Med. J. Cairo Univ., Vol. 92, No. 2, Accepted 19/5/2024
DOI: 10.22608/MJCU. 627-640, June 2024
www.medicaljournalofcairouniversity.net

Effect of Vitamin D and Calcium Supplementation on Immune & Inflammatory Responses of Adipose Tissue in Obese Male Albino Rats

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Abstract

Background: Obesity is a significant worldwide health challenge that reults in various complications as well as persistent low-grade inflammation.

Aim of Study: The current study aimed to investigate the possible effects of vitamin D supplementation with or without added calcium on; obesity indices, obesity-induced insulin resistance, resident immune cells and local inflammatory response.

Material and Methods: 32 adult male Wistar albino rats were assigned into 2 groups: (1) The Control group, and (2) Obesity group. Obesity was induced by giving rats high-fat diet (HFD) for four weeks. Obesity was confirmed by BMI. Vitamin D and calcium were supplemented to the obesity subgroups (2B,2C) for 4 weeks. At the end of the study, epididymal adipose tissue was collected and weighed. Serum active vitamin D, fasting insulin and glucose levels were measured, HOMA-IR was calculated then lipase enzyme gene expression, Uncoupling protein UCP2 gene expression, tumour necrosis factor-alpha: (TNF-α), Interleukin-6 (IL6), IL 10 and resident CD4 and CD8 T lymphocytes were measured in the adipose tissue.

Results: Supplementation of vitamin D significantly decreased BMI, weight gain, epididymal fat weight, fasting serum glucose and insulin levels, insulin resistance, adipose tissue TNF-alpha, IL-6 and CD8 T cells. However, it didn't cause a significant change in adipose tissue CD4 T cells. On the other hand, it significantly increased adipose tissue gene expression of UCP-2 and lipase enzyme, adipose tissue IL-10 and serum vitamin D levels. Co-supplementation of calcium together with vitamin D caused significant improvement in obesity indices, adipose tissue UCP2 and lipase enzyme gene expression as well as adipose tissue IL10 compared to vitamin D alone supplementation. However, no added difference was observed in other measured parameters.

Conclusion: Co-supplementation of vitamin D with calcium could have a synergistic effect on reducing body weight and combatting obesity-induced insulin resistance, adipose tissue inflammation and immune disorders.

Key Words: *Obesity – Vitamin D – Calcium – Immune and inflammatory response – Adipose tissue.*

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Introduction

OBESITY is a significantly increasing worldwide problem. It is a major health challenge that can lead to various metabolic complications including insulin resistance (IR), hyperlipidemia, hypertension, and atherosclerosis **[1]**.

A paradigm shift has taken place in understanding the biology of adipose tissue (AT). Adipose tissue is now regarded as an endocrine organ secreting numerous factors which exert specific functions in target tissues. AT seems to play a major & essential role as both a source and site of inflammation **[2]**.

Growing evidence suggests that the primary pathophysiological factor causing metabolic complications in obesity is adipocyte dysfunction. AT regulates metabolism through diverse immunological mechanisms that have given rise to a novel area of research known as immunometabolism **[3]**.

Adipose tissue and the immune system are intimately related. AT is now regarded as an active immunological organ with multiple roles in maintaining overall physiological homeostasis. AT contains immune cells that often exhibit functions different from similar cells in other parts of the body. These resident immune cells are crucial for preserving tissue and immune balance **[4]**. It has been reported that even mild degrees of overweight or obesity induce changes in the immunological function of adipose tissue and cause a shift in the morphologies and numbers of immune cells **[5]**.

Interestingly, it has been revealed that inflammation caused by obesity, mediated by the immune cells in adipose tissue, contributes to the development of obesity-induced IR **[6]**. In addition, obesity promotes the formation of AT fibrosis, a condition linked to IR **[7]**.

Vitamin D generally plays a significant role in regulating the immune system **[8]**. There is strong cellular evidence that vitamin D reduces inflammation **[9]**. Additionally, vitamin D plays a significant role in energy balance. The presence of nuclear and membrane vitamin D receptors in adipocytes indicates that the adipose tissue responds to vitamin D **[10]**. Low vitamin D levels have been suggested to be associated with obesity **[11]**. Nevertheless, there hasn't been enough evidence from clinical trials to conclude the effectiveness of vitamin D supplementation in obesity.

In addition, consistent evidence is present showing that calcium increases the oxidation of fat throughout the body and increases the outflow of fat in feces **[9]**. Some studies suggest that individuals with high calcium intake are less likely to be overweight or obese **[12]**.

However, it's still debatable if obesity and calcium are related. Moreover, vitamin D and calcium were suggested to influence glucose tolerance. The effects of combined vitamin D and calcium supplementation on glucose metabolism have not been extensively studied **[13]**.

The current study aims to investigate the possible effects of vitamin D supplementation with or without calcium on obesity indices, obesity-induced insulin resistance, resident immune cells and local inflammatory response.

Material and Methods

Ethical approval:

The study included thirty-two mature male Wistar albino rats (150-160g). Rats were bred and raised at the animal care unit of the Faculty of Medicine,-Cairo University during 2019. The duration of the study was 8 weeks. The animals were housed under ordinary living conditions (e.g., humidity, temperature, and light/dark cycles); with free access to rat chow and water throughout the study period. All animals' procedures were in accordance with the recommendations for the proper care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee, Cairo University (CU-IACUC) (Approval number: CU-III/F/83-17) in accordance with ARRIVE guidelines.

Animal groups and the study protocol:

The included rats were randomly assigned into 2 groups: (1) The control group (n=8); rats in this group were fed standard rat chow for eight weeks, 2) Obese group (n=24); rats of this group were fed high fat diet (HFD) for four weeks then after obesity was confirmed by BMI calculations, rats were subdivided randomly into the following groups for the next four weeks: Subgroup 2A: Obese rats receiving only HFD (n=8), subgroup 2B: Obese rats receiving HFD and vitamin D (10ug/kg/day) orally, once daily for four weeks (n=8), and subgroup 2C: Obese rats receiving HFD and vitamin D (10ug/kg/day) orally, once daily and Calcium carbonate (1gm/100 gm HFD) orally, daily for four weeks (n=8).

Composition of standard rat chow:

Its composition was 5.4% fat, 53.8% carbohydrate, 21.9% protein, 2.9% fibre, 6.6% minerals, added vitamins A, D, and E, and 0.02% cholesterol (350 kcal/100 g).

Induction of obesity:

In the current study, rats were given a high-fat diet (HFD) for four weeks in order to develop obesity. The HFD consisted of 60% Kcal fat of daily caloric intake **[14]**. Obesity was verified by calculating the Body mass index (BMI) for each group.

