Iranian Journal of Pharmaceutical Sciences
2024: 20 (1): 12- 21
https://journals.sbmu.ac.ir/IJPS

Original Article

Assessment of Variations in Particular Immunological and Biochemical Markers in Rats under a Standard and Non-Standard Lifestyle

Kardo Ibrahim Hama Sheikh[a], Shiva Khezri[a*], Ahmed Farhan Shallal[b*]

[a] *Department of Biology, Faculty of Science, Urmia University, Urmia, Iran,* [b]*Department Biology, College of Science, University of Raparin, Rania-Sulaymaniyah, Kurdistan region, Iraq*.

Abstract

This investigation aimed to assess the influence of vitamin D3 and calcium on certain immunological and biochemical factors in rats. Forty-eight male rats were assigned to eight distinct groups. There were two main groups. The first group had standard Diet-Fed rats (Vit. D3, Ca+2, Vit. D3, and Ca+2, Sunlight, and Fasting). The second group had high-fat diet-fed rats (HFD and HFD with Vit. D3 and Ca+2), also compared to the control group. The administration of calcium and vitamin D supplements lasted for six weeks. The levels of vitamin K, IL-10, TNF-α, IgM, and Osteocalcin were determined by applying ELISA. The administration of Vitamin D and calcium has been observed to significantly increase Vitamin D, Vitamin K, and Osteocalcin levels in the rats fed on the typical diet. In contrast, sunlight exposure and fasting for the same duration did not substantially impact serum vitamin D and Osteocalcin in rats fed a normal diet. Additionally, a significant reduction in the concentration of Vitamin K in the serum was detected in the experimental rats fed on a normal diet and subjected to sunlight and fasting. The administration of HFD for six weeks was found to provoke hyperglycemia in experimental rats. However, it did not elicit any significant influence on the concentration of vitamin D, vitamin K, and osteocalcin. Furthermore, using calcium and vitamin D for six weeks negatively impacted immune disturbances in rats consuming a normal diet (ND) or HFD by regulating anti-inflammatory cytokine (IL-10) secretion.

Keywords: TNF-α; ND; HFD; IL-10; Vitamin D; Vitamin K; Osteocalcin.

1. Introduction

Food is crucial to a person's overall health since it provides the necessary nutrients to support vital bodily functions. Its nutritional value has been evaluated throughout history by analyzing the nutrients it contains and the extent to which it can be digested, absorbed, and utilized [1]. Consuming food or food products is a vital and regular element of everyday living. Conceptions of well-being, wholesome sustenance, and meals are inextricably linked to the selection of edibles and are incorporated into communal existence, regulated by the

Corresponding Authors: Shiva Khezri, Department of Biology, Faculty of Science, Urmia University, Urmia, Iran. E-mails: sh.khezri@urmia.ac.ir. AND Ahmed Farhan Shallal, Department Biology, College of Science, University of Raparin, Rania-Sulaymaniyah, Kurdistan region, Iraq. E-mails: ahmed.farhan@uor.edu.krd.

Cite this article as: Sheikh KIH, Khezri Sh, Shallal AF, *Assessment of Variations in Particular Immunological and Biochemical Markers in Rats under a Standard and Non-Standard Lifestyle*, Iran. J. Pharm. Sci., 2024, 20 (1): 12- 21.
DOI: https://doi.org/10.22037/ijps.v20i1.44182

nutritional value of comestibles [2]. Health is of immense importance, and as a result, the state of one's health is significantly impacted by food consumption. Notably, consumers are becoming increasingly cognizant of this fact [3]. The uptake of a high-fat diet produces a desirable energy balance, causing a rise in body weight as it encourages fat accumulation rather than its oxidation for energy consumption.

