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Effect of Different Doses of Vitamin D_3 on Anxiety in Rats Without Vitamin D Deficiency: An Experimental Study

D Vitamini Eksikliği Olmayan Sıçanlarda Farklı Dozlarda D_3 Vitamininin Anksiyete Üzerine Etkisi: Deneysel Çalışma

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This study was prepared based on the findings of Haifia Masud's thesis study titled "The Effect of High Dose Vitamin D on the Sympathetic Skin Response in Rats" (Ankara: Başkent University; 2022) and Zahour Gamal Eddn Asmaeil's thesis study titled "The Effects of High Doses Vitamin D on Anxiety and Exploratory Activity Behaviours in Rats" (Ankara: Başkent University; 2021).
This study was presented as a summary orally in 46th National Physiology Congress of the Turkish Physiological Sciences Association in October 08-10, 2021, Online and a summary of the papers presented at the congress was published in the special issue of a journal "Acta Physiologica".

ABSTRACT Objective: The effects of vitamin D_3 on anxiety are controversial. In this study, the effects of two different doses of vitamin D_3 on anxiety in rats without vitamin D_3 deficiency were investigated by sympathetic skin response (SSR), elevated plus maze (EPM), and open field (OF) tests. SSR measures eccrine sweat gland activity innervated with sympathetic fibers. Increased skin conductance level (SCL, a parameter of SSR), decreased open arm duration at EPM and spent time in the peripheral area at OF show anxiety status. **Material and Methods:** The present study was conducted with 36 Wistar male rats (aged 6-8 weeks, 200-250 g). Normal water (Sham group, Group 1), 400IU vitamin D_3 (Group 2), and 1000IU vitamin D_3 (Group 3) were given to rats per day with oral gavage. After 8 weeks, tonic SCL (resting), phasic SCL (with auditory stimulus), and behavioral tests were measured. **Results:** The Tonic SCL of Group 2 and Group 3 were higher than the sham group. There was no significant difference between the tonic SCLs of Group 2 and Group 3. The phasic SCL of Group 2 was higher than Group 1. The time spent in the center at OF in Group 2 and Group 3 was significantly lower than in Group 1. Group 3 spent less time on the open arms of the EPM. **Conclusion:** Administration of different doses of vitamin D to rats without vitamin D_3 deficiency increased anxiety. This situation should be considered when giving/taking vitamin D_3 supplements.

Keywords: Calcitriol; rats; open field test; elevated plus maze; galvanic skin response

ÖZET Amaç: D3 vitamininin anksiyete üzerine etkileri tartışmalıdır. Bu çalışmada, D3 vitamini eksikliği olmayan sıçanlara iki farklı dozda D_3 vitamini uygulamasının anksiyeteye etkileri, sempatik deri cevabı (SDC), yüksek artı labirent (YAL) ve açık alan (AA) testleri ile araştırıldı. SDC, sempatik liflerle innerve edilen ekrin ter bezi aktivitesini ölçer. Deri iletkenlik seviyesinin (DİS, SDC'nın bir parametresi) artması, YAL'de açık kol süresinin azalması ve AA'da periferik alanda geçirilen sürenin artması anksiyete durumunu gösterir. **Gereç ve Yöntemler:** Bu çalışma 36 Wistar erkek sıçanda (6-8 haftalık, 200-250 g) gerçekleştirildi. Sıçanlara günde bir kez normal su (Sham grubu, Grup 1), 400 IU D3 vitamini (Grup 2) ve 1000 IU D_3 vitamini (Grup 3) oral gavajla verildi. 8 hafta sonra tonik DİS (dinlenme), fazik DİS (işitsel uyaranla) ve davranış testleri ölçüldü. **Bulgular:** Grup 2 ve Grup 3'ün tonik DİS'si sham grubuna göre daha yüksekti. Grup 2 ve 3 arasında tonik DİS açısından anlamlı bir fark yoktu. Grup 2'nin fazik DİS'si Grup 1'e göre daha yüksekti. Grup 2 ve Grup 3'te AA'da merkezde geçirilen süre Grup 1'e göre anlamlı derecede düşüktü. Grup 3, YAL'in açık kollarında daha az zaman harcadı. **Sonuç:** D_3 vitamini eksikliği olmayan sıçanlara farklı dozlarda D vitamini uygulanması anksiyeteyi artırdı. D_3 vitamini takviyesi verirken/alırken bu durum dikkate alınmalıdır.

