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Phytosterols do not change susceptibility to obesity, insulin resistance, and diabetes induced by a high-fat diet in mice

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Abstract

Most studies have focused on the cholesterol-lowering activity of phytosterols; however, other biological actions have also been attributed to these plant compounds. In this study, we investigated whether phytosterols could delay (progression phase) and/or reverse (regression phase) insulin resistance or type 2 diabetes mellitus in an experimental mouse model of diet-induced obesity, insulin resistance, and diabetes. Body mass, plasma lipid levels, insulin resistance, and hyperglycemia were determined. Phytosterol intake did not improve these metabolic parameters. Therefore, we were unable to substantiate any protective effect of phytosterol intake on diabetes development or regression in the mouse model used.

1. Introduction

Phytosterols/stanols reduce serum low-density lipoprotein cholesterol levels, and food products containing these plant compounds are widely used as a therapeutic dietary option to reduce hypercholesterolemia and atherosclerotic risk [1]. The hypocholesterolemic effect of phytosterols has been demonstrated in both humans and animals [2-4]. One mechanism that could be involved in this hypocholesterolemic action is the physical competition between phytosterols and cholesterol for incorporation into micelles, which compromises cholesterol absorption [5]. Although most studies have focused on the cholesterol-lowering activity of phytosterols, other biological properties such as antidiabetic agents [6-9] have also been attributed to these plant compounds. Accordingly, aloe vera–derived phytosterols ameliorated hyperglycemia in treated db/db type 2 diabetic mice [6]. Also, a phytostanol mixture induced improvement in glucose tolerance in fat Zucker rats [7]. Two stigmasterol-derived compounds extracted from the cashew plant produced a significant reduction in blood glucose levels when intravenously administered to dogs [8]. Furthermore, changes in intestinal cholesterol absorption could correlate with insulin sensitivity [10], as type 2 diabetic patients present increased cholesterol synthesis but decreased absorption [10].

To gain further insight into the potential therapeutic value of phytosterols or their derivatives in insulin resistance states, we sought to determine whether long-term treatment with phytosterols could delay (Progression phase) and/or reverse (Regression phase) insulin resistance or type 2 diabetes mellitus onset in a mouse model of diet-induced obesity, insulin resistance and diabetes.

2. Materials and methods

2.1. Mice and diets

Thirty-two male C57BL/6J mice, obtained from Jackson Laboratories (Bar Harbor, ME), were studied. Mice were housed in environmentally controlled conditions with a 12-hour light/dark cycle and free access to food, except during fasting periods, before blood sampling. In the progression phase, animals were fed either a control high-fat Western-type diet (C-WTD) (n = 24) (Mucedola srl,

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Table 1
Composition of C-WTD and 2% (wt/wt) P-WTD

Diet ingredient	C-WTD		P-WTD	
	g	kcal	g	kcal
Casein	195	780	195	780
DL-Methionine	3.0	12	3	12
Sucrose	342	1368	342	1368
Cornstarch	150	600	150	600
Beef tallow	210	1890	210	1890
Cholesterol	0.8	7.2	0.8	7.2
Cellulose	50	0	50	0
Mineral mix, AIN-76 (CA. 170915)	35	0	35	0
Calcium carbonate	4	0	4	0
Vitamin mix, Teklad (CA. 40060)	10	40	10	40
Phytosterols	0	0	20	0
Total	1000	4697	1020	4697
		kcal %		kcal %
Protein		16.8		16.8
Carbohydrate		42.8		42.8
Fat		40.4		40.4
TOTAL kcal		100		100

Settimo Milanese, Italy) or a 2% phytosterol-enriched Western-type diet (P-WTD) (wt/wt) (n = 8) for 32 weeks (Table 1) [3]. Western-type diet is known to cause obesity, insulin resistance, and diabetes [11]. In the regression phase, 16 mice that had been fed with the C-WTD diet for 32 weeks were randomized into 2 groups (n = 8 each) and fed a control chow diet (C-CD) or a phytosterol-enriched CD (P-CD) for 8 weeks. The C-CD was a Teklad Global 18% protein diet containing 18% protein, 5% fat, and 57% carbohydrate (Harlan Teklad, Madison, WI; catalog no. 2018, www.teklad.com) and P-CD was the 2% phytosterol-enriched C-CD diet. The phytosterol product was composed of 20% campesterol, 22% stigmasterol, and 41% β-sitosterol (Lipofoods, S.L., Gavà, Spain). More than 40 phytosterols, which are highly abundant in vegetable oils, seeds and cereals, have been identified. The ones used in this study (campesterol, stigmasterol, and β-sitosterol) are more than 95% present in vegetable extracts [12,13]. Body mass was recorded once a month throughout the study. Mice were individually housed in metabolic cages, and food consumption calculated over the 7 consecutive days at 1, 3, and 5 months of treatment. Mice were anesthetized with isoflurane gas and exsanguinated by cardiac puncture at the end of the study. Both kidneys and their adipose tissue were collected from each animal and visible white adipose tissue removed completely and weighed [11]. Epididymal fat pads were also removed and weighed [15]. All animal procedures were approved by the Institutional Animal Care Committee of the Hospital de la Santa Creu i Sant Pau.

