

**BAŞKENT UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHYSIOLOGY
MASTER'S PROGRAM**

**THE EFFECTS OF VITAMIN D AND ZINC ON ANXIETY AND
EXPLORATORY BEHAVIOURS IN RATS**

BY

AMNA ABDULSALAM BAROUD

MASTER'S THESIS

ANKARA- 2022

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THESIS SUPERVISOR

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ANKARA- 2022

BAŞKENT UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

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Finally, I recognize that Başkent University Research Council supported this work (Research number DA20/17) and Animal Experiments Ethics Committee at the Başkent University (permission number 20/15).

ÖZET

Amna Baroud, Sıçanlarda D Vitamini ve Çinkonun Anksiyete ve Keşif Etkinlik Davranışlarına Etkisi, Başkent Üniversitesi Sağlık Bilimleri Enstitüsü Fizyoloji Anabilim Dalı, Fizyoloji Tezli Yüksek Lisans Programı Yüksek Lisans Tezi, 2022

D vitamini lipofilik bir vitamindir Koronavirüs pandemisi sırasında birçok kişi virüse karşı bağışıklıklarını güçlendirmek için D Vitamini (D vit) ve çinko takviyeleri almaya başlamıştır. Ancak, D vit ve çinkonun anksiyete ve keşif davranışları üzerine etkilerini araştıran bir çalışmaya rastlanmamıştır. Bu çalışmada, sıçanlarda değişik dozlardaki D vit ve çinko takviyesinin, anksiyete ve keşif davranışı üzerindeki etkisinin açık alan testi ve yükseltilmiş artı labirent ile araştırılması amaçlanmaktadır.

Bu çalışmada 48 genç (8 haftalık) erkek Wistar sıçan eşit olarak dört gruba ayrıldı. 8 hafta boyunca günde bir kez olmak üzere Grup 1'e (sham grubu) normal su, Grup II'ye 400 IU/gün D vit, Grup III'e 1000IU/gün D vit gavajla verildi. Grup IV'e altı gün boyunca serum fizyolojik (ip) içinde çözülmüş olarak 30 mg/kg ZnSO₄ verildi. 8 hafta sonra, D vit gruplarına altı gün boyunca ZnSO₄ (30 mg/kg, serum fizyolojik içinde çözülmüş, ip) ve D vit (400 IU/gün ve 1000 IU/gün, ağızdan) birlikte verildi. Bu sürenin sonunda, sıçanlara açık alan testi (AAT) ve yükseltilmiş artı labirent testi (YAL) uygulandı.

Açık alan testinde Grup 2 ($p<0.013$) ve Grup 3'ün ($p<0.003$) merkezi alana giriş sayısı (MAG) ve Grup 2 ($p<0,039$) ve Grup 3'ün ($p<0,003$) merkez alanda geçirilen süreleri (MAGS) Grup 1'den düşüktü. Grup 2 ($p<0,000$), Grup 3 ($p<0,000$) ve Grup 4'de ($p<0,000$) gerinme sayıları (GS) ve Grup 2 ($p<0,000$), Grup 3 ($p<0,000$) ve Grup 4 ($p<0,004$)'ün dışkılama sayıları (DS) Grup 1'den anlamlı olarak düşüktü. YAL'de Grup 2'nin kapalı alanda geçirilen süresi (KAGS) tüm gruplar arasında en yüksekti. KAGS değeri Grup 2'de Grup 1'e ($p<0.01$) ve Grup 4'e ($p<0.01$) göre istatistiksel olarak daha yüksek, açık alanda geçirilen süre (AAGS) değeri Grup 2'de Grup 1'e göre istatistiksel olarak daha düşüktü ($p<0.04$). Grup 1'in açık kola giriş sayısı (AKG), en yüksek bulundu. Grup 2 ($p<0.01$) ve Grup 3'ün ($p<0.03$) AKG değerleri Grup 1'den istatistiksel olarak daha düşüktü.

AAT ve YAL sonuçlarımıza göre farklı dozlardaki D vit ile birlikte çinko verilen ratlarda anksiyojenik etkiler görüldü; ancak çinko tek başına uygulandığında anksiyeteyi etkilemedi.

Farklı inko dozlarının kaygı zerindeki etkisini arařtırmak iin daha fazla alıřmaya ihtiyaımız bulunmaktadır.

Anahtar Kelimeler: D Vitamini, inko, Anksiyete, Keřif aktivitesi, Sıan.

Bu alıřma, Bařkent niversitesi Arařtırma Kurulu tarafından DA20/17 nolu arařtırma projesi olarak desteklenmiř, Bařkent niversitesi Klinik Arařtırmalar Etik Kurulu tarafından 02/11/2020 tarihinde 20/15 karar numarası ile onaylanmıřtır.

ABSTRACT

Amna Baroud, The Effects of Vitamin D and Zinc on Anxiety and Exploratory Behaviours in Rats, Başkent University Institute of Health Sciences, Master's Thesis, 2022

Vitamin D is a lipophilic vitamin. During the coronavirus pandemic, many people started taking Vitamin D (vit D) and zinc supplements to increase their immunity against the virus. However, there is no study investigating the effects of vitamin D and zinc on anxiety and exploratory behaviors. In this study, it is aimed to investigate the effect of different doses of vitamin D and zinc supplementation on anxiety and exploratory behavior in rats by open field test and elevated plus maze.

In this study, 48 young (8 weeks old) male Wistar rats were equally divided into four groups. Normal water was given to Group 1 (sham group), 400 IU/day D vit to Group II, 1000 IU/day D vit to Group III, once a day for 8 weeks by gavage. 30 mg/kg ZnSO₄ dissolved in physiological saline (ip) was administered to group IV for six days. After 8 weeks, vit D groups were given concomitant ZnSO₄ (30 mg/kg, dissolved in saline, ip) and vit D (400 IU/day and 1000 IU/day, orally) for six days. At the end of this period, the rats underwent open field test (OFT) and elevated plus maze test (EPM).

In the open field test, the number of entrances to the central area (NEC) of Group 2 ($p<0.013$) and Group 3 ($p<0.003$) and the time spent in the central area (STC) of Group 2 ($p<0.039$) and Group 3 ($p<0.003$) was lower than Group 1. The number of rearing (NR) values for Group 2 ($p<0.000$), Group 3 ($p<0.000$) and the number of defecation (ND) values of Group 2 ($p<0.000$), Group 3 ($p<0.000$) and Group 4 ($p<0.004$) were significantly lower than Group 1. At EPM, Group 2 had the highest spent time in the closed area (STCA) value among all the groups. STCA value was statistically higher at Group 2 than Group 1 ($p<0.01$) and Group 4 ($p<0.01$), while spent time in the open area (STOA) value was statistically lower at Group 2 than Group 1 ($p<0.04$). Group 1 had the highest enter the open arm (EOA). EOA values for Group 2 ($p<0.01$) and Group 3 ($p<0.03$) were statistically lower than Group 1.

According to our OFT and EPM results, anxiogenic effects were observed in rats given different doses of vitamin D and zinc; however, zinc did not affect anxiety when

administered alone. We need more studies to investigate the effect of different doses of zinc on anxiety.

Key Words: Vitamin D, Zinc, Anxiety, Exploratory Activity, Rat

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CONTENTS

ACKNOWLEDGMENTS	I
ÖZET	II
ABSTRACT	IV
LIST OF TABLES	VI
LIST OF FIGURES	VII
LIST OF SYMBOLS AND ABBREVIATIONS	VIII
1. INTRODUCTION	1
2. GENERAL INFORMATION.....	5
2.1. Vitamin D.....	5
2.1.1. Discovery of Vitamin D	5
2.1.2. Forms and Sources of Vitamin D.....	5
2.1.3. Vitamin D Production and Metabolism	8
2.1.4. Physiological Effects of Vitamin D	9
2.1.5. Deficiency and Resistance of Vitamin D	10
2.1.6. Vitamin D Toxicity	11
2.2. Zinc.....	12
2.2.1. Sources of Zinc	12
2.2.2. Zinc metabolism	13
2.2.3. Physiological Effect of Zinc	14
2.2.4. Zinc Deficiency	15
2.2.5. Zinc Toxicity	17
2.2.6. Zinc and Anxiety	17
2.3. Anxiety and Exploratory Activity Models Used in Experimental Animals...	17
2.3.1. Anxiety.....	17
2.3.2 Exploratory Behavior Models	18
2.3.3. Models for Anxiety in Experimental Animals	19
2.3.3.1. Plus-Maze Test	20
2.3.3.1.1. Elevated Plus-Maze Test (EPM)	20
2.3.3.1.2 Elevated T-maze Test.....	21
2.3.3.1.3. Elevated Zero-Maze Test.....	22
2.3.3.2. Open Field Exploration Test:.....	23

3. MATERIALS AND METHODS.....	24
3.1. Experimental Animal and Laboratory Conditions	24
3.2. Experimental Groups and Applied substances	24
3.3. Open Field Test Apparatus	27
3.4. Elevated Plus Maze Apparatus.....	28
3.5. Statistical Evaluation	29
4. RESULTS	30
5. DISCUSSION.....	34
6. CONCLUSION.....	37
REFERENCES.....	38
APPENDICES	
APPENDIX 1: PROJECT APPROVAL	
APPENDIX 2: ETHICAL APPROVAL	

LIST OF TABLES

page

Table 2. 1. Classification of animal models of anxiety in terms of unconditioned reaction tests and conditioned response.....	19
Table 4.1. Open field test finding of groups.....	30
Table 4.2. Maximum-minimum values of groups for Open field test.....	31
Table 4.3. Elevated plus maze findings for groups.....	32
Table 4.4. Maximum-minimum value of groups for Elevated plus maze.....	33

LIST OF FIGURES

	page
Figure 2.1. Forms of vitamin D (vitamin D2 and vitamin D3) (8).....	6
Figure 2.2. Diagram illustrating different sources and forms of vitamin D (24)	7
Figure 2.3. Scheme for zinc distribution in the body (60).	14
Figure 2.4 Comparison of the effects of zinc intoxication versus deficiency (70)	16
Figure 2.5. Elevated Plus Maze (89)	20
Figure 2.6 Elevated T-maze (91).....	21
Figure 2.7 The Elevated Zero Maze (89).....	22
Figure 2.8 Open Field Test (97).....	23
Figure 3.1 Administration of oral vitamin D doses and zinc sulfate doses.....	25
Figure 3.2 Schedule of Study.....	26
Figure 3.3. Open Field Test Apparatus.....	27
Figure 3.4. Elevated Plus Maze Apparatus.....	28

