

Body Weight, Insulin Resistance, and Inflammatory Biomarkers in Rats Fed Normal-Fat, High-Fat, and Ketogenic Diets Supplemented with Vitamin D

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ABSTRACT

Ketogenic (KD) and high-fat (HFD) diets and vitamin D (VD) produce variable effects on insulin secretion and body weight (BW), but mechanisms remain unclear. We investigated the effects of normal fat diets (NFD), KD, and HFD with and without VD on BW and serum glucose, insulin, VD, insulin resistance, C-reactive protein, interleukin-6, and tumor necrosis factor-alpha in rats. Three isocaloric NFD, KD, and HFD containing respectively protein-carbohydrate-fat (NFD: 14.8%-75.7%-9.5%; KD:20.2%-10.3%-69.5%; HFD:15.2%-42.7%-42.0%) and three other similar diets but with (1000 IU/kg) VD were used. Forty-five adult male Sprague-Dawley rats were used, 5 rats were sacrificed at the start, remainders were randomly divided into NFD (n=15) and HFD (n=25), and fed for 8 weeks, then 5 rats from each were sacrificed. NFD remainders were divided into 2 subgroups (n=5) and fed NFD and NFD-VD, and HFD remainders were divided into 4 subgroups (n=5) and fed HFD, HFD-VD, KD, and KD-VD for further 8 weeks, then all rats were sacrificed. BW and food intake were measured, food conversion ratio (FCR) was calculated, and biological variables were determined following standard protocols. BW change and FCR (-15.6 ± -10.13 ; 0.033 ± 0.350 respectively) of rats fed KD-VD were the lowest ($P < 0.05$) compared to those fed KD (144.8 ± 1.47 ; 0.189 ± 0.050), HFD-VD (143.0 ± 8.49 ; 0.187 ± 0.100), HFD (155.8 ± 0.3 ; 0.203 ± 0.010), NFD-VD (142.8 ± 6.34 ; 0.183 ± 0.009), and NFD (51.0 ± 1.02 ; 0.074 ± 0.110) respectively. BW change correlated ($P < 0.01$) with food intake ($r = 0.752$), % carbohydrate ($r = 0.292$), and % fat ($r = 0.341$). None of the diets affected other biomarkers. Results clearly show BW-reducing effects for KD-VD that may be mediated by changes in food intake and dietary fat and carbohydrate proportion.

Keywords: Carbohydrate, Bodyweight, Insulin resistance, High-fat diet, Ketogenic diet, Obesity, Rat, Vitamin D.

INTRODUCTION

Obesity is rising worldwide at an alarming rate (Tremmel et al., 2017; Ajlouni et al., 2020). It is a risk factor for several chronic diseases such as insulin resistance, type 2 diabetes, several cancers, and

cardiovascular disease (Leggio *et al.*, 2018). Obesity is considered a state of chronic low-grade inflammation that increases the secretion of several pro-inflammatory factors mainly, C-reactive protein (CRP), interleukin 6 (IL)-6, and tumor necrosis factor-alpha (TNF)- α . This state is known to promote insulin resistance (Leggio *et al.*, 2018). Obesity

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is often associated with remarkable deterioration in eating patterns and behavior related to dietary overload in the form of high-fat diets (HFD) in combination with high carbohydrate intake (Bradley *et al.*, 2017).

Caloric restrictions, behavioral modification and exercise, and infrequency medication are the first-line management for obesity, but the results are not always encouraging (Wadden *et al.*, 2012; Freire, 2020). Variability in responses to obesity treatment is well-documented, but an understanding of why some individuals respond while others do not is limited (Ahmad and Al-Badarein, 2019). Short-term (3-7 days) HFD interventions increase adipose tissue and insulin resistance (Lee *et al.*, 2014), and continued consumption of HFD provokes adipose tissue dysfunction and inflammation state (Lee *et al.*, 2011). However, other studies failed to confirm this (Cummins *et al.*, 2014).

One controversial yet efficient approach to managing obesity has been the application of low-carbohydrate ketogenic diets (KD) (Lamont *et al.*, 2016; Gomez-Arbelaez *et al.*, 2018; Grandl *et al.*, 2018). This diet is a severe restriction of dietary carbohydrates, with a concurrent increase in dietary fat to compensate for the energy deficit resulting in a state of metabolic ketosis. While macronutrient ratios of different KD diets may vary, a typical KD diet contained approximately 20-25% of calories from protein, 60-65% of calories from fat, and 10-15% of calories from carbohydrates (Lamont *et al.*, 2016). This diet can lead to significant weight loss and long-term weight maintenance (Moreno *et al.*, 2016). It targets body fat mass and induces a minor or null reduction in resting metabolic rate, thus preventing weight regain (Gomez-Arbelaez *et al.*, 2018). Despite its beneficial effect on weight loss, KD promotes insulin resistance in humans and animals (Jornayvaz, 2011; Bielohuby *et al.*, 2013; Grandl *et al.*, 2018). KD also contributes to weight loss as well as improves glycemic control and metabolic parameters in type 2 diabetes (Aylward *et al.*, 2014). The reason for this variability could be due to the different experimental protocols and models, diets, and tissue specificity. KD and

HFD do not produce similar changes in insulin action or weight change (Asrih *et al.*, 2015). Whether the change in insulin response or secretion is a direct consequence of KD and HFD or due to other factors remains to be elucidated.

