symptoms of the disease in the early stages. Studies have confirmed the use of Anticholinergics, such as Benzhexol, and Benztropine, which are used in the treatment of symptoms of motor problems and tremors. (Hayes et al., 2019)

Studies by Wyant and colleagues have demonstrated the use of a number of medications to treat symptoms resulting from Huntington's disease, including tetrabenazine (TBZ), which is proven to be used in the treatment of chorea considered as the main symptomatic feature for HD, and studies have shown an effect of antipsychotics on patients' who are suffering from chorea status, among these antipsychotics: perphenazine, fluphenazine, and haloperidol. Also, even after the discovery of the genetic mutation that leads to Huntington's disease, no effective treatment for the disease has been found, and all treatments are still used only to treat symptoms. (Wyant et al., 2017)

2.2. Using Light As Alternative Treatment For Neurodegenerative Diseases

Phototherapy can be recognized by its capability to motivate photobiolo.gical mechanisms in cells. (Huang et al., 2011) Diabetes that depends on insulin, in addition to autoimmune diseases, including multiple sclerosis and also rheumatoid arthritis, are diseases that are positively affected by sunlight, as it has been shown through several studies that sunlight plays a protective factor for these diseases. (Avsar et al, 2015)

Studies indicate light therapy is used as a promising treatment for neurodegenerative diseases and chronic inflammation, as hypotheses refer to the mechanism of action of light therapy through the action of mitochondria cytochrome c oxidase (CcO) as a photoreceptor for Far-red/Near infra-red (FR/NIR) light. The process of light absorption by mitochondria cytochrome c oxidase leads to the restoration of the stimulation of mitochondrial gene transcription. (Lyons, 2015)

As a result of research carried out by scientists, it was found that there is a positive effect of low light laser therapy in various diseases, including neurodegenerative and autoimmune diseases. Research has shown that light helps heal wounds and reduce inflammation in addition to reducing pain and also supports cell survival and the formation of new proteins. It has been noted that low-level light has a better effect than high-level light, which may lead to inhibition and preservation of cell activity. (Hamblin, 2017)

Scientists also focused on the use of low-light laser therapy in cases of inflammation that may affect different tissues, including brain tissue, and also may

contribute to reducing oxidative stress and regulating antioxidant defenses. (Hamblin, 2017)

Among the various red spectral wavelengths, which have positive effects, the positive and beneficial effect was reached for wavelengths between (600-700 nanometers) and wavelengths between (770-1200 nanometers), while studies showed a negative effect for wavelengths between (700-770 nanometers). (Hamblin, 2017)

It was noted in the study by Amadeo and colleagues that there is a positive effect of using low-level laser therapy (LLLT) during aerobic exercise, and that it may be a therapeutic method in reducing inflammatory markers caused by cytokine agents such as TNF-α; However, no effect was observed using LLLT alone. (Amadio et al., 2015)

The study by Oron and his partner confirmed that the use of low-level laser therapy (LLLT) to treat the symptoms of Alzheimer's disease, especially beta-amyloid in experimental mice, leads to the activation of mesenchymal stem cells (MSCs) which is located in the bone marrow, these MSCs that contribute to the process of phagocytosis of toxic beta-amyloid, which leads to a decrease in the levels of toxic beta-amyloid in the brain of the laboratory mice and a change for the better in the cognitive and memory functions of the mice used in the experiments. (Oron and Oron, 2016)

Through the experiments carried out by Cho and colleagues using the 5XFAD mouse as a modified Alzheimer's model, it was shown that there is an effect of light-emitting diode (LED) photomodulation on the formation and accumulation of amyloid-beta in the brains of mice used in the experiments by reducing or preventing its accumulation, especially if phototherapy is used in the early stages. Photobiomodulation therapy also contributed to reducing the neurodegenerative condition leading to a decrease in anxiety and memory loss in the laboratory model. (Cho et al., 2020)

Studies by Pople and colleagues concluded that the use of photobiomodulation therapy reduces oxidative stress, decreases the concentration of amyloid plaques in the brains of mice used in experiments, minimizes apoptosis and neurodegeneration, in addition, to stimulates metabolic processes in the mitochondria. However, these results are still limited to laboratory animals, while human clinical trials still need more studies, with positive signs in the use of photobiomodulation for treatment. (Pople et al., 2020)

The review conducted by Salehpour and Hamblin concluded that the process of using photomodulation in the curing of neurodegenerative diseases, especially Parkinson's disease, can yield a good result, and this was demonstrated during experiments on animal models of Parkinson's disease by trying to preserve dopamine-producing neurons. Based on the positive results of laboratory experiments on animals for the use of photomodulation in the treatment of Parkinson's disease, scientists went to start clinical trials using the same technology on patients with the disease, and an improvement appeared in 75% of patients who underwent this experiment, while 25% did not make any change. In their case, the positive results included an improvement in movement, an amelioration in the ability to smell, in addition to an advance in the ability to speak and swallow, in addition to an improvement in the initial state of tremor. (Salehpour and Hamblin, 2020)

Moges and her colleagues sought to reveal the effect of light therapy on familial ALS, as it is known that light has effects on mitochondria by improving the energy produced in them and reducing the oxidation state. But what this study found is that there is no noticeable effect of treatment when using light on amyotrophic lateral sclerosis, except for a very small effect on the motor function of the mice used in the experiments, which were exposed to light in the early phases of the disease, and thus the study concluded the possibility of an effect of phototherapy on other degenerative diseases that are related to mitochondrial disorders. (Moges et al., 2009)

During the experiment which has done by Jeri-Anne Lyons, the use of the autoimmune encephalomyelitis (EAE) model was studied to examine the ability to the treatment of the photobiomodulation for MS. This study conducted on mice showed that there was a positive effect of light therapy with a wavelength of 670 nm with a decrease in cell death that was detected through immunohistochemical analysis. (Lyons, 2015)

A group of American researchers, led by Henderson, reached an invention; they tried to use the light therapy without any other agents or with a combination with other pharmacological agents as a novel treatment. They used this way to prompt treated the main nerve areas that lead to these dysfunctions or the development of these diseases as mitochondria and promote the production of neurotrophic agents. (Lyons, 2015)

This invention is also related to an attempt to determine the location of dysfunction, injury, or disease through the use of quantitative analysis of

neurophysiology data or quantitative analysis of imaging data, in order to focus the treatment on affected areas specifically, and with this process, a method is developed to examine the progress of the therapeutic benefit in a new way that has not been done before. (Lyons, 2015)

Over the past several years, lasers have been tried at low-level wavelengths in order to observe their effect on several diseases. Near-infrared radiation was tried to check its ability to modify intracellular restoration. Near-infrared radiation has been found to stimulate rapid wound healing, promote muscle reform, and the development of new blood vessels (Angiogenesis). (Morries and Henderson, 2018)

Current information indicates that near-infrared light, which has wavelengths between 600 -1200 nm, is very necessary to use as phototherapy. Where are the wavelengths, 1200-600 nm are absorbed by the cytochrome c oxidase through the respiratory chain of the mitochondria, which leads to an increase in the activity of the respiratory chain and this leads to an increase in energy production (ATP). (Morries and Henderson, 2018)

When we talk about infrared light, we have to mention its properties, considered as physical properties that have a connection with clinical use. Light is considered as a form of electromagnetic radiation which has the features of particles, and waves and is also characterized by the amount of energy content that it has. (Morries and Henderson, 2018)

There are a number of biological effects of near-infrared radiation, but it is also important to understand the mechanism of the physical interactions between light and tissues. These interactions are based on the premise that the greater wavelength of light, have the greater amount of energy that penetrates the tissues, and thus the greater number of photons that will enter the tissues. Through the laboratory studies, it appears that infrared light has the ability to induce the level of ATP, stimulate mitochondria, increase the nerve growth factors, change nitric oxide (NO) levels, raise synaptogenesis, turn on genes, and stimulate other processes. (Morries and Henderson, 2018)

The patent registered by Morries and his colleague which they used an opposite research methodology to the previous research which gave results that the low-level near-infrared therapy cannot penetrate different depths of tissues such as 2 mm from the skin and 3 cm from the skull. Therefore, near-infrared therapy is considered

ineffective in the treatment of neurodegenerative disorders, and moreover, these light wavelengths cannot enter the joints of muscle tissue. (Morries and Henderson, 2018)

Many studies indicate that the most common symptom of multiple sclerosis is fatigue, and more than 25% of patients consider that fatigue the most disabling sign, there are different forms for fatigue like the reduced function of the frontal cortex, dopaminergic neuropathy in the basal ganglia, cytokine stimulation in an ongoing state of inflammation in the brain. In the study by Mateen and her partners, the use of bright white light was investigated in the treatment of fatigue associated with multiple sclerosis, which may help save the costs of treating MS fatigue without surgical procedures. Through previous studies, it was shown that there is an effect of treating fatigue with light in several diseases, including traumatic brain injury, Parkinson's disease, cancer-related fatigue, and seasonal affective disorder. (Mateen et al., 2017)

It appears through many studies that were made after the discovery of the laser about forty years ago that light or near-infrared rays can be used to treat deep nerves, increase wound healing, reduce inflammation and stop tissue damage. But low-light therapy remains controversial. (Hamblin, 2018)

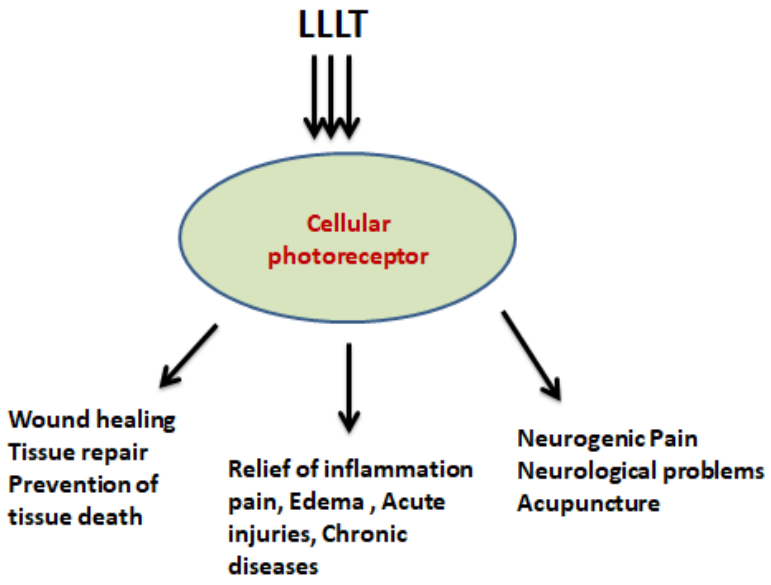

***Figure 5**: Diagram represents the main areas of application of light therapy (Hamblin, 2018).*

Through studies conducted by Karu, it was shown that there is a positive effect (which means there is a good effect for LLLT) of treatment with low laser light, through his experiment on cervical cancer cells, Escherichia coli cells, and HeLa cells. To ensure the effectiveness of low-light laser therapy, it must be tried on healthy cells to observe its effect on them and what stimuli occur in healthy cells when exposed to light. (Hamblin, 2018)

According to studies conducted by Yu et al., many activities were observed in cells after exposure to low-laser light therapy, and among those response activities were the increase in reproduction and migration, the increase in metabolism, as well as the increase in the synthesis and secretion of various proteins. These studies showed effects on more than one of these responses. (Hamblin, 2018)

Lots of animal models have been used to prove the effect of low-light laser therapy, which has an effect on many chronic conditions, and acute diseases. As a part of these studies, Byrnes et al. used the LLLT and especially 810nm diode laser to progress functionality and healing in a T9 dorsal hemi-section of the spinal cord in rats. While Anders et al., used the LLLT to the renovation of the facial nerve of rat which was destroyed and they found the best effect was by the wavelength of 633nm HeNe laser. (Hamblin, 2018)

In research experiments conducted by Whelan's, LED light with a wavelength of 670 nm was used to counteract damage to neurons caused by neurotoxins. Later on, experimenting with different wavelengths (670, 728, 770, 830, and 880 nm), the result was the emergence of the largest effect of wavelengths 670, 830 nm in conjunction with the near-infrared spectrum absorption of oxidized cytochrome C oxidase. (Hamblin, 2018)

The use of light therapy has demonstrated the important function of mitochondria which help sustain life through metabolism and energy production. Where the mitochondria can use as an energy generator that can produce the energy ATP by converting it from food molecules through an oxidative phosphorylation mechanism. (Huang et al., 2009)

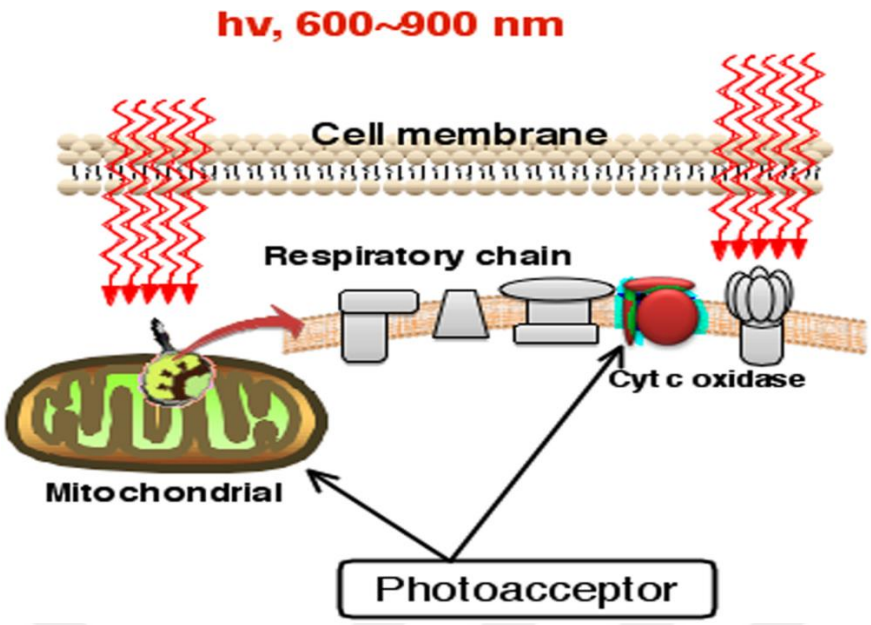

***Figure 6**: Figure demonstrating the absorption of red and NIR light by specific cellular chromophores or photoreceptors localized in the mitochondrial respiratory chain (Huang et al., 2009).*

Cytochrome c oxidase (Cco) is supposed to be the main photoreceptor for red-NIR light and this is suggested by many studies. The nitric oxide produced in mitochondria can prohibit respiration by connecting to Cco and competitively replacing the oxygen, particularly in the cells that suffer from a low level of oxygen or which are under stress. (Huang et al., 2009)