LIST OF SYMBOLS AND ABBREVIATIONS

ECA	entry number of closed arm
EOA	entry number of open arm
EPM	Elevated Plus-Maze
ETM	Elevated T maze
EZM	Elevated zero maze
FGF23	fibroblast growth factor 23
IU	international Unit
ND	number of defecation
NEC	number of entries to the central area
NR	number of rearing
OFT	Open field test
PTH	parathyroid hormone
STC	spent time in the central area
STCA	spent time in closed arm
STOA	spent time in open arm
VDBP	vitamin D binding protein
VDI	vitamin D intoxication
VDR	vitamin D receptor
Zip	zinc Irt-related proteins
Zn	zinc
ZnT	zinc transporter
7-DHC	7-dehydrocholesterol
25(OH)D	25-hydroxyvitamin D
1.25(OH)2D	1,25 dihydroxyvitamin D
24.25 (OH) 2D	24,25-dihydroxyvitamin D

1. INTRODUCTION

Initially, vitamin D was considered as a vitamin when it was discovered in the early twentieth century; however, it is now regarded as a prohormone. The human body can produce vitamin D, a distinguishing feature of this nutrient (1). It forms in the skin as a result of direct exposure to sunlight. This vitamin's dietary sources are limited, and its only rich dietary source is cod liver oil. It is also present in butter, cream, yoke, seafood, and liver in limited and different amounts (2). Vitamin D produces in the body when skin exposes to UVB radiation mainly because 7-dehydrocholesterol (7-DHC) photolyzes into pre-vitamin D₃. It is then subjected to thermally-mediated isomerization. Vitamin D₃ undergoes two consecutive hydroxylation after entry into the bloodstream to form the primary circulating source of vitamin 1,25-dihydroxy vitamin D (25(OH)D), vitamin D, 1,25-dihydroxy vitamin D (1,25(OH)₂D) D hormonal type (3). Vitamin D hydroxylates to 25(OH)D in the liver and kidney by 1- α -hydroxylase to produce the active hormone, resulting in 1,25(OH)₂D. It is a main circulating form of vitamin D, preceded by the hormonal vitamin D form (also called calcitriol).

Contrary to 25(OH)D levels, which significantly change because of food and sun exposure, 1,25(OH)₂D concentrations are tightly regulated. Parathyroid hormone (PTH) stimulates renal synthesis, and 1,25(OH)₂D controls it. Phosphate, calcium, and fibroblast growth factor 23 (FGF23) inhibit renal synthesis (4). The active metabolite performs through binding to its intra nuclear receptor that causes changes in the transcription of a wide variety of genes. Both are associated with calcium homeostasis and other functions (5).

It is already established that Vitamin D strongly influences the release and storage of calcium reserves and the calcium ions concentration in extracellular fluid. It also improves calcium absorption in the intestines and calcium secretion from the skeletal tissue. The primary regulator of calcium is the parathyroid hormone, which helps metabolize and synthesize Vitamin D. When the parathyroid gland senses low circulating calcium levels, it releases the parathyroid hormone (PTH) to raise circulating 1,25-dihydroxy vitamin D. It increases the phosphate release and calcium release in the small intestine that increases absorption of these minerals. It sends feedback to the parathyroid before chief cells identify elevated serum

calcium levels (6). Adequate serum 1,25(OH)₂D amounts are sufficient to assure the calcium supply needed for bone remodeling and osteoblast actions, which VDR mediates, such as osteonectin, osteopontin, and osteocalcin synthesis (5). Vitamin D plays a vital role in restoring the homeostasis of different biological processes. It also helps neuromuscular skeletal, immune, cardiovascular, and cutaneous systems. Also, it has anti-inflammatory, antibacterial, and tumor-suppressive properties (7). Skeletal metabolism and calcium homeostasis are critical vitamin D functions. Vitamin D deficiency may lead to skeletal issues, such as growth retardation and childhood rickets (8). In adults, vitamin D deficiency causes osteopenia, muscle weakness fracture, and osteoporosis (9).

Vitamin D may be harmful in high doses and has sporadic toxicity records. Hypervitaminosis D is characterized by elevated 25hydroxyvitamin D(25[OH]D) levels in the blood and hypercalcemia. Prolonged hypervitaminosis D can accumulate calcium in soft tissues (10). The calcification of blood vessels causes the hardening of the arteries. Ca²⁺ accumulation, especially in the kidneys and heart, causes anorexia, muscular weakness, vomiting, growth retardation, kidney stones, polyuria, and nephrolithiasis. Excessive vitamin D may cause depression of the CNS, which may further lead to coma and death if the case is severe (11).

Zinc, an essential trace element, plays a vital role in preserving human health throughout the human lifespan. In trace metal abundance, zinc is the second most abundant metal, and its prevalence is a result of its significant functions (12). Zinc is found as Zn²⁺ in the body in its free form, but it is connected to different systems and provides these structures with attributes that enable them to fulfill their responsibilities. Zinc is essential for forming bones, muscles, tissues, and cartilages. It enhances osteoblast function and reduces the activity of osteoclasts in bone cells (13). Zinc is essential in every cell and is vital for diverse processes, such as DNA synthesis, gene expression, hormone storage, enzymatic events, memory, neurotransmission, and cell repair (14). Zinc increases osteoblast activity and reduces the action of the bone cells of osteoclasts. Zinc can enhance cell proliferation, alkaline phosphatase activity, and osteogenic effects in osteoblastic cells. Also, it functions within the capacities of neuromodulators and neurotransmitters. Released from the presynaptic zone, it works in the region of neurons in the postsynaptic area; therefore, zinc is also linked to learning and memory.

In contrast to the nervous system, zinc deficiency results in poor learning, memory loss, and depression (13). Anti-oxidative protection is one of the most significant functions of zinc. For proper hormonal balance, zinc ions are essential and thus exert influence on the whole organism. Zinc ions are hormone components (such as thymus hormones). They control the synthesis of hormones and ensure that the hormone trails are appropriate (i.e., insulin) (15). Zinc also plays a significant part in diet regulation. Less appetite and body mass are also linked to minor zinc deficiency in humans and animals. Such characteristics can be observed in cases of zinc deficiency and patients with anorexia nervosa. Several clinical symptoms are associated with zinc deficiency itself. Marginal zinc deficiency, for example, can lead to depressed immunity, memory impairment, neurosensory disorders such as night blindness, a decrease in smell and taste, and a reduction in the production of male sperm. It results in a severely impaired immune system, causing skin rashes, increased frequency of infections, diarrhea, hair loss, and mental disorders (16). Although zinc is an essential element for a healthy life, it may be harmful to take excessive quantities of zinc. Copper and iron absorption are suppressed by excessive zinc absorption. Acute toxic effects of high zinc consumption include vomiting, nausea, weight loss, diarrhea, headaches, and stomach colic. A case study has shown extreme nausea and vomiting within 30 minutes after consuming 4 grams of zinc gluconate (17).

The commonly used apparatus is elevated plus maze (EPM), which helps study rodent behavior in studies that require assessment of anxiety or anti-anxiety after consuming a pharmacological drug (18).

The open-field test (OFT) is a commonly used method to examine rodent behavior, which assesses different pharmacological agents' effects on loco motor activity (19).

Several studies shed light on the effects of vitamin D and zinc deficiencies but only a few have investigated the link between impaired animal behavior and zinc deficiency. Zinc deficiency can lead to anxiety, irritability, depression, inappropriate social behavior, and emotional instability (20).

So far, no previous study has described the effects of high doses of vitamin D and zinc on anxiety and exploratory behavior based on elevated plus maze and open field tests in rats.

This study was aimed to investigate the effects of high vitamin D and zinc doses on rats' exploratory activity and anxiety.



2. GENERAL INFORMATION

2.1. Vitamin D

2.1.1. Discovery of Vitamin D:

The credit for the discovery of vitamin D goes to McCollum, who discovered it at the beginning of the twentieth century in the year 1922. Cod liver oil aeration and heating are effective methods to prevent the destruction of vitamin D synthesis. After the treatment, cod liver oil retained its ability to treat rickets. It was discovered that Professor McCollum was correct in assuming the activity in curing rickets was attributed to a newly discovered vitamin named "Vitamin D." This was how vitamin D became known as an essential nutrient. McCollum and Mellanby conducted experiments in 1922 (21), which resulted in the discovery of vitamin D, and they explored the influence of ultraviolet radiation on the skin. The researchers described the physiological actions that resulted in a healthy skeleton besides other benefits (22).

2.1.2. Forms and Sources of Vitamin D:

Since vitamin D is a prohormone steroid and a lipophilic vitamin, which affects the endocrine, paracrine and autocrine systems. Two chemically different forms of vitamin D were initially discovered: Vitamin D2 (Ergocalciferol) and Vitamin D3 (cholecalciferol), both forms can be synthesized non-enzymatically from the precursor 7-dehydrocholesterol (7-DHC) in the human skin if it is exposed to UVB radiation. Besides, it is found in a few animal-derived foods. Plants also produce Vitamin D2 when ergosterol is exposed to UVB radiation, which mostly happens in yeast and fungus. It is possible to take either D2 or D3 through diet or supplement, but it must be remembered that vitamin D3 is created in animal and human skin when the skin comes in contact with UVB radiation. There is a difference between the chemical structures of vitamins D2 and D3. The side-chain structure of vitamin D3 has an extra double bond, as Figure2.1 shows.

According to research, vitamins D2 and D3 are functionally similar. Although vitamin D2 is less stable than D3, their differences do not affect metabolism. Both vitamins D2 and D3 act as pro-hormones during metabolism, and regardless of the source, they go through the same metabolic process.