Additionally, the application of a high-fat diet (HFD) in albino rats has been noted to trigger the emergence of diabetes, hyperlipidemia, and oxidative stress, making it a significant model for replicating human obesity [4]. It has been widely accepted that the administration or prolonged consumption of a diet with high-fat content can elicit hyperphagia, hyperglycemia, hyperlipidemia, and insulin resistance, as well as a considerable augmentation in mammal body mass [5]. Consistent and daily consumption of vitamins is imperative for preserving and advancing optimal health. Typically, vitamin insufficiencies result in severe health complications, such as hindering normal growth and development. The significance of Vitamin D cannot be overstated, as it has a crucial role in the human body's functions. Its inadequacy can lead to numerous disorders, disrupting or reducing immune function [6, 7]. Besides, Vitamin D, a pivotal regulator of calcium metabolism, has fundamental processes for various physiological functions [8, 9]. In order to enhance the absorption and bioavailability of calcium in the bloodstream, it is often recommended to take it in conjunction with vitamin D3. Research conducted by Wasilewski et al. (2019) has demonstrated, through various studies, that calcium supplementation can improve bone mineral density and reduce the likelihood of fractures [10]. The regulatory effect of vitamins D and K2 maintains the body's homeostasis of phosphorus and calcium ions. Deficiencies in these nutrients can lead to harmful bone diseases, resulting in the recommendation for calcium supplementation with vitamin D. The combination of vitamins D and K2 confers benefits in promoting bone health and preventing bone disorders associated with aging, namely osteoporosis. Vitamin K2 as a therapeutic intervention can enhance bone health and mitigate the risk of fractures in individuals with osteoporosis, thereby amplifying the impact of calcium and vitamin D supplementation [11]. Thus, this investigation was conducted to study the comparative roles of calcium and vitamin D3 supplementation, either alone or in combination, on selected biochemical and immunological parameters in albino rats that were fed standard and high-fat, high-sugar diets for six weeks, following the statements mentioned above.

2. Materials and Methods

2.1. Animals breeding (Housing)

Rattus norvegicus species (male, eight weeks, and weighing between 150-200 grams) were used for the study, with a sample size of forty-eight. The rationale behind selecting male subjects was to avoid the physiological changes that occur during the female menstrual cycle, which repeats every 4-6 days [12]. The research was performed within the Department of Medical Laboratory Science animal facility at Raparin University's College of Science

between April and June 2022. The rodents were accommodated in plastic enclosures furnished with wooden shavings, and the number of rats per cage was six during the experimental period. The housing conditions were consistent with standard laboratory settings, including a photoperiod consisting of 12 hours of light followed by 12 hours of darkness, in addition to a temperature maintained at 22 ± 4 Cº. *Rattus norvegicus* was provided with standard pellets and tap water ad libitum. A standard pellet was created using a computer program that relied on Pico Lab. Rodent Diet 20 was used and contained oil-sun flower (4.4%), soya (25.6%), wheat (66.6%), salt (0.63%), limestone (1.5%), methionine (0.158%), trace elements (0.05%), and choline chloride (0.062%) [13]. The high-fat diet was formulated according to the procedures outlined in Ibrahim's study (2021). It was administered to the experimental rats as a standard diet. This diet contains a fat content of 12% in terms of energy, whereas the normal diet (ND) contains only 4% fat [14]. This study was approved by the Ethics Committee of Raparin University (Code: 7094-30-10-2023).

2.2. Experimental design

The rats were segregated into eight groups, each consisting of six individuals. The first group acted as a control group and was provided with a typical diet. The second group was given a conventional diet fortified with Vitamin D3, while the third group was given a conventional diet supplemented with Calcium. The fourth group was given a conventional diet augmented with Calcium and Vitamin D3. The fifth group was exposed to natural sunlight, while the sixth group was subjected to a period of abstention from food. The seventh group was given a high-fat diet (HFD: The high-fat diet was prepared following the methods delineated in the investigation carried out by Ibrahim (2021). It was administered to the experimental rats as a diet that was freely accessible and standardized. This specific diet consists of a lipid composition equivalent to 12% of the total energy, whereas the regular diet (ND) solely comprises 4% lipids [14]), and the eighth group was provided with an HFD that was fortified with both Calcium and Vitamin D3. Vitamin D was orally administered via gavage, utilizing a solution comprised of 50.000 IU combined with 50 ml of normal saline at a ratio of 1 ml per rat per week. On the other hand, calcium was provided through dietary supplementation at a concentration of 0.026% w/w. Supplementary intake of calcium and vitamin D was facilitated over six weeks.