There is increasing evidence that vitamin D (VD) deficiency may contribute to the onset of diabetes mellitus (Wimalawansa, 2018). The findings that relate VD receptor polymorphism to diabetes provide support for this evidence (Sentinelli *et al.*, 2016). There are also indications that VD supplementation may prevent the onset of type 2 diabetes (Sergeev, 2016). Some work has revealed that VD inhibits the production of proinflammatory-cytokines and is positively associated with circulating an anti-inflammatory biomarker (Kim *et al.*, 2013; Tiosano *et al.*, 2013). Obesity is known to associate with VD deficiency (Afzal *et al.*, 2014), although there is no consistent evidence for the causal relationship between these events (Pereira-Santos *et al.*, 2015).

Studies demonstrating the synergistic effects of KDs and VD on body composition are limited. Wortsman *et al.* (2000) were the first to provide strong evidence that VD may become sequestered within adipose tissue. Chronic ketoacidosis results in increased demand for bone minerals for buffering capacity and decreased renal conversion of 25 OH vitamin D to 1, 25 (OH)₂ vitamin D (Sampath *et al.*, 2007). There is only one published report that explored the effect of KD on the developing skeleton and demonstrated reduced bone mineral content in patients on KD when followed up for 15 months (Bergqvist *et al.*, 2008).

While KDs have received considerable attention as an effective weight-loss method, the underlined anti-adipogenic, anti-lipogenic, and anti-inflammatory mechanisms remain unclear. Studies on the combined effects of a KD and VD in human and animal models are also limited. Thus, we investigated the influence of normal-fat diets (NFD), KD, and HFD with and without VD on body weight and serum glucose, insulin, VD, insulin resistance, C-reactive protein, interleukin-6, and tumor necrosis factor in rats.

MATERIALS AND METHODS**Experimental diet preparation:**

Three isonitrogenous and isocaloric diets were prepared and assigned as normal-fat (NFD), high-fat Experimental diet mixtures were prepared according to Reeves (1997). The basic chemical composition of these diets is summarized in Tables (1,2)

(HFD), and ketogenic (KD) diets. Three other similar diets were prepared and supplemented with (1000 IU/ kg diet) vitamin D (Meerza *et al.*, 2012; Yin *et al.*, 2012).

Table 1. Macronutrient and energy content of the experimental diets.

Parameter	NFD	NFD-VD	HFD	HFD-VD	KD	KD-VD
Energy (kJ/100g)	378.2	378.2	378.2	378.2	378.2	378.2
Carbohydrate (%)	75.70	75.70	40.00	40.00	13.20	13.20
Protein (%)	14.80	14.80	14.80	14.80	14.80	14.80
Fat (%)	09.50	09.50	45.20	45.20	72.00	72.00

NFD: Normal-fat diet, HFD: High-fat diet, KD: ketogenic diet, -VD: supplemented with vitamin D

Table 2. The basic ingredient composition of the experimental diets (g/kg).

Parameter	NFD	NFD-VD	HFD	HFD-VD	KD	KD-VD
Egg albumin	140	140	140	140	140	140
Cornstarch	615.5	615.5	278	278	25	25
sucrose	100	100	100	100	100	100
Soybean oil	40	40	190	190	302.5	302.5
cellulose	50	50	237.5	237.5	378	378
Fat-soluble vitamins	4	4	4	4	4	4
Water-soluble vitamins	10	10	10	10	10	10
Mineral mixture	35	35	35	35	35	35
Biotin mixture	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Tert-Butylhydroquinone	0.008	0.008	0.008	0.008	0.008	0.008
VD (IU)	-	1000	-	1000	-	1000

NFD: Normal fat diet, HFD: High-fat diet, KD: ketogenic diet, -VD: supplemented with vitamin D.

Animals' experimentation: Adult male Sprague Dawley rats (200-250g) were used as an animal model in a randomized control design experiment. Rats (n=45) were

obtained from the experimental animal unit at Jordan University, Amman, Jordan. After an adaptation period of 1 week, 5 animals were sacrificed, plasma and serum were

obtained and kept frozen at -20 °C till analyzed. Rats (n=40) were then randomly divided into 2 main groups: the NFD group (n=15) and HFD group (n=25) and fed the corresponding diets for 8 weeks. At the end of this period, 5 rats from each group were sacrificed, plasma and serum were obtained. The remaining rats in the NFD group (n=10) were divided into 2 subgroups (n=5 each), one continued on the NFD and the other was fed NFD-VD. The remaining rats in the HFD group (n=20) were divided into 4 subgroups (n=5 each), one continued on the HFD and the others were fed HFD-VD, KD, and KD-VD. The feeding period for this part of the experiment was 8 weeks (Kennedy *et al.*, 2007).

