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ORIGINAL ARTICLE

THE PH OF DRINKING WATER ALTERS THE METABOLIC AND NEUROBEHAVIORAL RESPONSE OF RATS EXPOSED TO VITAMIN D3 OR PREDNISOLONE

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Summary

Background: The degree of blood pH affects the fundamental function of vitamin D, which is to maintain calcium and phosphorus as well as many other biochemical, neurological, and behavioural activities the difference in blood pH impacts the levels of calcium and vitamin D in the blood, and it is also in charge of ensuring that calcium levels are suitable and constant.
Objective: that blood pH affects the basic function of calcium and phosphorus and vitamin D, the difference in pH affects calcium and vitamin D levels in the blood and is also responsible for keeping calcium levels appropriate and stable.
Methods: 7 groups composed of 35 mature female rats were divided at random.
Results: After 24 hours, none of the animals died. While stress activity increased with alkaline water, vitamin D3 and calcium levels increased in the acid water group while their concentration decreased with prednisolone, while calcium levels did not change in the alkaline water group. The animals in the two groups of vitamin D3 and acidified water with or without prednisolone moved less in the open field. Along with increased oxidative stress, vitamin D3 with acid water also inhibited cholinesterase activity. The lipid profile in the blood is impacted by the vitamin D3 group in both acidic and alkaline water.
Conclusion: Prednisolone reduces serum levels of vitamin D3. And that acidic water directly affects the toxicity of vitamin D3, while alkaline water significantly reduces toxicity.

Key words: Vitamin D3; Toxicity; Acidic water; Prednisolone; Rats

Introduction

Human epithelial cells produce vitamin D by photochemical production, and it can also be consumed from a variety of food sources (1). The two main biological forms of vitamin D are D3 (cholecalciferol) and D2 (ergocalciferol) (2). Its synthesis in plants, yeast, and fungi as well as the skin's reaction to UV radiation are what make up the plant-derived version of ergocalciferol (3). Cholecalciferol, which comes from animals, can be produced in a non-enzymatic manner from the precursor 7-dehydrocholesterol (7-DHC) (4).

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This vitamin has been shown to influence bone metabolism and calcium balance. The body relies on it to absorb the minerals calcium and phosphorous, whether from foods or supplements, and this helps to strengthen the bones and protect them from fractures, fragility, and rickets. It plays a major role in improving mood and preventing mental disorders, such as depression. It is of great importance for kidney patients because it contributes to maintaining calcium levels in their bodies. In addition, it is necessary for patients with disorders, such as hypothyroidism, hypophosphatemia, and hypoparathyroidism, "parathyroid", as it strengthens immunity. It reduces the chances of developing chronic diseases, such as diabetes, some cancers and heart diseases (1, 4, 5). One of vitamin D's many tasks is to control the growth and operation of the neurological system, making it one of the most crucial vitamins the body requires (6). Vitamin D's ability to influence intracellular calcium homeostasis, the synthesis of neuromodulators, the generation and secretion of neurotransmitters, and the avoidance of oxidative damage to nerve tissue all contribute to its protective effects (7).

Vitamin D insufficiency is linked to the emergence of numerous pathological processes, according to clinical investigations, and may raise the likelihood of central nervous system (CNS) illnesses, including schizophrenia and multiple sclerosis. Vitamin D is also essential for sustaining health (8). Infections, immunological disorders, type 2 diabetes, metabolic syndrome, cardiovascular disease, and common malignancies are all significantly exacerbated by low vitamin D levels (9).

Because they interfere with numerous clinical symptoms of numerous disorders, their greater intake is linked to the emergence of numerous harmful effects that are rarely addressed. The increase in blood calcium is one of these noticeable effects. due to its quick photodegradation into a range of physiologically inert photoproducts, excessive exposure does not increase the synthesis of vitamin D (10, 11). Due to the limited available studies on the toxicity of vitamin D3 and its relationship to the degree of blood pH in animals, we chose to perform this study, and to do so, we established the following objectives. Rats' levels of nervous behaviour and motor activity, oxidative stress, and some biochemical alterations are all impacted by the interplay of vitamin D3 and prednisolone and their link to acid or alkaline water in subchronic toxicity.