2.3. Methods

Table 1 displays the different materials employed in the ongoing investigation.

Table 1: The chemicals and kits.

No.	Chemicals and kits	Supplier and Company
1	Glucose Kit	Roche-Germany
2	Vitamin D Kit	
3	Vitamin K Kit	Bioassay Technology Laboratory-China
4	Osteocalcin Kit	
5	TNF-α Kit	
6	Interleukin-10 kit	
7	Di-ethyl ether	Scharlau-Spain
8	Distilled water	Local preparation

After the completion of the six weeks, the rats underwent an overnight period of fasting and were administered anesthesia using Di-ethyl ether. Subsequently, a sample was collected via cardiac puncture and separated into cooled

tubes containing ethylenediaminetetraacetic acid (EDTA) (4.5 mM) as an anticoagulant for hematological analysis, and gel tubes for serum placed in a water bath (37◦C) for 15 minutes, before ultimately being centrifuged at 3000 rpm for a period of 30 minutes [15]. A complete blood count (CBC) analyzer was implemented to quantify the total count of white blood cells. In contrast, the automated biochemistry analyzer was utilized to evaluate serum glucose and vitamin D. The application of the Enzyme-Linked Immunosorbent Assay was utilized in the analysis of the concentrations of vitamin K, Immunoglobulin M (IgM), Interleukin-10, Tumor Necrosis Factor-alpha, and Osteocalcin.

2.4. Statistical analysis

Statistical Package for the Social Sciences (SPSS) was utilized to analyze the data. Results were expressed as Mean±standard error of the mean (mean±SEM). After analyzing variance (ANOVA), statistical differences were determined using Duncan's test. A P value of 0.05 was selected as the threshold for significance across all results.

3. Results and Discussion

An administration of calcium alone, as well as combinations of vitamin D and calcium with exposure to sunlight over six weeks, the outcome led to a significant rise ($P<0.05$) in the concentration of glucose in the blood serum among rats fed a standard diet, with respective values of 99.33±0.84, 101.00±1.06, and 98.67±1.64, as compared to the non-treated group (91.33±2.33). Conversely, treatment with vitamin D alone (94.83±1.53) and fasting did not yield statistically significant alterations (**Table 2**). Our study investigated the link between serum vitamin D3 concentrations and dietary interventions in rats. It was observed that rats receiving a normal diet exhibited no significant changes in their serum vitamin D3 levels when subjected to vitamin D3 and Ca+2 supplements, sunlight exposure, or fasting (51.48±14.01, 35.50±1.60, 47.28±13.00, 45.15±1.51, and 36.33±0.98, respectively) in comparison to untreated rats (32.83±1.32) (Table 2). The findings of this study suggest that vitamin D3 supplementation, either alone or in combination with calcium, results in a noteworthy increase ($P<0.05$) in vitamin K levels (70.62±2.04 and 64.12±1.14, respectively).

Table 2: Effects of Vitamins D3 and Ca+2 supplementations, sunlight exposure, and fasting. *significant

Parameters	Standard Diet-Fed Rats					
	Control	Treatment Groups				
		Vit. D_3	Ca^{+2}	Vit. D_3 & Ca^{+2}	Sunlight	Fasting
Sugar (gm/dl)	91.33±2.33 ^{a}	94.83±1.53 ^{a}	99.33±0.84 ^{bc}	101.00±1.06 ^{c}	98.67±1.64 ^{bc}	90.50±1.11 ^{a}
Vitamin D (ng/ml)	32.83±1.32 ^{a}	51.48±4.01 ^{a}	35.50±1.60 ^{a}	47.28±3.04 ^{a}	45.15±1.51 ^{a}	36.33±0.98 ^{a}
Vitamin K (ng/ml)	49.73±1.55 ^{b}	70.62±2.04 ^{d}	42.36±0.68 ^{a}	64.12±1.14 ^{c}	43.56±0.89 ^{a}	42.60±0.84 ^{a}
Osteocalcin (ng/ml)	22.83±1.30 ^{a}	26.67±1.43 ^{b}	36.00±0.57 ^{d}	37.83±0.83 ^{d}	25.67±0.66 ^{ab}	23.67±0.88 ^{ab}

The same letters mean no significant differences, while the different letters mean significant differences.

