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 one of the most important health challenges that causes several complications and low-grade chronic inflammation.

Aim of Study: The present 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: Thirty-two adult male Wistar albino rats were assigned into 2 groups: 1) The Control group, and 2) Obesity group. Obesity was induced by the administration of a 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 adipose tissue.

Results: Vitamin D supplementation causes a significant decrease in BMI, weight gain, epididymal fat weight, fasting serum glucose and insulin levels, insulin resistance, adipose tissue TNF-alpha, IL-6 and CD8 T cells but no effect on CD4 T cells was observed compared to subgroup 2A. However, it yielded a significant increase of adipose tissue UCP-2 and lipase enzyme gene expression, IL-10 and serum vitamin D levels.

Co-supplementation of calcium together with vitamin D causes significant improvement in obesity indices, adipose tissue UCP2 and lipase enzyme gene expression as well as 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 one of the most important health challenges that may cause several metabolic complications including insulin resistance (IR), hyperlipidemia, hypertension, and atherosclerosis **[1].**

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

Increasing evidence points to this adipocyte dysfunction as the key pathophysiological factor for metabolic complications in obesity. AT exerts metabolic control through various immunological mechanisms that instigated a new research field termed immunometabolism **[3].**

A close relationship exists between adipose tissue and the immune system. AT is now considered an active immune organ with numerous roles in total 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 modest levels of overweight/obesity elicit modifications in adipose tissue immune function and drive a shift in immune-cell phenotypes and numbers [5].

Interestingly, it has been revealed that obesity-induced inflammation, mediated by immune cells in adipose tissue, participates in the pathogenesis of obesity-induced IR [6]. In addition, obesity favours the development of AT fibrosis, a condition associated with IR [7].

Vitamin D is an important regulator of the immune system in general [8]. Good cellular evidence for vitamin D-reducing inflammation is present [9]. Vitamin D has also been found to be important in energy balance. Nuclear and membrane vitamin D receptors (VDRs) have been demonstrated in adipocytes, suggesting that AT is responsive to vitamin D [10]. Previous studies suggest that low vitamin D is associated with obesity [11]. However, clinical trials supporting the role of vitamin D supplementation in obesity have not been conclusive.

In addition, there is consistent data to support calcium increasing whole body fat oxidation and increasing fecal fat excretion [9]. Some studies show that people with high calcium intake have a lower chance of being overweight and obesity [12].

However, the relationship between calcium and obesity is still controversial. Moreover, vitamin D and calcium were suggested to affect glucose tolerance. Few studies have examined the effects of vitamin D plus calcium supplementation on glucose metabolism [13].

The present work aims to study the effect of vitamin D with or without calcium on obesity indices, obesity-induced insulin resistance, resident immune cells and local inflammatory response.

Material and Methods

1- Ethical Approval:

Thirty-two adult male Wistar albino rats (150-160g) were involved in the study. The animals were bred and raised at the animal care unit of the faculty of medicine, at Cairo University. The duration of the study was 8 weeks May – June 2020. 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.

2- 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 randomly subdivided 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).

3- 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 per 100g).

4- Induction of obesity:

Obesity was induced in the present study by feeding rats a high-fat diet (HFD) for four weeks. The HFD consisted of 60% Kcal fat of daily caloric intake [14]. Obesity was confirmed by measuring the Body mass index (BMI) for each group.

a- Vitamin D supplementation:

Vitamin D was supplemented in the subgroup 2B during the last four weeks of the study. Vitamin D was supplied by oral gavage 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 1 gm/100 HFD daily [16].

6- *Anthropometric measurements:*

- Body weight gain: All rats were weighed weekly until 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: Body weight and nose-anus length were measured for each rat then BMI was calculated by the following formula:

  BMI = Body weight (g)/length2 (cm^2) [17].

  - Length = Nose-to-anus length (cm)
  - (Obesity in rats is considered if BMI >0.5gm / cm^2).

7- *Sample collection:*

At the end of the experiment, after calculation of BMI, the rats were anaesthetized by 90mg/Kg ketamine - 10mg/Kg xylazine cocktail for removal

of epididymal fat pads and blood collection via retro-orbital venous plexus and then killed by cervical dislocation. The blood was centrifuged at 1000 g for 10 minutes. The serum was removed and stored at –80 degrees C for future analysis.

The serum was separated and used for estimation of 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).