Materials and Methods

Animals

In this study, white female rats that were roughly 35 in number and were kept in the animal house of the College of Veterinary Medicine, University of Mosul, were used. The rats were between 200 and 250 g in weight. Within the same group, the rats' weights were closely matched. The rats were given water and food and put in cages that were designed specifically for this purpose. In addition, the ventilation, temperature, lighting, and sleeping requirements were supplied (12).

Chemicals and medicines

The Italian company Sitron Pharmaceutical SpA produces vitamin D3, there is the medication prednisolone from American pharmacies. Trichloro Acetic Acid RICCA chemical company, Thiobarbituric Acid (TBA) firm, Na2HPO4 buffer, (5.5 thio-bas 2-nitrobenzoic acid DTNB), and sodium bicarbonate Commercial apple cider vinegar.

Ethical Approval

All ethical approvals for the humane treatment of laboratory animals were submitted by the College of Veterinary Medicine at the University of Mosul from UM.VET.2021.35.

Drinking water preparation

Using a pH meter, The pH of drinking water was determined to be more than 5 but less than 8, To adjust the pH, commercial apple cider vinegar and sodium bicarbonate were utilized.

Experiments

First, we intended to assess the median lethal dose (LD50) of vitamin D3 in rats using 6 animals (13).

There were 35 rats utilized in this experiment, which were randomly divided into 7 groups of 5 rats each.

- G1: Negative control (acid water)
- G2: Positive control (alkaline water)
- G3: Vitamin D3+ acid water
- G4: Vitamin D3+ Alkaline Water
- G5: Vitamin D3 + Prednisolone + Acid Water
- G6: Vitamin D3 + Prednisolone + Alkaline Water
- G7: prednisolone

Vitamine D3 100.000 IU\animal was given by muscular injection for a month, then prednisolone 5mg\kg for one week.

Following the fifth week period, the changes in the rats' neurobehavioral and motor activity were noted in the open field (number of cross squares and rearing), and then a number of times the head pocketing was carried out using a plastic surface with the radius of 30 cm and a height of 20 cm and contains 10 circular holes. The test was conducted by observing the animal and calculating the number of times the head was inserted into the holes. The duration of the test is 3 minutes for each animal. This test measures the animal's curiosity and familiarity with its surrounding (14). The rat was euthanized by ether to collect the blood serum, and blood was drawn from a vein in the inner corner of the eye and deposited in specialized tubes without the use of anticoagulants. centrifuged at 3000 rpm for 15 minutes, and then stored in special plastic tubes and frozen at -20°C for the completion of laboratory biochemical tests.

Measuring some biochemical indicators

Some biochemical parameters were measured in the blood serum of treated rats, and these measurements included:

- Vitamin D3 Kit (Elabscience, USA)
  This ELISA kit uses the Competitive-ELISA principle. The small ELISA plate provided in this kit has been pre-coated with VD3. During the reaction, the VD3 in the sample or standard competes with a fixed amount of VD3 for the solid-phase supporter sites in the VD3 biodiscovery Ab. The excess conjugate and unbound sample or form are washed, and an Avidin-Horseradish Peroxidase (HRP) conjugate is added to each pit of the microplate and then incubated. Then a TMB substrate solution was added to each hole. The enzymatic substrate reaction is terminated by the addition of the stop solution and the colour changes from blue to yellow. Optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of VD3 in the tested samples can be calculated by comparing the OD of the different samples.
- Calcium kit (BIOLAB, France).
  Total serum calcium reaction during 5 min at temperature and measured at a wavelength of 570 nm (550-590).
- Cholesterol kit (CHO21, Germany).
  The serum cholesterol concentration was calculated using the 311 Cobas C device manufactured by the German company Roche and based on the enzymatic method CHO21.
- Triglyceride kit (TRIGL, Germany).
  The concentration of triglycerides in the blood serum was calculated using the 311 Cobas C device from the German company Roche, based on the enzymatic method TRIGL.
- HDL measuring kit (Roche, Germany).
  The concentration of high-density lipoproteins in the serum was calculated using the 311 Cobas C device by the German company Roche, based on the enzymatic method contained in the HDLC4 measurement kit.
- Measurement of glutathione concentration in serum: method mentioned in Jamese et al. 1982, to determine glutathione concentration in serum (15).

The principle of interaction depends on the interaction between the DTNB reagent, which is the German reagent, with the glutathione present in the sample, forming a golden-yellow colour complex. The colour intensity of the complex depends on the concentration of glutathione in the sample, and this colour is measured using a spectrophotometer.

