BAŞKENT UNIVERSITY
HEALTH SCIENCE INSTITUTE
PHYSIOLOGY DEPARTMENT
PHYSIOLOGY MASTER'S PROGRAM

THE EFFECTS OF HIGH DOSES VITAMIN D ON ANXIETY AND EXPLORATORY ACTIVITY BEHAVIOURS IN RATS

BY

ZAHOUR GAMAL EDDN ASMAEIL

MASTER'S THESIS

ANKARA- 2021

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This study, which was prepared by Zahour Gamal Eddn Asmaeil within the framework of the Department of Physiology Master's Program, was accepted as the Master's Thesis by the following jury.

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ÖZET

ZAHOUR ASMAEIL, THE EFFECTS OF HIGH DOSES VITAMIN D ON ANXIETY AND EXPLORATORY ACTIVITY BEHAVIOURS IN RATS, BAŞKENT UNIVERSITY, INSTITUTE OF HEALTH SCIENCES / MASTER'S THESIS 2021

D vitamini, güneş ışığına maruz kalmaya tepki olarak deride üretilen yağda çözünen bir vitamindir. Kalsiyum ve fosfat homeostazının yanı sıra nöromüsküler ve immünolojik fonksiyon da dahil olmak üzere çeşitli süreçler için gereklidir. Anksiyete, keşif davranışı ve farklı D vitamini dozları arasındaki ilişkiyi incelemek için çok fazla çalışma yapılmamıştır. Mevcut çalışma, yükseltilmiş artı labirentini kullanarak sıçanlarda farklı D vitamini dozlarının anksiyete ile ilgili davranışlar üzerindeki etkisini değerlendirmeyi amaçlamaktadır. yöntemi ve açık alan testi kullanılarak sıçanların keşif davranışları ve genel aktiviteleri üzerine. Bu çalışmada 8 haftalık 36 adet genç erkek Wistar rat kullanıldı. Sham grubu, D vitamini grubu olarak 3 gruba ayrıldılar. Ad libitum 300 IU Vit D içeren yemle beslendiler. Sham grubu (grup 1) sonda ile normal su aldı (n=12). D vitamini grubu (grup 2) 400 IU D vitamini aldı ve son grup (grup 3) 1000 IU D vitamini (n=12) aldı. İlaçlar gruplara 8 hafta boyunca günde bir kez gavaj yoluyla verildi. 8 hafta sonra, sıçanlar yükseltilmiş artı labirent ve açık alan testlerinde test edildi. Açık alanda Grup 2 ($p<0.02$) ve Grup 3($p<0.05$) orta alanda geçirilen süre (STC) değerleri Grup 1'e göre çok daha yüksek bulundu. En düşük STP değeri Grup 2 ve Grup 3'te hemen hemen aynıydı. Yetiştirme sayısı Grup 2'de en yüksekti. Yükseltilmiş artı labirent testi değerlendirildiğinde. Grup 2'de diğer gruplara göre kapalı kolda geçirilen süre (STCA) değeri en düşük, açık kolda geçirilen süre (STOA) değeri en yüksek bulundu. kapalı kol giriş sayısı (ECA) değeri Grup 2'de G3'e göre istatistiksel olarak daha yüksekti ($p=0,05$).

Bulgularımız, 400 IU ve 100 IU D vitamini dozlarının ikisi arasında anlamlı bir fark olmaksızın anksiyojenik etkiler sergilediğini gösterdi. Sonuç olarak D vitamini takviyesinin artması anksiyetede artışa neden olur.

Anahtar Kelimeler: D Vitamini, Anksiyete, Açık alan testi, Sıçan, Yüksek artı labirent.

ABSTRACT

ZAHOUR ASMAEIL, THE EFFECTS OF HIGH DOSES VITAMIN D ON ANXIETY AND EXPLORATORY ACTIVITY BEHAVIOURS IN RATS, BAŞKENT UNIVERSITY, INSTITUTE OF HEALTH SCIENCES / MASTER'S THESIS 2021

Vitamin D is a fat-soluble vitamin produced in the skin in reaction to exposure to sunshine. It is necessary for a variety of processes, including calcium and phosphate homeostasis, as well as neuromuscular and immunological function. Not many studies have been conducted to examine the relationship between anxiety, exploratory behaviour, and different doses of vitamin D. The current study aimed to evaluate the impact of different doses of vitamin D on anxiety-related behaviours in rats using the elevated plus-maze method and on exploratory behaviour and general activity of rats by using the open field test. In this study, 8 weeks old age 36 young male Wistar rats were used. They were divided into 3 groups as sham group and two different doses of vitamin D groups. They were fed with feed containing 300 IU Vit D ad libitum. Sham group (Group 1) took normal water by gavage (n=12). The vitamin D group (Group 2) took 400IU vitamin D, and the last group (Group 3) took 1000IU vitamin D (n=12). The drugs were given once a day for 8 weeks to the groups by gavage. After 8 weeks, the rats were tested in the elevated plus-maze and open field tests. In the open field, spent time in the central area (STC) values of Group 2 ($p<0.02$) and Group 3 ($p<0.05$) were found to be much higher than Group 1. While the STC value of Group 3 was the lowest, the STP value was nearly the same in Group 2 and Group 3. The number of rearing was the highest in Group 2. When the elevated plus-maze test evaluated, there were no statistical differences between groups for the spent time in the closed arm (STCA) and spent time in the open arm (STOA), but in Group 2, STCA value was the lowest and STOA value was the highest from other groups. Entry number of closed arms (ECA) value was statistically higher in Group 2 than Group 3 ($p=0.05$).

Our findings showed that 400IU and 1000IU doses of vitamin D exhibited anxiogenic effects, with no significant difference between the two groups. As a result, an increase in vitamin D supplementation causes an increase in anxiety.

Key Words: Vitamin D, Anxiety, Open field test, Rat, Elevated plus-maze

The Başkent University Research Council supported this research with the DA20/19 research number

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LIST OF SYMBOLS AND ABBREVIATIONS

EPM	Elevated plus maze
EZM	Elevated zero maze
ETM	Elevated T maze
HPLC	High pressure liquid chromatography
IOM	Institute of Medicine
LC-MS/MS	Isotope dilution liquid chromatography tandem mass spectrometry
MED	Minimum erythemal dose
OFT	Open field test
PTH	Parathyroid hormone
UV	Ultra-violet
UVB	Ultraviolet-B irradiance
VDBP	Vitamin D binding protein
1,24,25(OH)3D	1,24,25 trihydroxy vitamin D
1,25(OH)2D	1,25 dihydroxy vitamin D
25(OH)D	25-hydroxyvitamin D
25 (OH)D3	25 hydroxyvitamin D3

1. INTRODUCTION

Vitamin D plays a critical part in bone metabolism and calcium (Ca) homeostasis [1]. It is also soluble in fats and is formed in the skin because of direct sunlight exposure. Dietary sources can supply the human body with some of its daily needs. Cod liver oil is another substantial supply of this vitamin. Additionally, it is contained in trace amounts in butter, cream, yolk, liver, and supplements. To increase the bioavailability of vitamin D, In the liver, it must be hydroxylated to 25 (OH) D then converted to 1,25 (OH)2D [2]. Vitamin D inhibits the release of parathyroid hormone (PTH), while PTH increases the production of vitamin D in the kidneys. PTH also regulates ionized calcium levels. Due to the effects of PTH on the stomach and bone, serum calcium is kept in a tightly controlled range [3].

The activated form of vitamin D (1,25(OH)2D) maintains phosphorus homeostasis and blood calcium since they are both necessary for neuromuscular functions the mineralization of bones [4]. Recent studies were mentioned a possible intricate relationship between brain growth vitamin D levels [5]. Vitamin D's neuroprotective effect is linked to its effects on neurotrophic factor production and release, prevention of oxidative damage to nervous tissue, intracellular calcium homeostasis, and neuromodulator synthesis [6]. Also, some research suggests a connection between vitamin D deficiency and different diseases, including bone conditions like rickets, osteomalacia, and osteoporosis [7], as well as other conditions such as type 1 and type 2 diabetes mellitus, cancer, obesity [8]. Vitamin D is hazardous in high dosages, and reports of toxicosis have been made on individuals who have taken quantities of vitamin D sporadically (sporadic hypervitaminosis D). Signs are (i) loss of appetite, (ii) nausea, (iii) vomiting, (iv) polyuria, (v) polydipsia, (vi) weariness, (vii) mental state alterations, and (viii) constipation. When hypervitaminosis D is referred to as high levels of 25-hydroxyvitamin D in the blood, it is characterized as high levels of 25-hydroxyvitamin D in the serum, hypercalcemia or hypercalciuria, or both (25[OH]D). High intake of vitamin D over a long period as it could lead to changes in the deposition of calcium in the soft tissues (particularly the heart and kidneys), and in more severe scenarios, death. [9].