Experimental diets and water were provided *ad libitum* during the study. Rats were individually housed in a well-ventilated room with a constant temperature of 25°C and a 12-hour dark/12-hour light cycle. Body weights were measured once every 2 weeks and food intake was measured every 3 days. At the end of the experimental period, rats were fasted for 8 hours and anesthetized by carbon dioxide. Blood was collected by cardiac puncture in a heparinized tube, centrifuged to obtain plasma, and kept frozen at -20°C till analysis. All the experiments involving animals were approved by the Institutional Animal Ethics Committee and carried out according to the recommended guidelines for animal care and use (National Research Council, 2011).

Biochemical analyses: Standard biochemical kits procedures were followed for the determination of glucose (glucose GOD-PAP, BIOLABS), insulin (MBS 760915 Rat Insulin ELISA Kit), vitamin D (MBS2503525 Vitamin D ELISA Kit), and the inflammatory biomarkers: C-reactive protein (Rat C-Reactive Protein ELISA 88-7501), interleukin 6 (Mouse IL-6 Uncoated ELISA 88-7064) and tumor necrosis factor-alpha (Rat Tumor Necrosis Factor ELISA Kit ®, lot number 1125150743, Ray-Bio, USA).

Insulin resistance was evaluated by the homeostasis model assessment of insulin resistance, [Insulin resistance (HOMA-IR) = fasting insulin (μ/ml) X fasting glucose (mmol/l)/ 22.5] according to Wareham *et al* (1995). Analyses were done in duplicates following the kits manufacturer's instructions and were performed at the nutritional biochemistry laboratories of the Department of Nutrition and Food Technology, University of Jordan, Amman, Jordan.

Statistical analysis: Statistical analyses were performed using (SAS version 9, USA). Data were presented as means ± standard deviation and differences between means were considered as significant at $P \leq 0.05$. Statistical significance for differences in variable means between study groups was computed using ANOVA repeated measures (Diet and time interaction) for weight change and food intake and one-way ANOVA for other variables, followed by turkeys post-hoc test for mean separation. Spearman rank correlation coefficient (r) was used to assess the relationship in the degree of change between weight, food intake, and type of macronutrients and for all variables.

RESULTS

Bodyweight and food intake

Rats fed the NFD gained significantly ($P \leq 0.05$) less weight and had less food intake after 16 weeks of the study (51.0 ± 1.0 , 691 ± 114 g) respectively, compared to other groups (Table 3). The KD-VD rats lost (15.6 ± 10.1 g) and had the lowest ($P \leq 0.05$) food intake (469.6 ± 226.3 g) compared to other groups. Weights of the other groups were significantly ($P < 0.05$) different from the beginning of the study with no significant differences ($P > 0.05$) between groups at week 16.

Table 3. Initial, final, and weight change (g), food intake (g), and food conversion ratio of rats fed different diets for 16 weeks.

	NFD	NFD-VD	HFD	HFD-VD	KD	KD-VD
Initial weight	228±30a	231±35a	233±28a	236± 32a	237± 34a	239± 32a
Final weight	279±36b	373± 36 a	388± 36a	379± 32 a	381± 24 a	223± 33b
Weight change	51.0±1.0b	142.8± 6.3 a	155.8± 0.3a	143.0± 8.5 a	144.8± 1.5a	-15.6± -10.1c
Food intake	691±114b	780± 151 a	768± 163a	773± 154 a	760± 148a	470± 226c
FCR	0.07±0.11b	0.18± 0.09 a	0.20± 0.01a	0.19± 0.10a	0.19± 0.05a	0.03± 0.35 c

Values are means ± standard deviation; FCR: food conversion ratio (weight gain/food intake); Values in rows with different superscripts are significantly different ($p < 0.05$), rats fed (NFD: Normal fat diet, HFD: High-fat diet, KD: ketogenic diet, -VD: supplemented with vitamin D), (n=5)/groups, Repeated measures ANOVA (Diet and time interaction, $P \leq 0.05$). Post hoc test Tukey-Kramer ($P \leq 0.05$).

Biochemical parameters

Findings demonstrated that rats fed the NFD and HFD in the first 8 weeks did not show significant ($P > 0.05$) differences in the mean value of serum glucose (6.1 ± 0.1 vs. 7.4 ± 0.1 mmol/l), insulin (15.3 ± 4.9 vs. 16.6 ± 7.6 μ UI/ml), HOMA-IR (0.42 ± 0.16 vs. 0.54 ± 0.23), vitamin D

(32.7 ± 4.6 vs. 32.1 ± 2.9 ng/ml), and CRP (1.8 ± 0.3 vs. 2.2 ± 0.5 mmol/l), TNF- α (1.9 ± 0.9 vs. 1.7 ± 1.3 mmol/l), IL6 (1.7 ± 0.3 vs. 1.8 ± 0.1 mmol/l) respectively (Figure 1).