Studies have shown that the effect of Low Light Laser Therapy (LLLT) applicable to detached mitochondria has various forms such as stimulation of RNA and protein formation, enhancement of NADH and ATP formation, an increase of oxygen consumption, formation of ATP, and increase of proton electrochemical potential. (Huang et al., 2009)

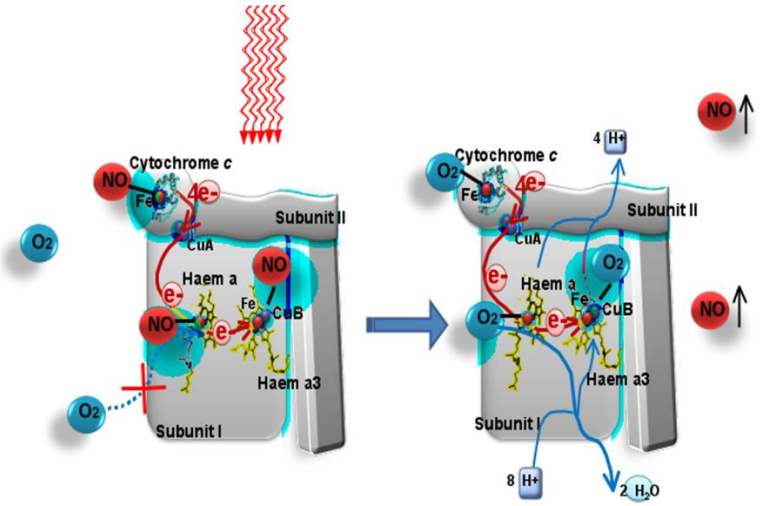

***Figure 7**: The scheme shows the photodissociation of NO from its binding sites on the heme-iron and copper centers (Huang et al, 2009).*

From the fig. (7) was observed respiration returns to the previous level due to the effect of light that leads to the release of nitric oxide from its binding sites of copper and iron in cytochrome c oxidase. which leads to restoring the binding of oxygen to these sites and thus an increase in the production of ATP. (Huang et al., 2009)

This scheme explains the photodissociation of NO from its binding sites on the heme-iron and copper centers where it inhibits oxygen binding and reduces necessary enzymatic activity, thus allowing an immediate influx of oxygen and resumption of respiration and generation of reactive oxygen species. (Huang et al., 2009)

Until now the mechanism of action of the LLLT is still unknown, but the review studies were done by Huang and his partners indicate that the use of LLLT with a low dose is better than a high dose to protect the different tissue cells. through the studies appeared there is increasing in cell proliferation like keratinocytes, lymphocytes, endothelial cells, and fibroblasts. Also, help in stopping cell death and increase the release of growth factor, more on that it can reinforce forming of new blood vessels, and induce collagen formation which can increase injury healing, restoration of injured nerves, and moreover different internal organs, also may help in reducing the inflammation and pain which happened by autoimmune disorders or degenerative illnesses. (Huang et al., 2009)

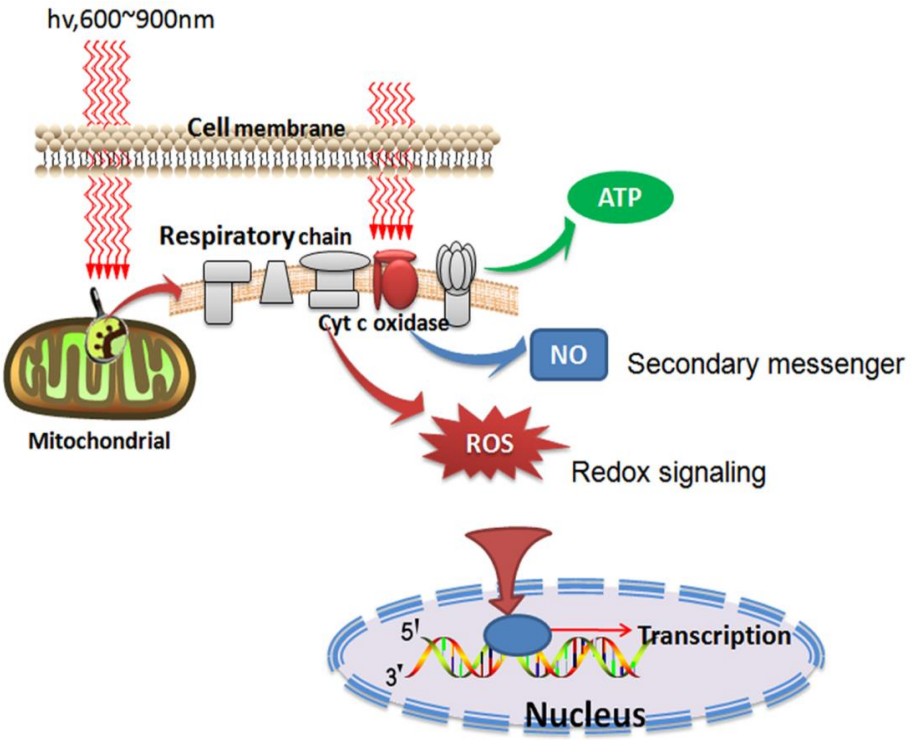

***Figure 8**: Figure shows some of the signaling trends that occur within cells after exposure to LLLT (Huang et al., 2009).*

Through figure (8), we can see the effects of LLLT after the absorption of photons into the mitochondria by chromophores at the cellular level, as it leads to an increase in the production of ATP and an increase in the respiration process, but during this process nitric oxide and reactive oxygen species (ROS) are also produced and these molecules are known as signaling molecules. (Huang et al., 2011)

Studies have shown that exposure of nerve cells to nitric oxide leads to a blocking of the axonal conduction within a period of time not exceeding minutes from exposure to it, and the prevention continues for hours if the exposure to nitric oxide remains. The axonal conduction is restored within minutes of stopping the effect of nitric oxide. The conduction blocking mechanism is still unknown, but there may be a direct effect on energy production in the mitochondria or ion channels, particularly sodium ion channels. There is strong evidence that nitric oxide is produced in greater concentration during the occurrence of multiple sclerosis. (Smith and McDonald, 1999)

The inflammatory impact of MS goes beyond central white matter to areas of high synaptic density, such as the cerebral cortex. There are also many factors with inflammation that disrupt the process of nerve transmissions, such as nitric oxide (NO), TNF-α, interleukin-1, interleukin-2, and IFN-γ. (Smith and McDonald, 1999)

Through the brain-derived neurotrophic factor (BDNF) and its multi-directional benefits in the brain, it appears that the use of low-level laser therapy (LLLT) may have benefits in treating mental and neurodegenerative diseases. (Xuan et al., 2014)

Studies conducted by Xuan and his partners in the use of low-level laser therapy have demonstrated that there are benefits to using it in treating several types of encephalopathies, and traumatic brain injury (TBI). The use of near-infrared radiation in the treatment of animals affected by the brain revealed a decrease in nerve inflammation, improvement in nerve performance, as well as an increase in the formation of neurons, and a reduction in the size of injury in the brain. (Xuan et al., 2014)

The process of using PHOTOBIOMODULATION depends on several factors, the most important of which are the number of repetitions of the treatment, the length of the light wave, the time period, and the form in which the light is delivered if it is pulsed or continuous, except for the energy density that reaches the cells. (Salehpour et al., 2018)

The review carried out by Salehpour and his colleagues concluded that the use of PHOTOBIOMODULATION therapy to the many benefits it gives to cells in preventing apoptosis, improving neuronal feeding processes, and raising antioxidant factors, besides working on improving blood flow through blood vessels and their formation. In addition to that inhibiting inflammatory processes that affect the nervous system, which prompted scientists to say that the use of light therapy will be one of the most used treatment methods in the future; if the clinical trials they are working on are successful, which are carried out through irradiation inside of the skull, mouth, nose and even through the ear canal. (Salehpour et al., 2018)

2.3. Using Vitamin D3 As a Treatment For Neurodegenerative Diseases

So far, the links of vitamin D to the nervous system and diseases that affect it, such as Parkinson's, Alzheimer's, multiple sclerosis, and other diseases, are not

completely clear. Studies also indicate the association of vitamin D with many brain functions and growth, and the contribution of vitamin D to synaptic plasticity and protection of nerves, in addition to the connections between neuronal circuits. (Bivona et al., 2019)

Vitamin D is a secosteroid hormone that can be obtained from food, but the main source of it from exposure to sunlight, as more than 80% of the vitamin is synthesized in the skin. (Breuer et al., 2018)

One of the most prominent functions that vitamin D performs is regulating calcium physiology, but it has many additional important functions in regulating brain function and growth, controlling blood pressure, and regulating programmed cell death and reproduction, as well as controlling the immune response and discrimination of immune cells, in addition to controlling the level of insulin in the blood. (Ascherio et al., 2010)

Studies indicate that the primary function of vitamin D is to regulate the homeostasis of calcium and phosphate and to maintain bone homeostasis, in addition to recent studies showing the link of vitamin D in cognitive and neurological processes. The study, conducted by Lin and colleagues, aimed to ascertain the relationship of vitamin D in the treatment of cognitive impairment resulting from Alzheimer's disease. The researchers concluded that there is a positive effect of vitamin D in improving memory in Alzheimer's disease, which may be through the regulation of Collapsin response mediator protein-2 (CRMP2) by reducing the state of phosphorylation that occurs in it. Previous studies indicate that an increase in phosphorylated CRMP2 may lead to the emergence of Alzheimer's disease, a condition that appears even before amyloid plaques, in addition to the possibility of protecting the central nervous system from various disorders that may affect it. (Lin et al., 2020)

The toxic effects of Aβ on neurons appear through the state of oxidative stress and cholinergic nerve destruction, in addition to the inflammatory diagnosis of neurons, and studies have shown that vitamin D may play a positive role in the process of removing amyloid plaques by phagocytosis. (Jia et al., 2019)

The study conducted by Jia and colleagues revealed that vitamin D has a beneficial effect in improving cognitive functions, which is one of the most prominent features of Alzheimer's disease, in addition to reducing the state of accumulation of Aβ protein and even working to remove it, and the study concluded that vitamin D

could be used as a new and promising treatment in Alzheimer's disease treatment. (Jia et al., 2019)

Through the experiments carried out by Morello and her colleagues, it was found that the process of using vitamin D in the treatment of neurodegenerative diseases, especially Alzheimer’s disease, has an effective role as it contributes to promoting the process of neurogenesis and improving cognitive functions; As taking vitamin D in the early stages of the disease showed a positive effect on female experimental mice used as a model for Alzheimer's disease. (Morello et al., 2018)

It was shown through the review conducted by Lv and his colleagues that vitamin D plays important functions in the central nervous system by contributing to providing protection for neurons, increasing the number of neurons, and working on their differentiation and arranging immune processes. The review also confirmed the presence of receptors for vitamin D in the human brain, as well as enzymes that contribute to its neurometabolic. (Lv et al., 2020)

As a result of the studies, it appeared that there is a direct or indirect relationship between the incidence of Parkinson's disease and vitamin D levels; Whereas vitamin D contributes to the protection of dopaminergic neurons, the mechanism is still unknown. (Lv et al., 2020)

Research has confirmed that taking vitamin D in young people with Parkinson's disease leads to an improvement in the patient's balance. In other studies, it appeared that taking vitamin D supplements contributes to improving the balance and strength of elderly patients, in addition, try to delay the development of the disease for a short period of time. Vitamin D also contributes to many functions related to the skeletal, muscular, and nervous systems, which leads to the possibility of an effect of the vitamin on Parkinson's disease and its symptoms. (Lv et al., 2020)

Zhou's review of several studies revealed that exposure to sunlight for at least 15 minutes per day may lead to the prevention of various neurodegenerative diseases, including Parkinson's disease, as exposure to sunlight leads to an increase in the concentration of vitamin D in the blood serum. Thus, it appears that vitamin D has a role in the prevention rather than treatment of Parkinson's disease. It also appeared that the process of using vitamin D supplements does not have any therapeutic effects on the motor functions of patients with Parkinson's. (Zhou et al., 2019)

Research revealed that vitamin D can be used in the treatment of amyotrophic lateral sclerosis (ALS), but because the disease occurs as a result of several factors, it is not reasonable for vitamin D to be a general treatment, but its role is limited to specific factors. (Karam and Scelsa, 2011)

Histopathology of people with amyotrophic lateral sclerosis showed low levels of various neurotrophic factors such as insulin-like growth factor-I (IGF-I) and glial cell line-derived factor. Vitamin D supports insulin-like growth factor-I (IGF-I) by increasing its receptors, thus extending the lifespan of ALS. (Karam and Scelsa, 2011)

Vitamin D can also improve and stimulate the building of new axons in ALS patients. Through research, it was observed that some neurons are less exposed to motor degeneration, and this may be a result of an increase in calcium-binding proteins (CBPs), and as a result of research in this area, scientists have concluded that the process of giving vitamin D to people with ALS is likely to increase the expression of CBPs in impaired motor neurons and thus increases resistance to disease. (Karam and Scelsa, 2011)

Studies by Molnár and his associates showed that the process of using vitamin D in the treatment of motor impairment caused by Huntington's disease was not significantly effective, but it was proven that vitamin D led to an increase in the lifespan of the experimental animals, which indicates that vitamin D It may have a useful complementary role on the clinical symptoms of Huntington's disease. (Molnár et al., 2016)

Studies conducted in the northern regions of Scotland have shown that there is an effect of vitamin D and ultraviolet light on the various immune processes of the body but for each factor separately. Through the conduct of experiments and studies, it was shown that there are significant effects of vitamin D on the immune system, in addition to the low level of vitamin D in people with the clinically isolated syndrome (CIS) increases the possibility of developing multiple sclerosis. (Breuer et al., 2018)

Through some new studies, a correlation was appeared between gray matter volume and brain volume with the amount of vitamin D, in addition to its relationship to the clinically isolated syndrome, which is the earliest case of multiple sclerosis, in addition to the formation of new lesions that appear on magnetic resonance imaging (MRI). (Abbatemarcoa et al., 2019)

It also appeared that the lower the patient's vitamin D level, the more severe the relapse. It was observed in a study conducted between 2000-2014 in various countries of the world that among the most prominent factors causing the risk of developing multiple sclerosis is a lack of vitamin D, in addition to the high percentage of people who suffering from obesity, also who is suffering from low levels of vitamin D and lack of exposure to sunlight. (Luque-C´ordoba and D.Luque de Castro, 2016)

Studies have revealed that vitamin D and its receptors are working as a key molecule in brain development, formation of nerve growth factor and protection from anxiety, in addition to stimulating neurotrophic factors. (Luque-C´ordoba and D.Luque de Castro, 2016)