There are pieces of evidence that low levels of vitamin D can contribute to anxiety, based on the findings of previous studies [10]. There have only been a few research types that established a connection between high doses of vitamin D and anxiety.

In recent years, it has been confirmed a link between anxiety and vitamin D deficiency because many people are spending less time in the sun [10]. A recent study of emerging risk factors for mood disorders in the general population suggests that vitamin D deficiency may have a significant impact on the overall body health [11].

Elevated plus maze (EPM) and open field (OF) tests are used in the research of anxiety in experimental animals. There is an increasing amount of research that suggests a substantial link between vitamin D insufficiency and emotional disorders such as sadness and anxiety. It has been shown in some of these studies vitamin D deficiency increase anxiety [12] and there are some studies using vitamin D in treatment, claiming that it reduces anxiety in humans [13]

In this study, we aimed to investigate the effects of high doses of vitamin D on anxiety-related behaviors. Their effect on anxiety in rats was investigated by the EPM test. In addition, we aimed to detect the effects of high vitamin D doses on exploratory behavior and overall activity of rats via the OFT used in this research.

2. GENERAL INFORMATION

2.1. Vitamin D:

Vitamin D is considered a steroid hormone that can be obtained through sun exposure, nutrition, or even supplements [14]. It is internationally recognized for its significance in the promotion of bone mineral metabolic health and, because of its antirachitic properties, was historically known as the "sunshine" vitamin. Vitamin D has been researched in recent years for its possible function in cancer prevention, cardiovascular disorders and, other conditions [15].

2.1.1. Forms of Vitamin D:

Vitamin D2 (ergocalciferol) and D3 (cholecalciferol) are the mainly two biological forms of Vitamin D. [16]. Ergocalciferol is a plant-derived form and can be synthesized in plants, yeast, fungi, and mushrooms in response to ultraviolet irradiation [17]. The animal-derived form is cholecalciferol, which can be synthesized non-enzymatically from the precursor 7-dehydrocholesterol (7-DHC). Pre-vitamin D is produced when ultraviolet B (UVB) rays strike the skin, which is an unstable intermediate that must be thermally transformed into vitamin D3 before it can be absorbed by the body (Figure 2.1) [18].

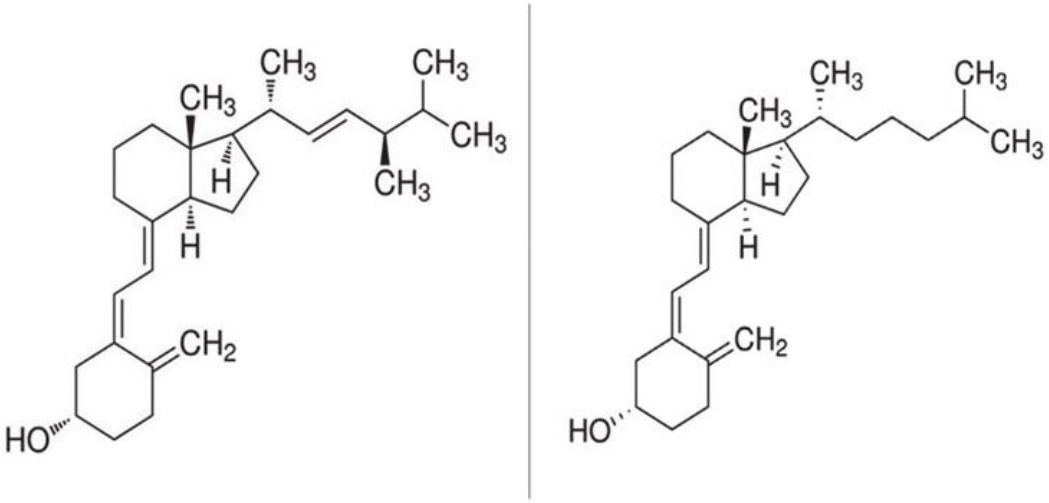

Figure 2. 1. Chemical structures of vitamin D3 (Right) and vitamin D2 (Left) [18]

2.1.2. Sources of Vitamin D:

Sunlight exposure, supplements, and nutrition are the main sources of providing vitamin D for the body.

2.1.2.1. Sunlight Irradiation:

After being exposed to sunlight, solar UVB radiation penetrates the skin and converts 7-dehydrocholesterol to pre-vitamin D3, which later transforms into vitamin D3. The amount of UV radiation emitted by the comparable skin length is determined by latitude, altitude, air quality, cloud coverage, daylight hours, and the latitudes of the month of the year. Furthermore, the effects of UVB on the skin are influenced by clothing and the use of sun protection. Vitamin D3 does not become intoxicated as a result of excessive sun exposure. [18,19]

2.1.2.2.Dietary sources :

Since vitamin D is not present in all foods, diet is not considered the main source of it [20]. It is doubtful that people can receive adequate vitamin D from their diet. Unlike skin synthesis, dietary consumption provides Ergocalciferol and Cholecalciferol. Vitamin D-rich food includes oily fish, eggs, cereals, mushrooms, and powdered milk. However, consumption of these items appears to be modest in poor countries [21].

2.1.2.3.Vitamin D supplementation:

Many low-cost additional forms of vitamin D, such as vitamin D3 and vitamin D2, are available over the counter [22] in a variety of licensed drugs and food products, such as calcium supplements and fish oil products with up to 500 IU (12.5 g). 400 IU (10g) multivitamins, as well as oil formulations, are widely available in capsules and tablets with 100,000 IU (2,500g) [23]. Recommended vitamin D intake levels are shown in Table 2.1.

Table 2. 1 Vitamin D intake recommendations from the Institute of Medicine vs. the Endocrine Society [24]

Age group	Recommended Dietary Allowance (RDA) per day	Tolerable Upper Intake Level (UL) per day
Infants 0-6 months	400 IU (10 mcg)*	1000 IU (25 mcg)
Infants 7-12 months	400 IU (10 mcg) *	1500 IU (38 mcg)
Children 1-3 years	600 IU (15 mcg)	2500 IU (63 mcg)
Children 4-8 years	600 IU (15 mcg)	3000 IU (75 mcg)
Children and Adults 9-70 years	600 IU (15 mcg)	4000 IU (100 mcg)
Adults > 70 years	800 IU (20 mcg)	4000 IU (100 mcg)
Pregnancy & Lactation	600 IU (15 mcg)	4000 IU (100 mcg)
* Adequate Intake rather than Recommended Dietary Allowance.		

The suggested quantities of vitamin D3 to be supplied per kg food for rats are 300IU. [25]

2.1.3. Vitamin D Metabolism:

Vitamin D requirements can be provided by endogenously (via sunshine exposure) and exogenously (from diet and supplements). However, it makes no difference how we obtain vitamin D: by skin synthetizations, diet, or supplementation [26]. All vitamin D metabolites has low water solubility; they must be delivered by binding to plasma proteins such as the Vitamin D Binding Protein (VDBP) [27]. Before the inactive form of vitamin D3 is hydroxylated, it has to be attached to the VDBP, which carries inactive D2 and D3 to the liver through the blood and lymph, then the 25-hydroxylase enzyme hydroxylases both vitamin D2 and vitamin D3, causing the formation of 25-hydroxyvitamin D (25[OH]D) [28]. As the most abundant circulating form of vitamin D, 25-hydroxyvitamin D (25-OHD) blood levels are frequently used to estimate total body reserves of vitamin D. It is, however, physiologically inactive and must be hydroxylated in the kidney in order to be converted into the biologically active 1,25-dihydroxyvitamin D (1,25[OH2] D) (Figure 2.2) [29]. Endocytic receptors, such as megalin and cubilin, can be found in the kidney's proximal tubule cells. They are in charge of VBP reabsorption, which leads to the intracellular conversion of 25(OH)D to 1,25(OH2) D in the presence of 1 α-hydroxylase [30].