In contrast, treatment with calcium alone, exposure to sunlight, and fasting led to a significant reduction (P<0.05) in vitamin K levels (42.36±0.68, 43.56±0.89, and 42.60±0.84, respectively) as compared to the control group (49.73±1.55) (Table 2). The levels of Osteocalcin exhibited a significance elevating (P<0.05) was observed in the groups subjected to treatment with vitamin D3 and calcium, both independently and in combination, with values of 26.67±1.43, 36.00±0.57, and 37.83±0.83, respectively, in comparison to the group of rats that were not treated (22.83±.30). Conversely, the groups subjected to sunlight exposure and fasting did not exhibit significant alterations, with values of 25.67±0.66 and 23.67±0.88, respectively (Table 2). Significantly, the serum glucose level was elevated (P<0.05) by the use of a high-fat diet (226.5±2.98) in comparison to rats that were given a standard diet (91.33±2.33). However, the use of a vitamin D-calcium treatment along with the high-fat diet resulted in a significant reduction (P<0.05) in the concentration of glucose (199.67±2.48) when comparing rats that were fed a diet high in fat without any treatment (91.33±2.33) (**Table 3**). The study outcomes indicate no noteworthy variation in the serum vitamin D levels (39.66±1.05) between the group that consumed a diet high in fats and the group that consumed a diet following standard guidelines (32.83±1.32). Moreover, the concurrent administration of vitamin D and calcium to rats fed a high-fat diet (57.95±15.85) did not yield a notable alteration in vitamin D levels compared to the high-fat diet group (Table 3). Vitamin K levels in serum indicate any notable disparity among the groups regarding the standard and high-fat diets, which were found to be 49.73±1.55 and 49.88±0.34, respectively. However, when rats on a high-fat diet were given co-treatment of vitamin D and calcium, there was a remarkable increase (P<0.05) in their serum vitamin K levels, which were measured at 64.35 ± 0.88, in contrast to the high-fat diet group that was not subjected to any treatment (Table 3). No noteworthy alterations in the levels of Osteocalcin detected in the serum were noted among rats that ingested a diet with high-fat content (24.00±0.89) compared to rats that consumed a normal diet (22.83±1.30). Nevertheless, the co-administration of vitamin D and calcium was significantly related to a significant (P<0.05) increase in Osteocalcin concentrations (37.50±1.11) (Table 3). In this study, rats were subjected to varying treatments, including vitamin D, vitamin D with calcium, and fasting.

Table 3: Effects of Vitamin D_3 and Ca^{+2} co-supplementations on some biochemical aspects in rats fed on a high-fat fat-diet (Mean ± SE).

Parameters	Control	High Fat Diet-Fed Rats	
		HFD	Treatment Group
			Vit. D_3 & Ca^{+2}
Sugar (gm/dl)	91.33 ± 2.33 [a]	226.5 ± 2.98 [c]	199.67 ± 2.48 [b]
Vitamin D (ng/ml)	32.83 ± 1.32 [a]	39.66 ± 1.05 [a]	57.95 ± 5.85 [a]
Vitamin K (ng/ml)	49.73 ± 1.55 [a]	49.88 ± 0.34 [a]	64.35 ± 0.88 [b]
Osteocalcin (ng/ml)	22.83 ± 1.30 [a]	24.00 ± 0.89 [a]	37.50 ± 1.11 [b]

The same letters mean no significant differences, while the different letters mean significant differences.

Table 4: Effects of Vitamins D_3 and Ca^{+2} co-supplementations on some biochemical aspects in rats fed on a high-fat diet (Mean ± SE).