Experimental drugs:

a- *Vitamin D supplementation:*

Vitamin D was supplemented in subgroup 2B during the last four weeks of the experimental period using oral gavage. Vitamin D was dissolved in corn oil vehicle at a dose of 10ug/kg/day **[15]**.

b- *Calcium carbonate supplementation:*

Calcium carbonate was co-supplemented with vitamin D in subgroup 2C during the last four weeks of the study. Calcium was supplied in the form of calcium carbonate at a dose of 1gm/100gm HFD daily **[16]**.

Anthropometric measurements:

- Body weight gain: All rats were weighed once a week till the end of the experiment.
- Epididymal fat weight: At the end of the experimental period, Rats were anaesthetized, the abdominal cavity of each rat was opened, and the epididymal fat pads were removed and weighed.
- Calculation of BMI: We measured nose to anus length and body weight for each rat then BMI was calculated using the formula:

  BMI = Body weight (g)/length^2 (cm^2) [17].

  * Length = Nose-anus length (cm).

  * (In rats, obesity is defined as BMI greater than 0.5gm/cm^2).

Sample collection:

At the end of the experimental period, after calculation of BMI, the rats were anaesthetized by 90mg/Kg ketamine-10mg/Kg xylazine cocktail for removal of epididymal fat pads and blood was col-

lected from retro-orbital venous plexus and then rats were killed by cervical dislocation. Serum was separated after blood was centrifuged, then stored at –80 degrees C for further analysis. Serum was used for measuring Active vitamin D level (by ELISA technique), Fasting insulin level (by ELISA technique). Fasting glucose level (by glucose oxidase enzymatic technique). Calculation of insulin resistance index (HOMA-IR) using the following formula:

$$\text{HOMA-IR.} = \frac{\text{Fasting insulin } (\mu\text{IU/ml}) \times \text{Fasting glucose (mmol/L)}}{22.5}$$

A sample of the adipose tissue was collected from each group and subjected to enzymatic degradation to be used for estimation of: Lipase enzyme gene expression (by PCR technique). Uncoupling protein UCP2 gene expression (by PCR technique). Inflammatory markers: tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL6) (by ELISA technique), Anti-inflammatory marker: IL 10 (by ELISA technique), Resident CD4 and CD8 T lymphocytes gene expression (by PCR technique).

Statistical analysis:

Data were coded and entered using SPSS version 25. Quantitative variables were summarized using means and standard deviations, while categorical variables were summarized using frequencies (number of cases) and relative frequencies (percentages). Group comparisons were conducted using analysis of variance (ANOVA) with a post hoc test for multiple comparisons. The Pearson correlation coefficient was utilized to perform correlations between quantitative variables. A *p*-value of less than 0.05 was considered statistically significant.

Results

Obesity indices (weight gain, BMI & epididymal fat weight) & serum vitamin D:

As observed in Table (1) and Fig. (1), HFD-induced obesity caused a significant increase (*p*-value <0.05) in the mean value of the body weight gain in the obese subgroups 2A, 2B & 2C (147.5±4.63 gm, 136.25±5.18 gm & 127.5±7.07 gm respectively) compared to their corresponding value in the control group1(110±0 gm).

Interestingly, administration of vitamin D alone or combined with calcium in subgroups 2B & 2C respectively resulted in a significant decrease (*p*-value <0.05) in the mean value of the body weight gain when compared with their corresponding value in the obese subgroup 2A. Moreover, the addition of calcium to vitamin D in subgroup 2C yielded a significant decrease (*p*-value <0.05) in the mean value of the body weight gain when compared with its corresponding value in the obese subgroup 2B.

Additionally, a significant rise (*p*-value <0.05) in the mean value of BMI was observed in the obese subgroups 2A, 2B & 2C (as shown in Table 1 and Fig. 1) compared to their corresponding value in the control group1 (0.73±0.08, 0.67±0.05 & 0.59±0.04 gm/cm^2 versus 0.45±0 gm/cm^2 respectively).

Table (1): Comparison of the mean values of body weight gain, BMI, epididymal fat weight & serum vitamin D among the studied groups.

	Group 1	Group 2A	Group 2B	Group 2C
Body weight gain (gm)	110±0	147.5±4.63*	136.25±5.18*#	127.5±7.07*#$
Body mass index (BMI) (gm/cm^2)	0.45±0	0.73±0.08*	0.67±0.05*#	0.59±0.04*#$
Epididymal fat weight (gm)	4.14±0.12	5.75±0.32*	4.7±0.13*#	4.56±0.12*#$
Serum vitamin D (ng/ml)	10.96±3.03	2.84±0.84*	7.98±1.2*#	8.62±1.15*#

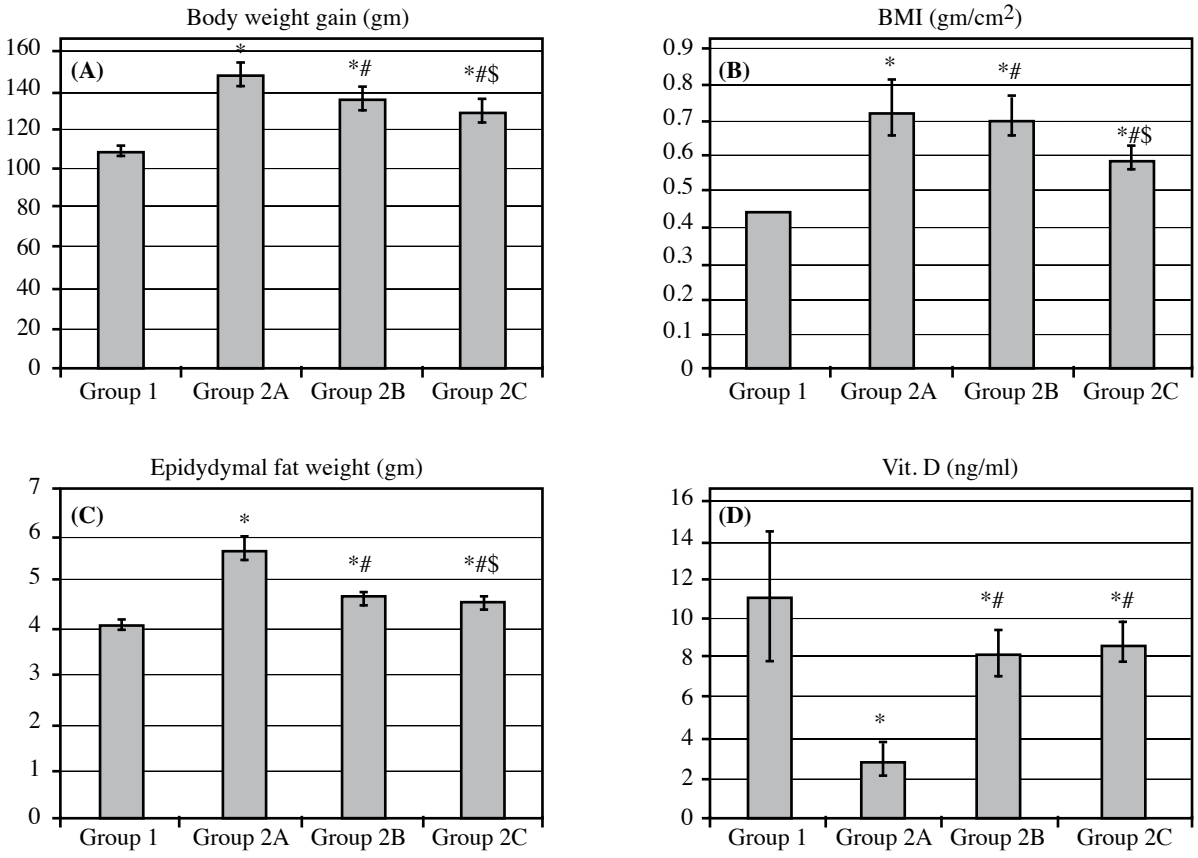
Values are presented as mean ± SD.