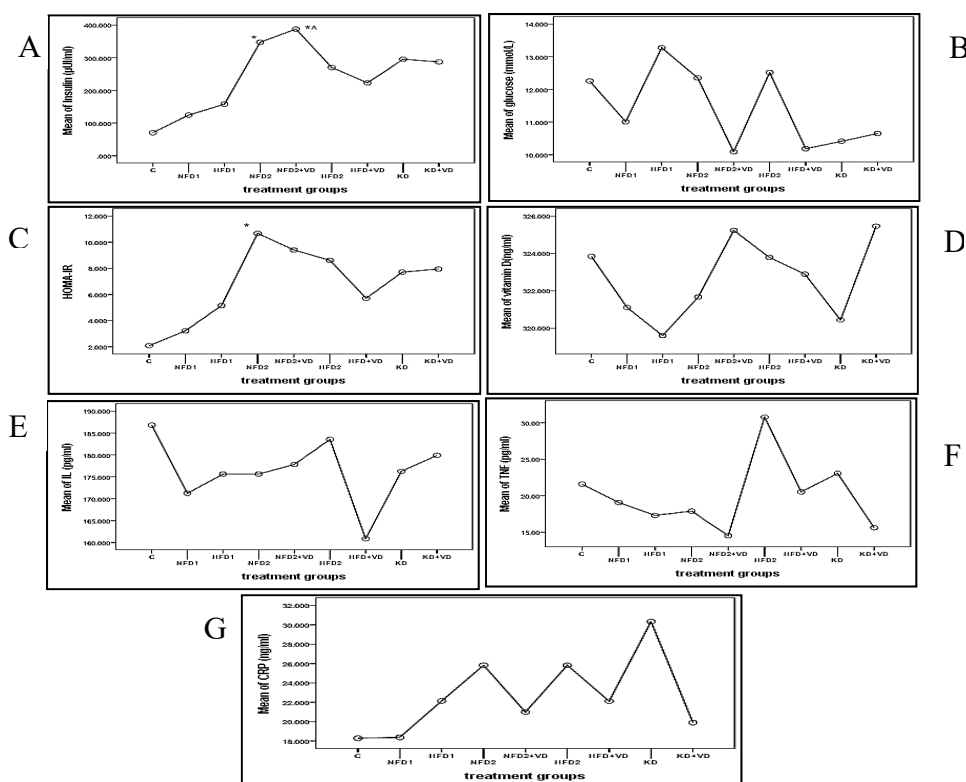


Figure 1. Concentrations of fasting (A) plasma insulin (B) serum glucose (C) HOMA-IR (D) plasma vitamin D (E) plasma IL6 (F) plasma TNF (G) plasma CRP of rats fed different diets for 16 weeks.

Values are means; *the mean difference is significant from C group; ^ the mean difference is significant from NFD1 group; rats fed (C: control, NFD: Normal-fat diet, HFD: High-fat diet, KD: ketogenic-diet, - VD: supplemented with vitamin D, 1: at the end of 8 weeks/ 2: at the end of week 16. CRP: C reactive protein; TNF α : tumor necrosis factor; IL6: interleukin 6), (n=5)/groups, one-way ANOVA. Post hoc test Tukey-Kramer). Insulin resistance (HOMA-IR) = fasting insulin (μ UI/ml) X fasting glucose (mmol/l)/22.5.

At the end of 16 weeks of dietary interventions (Figure 1), no significant ($P>0.05$) differences were found between NFD, NFD-VD, HFD, HFD-VD, KD, and KD-VD respectively, in the mean values of serum glucose (6.9 ± 0.1 , 5.6 ± 0.1 , 6.9 ± 0.1 , 5.7 ± 0.1 , 5.8 ± 0.1 , 5.9 ± 0.1 mmol/l), insulin (20.0 ± 4.3 , 22.4 ± 3.1 , 19 ± 6.9 , 18.0 ± 6.3 , 19.1 ± 5.8 , 19.7 ± 4.7 μ UI/ml), HOMA-IR (0.61 ± 0.13 , 0.56 ± 0.11 , 0.58 ± 0.28 , 0.46 ± 0.15 , 0.49 ± 0.13 , 0.52 ± 0.15), VD (31.8 ± 2.4 , 32.6 ± 1.5 , 32.7 ± 5.1 , 32.5 ± 6.1 , 32.5 ± 9.7 , 32.7 ± 2.2 ng/ml), CRP (2.6 ± 0.8 , 2.1 ± 0.3 , 2.6 ± 1.3 , 2.2 ± 0.61 , 3.0 ± 0.4 , 2.0 ± 1.1 mmol/l), TNF- α (1.8 ± 0.8 , 1.5 ± 0.7 , 3.1 ± 1.9 , 2.1 ± 1.3 , 2.3 ± 1.1 , 1.6 ± 0.9 mmol/l), and IL6 (1.8 ± 0.1 , 1.8 ± 0.3 , 1.8 ± 0.0 , 1.6 ± 0.3 , 1.8 ± 0.2 , 1.8 ± 0.1 mmol/l). There was a 4-fold ($P\leq0.05$) increase in the plasma insulin in rats fed NFD2 and NFD+VD2 compared control with no significant ($P>0.05$) differences were shown between the groups within the two experimental periods.