Calculated as the product of fasting insulin (microunits/ml) and fasting glucose (mMol/L) divided by 22.5. A lower index indicates greater insulin sensitivity.

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

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).

8- *Statistical analysis:*

Data were coded and entered using the statistical package SPSS version 25. Data was summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test. Correlations between quantitative variables were done using the Pearson correlation coefficient (Chan, 2003b). *p*-values less than 0.05 were considered statistically significant.

Results

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

As observed in Table (1) and Fig. (1), induction of obesity by HFD resulted in 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±4.63gm, 136.25±5.18gm & 127.5±7.07gm respectively) compared to their corresponding value in the control group 1(110±0gm).

Interestingly, vitamin D supplementation 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 increase (*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 group 1 (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*#

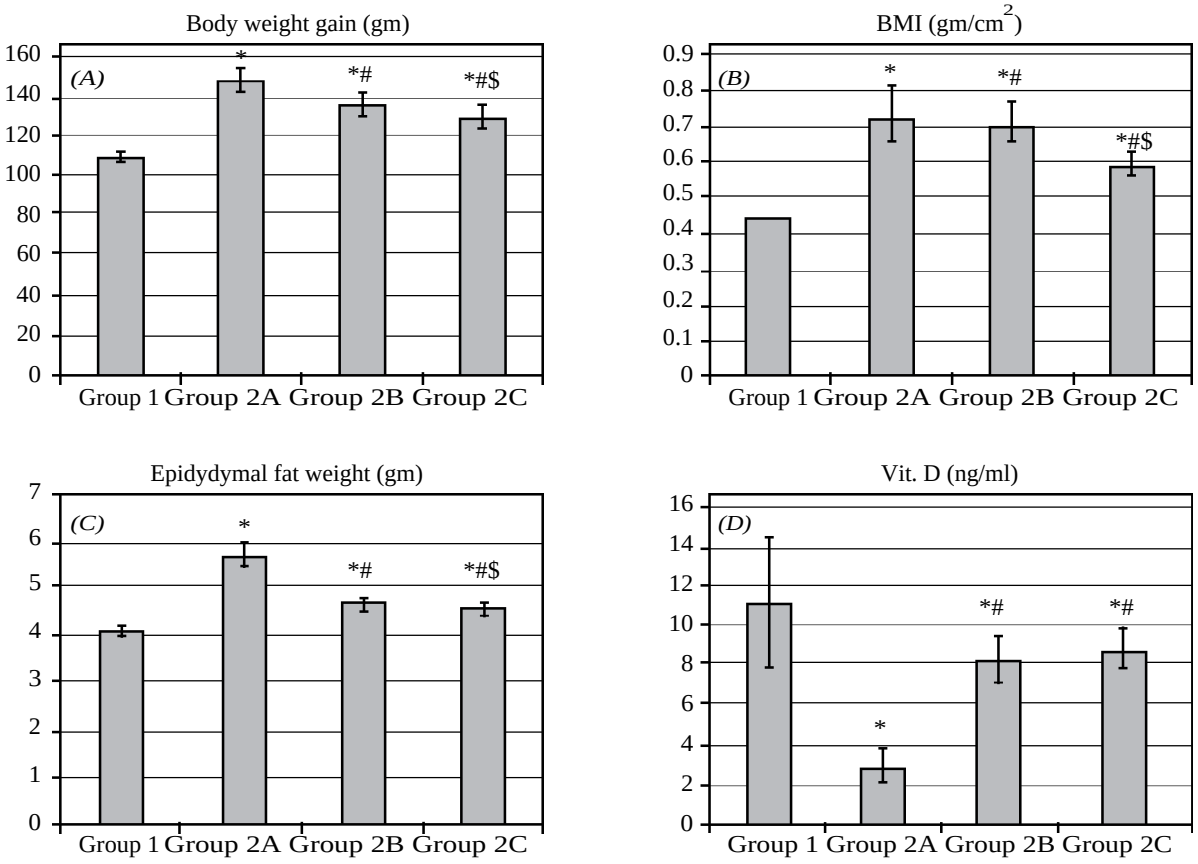
Values are presented as mean ± SD.

*: 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, 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 BMI when compared to their corresponding value in the obese subgroup 2A (0.67±0.05 & 0.59±0.04gm/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±0.04gm/cm^2 versus 0.67±0.05gm/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, vitamin D supplementation alone or co-supplemented 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 sedentary 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), induction of obesity by HFD 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).