*: Statistically significant compared to the corresponding value in group 1 (p<0.05).

#: Statistically significant compared to the corresponding value in group 2A (p<0.05).

$: Statistically significant compared to the corresponding value in group 2B (p<0.05).

Fig. (1): (A) Comparison of the mean values of body weight gain (gm), (B) Body mass index (gm/cm^2), (C) Epididymal fat weight (gm) and (D) Serum vitamin D (ng/ml) among the studied groups.



Values are presented as mean ± SD.
*: Statistically significant compared to the corresponding value in group 1 (p<0.05).
#: Statistically significant compared to the corresponding value in group 2A (p<0.05).
$: Statistically significant compared to the corresponding value in group 2B (p<0.05).

However, supplementation of vitamin D alone or combined with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in the mean value of the BMI when compared to their corresponding value in the obese subgroup 2A (0.67±0.05 & 0.59±0.04 gm/cm^2 versus 0.73±0.08 gm/cm^2 respectively). Moreover, the addition of calcium to vitamin D in subgroup 2C yielded a significant decrease (p-value <0.05) in the mean value of the BMI when compared to its corresponding value in the obese subgroup 2B (0.59±0.04 gm/cm^2 versus 0.67±0.05 gm/cm^2 respectively).

Indeed, a significant increase (p-value <0.05) in the mean value of epididymal fat weight was observed in the obese subgroups 2A, 2B & 2C (as shown in Table 1 and Fig. 1) compared to their corresponding value in the control group 1 (5.75±0.32, 4.7±0.13 & 4.56±0.12gm versus 4.14±0.12gm respectively).

Interestingly, supplementation of vitamin D alone or co-supplementation with calcium in subgroups 2B & 2C respectively resulted in a significant decrease (p-value <0.05) in the mean value of the epididymal fat weight when compared with their corresponding value in the obese subgroup 2A (4.7±0.13 & 4.56±0.12gm versus 5.75±0.32gm respectively).

Moreover, co-supplementation of calcium to vitamin D in subgroup 2C yielded a significant decrease (p-value <0.05) in the mean value of the epididymal fat weight when compared with its corresponding value in the obese subgroup 2B (4.56±0.12gm versus 4.7±0.13gm respectively).

Furthermore, Serum vitamin D was significantly decreased (p-value <0.05) in the obese subgroups 2A, 2B & 2C compared to their corresponding value in the control group (2.84±0.84ng/ml, 7.98±1.2ng/ml and 8.62±1.15ng/ml versus 10.96±3.03ng/ml respectively).

As expected, vitamin D supplementation alone or combined with calcium in subgroups 2B & 2C respectively resulted in a significant increase (p-value <0.05) in serum vitamin D when compared with their corresponding value in the obese subgroup 2A. However, no statistically significant difference was observed between subgroups 2B & 2C.

Adipose tissue uncoupling protein UCP2 and lipase enzyme gene expression:

As observed in Table (2) and Fig. (2), HFD-induced obesity resulted in a significant decrease (p-value <0.05) in the mean value of the adipose tissue UCP2 gene expression in the obese subgroup 2A compared to its corresponding value in the control group 1 (0.48±0.1 versus 1±0.19 respectively).

Table (2): Comparison of the mean values of adipose tissue uncoupling protein UCP2 and lipase enzyme gene expression among the studied groups.

	Group 1	Group 2A	Group 2B	Group 2C
UCP2 gene expression	1.±0.19	0.48±0.1 *	1.62±0.11*#	3.43±0.21 *#$
Lipase gene expression	1.38±0.1	0.74±0.05*	2.4±0.09*#	4.41±0.11*#$

Values are presented as mean ± SD.
*: Statistically significant compared to the corresponding value in group 1 (p<0.05).
#: Statistically significant compared to the corresponding value in group 2A (p<0.05).
$: Statistically significant compared to the corresponding value in group 2B (p<0.05).

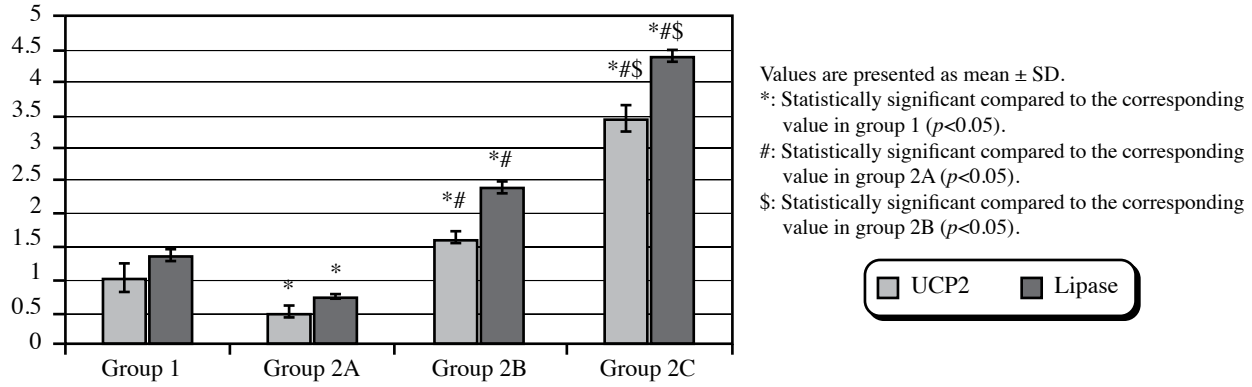


Fig. (2): Comparison of adipose tissue uncoupling protein UCP2 and lipase enzyme gene expression among the studied groups.