Parameters	Standard Diet-Fed Rats					
	Control	Treatment Groups				
		Vit. D_3	Ca^{+2}	Vit. D_3 & Ca^{+2}	Sunlight	Fasting
Total WBC (Cell/cumm^3)	5±0.11^{ab}	8.9±0.1^{d}	5.21±0.11^{b}	9.28±0.08^{e}	4.85±0.08^{a}	7.81±0.17^{c}
IL-10 (pg/ml)	0.04±0.02^{ab}	0.07±0.001^{b}	0.05±0.001^{b}	0.07±0.001^{b}	0.01±0.001^{a}	0.01±0.001^{a}
TNF- α (ng/L)	48.68±0.43^{c}	53.52±0.54^{d}	54.22±0.86^{d}	63.21±0.84^{e}	46.76±0.32^{b}	43.29±0.59^{a}
IgM (ng/ml)	5.83±0.47^{a}	9.17±0.47^{b}	7.17±0.30^{a}	10.67±0.66^{c}	8.83±0.40^{b}	9.67±0.49^{bc}

The same letters mean no significant differences, while the different letters mean significant differences.

The results indicated there was a significant rise ($P<0.05$) observed in the overall leukocyte count in the rats that underwent these treatments (8.900±0.100, 9.283±0.087, and 7.817±0.177, respectively), in contrast to the group that was not treated. On the contrary, the groups treated with calcium and sunlight (5.217±0.110 and 4.850±0.084, respectively) did not exhibit significant changes (**Table 4**). No statistically significant alteration was observed in the levels of serum interleukin-10 across all treatments when compared to a control group exhibiting standard levels (Table 4). TNF-α values were observed to be significantly elevated ($P<0.05$) in the vitamin D and calcium groups after comparing to the control group, separately and in combination therapy modes (53.520±0.54, 54.223±0.86 and 63.211±0.84 respectively). Conversely, a significant reduction (p-value<0.05) was noted among the experimental groups subjected to exposure to sunlight and fasting (46.768±0.32 and 43.296±0.59, respectively) (Table 4). A noteworthy increase ($P<0.05$) in the concentrations of immunoglobulins in the blood serum was recorded in all treatment groups except the calcium-treated group (7.17±0.30), which did not exhibit any significant changes (Table 4). There was no statistically significant difference ($P>0.05$) in the total white blood cell (WBC) parameter between the rats from the normal control group and the high-fat diet (HFD) group, which were fed a standard and high-fat diet, respectively, with mean values of 5.000±0.115 and 5.333±0.14. Compared to the high-fat diet (HFD) group, the co-treatment of Vitamin D3 and Calcium resulted in a statistically significant increase ($P<0.05$) in the total count of white blood cells (**Table 5**). There were no noteworthy alterations observed in the IL-10 levels between the cohorts that were given the standard diet, those that were exclusively fed a high-fat diet, and those that received a high-fat diet along with vitamin D and calcium supplementation (0.044±0.029, 0.014±0.00, and 0.060±0.00, respectively) (Table 5). The consumption of a high-fat diet for six weeks was found to be significantly associated ($P<0.05$) with an increase in the parameters of both TNF- α and IgM (55.24±0.49 and 2.33±0.49 respectively), as compared to rats that were fed a normal diet (48.68±0.43 and 5.83±0.47 respectively).

Table 5: Effects of Vitamins D_3 and Ca^{+2} co-supplementations on immunological aspects in rats fed on a high-fat diet (Mean ± SE).

Parameters	Control	High Fat Diet-Fed Rats	
		HFD	Treatment Group
			Vit. D_3 & Ca^{+2}
Total WBC (Cell/cumm^3)	5.000 ± 0.115 [a]	5.333 ± 0.14 [a]	8.683± 0.21 [b]
IL-10 (pg/ml)	0.044 ± 0.020 [a]	0.014 ± 0.01 [a]	0.060 ± 0.01 [a]
TNF- α (ng/L)	48.68 ± 0.43 [a]	55.24 ± 0.49 [b]	56.93 ± 0.72 [b]
IgM (ng/ml)	5.83 ± 0.47 [a]	12.33 ± 0.49 [b]	11.00 ± 0.68 [b]

The same letters mean no significant differences, while the different letters mean significant differences.

However, the combined effects of vitamin D and calcium on rats that were fed a high-fat diet did not yield significant outcomes for TNF- α and IgM (56.93±0.72 and 11.00±0.68 respectively), as compared to rats that were fed a high-fat diet alone (**Table 5**).