Anahtar Kelimeler: Kalsitriol; sıçanlar; açık alan testi; yükseltilmiş artı labirent; galvanik deri yanıtı

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Many articles have been published indicating that depression and anxiety may occur in the case of vitamin D_3 (calcitriol) deficiency. Studies are showing that vitamin D_3 levels are low in patients with anxiety disorders.[1] Many studies have been conducted showing that vitamin D_3 supplementation has a curative effect on anxiety and depression in people with different diseases or that vitamin D_3 has no effect.[2-5] A study reports that vitamin D_3 does not affect mood in healthy young people without mood disorders.[6]

Many studies show the anxiolytic effect of vitamin D_3 in animal models.[7] A decrease in the level of vitamin D_3 in the blood causes structural changes in the animal brain, causing anxiety and depression.[8] Vitamin D deficiency impairs neurobehavioral development in animals, and mice lacking the vitamin D receptor gene show increased anxiety-like behavior.[9,10] Vitamin D_3 supplementation improved depression and anxiety like behavior in ovariectomized rats.[11,12] Bakhtiari-Dovvombaygi et al. found that vitamin D_3 improved unpredictable chronic mild stress in elevated plus maze (EPM), and open field (OF).[13]

However, when vitamin D_3 was administered intraperitoneally to mice, no anxiolytic effect was observed in the elevated plus maze.[14] No anxiolytic effect was found in the open field when intraperitoneal 5 and 10 μg/kg vitamin D_3 was administered twice a week to rats exposed to chronic mild stress for 3 weeks.[15] The lack of difference in ascending plus maze performance of mice fed a vitamin D_3-deficient diet suggests that vitamin D_3 does not affect anxiety.[16,17]

Although the possible anxiolytic effect of vitamin D_3 has been demonstrated in many studies, the effects of different doses of vitamin D_3 on anxiety in humans and rodents are still controversial. Therefore, there is a need to study new models with different doses of vitamin D_3 in animal anxiety models.

In this study, the effect of vitamin D on anxiety was investigated by sympathetic skin response (SSR, galvanic skin response). SSR is an electrophysiological autonomic test that reflects sudomotor activity function resulting from autonomic peripheral sympathetic cholinergic stimulation.[18,19] Skin resistance (SR) or skin conductance is used to evaluate sympathetic skin response. The sympathetic skin conductance we measured in our study is a reliable method frequently used to measure anxiety and attention.[20] Increased eccrine sweat gland activity reflects increased anxiety. A parameter of SSR is skin conductance level (SCL). The increase in SCL indicates increased sympathetic activity and eccrine sweat gland activity.[21]

The presented study aimed to investigate the effect of two doses of vitamin D_3 supplementation on anxiety and exploratory behavior with sympathetic skin conductance response, elevated plus-maze, and open field test in rats without vitamin D_3 deficiency.

MATERIAL AND METHODS

ANIMALS

Thirty-six Wistar rats (male; aged 6-8 weeks, 200-250 g), were obtained from Experimental Research Center. Animals were housed at controlled room temperature (21±2°C), humidity (45±10%), a 12-h light/dark cycle, and under sterilized conditions. Rats were given standard animal chow and filtered water before and throughout the experiment. Ethical approvals, numbered 20/18 and 20/19 were obtained from the Animal Experiments Ethics Committee of the Başkent University. The study was conducted under the principles of the Helsinki Declaration.

PROTOCOL OF THE EXPERIMENTAL DESIGN

After 2 weeks of acclimatization, thirty-six rats were randomly divided into 3 groups of 12 rats each. Group 1 (Sham) was given normal drinking water. Group 2, 400 IU vitamin D_3 was given in drinking normal water. Group 3, 1000 IU vitamin D_3 was given in drinking normal water. Drinking water and vitamin D_3 were orally administered via a gavage tube.[22] A water-soluble form of Devit-3 oral drops (50,000 IU Vitamin D_3/15 ml drops) was used (Deva A.Ş, Türkiye).