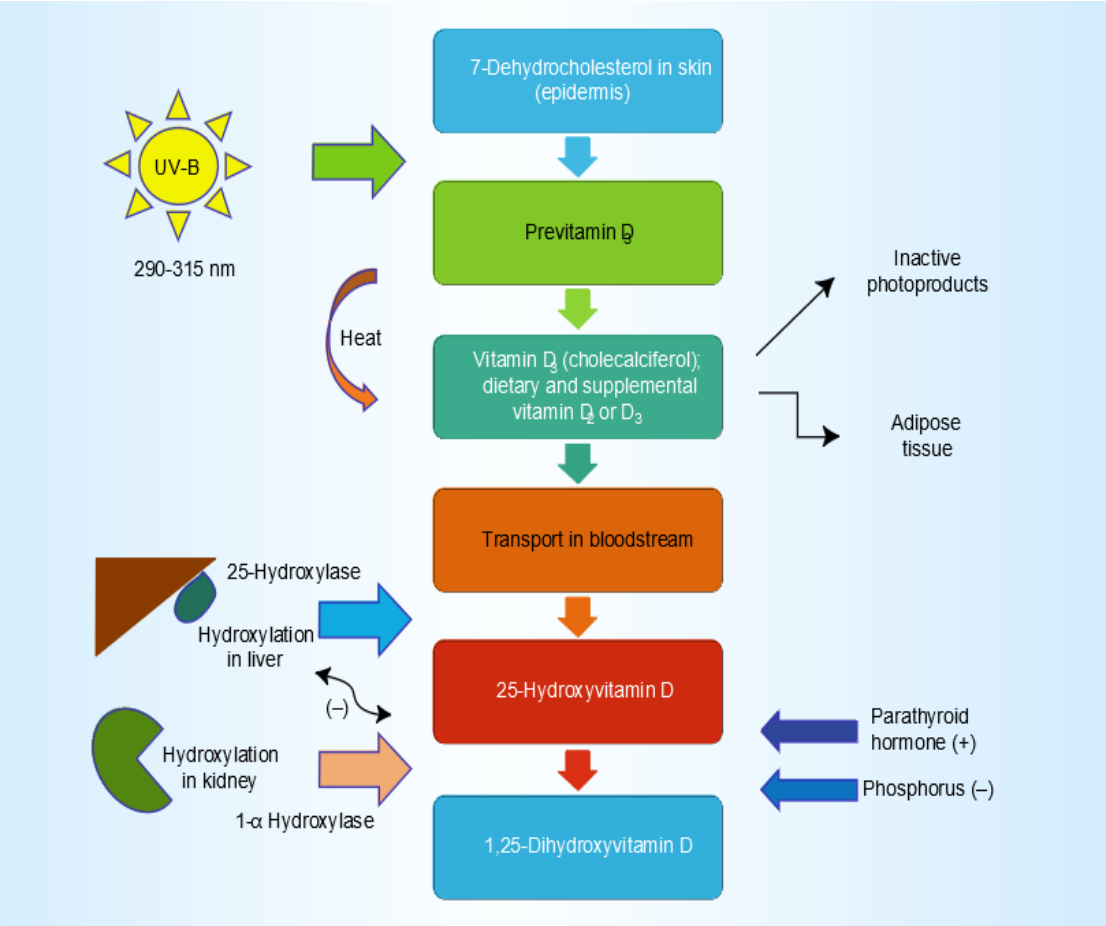

Figure 2. 2. Photobiosynthesis of Vitamin D [14]

2.1.4. Regulation of Vitamin D Metabolism and Functions:

Vitamin D is major function is to keep calcium, phosphorus, and bone homeostasis. Vitamin D is responsible for maintaining adequate and regular calcium levels. Its deficiency has a significant role in the development of bone mineralization illnesses such as osteoporosis and fractures. low blood calcium levels induce the parathyroid gland to emit PTH which triggers 1-hydroxylase in the kidneys to produce 1,25(OH)2D which elevates calcium levels by three different mechanisms. [31] increases bone mobilization through increased intestinal absorption interacting with PTH to stimulate reabsorption in the kidneys and boosting intestinal absorption although it is suggested that bone cells can convert 25(OH)D to 1,25(OH)2D when calcium availability is limited dietary calcium is usually favored over bone mobilization [32]. A single vitamin D receptor is responsible for vitamin D hormone functions and is found in osteoblasts, enterocytes, and renal tubular cells as target cells. It was recently discovered that tissues and cells that do not regulate calcium and

phosphate metabolism have 1,25(OH)2D3 receptors. This involves parathyroid gland cells, the colon, the ovaries, the pituitary gland, promyelocytes, skin keratinocytes, and lymphocytes. This demonstrates that the repercussions of vitamin D insufficiency extend beyond bone metabolism [33]. Vitamin D deficiency is linked to the pathophysiology of different disorders, including hypertension, diabetes mellitus, tumor growth, autoimmune diseases, mood disorders (depression, anxiety, stress) and infections. [34]

2.1.5 Measurement of Vitamin D Metabolites:

One of the most prevalent vitamin D metabolites is 25(OH)D. So, its serum or plasma concentrations are frequently employed to assess vitamin D status. If valid measurements are conducted at room temperature and after up to four freeze-thaw cycles, the concentration of 25(OH)D in serum samples is stable [35]. 25(OH)D concentrations can be determined using immunoassays developed in the early 1980s. To assess the quantity of 25(OH)D, competitive protein binding assay and other methods can all be utilized [36]. LC-MS/MS, in contrast to the majority of commercial immunoassays, is capable of concurrently measuring 25(OH)D2 and 25(OH)D3 (total 25(OH)D = 25(OH)D2 + 25(OH)D3) and is thus the gold standard method for evaluating vitamin D status [37]. Additionally, LC-MS/MS may be utilized to quantify vitamin D metabolites 24,25(OH)2D2 and 24,25(OH)2D3 reliably. 1,25(OH)2D is frequently assessed using an enzyme-linked immunosorbent assay or a chemiluminescent immunoassay, which is especially difficult considering that circulating levels are hundreds of times lower than those of its precursor 25(OH)D [36].

2.1.6. Meaning of Vitamin D Deficiency:

A low level of 25(OH)D in the blood is indicates to be a deficiency of vitamin D, as it is widely used in assessing the level of vitamin D in the blood. The explanation for this is that 25(OH)D has a relatively long half-life of around 2-4 weeks. [38]. Furthermore, since the 25-hydroxylation stage is thought to be poorly regulated by metabolism, 25(OH)D should represent vitamin D intake [39]. Low level of 25(OH)D in results in vitamin D deficiency. There is no agreement on what constitutes a normal 25(OH)D concentration. [40] There are currently numerous recommendations, it has been proposed that vitamin D status be characterized according to 25(OH)D concentration in humans (Table 2.2) [41] as follows:

deficiency (less than 20 ng/ml), insufficiency (21–29 ng/ml), sufficiency (30–100 ng/ml).

Other Institute of Medicine (IOM) guidelines, on the other hand, state that serum 25(OH)D levels between 16 and 20 ng/ml (40 and 50 nmol/l, respectively) are regarded sufficient, and that levels exceeding 50 ng/ml (125 nmol/l) should be evaluated for possible harmful effects on health (Table 2.2.) [42].

Table 2. 2 The range of plasma 25(OH)D values recommended by the Endocrine Society [41].

Vitamin D status	**Endocrine Society**	**Institute of Medicine**
Deficient	below 20 ng/ml (50 nmol/l)	below 16 ng/ml (40 nmol/l)
insufficient	21 – 29 ng/ml (52 – 72 nmol/l)	
Sufficient	30 – 100 ng/ml (75 – 250 nmol/l)	16 – 20 ng/ml (40 – 50 nmol/l)

2.1.7. Toxicity of vitamin D:

High vitamin D doses could possibly lead to hypercalcemia. During early stages of vitamin D poisoning, people have gastrointestinal difficulties such as anorexia, diarrhea, constipation, nausea, and vomiting. Furthermore, numerous additional problems, such as aching bones, fatigue, headaches, a racing heartbeat, a decrease in appetite, and muscular and joint discomfort, commonly occur in conjunction with the aforementioned symptoms. Kidney stone illness can frequently be found in those who wake at night to urinate, accompanied by increased thirst, weariness, anxiety, and itching [43]. The quantity of vitamin D produced by one MED is multiplied by 10,000–25,000 IU of oral vitamin D, the amount of UVB radiation required for vitamin D sufficiency may be calculated [44]. The MED refers to the amount of time it takes for the skin to turn pink. The time duration differs

according to the location, skin pigmentation, age, and body fat percentage. It is worth mentioning that sunlight eliminates any additional vitamin D [45].