Interestingly, supplementation of vitamin D alone or combined with calcium in subgroups 2B & 2C resulted in a significant elevation (p-value <0.05) in the mean value of the adipose tissue UCP2 gene expression when compared to their corresponding value in the obese subgroup 2A (1.62 ±0.11 & 3.43±0.21versus 0.48±0.1 respectively). Not only this, they even become significantly higher than the corresponding value in the normal control group 1 (1.62 ±0.11 & 3.43±0.21 versus 1±0.19 respectively).

Moreover, co-supplementation of calcium with vitamin D in subgroup 2C yielded a significant increase (p-value <0.05) in the mean value of the adipose tissue UCP2 gene expression when compared with its corresponding value in the obese subgroup 2B (3.43±0.21versus 1.62±0.11 respectively).

Moreover, induction of obesity by HFD resulted in a significant drop (p-value <0.05) in the mean value of the adipose tissue lipase enzyme gene expression in the obese subgroup 2A compared to its corresponding value in the control group 1 (0.74±0.05 versus 1.38±0.1 respectively).

However, supplementation of vitamin D alone or combined with calcium in subgroups 2B & 2C resulted in a significant rise (p-value <0.05) in the mean value of the adipose tissue lipase enzyme gene expression when compared to their corresponding value in the obese subgroup 2A (2.4 ±0.09 & 4.41±0.11 versus 0.74±0.05 respectively). Not only this, they even become significantly higher than the corresponding value in the normal control group 1 (2.4 ±0.09 & 4.41±0.11 versus 1.38±0.1 respectively).

Moreover, co-supplementation of calcium with vitamin D in subgroup 2C yielded a significant increase (p-value <0.05) in the mean value of the adipose tissue lipase enzyme gene expression when compared to its corresponding value in the obese subgroup 2B (4.41±0.11 versus 2.4±0.09 respectively).

Serum fasting glucose, fasting insulin and HOMA-IR:

As revealed in Table (3) and Fig. (3), induction of obesity by HFD resulted in a significant increase (p-value <0.05) in the mean value of the serum fasting glucose in the obese subgroups 2A, 2B & 2C compared to their corresponding value in the control group 1 (16.29±1.91, 9.94±1.31 & 9.25±0.97mmol/L versus 5.72±0.97mmol/L respectively).

Table (3): Comparison of the mean values of the serum fasting glucose, fasting insulin and HOMA-IR among the studied groups.

	Group 1	Group 2A	Group 2B	Group 2C
Glucose (mmol/L)	5.72±0.97	16.29±1.91*	9.94±1.31*#	9.25±0.97*#
Insulin (mIU/L)	8.79±0.94	21.49±1.89*	14.12±1.34*#	12.95±0.47*#
HOMA-IR	2.25±0.55	15.61±2.76*	6.25±1*#	5.31±0.51*#

Values are presented as mean ± SD.

*: Statistically significant compared to the corresponding value in group 1 (p<0.05).

#: Statistically significant compared to the corresponding value in group 2A (p<0.05).

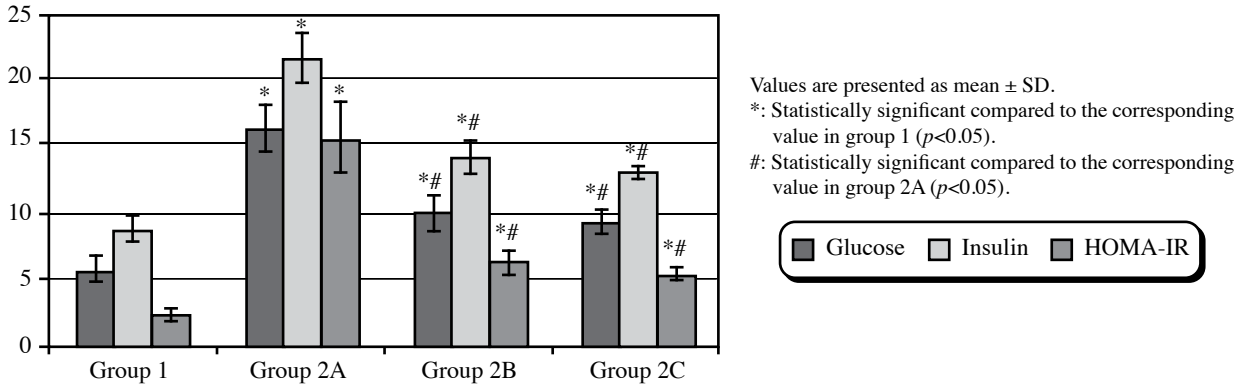


Fig. (3): Comparison of the mean values of the serum fasting glucose (mmol/L), fasting insulin (mIU/L) and HOMA-IR among the studied groups.

Interestingly, vitamin D supplementation alone or combined with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in the mean value of the serum fasting glucose when compared with their corresponding value in the obese subgroup 2A (9.94±1.31 & 9.25±0.97mmol/L versus 16.29±1.91mmol/L respectively).

However, no statistically significant difference was observed in the mean value of the serum fasting glucose between subgroups 2B & 2C denoting no significant effect for adding calcium to vitamin D (9.94±1.31mmol/L versus 9.25±0.97mmol/L respectively).

Additionally, induction of obesity by HFD resulted in a significant increase (p-value <0.05) in the mean value of the serum fasting insulin in the obese subgroups 2A, 2B & 2C (as shown in Table 3 and Fig. 3) compared to their corresponding value in the control group 1 (21.49±1.89, 14.12±1.34 & 12.95±0.47mIU/L versus 8.79±0.94mIU/L respectively).

However, supplementation of vitamin D alone or co-supplemented with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in the mean value of the serum fasting insulin when compared to their corresponding value in the obese subgroup 2A (14.12±1.34 & 12.95±0.47mIU/L versus 21.49±1.89mIU/L respectively).

No statistically significant difference was observed in the mean value of the serum fasting insulin between subgroups 2B & 2C denoting no significant effect for adding calcium to vitamin D (12.95±0.47mIU/L versus 14.12±1.34mIU/L respectively).

Furthermore, the mean value of HOMA-IR (as shown in Table 3 and Fig. 3) was significantly (p-value <0.05) increased by HFD in the obese subgroups 2A, 2B & 2C compared to their corresponding value in the control group 1 (15.61±2.76, 6.25±1 & 5.31±0.51 versus 2.25±0.55 respectively).