Correlation analyses

Correlation analyses revealed a significant positive association ($r = 0.75$, $P.01$) between the change in body weight and food intake (Table 4). A similar positive association was observed between the change in body weight and the proportion of carbohydrate ($r = 0.292$, $P < 0.01$) and fat ($r = 0.341$, $P<0.01$) in the die. No significant ($P>0.05$) correlations were found among NFD, NFD-VD, HFD, HFD-VD, KD, and KD-VD for glucose, insulin, HOMA-IR, IL6, TNF, and CRP (Table 5).

Table 4. Overall correlation coefficients among food intake, body weight change, and dietary fat and carbohydrate proportions (n=40).

Variables	Food intake	Carbohydrate (%)	Fat (%)
Carbohydrate (%)	0.495**		
Fat (%)	0.337**	-0.651**	
Weight change	0.752**	0.292**	0.341**

** Significant at $P<0.01$

Table 5. Overall correlation coefficients among studied variables (n=45).

Variable	Insulin	Glucose	HOMA-IR	Vitamin D	CRP	IL6
Glucose	-0.08					
HOMA-IR	0.95	0.20				
Vitamin D	0.11	0.12	0.15			
CRP	0.30	0.18	0.36	-0.15		
IL6	-0.15	0.15	-0.12	-0.01	0.04	
TNF α	-0.15	0.22	-0.13	-0.01	-0.19	0.31

All correlations (P>0.05). CRP: C reactive protein; TNF α : tumor necrosis factor; IL6: interleukin 6 Insulin resistance (HOMA-IR) = fasting insulin (μ UI/ml) X fasting glucose (mmol/l)/22.5. are not significant,

DISCUSSION

This research aimed to investigate the influence of NFD, KD, and HFD with and without VD on body weight, food intake, serum glucose, insulin, HOMA-IR, vitamin D, and the inflammatory biomarkers CRP, IL-6, and TNF- α in rats. These diets contained equivalent proportions of calories, protein, and micronutrients. Soybean oil was used as the primary source of fat in the experimental diets. This oil is composed of five fatty acids: palmitic acid (10%), stearic acid (4%), oleic acid (18%), linoleic acid (55%), and linolenic acid (13%), 86% of them is unsaturated fatty acids (Clement and Cahoon 2009). Compared to other diets, KD-VD was the only one to show a significant reduction in body weight, food intake, and FCR with no changes in the other variables.

The similarity of weight gain of rats fed KD compared with those fed HFD and NFD may be attributed to the increased food intake and thus food conversion ratio observed in all groups. However, studies in obese or non-obese rodents have shown KD is efficient for weight loss (Kennedy *et al.*, 2007; Badman *et al.*, 2009). In a study by Garbow *et al.* (2011), after 12 weeks, KD led to significantly less weight gain compared to chow-fed and high-simple-carbohydrate high-fat Western diet-fed mice and reduced lean mass in KD-fed mice compared to chow-fed mice. In another study, accumulation of visceral fat

mass was significantly higher (at least 30%) in rats fed KD with two compositions: a high-protein “Atkins-style” or a low-protein diet, both of which contained low-carbohydrate and high-fat content, compared to chow-fed controls, after four weeks of dieting (Bielohuby *et al.*, 2013).

The similarity in weight gain between study groups may also be attributed to the use of adult rats, while the levels of ketone bodies in such or aged rats matched those of the young by week 4, this perhaps indicated that adult rats take longer time to initiate ketosis (Jornayvaz *et al.*, 2011). While the mechanism of delayed ketosis in adult or aged rats is unknown, this finding demonstrates that short-term dietary intervention may be insufficient to produce measurable physiological changes in old animals (Hernandez *et al.*, 2020).

In this study, the ketogenic ratio of the KD was 1.4 as calculated according to Withrow's equation (Withrow, 1980). For this reason, the current KD may be a characteristic of HFD with a different macronutrient ratio that may need a longer time to induce ketosis than the intervention time. On the other hand, Lecomte *et al.* (2015) showed that the HFD fed rats gained more weight than controls three weeks after dietary intervention. The weight change of HFD-fed rats in this study was not significantly different from those fed NFD. The fact that daily caloric intake in all study groups over 16 weeks was not significantly different appears to be consistent with the

weight data. A possible explanation of this phenomenon might be the animals' adaptation to various HFD diets and fat sensitivity. In this context, variations in the experimental design of HFD studies with different types, fatty acid composition, and contents of dietary fats (40–60% of total energy intake), and differences in the HFD study duration (8–20 weeks) are among the reported confounding factors (Jornayvaz *et al.*, 2011; Garbow *et al.*, 2011; Lecomte *et al.*, 2015; Hernandez *et al.*, 2020). Another possible explanation may be dietary fiber and its potential as a satiating food component. It has been reported that the inclusion of insoluble dietary fiber in HFD protects against excessive body weight gain in animal models (Clark and Slavin, 2013; Trigueros *et al.*, 2013; Rebello *et al.*, 2014). Nonetheless, there is a lack of evidence for the long-term effects of dietary fiber in obese subjects.