2.1.8. Vitamin D and Anxiety:

The role of vitamin D is to participate in the processes of development and growth, as well as to maintain the normal structure of the skeleton by regulating calcium metabolism in the body that occurs as a result of increased calcium absorption in the intestine [46]. However, Vitamin D is an active neurosteroid that is thought to modulate a wide range of brain processes. where it is synthesized in the brain and crosses the blood-brain barrier. also, vitamin D receptors have been found on neurons and glia in numerous parts of the human brain, including those involved in emotional regulation. [47] As previously noted, vitamin D's most well-known role is to maintain blood calcium and phosphorus balance, so a rise in vitamin D levels results in hypercalcemia, which causes neuropsychiatric dysfunction. previous research has found the patients with mild cases of hypercalcemia may show different cognitive abnormalities, whereas patients with severe cases of hypercalcemia may present with altered mental state, psychosis, and confusion. [48]

Many research demonstrates a link between hypovitaminosis D and cognitive fundamental and executive role, depression, bipolar disorder, and schizophrenia. Vitamin D enables neuronal receptors in regions involved with behavior control, encourages neurotrophic release and protects the brain with buffering anti-oxidant and anti-amative defenses against vascular injury. [49] Also, vitamin d regulates the release of nerve growth factor (NGF) which is a chemical for the neuronal survival of the hippocampus and cortical neurons [50]. Vitamin D may have autocrine/paracrine characteristics in the human brain, based on the extensive distribution of 1-Alpha-Hydroxylase [51].

2.2. Anxiety and Exploratory Behavior:

Anxiety is a common reaction to stress and is defined as a feeling of worry, uneasiness, or fear about the condition in one's life. Anxiety can present itself in a variety of ways. Anxiety and depression are frequently linked, as both have a destructive effect on one’s life. While depression and anxiety are often treatable with medication, the majority of people do not seek treatment for these conditions Individuals suffering from anxiety disorders have a tendency repeat negative thoughts and fears, which prompts them to avoid specific situations out of dread [52]. Anxiety issues affect women more than twice as much as men. Social

phobia disorder, and specific phobia disorder are different forms of anxiety disorders. Anxiety manifests itself physically in a variety of ways, including tension, sweating, elevated heart rate and blood pressure, trembling, dizziness, and rapid heartbeat. [53].

Exploratory behavior, which enables the gathering of information about novel objects, is critical for survival due to the increased opportunities for finding food, water, a mating partner, and shelter that it provides. At any one-time, exploratory behavior is affected by conflicting motivations to investigate a harmful novel area or to remain in safe and familiar settings. So, an animal's behavior in a novel habitat is always impacted by both curiosity and neophobia. Exploration-based behavioral tests in mice are frequently used to assess the anxiety-related effects of psychoactive medicines where it was discovered that animals' exploratory behavior is affected by extreme anxiety. [54].

2.2.1 Animal Models of Anxiety:

Animal models of psychiatric disorders try to show different aspects of the human experience, ranging from behavioral changes that indicate the emotional state to disease etiology and treatment effects. Animal models are "experimental preparations developed in one species for the aim of understanding phenomena occurring in another species," according to McKinney [55]. animal models can aid in the understanding of molecular systems involved in anxiety, as well as the screening and development of new drugs for their treatment that would be unfeasible in humans. The genetic foundation of anxiety has been established through human studies, and animal studies have been utilized to try to better define its genetic determinants. Animal models in the realm of anxiety research can be divided into two categories(i) models based on unconditioned responses; and (ii) models based on conditioned responses. The first category includes models based on rodent exploratory behavior, models based on rodent social behavior or nonhuman primates, and models based on the somatic stress response. Other paradigms that do not neatly fit into any of the other divisions, such as anxiety, are summarized in the fourth group [56].

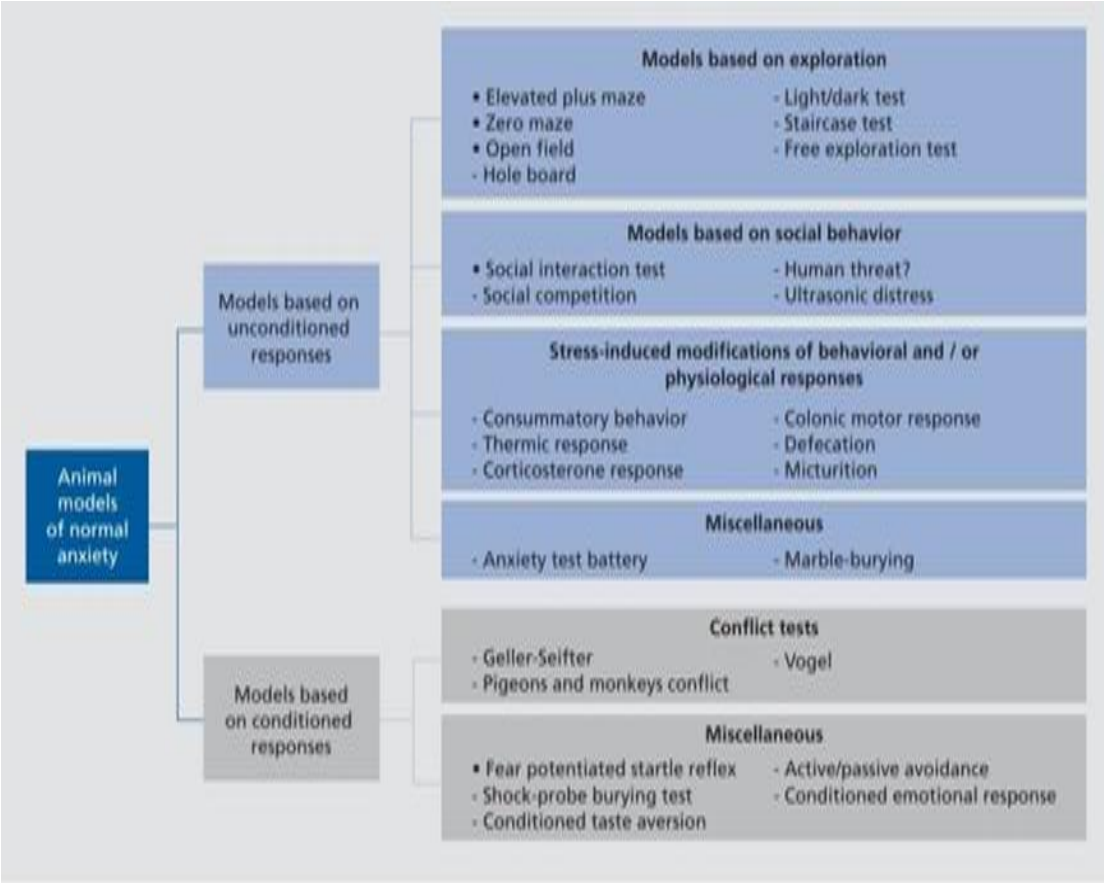

Figure 2. 3. Animal models of normal and state anxiety are classified. Models are divided into two types for ease of understanding: Unconditioned reaction tests and conditioned response tests are also available. is used to denote the tests in this article. [56]

2.2.1.1. Models Based on Exploratory Behavior:

2.2.1.1.1 Plus-Maze:

2.2.1.1.1.1 Elevated Plus-Maze:

Anxiety animal models such as the elevated plus-maze are unquestionably among the most commonly employed in current preclinical anxiety investigation. The test was developed as a result of the early finding that now in mazes with closed and open alleyways, in enclosed alleys, rats routinely demonstrate higher levels of exploration than open alleyways. However, when given a choice between alley types, rats choose to avoid those that do not have walls around them. This discovery resulted in the first investigations into the possible utility of an elevated 'X'-maze as paradigm of anxiety, which was conducted some 30 years later [57]. These researchers used a straightforward maze configuration that was elevated

two feet above the floor level and comprised two open and enclosed arms. Researchers also verified that rats avoid open arms, but they also demonstrated that diazepam reduced open arm avoidance while picrotoxin increases it [58]. Following this initial report, Comprehensive assessments of the test (that called as the elevated 'plus'-maze) to usage with rodents were conducted out, and more current studies suggest that it may be beneficial for a variety of other species. Beginning with the plus-maze, spatiotemporal measures of anxiety (i.e., the time spent in open arms as a percent or ratio of total arm time, and the frequency of open arm entries as a percent or ratio of total arm entries) have been the primary indices used to assess anxiety, while the total number of entries in all arms have frequently been used to measure general activity [59].

Figure 2. 4. Elevated Plus Maze [60].

2.2.1.1.1.2. Elevated Zero Maze:

It is a recently developed device of the plus-maze. this test, like the elevated-plus maze, is predicated on two opposing inherent tendencies: investigating a new environment and avoiding elevated and open spaces that represent predator risk circumstances. It consists of a circular elevated platform with two open regions opposite each other and two closed regions animals are put in one of the closed regions chosen as the starting region and anxiety-related behaviors are assessed both manually and through a video system [61].