However, supplementation of vitamin D alone or co-supplemented with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in HOMA-IR when compared to their corresponding value in the obese subgroup 2A (6.25±1 & 5.31±0.51 versus 15.61±2.76 respectively).

No statistically significant difference was observed between subgroups 2B & 2C denoting no significant effect for adding calcium to vitamin D (6.25±1 versus 5.31±0.51 respectively).

Adipose tissue inflammatory indices: inflammatory markers (TNFα, IL-6) and anti-inflammatory marker (IL-10):

As observed in Table (4) and Fig. (4), induction of obesity by HFD resulted in a significant increase (p-value <0.05) in the mean values of both adipose tissue inflammatory markers; TNF-alpha and IL-6 in the obese subgroups 2A, 2B & 2C compared to their corresponding values in the control group 1 (TNF-alpha: 107.79±16.43, 68.94±5.51 & 56.29±9.52 pg/ml versus 14.72±1.56 pg/ml respectively; IL-6: 135.61±5.58, 80.74±5.86 & 72.52±11.02 pg/ml versus 31.08±5.72 pg/ml respectively).

Table (4): Comparison of the mean values of adipose tissue tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) among the studied groups.

	Group 1	Group 2A	Group 2B	Group 2C
TNF-α (pg/ml)	14.72±1.56	107.79±16.*	68.94±5.51*#	56.29±9.52*#
IL-6 (pg/ml)	31.08±5.72	135.61±5.5*	80.74±5.86*#	72.52±11.02*#
IL-10 (pg/ml)	144.59±10.7	56.1±4.73*	103.18±11.43*#	117.95±5.13*#$

Values are presented as mean ± SD.
*: Statistically significant compared to the corresponding value in group 1 (p<0.05).
#: Statistically significant compared to the corresponding value in group 2A (p<0.05).
$: Statistically significant compared to the corresponding value in group 2B (p<0.05).

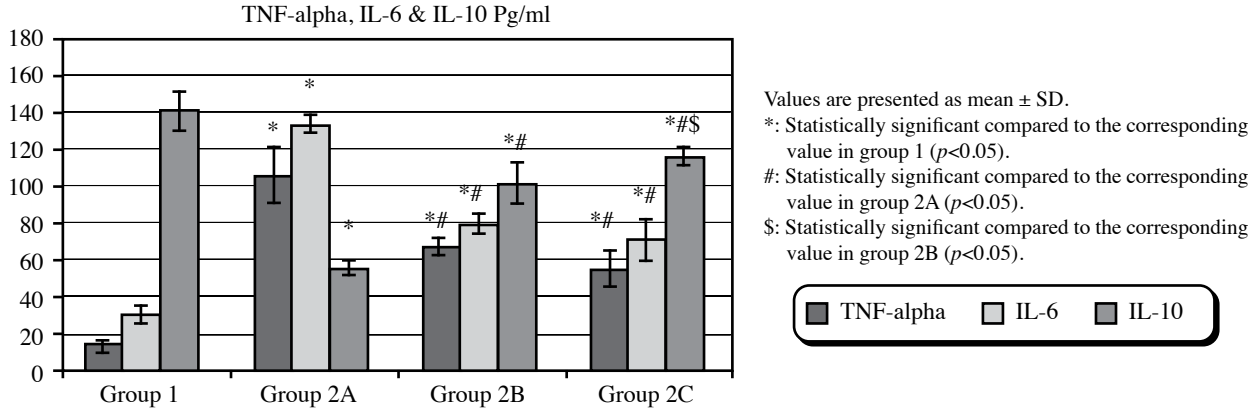


Fig. (4): Comparison of the mean values of adipose tissue tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) among the studied groups.

Interestingly, supplementation of vitamin D alone or combined with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in the mean values of both adipose tissue inflammatory markers; TNF-alpha and IL-6 when compared to their corresponding value in the obese subgroup 2A (TNF-alpha: 68.94±5.51 & 56.29±9.52 pg/ml versus 107.79±16.43pg/ml respectively; IL-6: 80.74±5.86 & 72.52±11.02pg/ml versus 135.61±5.58 pg/ml respectively).

However, no statistically significant difference was observed in the mean values of both adipose tissue TNF-alpha and IL-6 between subgroups 2B & 2C denoting no significant effect for adding calcium to vitamin D (TNF-alpha: 68.94±5.51pg/ml versus 56.29±9.52pg/ml respectively; IL-6: 80.74±5.86pg/ml versus 72.52±11.02pg/ml respectively).

Contrarily, induction of obesity by HFD resulted in a significant decrease (p-value <0.05) in the mean value of the adipose tissue anti-inflammatory marker IL-10 in the obese subgroups 2A, 2B & 2C compared with their corresponding value in the control group1 (56.1±4.73, 103.18±11.43 & 117.95±5.13 pg/ml versus 144.59±10.7 pg/ml respectively).

However, supplementation of vitamin D alone or co-supplemented with calcium in subgroups 2B & 2C resulted in a significant increase (p-value <0.05) in the mean value of the adipose tissue IL-10 when compared with its corresponding value in the obese subgroup 2A (103.18±11.43 & 117.95±5.13pg/ml versus 56.1±4.73pg/ml respectively).

Moreover, co-supplementation of calcium with vitamin D in subgroup 2C yielded a significant rise (p-value <0.05) in the mean value of the adipose tissue anti-inflammatory marker IL-10 when compared to its corresponding value in the obese subgroup 2B (117.95±5.13 pg/ml versus 103.18±11.43 13 pg/ml respectively).

Adipose tissue CD4 and CD8:

As observed in Table (5) and Fig. (5), induction of obesity by HFD resulted in adipose tissue immune cell infiltration with a significant increase (p-value <0.05) in the mean values of both the adipose tissue CD4 and CD8 cells in the obese subgroups 2A, 2B & 2C compared to their corresponding value in the control group 1 (CD4: 51.5±10.86, 49.5±7.07 & 49.75±8.03% versus 26.5±6.63% respectively; CD8: 62.12±12.24, 45.12±7.04 & 38.75±6.63 versus 19.5±3.42 respectively).

Table (5): Comparison of the mean values of adipose tissue CD4 (%), and CD8 (%) among the studied groups.

	Group 1	Group 2A	Group 2B	Group 2C
CD4 (%)	26.5±6.63	51.5±10.86*	49.5±7.07*	49.75±8.03*
CD8 (%)	19.5±3.42	62.12±12.24*	45.12±7.04*#	38.75±6.63*#

Values are presented as mean ± SD.

*: Statistically significant compared to the corresponding value in group 1 (p<0.05).

#: Statistically significant compared to the corresponding value in group 2A (p<0.05).