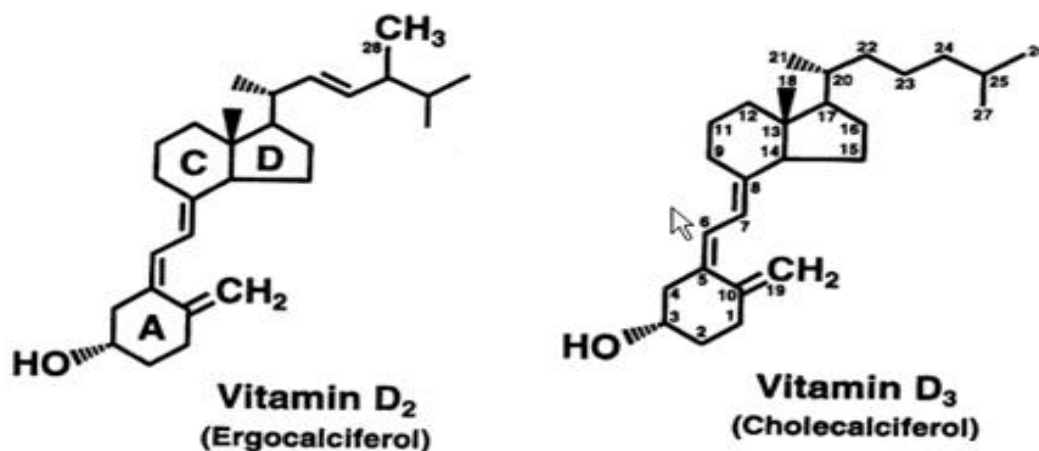


Figure 2.1. Forms of vitamin D (vitamin D2 and vitamin D3) (8)

So far, only three sources of vitamin D are known: diet, sunlight, and supplement. Vitamin D2 is plant-derived to be synthesized in yeast, fungi, and plants. Vitamin D3 is animal-derived (cholecalciferol) that can be non-enzymatically synthesized from the precursor 7-dehydrocholesterol (7-DHC), and it exists in fish, eggs, and meat, which are its nutritional sources in the natural forms (23). Sunlight delivers a single form of vitamin D (D3), but the dietary sources provide both forms, as Figure 2.2 (8) indicates, but many experts consider both forms equivalent and interchangeable (24). Exogenous vitamin D form is packaged in chylomicrons, which are transferred to the liver. The primary vitamin D source (up to 95%) is ultraviolet B radiation. Vitamin D3 endogenously synthesizes in the skin. When human skin exposes to UVB radiation, 7-dehydrocholesterol (pro vitamin D3) converts into pre-vitamin D3 (also called calciferol). It further converts into vitamin D3 through thermal isomerization of the epidermis. As mentioned earlier, vitamin D3 produces in the skin; so its quantity depends on the quality and amount of UVB radiation that reaches the dermis. Vitamin D production also depends on 7-dehydrocholesterol availability and skin characteristics (25). Research shows that the efficiency of each wavelength to synthesize vitamin D in the skin should be within the median wavelength range of 290-315nm. 15-20-minute whole-body exposure to UVB radiation produces approximately 250µm vitamin D (equivalent to 10,000 IU) (24).

UVB rays photoisomerize 7DHC into pre-vitamin D3 within the skin cells. And then, pre-vitamin D3 heat-isomerizes and converts into vitamin D3 in the skin, which takes several hours. The vitamin synthesis effectiveness depends on the skin condition and age. Moreover, the solar zenith angle also controls the availability of UVB. Thus season, latitude, time of day, sunscreen use, skin pigmentation, and lifestyle are the primary determinants of the amount of vitamin D produced in the body (26).

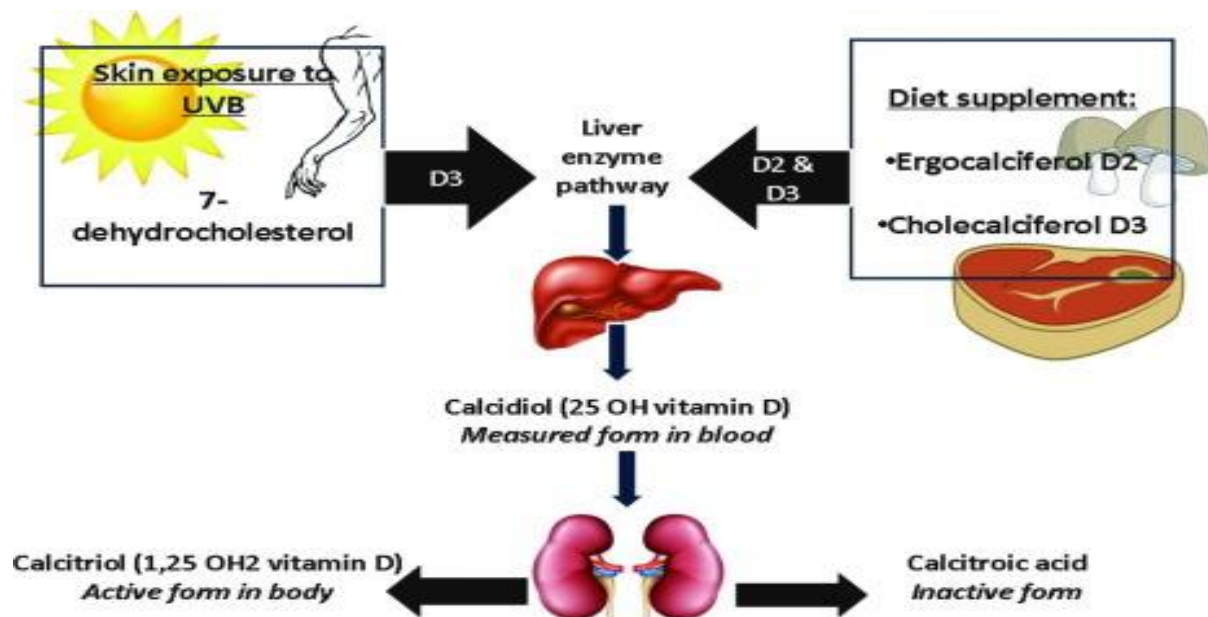


Figure 2.2. Diagram illustrating different sources and forms of vitamin D (24)

2.1.3. Vitamin D Production and Metabolism:

It is typically produced in the skin when an active photolytic process occurs, making pre-vitamin D using a derivative of cholesterol called 7-dehydrocholesterol. It slowly isomerizes into vitamin D. Vitamin D₃ is the natural vitamin D form, which releases in the skin, but vitamin D₂ forms through ergo sterol irradiation. It is present in plankton in modest levels under normal conditions and utilized to produce vitamin D₂ through the mold ergot (containing up to 2% ergo sterol). Now is the right time to get away from the concept that vitamin D is a vitamin. It functions more like a hormone. It is significant for everybody in all stages of life. Vitamin D is required for bone growth and various other physiologic processes. If it is used in appropriate quantities, it may be helpful to prevent several degenerative disorders, and in some cases, it also acts as an anticancer agent (27). First, the liver hydroxylates vitamin D into calcitriol and then secretes it into the blood plasma. The calcitriol gets bound to vitamin D binding protein (DBP). It circulates in the blood (28), where it acts as a reservoir to hydroxylate 1,25-dihydroxy vitamin D or 24,25-dihydroxy vitamin D (24,25(OH)₂D), which forms physiologically active Vitamin D. The enzyme 1 α -hydroxylase (CYP27B1) transforms 25(OH) D into 1,25 (OH) ₂D. Another enzyme 24-hydroxylase (CYP24), converts 25 (OH) ₂D into 24,25(OH)₂D in the extra-renal tissues and kidneys. 1,25(OH)₂D is the endocrine modulator for calcium and phosphate homeostasis in the kidney. Kidneys secrete it into the lymph and blood and transfer it in the DBP-bound condition to tissues involved in Ca⁺² and P supply, including parathyroid glands, intestine, kidneys, and bones. As compared to 25 (OH) D, 1,25 (OH) ₂D has a substantially shorter life (4–6 hours) (29). 1,25 (OH) ₂D causes genetic and non-genetic responses in cells for Ca⁺², P homeostasis system, and several target tissues through interactions with vitamin D receptors (VDRs). Extrarenal cells and endocytic receptors exist in the kidneys, and 25(OH) D hydroxylation acts as an alternative to 24,25(OH) ₂D (30). It attaches to VDBP and has a high plasma level. Vitamin D intoxication prevention is the initial stage of the metabolic pathway which inactivates and degrades 25(OH)D. At this point, this metabolite starts its functional role, but 1,25 (OH) ₂D is the primary metabolite, which is primarily responsible for the biological effects of vitamin D (29). When the converted vitamin D generates and reaches the liver and bloodstream, it links to a vitamin D protein called DBP. The liver produces DBP to vitamin D and metabolites it. The 25(OH)D absorption from food

moves straight to the lymph or portal vein, and then carried to the liver. In the blood, DBP availability is 1000 times more than the need (31).

2.1.4. Physiological Effects of Vitamin D:

Vitamin D is significant for preserving extracellular calcium ion levels in the body. For proper functioning, many neuromuscular tasks and metabolic processes require extracellular calcium. It affects the calcium levels by regulating calcium absorption in the intestine and directly affecting bone and secretion of parathyroid hormone (PTH) (32). Vitamin D 1,25(OH)₂D binds to the retinoid X receptor and nuclear vitamin D receptor (VDR) to regulate gene transcription (33). There is a single vitamin D receptor responsible for vitamin D hormone functions. It is found in enterocytes, renal tubular cells, and osteoblasts. Recently, it was discovered that cells and tissues, which do not regulate calcium and phosphate metabolism, have 1,25(OH)₂D₃ receptors. It involves parathyroid gland cells, the ovaries, the colon, promyelocytes, the pituitary gland, lymphocytes, and skin keratinocytes. Studies show that vitamin D insufficiency repercussions extend beyond bone metabolism (27). Vitamin D maintains bone metabolism, the development and division of cells, and calcium and phosphorus homeostasis. The VDR is also found in the nuclei of various other tissues, which require calcium and phosphate metabolism regulation. Both vitamin D and 1,25(OH)₂D regulate the immune system. They are also clearly identified in activated immune system T cells, epidermal keratinocytes, monocytes, antigen-presenting cells, and macrophages (34). Vitamin D is essential in various biological systems, such as skeletal, neuromuscular, cardiovascular, immune, and cutaneous systems. Moreover, vitamin D also has tumor-suppressive, antibacterial, and anti-inflammatory properties (7). Vitamin D may also help prevent diabetes because of the presence of VDR in pancreatic beta cells and calcitriol's stimulatory action on insulin production. It reduces insulin resistance in muscles and inflammation associated with insulin resistance. The presence of 1-hydroxylase and VDR in the brain activates brain development and physiological brain processes. Vitamin D deficiency is linked to different disorders, including metabolic syndromes, infections, mood disorders (anxiety/depression), and Alzheimer's disease (35).