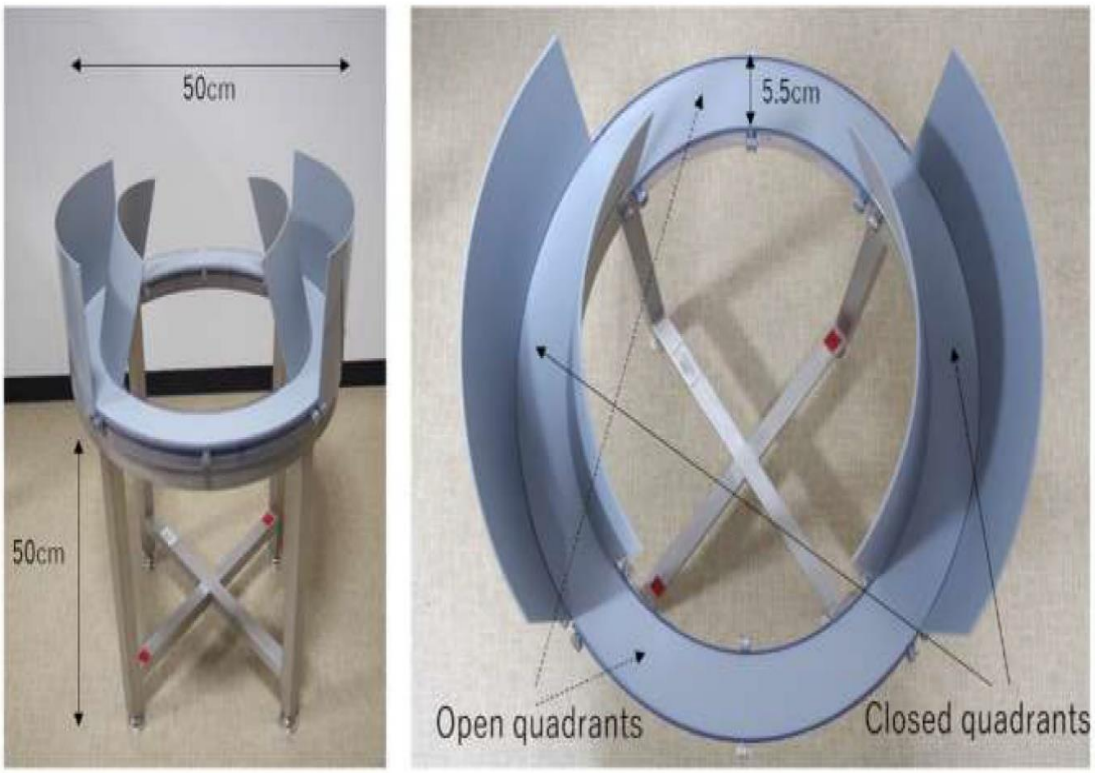

Figure 2. 5. The elevated zero maze (EZM) is seen from the side (left) and from above (right) [62].

2.2.1.1.1.3. Elevated T-maze test:

The elevated T labyrinth is a 3-armed experimental setup made of Plexiglas of the same size (50x12cm) at a length of 50 centimeters above the ground. One of them is closed with 40 cm high side walls made of Plexiglas and is placed perpendicular to two open arms standing opposite each other. T maze with different sizes for mouse and rat is used for rats. The Elevated T-maze takes use of the subjects' natural dread of heights and open spaces. Anxiety in rodents can be assessed by measuring inhibitory avoidance in the face of conflicts, whereas panic can be assessed by conducting one-way escape tests on the Elevated T-maze [63].

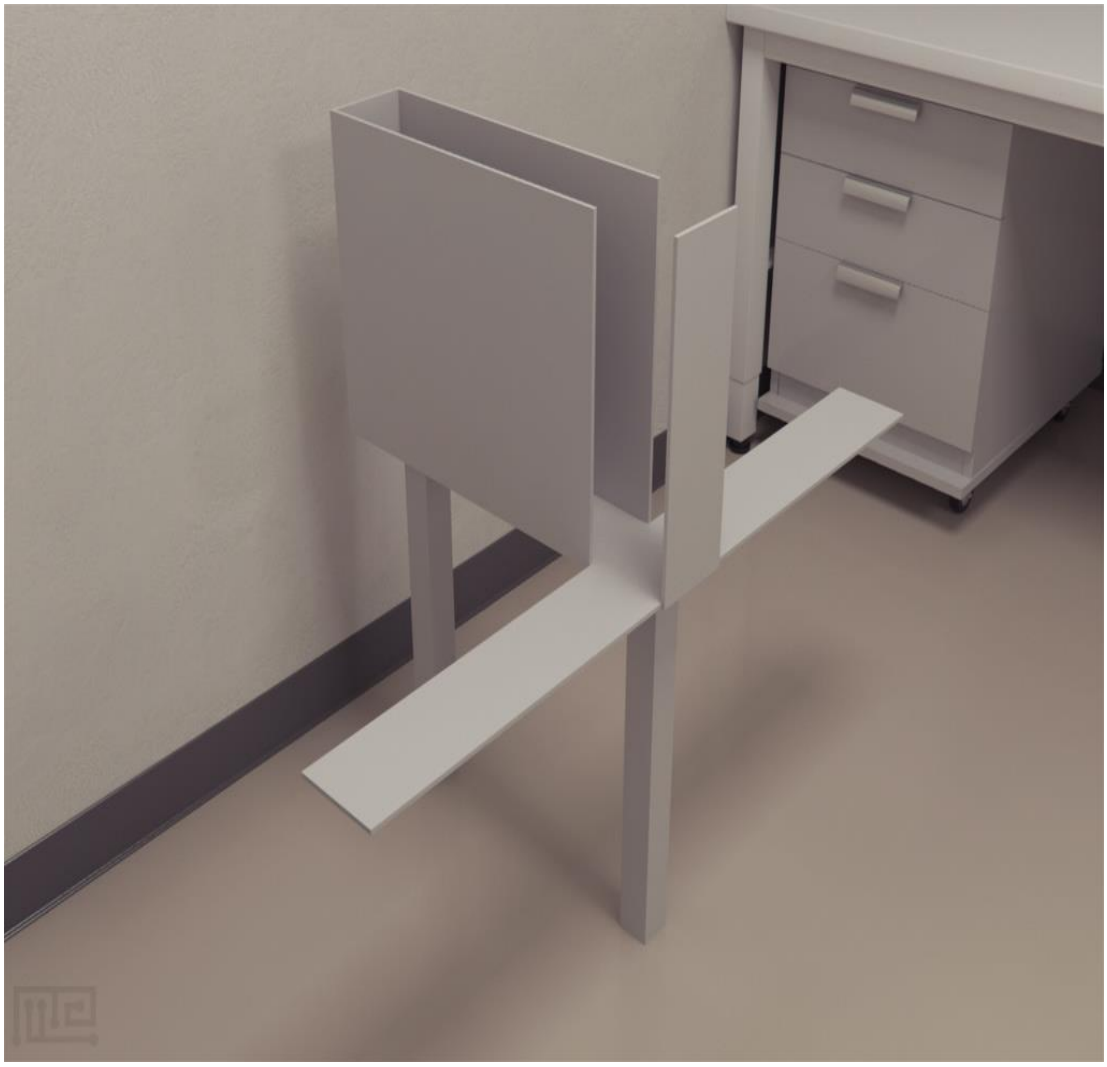

Figure 2. 6. Elevated T-maze. [64]

2.2.1.1.2. Open field test:

The Open Field Maze (OFM) was created in 1934 as a test to assess emotionality in rodents. It has become one of the most commonly utilized measurements of behavior in animal psychology. It allows for a simple and quick assessment of well-defined behaviors while requiring minimal training for the test participant and little to no specialist training for the person conducting the test. These characteristics have led to the open field maze's widespread use in studies involving different animal species [65]. Its popularity stems in part from the psychological and physiological benefits it provides. Considered Rodents are nocturnal animals that like to stay in the dark and avoid strong light. This must be considered while doing an open field test, which is a frequent observational strategy. The animal is removed from its cage and placed in an unfamiliar, well-lit arena that allows it to walk about freely [66]. Due to its photophobia, the animal avoids brightly lighted open regions and prefers to remain close to the walls. The region is separated into peripheral and core sections, which may be used to monitor movement and rearing. Exploratory or locomotor behavior is evaluated, as well as an autonomic activity like urination and faces. Infrared beam array technologies are used to track automated movement, rearing, and time spent in specific parts of the open field. Furthermore, comparable to the elevated plus-maze, the open field behavior is highly responsive to a variety of internal and external stimuli. [67].