The assay was done by adding 0.5 ml of the serum of each sample in a clean test tube, and 0.5 ml of distilled water was placed in the Blank tube, then 2 ml of sodium phosphate buffer solution prepared on top was added to each tube, then the tubes were shaken well, then 0.5 ml of the solution was added. DTNB detector to each tube, then shake the tubes well and then leave for 5 minutes, after which the optical absorption of the samples was measured against the efficient sample by means of a spectrophotometer at a wavelength of 412 nm.

- Measurement of MDA concentration in serum: A method was used based on the interaction between malondialdehyde and thiobarbituric acid to form the MDA-TBA2 complex that is absorbed in the wavelength of 352 nm (16).

- Studying the activity of cholinesterase in the serum. The modified electrometric method was used to measure the activity of acetylcholine esterase (17). according to the following steps: 3 ml of distilled water is placed in a glass beaker with a capacity of 10 ml, then 0.2 ml of plasma is added to it, then 3 ml of a phosphate buffer solution PH equal to 8.1 and mix well, then the initial Ph is measured by a pH-meter, then 0.1 ml of 7.5% acetylcholine iodide solution is added as a substance The mixture is then transferred to the water bath set at 37°C and incubated for 30 minutes. After the sample is removed from the incubator, the secondary Ph is measured, then the change in Ph is calculated and the difference between them is calculated within 30 minutes. This result reflects the activity of the yeast in the sample used. The efficient sample contains all solutions except plasma.
  Change in pH of samples/30 min = Ph2-Ph1
  Change in pH efficiency/30 min = Ph2-Ph1
  Change in pH /30 min = Ph2-Ph1-Ph efficient

Statistical analysis

Parametric data were statistically examined using the SPSS program, one-way analysis of variance test (ANOVA) software, and then subjected to the LSD test. The Mann-Whitney test was applied to the non-parametric data, and the level of significant difference was at a probability level less than p 0.05.

Results

We try to determine the rat LD50 for the median lethal dosage of vitamin D3.

With a wide range of lethal dosages of vitamin D3 ranging from 400.000 to 600.000 IU/animal based on the injection volume in rats, which is 2 ml/kg, none of these toxic reactions occurred when the vitamin was given orally or intramuscularly to rats. Doses any mortality following 24 hours of injection. After 24 hours, certain toxic consequences on the animal were noticed. These symptoms included increased movement and low weight, as well as the death of some rats given very high doses after 4-5 days of therapy. Vitamin D3 generally does not cause any mortality in acute cases within 24 hours).

Effect the PH of drinking water on the vitamin D3 toxicity with or without prednisolone on motor neurobehavioral:

Rats were tested for neurobehavioral and motor activity in an open field, and the results showed a significant increase in neurobehavioral in vitamin D3 and alkaline water and a significant decrease in the number of squares the rats crossed, the number of rearing, and the number of times they stuck their heads into holes. The animal activity is improved by using prednisone, but it still differs from the positive and the negative controls (Table 1).

It was discovered that all groups had significantly higher vitamin D3 levels than the positive and negative controls. Vitamin D3 levels were lower after prednisolone treatment, but they were still greater than in the control

groups. The calcium level greatly increased in the groups treated with vitamin D3 and acidic water, whereas it dramatically dropped when prednisolone was used (Table 2).

Table 1. Neurobehavioral and motor test results of using prednisolone and/or high doses of vitamin D3.

Groups (n=5 rats)	Number of Squares	rearing	Number of Pocking
Negative control (acid water)	78 ± 5	16 ± 3	6 ± 1.2
Positive control (alkaline water)	70 ± 05	16 ± 4	5 ± 1.2
Vitamin D3 + acid water	54 ± 2*#	12 ± 2*#	3 ± 1*#
Vitamin D3 + Alkaline Water	80 ± 4#	25 ± 2*#	7 ± 1#
Vitamin D3 + Prednisolone + Acid Water	67 ± 4*a	12 ± 2*#a	3 ± 1.5*#
Vitamin D3 + Prednisolone + Alkaline Water	60 ± 4*a	13 ± 2*#a	4 ± 1.5*
Prednisolone	69 ± 6	14 ± 4	6 ± 2

*represents a difference from the negative control group
represents a difference from the positive control group
a represents a difference from the Vit D3+ acidic water

Table 2. Plasma levels of vitamin D3 and Ca in studied groups after therapy.