It has been shown that plasma VD levels inversely correlate to several indices of obesity such as body mass index, fat mass, and waist circumference (García *et al.*, 2009; Cheng *et al.*, 2010). It has been also shown that increased dietary intake of VD raises its plasma levels and is associated with lower visceral adiposity (Caron-Jobin *et al.*, 2011). In this study, plasma VD levels did not differ between different experimental groups. The fact that the adipose tissue is the main body storage site of VD and its metabolites may provide a plausible explanation for the current results. This fact has prompted the opinion that VD and its metabolites may get sequestered in the excess fat mass (Wortsman *et al.*, 2000).

However, the physiological mechanisms underlying the above-stated opinion have not been brought forward. Nevertheless, as pointed out in the study of Drincic *et al.* (2012), it might just be that in individuals with a higher body mass, 25(OH) VD is simply diluted in a higher volume, so they would require higher VD input than lean individuals to achieve a sufficient 25(OH) VD status. It is presumed that the excess VD is stored in body fat as a native compound and is slowly released (Brouwer *et al.*, 1998; Heaney *et al.*, 2008). Thus, high VD doses may result in flatter slopes compared with those resulting from low VD doses. Therefore, this may result in a longer apparent

half-life of 25(OH) VD. This mechanistic pathway may also explain the widely reported variable half-life in response to different doses of VD supplementation. For this reason, the current result showed no effect due to being supplemented rats after 8 weeks when the bodyweight increased in rats fed with VD (1000 IU/kg diet) treatment dose (Brouwer *et al.*, 1998; Heaney *et al.*, 2008; Drincic *et al.*, 2012).

Furthermore, the baseline 25(OH) VD concentration has been consistently shown to make a significant contribution to the variance in 25(OH) VD response to VD supplementation (Aloia *et al.*, 2008; Zhao *et al.*, 2012; Mazahery *et al.*, 2015). The hepatic hydroxylation of VD is a saturable process (Barger-Lux *et al.*, 1998). Thus, the response of plasma VD to VD supplementation may be affected by the baseline concentration of 25(OH) VD that was in the normal range in this study. However, in the current study, experimental diets were supplemented with poly- and mono-unsaturated fatty acids-rich soybean oil. It has been found that dietary fat composition markedly affects VD response to VD supplementation (Niramitmahapanya *et al.*, 2011). The mechanisms by which the type of fatty acids may influence vitamin D response are not known. Niramitmahapanya *et al.* (2011) suggested that fatty acids such as linoleic and linolenic acid may increase the solubility of VD in the micelles and increase their size. As a consequence, VD may stay longer in the micelles and may have difficulty in passing the intestinal mucosa. Decreased plasma 25(OH) VD levels could also result from VD dysmetabolism during obesity development (Niramitmahapanya *et al.*, 2011).

Obesity is often associated with the development of insulin resistance and type 2 diabetes. Notably, as already discussed, all rat groups increased in weight, but without the concomitant occurrence of insulin resistance. Mice fed KD for 12 weeks remained euglycemic with reduced serum insulin and HOMA-IR indices and exhibited glucose intolerance, as assessed by intraperitoneal glucose tolerance tests (Garbow *et al.*, 2011). However, Garbow *et al.* (2011) observed that, despite mild hepatic steatosis, systemic response to insulin was preserved, unlike in other studies. The authors explained this discrepancy by a

relatively reduced lean body mass in KD-fed mice, resulting in a higher insulin dose in insulin-tolerance tests.

Also, hepatic insulin resistance may confer a smaller contribution to overall glucose homeostasis than peripheral glucose disposal. In studies using rats, KD also induced glucose intolerance and insulin resistance (Kinzig *et al.*, 2010; Bielohuby *et al.*, 2013) despite reduced glucose and insulin levels. In the same studies, short-term KD feeding in rats was also associated with decreased β cell mass, but this effect could be due to a lower lean body mass of KD-fed rats (Bielohuby *et al.*, 2013). Interestingly, after only 6-days of KD feeding, mice showed impairments in glucose tolerance and insulin sensitivity; this was attributed to a possible adaptation to maintain blood glucose levels against insufficient amounts of carbohydrates (Murata *et al.*, 2013). In this case, insulin signaling was impaired only in white adipose tissue but not in the liver and muscle. The authors suggested that this impairment in white adipose tissue could not be the only culprit for whole-body glucose intolerance in KD-fed mice. The possible role of KD in inducing insulin resistance is nevertheless controversial. Indeed, several authors reported that long-term KD-fed mice had normal glucose tolerance, lower baseline insulin levels, and improved insulin sensitivity (Douris *et al.*, 2015; Holland *et al.*, 2016).

In humans, the effect of KD on glucose homeostasis is more controversial and is notably dependent on whether or not type 2 diabetes is present (Foster *et al.*, 2003; Westman *et al.*, 2007; Johnstone *et al.*, 2008). In this study, we did not assess the difference in body composition. If there is a decrease in a lean mass known to affect insulin sensitivity or organ-specific insulin resistance, the unaffected glucose and insulin levels will keep lowering glucagon levels that were also not tested. Interestingly, in a study on mice, the alpha cell mass decreased considerably in line with the reduced glucagon levels after 5-weeks of KD intervention (Jornayvaz *et al.*, 2011). In relative terms, this decreased alpha cell mass was even more pronounced than the reduction in beta-cell mass that caused a marked decrease in the alpha-to-beta cell ratio. Low glucagon levels reduced hepatic gluconeogenesis and prevented hyperglycemia in KD-fed mice. Whether this change in

alpha cell mass is a direct consequence of KD or a response to counteract glucose intolerance remains to be elucidated.