Figure 2. 7. Open field test [68].

3. MATERIALS AND METHODS

3.1. Experimental Animal:

We used a total of 36 male Wistar rats that were 8 weeks old. Animals were supplied from the Başkent University Experimental Animal Breeding Center. They were placed in normal cages and provided food and water ad libitum. All animal tests were conducted in Başkent University, Experimental Animal Research Center. The tests were conducted with the agreement of Başkent University's Animal Experiments Ethics Committee (20/17).

3.2. Experimental Groups:

Equal numbers of rats were assigned to three groups (n = 12).

- Sham group (Group 1) [n=12]: Each rat received normal drinking water via oral gavage.
- Vitamin D at a 400IU dose (Group 2) [n=12]: Each rat received 400 IU/day of vitamin D via oral gavage [69].
- Vitamin D at a 1000IU dose (Group 3) [n=12]: Each rat received 1000 IU/day of vitamin D via oral gavage [70].
- Vitamin which we used in experiment Devit-3 oral drops, each 15 ml drop contains 50,000 IU of vitamin D3 (Deva Holding A.Ş, Turkey). The active ingredient cholecalciferol (vitamin D3) is produced from sheep's wool fat.
- For eight weeks, the groups received the drugs once daily. After this time, the rats are tested. The study's schedule was shown in (Figure 3.1).

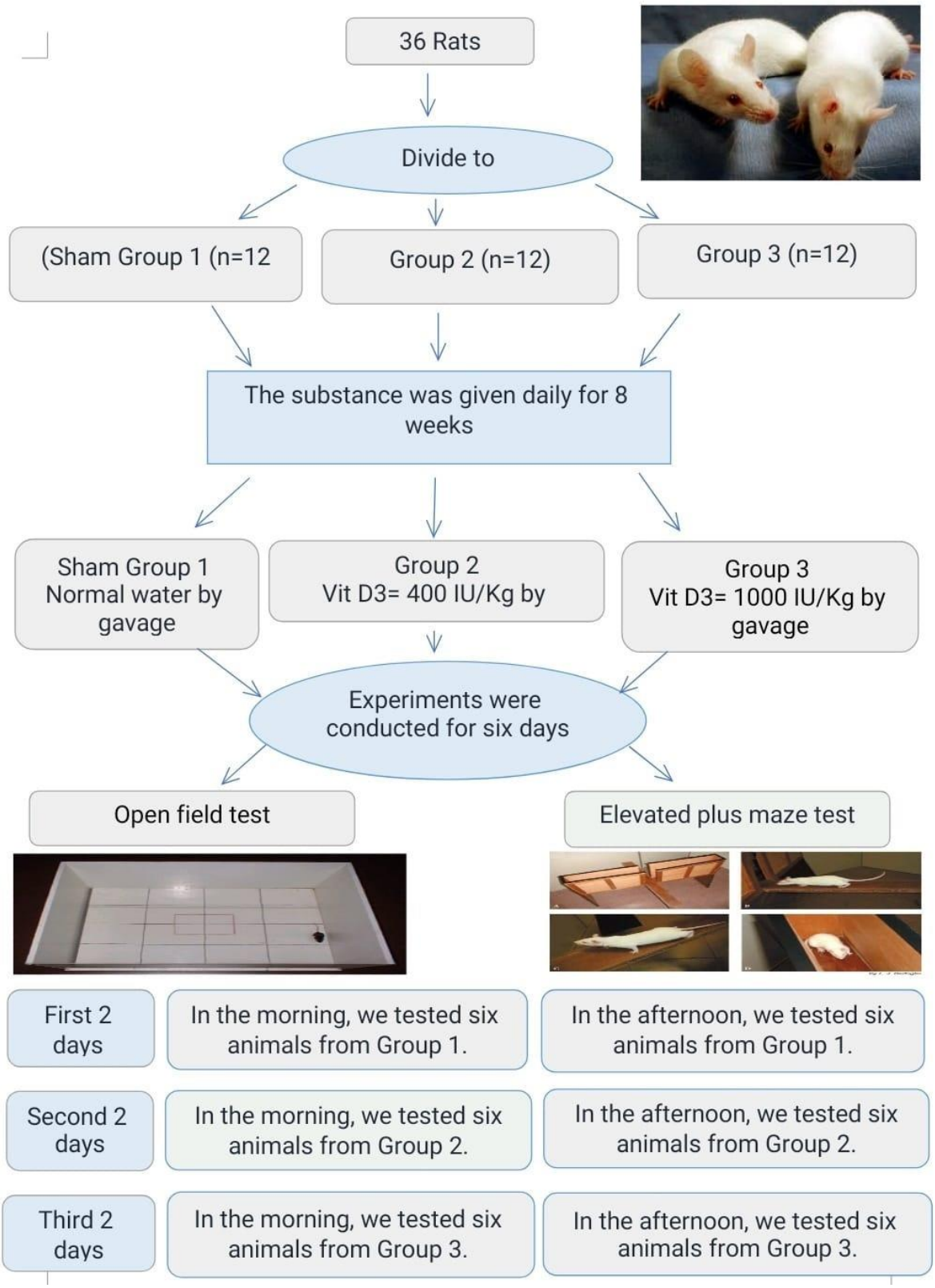

Figure 3. 1. The Schedule of the Study

3.3. Open Field Test:

We did the open field test after completing the period of giving the vitamin D [8 weeks] for 2 days in the morning consecutively for each group starting with the sham group in the first 2 days then the low-dose group on the second 2 days and high dose group in the third 2 days. During this test, each rat was placed in the corner of the box (OFT apparatus consists of a wood box (100 × 100 × 36 cm3) and the test period lasted 10 minutes. A video recording system was used to determine and quantify the overall distance traveled, the number of reared, and the number of defecations. After testing each rat for ten minutes, the device was cleaned with alcohol before began the new test (figure 3.3) [71].

Figure 3. 2. Open Field Test Apparatus

3.4. Elevated Plus Maze:

We did the EPM test after completing the period of giving the vitamin D [8 weeks] for 2 days in the afternoon consecutively for each group starting with the sham group in the first 2 days then the low-dose group on the second 2 days and high dose group in the third 2 days. where the EPM apparatus consists of two open arms (48 cm long, 10 cm broad each) that are perpendicular to two arms open arms that have the same size, that are enclosed by 50-cm high walls. Each open and closed arm is divided into three parts (16 × 10 cm each) and a Centre open segment that connects the two arms. the rat was first placed in the central segment of the EPM, facing one of the open arms the experiment was started after a 5-minute habituation period. An exploratory activity measurement was recorded for the number of open and closed segments crossed by each rat. The proportion of entries into open arms was computed using the following formula: percent entries= (entries into open arms/total number of entries × 100). Similarly, the percentage of time spent in open arms was calculated as follows: percent time= (time in open arms/ (time in open arms + time in closed arms) × 100) [72]. After each rat was tested for 5 minutes, alcohol was used to clean the apparatus.

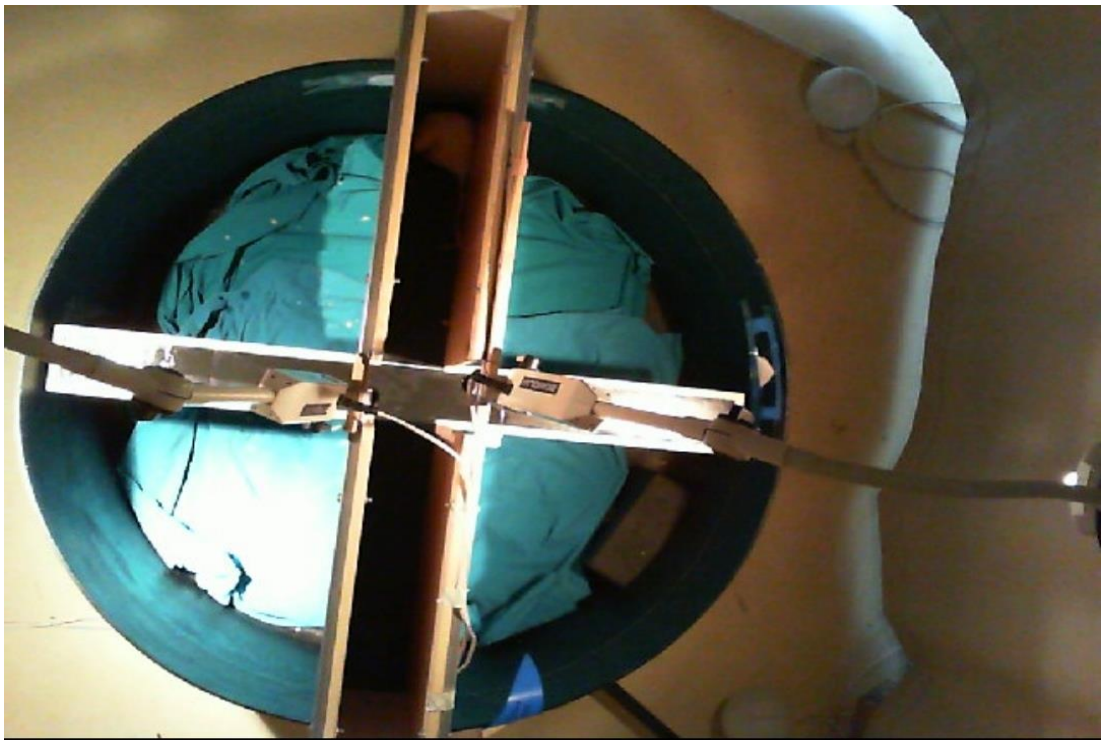

Figure 3. 3. Elevated Plus Maze Apparatus.