2.1.5. Deficiency and Resistance of Vitamin D:

Vitamin D deficiency or resistance has been a problem since the early nineteenth century. Even now, it continues to be a major public health concern worldwide despite technological advances and lifestyle changes. Low 25(OH)D levels mean vitamin D deficiency that affects all age groups and races. It does not just affect children, as previously assumed. Vitamin D deficiency decreases calcium and phosphorus absorption in the gut during the early vitamin D deficient stages. Many studies have found that vitamin D deficiency/insufficiency is linked to several chronic and acute illnesses. Hypovitaminosis D reduces when there is less sunshine exposure, which is a primary cause of this condition in babies and children because it causes rickets and growth retardation. Vitamin D insufficiency in adults and older people may result in osteomalacia linked with deficient osteoid mineralization and expanded osteoid seams, increasing the risk of bone fractures (36). Vitamin D deficiency is also linked to hip fractures and pathogenesis of osteoporosis that results in secondary hyperparathyroidism, increased cortical bone loss, and decreased bone mineralization (32). Vitamin D deficiency has long been related to muscle fatigue in the limbs because skeletal muscles contain a vitamin D receptor. It also results in hypocalcemic convulsion and muscle weaknesses in the respiratory system, limbs, and heart, mainly because of skeletal mineralization defects. Serious mineralization issues further lead to increased body sway and a high risk of falling. In adults, vitamin D deficiency can result in skeletal mineralization issues, which provide little structural protection for the periosteal coating. Different conditions, including mental diseases, depression, cancer, type 2 diabetes, and multiple sclerosis, are linked with vitamin D deficiency. Vitamin D deficiency may lead to a higher preeclampsia risk during pregnancy (9).

Several studies have also found a link between vitamin D levels and depression, bipolar disorder, and schizophrenia. The few treatment trials on the effect of vitamin D supplementation on mood and psychiatric disorders have provided mixed results, probably due to the fact that most have used low doses of vitamin D. Vitamin D enables neuronal receptors in regions involved with behavior control, encourages neurotrophic release and protects the brain with buffering anti-oxidant and anti-apoptotic defenses against vascular injury. As a result, it appears increasingly likely that vitamin D influences cognition, mood, and behavior by acting directly on the brain and indirectly via multiple physiologic pathways (37).

2.1.6. Vitamin D Toxicity:

Toxic vitamin D levels do not occur because of prolonged sun exposure. Vitamin D intoxication (VDI) arises when healthcare specialists recommend large vitamin D dosages without regularly monitoring vitamin D levels.

Hypervitaminosis D differs from vitamin D intoxication (VDI). Serum levels more than 375 nmol /L (or 150ng/ml) are considered as the case of VDI. Higher vitamin D levels in the blood (more than 250 nmol/L or 100 ng/ml) are regarded as hypervitaminosis (38). Excessive vitamin D consumption for weeks or months results in severe toxicity in animals and humans (11). Hydroxyvitamin D (25OHD) levels increase, leading to hypercalcemia. Long-term hypervitaminosis D leads to calcium accumulation in soft tissues, which leads to signs of clinical toxicity and imbalance of bone metabolism and calcium homeostasis (39). During early vitamin D toxicity stages, gastrointestinal disorders occur, including vomiting, nausea, diarrhea, and decreased appetite. Other symptoms include a racing heartbeat, drowsiness, joint pain, and muscular weakness, which means that artery hardening takes place because of calcification of tissues and blood vessels, nephrolithiasis (Ca²⁺ deposition in kidneys), CNS depression, growth retardation, polyuria, itching, and bone pain which may lead to coma or even death (11).

2.2. Zinc

Raulin, in 1869, discovered the importance of zinc in biological systems for the first time by studies of *Aspergillus Niger* growth (40). For the first time, zinc was revealed as necessary for the growth of rats in 1933. Human experiments, conducted three decades ago in the Middle East, proved that it was also true for humans (40). The first human zinc deficiency was discovered in 1963 (41). Zinc is an essential mineral added to most multivitamins because it performs several physiological roles: antioxidant, ant apoptotic, anti-inflammatory, and neuromodulator (42). Zinc is present in the body in its free form as Zn^{+2} , but by binding to different structures, it endows these structures with properties that allow them to perform their functions (13). It functions as a support system for proteins, and it has approximately 300 enzymatic processes. Zinc performs numerous bioactive roles, including its transportation across the biological membrane. It requires several specific systems. Zinc transport proteins are necessary for physiological uses of zinc, specifically Zn transporter (ZnT) and Zrt- and Irt-related proteins. Zinc contributes to several physiological/cellular functions (immune, reproductive, endocrine, neuronal, and skeletal systems) by controlling zinc homeostasis (43). A study shows its importance for various physical functions, including reproduction (44).

2.2.1. Sources of Zinc:

Zinc is a trace element that performs structural, regulation, and catalytic activities needed by over 200 metalloenzymes involved in many biochemical pathways. Zinc is found in many legumes, animal products, dairy products, and whole grains (45). Oysters are packed with more zinc than any other food, and other major zinc sources include red meat, specifically lamb, liver, and beef, which have very high zinc contents. Other good zinc sources are beans, seeds, and berries. Other zinc sources are cereals, pumpkin seeds, almonds, mushrooms, grains, and sunflower seeds (46). In plants, zinc varies because that depends on the quantities of zinc in the soil. Plant-based foods, which contain the most zinc, include wheat (bran) and several seeds, including poppy, sesame, mustard, and celery (47). Zinc is normally added to various drugs, including the whole drug category branded as homeopathic medicines and the over-the-counter drug range manufactured to prevent and treat diseases. Various zinc forms are added in supplements, such as zinc gluconate, zinc sulfate, and zinc acetate. The quantity of elemental zinc varies in supplements based on the type of supplement. Scientists have still not released a

clear guideline regarding the variations in different zinc types' bioavailability, absorption, or tolerability (48). In humans, normal zinc serum concentrations range is 60-110 mg/dL (49).

2.2.2. Zinc metabolism:

During digestion, zinc is absorbed from the food in the form of free ions that bind to endogenously-secreted exogenous substances in the intestinal lumen, leading to trans-cellular absorption in the duodenum's distal part, the beginning of the jejunum (50). Zinc transfer has flow dynamics that transfer into the enterocyte. They indicate that a particular transport mechanism exists. Zinc may also be absorbed through a passive route in high intake levels. The zinc transporter proteins, such as protein-1 (ZnTP-1), can promote zinc's passage in the enterocyte's basolateral membrane and into the portal circulation system (51). The portal system facilitates direct zinc transport from the gut to the liver and is rapidly absorbed and released into the circulatory system (52). Approximately 70% of zinc circulation is linked to albumin, so the variables, which affect albumin concentration, have a secondary influence on the zinc levels in the serum. In pregnancy, plasma volume elevates, and the zinc content in the serum reduces in concert with albumin. Zinc levels in the blood decrease due to hypoalbuminemia in the conditions of aging and protein-energy malnutrition. In the blood, zinc levels are influenced by factors that influence its absorption through tissues, such as traumatic injury and infection, which increase cortisol and cytokine secretion (for example, interleukin 6). Serum zinc constitutes only 0.1% of the total zinc in the body. Still, zinc circulation is rapidly replenished in the body (approximately 150 times/day) for satisfying the needs of the body's tissues. In 24 hours, the bloodstream and other tissues exchange almost one-fourth to one-third of the total zinc present in the body (53). The digestive system consumes nearly half of the body's total zinc. After a meal, the pancreas secretes significant zinc quantities (3–5mg) in the gut, and besides, zinc is found in substantial levels in the intestinal and biliary secretions (54). The total endogenous gastrointestinal zinc secretion can exceed the dietary zinc intake, but it is essential to understand that zinc secretion into the gut largely reabsorbs. This process is necessary to improve a healthy zinc balance. The mechanisms that eliminate zinc include urine, which accounts for 15% of the total zinc losses, epithelial cell desquamation, sperm, sweat, menstruation blood, and hair. Menstruation blood accounts for almost 17% of zinc excretion (55). The intrinsic zinc excreted through feces is less than 1 mg/d (56). Generally,

the endogenous zinc, which is eliminated in the stool, increases because the total zinc absorption increases. The endogenous zinc excreted in the stool decreases if either zinc demands increase or dietary zinc intake decreases due to breastfeeding or growing, respectively (57). When the amount of dietary zinc decreases, the person enters a period of negative zinc balance and remains in it until regaining the minimum levels of zinc required to balance the zinc levels (53). In adults, the total body zinc ranges from 2–3 g. Almost 60% of zinc exists in the skeletal muscle, 5% in the liver and skin, and 30% in the bone. The remaining 2–3% exists in other tissues (58) (Fig. 3). It is important to note that serum zinc accounts for only 0.1% zinc, 20% is closely bound to α 2-macroglobulin, and 80% is loosely bound to albumin (59).