It has been shown through studies that it is possible that a decrease in vitamin D levels may occur as a result of genetic disorders and not only due to lack of exposure to sunlight or food, as imbalances have appeared in the genes, to be more clear CYP27B1, encoding 1α-hydroxylase, the enzyme which converts the diversion of Vit D to its active metabolite, and CYP24A1, encoding 24-hydroxylase, which is the enzyme that changing the active Vit D to its inactive metabolite calcitroic acid, thus increasing the risk of developing multiple sclerosis. In a study conducted on a group of twins, the importance of people who have a genetic potential should be exposed to sunlight, and this situation gives indications that vitamin D deficiency may be a genetic and / or environmental factor in multiple sclerosis. (Breuer et al., 2018)

It was also shown through a study conducted by Cantorna and his partners that giving an infected animal with dihydroxymetabolite supplementation at the beginning of its infection with EAE leads to prevents the development of EAE, and if stops to give the supplementation these leading to the return of disease progression. Another result appeared indicating that the ultraviolet light working onto stopping the development of EAE without Vitamin D supplementation is needed but the supplementations have effects, this study is indicative of UV independence in preventing EAE. (Luque-C´ordoba and D.Luque de Castro, 2016)

Studies conducted by Ascherio and colleagues have shown that black-skinned people are more deficient in 25-hydroxyvitamin D than white-skinned people, which leads most of the time to vitamin D deficiency due to melanin in human skin responsible for absorbing ultraviolet rays. (Ascherio et al., 2010)

Vitamin D is primarily present as colecalciferol (Vitamin D3) in two sources: diet and skin exposure to UV rays. The length of the ultraviolet rays ranges between 290-315 nanometers, and these rays photolysis 7-dihydrocholesterol and converts it to pre-vitamin D3 in the skin and then turns into colecalciferol. Colecalciferol and ergocalciferol (vitamin D2) can also be obtained from food sources (such as tuna, salmon, milk, orange juice, etc.) in addition to vitamin supplements. Food sources do not provide large amounts of colecalciferol, while exposure to UV rays provides massive amounts compared to food. (Ascherio et al., 2010)

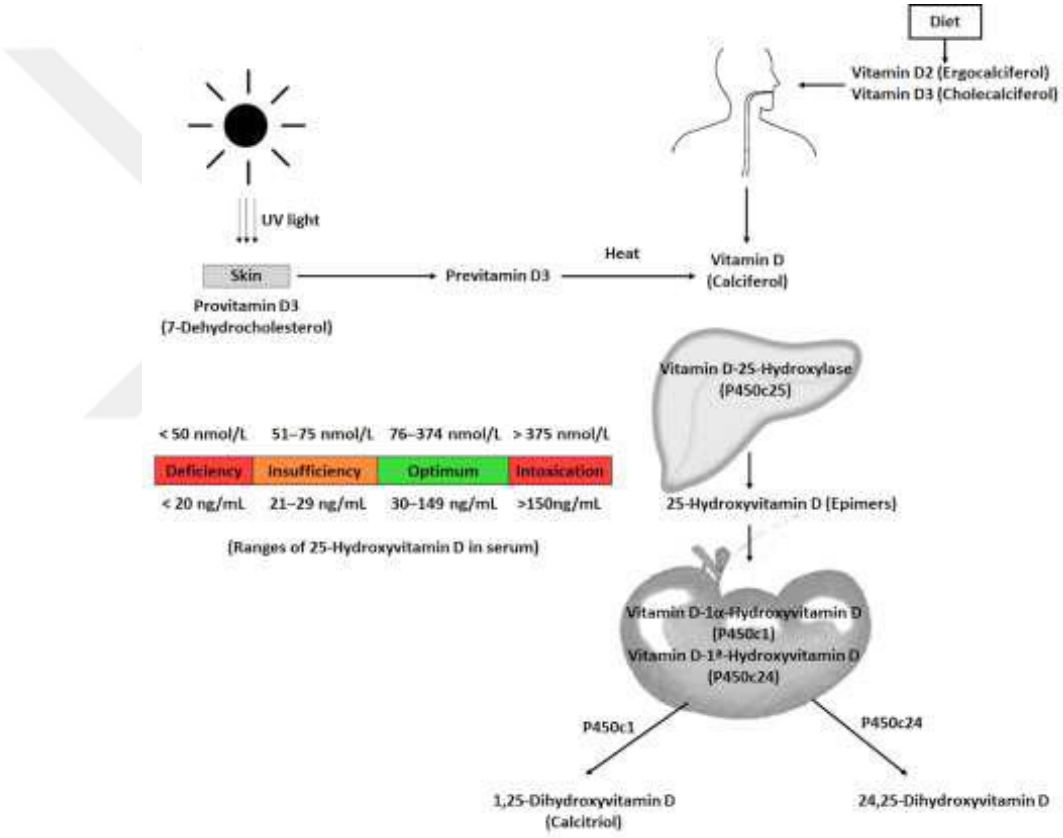

***Figure 9**: Sources and metabolism of vitamin D in the human body (Ascherio et al, 2010).*

Through figure (9) above, we can see the forms in which vitamin D is present, the two forms in which vitamin D is present are considered inactive, cholecalciferol and ergocalciferol. Vitamin D is converted to its active form in the liver, which is 25-hydroxyvitamin D by enzymes, then another hydroxyl process is performed, either in

the kidneys by parathyroid hormones or other tissues, and thus we get the active form of vitamin D which is (1, 25 - dehydrox (vitamin D) or what is also called calcitriol if obtained from vitamin D3. (Ascherio et al., 2010)

2.4. Objective

The aim of this study is to investigate the possible effects of laser with wavelength 660nm at different values of power output and vitamin D on the cell survival and function in SH-SY5Y cells which was treated with TNF-α as a toxic agent.

Chapter 3

Methodology

3.1. Materials

3.1.1. Kits and chemicals. All chemicals were used in this experiment are listed in the table 3.1. All kits were used are listed in the table 3.2.

Table 1: List of chemicals

Chemical product	Brand	Catalog number
Ethanol 96%	ISOLAB/Germany	920.026.2500
Phosphate buffered saline (PBS)	Gibco/USA	70011-044
Devit-3 (50000 IU/15ml)	DEVA HOLDING/Turkey	A092299
Penicillin-Streptomycin	Gibco/ USA	15140-122
Trypsin	Gibco/UK	25200-056
Fetal bovine serum (FBS)	Gibco/UK	10270-106
DMEM	Gibco/ USA	41966-029

Table 2: List of kits

Kit	Brand	Catalog number
Lactate dehydrogenase (LDH)	Sigma-Alderich/USA	MAK066
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid. (MTT)	Sigma-Alderich/USA	CT02

3.1.2. Equipment. All equipment which used in this experiment are listed in the table 3.3.

Table 3: List of equipment

Equipment	Brand
DMIL LED light microscope	Leica/Germany
Universal 320R centrifuge	Hettich Zentrifugen/ Germany
Incubator	Panasonic/Japan
Laminar flow hood (Telstar Bio II advance)	Telstar®/USA
Serological pipettes	SARSTEDT/Germany
Micro pipette (0.2-2 µl)	DRAGON LAB/China
Micro pipette (0.2-10 µl)	DRAGON LAB/China
Micro pipette (20-200µl)	DRAGON LAB/China
Micro pipette (100-1000µl)	DRAGON LAB/China
Micro pipette tips	GlobalRoll®/China
T25, T75 flasks	SPL life sciences/Korea
96 wells plats	VWR®/China
Micro plate reader	Hidex/Finland
Gloves	Broche® Turkey
Red laser at 660nm	Changchun New Industries Optoelectronics Technology/China

Falcon tube 15ml	LP italiana SPA/ Italy
laboratory water bath	WiseBath®/Germany
Refrigerator	PROFiLO/Turkey
Cell cytometer	HIRSCHMANN/Germany

3.2. Methods

3.2.1. SH-SY5Y human neuroblastoma cells source. It's a commercially available cell line, it was purchased from American Type Culture Corporation (ATCC) (CRL-2266, ATCC, USA).

3.2.2. Preparation of culture medium. Complete DMEM was prepared as a net volume, this complete DMEM contain 10% FBS (v/v) and 1% of Penicillin-Streptomycin (v/v).

3.2.3. Culturing and propagating of the SH-SY5Y cell line. After the complete DMEM was prepared, subculturing the SH-SY5Y cells was started in this medium at T25 flask by removing all of the old medium then 3-5ml of 1X PBS was added and move it gently to wash all of the surface of T25 flask to remove all the ruminant proteins that may causing late in detaching the cells from the surface of the T25 flask, after that the 3-5ml of PBS was removed and 500µl of trypsin enzyme was added to cut the root of the cells from the bottom of the flask and detached the cells, then washing the surface of the flask by added 10ml of complete DMEM and pipetted the surface several times to ensure all of the cells were detached, later the cells was transferred which were detached in the medium to 15ml of Falcon tube then centrifuge for 5minutes at 1500 rpm and 4°c. After that the supernatant was removed and resuspended the pellet (cells) by 10ml of complete DMEM then transferred the mixture to a new T25 flask with a specific dilution factor which was decided and incubate at 37°C, 5% of CO_2 and 95% O_2 in the incubator overnight.

3.2.4. Cell freezing. The freezing medium was prepared as the same of the complete DMEM with only change in the concentration of FBS which was 20% and 5% of DMSO was added to the medium which working as a cryoprotective agent and the volume of cells was divided into vials which was transferred to -20°c for two hours then to deep freezer at -80°c until the second day, and in the next day was transferred to liquid nitrogen.

3.2.5. Cell counting. The method which was used to determine the number of SH-SY5Y cells in each well in the 96 well plates to get more specific results. The number of cells was calculated by using this equation.

Conc. of cells/ml = No. Viable cells × Dilution factor × 10^4

The dilution factor was calculated by this equation

Dilution factor $= \frac{Total\ volume}{Cell\ suspention\ volume}$

Total volume= Volume of Trypan blue + Cells suspension

3.2.6. Power density calculations

Table 3.4: Power density calculations

Power (mW)	Beam Radius (cm)	Beam Area (cm2)	Power Density (mW/cm2)	Energy Density (J/cm2)	Time of Irradiation (s)
10	0.4	0.5024	19.90	1	50
20	0.4	0.5024	39.81	1	25
30	0.4	0.5024	59.71	1	17

When the power output increased this means the power density will be increased and the time during which the cells are exposed to the laser will be reduced in order to preserve the cells from the damage that may be caused to them as a result of the amount of energy they will be exposed to.

3.2.7. Preparations of plates for the experiments. After the cells were collected and centrifuged onto 1500 rpm at 4°c for 5 minutes, the supernatant was removed and the pellet was resuspended with 10 ml of complete DMEM, then the hemocytometer calculations were made to adjust the number of the cells in each well. The volume was transferred from cell suspension completed until 200µl with complete DMEM, then the plates were incubated for 24hrs.

After 24hrs from incubation the Vit. D3 was added to the wells where were applied by adding 333IU/ml, and the TNF-α was added to the wells with 30ng/ml concentration where were applied on by removing 2µl from the medium in that's wells and the 2µl of TNF-α was added, and the laser was applied on the well were planned to apply on with specific calculation returning to the (***Table 4***) depending on the workgroups. Finally, the cells were incubated for 24hr. The incubation period for the cells from the beginning of the experiment was 96hrs.

3.2.8. Groups of work

1. Cells alone
2. Cells + TNF-α
3. Cells + Vit.D3
4. Cells + TNF-α+Vit.D3
5. Cells + Laser
6. Cells + TNF-α + Laser
7. Cells + Laser + TNF-α
8. Cells + Laser + Vit.D3
9. Cells + TNF-α + Vit.D3 + Laser
10. Cells + Laser + Vit.D3 + TNF-α

3.2.9. The protocol of MTT assay. The MTT was prepared with concentration 5mgl/ml which was done by dissolve each 5mg of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid powder in 1ml of 1x PBS and ensure that there is no recipient in the mixture, in total 20ml of MTT mixture was prepared. The protocol was done by easy and fast steps:

- 50µl of the medium inside the wells was removed

- 50µl of the MTT mixture was added to each well and was mixed by pipetting
- The plate was incubated for 30 minutes inside the incubator at 37°c and 5% CO_2 and dark (MTT mixture sensitive to light)
- 150µl of solvent solution for MTT was added to each well and mixed well
- The blanks contain only complete DMEM and MTT mixture and MTT solvent solution
- The last step microplate reader was used to collect the data by using 570nm of optical density.

  % Of cell viability or toxicity = (absorbance of sample - absorbance of control) *100

3.2.10. The protocol of Lactate dehydrogenase (LDH) assay

- In the beginning the catalyst was dissolved in 1ml od double distilled water, and the reaction mixture was prepared for 200 reactions which was prepared by added 22.5ml of the dye solution to 500µl of the catalyst. The protocol was done by simple steps:
- 5µl of the lysis solution was added to each well which contain the cells
- The plate was incubated for 15 minutes inside the incubator at 37°c and 5% CO_2
- 100µl of the reaction solution was added to each well contain the cells
- The plate was incubated for 10 minutes at room temperature and at dark place (the reaction solution of LDH sensitive to light)
- 50µl of stop solution was added to each well contain the cells
- The blanks contain just the reaction solution and the stop solution, there is no DMEM
- The plate was shaken for 10 seconds and the measurement by microplate reader was done at 490-492nm of optical density.

3.2.11. Statistically analysis. The data have been statistically analyzed by using SPSS software package. One-way ANOVA test was implemented with a Tukey post-hoc test and P-value which is less than 0.05 was taken as a limit for statistical significance.