Groups (n=5 rats)	D3 vitamin D3(ng/l)	Ca (mmol\l)
Negative control (acid water)	27.4 ± 4.01	2.3 ± 0.08
Positive control (alkaline water)	42.4 ± 3.01*	2.7 ± 0.02
Vitamin D3 + acid water	126 ± 5.01*#	4.0 ± 1.02*#
Vitamin D3 + Alkaline Water	117.6 ± 8.01*#	1.2 ± 0.009
Vitamin D3 + Prednisolone + Acid Water	100 ± 4.01*#a	2.4 ± 0.03a
Vitamin D3 + Prednisolone + Alkaline Water	73.1 ± 3.01*#a	2.3 ± 0.04
Prednisolone	39.1 ± 2.01ab	2.4 ± 0.06

*represents a difference from the negative control group
represents a difference from the positive control group
a represents a difference from the Vit D3+ acidic water
b represents a difference from the Vit D3+ acidic water+ prednisolone

Malondialdehyde levels and cholinesterase activity were both increased by vitamin D3 and acid water, compared to the group given prednisolone and vitamin D3 as well as the positive and negative control (Table 3).

Table 3. biochemical parameters in the studied groups after therapy.

Groups (n=5 rats)	MDA(nmol\l)	GSH (mmol\l)	ACHE activity
Negative control (acid water)	2.47 ± 0.02	0.48 ± 0.03*	0.2 ± 0.005*
Positive control (alkaline water)	2.72 ± 0.008	0.59 ± 0.02	0.37 ± 0.009
Vitamin D3 + acid water	6.63 ± 0.04*#	0.41 ± 0.01*	0.05 ± 0.01*#
Vitamin D3 + Alkaline Water	2.70 ± 0.009	0.76 ± 0.009	0.44 ± 0.02
Vitamin D3 + Prednisolone + Acid Water	2.32 ± 0.021	0.39 ± 0.01	0.29 ± 0.003a
Vitamin D3 + Prednisolone + Alkaline Water	3.71 ± 0.01a	0.70 ± 0.02	0.29 ± 0.01
Prednisolone	3.33 ± 0.009a	1.01 ± 0.01	0.34 ± 0.01

*represents a difference from the negative control group
represents a difference from the positive control group
a represents a difference from the Vit D3+ acidic water

Compared to the negative and positive controls, the level of triglycerides was considerably higher in the group treated with vitamin D3 and acid water, while cholesterol fell in the same group compared to the negative control group, and a reduction in high-density lipoprotein levels in the group treated with or without prednisolone in comparison to the negative and positive control group, cholesterol decreased significantly in alkaline water and vitamin D3 with prednisolone or alone (Table 4).

Table 4. Lipid profile of the studied groups after therapy.

parameters / Groups (n=5 rats)	TG (mmol\l)	TC (mmol\l)	VLDL (mmol\l)	HDL (mmol\l)
Negative control (acid water)	0.7 ± 0.03	1.4 ± 0.02	1.3 ± 0.02	1.2 ± 0.01
Positive control (alkaline water)	0.7 ± 0.1	2.8 ± 0.1	1.4 ± 0.3	1.6 ± 0.1
Vitamin D3 + acid water	1.2 ± 0.03*#	1.2 ± 0.01*	0.8 ± 0.01	1.0 ± 0.02
Vitamin D3 + Alkaline Water	0.6 ± 0.2	0.8 ± 0.2*#	2.0 ± 0.3	0.2 ± 0.01*#
Vitamin D3 + Prednisolone + Acid Water	1.2 ± 0.5	1.2 ± 0.2	0.7 ± 0.01*#	1.6 ± 0.3
Vitamin D3 + Prednisolone + Alkaline Water	0.8 ± 0.2	0.5 ± 0.02*#	1.3 ± 0.1	0.1 ± 0.1#
Prednisolone	1.0 ± 0.2	1.2 ± 0.1	0.6 ± 0.2	1.0 ± 0.2

*represents a difference from the negative control group
represents a difference from the positive control group

Discussion

After receiving medication for 24 hours, none of the animals was killed by these high doses. This outcome could be explained by the fact that vitamin D is absorbed over several days and builds up in fatty tissue until it reaches the blood level at which toxicity and death symptoms start to manifest. It appears that calcium is the key factor contributing to vitamin D intoxication (2).