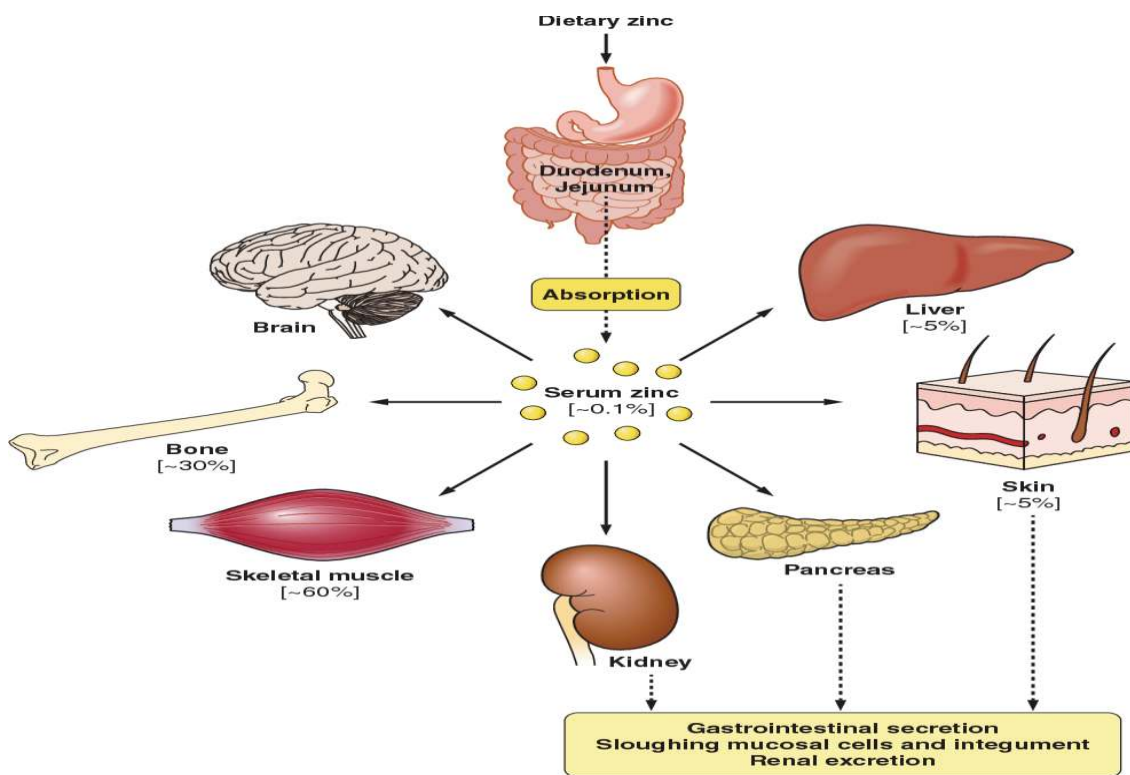


Figure 2.3. Scheme for zinc distribution in the body (60).

2.2.3. Physiological Effect of Zinc:

Zinc is a trace dietary element, an intracellular metal that involves several metabolic mechanisms, acting as a catalyst, structural component, or regulatory ion (60). The biological importance of zinc lies within the homeostatic systems that regulate absorption and cellular uptake diffusion in the intracellular compartments, macromolecules, and excretion. The mentioned biochemical processes help maintain a broad range of functions that depend on zinc

proteins and enzymes, ranging from involvement in intermediary metabolisms, such as the membrane structure of platelets and erythrocytes. In this context, it is important to know that homeostatic mechanisms affect the gastrointestinal tracts by zinc absorption in the skin and kidney absorption besides zinc excretion and transport in the plasma (61). Zinc regulates many cell-signaling pathways by modulating kinase, phosphorylase activity, neurobehavioral development, and reproductive function (62). Zinc is essential for insulin release, synthesis, secretion, and storage. If zinc is added to the human diet, it may help a person get the required insulin because it makes the insulin receptor tyrosine kinase work more efficiently. Low zinc may lead to diabetes (types I and II). Zinc supplements may enhance insulin secretion (63). Zinc is also essential in the regulation of nutrition. It helps the thyroid hormone thyroxine transform into triiodothyronine in animals and humans, so insufficient zinc can lead to hypothyroidism. It is a common endocrine disease because of decreased thyroid hormone production.

In animals, zinc deficiency reduces thymulin activity. Thymic epithelial cells produce nonapeptide thymulin, which requires zinc for biological action. It is needed for mature T-helper cells, which result in lower T-helper 1 (Th-1) cytokine levels in zinc-deficient people (64). Moreover, it significantly affects the inflammatory response, specifically in the final part of this process. Inflammatory processes, specifically sepsis, involve alteration in homeostasis, zinc metabolism, and zinc transfer from one organ to another (65). Thus, zinc also controls sex hormones in males and females, enhancing oxytocin stability. Zinc is essential for oxytocin that attaches to its receptor on cells and has a vital function in the brain during all the stages of life (66). Zinc controls brain excitability because zinc deposits in some synaptic vesicles by glutamatergic neurons. It plays a vital role in synaptic plasticity, which leads to improved learning. It can act as a neurotoxic even though zinc homeostasis is necessary for a healthy brain and the whole nervous system (67).

2.2.4. Zinc Deficiency:

According to estimates, nearly two billion individuals living in the developing world are zinc deficient. Lack of appetite, reduced immunological function, and growth retardation are significant consequences of zinc deficiency. Men have diarrhea, hair loss, hypogonadism, delayed sexual development, and eye and skin diseases in severe cases. Other symptoms

include delayed wound healing, weight loss, cognitive impairment, and taste disturbances (17). Zinc insufficiency occurs because of inadequate zinc intake/absorption, zinc losses, or increased zinc need (68, 69). Clinical zinc deficiency ranges from mild to severe symptoms, as Figure 2.4 shows (70).

Zinc deficiency may cause acrodermatitis, malabsorption, chronic liver disease, enteropathy, sickle cell anemia, glomerulonephritis, cancers, diabetes, and other chronic diseases (71). It increases infection and diarrhea in children, leading to approximately 800,000 deaths every year. In severe cases, zinc deficiency impairs immune function, and in mild to moderate cases of zinc deficiency, natural killer cell activity, complement activity, and macrophage and neutrophil functions are affected (17). Moreover, people with reduced zinc levels often report reduced response to lymphocyte proliferation and mitogens besides other adverse immune changes. Still, the mentioned conditions can be corrected by appropriate zinc supplements (72,73). If zinc deficiency occurs during the stages of brain growth, it results in persistent behavior and cognitive learning issues. In other words, zinc deficiency leads to disturbing behavior in adults, contributing to the age-dependent reduction in cognitive functions. Zinc homeostasis changes are extensively studied concerning brain disorders, but zinc deficiency leads to several conditions affecting all the body systems (16).

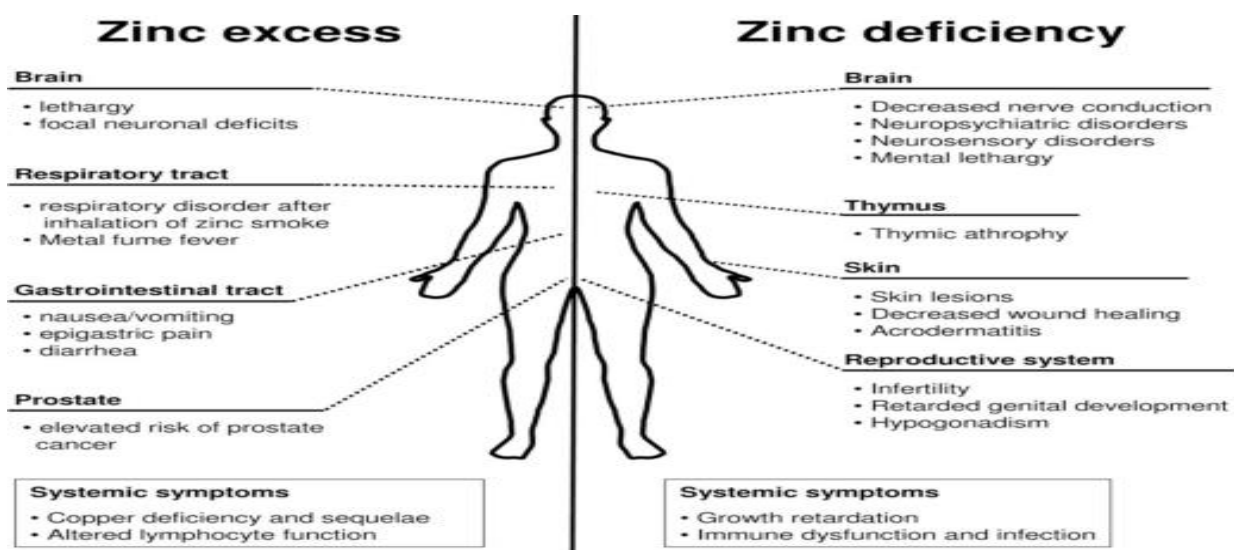


Figure 2.4. Comparison of the effects of zinc intoxication versus deficiency (70)

2.2.5. Zinc Toxicity:

Zinc enters the human body through three primary routes: inhalation, dermal exposure, and ingestion (74). Each type of exposure affects different body parts (Figure 2.4). Because zinc is an essential trace element, oral absorption of small doses is needed for survival. Excessive zinc absorption reduces the absorption of copper with iron. It may cause vomiting, nausea, stomach discomfort, lack of appetite, diarrhea, or headache in case of overdose (75).

Approximately 225–450 mg zinc overdose in adult human bodies may result in immediate vomiting. Chronic zinc overdose in the 100–300 mg zinc/day range for individuals may lead to copper deficiency (76) and variations in serum lipoprotein levels and immune response. Some mentioned side effects also occur when doses are lower (for example, 50 mg zinc/day), but in this case, the data are conflicting and require confirmation (77,78).

2.2.6. Zinc and Anxiety:

The medical community has recognized zinc as an essential trace element as one of the cures of depression and anxiety. It is needed to perform many body functions, such as transcription, protein biosynthesis, and repeatability, to affect cell differentiation and reproduction (79). Zinc deficiency has a significant influence on the development process of depression, and it has been observed that zinc restoration reverses depression symptoms. Research studies show that zinc deficiency may be a possible reason behind anxiogenic-like behavior, so it can be one of the reasons behind the depressive disorder. It was noted that zinc deficiency increases anxiety-like behaviors in rats (80).

2.3. Anxiety and Exploratory Activity Models Used in Experimental Animals:

2.3.1. Anxiety

Anxiety is an aversive emotional reaction to a threat or the chance of a threat; however, when this emotion is inappropriate, intense, and continuous and is not proportionate to the nature of the danger, it is described as pathological. Anxiety is generally accompanied by emotional stress in reaction to hazardous situations, including behavioral, expressive, and physiological aspects such as avoiding the risk source, elevating blood pressure, and

developing defensive postures. It is found that anxiety has a link with different psychiatric conditions, such as panic attacks, depression, general anxiety disorder, phobias, post-traumatic stress disorder, and obsessive-compulsive disorder (81,82).