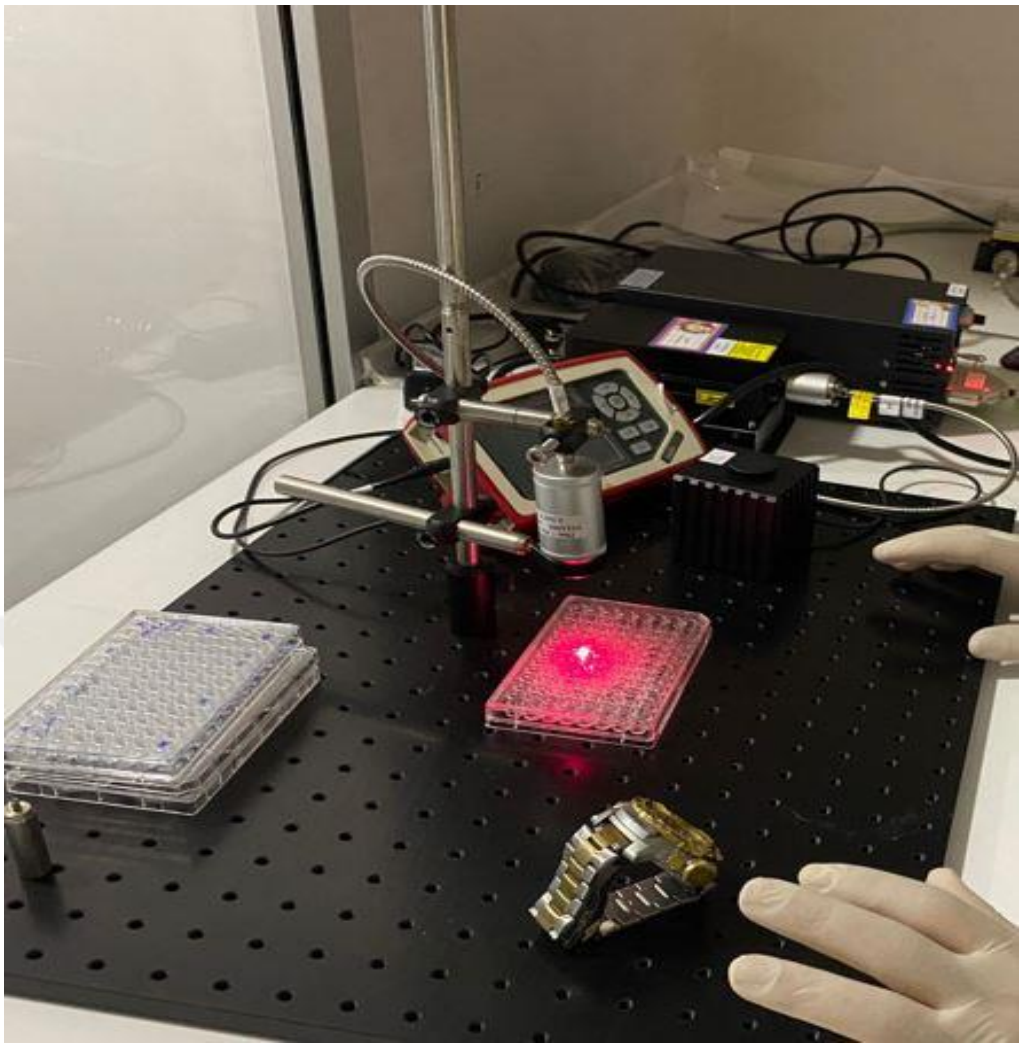

Figure 10: *Laser device at 660nm*

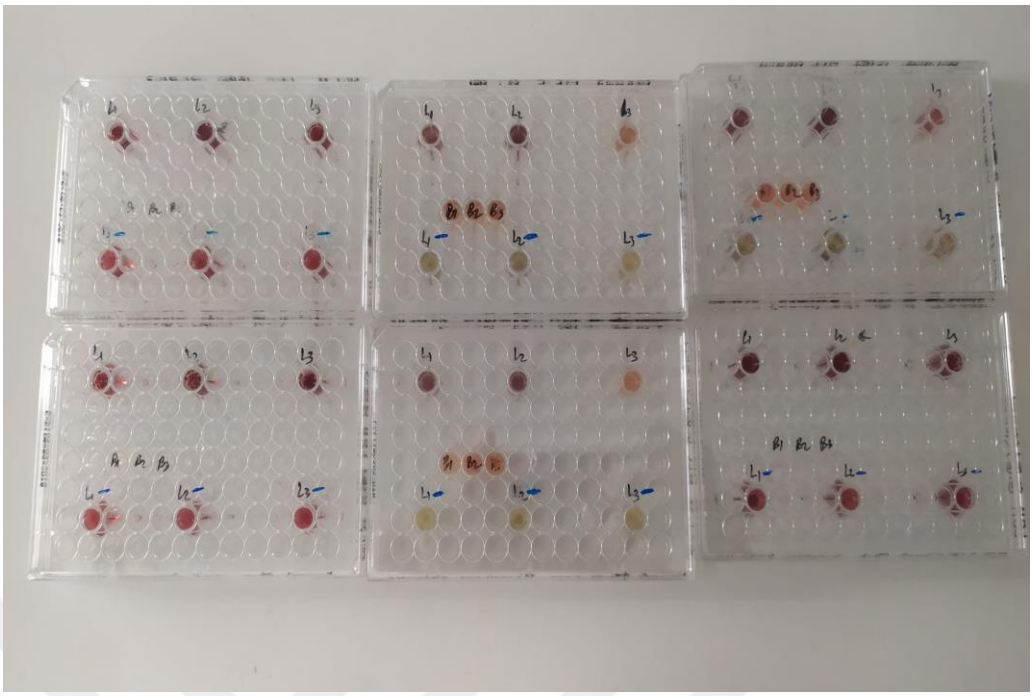

Figure 11: *Experimental plates*

Chapter 4

Findings

When the experiments were conducted, the results were as shown in the following graphs. The first figure shows the SH-SY5Y cells in a normal situation when was cultured in complete DMEM.

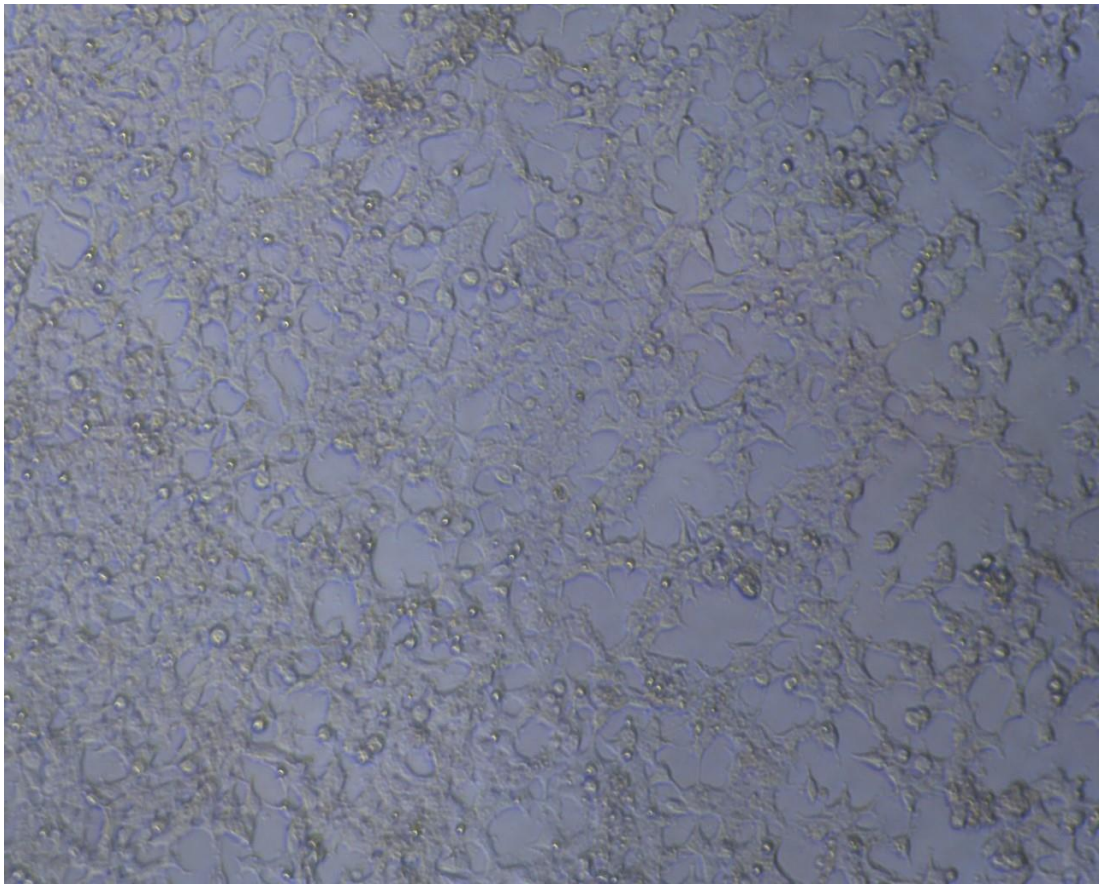

***Figure 12**: SH-SY5Y cells in a normal situation at 20x magnification.*

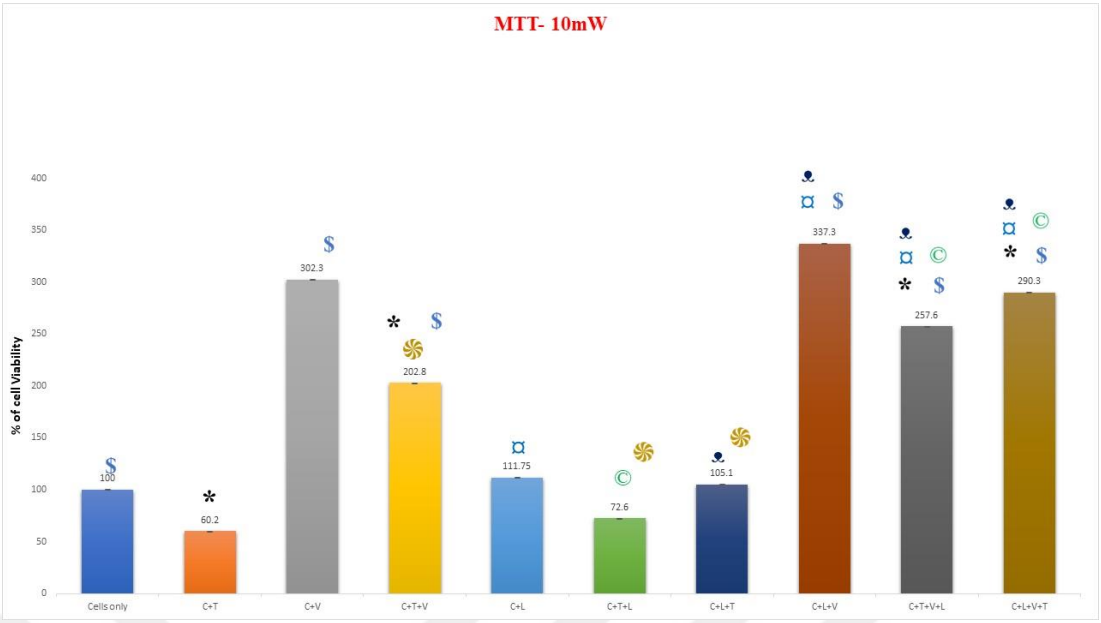

Figure 13: *MTT assay results for wavelength 660nm at power output 10mW.* (Group 1=$, group 2=*, group 4=֍, group 5=¤, group 6=©, group 7= ๏)

When MTT assay was done on plates that were exposed to the power output at 10mW there was a statistically significant difference as was observed at (**figure 13**) between group 1 which contains cells alone with group 3 having cells were treated with Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 202.3% in group 3). Also, there was a statistically significant difference between group 1 and group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 102.8% in group 4). Moreover, a statistically significant difference was found between group 1 and group 8 which contains cells that were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 237.3% in group 8). In addition to that, there was a statistically significant difference between group 1 and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and the P-value was P<0.001 (the increase in cell viability was 157.6% in group 9). Also, there was a statistically significant difference between group 1 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 190.3% in group10).

When MTT assay was done on plates that were exposed to the power output at 10mW there was a statistically significant difference as was observed at (**figure 13**)

between group 2 containing cells were treated with TNF-α and-group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 142.6% in group 4). In addition to that, there was a statistically significant difference between the second group and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and the P-value was P<0.001 (the increase in cell viability was 197.4% in group 9). Also, there was a statistically significant difference between group 2 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 230% in group10). Moreover, there was an effect between group 2 and group 6 holding cells treated with TNF-α then exposed to laser and this effect was not statistically significant (the increase in cell viability was 12.4% in group 6). Also, there was an effect but there was no significant difference between group 2 and group 7 which consists of cells that were exposed to laser then treated with TNF-α (the increase in cell viability was 44.9% in group 7).

When MTT assay was done on plates that were exposed to the power output at 10mW there was no statistically significant difference as was observed at (**figure 13**) between group 3 which contains cells that were treated with Vit. D3 and the other groups. There was an effect between group 3 and group 4 containing cells that were treated with TNF-α then with Vit.D3 (the decrease in cell viability when TNF-α was added at group 4 was 99.5%). Also, there was no significant difference between group 3 and group 8 which contains cells exposed to laser and then treated with Vit.D3 (the increase in cell viability when the laser was exposed at group 8 was 35%). In addition to that, there was an effect between group 3 and group 9 which consists of cells ware treated with TNF-α and Vit.D3 then exposed to laser and no statistically significant difference (the decrease in cell viability at group 9 was 44.7%). Moreover, there was an effect but no significant difference between group 3 and group 10 which holds cells that exposed to laser as a protective agent then treated with Vit.D3 and TNF-α (the decrease in cell viability at group 10 was 12%).

When MTT assay was done on plates that were exposed to the power output at 10mW there was a statistically significant difference as was observed at (**figure 13**) between group 4 which holds cells treated with TNF-α and Vit.D3 and group 6 containing cells that were treated with TNF-α then exposed to laser and the P-value was P<0.001(the decrease in cell viability was 130.2% in group 6). In addition to that,

there was a statistically significant difference between group 4 and group 7 holding cells were exposed to laser then treated with TNF-α and the P-value was P<0.001(the decrease in cell viability was 97.7% in group 7). Moreover, there was no statistically significant difference between group 4 and group 9 containing cells treated with TNF-α then Vit.D3 and exposed to laser (the increase in cell viability was 54.8% in group 9). Also, there was no statistically significant difference between group 4 and group 10 which contains cells that were exposed to laser as a protective effect then treated with Vit.D3 and TNF-α, and the increase in cell viability was 87.5% at group 10.

When MTT assay was done on plates that were exposed to the power output at 10mW there was no statistically significant difference as was observed at (**figure 13**) between group 5 which contains cells exposed to laser with group 6 which contains cells treated with TNF-α then exposed to laser and the decreasing for cell viability was 39.2% in group 6. Also, there was an effect but not statistically significant between-group 5 and group 7 holding cells were exposed to laser as a protective factor then treated with TNF-α and the decrease in cell viability was 6.7% in group 7. In addition to that, there was a statistically significant difference between group 5 and group 8 containing cells exposed to laser then Vit. D3 was added and the P-value was P<0.001(the increase in cell viability was 225.6% in group 8). Moreover, a statistically significant difference was found between group 5 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 145.9% in group 9). Also, there was a statistically significant difference between group 5 and group 10 containing cells were exposed to laser and Vit. D3 was added then applied TNF-α and the P-value was P<0.001 (the increase in cell viability was 178.5% in group 10).

When MTT assay was done on plates which were exposed the power output at 10mW there was a statistically significant difference as was observed at (**figure 13**) between group 6 which contains cells were TNF-α applied and exposed to laser with group 9 which holds cells were treated by TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 185% in group 9). Also, a statistically significant difference was found between group 6 and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001 (the increase in cell viability was 217.7% in group 10). In addition to that, there was an effect between group 6 and group 7 which

contains cells that were exposed to laser as a protective agent this effect was not statistically significant and the increase in cell viability was 32.5% in group 7. These results showed that the protective effect for the laser at group 7 from TNF- alpha was better than the treatment effect at group 6.