Reduced cholinergic transmission, which in turn affects learning and cognition as seen in rats, may be the cause of decreased motor activity and neurological behaviour in open field rats when using acidic water with vitamin D3. Other possible causes include the influence of several neurotransmitters necessary for integrating learning and memory functions in the brain, including acetylcholine. Vitamin D3 overdose can have toxic and inflammatory consequences as well as affect motor development (18).

While the outcome of neurological behaviour was the opposite when using alkaline water with vitamin D3, this suggests that 1,25 (OH)2 D3 is a significant factor that modifies the synthesis of some neurotransmitters, such as acetylcholine, by increasing the gene expression of the enzyme choline acetyltransferase (CAT) (19). Additionally, increased calcium (Ca) ions in neurons contribute to the increased release of glutamate, the fusing of synaptic vesicles with the presynaptic membrane and the release of transmitters are caused by higher concentrations of these ions in the cytosol (20).

The high level of vitamin D3 in the blood with acidic water showed toxic effects that were recorded for the first time to our knowledge, as we did not find any scientific articles studying this topic, and our study showed that the degree of acidity of drinking water is reflected in the blood, which has a critical role in dealing with high doses of Vitamin D3.

One of our explanations for some of these results is that the acidic environment impedes the functioning of vitamin D3, which was observed to have toxic effects when high levels of vitamin D3 were combined with acidic water. To our knowledge, this was the first time this had been documented.

It is believed that what occurs when the blood's acidic environment has high levels of vitamin D is comparable to what occurs when there is a deficiency in vitamin D (21).

To avoid oxidative stress, 1,25-(OH)D has been demonstrated to raise glutathione levels in neurons in studies using mice. The antioxidant glutathione (GSH), which is produced by neurons and astrocytes, is crucial for defending cells against ROS and the oxidative stress that causes apoptosis (20).

The neuroprotection by the active form of vitamin D reduces cholesterol by preserving the function of vascular endothelial cells and prevents oxidative damage to the central nervous system (22).

When vitamin D3 levels were measured in the group that had been exposed to acidic water, were found to have significantly increased and to have several hazardous symptoms at several levels, where calcium levels were also high. Vitamin D receptors are present in the small and large intestines. Hypercalcemia can result from a high vitamin D intake. Vitamin D levels are associated with its ability to protect neurons from oxidative damage and promote the release of neurotrophic factors. prevention of oxidative damage to the nervous system and calcium homeostasis. Intracellular and neuronal synthesis (23). The high level of malondialdehyde and the decline in glutathione were evidence that raising vitamin D levels had a variety of effects, including an increase in oxidative stress.

As normal physiological pathways might be interrupted and cell death can occur, high calcium concentrations may play a role in oxidative stress (19). Ca signalling mostly accounts for the effect. When there is oxidative stress, Ca crosses the cell membrane from the external environment and the endoplasmic reticulum or sarcoplasmic reticulum into the cytoplasm. Ca enters the mitochondria and nucleus in large quantities when it is present in the cytoplasm in high concentrations. Ca speeds up and interferes with normal metabolism in mitochondria, causing cell death and the production of free radicals (24).

Although the amount of vitamin D3 was decreased by prednisolone, it remained higher than in the two control groups, and this is because prednisolone caused a reduction in the half-life of vitamin D3 in plasma, a reduction in the accumulation of vitamin metabolite (24), Bioactive D3, and a rapid emergence of inactive bioactive metabolic. This led to a continued decrease in the activity and behaviour of the animals when prednisolone was combined with vitamin D3 and acid.

Prednisolone's ability to lower vitamin D3 because it affects its kinetics in the blood and it has been established in humans that glucocorticoids have a significant effect in reducing calcium in cases characterised by taking a large dose of vitamin D and/or hypersensitivity to vitamin D, apparently by reducing intestinal calcium absorption, is consistent with our findings even though the level of calcium was decreased compared to the group not treated with prednisolone (21).

Conclusion

According to the results of this investigation, vitamin D3 toxicity is directly influenced by the acidity of drinking water, while basal water lessens its toxicity and prednisolone lowers its concentration without preventing it. This was kept track of using measures of nervous behaviour and motor activity as well as some indicators of oxidative stress and some other biochemical parameters in rats.

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Conflict of interest

There are no competing interests.

Adherence to Ethical Standards

All ethical approvals for the humane treatment of laboratory animals were submitted by the College of Veterinary Medicine at the University of Mosul from UM.VET.2021.35.

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