3.5. Statistical Evaluation:

The data is evaluated using descriptive statistics and mean comparisons between all study groups. One-way analysis of variance (ANOVA) was used to examine anxiety and exploratory activity, followed by Tukey's multiple ranges to understand the specific mean differences and statistical significance ($p < 0.05$).

All statistical analyses were regarded statistically significant when $p \leq 0.05$, and highly significant when $p < 0.001$.

4. RESULTS

4.1. Open Field Tests Results

We showed the open field test parameters as mean ± SE in Table 3.1.

Table 4. 1 Open Field Test Finding of Groups

	Group 1[a] (n=12)	Group 2[b] (n=12)	Group 3[c] (n=12)	F	p
NEC	1.83 ± 0.71	1.25 ± 0.50[a]	0.50± 0.67[a]	1.65	0.20
STC	4.83 ± 1.23	1.50 ± 0.41[a]	1.25 ± 0.50[a]	6.12	0.005
STP	593.33± 2.42	598.50± 0.41[a]	598.75± 0.50[a]	4.44	0.02
NR	11.83 ± 1.08	18.33 ± 1.84[a]	11.66 ± 1.56[b]	6.14	0.005
ND	3.66 ± 0.41	2.50 ± 0.37	2.66 ± 0.37	2.61	0.08

Group 1=a: Sham group, Group 2=b: 400IU Vitamin D, Group 3=c: 1000IU Vitamin D, NEC: Number of entering to the central area, STC: Spent time in the central area, STP: Spent time in the peripheral area, NR: Number of rearing, ND: Number of defecations. ([a], this mean is different from Group 1)

There were significant differences among a group in term of open field test value except for NEC and ND (Table 4.1).

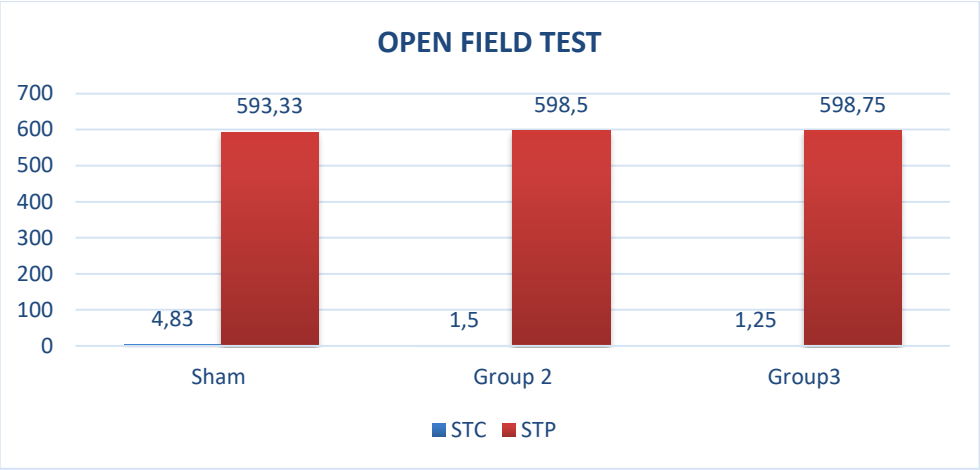

Figure 3. 4. Comparison of spent time in center and in peripheral between all groups.

STC values of Group 2 ($p<0.02$) and Group 3 ($p<0.01$) were statistically lower than group 1. On the contrary, the STP values of Group 2 ($p<0.05$) and Group 3 ($p<0.04$) were higher than group 1. While the STC value of Group 3 was the lowest, the STP value was nearly the same in Group 2 and Group 3(Figure 3.4).

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The NR value was the highest in Group 2. There was a difference between Group 1 and Group 2 ($p<0.01$) and between Group 2 and group 3 ($p<0.01$) in terms of NR.

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There were no differences among the groups for NEC and ND.

4.2. Elevated Plus Maze Results

We showed the elevated plus-maze test findings as mean ± SE at Table 3.2.

Table 4. 2 The elevated plus-maze finding of groups (mean ± SE)

	Group 1[a] (n=12)	Group 2[b] (n=12)	Group 3[c] (n=12)	F	p
STCA	217.16± 33.10	196.33± 19.98	234.58 ± 24.3	0.52	0.59
STOA	82.83 ± 33.1	103.66 ± 19.98	40.41 ± 12.22	1.89	0.16
ECA	1.50 ± 0.33	2.25 ± 0.37 [c]	1.16 ± 0.16	3.30	0.04
EOA	1.25 ± 0.21	1.33 ± 0.22	0.83 ± 0.24	1.38	0.26
Formula of Entry	47,46 ± 8,71	43.05 ± 6.09	29.16 ± 7.43	1.62	0.21
Formula of Time/Sec	25.00 ± 10.49	25.24 ± 5.85	13.47 ± 4.07	0.84	0.43

Group 1: Sham group, Group 2: 400 IU vitamin D, Group 3: 1000 IU vitamin D, STCA: Spent time in the closed arm, STOA: Spent time in open arm, ECA: Entry number of the closed arm, EOA: Entry number of open arms

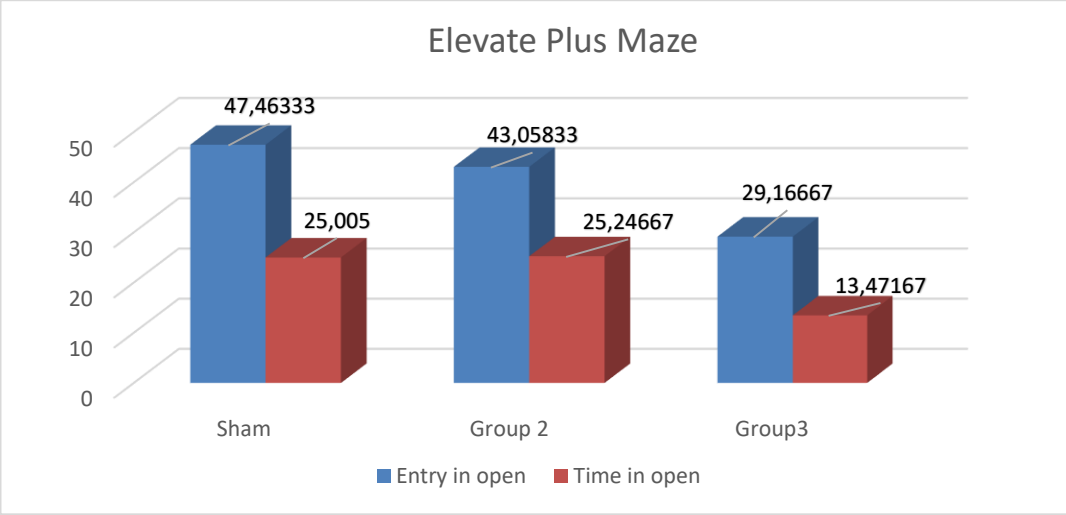

Figure 3. 5. Comparison by percentage of spent time and number of enter in the open arms.

In Group 2, the STCA value was the lowest and the STOA value was the highest from other groups.

ECA value was statistically higher in Group 2 than Group 3. There were no statistical differences between groups for EOA value, for the formula of entry and for a formula of time among groups.