When MTT assay was done on plates which were exposed the power output at 10mW there was a statistically significant difference as was observed at (**figure 13**) between group 7which contains cells were exposed to laser and treated with TNF-α and with group 8 containing cells were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 232.2% in group 8). Also, there was a statistically significant difference between group 7 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 152.5% in group 9). In addition to that, there was a statistically significant difference between group 7 and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001(the increase in cells viability was 185.2% in group 10).

When MTT assay was done on plates which were exposed the power output at 10mW there was no statistically significant difference as was observed at (**figure 13**) between group 8 which contains cells were exposed to laser then Vit. D3 was added and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α (the decrease in cells viability was 47.1% in group 10).

When MTT assay was done on plates that were exposed to the power output at 10mW there was an effect but not statistically significant between-group 9 contains cells that were treated with TNF-α and Vit. D3 was added and exposed to laser as a treatment with group 10 which contains cells that were exposed to laser as a protective agent and Vit. D was added then treated with TNF-α and the increase in cell proliferation in group 10 was 32.6%.

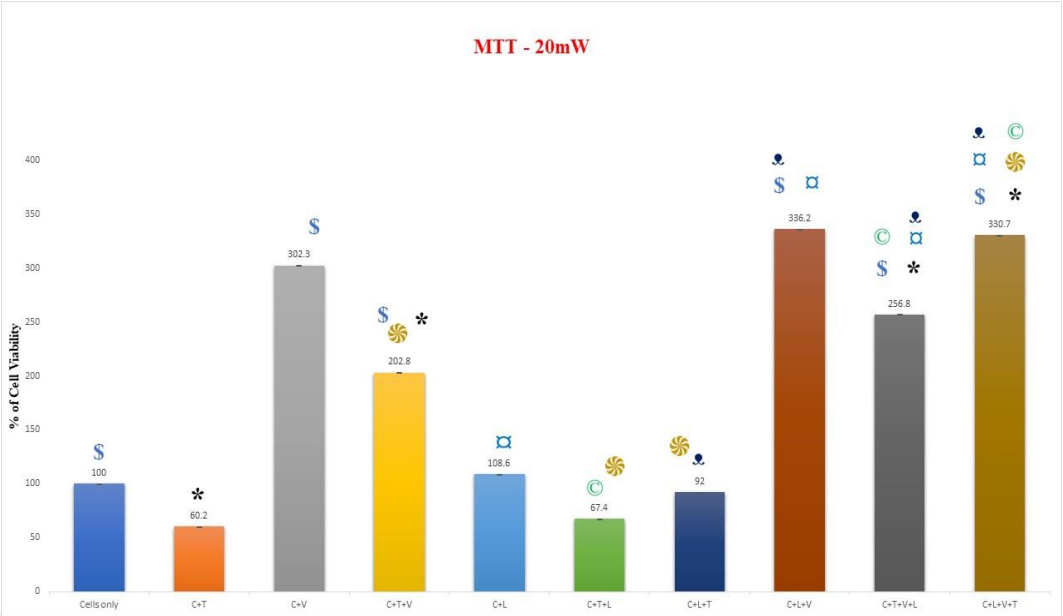

***Figure 14**: MTT assay result for wavelength 660nm at power output 20mW.*
(Group 1=$, group 2=*, group 4=֍, group 5=¤, group 6=©, group 7= ๏)

When MTT assay was done on plates that were exposed to the power output at 20mW there was a statistically significant difference as was observed at (**figure 14**) between group 1 which contains cells alone with group 3 having cells were treated with Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 202.3% in group 3). Also, there was a statistically significant difference between group 1 and group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 102.8% in group 4). Moreover, a statistically significant difference was found between group 1 and group 8 which contains cells that were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 236.2% in group 8). In addition to that, there was a statistically significant difference between group 1 and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and the P-value was P<0.001 (the increase in cell viability was 156.8% in group 9). Also, there was a statistically significant difference between group 1 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 230.7% in group10).

When MTT assay was done on plates that were exposed to the power output at 20mW there was a statistically significant difference as was observed at (**figure 14**) between group 2 containing cells were treated with TNF-α and group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 142.6% in group 4). In addition to that, there was a statistically significant difference between the second group and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and the P-value was P<0.001 (the increase in cell viability was 196.6% in group 9). Also, there was a statistically significant difference between group 2 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 270.5% in group10). Moreover, there was an effect between group 2 and group 6 holding cells treated with TNF-α then exposed to laser and this effect was not statistically significant (the increase in cell viability was 7.2% in group 6). Also, there was an effect but there was no significant difference between group 2 and group 7 which consists of cells that were exposed to laser then treated with TNF-α (the increase in cell viability was 31.8% in group 7).

When MTT assay was done on plates that were exposed to the power output at 20mW there was no statistically significant difference as was observed at (**figure 14**) between group 3 which contains cells that were treated with Vit. D3 and the other groups. There was an effect between group 3 and group 4 containing cells that were treated with TNF-α then with Vit.D3 (the decrease in cell viability when TNF-α was added at group 4 was 99.5%). Also, there was no significant difference between group 3 and group 8 which contains cells exposed to laser and then treated with Vit.D3 (the increase in cell viability when the laser was added at group 8 was 33.9%). In addition to that, there was an effect between group 3 and group 9 which consists of cells ware treated with TNF-α and Vit.D3 then exposed to laser and no statistically significant difference (the decrease in cell viability at group 9 was 45.5%). Moreover, there was an effect but no significant difference between group 3 and group 10 which holds cells that exposed to laser as a protective agent then treated with Vit.D3 and TNF-α (the increase in cell viability at group 10 was 28.4%).

When MTT assay was done on plates that were exposed to the power output at 20mW there was a statistically significant difference as was observed at (**figure 14**) between group 4 which holds cells treated with TNF-α and Vit.D3 and group 6

containing cells that were treated with TNF-α then exposed to laser and the P-value was P<0.001(the decrease in cell viability was 135.4% in group 6). In addition to that, there was a statistically significant difference between group 4 and group 7 holding cells were exposed to laser then treated with TNF-α and the P-value was P<0.001(the decrease in cell viability was 110.8% in group 7). Moreover, there was no statistically significant difference between group 4 and group 9 containing cells treated with TNF-α then Vit.D3 and exposed to laser (the increase in cell viability was 54% in group 9). Also, there was a statistically significant difference between group 4 and group 10 which contains cells that were exposed to laser as a protective effect then treated with Vit.D3 and TNF-α, and the P-value was P<0.05, and the increase in cell viability was 127.9% at group 10.

When MTT assay was done on plates that were exposed to the power output at 20mW there was no statistically significant difference as was observed at (**figure 14**) between group 5 which contains cells exposed to laser with group 6 which contains cells treated with TNF-α then exposed to laser and the decreasing for cell viability was 41.2% in group 6. Also, there was an effect but not statistically significant between group 5 and group 7 holding cells were exposed to laser as a protective factor then treated with TNF-α, and the decrease in cell viability was 16.6% in group 7. In addition to that, there was a statistically significant difference between group 5 and group 8 containing cells exposed to laser then Vit. D3 was added and the P-value was P<0.001(the increase in cell viability was 227.6% in group 8). Moreover, a statistically significant difference was found between group 5 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 148.2% in group 9). Also, there was a statistically significant difference between group 5 and group 10 containing cells were exposed to laser and Vit. D3 was added then applied TNF-α and the P-value was P<0.001 (the increase in cell viability was 222.1% in group 10).

When MTT assay was done on plates which were exposed the power output at 20mW there was a statistically significant difference as was observed at (**figure 14**) between group 6 which contains cells were TNF-α applied and exposed to laser with group 9 which holds cells were treated by TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 189.4% in group 9). Also, a statistically significant difference was found between group 6 and

group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001 (the increase in cell viability was 263.3% in group 10). In addition to that, there was an effect between group 6 and group 7 which contains cells that were exposed to laser as a protective agent this effect was not statistically significant and the increase in cell viability was 24.6% in group 7. These results showed that the protective effect for the laser at group 7 from TNF- alpha was better than the treatment effect at group 6.

When MTT assay was done on plates which were exposed the power output at 20mW there was a statistically significant difference as was observed at (**figure 14**) between group 7which contains cells were exposed to laser and treated with TNF-α and with group 8 containing cells were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 244.2% in group 8). Also, there was a statistically significant difference between group 7 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 164.8% in group 9). In addition to that, there was a statistically significant difference between group 7 and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001(the increase in cells viability was 238.7% in group 10).

When MTT assay was done on plates which were exposed the power output at 20mW there was no statistically significant difference between group 8 which contains cells were exposed to laser then Vit. D3 was added and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α (the decrease in cells viability was 5.5% in group 10).

When MTT assay was done on plates that were exposed to the power output at 20mW there was an effect but not statistically significant between-group 9 contains cells that were treated with TNF-α and Vit. D3 was added and exposed to laser as a treatment with group 10 which contains cells that were exposed to laser as a protective agent and Vit. D was added then treated with TNF-α and the increase in cell proliferation in group 10 was 73.9%.

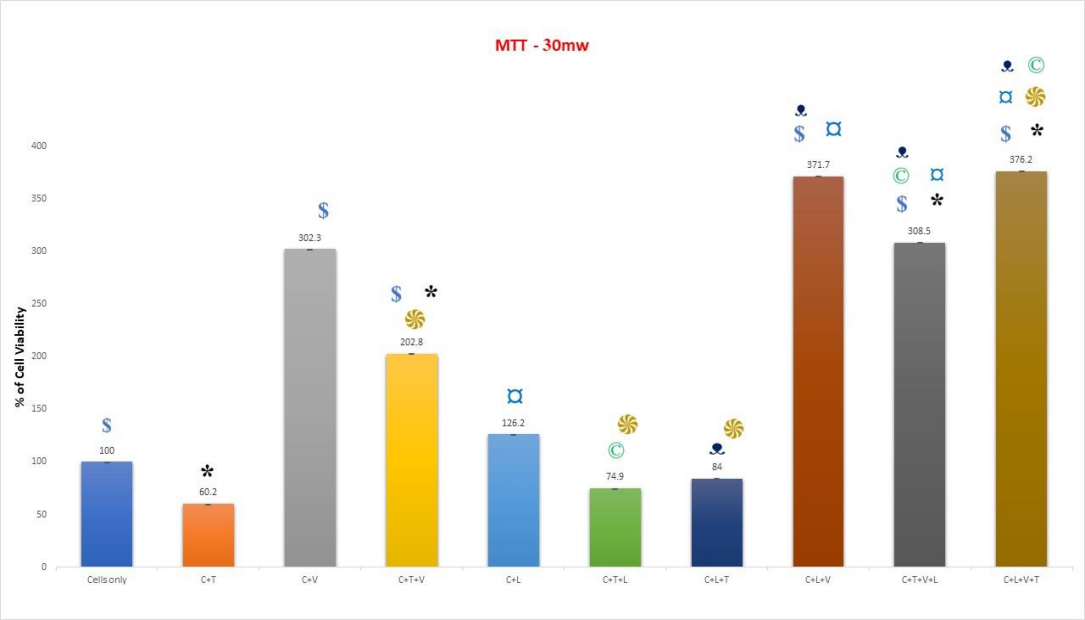

***Figure 15**: MTT assay result for wavelength 660nm at power output 30mW.*
(Group 1=$, group 2=*, group 4=֍, group 5=¤, group 6=©, group 7= ๏)

When MTT assay was done on plates that were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 15**) between group 1 which contains cells alone with group 3 having cells were treated with Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 202.3% in group 3). Also, there was a statistically significant difference between group 1 and group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 102.8% in group 4). Moreover, a statistically significant difference was found between group 1 and group 8 which contains cells that were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 271.7% in group 8). In addition to that, there was a statistically significant difference between group 1 and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and

the P-value was P<0.001 (the increase in cell viability was 208.5% in group 9). Also, there was a statistically significant difference between group 1 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 276.2% in group10).

When MTT assay was done on plates that were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 15**) between group 2 containing cells were treated with TNF-α and group 4 holding cells that were treated with TNF-α then applied Vit. D3 and the P-value was P<0.001 (the increase in cell viability was 142.6% in group 4). In addition to that, there was a statistically significant difference between the second group and group 9 which contains cells that were treated with TNF-α then Vit. D3 was added then exposed to laser and the P-value was P<0.001 (the increase in cell viability was 248.3% in group 9). Also, there was a statistically significant difference between group 2 and group 10 containing cells were exposed to laser then applied Vit. D3 and TNF-α and the P-value was P<0.001 (the increase in cell viability was 316% in group10). Moreover, there was an effect between group 2 and group 6 holding cells treated with TNF-α then exposed to laser and this effect was not statistically significant (the increase in cell viability was 14.7 in group 6). Also, there was an effect but there was no significant difference between group 2 and group 7 which consists of cells that were exposed to laser then treated with TNF-α (the increase in cell viability was 23.8% in group 7).

When MTT assay was done on plates that were exposed to the power output at 30mW there was no statistically significant difference as was observed at (**figure 15**) between group 3 which contains cells that were treated with Vit. D3 and the other groups. There was an effect between group 3 and group 4 containing cells that were treated with TNF-α then with Vit.D3 (the decrease in cell viability when TNF-α was added at group 4 was 99.5%). Also, there was no significant difference between group 3 and group 8 which contains cells exposed to laser and then treated with Vit.D3 (the increase in cell viability when the laser was added at group 8 was 69.4%). In addition to that, there was an effect between group 3 and group 9 which consists of cells ware treated with TNF-α and Vit.D3 then exposed to laser and no statistically significant difference (the increase in cell viability at group 9 was 6.2%). Moreover, there was an effect but no significant difference between group 3 and group 10 which holds cells

that exposed to laser as a protective agent then treated with Vit.D3 and TNF-α (the increase in cell viability at group 10 was 73.9%).

When MTT assay was done on plates that were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 15**) between group 4 which holds cells treated with TNF-α and Vit.D3 and group 6 containing cells that were treated with TNF-α then exposed to laser and the P-value was $P<0.001$(the decrease in cell viability was 127.9% in group 6). In addition to that, there was a statistically significant difference between group 4 and group 7 holding cells were exposed to laser then treated with TNF-α and the P-value was $P<0.001$(the decrease in cell viability was 118.8% in group 7). Moreover, there was no statistically significant difference between group 4 and group 9 containing cells treated with TNF-α then Vit.D3 and exposed to laser (the increase in cell viability was 105.7% in group 9). Also, there was a statistically significant difference between group 4 and group 10 which contains cells that were exposed to laser as a protective effect then treated with Vit.D3 and TNF-α, and the P-value was $P<0.05$ and the increase in cell viability was 173.4% at group 10.