Burcelin *et al.* (2002) showed that long-term feeding of HFD dramatically decreased glucose turnover and clearance in mice. Glucose utilization in the presence and absence of insulin was similar in muscles isolated from the different groups of studied mice, indicating that glucose clearance could be regulated by a factor distinct from insulin, like adipokines (Burcelin *et al.*, 2002). Importantly, in the current study, none of the HFD-fed rats became insulin resistant when measured by the HOMA-IR index. Even though the diet change from HFD to KD and KD+VD did not affect HOMA-IR. The possible effect of insoluble fiber contained in the experimental diets may explain these results. In their controlled-intervention study, Weickert *et al.* (2011) indicated that high intakes of insoluble fiber improve insulin resistance independently of weight loss through modifications of dietary protein absorption.

In a randomized, controlled, 18-weeks trial with 111 group-matched, overweight adults with one or more further metabolic risk factors, Weickert *et al.* (2011) compared the effects of isoenergetic diets supplemented with varying levels of cereal fiber and plant-based protein on whole-body and hepatic insulin resistance. After six weeks of treatment, IR expressed as M-value was 25% lower in participants who consumed a high fiber diet than those who ate a high-protein diet. Supplementation of HFD with omega-3 fatty acids prevented high-fat diet-induced insulin resistance by reducing inflammasome-dependent inflammation and increasing the secretion of leptin and adiponectin, both of which exert insulin-sensitizing effects in rodents (Yan *et al.*, 2013). Intubation of eicosapentaenoic acid reduced fasting insulin and insulin resistance without affecting blood glucose in male rats fed control and high-fat diets (Pérez-Matute *et al.*, 2007).

Vitamin D also seems to have a direct influence on insulin sensitivity or resistance. Several studies have shown an increase in insulin sensitivity through stimulation of expression of insulin receptors on target tissues (Maestro *et al.*, 2000) and the regulation of cellular calcium concentration in skeletal muscle cells, that might enhance glucose transport through the membrane via the

recruitment of glucose transporter 4 (Wei *et al.*, 2008; Muscogiuri *et al.*, 2012). Vitamin D could also enhance insulin release by regulating the beta-cell intracytoplasmic calcium concentration (Muscogiuri *et al.*, 2012). Vitamin D status was inversely associated with the incidence of type 2 diabetes mellitus in a longitudinal study (Mitri and Pittas, 2014). Furthermore, two meta-analyses of longitudinal observational studies confirmed these results (Afzal *et al.*, 2013; Song *et al.*, 2013). Nevertheless, age, diet, and lifestyle are among several confounding factors that may influence VD status and diabetes incidence (Pittas *et al.*, 2009; Mitri *et al.*, 2011; Mitri and Pittas, 2014).

Furthermore, no change in plasma cytokine concentrations in the current study; this result is not in line with the observation that short-term KD in mice is associated with increased inflammatory markers in the liver and adipose tissue (Badman *et al.*, 2009) and macrophage infiltration in the liver (Garbow *et al.*, 2011). This discrepancy may be due to the polyunsaturated fatty acid used. Rosenbaum *et al.* (2019) found that circulating CRP but not the inflammatory cytokine IL-6 was significantly higher on the KD. Ketosis in mice seemed to increase tissue-specific liver and white adipose tissue expression of inflammatory cytokines TNF- α , IL-6, and macrophage markers (Garbow *et al.*, 2011). Outpatient studies of low-carbohydrate diets in humans have found significant increases in plasma CRP but not IL-6 (Song *et al.*, 2016). In subjects with type 2 diabetes, the advice to follow a lower carbohydrate diet decreased IL-6 but not CRP concentrations (Jonasson *et al.*, 2014).

In humans, several researchers have attempted to link ω 3 polyunsaturated fatty acid intake with the inflammatory response without reaching consistent conclusions. A one-year dietary supplementation with ω 3 polyunsaturated fatty acids did not modify the circulating cytokine levels in healthy volunteers (Blok *et al.*, 1997). Conversely, other studies reported a marked decrease in inflammatory markers after ω 3 polyunsaturated fatty acid

supplementation (Cooper *et al.*, 1993; James *et al.*, 2000). It is important to note that the blood inflammatory profile appears to be less representative than that of the adipose tissue (Balvers *et al.*, 2015). Furthermore, the level of nutritional ketosis may be an important factor in the regulation of adiponectin expression, knowing that ketone bodies influence adiponectin activity (Makki *et al.*, 2013). The authors found a negative correlation between adiponectin and pro-inflammatory cytokine TNF- α (Makki *et al.*, 2013). Adiponectin can be a pro-inflammatory cytokine expresser, including TNF- α and IL-6. Further studies are needed to assess body ketosis and serum adiponectin (Awazawa *et al.*, 2011; Nigro *et al.*, 2013).