In anxiety disorders, stress hormones, including cortisol and adrenaline, are linked to physical symptoms, and the mentioned chemicals affect almost every body part. Untreated anxiety problems lead to depression that causes hypertension, breathing difficulty, increased heart rate, sweating, shortness of breath, irritability, increased muscle tension, and lower intestinal blood flow that sometimes causes vomiting or headaches (83). After evaluating anxiety-related behavior in animals, it has been presumed that animal anxiety is similar to human anxiety. In rodents, different behavioral and physiological patterns indicate anxiety in the form of behavioral and peripheral alterations, which follow increased sympathetic nerve activity (83).

2.3.2 Exploratory Behavior Models:

When confronted with an unfamiliar area, animals frequently display behavior patterns broadly categorized as exploration, such as moving around the area, being drawn to novelty and touching or sniffing new products. Exploration may provide information regarding food, possible shelters, and mating chances to an animal. By proceeding and surviving in new surroundings, the chances of a risk or an attack from another animal increase for an animal.

Conflicting motivations affect exploratory behavior and urge an animal to investigate a harmful novel area for safety. Thus, curiosity and neophobia always affect an animal's behavior in an unknown habitat. It is essential to clarify that an animal's aversion to approaching a new object or place is called neophobia. Both neophilia and neophobia can be described as a wish to discover something despite fear of it. Behavioral tests are frequently conducted on mice and rat to assess the psychoactive medicines' anxiety-related effects. The most widely used exploratory behavior test is the open field test conducted on laboratory rats (84).

2.3.3. Models for Anxiety in Experimental Animals:

The animal models aim at "experimental preparations" in a species to understand the phenomena occurring in another species (termed as "paradigm"). Animal models are created in a non-human species to understand human pathology, physiology, or behaviors. The animal models of psychological disorders fall in one of the following two groups. The simplest models deal with psychotropic medication or other therapeutic evaluations, and it is valid for most cases designed to replicate a particular sign or symptom (behavioral similarity models).

On the other hand, "mechanistic" or "theory-driven" paradigms (according to McKinney's language) measure the etiology, genetic factors, and neurochemicals behind anxiety disorders (85). There are two types of animal models for anxiety: conditioned and unconditioned. (Table 1)

The first group shows the animal's conditioned responses to frequently painful and uncomfortable circumstances (for example, shocking their feet with an electric charge). In the second group, we can find ethological models involving the animal's natural or spontaneous responses (such as avoidance, flight, or freezing) causing discomfort and pain (for example, exposure to a new and luminous test room or a predator) (86).

Table 2. 1. Classification of animal models of anxiety in terms of unconditioned reaction tests and conditioned response

Conditioned responses	Unconditioned responses
Geller-Seifter conflict (GS)	Elevated plus maze (zero/T maze)
Vogel conflict Four-plate test (FPT)	Light/dark exploration (L/D)
Conditioned emotional response (CER)	Social interaction
Conditioned taste aversion (CTA)	Open field
Fear-potentiated startle	Ultrasonic vocalization (pain or separation)
Defensive burying	Fear/anxiety-defense test batteries
Active/passive avoidance	Staircase test Hole board
Electrical brain stimulation (dPAG)	Predator

2.3.3.1. PLUS-MAZE:

2.3.3.1.1. Elevated Plus-Maze Test (EPM):

EPM is a standard anxiety-related behaviour test initially designed for use with rats, but the recent list of animals includes guinea pigs, mice, hamsters, gerbils, and voles. 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 in 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 a paradigm of anxiety, which was conducted some 30 years (87). EPM has the shape of a cross or plus that has two open elevated arms facing each other, and they separate by two arms of the same size and a centre square (figure 2.5). In the test, a mouse or rat is placed in the middle area and then left to explore the maze for a set amount of time. The amount of time spent in the walled arms vs the amount of time spent in the open arms indicates worry or fear. The test is based on rodents' natural tendency to avoid open or elevated situations, which is balanced by their innate drive to investigate new areas. In theory, a less worried mouse will spend more time in the open, more exposed arms of the maze, whereas an anxious mouse will spend more time in the closed arms (88).



Figure 2.5. Elevated Plus Maze (89)

2.3.3.1.2 Elevated T-maze Test:

It is an anxiety measurement method in an elevated mechanism with two arms: an open arm and a closed one. T-maze consists of three identical plastic arms (50x12 cm) placed 50 cm above the ground. One of these arms is closed with 40 cm high plastic sidewalls and is set in a perpendicular position with respect to the other two open arms standing opposite to each other. Different sizes of T-maze are used for mice and rats. This ETM offers an effective model to measure two aversively motivated behaviors: one-way escape and inhibitory avoidance. They may be linked to innate fear (panic disorder, unconditioned fear) and anticipatory anxiety (conditioned fear) (90). The elevated T maze is shown in Figure 2.6 (91).

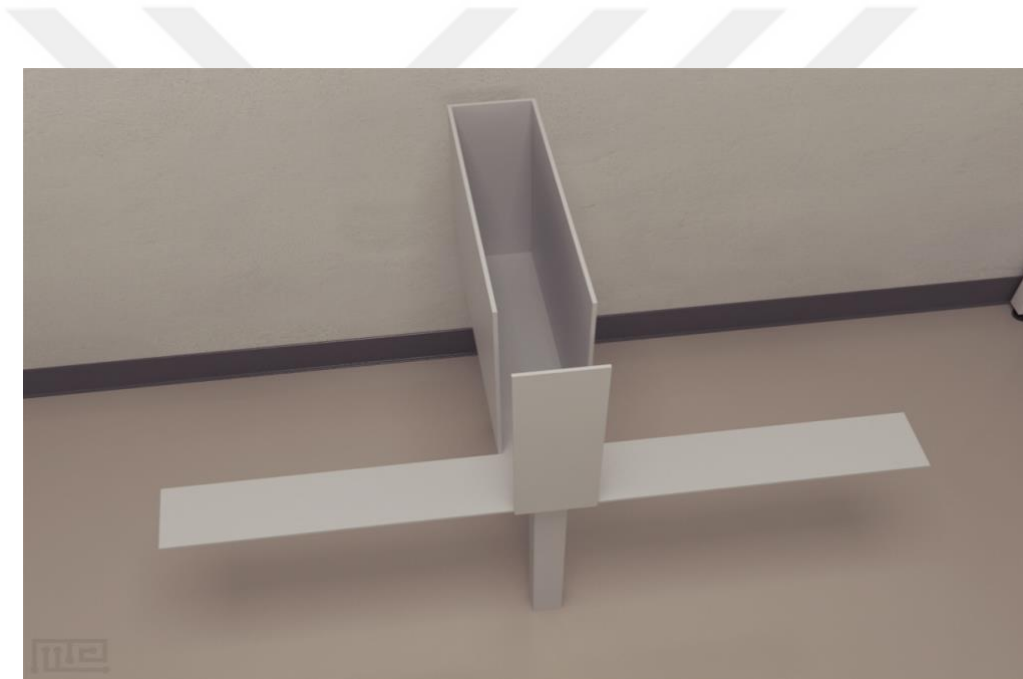


Figure 2.6. Elevated T-maze (91)

2.3.3.1.3. Elevated Zero-Maze Test:

The elevated Zero-maze or X-maze test is an anxiety model based on Montgomery's classic series of experiments. It is a recently developed plus-maze device. Like the elevated-plus maze, the conventional apparatus consists of a cross-shaped elevated platform with two opposing pairs of enclosed and open arms elevated 50 cm above the ground. The elevated zero maze can be used with a video tracking system. Anxiety-related behavior is assessed in this case by the degree to which the rodents avoid the maze's unenclosed areas (92). The elevated zero maze is shown in Figure 2.7 (89)



Figure 2.7. The Elevated Zero Maze (89)

2.3.3.2. Open Field Exploration Test:

The open-field test, developed by Calvin S. Hall, is an experimental test used in scientific research to assess general locomotor activity levels, anxiety, and readiness to explore in animals (typically rats) (93). The open-field test can be used to evaluate memory by assessing the animal's ability to recognize a stimulus or object. The novel object recognition test is another animal test that uses the same concept to assess memory. The open field is a surrounded by wall field with no way out. A grid and square crossings are commonly used to mark the field. To distinguish it from the other squares, the center of the field is marked with a distinct hue. Infrared beams or video cameras with related software can be employed in current open field devices to automate the assessment procedure (94). In this test, the number of entering the center square and rearing is counted (the frequency with which the rodent stands on its hind legs in the field). Unsupported raising and related behavior in which the animal's forepaws rest against the walls of the enclosure have different underlying genetic and neurological causes, and unsupported rearing may be a more direct indicator of fear (95). Animals such as rats and mice display a natural aversion to brightly lit open areas. Reduced anxiety leads to increased exploratory behavior. Anxiety causes decreased mobility and a tendency for staying near the field's walls (96).



Figure 2.8. Open Field Test (97)

3. MATERIALS AND METHODS

3.1. Experimental Animal and Laboratory Conditions:

We used 48 male eight weeks old Wistar rats for the research. Animals were obtained from Başkent University Experimental Research Center, and they were housed in standard cages where unlimited amounts of water and feed were available. The researcher conducted all the animal experiments in the premises of Animal Experiment Laboratory, Faculty of Medicine, Department of Physiology, Başkent University. After obtaining permission from the Animal Experiments Ethics Committee, Başkent University (20/15), the experiments were performed.

3.2. Experimental Groups and Applied substances:

The rats were divided into four groups in equal numbers (n = 12).

- Sham group (Group 1) [n=12]: Each rat took orally provided during the trial.
- Medium dose Vitamin D and Zinc group (Group 2) [n=12]: Each rat received an oral dose of vitamin D, and the quantity was 400 [IU]/day (98) for eight weeks and then ZnSO₄ dissolved in the serum form intraperitoneally in the doses of 30 mg/kg with oral doses of vitamin D for six days, as Figure 3.1 indicates.
- High dose Vitamin D and zinc group (Group 3) [n=12]: Each rat was orally provided 1000[IU]/day doses (99) for eight weeks, and then ZnSO₄ was given to them in the serum form intraperitoneally in 30 mg/kg doses with oral doses of vitamin D for six days, as Figure 3.1 shows.
- Zinc group (Group 4) [n=12]: Each rat was given only ZnSO₄ in the serum form in 30 mg/kg doses for six days (100).