The vitamin D_3 was applied to the animals once a day for 8 weeks.[22] SSR (between 9:00 and 12:00 a.m.), EPM, and OF tests (between 01:00 and 03:00 p.m.) were done for each rat within 24 hours after the last vitamin D_3 application (Figure 1).

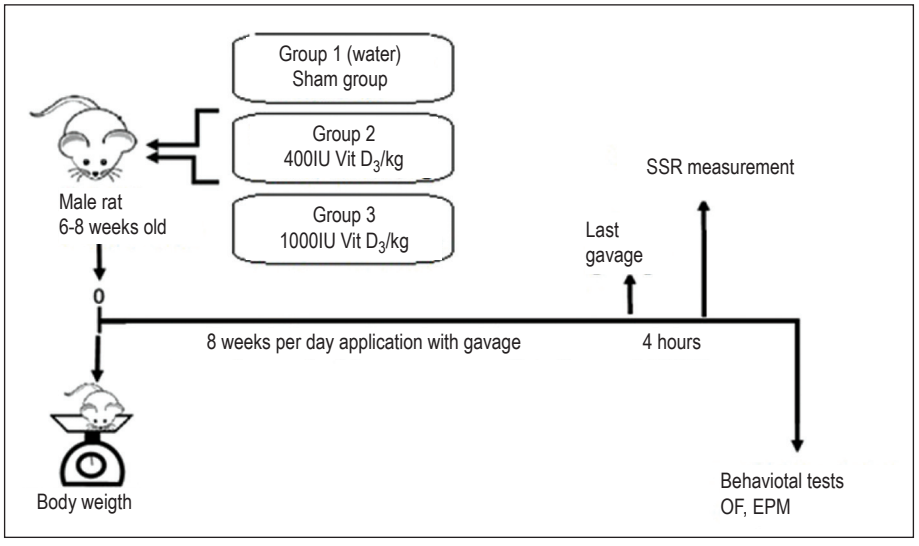

FIGURE 1: Schedule of the study. SSR: Sympathetic skin response; OF: Open field; EPM: Elevated plus maze.

SSR RECORDINGS

SSR was recorded immediately after 8 weeks of completing the period of giving the vitamin D_3. Rats were placed in the immobilizer, excluding the tail and hind limbs. Skin conductivity signals recorded with the MP30 system were passed through an Analog/Digital converter. The plantar surfaces of the back of two extremities of the rat were wiped with 70% alcohol and 0.8 cm diameter Silver/Silver Chloride (Ag/AgCl) was placed. Gel was filled into electrodes. Electrodes were connected to the MP30 system with an SSR transducer. After the electrodes were attached, the animal was allowed to calm down in the room which was kept dark and silent for 5 minutes.

Tonic recordings: Tonic recordings were taken without stimulation during the 2-minute.

Phasic recording: At the end of the tonic recordings, phasic recordings were taken for 10 minutes together with 15 sound stimuli. The stimuli were randomly given at changing intervals between 30-80 seconds without pausing. A stimulus generator was used for sound stimuli that generate sympathetic skin responses. The sounds of 1000Hz frequency and 90 dB intensity generated from the stimulus generator were played to the animals with two speakers installed in the experimental room.[21,23]

After the recording process was completed, the animal was taken out of the immobilizer and returned to its cage.

Skin conductance levels (SCL) were from SSR recordings and analyzed with special software of the MP30 system on the registered PC. SCL was analyzed with a cursor of our system and the mean of SCL was calculated. Analyses of skin conductance are evaluated as micromho (μmho).

OPEN FIELD TEST (OFT)

The OFT is used for anxiety-related behavior and locomotor activity in rats.[24,25] The test was constructed in a white plexiglass box (100x100 cm), and surrounded by 40 cm walls. The floor is divided into 25 squares; 9 squares in the middle as the center area and 16 squares in the periphery.

After the 5-minute habituation period, the rat was released to the corner of the open field and allowed to explore the area freely for 10 min. All anxiety parameters which are the time spent in the center area, the number of entering the center area, the time spent in the periphery area, the number of rearing, number of defecation (ND) were recorded by a video camera and measured after the experiment. The device was then cleaned with a 20% alcohol solution for each rat.