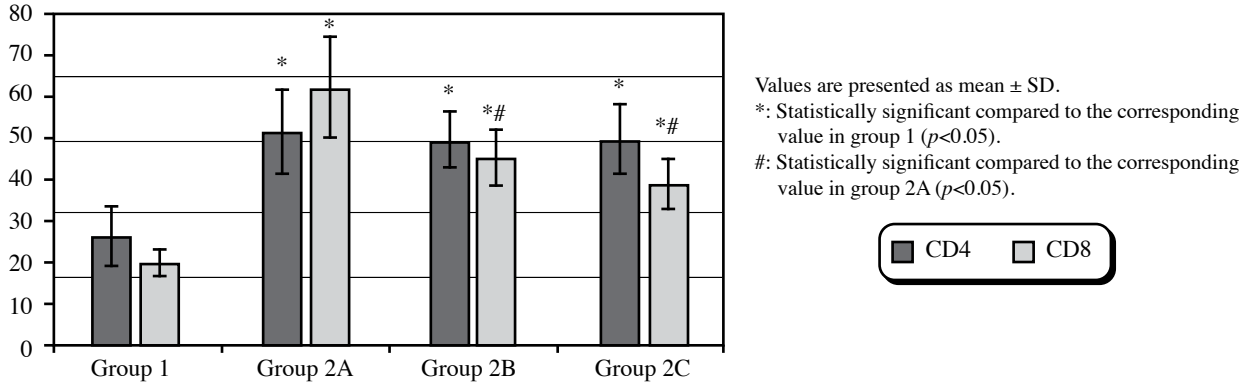


Fig. (5): Comparison of the mean values of adipose tissue CD4 and CD8 among the studied groups.

Interestingly, supplementation of vitamin D alone or combined with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in the mean value of adipose tissue CD8% when compared with their corresponding value in the obese subgroup 2A (45.12±7.04 & 38.75±6.63% versus 62.12±12.24% respectively). However, the mean value of the adipose tissue CD4% didn't change significantly among the 3 groups (2A: 51.5±10.86, 2B: 49.5±7.07 & 2C: 49.75±8.03%).

Moreover, no statistically significant difference was observed in the mean value of the adipose tissue CD8% between subgroups 2B & 2C denoting no significant effect for adding calcium to vitamin D (45.12±7.04% versus 38.75±6.63% respectively).

Discussion

The current study was conducted to investigate the effect of vitamin D supplementation with or without added calcium in obese rats, to explore the crosstalk between the local inflammatory and immune responses in adipose tissue & their contribution to the development of obesity-induced insulin resistance.

As expected, in the current study, HFD feeding yielded a significant increase in the obesity indices when compared to their corresponding values in the control group. This was accompanied by a significant drop in serum vitamin D.

Results of the current study could be explained by the reduced bioavailability of vitamin D in obese individuals being trapped in the expanded adipose tissues with a subsequent decrease in the formation of calcitriol [18]. Another hypothesis for low vitamin D concentrations is that sedentary lifestyles are common in obese individuals who tend to have lower levels of physical activity, resulting in reduced sunlight exposure and decreased endogenous vitamin D synthesis [19].

Other interrelated hypotheses include hepatic steatosis developing in obesity which impairs the metabolism of vitamin D and production of 25- hydroxy vitamin D [20]. Also increased leptin levels hinder the synthesis of 25-hydroxy vitamin D by influencing Vitamin D receptors [21].

Evidence shows that obesity both influences and is influenced by vitamin D metabolism, storage, and

action. Low Vitamin D status is likely to contribute directly to the development of overweight and obesity, according to Mehmood & Papandreou **[22]**. A diet low in vitamin D would change the responses of genes linked to lipolysis, lipogenesis, and adipocyte differentiation **[23]**; as a result, it may be the underlying cause of a worsening of obesity.

In the current study, supplementation of vitamin D significantly decreased the obesity indices, accompanied by a significant rise in serum Vitamin D levels. Consistent with our results, Rosenblum et al. **[24]** demonstrated that supplementation of vitamin D can significantly reduce visceral adipose tissues in obese individuals.

Vitamin D3 supplementation reduces weight gain caused by HFD by increasing lipid oxidation **[25]**. Calcitriol administration modulates peroxisome proliferator-activated receptor α (PPARα), preventing body weight increase caused by a high-fat diet by reducing lipogenesis **[26]**.

Moreover, parathyroid hormone (PTH), which increases intracellular calcium and encourages fat storage in adipose tissue is directly suppressed by vitamin D **[27]**. Also, lower parathyroid hormone levels can lead to weight loss through thermogenesis and lipolysis mediated by the sympathetic nervous system **[28]**. Moreover, 1,25- dihydroxy vitamin D can induce apoptosis in adipocytes **[29]**.

In contrast, some studies found that taking vitamin D supplements had no effect on weight or other body composition metrics **[30]**. Also, Salehpour et al. **[31]** observed no difference in waist circumference or body weight, but they did observe a higher reduction in fat mass in individuals receiving vitamin D compared to the placebo group. It is challenging to draw conclusions from these studies since the majority of them utilized low-dose vitamin D or the subjects may have been taking other vitamin D supplements during the study.

A growing body of evidence has shown that supplementing animals with calcium in their diets has anti-obesity effects **[32]**. However, the effects of calcium supplementation combined with vitamin D or alone, on weight management and metabolic profiles have not been conclusively shown by the findings from randomized controlled trials.

In the present study, co-supplementation of vitamin D and calcium resulted in a further significant decrease in the obesity indices compared to obese rats receiving vitamin D only, which reveals the valuable effect of calcium co-administration along with vitamin D on obesity.

Regulation of adipocyte death (apoptosis), adipogenesis and lipid metabolism are among the proposed mechanisms by which calcium and vitamin D prevent obesity. Additionally, there is evidence that dietary calcium increases the excretion of fecal fat **[28]**. In line with our results, rats fed with chow supplemented with calcium significantly reduced body weight and visceral adipose tissue depots in the epididymus, retroperitonium, and mesentery compared to standard chow fed rats **[33]**. Contrarily, the weight of obese women was not significantly affected by calcium and vitamin D supplements in another clinical trial **[34]**.

Interest has been growing in understanding the mechanism of action of vitamin D and Calcium supplementation on obesity indices. Uncoupling proteins (UCPs) are emerging as new molecular targets for boosting energy expenditure, which could be beneficial for obese patients who find it difficult to follow a regular diet and exercise regimen. In particular, UCP2 is considered a promising candidate gene for obesity and and type 2 diabetes, as it is located on areas linked to obesity and hyperinsulinemia on mouse chromosome 7 and human chromosome 11q13 **[35]**.