5. DISCUSSION

In our study, we wanted to determine the effect of Vitamin D on anxiety in rats by using a variety of doses. Because of the increasing amount of research that suggests a substantial link between VD insufficiency and emotional disorders such as sadness and anxiety, we thought that extra vitamin D usage would prevent anxiety. Where serum 25(OH) D deficiency might cause anxiety and depression symptoms, and lower serum 25(OH) D levels could result in severe anxiety at baseline in older people [73, 74] since vitamin D can be produced naturally by human skin under suitable conditions, many people would easily exceed their daily recommended dose by taking unnecessary supplements. This could lead to undesirable side effects and possibly anxiogenic effect as we will discuss later.

The investigation was conducted to observe how different doses of vitamin D could result in behavioural changes and anxiety in rats, through both open field and elevated plus-maze tests. The results will help us understand better how vitamin D could affect deeply our mood and psychological behaviors in the future.

By conducting the OFT, we could notice some behavioural changes among the three groups. Rats receiving both doses of vitamin D spent the most time in the peripheral area and preferred not to explore the central area and group 3 showed the least exploring activity compared with group 1 and group 2. The time spent in the peripheral area in OFT was used as a sign of anxiety [75]. These results are consistent with (Burne, Thomas HJ, et al. (2006), where unrestricted DVD-deficient rats spent more time on the wall sides in the open field and spent less time on its corners and centre than control rats [76]. Fedotova et al. (2017) assessed also the effectiveness of chronic administration of vitamin D using the different doses through the subcutaneous route. In his study, the administration was done once a day for 14 days consecutively. Contrary to our results, this test showed that only the doses of 1.0 and 2.5 mg/kg per day showed anxiolytic effects, keeping in mind that the rats were adults and had their ovaries removed [77]. While the rats that took higher-dose vitamin D (G 3) in the EPM test had the lowest number of entries in the open arm, rats prefer to stay in the enclosed arms of the maze and avoid the open arms. This is owing to rats' natural dislike to open spaces, as well as the maze's height [78]. These results are consistent with (Fu, Lin, et al., 2017) an animal study that discovered that adulthood vitamin D deficiency was linked to mood-changing behaviours, were found the time of entry into the open arms was reduced in VDD diet-fed mice than in control animals [79], Baranenko et al. (2019) demonstrated

that the treatment with VD3 (5.0 mg/kg) in ovariectomized rats significantly reduced anxiety-like behaviour in the EPM [80]. The dose difference between that study and ours may be due to the fact that the rats used were ovariectomized not normal rats. However, both vitamin D overdosing and deficiency can alter the social behaviour of the rats.

Vitamin D is important for the metabolism of both phosphate and calcium in living beings. The main purpose of vitamin D is to ensure that the body absorbs calcium. Because of its vital role in preserving the basic structure of the organism) i.e., musculoskeletal health, Previous research showed that vitamin D is one of the components that found its receptor across the body, even the brain in the same areas related to depression and anxiety [81], where they expressed that vitamin D, activates genes that regulate the immune system and releases neurotransmitters that influence brain functions [82]. Also, because vitamin D is the most important factor in calcium metabolism, high consumption of vitamin D can lead to hypercalcemia as it can increase the level of calcium in the blood [83].

Hypercalcemia caused by a high intake of vitamin D over a long period has been linked to neuropsychiatric problems. Patients with hypercalcemia usually have anxiety, but patients with severe hypercalcemia have changed mental status [84]. The mechanism of hypercalcemia-induced psychosis is still unknown. This can be explained as a result of variations in monoamine levels in the central nervous system and glutaminergic excitotoxicity via N-Methyl-D-aspartate receptors could explain it, though [85].

Vitamin D is generated in the brain and then passes through the blood-brain barrier to operating directly on cells that contain its nuclear receptor [86]. Vitamin D metabolites may alter the behaviour of cognitive functions through their particular neuroprotective effects, according to recent studies. In tests testing cognitive functioning, people with low vitamin D levels do worse [87], while others show that those with appropriate vitamin and micronutrient concentrations have improved cognitive skills [88].

According to some research, dopamine appears to have an essential role in anxiety modulation in several areas of the brain, according to studies. The mesolimbic, mesocortical, and nigrostriatal dopaminergic systems have been implicated in anxiety [89].

In certain studies, vitamin D has been found to have a neuroprotective impact on dopaminergic pathways in the adult brain. For example, when 6-hydroxydopamine (6-OHDA) is given to rats that have been pre-treated for one week with 1,25(OH) 2D3, the dopaminergic function in the treated rats is intact. [90]. Increased dopamine (DA) and

alterations in the caudate-putamen and hypothalamus were seen in the brainstem of rats given a single dose of vitamin D postnatal [91]. In vitro, 1,25(OH)2D3 promotes tyrosine hydroxylase production in adrenal medullary cells. [92] Vitamin D has been demonstrated to increase the production of tyrosine hydroxylase, hinting that it may have an impact on dopaminergic functions [93].

Serotonin 5-hydroxytryptamine or 5-HT is a neurotransmitter in 1948, it was discovered. that is hypothesized to play a role in pain sleep appetite anxiety depression and migraines among other things serotonin is made in the body from tryptophan which is converted to 5-HT by a variety of enzymes [94]. Serum serotonin levels increased significantly in the vitamin D-treated group compared to the non-treated group in a previous study this could be due to vitamin D's role in increased transcription of the tryptophan hydroxylase-2 gene which results in increased conversion tryptophan to serotonin in the brain [95]. As a result, in our study, we expect that high dose vitamin D intake might cause excess release of serotonin in the central nervous system and this release causes a disorder known as serotonin syndrome [96]. Serotonin poisoning is characterized by neuromuscular excitation altered mental state and autonomic stimulation [97].

6. CONCLUSION

According to our findings from open field test and elevated plus maze both different doses of Vitamin D showed anxiogenic effects. The mechanisms by which vitamin D increases anxiety are currently unknown, and additional research is required.

In the light of past findings and conclusions, this study's findings resulted in the following recommendations:

1- Use different vitamin D doses of low than 1000 IU are administered to experimental animals in order to determine the effects and changes that are expected to occur for anxiolytic effect.

2- To achieve better findings in future anxiolytic effect research, it is preferable to increase the number of experimental rats and shorten the experiment length to less than two months.

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APPENDIX (I)

1993
BAŞKENT ÜNİVERSİTESİ
Tıp ve Sağlık Bilimleri Araştırma Kurulu

TS-EN-ISO 9001
KALİTE SİSTEM BELGESİ

Sayı : 94603339-604.01.02/ 33348 18/11/2020
Konu : Proje Onayı

FİZYOLOJİ ANABİLİM DALINA

Fizyoloji Anabilim Dalında görev yapmakta olan Prof. Dr. Nazan Dolu'nun danışmanlığında Sağlık Bilimleri Enstitüsü / Fizyoloji Tezli Yüksek Lisans Programı öğrencisi Zahour Gamal Eddn Habib Asmaeil'in sorumluluğunda yürütülecek olan DA20/19 nolu "Effects of high doses of vitamin D on anxiety and exploratory activity behaviours in rats" başlıklı araştırma projesi Kurulumuz ve Hayvan Deneyleri Yerel Etik Kurulunun 02/11/2020 tarih ve 20/17 sayılı kararı ile uygun görülmüştür. Projenin başlama tarihi ile çalışmanın sunulduğu kongre ve yayımlandığı dergi konusunda Kurulumuza bilgi verilmesini rica ederim.

e-imzalıdır

Kurul Başkanı

Not: Çalışma bildiri ve/veya makale haline geldiğinde "Gereç ve Yöntem" bölümüne aşağıdaki ifadelerden uygun olanının eklenmesi gerekmektedir.

— Bu çalışma Başkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmış (Proje no:…) ve Başkent Üniversitesi Araştırma Fonunca desteklenmiştir.

— This study was approved by Baskent University Ethical Committee for Experimental Resarch on Animals (Project no:…) and supported by Baskent University Research Fund.

DAĞITIM
Sağlık Bilimleri Enstitüsü Müdürlüğüne
Fizyoloji Anabilim Dalına

APPENDIX (2)

1993
BAŞKENT UNIVERSITY

LOCAL ETHICS COMMITTEE FOR ANIMAL EXPERIMENTS DECISION		
SESSION NO	**DECISION NO**	**DATE OF DECISION**
09	20/17	02/11/2020

Project DA20/19 no entitled "Effects of high doses of vitamin D on anxiety and exploratory activity behaviours in rats" pending to be conducted by Nazan Dolu with the Department of Physiology has been reviewed and unanimously approved by the Local Ethics Committee for Animal Experiments.