Figure 3.1. Administration of oral vitamin D doses and zinc sulfate doses

Recommended levels of vitamins D3 per kg diet for rats is 300IU (101).

Vitamin D, which we used in the experiment, was in the form of 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 the fat obtained from the sheep's wool.

The substances were administered to the groups once a day for eight weeks and six days. At the end of this period, the rats are tested using an open field test (OFT) and elevated plus maze (EPM) .We tested them and noted readings.

The study's timetable is shown in Figure 3.2.

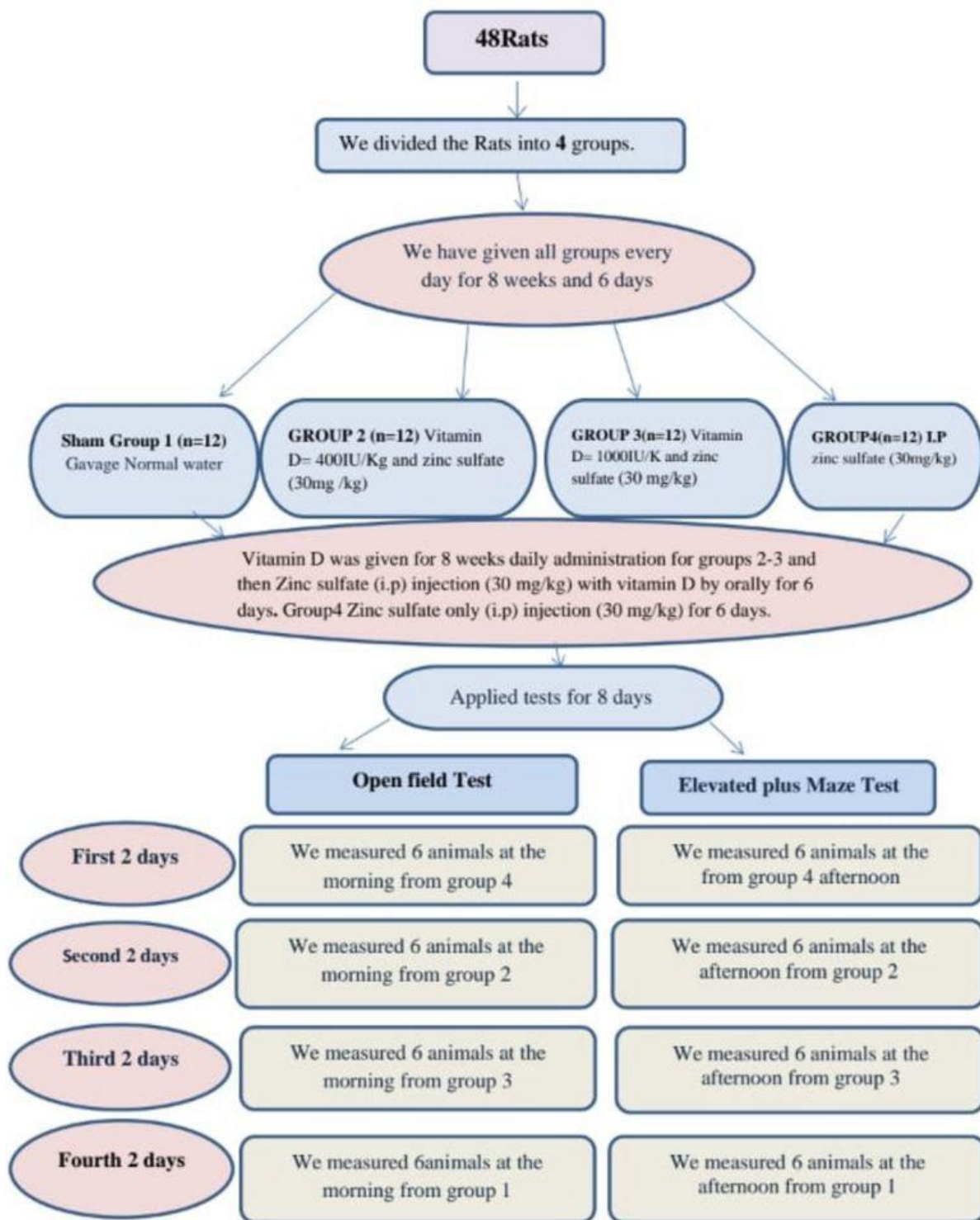


Figure 3.2. Schedule of Study

3.3. Open Field Test Apparatus:

Immediately after completing the vitamin D doses and zinc doses to rats [8 weeks six days], we conducted the OFT test. We started with the zinc group during the first two days, the medium-dose group in the second 2-day period, high-dose group in the third 2-day period, and the sham group in the fourth 2-day period. (OFT apparatus is mainly a 100×100×36cm³ wooden box and the testing period lasted for 10 minutes). As a part of this test, we placed individual rats in the corner of a square. We captured the activity using a video camera to score the following behavioral parameters for 10 min to determine the overall distance traveled, the number of defecations, and the number of the reared. After testing each rat for ten minutes, we carefully cleaned the field with alcohol (86), as Figure 3.3 indicates.



Figure 3.3. Open Field Test Apparatus

3.4. Elevated Plus Maze Apparatus:

We conducted the EPM test immediately after giving the vitamin D and zinc [8 weeks six days] in 8 days. We did it consecutively in the afternoon, starting with the zinc group in the first two days then the medium-dose group in the second two days. Then, high dose group in the third two days and the last sham group in the fourth two days. The EPM apparatus consists of two open arms (48 cm long, 10cm broad each) perpendicular to two open arms that have the same size, and 50 cm high walls enclosed them. Each open and closed arm is divided into three parts (16 × 10cm each) and a central open segment that connects the two arms. The rat was first placed in the main segment of the EPM, facing one of the open arms (18) of the experiment that we started after a 5-minute habituation period. During the 5-minute investigation, we recorded the rats' behavior for the number of open and closed segments each rat crossed. 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 time spent in open arms was calculated as follows: percent time= (time in open arms/ (time in open arms + time in closed arms) × 100). All tests can be recorded using a video camera, and we carefully cleaned the maze with alcohol (102), as Figure 4.3 shows.



Figure 3.4. 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

We started our study with 48 rat (12 rat per group). During the tenure of the experiment, a total of 5 of them died (2 from Group 2, 2 from Group 3, and 1 from Group 4).

4.1. Open Field Tests Results

Table 4.1. Open Field Test Finding of Group

	Group 1 ^a (n=12)	Group 2 ^b (n=12)	Group 3 ^c (n=12)	Group 4 ^d (n=12)	X ²	P
NEC	1.83 ± 0.71	0.25 ± 0.13 ^a	0.08 ± 0.08 ^a	0.5 ± 0.23	12.27	0.007
STC	6.25 ± 2.46	1.08 ± 0.57 ^a	0.25 ± 0.25 ^a	1.66 ± 0.83	11.13	0.011
STP	593.75 ± 2.46	498.91 ± 67.27	499.75± 67.38	548.5± 49.87	3.1	0.374
NR	11.83 ± 01.08	4.66 ± 0.86 ^a	3.83 ± 0.86 ^a	3.66 ± 0.65 ^a	23.93	0.000
ND	3.66 ± 0.41	1.08 ± 0.35 ^a	0.66 ± 0.41 ^a	1.58 ± 0.41 ^a	20.24	0.000

Group 1= Sham group (a), Group 2= Group receiving medium Vitamin D and Zinc doses (b), Group 3= Group receiving high doses of vitamin D and zinc (c), Group 4= Group receiving only zinc (d)

NEC: Number of entrances 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)

Table 4.2. Maximum-Minimum values of Groups for Open Field Test

	Group 1 (n=12)	Group 2 (n=12)	Group 3 (n=12)	Group 4 (n=12)
NEC	0.0- 9.0	0.0- 1.0	0.0- 1.0	0.0- 2.0
STC	0.0- 30.00	0.0- 5.00	0.0- 3.00	0.0- 8.0
STP	570.0- 600.0	0.0- 600.0	0.0- 600.0	0.0- 600.0
NR	7.0- 19.00	0.0- 8.00	0.0- 9.00	0.0- 7.00
ND	2.0- 7.0	0.0- 4.0	0.0- 5.0	0.0- 4.0

Group 1= Sham group, Group 2= Group receiving medium doses of Vitamin D and Zinc doses, Group 3= Group receiving high doses of vitamin D and zinc, Group 4= Group receiving only zinc.

NEC: Number of rat's entrance 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

There were significant differences among the group regarding open field test values except for STP (Table 4.1).

It was found that NEC values for Group 2 ($p<0.013$) and Group 3 ($p<0.003$), STC values for Group 2 ($p<0.039$), and Group 3 ($p<0.003$) were lower than Group 1.

NR values for Group 2 ($p<0.000$), Group 3 ($p<0.000$), and Group 4 ($p<0.000$) and ND values for Group 2 ($p<0.000$), Group 3 ($p<0.000$), and Group 4 ($p<0.004$) were lower the Group 1 values.

There was no statistical difference among groups for STP.

4.2. Elevated Plus-Maze Results

We showed mean $\pm$ SE of the elevated plus-maze test in Table 4.3 and maximum-minimum value in Table 4.4.

Table 4.3. Elevated Plus Maze Findings for Groups

	Group 1 ^a (n=12)	Group 2 ^b (n=12)	Group 3 ^c (n=12)	Group 4 ^d (n=12)	X ²	P
STCA	225.0 $\pm$ 31.47	286.0 $\pm$ 8.02 ^a	229.08 $\pm$ 32.80	222.33 $\pm$ 31.27 ^b	6.59	0.08
STOA	75.0 $\pm$ 31.47	14.08 $\pm$ 8.02 ^a	71.0 $\pm$ 32.40 ^a	52.66 $\pm$ 24.33	8.86	0.03
ECA	1.50 $\pm$ 0.33	1.08 $\pm$ 0.14	1.25 $\pm$ 0.27	1.33 $\pm$ 0.28	0.78	0.85
EOA	1.25 $\pm$ 0.21	0.41 $\pm$ 0.14 ^a	0.58 $\pm$ 0.31 ^a	1.00 $\pm$ 0.27	8.86	0.03
Formula of Entry	47.46 $\pm$ 8.71	19.38 $\pm$ 7.3 ^a	11.66 $\pm$ 6.13 ^a	35.27 $\pm$ 8.82	11.08	0.01
Formula of Time	25.0 $\pm$ 10.5	7.08 $\pm$ 3.31 ^a	8.61 $\pm$ 4.07 ^a	18.95 $\pm$ 7.98	7.95	0.04

Group 1= Sham group, Group 2= Group receiving medium Vitamin D and Zinc doses, Group 3= Group receiving high doses of vitamin D and zinc, Group 4= Group receiving only zinc.