ELEVATED PLUS MAZE

EPM is a reliable test for measuring anxiety in rodents.[24] EPM consists of a central area (10 cmx10 cm), two open (50 cmx10 cm), and two closed arms (50 cmx10 cmx20 cm) opening towards this area. The device is 50 cm above the ground. The arms are shaped like plus signs.

The testing was performed under a dim red light. The rat was placed in the center of the maze at the beginning of the test, facing one of the open arms. During 5 minutes, the time spent in open and closed arms and the number of entries into each arm were recorded. All experiment was recorded by a video camera mounted on the top of the EPM test device. The maze was cleaned with 20% alcohol after each test. The following formula was used for calculating the percentage of open-arm entries and the time spent in open-arm.[24,25]

Time spent in open arm (%)=(Time in open arm/Time in open arm+Time in the closed arm)x100

Open arms entries (%)=(Open arms entries/ Open arms entries+Closed arms entries)x100

STATISTICAL EVALUATION

SPSS 21.0 was used for statistical analysis (IBM, Armonk, NY, ABD). Statistical analyses were done using analysis of variance test and Bonferroni's post hoc test was applied. The results were expressed as the mean±standard error. All statistical analyses were regarded as statistically significant when p≤0.05.

RESULTS

SSR FINDINGS

Tonic SCL findings: It was found a significant difference between Group 1 and Group 2 (p<0.02), Group 1 and Group 3 (p<0.02). Group 3 had the highest SCL, so anxiety. There was no significant difference between Group 2 and Group 3 for tonic SCL (Table 1).

Phasic SCL findings: Phasic SCL was statistically different for all groups. When we compared groups, Group 1 and Group 2 (p<0.00), Group 1 and Group 3 (p<0.00), Group 2 and Group 3 (p<0.00) were significantly different. Group 2 had the highest SCL or anxiety level (Table 1).

TABLE 1: Tonic and phasic SCL values of the groups.

SCL (μmho)	Group 1 (n=12)	Group 2 (n=12)	Group 3 (n=12)	F	p value
Tonic SCL	8.43±0.80	14.78±1.72	14.93±0.68	10.104	0.000
Phasic SCL	8.30±0.87	18.37±1.33	14.99±0.63	28.154	0.000

SCL: Skin conductance level; Group 1: Sham group; Group 2: 400IU vitamin D_3; Group 3: 1000IU vitamin D_3.

TABLE 2: The test results obtained an open field in rats ($\bar{X}$±SE).

	Group 1[a] (n=12)	Group 2[b] (n=12)	Group 3[c] (n=12)	F	p value
NEC	1.83±0.71	1.25±0.50[a]	1.50±0.67	1.65	0.20
STC (sec)	4.83±1.23	1.50±0.41[a]	1.25±0.50[a]	6.12	0.005
STP (sec)	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

SE: Standard error; Group 1[a]: Sham group; Group 2[b]: 400IU vitamin D_3; Group 3[c]: 1000IU vitamin D_3; NEC: Number of entering 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.

OPEN FIELD TEST FINDINGS

As seen in Table 2, there was a statistical difference between the groups in open field parameters except for the number of times entering the central area and the ND.

The time spent by rats in Group 2 (p<0.02) and Group 3 (p<0.01) in the central region was significantly less than in Group 1. Naturally, rats in Group 2 (p<0.05) and Group 3 (p<0.04) spent more time in the peripheral area than Group 1. While spent time in the peripheral area was almost the same in Group 2 and Group 3 rats, Group 3 spent significantly more time in the central area than the other group.

The rearing number was higher in Group 2. For the rearing number, statistically significant differences were found between Group 1 and Group 2 (p<0.01) and between Group 2 and Group 3 (p<0.01).

ELEVATED PLUS MAZE FINDINGS

The analysis did not indicate the effect of vitamin D_3 supplementation on EPM measurements except for the entry number to the closed arm. Group 2 had a higher entry number to the closed arm than Group 3.

TABLE 3: The test results obtained at elevated plus-maze in rats ($\bar{X}$±SE).