When MTT assay was done on plates that were exposed to the power output at 30mW there was no statistically significant difference as was observed at (**figure 15**) between group 5 which contains cells exposed to laser with group 6 which contains cells treated with TNF-α then exposed to laser and the decreasing for cell viability was 51.6% in group 6. Also, there was an effect but not statistically significant between group 5 and group 7 holding cells were exposed to laser as a protective factor then treated with TNF-α, and the decrease in cell viability was 42.2% in group 7. In addition to that, there was a statistically significant difference between group 5 and group 8 containing cells exposed to laser then Vit. D3 was added and the P-value was $P<0.001$(the increase in cell viability was 245.5% in group 8). Moreover, a statistically significant difference was found between group 5 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was $P<0.001$ (the increase in cell viability was 182.3% in group 9). Also, there was a statistically significant difference between group 5 and group 10 containing cells were exposed to laser and Vit. D3 was added then applied TNF-α and the P-value was $P<0.001$ (the increase in cell viability was 250% in group 10).

When MTT assay was done on plates which were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 15**) between group 6 which contains cells were TNF-α applied and exposed to laser with group 9 which holds cells were treated by TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 233.6% in group 9). Also, a statistically significant difference was found between group 6 and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001 (the increase in cell viability was 301.3% in group 10). In addition to that, there was an effect between group 6 and group 7 which contains cells that were exposed to laser as a protective agent this effect was not statistically significant and the increase in cell viability was 9.1% in group 7. These results showed that the protective effect for the laser at group 7 from TNF- alpha was better than the treatment effect at group 6.

When MTT assay was done on plates which were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 15**) between group 7which contains cells were exposed to laser and treated with TNF-α and with group 8 containing cells were exposed to laser then Vit. D3 was added and the P-value was P<0.001 (the increase in cell viability was 287.7% in group 8). Also, there was a statistically significant difference between group 7 and group 9 which holds cells were treated with TNF-α and Vit. D3 was added then exposed to the laser and the P-value was P<0.001 (the increase in cell viability was 224.5% in group 9). In addition to that, there was a statistically significant difference between group 7 and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α and the P-value was P<0.001(the increase in cells viability was 292.2% in group 10).

When MTT assay was done on plates which were exposed the power output at 30mW there was no statistically significant difference between group 8 which contains cells were exposed to laser then Vit. D3 was added and group 10 containing cells were exposed to laser and Vit. D3 was added then treated with TNF-α (the decrease in cells viability was 4.5% in group 10).

When MTT assay was done on plates that were exposed to the power output at 30mW there was an effect but not statistically significant between-group 9 contains cells that were treated with TNF-α and Vit. D3 was added and exposed to laser as a

treatment with group 10 which contains cells that were exposed to laser as a protective agent and Vit. D was added then treated with TNF-α and the increase in cell proliferation in group 10 was 67.7%.

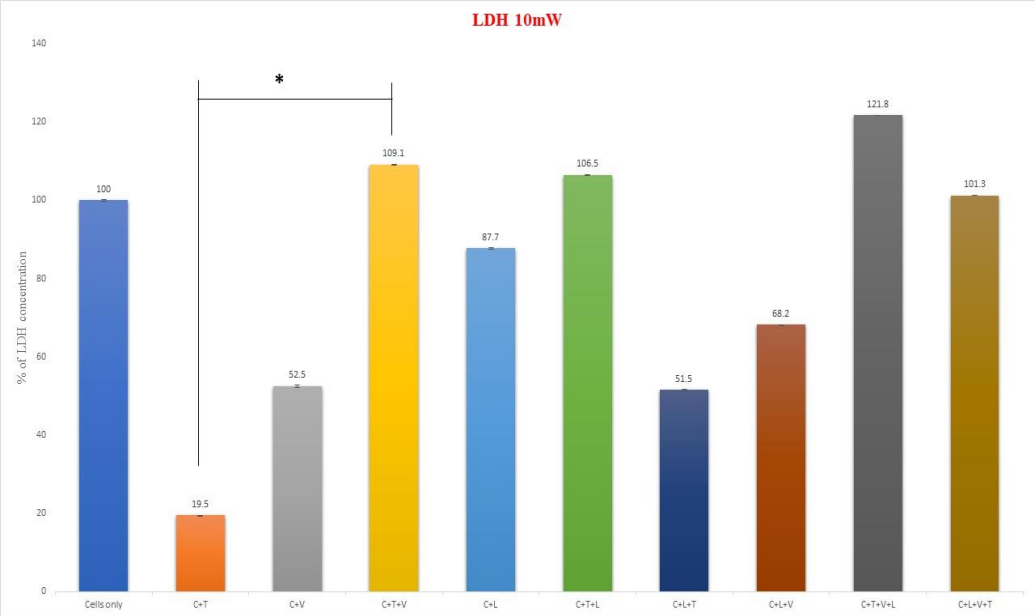

***Figure 16**: Lactate dehydrogenase assay result for wavelength 660nm at power output 10mW.*

When LDH assay was done on plates that were exposed to the power output at 10mw there was a statistically significant difference as was observed at (**figure 16**) between group 2 which contains cells that were treated by TNF-α and with group 4 consisting of cells that were treated with TNF-α then Vit. D3 was added and the P-value was $P<0.05$(the increase in the concentration of LDH enzyme was 89.6% in group 4).

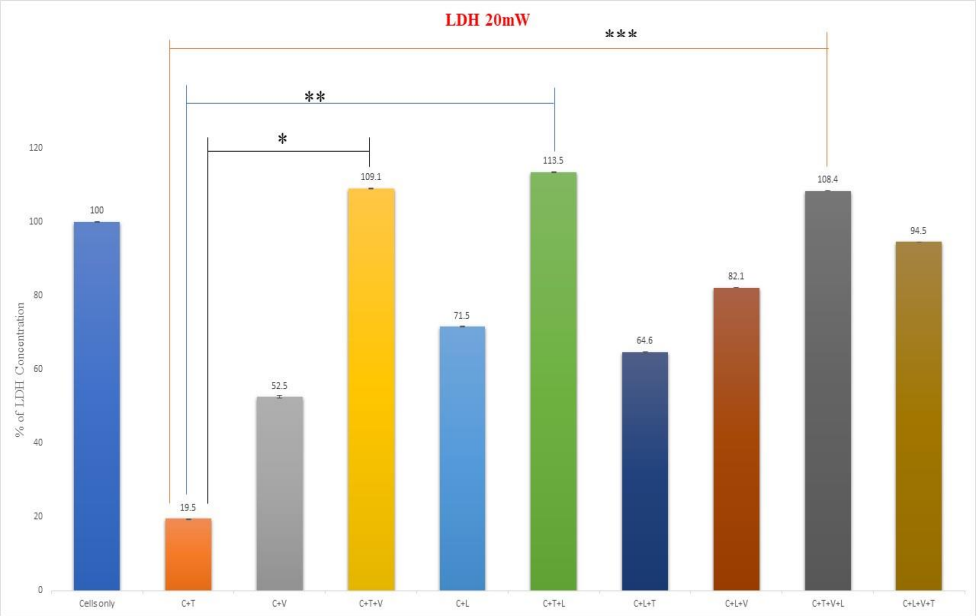

***Figure 16**: Lactate dehydrogenase assay result for wavelength 660nm at power output 20mW.*

When LDH assay was done on plates that were exposed the power output at 20mw there was a statistically significant difference as was observed (**figure 17**) between group 2 which contains cells that were treated by TNF-α and with group 4 consisting of cells that were treated with TNF-α then Vit. D3 was added and the P-value was P<0.05(the increase in the concentration of LDH enzyme was 89.6% in group 4). Also, there was a statistically significant difference between group 2 and group 6 which contains cells that were treated with TNF-α then exposed to laser and the P-value was P<0.05(the increase in the concentration of LDH enzyme was 94% in group 6). In addition to that, there was a statistically significant difference between the second group and group 9 which consisted of cells that were treated with TNF-α then Vit. D3 was added and then exposed to laser and the P-value was P<0.05(the increase in the concentration of LDH enzyme was 88.9% in group 9).

Another good result but without a statistically significant difference was discovered as was observed (**figure 17**) between group 6 which contain cells that were treated with TNF-α then exposed to laser as a treatment agent and with group 7 which holds cells were exposed to laser as a protective agent then treated by TNF-α (the decrease in the concentration of LDH enzyme was 48.9% in group 7). Also, in group

9 which contains cells that were treated with TNF-α and Vit. D3 was added and exposed to laser as a treatment with group 10 which contains cells that were exposed to laser as a protective agent and Vit. D was added then treated with TNF-α and the decrease in the concentration of LDH enzyme in group 10 was 13.9%.

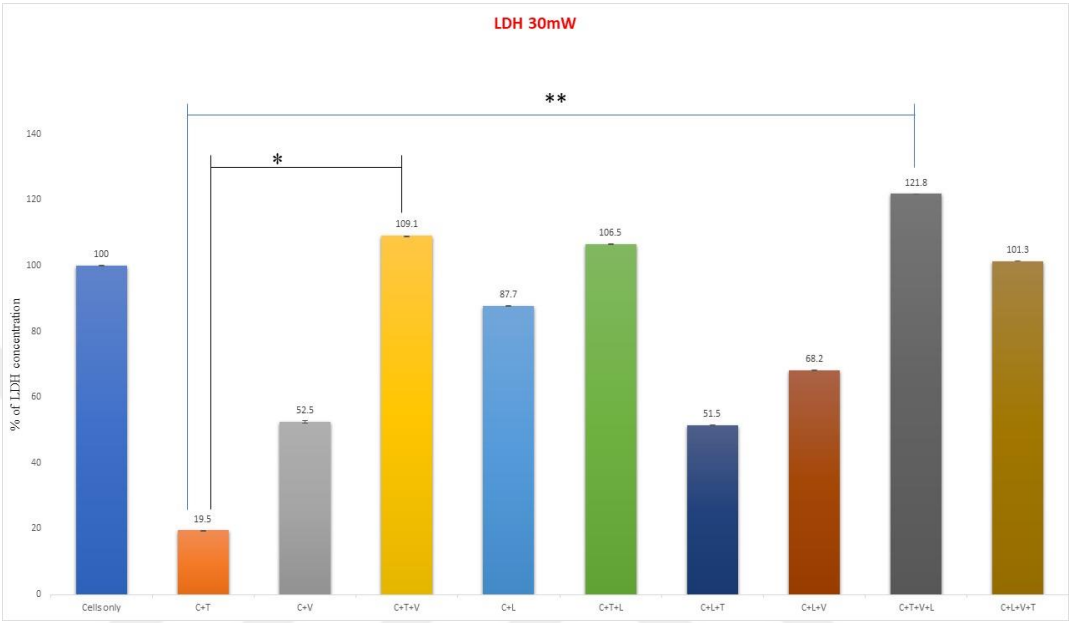

***Figure 18**: Lactate dehydrogenase assay result for wavelength 660nm at power output 30mW.*

When LDH assay was done on plates that were exposed to the power output at 30mW there was a statistically significant difference as was observed at (**figure 18**) between group 2 which contains cells that were treated by TNF-α and with group 4 consisting of cells that were treated with TNF-α then Vit. D3 was added and the P-value was $P<0.05$(the increase in the concentration of LDH enzyme was 89.6% in group 4). Also, there was a statistically significant difference between the second group and group 9 which consisted of cells that were treated with TNF-α then Vit. D3 was added and then exposed to laser and the P-value was $P<0.05$(the increase in the concentration of LDH enzyme was 102.3% in group 9).

Another good result but without a statistically significant difference was discovered between group 6 which contain cells that were treated with TNF-α then exposed to laser as a treatment agent and with group 7 which holds cells were exposed to laser as a protective agent then treated by TNF-α (the decrease in the concentration

of LDH enzyme was 55% in group 7). Also, in group 9 which contains cells that were treated with TNF-α and Vit. D3 was added and exposed to laser as a treatment with group 10 which contains cells that were exposed to laser as a protective agent and Vit. D was added then treated with TNF-α and the decrease in LDH enzyme concentration in group 10 was 20.5%.

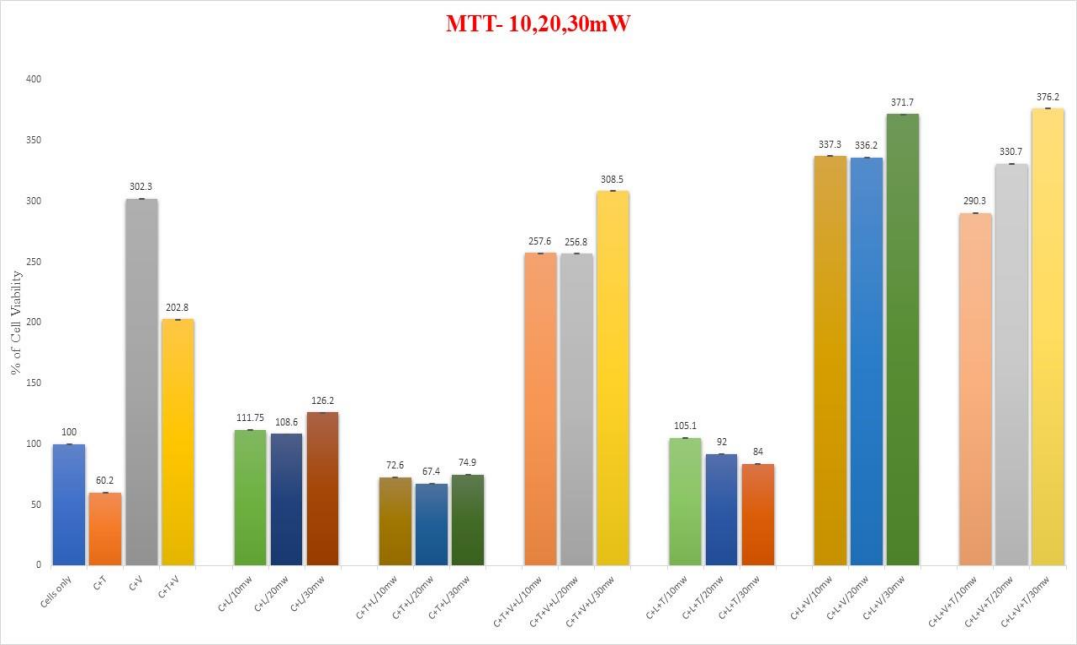

***Figure 19**: MTT assay result for wavelength 660nm comparing at power output 10,20,30mW.*

When MTT assay was done on plates that were exposed to different power outputs at 10,20,30mw there was no statistically significant difference between the groups as was observed at (**figure 19**), but there was an effect between group 7 which contains cells were exposed to laser at 30mW with incubation time 96hrs, and group 6 holding cells were exposed to laser at 20mW with incubation time 96hrs, and the cell viability was increased by 17.6% when 30mW was used than when 20mW was used, but this effect was not statistically significant. Also, group 7 with group 5 containing cells were exposed to laser at 10mW with an incubation time of 96hrs. Cell viability was decreased by 14.5% when 10mW was used than when 30mw was used, but this effect was not statistically significant.