STCA: Spent time in the closed arm, STOA: Spent time in open arm, ECA: Entry number for the closed arm, EOA: Entry numbers for open arm.

Formula of time = (time in open arm/ (time in open arm +time in closed arm) $\times$ 100).

Table 4.4. Maximum-Minimum value of Groups for Elevated Plus Maze

	Group 1 (n=12)	Group 2 (n=12)	Group 3 (n=12)	Group 4 (n=12)
STCA	0.0- 300.0	210.0-300.0	0.0- 300.0	0.0- 300.0
STOA	0.0- 300.0	0.0- 90.0	0.0- 110.0	0.0- 300.0
ECA	0.0- 4.0	0.0- 2.0	0.0- 3.0	0.0- 3.0
EOA	0.0- 3.00	0.0- 1.0	0.0- 3.0	0.0- 3.0
Formula of Entry	0.0- 100.0	0.0- 66.6	0.0- 50.0	0.0- 100.0
Formula of Time	0.0- 100.0	0.0- 32.7	0.0- 61.7	0.0- 100.0

Group 1= Sham group, Group 2= Group receiving medium Vitamin D and Zinc doses, Group 3= Group receiving high doses of vitamin D and zinc, Group 4= Group receiving only zinc.

There were significant differences among groups in terms of EPM value except for STCA and ECA (Table 4.4).

Group 2 had the highest STCA value among all the groups. STCA value was statistically higher for Group 2 as compared to Group 1 ($p<0.01$) and Group 4 ($p<0.01$), while STOA value was statistically lower for Group 2 than Group 1 ($p<0.04$).

Group 1 had the highest EOA value among all the groups. EOA values for Group 2 ($p<0.01$) and Group 3 ($p<0.03$) were statistically lower than Group 1. There is no statistical difference among groups for ECA values.

There was no statistical difference between Group 1 and 4, and their values were almost the same.

The formula of entry number and formula of time for Group 2 ($p<0.02$, $p<0.05$, respectively) and Group 3 were statistically lower as compared to Group 1 ($p<0.004$, $p<0.01$, respectively) (Table 4.4).

5.DISCUSSION

The current study used an open field test and elevated plus-maze test to see how different doses of vitamin D (400 IU/kg, 1000 IU/kg) for eight weeks and zinc (30 mg/kg) for six days affected rats' anxiety and exploratory behaviors. The finding of our experiment indicates the anxiogenic influence of co-administration of vitamin D and zinc and there was not effect of zinc administration alone on anxiety.

Vitamin D is becoming more widely recognized as a critical neurosteroid that has a variety of functions in the brain (103). In the blood, vitamin D passes the blood-brain barrier and reaches the glial and neuronal cells, and then it transforms into 1,25(OH) 2D, the active vitamin D form (104).

We know that calcitriol is an active vitamin D form that binds itself to the vitamin D receptor (VDR) that mostly exists in the nuclei of the target cells (105). Since VDR is a nuclear steroid receptor, it acts within the brain. Researchers have discovered vitamin D production and destruction processes in the brain and located VDR in different brain regions (103). Moreover, serotonin, dopamine, and norepinephrine are neurotransmitters involved in mood regulation. Vitamin D plays a role in synthesizing the mentioned neurotransmitters because it regulates the gene expression of tyrosine hydroxylase (involved in synthesizing the mentioned neurotransmitters) (106).

Vitamin D insufficiency has been linked to anxiety and depressive-like behavior in animals and memory and learning impairment. The Elevated Plus-Maze Test was used by a group of researchers to investigate the link between anxiety-related behavior and vitamin D. The vitamin D deficient rat exhibited fewer crossings in the open arms of the maze, but not in the closed arms, indicating that they were exhibiting anxiety-like behavior by avoiding open spaces. Furthermore, the vitamin D deficient rat were hyperactive during the Open Field Test, crossing more squares and traveling longer distances than the controls. This type of hyperactivity is linked to anxiety and restlessness (107).

Vitamin D₃ is a 1,25(OH)₂D₃ precursor linked to neuron metabolism in the brain (108). Vitamin D has a variety of biological impacts in the neurological system, including suppression

of induced nitric oxide synthase synthesis, which is responsible for the synthesis of nitric oxide and down-regulation of L-type calcium channel expression in hippocampal neurons (109,110). Nitric oxide (NO) has implications in various physiological and pathological brain functions, including stress reactions in hippocampus formation. NO is the tiniest and most versatile bioactive molecule ever discovered, and it is produced in the central nervous system (CNS) and a neurotransmitter (111). And because of its role in inflammation and oxidative stress, NO may control anxiety-like behavior or decrease anxiety (112). Studies show that NO had a modulatory influence on pain-induced alterations in anxiety when rodent pain models were used (113). Following this finding, we expect the high and low doses of vitamin D supplementation to decrease NO syntheses, which may cause anxiety in these groups.

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. Vitamin D has been shown to stimulate the production of tyrosine hydroxylase, implying that it may have an effect on dopaminergic functions. (114).

Severe hypercalcemia is a typical clinical symptom of vitamin D overdose (115). Neuropsychiatric symptoms such as mood and cognitive problems and severe psychosis and anxiety have been linked to hypercalcemia or high calcium levels, which may cause neuronal death due to glutaminergic excitotoxicity and dopaminergic, serotonergic dysfunction (116). Consequently, the anxiogenic effects of increased vitamin D supplements are supported.

Zinc, a trace metal, is important for the central nervous system's development and maintenance throughout life, from early newborn brain development to adult brain function maintenance (117). Neurons release zinc required for synaptogenesis, neurogenesis, neuronal development, and neurotransmission (118). Zinc-containing neurons are plentiful in cortical regions, the hippocampus, lateral septum, paraventricular nucleus of the hypothalamus, and amygdala, all linked to anxiety and depression. It indicates that zinc plays a role in anxiety (119).

The effects of zinc supplementation and deficiency on anxiety and depression have shown mixed outcomes in research. Short and acute supplementation paradigms are frequently used in studies that show zinc supplementation reduces anxiety and depression-like behavior

(120). Animal model research has found a link between zinc deficiency and cognitive disorders like depression and anxiety. Zinc deficiency has been demonstrated to cause depression- and anxiety-like symptoms, and supplementation has been used to treat severe depression. Zinc supplementation promotes antidepressant drug effectiveness in depressed individuals, and it may play an important role in treatment-resistant patients (121). On the other hand, studies that use chronic, pre and post-natal paradigms have revealed that excess zinc causes spatial memory deficiencies and decreases the ability to extinguish fear (122,123)

According to several studies, the role of antioxidative mechanisms in vitamin D₃–induced neuroprotection against zinc-induced oxidative stress was investigated. zinc supplementation does not induce depression or anxiety-like behaviors in rats (124,125). On the other hand, these investigations either use a single acute dose of zinc or a 14-day chronic zinc paradigm. A 14-day dosage may not be sufficient to be considered chronic when determining whether zinc supplementation is safe for long-term use.

Although both preclinical and clinical evidence strongly emphasizes the role of zinc in the pathogenesis and treatment of depression, the mechanisms behind zinc's antidepressant action are currently being researched (125).

6. CONCLUSION

We tested doses of vitamin D (400IU/1000IU) and zinc (30mg/kg) on rat and conducted elevated plus maze and open field tests to evaluate the impact of vitamin D on anxiety. The findings show that "medium doses of vitamin D and zinc" and "high doses of vitamin D and zinc" have anxiogenic effects. Zinc alone showed no effect on anxiety in rat. They have a similar effect as the sham group's rat, which received no dose of zinc or vitamin D. We need further studies to explore the impact of zinc on anxiety with different doses.

Based on the observations and findings of this study, we can give the following recommendations:

1. There is a need to improve the scale of this study. Increasing the number of rat and adding another vitamin D group without zinc administration is preferable to obtain more evidence and make better comparisons in future studies to find better results.
2. There is a need to try higher doses of vitamin D (more than 1000 IU) and different doses of zinc on experimental animals to determine their effects.

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APPENDIX 1: PROJECT APPROVAL



Sayı : 94603339-604.01.02/ 33352
Konu : Proje Onayı

18/11/2020

SAĞLIK BİLİMLERİ ENSTİTÜSÜ MÜDÜRLÜĞÜNE

Fizyoloji Anabilim Dalında görev yapmakta olan Prof. Dr. Nazan Dolu'nun danışmanlığında Sağlık Bilimleri Enstitüsü / Fizyoloji Tezli Yüksek Lisans Programı öğrencisi Amna Abdulsalam Barood'un sorumluluğunda yürütülecek olan DA20/17 nolu "The effect of vitamin D and zinc on anxiety and exploratory behaviours in rats" başlıklı araştırma projesi Kurulumuz ve Hayvan Deneyleri Yerel Etik Kurulunun 02/11/2020 tarih ve 20/15 sayılı kararı ile uygun görülmüştür. Projenin başlama tarihi ile çalışmanın sunulduğu kongre ve yayımlandığı dergi konusunda Kurulumuza bilgi verilmesini rica ederim.

e-imzalıdır

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 Research on Animals (Project no:...) and supported by Baskent University Research Fund.

DAĞITIM

Sağlık Bilimleri Enstitüsü Müdürlüğüne
Fizyoloji Anabilim Dalına

APPENDIX 2: ETHICAL APPROVAL


1993
BAŞKENT UNIVERSITY

LOCAL ETHICS COMMITTEE FOR ANIMAL EXPERIMENTS DECISION		
SESSION NO	DECISION NO	DATE OF DECISION
09	20/15	02/11/2020

Project DA20/17 no entitled "The effect of vitamin D and zinc on anxiety and exploratory 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.