	Group 1[a] (n=12)	Group 2[b] (n=12)	Group 3[c] (n=12)	F	p value
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
F1	47.46±8,71	43.05±6.09	29.16±7.43	1.62	0.21
F2	25.00±10.49	25.24±5.85	13.47±4.07	0.84	0.43

SE: Standard error; Group 1: Sham group; Group 2: 400IU vitamin D_3; Group 3: 1000IU vitamin D_3; STCA: Spent time in closed arm; STOA: Spent time in open arm; ECA: Entry number to the closed arms; EOA: Entry number to the open arms; F1: The percentage of the number of entries into the open arms; F2: The percentage of time spent in the open arms (sec).
a: Different from Group 1, b: Different from Group 2, c: Different from Group 3.

While spent time in the closed arm was smaller than Group 1, there was no statistical difference.

Although there was no significant difference among the groups for the percentage of time spent in the open arms and the percentage of the number of entries into the open arms, it was lower in Group 3 than in the other groups (Table 3).

DISCUSSION

The findings reported here provide further evidence of the role of vitamin D_3 in controlling anxiety-like behaviors along with its effects on the autonomic nervous system. In this sense, our results were the first study to investigate the relationship of these vitamin D_3-mediated behavioral responses with the sympathetic nervous system and to show that anxiety-like behavior occurs by increasing the SSR. Findings also obtained in the OFT and EPM indicate that vitamin D_3 may increase anxiety-like behaviors.

Studies on the effect of vitamin D_3 on the sympathetic nervous system are limited in number. No study on vitamin D_3 and sympathetic skin response has been found in the literature. When we look at the relationship between blood pressure and vitamin D to understand the relationship between sympathetic autonomic functions and vitamin D_3, it has been observed that if vitamin D_3 is given to people with vitamin D_3 deficiency, the increase in blood pressure returns to normal.[26] In another study, it was found that vitamin D_3 application and blood pressure were not related.[27]

In our study, rats at both doses of vitamin D_3 preferred the periphery much more than the central region in the OFT. Rodents tend to remain in close contact with the walls of the open field in which they are housed. This behavior is likely a result of rodents' tendency to avoid open, unknown, and potentially dangerous spaces. It is believed to be phylogenetically related to anxiety and fear responses. In OFT, increased time spent in the periphery is used as a symptom of anxiety.[28] This showed us that vitamin D_3 does not have an anxiolytic effect.

In Burne et al. conducted their study on male and female offspring of vitamin D deficient mothers and on age-matched, sex-matched controls without vitamin D deficiency. The offspring were tested in an open field for 30 minutes at 8 weeks of age. Half of the rats were restrained by holding them in a towel for 5 minutes immediately before the open field test. Unrestricted vitamin D-deficient mice spent more time near walls in the OF than control mice and no gender differences were observed.[29] Fedotova et al. showed that subcutaneous vitamin D had an anxiolytic effect when administered to ovariectomized adult rats at 1.0 and 2.5 mg/kg/day doses.[30] These studies show us that the effect of vitamin D_3 may vary depending on age, dose, or method of administration (subcutaneous, oral, etc.).

Our study found that rats receiving different doses of vitamin D_3 showed anxiety behavior by spending less time in the EPM and OF. These findings contradict the study showing that vitamin D-deficient rats had reduced open-arm entry time.[9]

Baranenko et al. showed that vitamin D_3 (5.0 mg/kg) supplementation reduced anxiety levels in the EPM with ovariectomized rats.[31] These studies show that not only vitamin D_3 deficiency but also high doses of vitamin D_3 can cause anxiety.

The limitations of the study include that it was conducted on rats rather than humans, that it was not conducted over a wider dose range, and that it was not followed up for a long time.

CONCLUSION

Our results suggested that supplementation of the two different doses of vitamin D to rats without vitamin D_3 deficiency increased anxiety. The anxiety-inducing effect of people without vitamin D_3 deficiency should be taken into consideration when giving/taking vitamin D_3 supplements.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Design:*** *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Control/Supervision:*** *Nazan Dolu;* ***Data Collection and/or Processing:*** *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Analysis and/or Interpretation:*** *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Literature Review:*** *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Writing the Article:*** *Nazan Dolu, Haifa Masud, Zahour Gamal Eddn Asmaeil;* ***Critical Review:*** *Nazan Dolu;* ***References and Fundings:*** *Nazan Dolu;* ***Materials:*** *Nazan Dolu.*

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