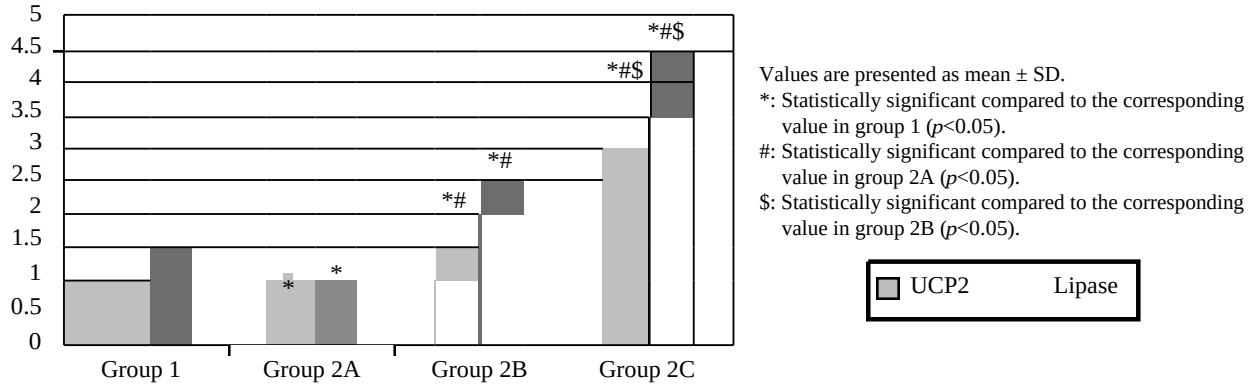


Fig. (2): Comparison of adipose tissue uncoupling protein UCP2 and lipase enzyme gene expression among the studied groups.

Interestingly, vitamin D supplementation alone or combined with calcium in subgroups 2B & 2C resulted in a significant increase (p-value <0.05) in the mean value of the adipose tissue UCP2 gene expression when compared with their corresponding value in the obese sedentary 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±0.21versus 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±0.21 versus 1.62 ±0.11 respectively).

Additionally, induction of obesity by HFD resulted in a significant decrease (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, vitamin D supplementation alone or combined with calcium in subgroups 2B & 2C resulted in a significant increase (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 with 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).
$: Statistically significant compared to the corresponding value in group 2B (p<0.05).

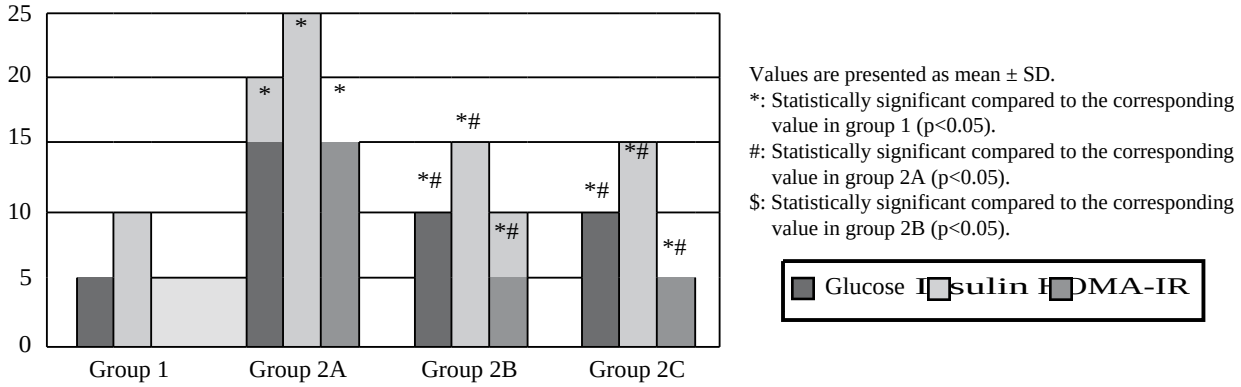


Fig. (3): Comparison of the mean values of the serum fasting glucose (mmol/L), fasting insulin (mIU/L) and HOMA-IR among the studied groups.