Also, there was an effect between group 10 which contains cells, TNF-α then were exposed to laser at 30mW, and group 9 holding cells, TNF-α then were exposed to laser at 20mW, and the cell viability was increased by 7.5% when 30mW was used than when 20mw was used, but this effect was not statistically significant. Also, group 10 with group 8 containing cells, TNF-α were exposed to laser at 10mW. Cell viability was decreased by 2.3% when 10mW was used than when 30mw was used, but this effect was not statistically significant.

Additionally, there was an effect between group 13 which contains cells, TNF-α, Vit. D was exposed to laser at 30mW power output with groups 12 holding cells, TNF-α, Vit. D3 was exposed to laser at 20mW and the increase in cell viability was 51.7% in group 13. Also, there was an effect between groups 13 and 11 which holds cells were treated with TNF-α and Vit. D3 then were exposed to laser at 10mW and the increase in cell viability was 50.9% when 30mW was used.

When MTT assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant difference between the groups, but there was an effect between group 14 which contains cells that were exposed to laser at 10mW and then applied TNF-α with group 15 that's including cells were exposed to laser at 20mW then applied TNF-α and the increase in cell viability was 13.1% when 10mW used. Also, no statistically significant difference between the groups, but there was an effect between group 14 and group 16 containing cells were exposed to laser at 30mW then applied TNF-α and the increase in cell viability was 21.1% when 10mW was used.

When MTT assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant difference between the groups, but there was an effect between group 19 which contains cells that were exposed to laser at 30mW and then applied Vit. D3 with group 17 that's including cells were exposed to laser at 10mW then applied Vit. D3 and the increase in cell viability was 34.4% when 30mW was used. Also, there was an effect between group 19 and group 18 containing cells were exposed to laser at 20mW then applied Vit. D3 and the increase in cell viability was 35.5% when 30mW was used and this effect was not a significant one.

When MTT assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant

difference between the groups, but there was an effect between group 22 which contains cells that were exposed to laser at 30mW and then applied Vit. D3 and TNF-α, and groups 20 that's including cells were exposed to laser at 10mW then applied Vit. D3, TNF-α, and the increase in cell viability was 85.9% when 30mW was used. Also, there was an effect between group 22 and group 21 which contains cells that were exposed to laser at 20mW then applied Vit. D3, TNF-α, and the increase in cell viability was 45.5% when 30mW was used.

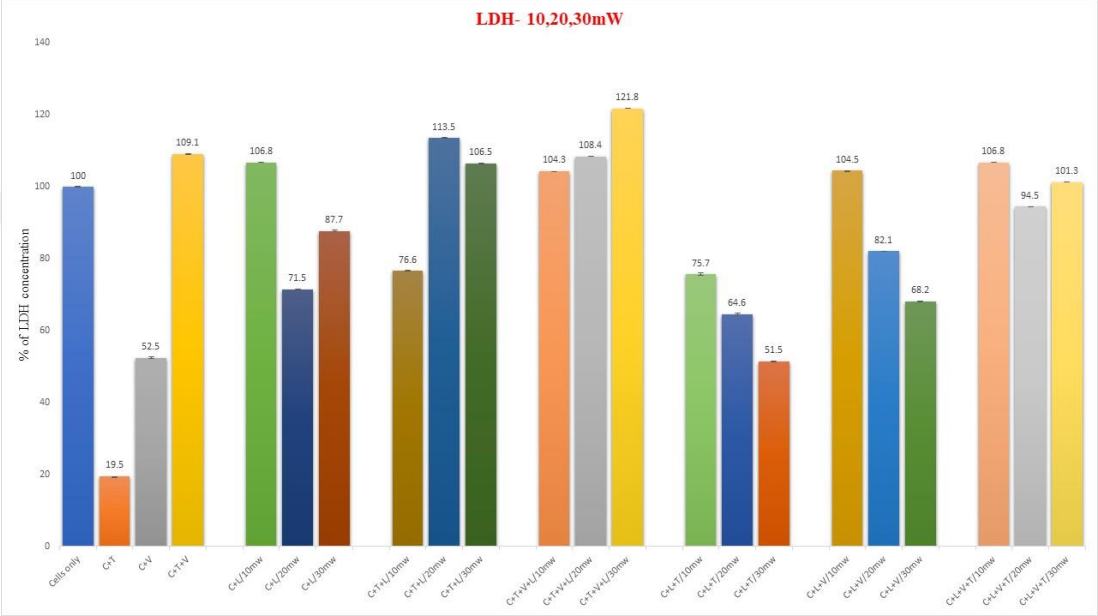

***Figure 20**: LDH assay result for wavelength 660nm comparing at power output 10,20,30mW.*

When LDH assay was done on plates that were exposed to the different power outputs at 10,20,30mW there was no statistically significant difference between the groups as was observed at (**figure 20**), but there was an effect between group 6 which consisted of cells were exposed to laser at 20mW, and group 5 holding cells were exposed to laser at 10mW, and the LDH enzyme concentration was decreased by 35.3% when 20mW was used than when 10mW was used, but this effect was not statistically significant. Also, group 6 with group 7 containing cells were exposed to laser at 30mW with an incubation time of 96hrs. LDH enzyme concentration was decreased by 16.2% when 20mW was used than when 30mW, but this effect was not statistically significant.

When LDH assay was done on plates which were exposed to different power outputs at 10,20,30mW there was no statistically significant difference between the groups, but there was an effect between group 8 which contains cells, TNF-α were exposed to laser at 10mW power output with groups 9 holding cells, TNF-α were exposed to laser at 20mW and the decrease in LDH enzyme concentration was 36.9% when 10mW was used. Also, there was an effect between group 8 and group 10 containing cells were that treated with TNF-α then exposed to laser at 30mW and the decrease in LDH enzyme concentration was 29.9% when 10mW was used.

In addition to that, there was an effect between group 11 which contains cells, TNF-α and Vit. D exposed to laser at 10mW power output with groups 12 holding cells, TNF-α and Vit. D3 was exposed to laser at 20mW and the decrease in LDH enzyme concentration was 4.1% when 10mW was used. Moreover, an effect was found but not a significant difference between groups 11 and 13 containing cells, TNF-α and Vit. D3 was exposed to laser at 30mW and the decrease in LDH enzyme concentration was 17.5% when 10mW power output was used.

When LDH assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant difference between the groups, but there was an effect between group 16 which contains cells that were exposed to laser at 30mW and then applied TNF-α with group 14 that's including cells were exposed to laser at 10mW then added TNF-α and the decrease in LDH enzyme concentration was 24.2% when 30mW was used. Also, an effect between group 16 and group 15 which contains cells was exposed to laser at 20mW then applied TNF-α and the decrease in LDH enzyme concentration was 13.1% when 30mW was used.

When LDH assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant difference between the groups, but there was an effect between group 19 which contains cells that were exposed to laser at 30mW and then applied Vit. D3 with groups 17 that's including cells were exposed to laser at 10mW then added Vit. D3 and the decrease in LDH enzyme concentration was 36.3% when 30mW was used. Also, there is an effect between groups 19 and group 18 which holds cells were exposed to laser at 20mW then applied Vit. D3 and the decrease in LDH enzyme concentration was 13.1% when 30mW was used.

When LDH assay was done on plates that were exposed to different power outputs at 10,20,30mW as a protective agent there was no statistically significant difference between the groups, but there was an effect between group 21 which contains cells that were exposed to laser at 20mW and then applied Vit. D3 and TNF-α, and groups 20 that's including cells were exposed to laser at 10mW then added Vit. D3 and TNF-α and decrease in LDH enzyme concentration was 12.3% when 20mW was used. Also, there was an effect but not a statistical one between group 21 and group 22 which contains cells that were exposed to laser at 30mW then applied Vit. D3 and TNF-α and decrease in LDH enzyme concentration was 6.75% when 20mW was used.

Chapter 5

Discussions and Conclusions

5.1. Discussion

Neurodegenerative diseases that affect the human nervous system are considered one of the most complex diseases, especially since humanity witnessing development in all fields, and for this reason, many researchers work to find effective treatment methods for them. Among the methods that scientists have been focusing on today is the use of lasers and UV light in the treatment of various types of diseases, including neurodegenerative diseases.

In this study, the effect of TNF-alpha as a toxic agent on SH-SY5Y cells and the efficacy of laser beams at a wavelength of 660 nm with power outputs of 10, 20, 30 mW and in the presence of vitamin D were investigated as the main goal.

The function of the MTT test is that it measures the redox and oxidation activities in living cells, as the process of increasing the uptake of MTT and turning it into a purple color indicates the survival of cells, while the LDH test measures the concentration of LDH enzyme released from cells. When the plasma membrane bursts, an increase in the enzyme concentration indicates an increase in cell death. In our study, the intracellular LDH concentration was measured after cells were lysed by using lysis buffer. By this way, LDH was used as an indicator for the energy metabolism of the cell instead of cytotoxicity.

Depending on the MTT results which were observed, the TNF-α decreases the cell viability (cell number ↓), and the TNF-α leads to a decrease in the LDH enzyme concentration because of the number of cells was decreased as indicated by MTT data.

The protective effect for the laser was shown when the cells were exposed to the laser before the TNF-alpha was added to the wells, while the

treatment effect occurred when the incubated cells were treated with the TNF-α then exposed to the laser at different power outputs.

The results showed that there was a protective effect more than the treatment effect for the laser at the different power outputs of 10,20,30mW in TNF-α groups (***Figure 19 & Figure 20***). When MTT and LDH assays were done on SH-SY5Y cells, it appeared that the protective effect of the laser has observed on the cells more than the treatment effect, with different percentages but without a statistically significant difference.

The laser works to increase and maintain cell survival, and this was demonstrated by the results of the MTT test. MTT demonstrated an increase in the concentration of formazan by increasing the number of cells that absorbed the dye. The LDH assay of cells showed there was a decrease in the LDH enzyme concentration when the laser was used as a protective agent from TNF-alpha more than when it was used as a treatment agent from TNF-alpha which was used as a toxic agent.

The concentration of LDH enzyme was the lowest in the cells when applying the laser at a power output of 30mW as a protective factor than 20mW and 10mW; the arrangement was as follows (10>20>30mW). But the opposite happened when applying the MTT test, where the effect of cell viability was higher as follows (10>20>30mW).

Also, exposing cells to laser without adding TNF-α or Vit. D3 led to an increase in the proliferation of cells in MTT at 30mW more than 10mW and 20mW. The arrangement was as follows (30>10>20mW), but this effect was not statically significant. On the other hand, in LDH assay 20mW is better in decreasing the concentration of LDH than 30mW and 10mW and the arrangement was as follows (10>30>20mW) and this effect was not statically significant.

Moreover, when the laser was used as a protective factor from toxicity caused by tumor necrosis factor the best power output was for 10mW after 20mW and 30mW at MTT assay, and the arrangement was as follows (10>20>30mW) and this effect was not statically significant. But when the laser was used as a treatment factor, the best effect was for 30mW after

10mW and 20mW, and the arrangement was as follows (30>10>20mW) and this effect again was not statically significant.

In addition to that, the MTT assay at the different power outputs showed when TNF-α was added as a toxic agent and treated with Vit.D3 then the laser used the best power output was for 30mW then 10mW and 20mW, and the arrangement was as follows (30>10>20mW) and this effect was not statically significant. While in LDH assay on group which contains the cells were treated with TNF-α then Vit.D3 and exposed to laser the lowest concentration of LDH was at 10mW then 20mW and 30mW, the arrangement was as follows (30>20>10mW) and this effect was not statically significant.

Also, the MTT assay at the different power outputs showed when the laser was used as a protective agent then Vit.D3 was added and treated with TNF-α as a toxic agent the best power output was for 30mW then 20mW and 10mW, the arrangement was as follows (30>20>10mW) and this effect was not statically significant. While in LDH assay applied on group which contains the cells were exposed to laser as a protective factor then treated with Vit.D3 and TNF-α the lowest concentration of LDH was at 20mW then 30mW and 10mW, the arrangement was as follows (10>30>20mW) and this effect was not statically significant.

Moreover, we can see from the results in the MTT assay that the 30mW when used as a protective or treating agent in the presence of TNF-α and Vit.D3 exhibited the best proliferation rate and highest cells viability with the other power outputs. In LDH assay there was a difference where is when the laser was used as a treatment agent from the toxic effect of TNF-α and Vit.D3 was added the lowest concentration of LDH was at 10mW. When the laser was used as a protective agent for the cells then Vit.D3 was added and treated with TNF-α the lowest concentration of LDH was at 20mW and these effects were not statistically significant.

In addition to that, we can see from the results in the MTT assay when the laser used then Vit.D3 has been added the best level of cell viability was at 30mW then 10mW and 20mW, and the arrangement was as follows (30>10>20mW) and this effect was not statically significant. While in LDH

assay the results were as follows (10>20>30mW) in increasing LDH concentration and these effects were not statistically significant.

Moreover, we can observe from the results in MTT assay when the cells were treated with TNF-alpha and Vit. D3 then the laser was used as a treatment factor, the best level for cell viability was at a power output 30mW and the arrangement follows (30>10>20mW) and this effect was not statistically significant. While in LDH assay the results were follows (30>20>10mW) in increasing the LDH concentration and the effects were not statistically significant.

Also, we can see from the results in the MTT assay when the cells were exposed to laser as a protective agent and then treated with Vit. D3 and TNF-alpha, the best level for cell viability was at power output 30mW and the arrangement follows (30>20>10mW) and this effect was not statistically significant. While in LDH assay the results were follows (10>30>20mW) in increasing the LDH concentration and the effects were not statistically significant.

When the three different power outputs were compared with each other, the results which were gotten by MTT indicate that the 30mW showed the greatest effect in all experiments, but in the experiments when the laser was used as a protective agent the 10mW had the greatest effect.

When considering the viability data from MTT analyses, it has been concluded that the 30mW power output, the highest output in this study, provided the highest cell viability except C+L+T group. However, as the power output decreases to 20 and/or 10mW, the cell viabilities did not exhibit the linear trend. One of the possible reasons about this observed nonlinearity would be that 10mW could have a specific effect on the cells when TNF-alpha toxicity was applied. As is known from the relevant scientific literature that 10mW power output has been widely used not to give the thermal harm to the cells. Another possible reason about the nonlinearity could be the nonlinear dynamical nature of living systems including cells, it would not be reasonable to consider that nonlinear dynamical systems respond to external stimuli in a linear way.

For LDH data two experimental groups, C+L+T and C+L+V, exhibited a linear increase as the power output was linearly reduced. On the other hand, for the C+T+V+L group, the amount of intracellular LDH increased as the power output of the laser increased. The rest of the experimental groups in LDH analyses showed a nonlinearity. For the C+T+V+L group, the possbile reason about the reverse orientation would be the addition of TNF-alpha first and then the laser. The other nonlinear behavior could be caused by the nonlinear dynamical nature of living systems just like in the case of MTT data.