In the present study, consumption of HFD caused a significant reduction in adipose tissue UCP2 gene expression which might contribute to the increased adipose mass in our obese rats. However, supplementation of vitamin D resulted in increased UCP2 expression which was even more prominent with the addition of calcium supplementation. Findings of the current study are consistent with that of Mahadik et al., **[36]** who observed desreased UCP2 gene expression and its correlation with HOMA-IR and obesity related parameters in obese and diabetic patients.

Obesity and its related metabolic diseases may be prevented or treated by targeting fat mobilization by decreasing intracellular fat contents and boosting lipolysis.According to results of the present study, mRNA expression of (HSL) enzyme decreased significantly in HFD group compared to that of the control group. On the other hand, lipase enzyme gene expression significantly increased in response to vitamin D treatment and this effect was even more pronounced with the addition of calcium to vitamin D supplementation.

These results support Beydoun et al. **[37]** who illustrated that treatment with 1,25(OH)2D enhances fat mobilization by decreasing intracellular fat contents and raising basal and isoproterenol-stimulated lipolysis. In that study, 1,25(OH)2D increased the expression of the lipolytic enzymes HSL and LPL.

In line with our results, dietary calcium supplementation produced a protective effect against obesity induced by high fat diet in mice by lowering the lipid content intracellularly, upregulating lipolysis gene HSL expression and down regulating lipogenesis genes expression including FAS and LPL **[38]**. Moreover, a meal high in calcium is believed to enhance fat oxidation, encourage apoptosis of fat cell,

and decrease lipid absorption by forming insoluble calcium-fatty acid soaps in the intestine **[39]**.

Moreover, induction of obesity in this study resulted in a significant rise in serum glucose, insulin and HOMA-IR when compared to the control group 1. However, these parameters significantly decreased with vitamin D administration. Noteworthy, there was no significant difference when calcium was added to vitamin D in subgroup 2C.

These findings could be explained by elevated cytokines secretions, dyslipidemia and aberrant adipocyte signaling resulting from fatty acid release into the portal and systemic circulations which is usually associated with visceral obesity **[40]**. However, vitamin D by regulating cytokine expression and activity, mitigates the systemic immune response and enhances the expression of insulin receptors peripherally **[41]**.

In accordance with our results, mice given high calcium, high vitamin D3, or high calcium + vitamin D3, all had higher levels of adiponectin, the hormone that makes adipocytes more sensitive to insulin, and lower plasma concentrations of glucose and insulin **[42]**.

Obesity is tightly linked to systemic chronic inflammation. Notably, the expression of inflammatory genes has been reported to be selectively activated in adipose tissues during the early stages of obesity **[43]**. In the current study the adipose tissue pro-inflammatory markers TNF-alpha, and IL6 increased significantly while IL-10, an anti-inflammatory marker, decreased significantly in the obese untreated rats. However, vitamin D supplementation resulted in a favourable anti-inflammatory effect since TNF-alpha and IL-6 decreased significantly while IL-10 increased significantly. Notably, When calcium was co-supplemented with vitamin D, levels of IL-10 significantly increased while TNF-alpha and IL-6 didn't differ significantly.

Vitamin D has anti-inflammatory actions by inhibiting the NF-KB and the signaling pathways of mitogen-activated protein kinase **[44]**, and by reducing the expression of toll-like receptors **[45]**. These receptors are transmembrane proteins that initiate TNF-alpha-activating classical cascade reactions **[46]**.

T cells are believed to play a crucial role in causing inflammation in adipose tissue. T cell accumulation has been shown to occur in obese adipose tissue in both mouse and human **[47]**, occurring even before the accumulation of macrophages **[48]**. It has been established that the CD4+/CD8+ ratio, which is typically 2:1 in normal mice and humans, is a crucial metric for assessing the intrinsic immune system's immunomodulation status and responsiveness to homeostasis **[49]**.

Results of the present study showed evidence of visceral adipose tissue immune cell infiltration, indicated by a highly significant increase in both CD4% and CD8% but the increase in cytotoxic CD8% was greater than that of CD4 thereby reversing the CD4/CD8 ratio (<1). Interestingly on administration of vitamin D, CD8% decreased significantly with no significant change in CD4% there by CD4/CD8 ratio returned to its control ratio (>1). Noteworthy, the addition of Calcium to vitamin D showed no significant difference.

These results are consistent with an in vivo study suggesting that supplementing with 1,25(OH)2D3 suppresses T lymphocyte proliferation, lowers immunological organ indices and lowers the CD4+/CD8+ T cell ratio in an adjuvant arthritis model **[50]**.

The exact mechanisms by which vitamin D affects the immune system are not fully understood. It has been demonstrated that T cells express vitamin D receptors **[51]**. Moreover, the vitamin D activating enzyme Cyp27B1 is expressed by activated T cells **[52]**. These findings suggest that T cells are not only targets of 1,25(OH)2D3 but can also produce it locally. The cellular immune response is mediated by IL-2, which is secreted by type 1 helper T cells. It also has the ability to stimulate the growth of T, B, and natural killer cells **[53]**. IL-2 might have a role in how vitamin D regulates the immune system. One study revealed that 1,25(OH)2D3 therapy increased the amount of IL-2 released by CD4+ T cells **[54]**.

Conclusion:

Supplementation of vitamin D is effective in reducing obesity indices and combatting obesity-induced insulin resistance, local adipose tissue inflammation and immune dysfunction. Combining dietary Calcium with vitamin D added no benefit to some of the studied biomarkers such as insulin resistance. However, it was beneficial in increasing local adipose tissue anti-inflammatory IL10 levels and reducing the obesity indices probably by significantly upregulating adipose tissue lipase and UCP2 gene expression.

Competing interests: None to be declared.

Funding: This research was not funded.

Author contributions: All the experiments were conducted at the Physiology and Biochemistry Departments of the Faculty of Medicine, Cairo University, Egypt. Reham A. Demerdash, Maha M. Sabry, Heba S. Shoukry and Nagwa M. Ramadan, all shared in the designation, conduction, interpretation of the data, and preparation of the manuscript. All persons designated as authors are qualified for authorship. All authors have approved the final version of the manuscript.

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تأثير فيتامين د والكالسيوم كمكملات غذائية على الاستجابة المناعية والالتهابية للنسيج الدهنى فى ذكور الفئران البيضاء المصابة بالسمنة

الخلفيه البحثيـة: ترتبط السمنة الحشوية بالأمراض الأيضية المزمنة، مثل مقاومة الإنسولين ومرض السكرى من النوع الثانى وحتى الآن لم يتم توضيح الآلية التى تربط السمنة بهذه الأمراض المصاحبة بشكل كامل، ولكن تشير مجموعه متزايدة من الأدلة إلى احتمال تجمع لحالة التهابية مما يؤدى إلى التهاب مزمن وعمليات أكسدة موضعيه داخل الأنسجة الدهنية.