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, vitamin D supplementation 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 with 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 Figure 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, vitamin D supplementation alone or co-supplemented with calcium in subgroups 2B & 2C resulted in a significant decrease (p-value <0.05) in HOMA-IR when compared with their corresponding value in the obese sedentary 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±9.52pg/ml versus 14.72±1.56pg/ml respectively; IL-6: 135.61±5.58, 80.74±5.86 & 72.52± 11.02pg/ml versus 31.08±5.72pg/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).

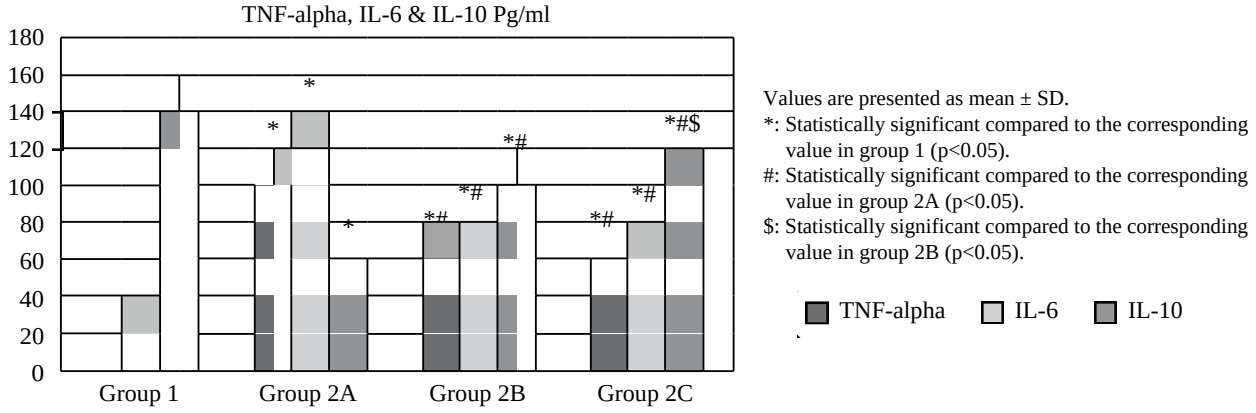


Fig. (4): Comparison of the mean values of adipose tissue tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-10 (IL-10) among the studied groups.

Interestingly, vitamin D supplementation 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 with their corresponding value in the obese subgroup 2A (TNF-alpha: 68.94±5.51 & 56.29±9.52pg/ml versus 107.79±16.43pg/ml respectively; IL-6: 80.74±5.86 & 72.52±11.02pg/ml versus135.61±5.58pg/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).

On the other hand, 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 to their corresponding value in the control group 1 (56.1±4.73, 103.18±11.43 & 117.95±5.13pg/ml versus 144.59±10.7pg/ml respectively).

However, vitamin D supplementation 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 increase (p-value <0.05) in the mean value of the adipose tissue anti-inflammatory marker IL-10 when compared with its corresponding value in the obese subgroup 2B (117.95±5.13pg/ml versus 103.18±11.43 13pg/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).

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

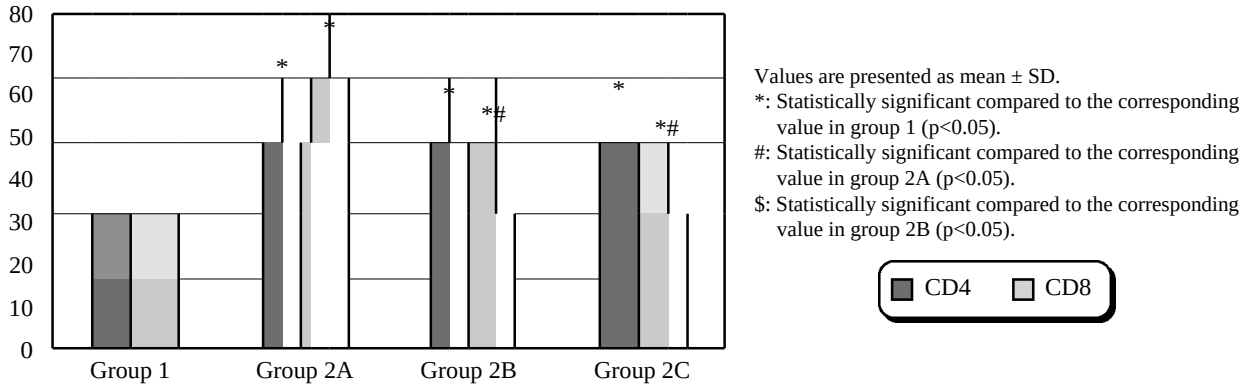


Fig. (5): Comparison of the mean values of adipose tissue CD4 and CD8 among the studied groups.