A diet primarily consisting of refined carbohydrates and saturated fatty acids, with a lack of fibers, minerals, and antioxidant micronutrients, is considered unhealthy and is identified as a contributing risk factor for various diseases [16]. The utilization of dietary supplements has experienced a substantial surge over the last two decades and has become increasingly prevalent in contemporary times to augment physical fitness and regeneration or enhance overall health and well-being [16, 17]. The findings of this study indicate that the serum glucose levels of rats fed a standard diet showed a significant increase in both the calcium and vitamin D treatment groups and the sunlight-exposed groups. However, these levels did not reach the values characteristic of diabetic or hyperglycemic conditions. On the contrary, rats that were subjected to a high-fat diet (HFD) for six weeks exhibited hyperglycemia and eventually developed diabetes when compared to their counterparts who were provided with a standard diet.

Additionally, administering vitamin D and calcium in conjunction resulted in a notable reduction in serum glucose levels in rats subjected to a high-fat diet. The present data exhibited a parallel trend with previous research [18], as noted by Mitrašinović-Brulić et al., wherein the administration of vit-D3 displayed a definitive impact on regulating induced hyperglycemia by reducing serum glucose levels. This subsequently resulted in an indirect influence on insulin secretion through the regulation of calcium transport across the cell membrane. The prolonged administration of a high-fat diet to rodents resulted in visceral adiposity, hyperglycemia, dyslipidemia, insulin resistance, and hepatic steatosis, all clearly associated with human obesity [19]. The potential beneficial impact of vitamin D on insulin resistance and glucose metabolism may be attributed to its ability to activate insulin receptors, regulate calcium homeostasis, modulate cytokine expression, and clarify insulin action in both animal models and cell cultures [20]. The present investigation has demonstrated the beneficial impact of exposure to solar irradiation and refraining from ingestion in maintaining elevated serum vitamin D levels, as confirmed by the obtained outcomes. These approaches could potentially

be recommended to safeguard against or manage pathological conditions that may arise due to vitamin D deficiency. According to [21], the combined effects of vitamin D and vitamin K involve enhancing bone mineralization and improving cardiovascular health. The positive impact of vitamin K on bone mineralization has been expounded [22]. Tsugawa and Shiraki have also elucidated the interactions between vitamin D and K concerning Osteocalcin. Gene expression of Osteocalcin is probably augmented by 1, 25 (OH) 2D, after which the Osteocalcin molecule undergoes a posttranslational modification by vitamin K. The data concerning the quantification of white blood cells, TNF-alpha, and IgM presented a significant increase in the groups that underwent treatment with calcium and vitamin D in both feeding styles and treatment strategies. However, no significant modification was observed in serum IL-10 in either feeding model [23]. Studies conducted by [24-26] report that vitamin D and calcium's proposed potent immunomodulatory effects are primarily due to identifying vitamin D receptors in immune system cells and observing that activated dendritic cells generate vitamin D hormones.

The alterations observed in the inflammatory markers within our study were consistent with prior research. It is plausible that leukocytosis may be attributed to an increase in both IL-6 and TNF-α, which stimulates the release and mobilization of bone marrow neutrophils. Furthermore, neutrophils may be redistributed from surrounding tissues into circulation during inflammatory conditions [27]. The focus of immunological changes encompasses multiple nutritional, metabolic, and endocrine factors [28]. Various studies have established a correlation between HFD consumption and metabolic and immunological complications. In light of the presented findings, it has been suggested that HFD intake increases inflammatory cytokine levels. Furthermore, HFD consumption stimulates adipogenesis, which contributes to obesity. This, in turn, leads to oxidative stress, resulting in lipid peroxidation, cellular injury, and, ultimately, the release of cytokines, specifically TNF-a [29, 30].

4. Conclusion

The positive impacts of calcium and vitamin D interventions on immune and physiological functions were observed in experiments conducted on both standard and high-fat diet feeding.

Acknowledgments

The present research was conducted jointly between Urmia University and the University of Raparin, Rania-Sulaymaniyah, Kurdistan region - Iraq, and we appreciate their support.

Conflict of interest

The authors declare to have no conflict of interest.

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