يلعب التهاب الأنسجة الدهنية دورا أساسياً فى حدوث المضاعفات الأيضية المرتبطة بالسمنة. وقد لوحظت الخلايا المناعية المقيمة فى الأنسجة الدهنية، وتم تقدير المساهمة المحتملة لهذه الخلايا فى خلل التمثيل الغذائى فى السنوات الأخيرة.

تشير الدلائل المتزايدة إلى هذا الخلل فى الخلايا الدهنية باعتباره العامل الباثوفسيولوجى الرئيسى للمضاعفات الأيضية فى مرض السمنة. ينبغى اعتماد استراتيجيات وقائية وعلاجية جديدة لمكافحة زيادة الإصابة بالسمنة وأمراض الأيض المصاحبة لها.

الهدف من البحث: دراسة تأثير فيتامين (د) مع أو بدون الكالسيوم كمكملات غذائية على وزن الجسم، مقاومة الأنسولين التى تسببها السمنة، الخلايا المناعية المقيمة فى الأنسجة الدهنية والاستجابة الالتهابية فى الأنسجة الدهنية فى مرض السمنة التى يسببها النظام الغذائى الغنى بالدهون. كما تهدف الدراسة إلى توضيح التفاعل بين التأثير المناعى لفيتامين (د) والكالسيوم واستكشاف إمكاناتهم العلاجية المحتملة فى السمنة وما تسببه من مقاومة للأنسولين.

طرق البحث: تم تقسيم ٣٢ من ذكور الفئران البيضاء البالغه إلى مجموعتين وهم المجموعة الضابطة (تضمنت ٨ فئران) والمجموعة البدينة (تضمنت ٢٤ فأرا) وقد تم تقسيم الفئران فى هذه المجموعة بشكل عشوائى إلى ثلاث مجموعات فرعيه تضمنت كل منها ٨ فئران.

– المجموعة الفرعية (2A): تم تغذيتها نظام غذائى عالى الدهون فقط لمدة أربعة أسابيع.

– المجموعة الفرعية (2B): تم تغذيتها نظام غذائى عالى الدهون وتلقت فيتامين د (١٠ ميكروجرام / كجم / يوم) عن طريق الفم مرة واحدة يومياً لمدة أربعة أسابيع.

– المجموعة الفرعية (2C): تم تغذيتها نظام غذائى عالى الدهون وتلقت فيتامين د (١٠ ميكروجرام / كجم / يوم) وكربونات الكالسيوم عن طريق الفم (١ جرام/١٠٠ جم غذاء عالى الدهون) لمدة أربعة أسابيع.

وفى نهاية الفترة التجريبية، تم جمع عينات الدم والأنسجة الدهنية البربخية من كل مجموعة واختبارها. تم فصل المصل واستخدامه لقياس: مستوى فيتامين (د) النشط، مستوى الأنسولين والجلوكوز فى الدم الصائم، حساب معامل مقاومة الإنسولين (HOMA–IR) فى حين تم جمع و وزن الأنسجة الدهنية البربخية واستخدمت عينة منها فى قياس التعبير الجينى لإنزيم الليباز ولبروتين UCP2، علامات الالتهاب: عامل نخر الورم ألفا (TNF–α)، إنترلوكين–٦ (IL6) والعلامة مضادة الالتهاب: IL ١٠ ونسبة الخلايا اللمفاوية المقيمة CD4 و CD8 .

النتائج: أظهرت نتائج الدراسة الحالية أن تناول فيتامين (د) كمكمل غذائى أسفرعن انخفاض كبير فى متوسط قيم كل من مؤشر كتلة الجسم، زيادة الوزن، وزن الدهون البربخية، مستوى الجلوكوز فى مصل الدم الصائم، مستوى الأنسولين فى مصل الدم الصائم، مقاومة الأنسولين، عامل نخر الورم ألفا (TNF-α)، إنترلوكين-٦ (IL6)، نسبة الخلايا اللمفاوية المقيمة (CD8) فى النسيج الدهنى ولكن لم يلاحظ له أى تأثير على الخلايا اللمفاوية CD4 بالمقارنة مع القيم المقابلة لهم فى المجموعة الفرعية 2A، بينما أسفر عن زيادة كبيرة فى متوسط قيم التعبير الجينى لبروتين UCP-2 وانزيم الليباز وإنترلوكين-١٠ (IL10) فى النسيج الدهنى ومستوى فيتامين (د) فى مصل الدم مما يدل على الآثار الإيجابية لفيتامين (د) كمكمل غذائى على السمنة التى يسببها النظام الغذائى العالى الدهون، حيث حسن توازن الطاقة بالجسم وأثر بشكل خاص على السمنة الحشوية وحسن أيضا مقاومة الإنسولين وفوق كل ذلك عدل الحالة الالتهابية الموضعية والخلل المناعى داخل الأنسجة الدهنية والذى يعد مسؤولا عن المضاعفات الأيضية فى السمنة.

علاوة على ذلك، فقد لوحظ أن تناول الكالسيوم كمكمل غذائى بالإضافة إلي فيتامين (د) أسفرعن انخفاض كبير فى متوسط قيم كل من مؤشر كتلة الجسم، زيادة الوزن، و وزن الدهون البربخية بينما لوحظت زيادة كبيرة فى متوسط قيم التعبير الجينى لبروتين UCP-2 وانزيم الليباز وإنترلوكين-١٠ (IL10) فى النسيج الدهنى مقارنة مع القيم المقابلة لهم فى المجموعة الفرعية 2A. من ناحية أخرى، لم يكن له أى تأثير على متوسط قيم كل من مستوى الجلوكوز فى مصل الدم الصائم، مستوى الأنسولين فى مصل الدم الصائم، مقاومة الأنسولين، عامل نخر الورم ألفا (TNF-α)، إنترلوكين-٦ (IL6)، نسبة الخلايا اللمفاوية المقيمة (CD4) و(CD8) فى النسيج الدهنى مقارنة مع القيم المقابلة لهم فى المجموعة الفرعية 2A.

الاستنتاج: زيادة فيتامين (د)عن طريق المكملات فعال فى تقليل الوزن، مقاومة الإنسولين المصاحبة للسمنة وكذلك الحالة الالتهابية الموضعية والخلل المناعى داخل الأنسجة الدهنية. وقد يكون تناول الكالسيوم كمكمل غذائى بالإضافة إلى فيتامين (د) له تأثير معضد.