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 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 3B & 3C 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 with their corresponding values in the control group. This was accompanied by a significant decrease in serum vitamin D.

The results of the present study could be explained by the decreased bioavailability of vitamin D in obesity being trapped in the expanded adipose tissues with a subsequent decrease in the formation of calcitriol *[18]*. Another hypothesis explaining low vitamin D concentrations by the fact that obese people tend to have a sedentary lifestyle and 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 vitamin D metabolism and 25(OH)D synthesis *[20]*. Also, increased leptin levels hinder the synthesis of 25(OH)D by influencing VDR receptors *[21]*.

There is evidence that VD metabolism, storage, and action both influence and are influenced by adiposity. Mehmood & Papandreou *[22]* indicated

that low VD status is likely to contribute per se to the development of overweight/obesity. A deficient supply of VD in diet would alter responses of the genes, related to adipocyte differentiation, lipolysis and lipogenesis *[23]* and therefore could be an underlying cause of obesity exacerbation.

In the present study, vitamin D supplementation significantly decreased the obesity indices, accompanied by a significant increase in serum Vitamin D levels. Consistent with our results, Rosenblum et al. *[24]* have shown that vitamin D supplementation can decrease visceral adipose tissues significantly in obese people.

VD3 supplementation limits weight gain induced by high-fat diets due to increased lipid oxidation *[25]*. Administration of calcitriol regulates peroxisome proliferator-activated receptor α (PPARα), which prevents HFD-induced body weight gain by inhibiting lipogenesis *[26]*.

Moreover, Vitamin D directly suppresses PTH hormone, which promotes fat accumulation in adipose tissue by increasing intracellular calcium *[27]*. Lower PTH levels can also lead to weight loss through sympathetic nervous system-mediated thermogenesis and lipolysis *[28]*. Moreover, 1,25-dihydroxyvitamin D can induce apoptosis in adipocytes *[29]*.

In contrast, some studies reported no changes in weight and other measures of body composition following vitamin D supplementation *[30]*. Similarly, Salehpour et al. *[31]* found no difference in weight and waist circumference, but they did find a greater decrease in fat mass in those treated with vitamin D compared with placebo. Unfortunately, most of these studies involved low-dose vitamin D supplementation or study subjects could have been taking other vitamin D supplementation during the study, so it is difficult to conclude these studies.

A growing body of evidence has demonstrated that dietary calcium supplementation exerts anti-obesity effects in various animals *[32]*. However, findings from randomized controlled trials evaluating the impact of calcium supplementation, with or without vitamin D, on weight management and metabolic profiles remain inconclusive.

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

The suggested anti-obesity mechanisms of Ca and vitamin D include the regulation of adipocyte death (apoptosis), adipogenesis and lipid metabolism. Dietary Ca has been also shown to increase fecal fat excretion *[28]*. In line with our results, rats fed with chow supplemented with calcium significantly reduced body mass and visceral adipose tissue (VAT) mass in the epididymal, retroperitoneal, and mesenteric depots compared to standard chow fed rats *[33]*. In contrast to our findings, supplementation with calcium and vitamin D in another clinical trial did not significantly affect the weight of obese women *[34]*.

There has been increasing interest regarding the mechanism of action of vitamin D and Calcium supplementation on obesity indices. Uncoupling proteins (UCPs) provide new molecular targets for increasing energy expenditure, for the treatment of obese patients for whom dietary restriction and exercise are difficult. In particular, UCP2 has been hypothesized as a promising candidate gene for obesity and T2DM, as it is located on human chromosome 11q13 and mouse chromosome 7, regions associated with obesity and hyperinsulinemia *[35]*.

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

Targeting fat mobilization by reducing intracellular fat contents and increasing lipolysis, has been considered for the prevention and/or treatment of obesity and its associated metabolic disorders. Results of the current study show that the mRNA expression of (HSL) enzyme decreased significantly in the HFD group compared with that of the control group. However, vitamin D treatment was accompanied by a significant increase in lipase enzyme gene expression 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 1,25(OH)2D treatment increases fat mobilization by reducing intracellular fat contents and increasing both basal and isoproterenol-stimulated lipolysis. In that study, lipolytic enzymes HSL and LPL were upregulated by 1,25(OH)2D.