CONCLUSIONS

Rats fed the NFD, HFD in the first eight weeks did not show significant differences in the mean values of serum glucose, insulin, HOMA-IR, VD, CRP, TNF- α , IL6. Following the intervention at the end of the second 8 weeks, body weight and feed efficiency were lowest in KD supplemented with VD rats' group. In addition to VD, the correlation showed that weight change was affected by food intake and fat and carbohydrate proportions in the diet. None of the other assessed inflammatory biomarkers or serum VD, insulin, and glucose was affected by the diets or VD supplementation. These results support that VD supplemented KD decreases body weight and feed efficiency in dietary-induced obesity in rats.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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وزن الجسم ومقاومة الأنسولين والمؤشرات الحيوية الالتهابية في جرذان مغذاة بأنظمة غذائية

دهنية طبيعية وعالية الدهون وكيوتونية مزودة بفيتامين د

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ملخص

قد تبين أن الأنظمة الغذائية الكيتونية (KD) وعالية الدهون (HFD) تنتج تأثيرات متغيرة على إفراز الأنسولين ووزن الجسم، بالرغم من أن الأسباب لا تزال غير واضحة، وأن دور فيتامين (د) (VD) في هذه المسألة الأيضية غير مؤكد. لقد قمنا بدراسة تأثير الأنظمة الغذائية الدهنية الطبيعية (NFD) و KD و HFD مع VD وبدونه على وزن الجسم وغلوكوز الدم والأنسولين و VD ومقاومة الأنسولين والبروتين النشط والإنترلوكين 6 وعامل النخر السرطاني في الجرذان. وتم تحضير ثلاث أنظمة غذائية ذات محتوى متساو من النيتروجين والطاقة تمثل تلك NFD و KD و HFD وتحتوي على التوالي من الدهون والكربوهيدرات والبروتين: (NFD: 9.5% و 75.7% و 14.8%) و (KD: 69.5% و 10.3% و 20.2%) و (HFD: 42.0% و 42.2% و 15.2%). وتم تحضير ثلاث أنظمة غذائية أخرى مماثلة لكن تم تزويدها ب VD (1000 وحدة دولية/كغم). تم استخدام خمسة وأربعين جرذاً ذكراً من نوع سبراغ داوولي بعمر 8-9 أسابيع تم التضحية بخمسة جرذان في بداية التجربة من أجل المتغيرات الأولية. وتم تقسيم الجرذان (ن=40) بشكل عشوائي إلى مجموعتين NFD (ن=15) و HFD (ن=25) وتم تغذيتها بالأنظمة الغذائية المقابلة لمدة ثمانية أسابيع. وتم التضحية بخمسة جرذان من كل مجموعة في نهاية هذه المدة، وتم الحصول على مصل الدم. وتم تقسيم جرذان مجموعة NFD (ن=10) إلى مجموعتين فرعيتين (ن=5 لكل مجموعة)، واحدة منها استمرت على النظام الغذائي NFD والأخرى تغذت على نفس النظام الغذائي المزود ب VD ، كما تم تقسيم جرذان مجموعة HFD (ن=20) إلى أربع مجموعات فرعية (ن=5 لكل مجموعة)، واحدة منها استمرت على النظام الغذائي HFD والأخرى تغذت على الأنظمة الغذائية التالية: KD و HFD المزود ب VD و KD المزود ب VD . وقد تم تغذية جميع الفئران بالأنظمة الغذائية المقابلة لمدة 8 أسابيع أخرى، وبعد ذلك تم التضحية بجميع الجرذان، وتم الحصول على مصل الدم. وتم قياس وزن الجسم والغذاء المتناول أسبوعياً وحُسب معدل التحويل الغذائي. وبعد التدخل الغذائي، وكان التغير في الوزن (-10.13 ± 15.6 غم) ومعدل التحويل الغذائي (0.033 ± 0.350) هما الأدنى ($P < 0.05$) في الجرذان التي تغذت على النظام الغذائي KD المزود ب VD مقارنة على التوالي بالجرذان التي تغذت على النظم الغذائية KD (144.8 ± 1.47 و 0.189 ± 0.050) و HFD المزود ب VD (143.0 ± 8.49 و 0.187 ± 0.100) و HFD (155.8 ± 0.300 و 0.203 ± 0.010) و NFD المزود ب VD (142.8 ± 6.34 و 0.183 ± 0.009) و NFD (51.0 ± 1.02 و 0.074 ± 0.110). وارتبط التغير في الوزن بشكل معنوي ($P < 0.01$) مع المتناول من الغذاء ($r=0.752$) ونسب الكربوهيدرات ($r=0.292$) والدهون ($r=0.341$) ولم تتأثر أي من المتغيرات الحيوية الأخرى. وتدعم نتائج هذه الدراسة دور ال KD المزود بال VD في تخفيض وزن الجسم في الجرذان مما قد يؤدي الى تغيرات بالكميات المتناولة من الغذاء ونسب الدهون والكربوهيدرات الغذائية.

الكلمات الدالة: الكربوهيدرات، وزن الجسم، مقاومة الأنسولين، غذاء عالي الدهون، غذاء كيتوني، السمنة، الجرذ، فيتامين (د).