The LDH data were obtained after the lysis of the cells by using the cell lysis buffer provided in the kit. By doing so, the LDH data showed the amount of the enzyme present in the cells, not in the cell culture medium which would be released from dying cells. It is also known from the scientific literature that the amount of the LDH enzyme inside the cells can be used as one of the indicators of the anaerobic energy metabolism, so our LDH data indicate a possible metabolic shift of the cells from aerobic to anaerobic energy metabolism when treated with laser.

Overall, our results indicate a possible positive effect of using lasers in the treatment of neuroblastoma cell line that have been exposed to tumor necrosis factor or even to protect from it.

5.2. Conclusion

As we know from the literature, 660nm affects directly the mitochondria and cytochrome C oxidase and this effect leads to an increase in the production of ATP. The results showed there was a good effect for 10mW power output in protecting the cells from the effect of TNF-α which may directly work on cytochrome C oxidase and this increases the production of ATP, when the laser was used as a treatment agent from TNF-α the 30mW power output had the best effect in increasing cell viability at MTT assay. Also, the LDH enzyme concentration was decreased in the cell by the effect of laser.

REFERENCES

Al-Gayyar, M. M., Elsherbiny, N. M. 2013. Contribution of TNF-*α* to the development of retinal neurodegenerative disorders. *Eur. Cytokine Netw*, **24**(1), pp. 27-36.

Amadio, E. M., Serra, A. J., Guaraldo, S. A., Silva, J. A. Jr., Antônio, E. L., Silva, F., Portes, L. A., Tucci, P. J., Leal-Junior, E. C., de Carvalho Pde, T. 2015. The action of pre-exercise low-level laser therapy (LLLT) on the expression of IL-6 and TNF-α proteins and on the functional fitness of elderly rats subjected to aerobic training. *Lasers in Medical Science*, **30**(3), pp. 1127-1134.

Ascherio, A., Munger, K. L., & Simon, K. C., 2010. Vitamin D and multiple sclerosis. *Lancet Neurol*, **9**, pp. 599–612.

Avsar, T., Durası, i. M., Uygunoğlu, U., Tütüncü, M., Demirci, N. O., Saip, S., Sezerman, U., Siva, A., Turanlı, E. T., 2015. CSF Proteomics Identifies Specific and Shared Pathways for Multiple Sclerosis Clinical Subtypes. *PLoS ONE*. **10**(5), pp. e0122045.

Berman, T. Bayati, A. 2018. What are Neurodegenerative Diseases and How Do They Affect the Brain?. *Front. Young Minds*, **6**(70).

Bivona, G., Gambino, C. M., Iacolino, G., Ciaccio, M. 2019. Vitamin D and the nervous system. *Neurological research*, **41**(9), pp. 827-835.

Breijyeh, Z., Karaman, R. 2020. Comprehensive Review on Alzheimer's Disease: Causes and Treatment. *Molecules*, **25**(24), pp. 5789.

Budd Haeberlein, S. L., Harris, T. J. 2015. Promising Targets for the Treatment of Neurodegenerative Diseases. *Clinical Pharmacology and Theraputics*, **98**(5), pp. 492-501.

Burré, J., Sharma, M., & Südhof, T. C. 2018. Cell Biology and Pathophysiology of α-Synuclein. *Cold Spring Harbor Perspective in Medicine*, **8**(3), pp. a024091.

Chen, X., Nie, X., Mao, J., Zhang, Y., Yin, K., Jiang, S. 2018. Perfluorooctanesulfonate induces neuroinflammation through the secretion of TNF-α mediated by the JAK2/STAT3 pathway. *Neurotoxicology*, **66**, pp. 32-42.

Cho, G. M., Lee, S. Y., Park, J. H., Kim, M. J., Park, K. J., Choi, B. T., Shin, Y. I., Kim, N. G., Shin, H. K. 2020. Photobiomodulation Using a Low-Level Light-Emitting Diode Improves Cognitive Dysfunction in the 5XFAD Mouse Model of Alzheimer's Disease. *The journals of Gerontology series A, Biological Science and Medical Science*, **75**(4), pp. 631-639.

Clark, I. A., Vissel, B. 2016. Excess cerebral TNF causing glutamate excitotoxicity rationalizes treatment of neurodegenerative diseases and neurogenic pain by anti-TNF agents. *Journal of Neuroinflammation*, **13**(1), pp. 236-251.

Clark, I.A., Vissel, B. 2018. Therapeutic implications of how TNF links apolipoprotein E, phosphorylated tau, α-synuclein, amyloid-β and insulin resistance in neurodegenerative diseases. *British Journal of Pharmacology*, **175**(20), pp. 3859-3875.

Coppedè, F., Mancuso, M., Siciliano, G., Migliore, L., Murri, L. 2006. Genes and the environment in neurodegeneration. *Biosci Rep*, **26**(5), pp. 341-367.

Cummings, J. L., Tong, G., Ballard, C. 2019. Treatment Combinations for Alzheimer's Disease: Current and Future Pharmacotherapy Options. *Journal of Alzheimer's Disease*, **67**(3), pp. 779-794.

de Araújo Boleti, A. P., de Oliveira Flores, T. M., Moreno, S.E., Anjos, L. D., Mortari, M.R., Migliolo, L. 2020. Neuroinflammation: An overview of neurodegenerative and

metabolic diseases and of biotechnological studies. *Neurochemistry International*, **136**, pp. 104714.

de la Torre, J. C. 2017. Treating cognitive impairment with transcranial low level laser therapy. *Journal of Photochemistry and Photobiology B*, **168**, pp. 149-155.

Erkkinen, M. G., Kim, M. O., Geschwind, M. D. 2018. Clinical Neurology and Epidemiology of the Major Neurodegenerative Diseases. *Cold Spring Harb Perspect Biol*, **10**(4), pp. a033118.

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., Gage, F. H. 2010. Mechanisms underlying inflammation in neurodegeneration. *Cell*. **140**(6), pp. 918-934.

Han, F., Lu, P. 2020. Introduction for Stem Cell-Based Therapy for Neurodegenerative Diseases. *Advances in Experimental Medicine and Biology*, **1266**, pp. 1-8.

Hayes, M. W., Fung, V. S., Kimber, T. E., O'Sullivan, J. D. 2019. Updates and advances in the treatment of Parkinson disease. *The medical journal of Australia*, **211**(6), pp. 277-283.

Hamblin, M. R. 2017. Mechanisms and applications of the anti-inflammatory effects of photobiomodulation. *AIMS Biophysics*, **4**(3), pp. 337-361.

Hou, Y., Dan, X., Babbar, M., Wei, Y., Hasselbalch, S. G., Croteau. D. L., Bohr, V. A. 2019. Ageing as a risk factor for neurodegenerative disease. *Nature Reviews Neurology*, **15**(10), pp. 565-581.

Huang, Y.Y., Chen, A., Carroll, J. D., Hamblin, M. R., 2009. Biphasic dose response in low level light therapy, *Dose response journal*. **7,** pp. 358-383.

Huang, Y.Y., Sharma, S. K., Carroll, J. D., Hamblin, M. R., 2011. Biphasic dose response in low level light therapy - an update. *Dose response journal*. **9,** 602-618.

Idriss, H. T., Naismith , J. H. 2000. TNF-α and the TNF receptor superfamily: structure-function relationship(s). *Microscopy resaerch and technique*, 50(3), pp. 184-195.

Jia, J., Hu, J., Huo, X., Miao, R., Zhang, Y., Ma, F. 2019. Effects of vitamin D supplementation on cognitive function and blood Aβ-related biomarkers in older adults with Alzheimer's disease: a randomised, double-blind, placebo-controlled trial. *Journal of Neurology Neurosurgery and Psychiatry*, **90**(12), pp. 1347-1352.

Karam, C., & Scelsa, S. N. 2011. Can vitamin D delay the progression of ALS?. *Medical Hypotheses*, **76**(5), pp. 643-645.

Lin, F. Y., Lin, Y. F., Lin, Y. S., Yang, C. M., Wang, C. C., Hsiao, Y. H. 2020. Relative D3 vitamin deficiency and consequent cognitive impairment in an animal model of Alzheimer's disease: Potential involvement of collapsin response mediator protein-2. *Neuropharmacology*, **164**, pp. 107910.

Liu, H., Huang, H., Li, R., Bi, W., Feng, L., Lingling, E. Hu, M., Wen, W. 2019. Mitophagy protects SH-SY5Y neuroblastoma cells against the TNFα-induced inflammatory injury: Involvement of microRNA-145 and Bnip3. *Biomed Pharmacother*, **109**, pp. 957-968.

Luque-C´ordoba, D., D.Luque de Castro, M., 2016. Metabolomics: a potential way to know the role of vitamin D on multiple sclerosis, *Journal of pharmaceutical and biomedical analysis*. **136**, pp. 22-31.

Lv, L., Tan, X., Peng, X., Bai, R., Xiao, Q., Zou, T., Tan, J., Zhang, H., Wang, C. 2020. The relationships of vitamin D, vitamin D receptor gene polymorphisms, and vitamin D supplementation with Parkinson's disease. *Translation Neurodegeneration*, **9**(1), pp. 34-46.

Lyons, J.A., 2015. Light therapy to treat autoimmune disease. University of Wisconsin-Milwaukee Milwaukee. *SPIENewsroom*. https://www.researchgate.net/publication/275724755 [23 Dec. 2015].

Matilla-Dueñas, A., Corral-Juan, M., Rodríguez-Palmero Seuma, A., Vilas, D., Ispierto, L., Morais, S., Sequeiros, J., Alonso, I., Volpini, V., Serrano-Munuera, C., Pintos-Morell, G., Álvarez, R., Sánchez, I. 2017. Rare Neurodegenerative Diseases: Clinical and Genetic Update. *Adv Exp Med Biol*.**1031**, pp. 443-496.

Moges, H., Vasconcelos, O. M., Campbell, W. W., Borke, R. C., McCoy, J. A., Kaczmarczyk, L., Feng, J., Anders, J. J. 2009. Light therapy and supplementary Riboflavin in the SOD1 transgenic mouse model of familial amyotrophic lateral sclerosis (FALS). *Lasers in surgery and medicine*, **41**(1), pp. 52-59.

Molnár, M. F., Török, R., Szalárdy, L., Sümegi, E., Vécsei, L., Klivényi, P. 2016. High-dose 1,25-dihydroxyvitamin D supplementation elongates the lifespan of Huntington's disease transgenic mice. *Acta Neurobiologiae Expermentalis* (Wars), **76**(3), pp. 176-181.

Morello, M., Landel, V., Lacassagne, E., Baranger, K., Annweiler, C., Féron, F., Millet, P. 2018. Vitamin D Improves Neurogenesis and Cognition in a Mouse Model of Alzheimer's Disease. *Molecular Neurobiology*, **55**(8), pp. 6463-6479.

Muralidar, S., Ambi, S. V., Sekaran, S. 2020. Thirumalai D, Palaniappan B. Role of tau protein in Alzheimer's disease: The prime pathological player. *International Journal of Biological Macromolecules*, **163**, pp.1599-1617.

Olmos, G., Lladó, J. 2014. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. *Mediators of Inflammation*, **2014**, pp. 1-12.

Oron, A., Oron, U. 2016. Low-Level Laser Therapy to the Bone Marrow Ameliorates Neurodegenerative Disease Progression in a Mouse Model of Alzheimer's Disease: A Minireview. *Photomed Laser Surg*, **34**(12), pp. 627-630

Olsson, T., Barcellos, L., Alfredsson, L., 2017. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Macmillan Publishers Limited, part of Springer Natur*, **13**, pp. 25-36.

Pople, C. B., Meng, Y., Li, D. Z., Bigioni, L., Davidson, B., Vecchio, L. M., Hamani, C., Rabin, J. S., Lipsman, N. 2020. Neuromodulation in the Treatment of Alzheimer's Disease: Current and Emerging Approaches. *Journal of Alzheimer's Disease*, **78**(4), pp. 1299-1313.

Pregi, N., Wenker, S., Vittori, D., Leirós, C. P., Nesse, A. 2009. TNF-alpha-induced apoptosis is prevented by erythropoietin treatment on SH-SY5Y cells. *Experimental Cell Research*, **315**(3), pp. 419-31.

Ransohoff, R. M. 2016. How neuroinflammation contributes to neurodegeneration, *Science*. **353**(6301), pp. 777-783.

Rocha, E. M., De Miranda, B., Sanders, L. H. 2018. Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. *Neurobiology of Disease*, **109**(Pt B), pp. 249-257.

Salehpour, F., & Hamblin, M. R. 2020. Photobiomodulation for Parkinson's Disease in Animal Models: A Systematic Review. *Biomolecules*, **10**(4), pp. 610-628.

Salehpour, F., Mahmoudi, J., Kamari, F., Sadigh-Eteghad, S., Rasta, S. H., Hamblin, M. R. 2018. Brain Photobiomodulation Therapy: a Narrative Review. *Molecular Neurobiology*, **55**(8), pp. 6601-6636.

Smith, K. J., McDonald, W. I. 1999. The pathophysiology of multiple sclerosis: the mechanisms underlying the production of symptoms and the natural history of the disease. Philos Trans R Soc Lond B Biol Sci. 354(1390), pp.1649-1673.

Star, B., J., Vogel, D., Y., D., Kipp, M., Puentes, F., David Baker, D., & Amor, S., 2012. In vitro and in vivo models of Multiple Sclerosis. *CNS & Neurological Disorders - Drug Targets*, **11**(5), pp. 570-588.

Stephenson, J., Nutma, E., van der Valk, P., Amor, S. 2018. Inflammation in CNS neurodegenerative diseases. *Immunology*, **154**(2), pp. 204-219.

Walker, F. O. 2007. Huntington's disease. *Lancet*, **369**(9557), pp. 218-228.

Xicoy, H., Wieringa, B., Martens, G. J. 2017. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration*, **12**(1), pp. 10-20.

Wyant, K. J., Ridder, A. J., & Dayalu, P. 2017. Huntington's Disease-Update on Treatments. *Current Neurology and Neuroscience Reports*, **17**(4), pp. 33-43.

Zhou, L., Miranda-Saksena, M., Saksena, N.K. 2013. Viruses and neurodegeneration. *Virology journal*, **10**, pp. 172-188.

Zhou, Y., Sun, Y., Ma, Q. H., Liu, Y. 2018. Alzheimer's disease: amyloid-based pathogenesis and potential therapies. *Cell Stress*, **2**(7), pp. 150-161.

Zhou, Z., Zhou, R., Zhang, Z., Li, K. 2019. The Association Between Vitamin D Status, Vitamin D Supplementation, Sunlight Exposure, and Parkinson's Disease: A Systematic Review and Meta-Analysis. *Medical Science Monitor*, **25**, pp. 666-674.