Consistent with our results, dietary calcium supplementation induced a protective effect against HFD-induced obesity in mice by reducing intracellular lipid content, decreasing the expression of lipogenesis genes, such as FAS and LPL, and increasing the expression of lipolysis gene HSL *[38]*. Moreover, a calcium-rich diet is thought to increase fat oxidation, promote fat cell apoptosis, and reduce

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

Moreover, induction of obesity in this study resulted in a significant increase in serum glucose, insulin and HOMA-IR when compared with control group 1. However, vitamin D supplementation caused a significant decrease in these parameters. Noteworthy, no significant difference was observed in adding calcium to vitamin D in subgroup 2C.

These findings could be explained by dyslipidemia, increased cytokines secretions, and abnormal signaling of adipocytes resulting from the release of fatty acid to the portal and systemic circulation which is usually associated with visceral obesity [40]. However, vitamin D increases the expression of insulin receptors in peripheral cells and counteracts the systemic immune response by modulating cytokine expression and activity [41].

In accordance with our results, mice fed high levels of vitamin D3, high calcium or high calcium plus vitamin D3, all had lower plasma concentrations of glucose and insulin along with increased levels of adiponectin, the hormone that sensitizes adipocytes to insulin [42].

Obesity is tightly linked to systemic chronic inflammation. Notably, it has been reported that inflammatory gene expression is selectively activated in adipose tissues during the early stages of obesity [43]. Results of the present study reported a significant increase in the adipose tissue pro-inflammatory markers TNF-alpha, and IL6 while the anti-inflammatory marker IL-10 was significantly decreased in the obese untreated rats. However, vitamin D supplementation resulted in a favourable anti-inflammatory effect with a significant decrease in TNF-alpha and IL-6 and a significant increase in IL-10. Co-supplementation of calcium with vitamin D, no significant difference was observed in TNF-alpha or IL-6 while there was a significant increase in levels of IL-10.

Vitamin D exerts anti-inflammatory effects mediated by the inhibition of the NF-κB and mitogen-activated protein kinase signaling pathways [44], and reduced toll-like receptor expression [45]. The latter are transmembrane proteins that trigger classical cascade reactions leading to the activation of TNF-alpha [46].

T cells are believed to play an important role in initiating inflammation in adipose tissue. Accumulation of T cells has been observed in both mouse and human obese adipose tissue [47], occurring even before the accumulation of macrophages [48]. The CD4+/CD8+ ratio (normally 2:1 in healthy humans and mice) has been recognized as an important indicator for evaluating the state of immunomodulation and response to homeostasis of the intrinsic immune system [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,

Vitamin D supplementation resulted in a significant decrease in CD8% with no significant change in CD4% thereby 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 1,25(OH)2D3 supplementation inhibits the proliferation of T lymphocytes, reduces the immune organ indexes and decreases the ratio of CD4+/CD8+ T cells in a model of adjuvant arthritis [50].

How vitamin D affects the immune system has not been fully elucidated. Previous studies have demonstrated that T cells express VDR [51]. In addition, activated T cells express Cyp27B1 (vitamin D activating enzyme) [52]. Those studies suggest that T cells are not only the targets of 1,25(OH)2D3 but are also able to produce 1,25(OH)2D3 locally. IL-2, secreted by type 1 helper T cells, mediates the cellular immune response and can induce the proliferation of T, B and natural killer cells [53]. IL-2 may participate in the regulatory effects of vitamin D on the immune system. A study showed that IL-2 secreted by CD4+ T cells was enhanced by 1,25(OH)2D3 treatment [54].

Conclusion:

Vitamin D supplementation 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 antiinflammatory IL8 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 البحثيه: 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 أساسيا 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 الى 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 عالي الدهون فقط لمدة أربعة أسابيع.
- المجموعة الفرعية (2B): تم تغذيتها نظام غذائى عالى الدهون وتلقت فيتامين د الفم، لمدة أربعة أسابيع.
- المجموعة الفرعية (2C): تم تغذيتها نظام غذائى عالى الدهون وتلقت فيتامين د.

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 في الدم الصائم، حساب معامل مقاومة الإنسولين (HOMA–IR) فى حين تم جمع و وزن الأنسجة الدهنية البربخية واستخدمت عينة منها فى قياس التعبير الجيني 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 أي 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.