2.2. Plasma lipid analyses

Plasma total cholesterol, HDL cholesterol, and triglycerides were determined enzymatically with commercial kits (Roche Diagnostics, Rotkreuz, Switzerland). Nonesterified fatty acids (NEFA) were determined enzymatically using a commercial kit from Wako (NEFA C, Wako Chemicals, Neuss, Germany). Triglyceride determinations were corrected for the free glycerol present in plasma (Sigma, St Louis, MO). These determinations were performed on an BM/HITACHI 911 autoanalyzer (Roche Diagnostics) [14].

2.3. Glucose and insulin measurements

Mice were fasted for 12 hours before glucose and insulin determinations. Plasma glucose concentrations were measured monthly throughout the study period using an Accucheck one-touch blood glucose meter (Roche Diagnostics, Rotkreuz, Switzerland). At the end of the progression and regression phases, 12-hour fasting plasma insulin levels were measured by a commercial radioimmunoassay that incorporates an antibody to rat insulin (RI-13K, Linco, St Charles, MO) [15]. Also, glucose tolerance tests and insulin sensitivity tests were carried out after a 12-hour fast at the time points indicated below. Glucose tolerance tests were performed by intraperitoneal administration of glucose (1 mg/g of body mass) and measurement of plasma glucose at $t = 0$ (baseline) and 20, 40,

Table 2
Anthropometric and biochemical measurements performed in C57BL/6J mice before and after being fed with a Western-type diet with (P-WTD) or without phytosterols (C-WTD) during 32 weeks (progression phase) and before and after being fed with a regular CD with (P-CD) or without phytosterols (C-CD) during 8 weeks (regression phase)

	Progression phase		Regression phase	
	C-WTD	P-WTD	C-CD	P-CD
No. of mice	8	8	8	8
Body mass (g)	41.5 ± 2.4	40.7 ± 1.7	35.1 ± 1.1	36.5 ± 1.3
Epididymal fat/body mass	0.051 ± 0.004	0.048 ± 0.005	0.023 ± 0.002	0.034 ± 0.004
Renal fat/body mass	0.016 ± 0.002	0.014 ± 0.002	0.007 ± 0.001	0.011 ± 0.002
Total cholesterol (mmol/L)	3.6 ± 0.3	2.4 ± 0.1*	2.6 ± 0.1	2.3 ± 0.1
HDL cholesterol (mmol/L)	2.9 ± 0.2	1.9 ± 0.1*	1.9 ± 0.1	1.7 ± 0.1
Triglycerides (mmol/L)	0.3 ± 0.03	0.3 ± 0.05	0.4 ± 0.07	0.4 ± 0.07
NEFA (mmol/L)	0.5 ± 0.02	0.5 ± 0.02	0.8 ± 0.04	0.7 ± 0.03
Glucose (mmol/L)	11.5 ± 0.5	11.7 ± 0.6	9.6 ± 1.1	9.5 ± 0.7
Plasma insulin (μU/mL)	19.3 ± 2.9	12.8 ± 2.8	10.7 ± 1.0	13.2 ± 2.1

Results are shown as means ± SEM. Unpaired t test was used for comparison of the experimental groups.
* $P < .05$, significantly different from C-WTD mice.

60, and 120 minutes after glucose administration [15]. To evaluate the overall glucose exposure, the area under the concentration curve (AUC) was calculated. Insulin sensitivity tests were performed in a similar way, except for the injection of porcine insulin at a dose of 0.5 IU/kg (Wak-Chemie Medical, Bad Homburg, Germany) [15].

2.4. Statistical analyses

Unpaired *t* test was used to compare data obtained from phytosterol-fed and control-fed animals after performing normality testing. Repeated-measures 2-way analysis of variance was used to compare daily food intake, body mass, and blood glucose over time in phytosterol-fed and control-fed mice. A Bonferroni posttest was used to compare replicate means of phytosterol- and control-treated mice at each time point analyzed. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used for all statistical analyses. All values are expressed as mean ± SEM. A *P* value of less than .05 was considered statistically significant.

3. Results

3.1. Progression phase

As expected, in the progression phase, the mice gained weight rapidly; however, after the 32-week diet period, neither mass of renal nor epididymal fat deposits were affected by phytosterol intake (Table 2 and Fig. 1A). No difference in weight gain was observed between phytosterol-treated mice and control littermates (Fig. 1A). Furthermore, phytosterol intake did not alter average diet consumption at the time points analyzed (4.1 ± 0.4 vs 3.7 ± 0.04 in control mice, 3.7 ± 0.5 vs 4.1 ± 0.3 in control mice, and 4.7 ± 0.8 vs 4.3 ± 0.1 g/d mouse in control mice at 1, 3, and 5 months of treatment, respectively).

Interestingly, phytosterols lowered plasma total cholesterol levels due to a decrease in HDL cholesterol (Table 2). In the progression phase, the high-fat WTD increased fasting plasma glucose concentrations similarly in both experimental groups regardless of phytosterol intake (Fig. 1B). As shown in Table 2, fasting plasma insulin content did not differ either. The AUC of glucose in glucose tolerance and insulin sensitivity tests did not differ between phytosterol-treated and nontreated mice, thereby indicating similar glucose clearance and insulin response (Fig. 2). Diabetes was diagnosed when glucose levels exceeded 11.1 mmol/L in 2 consecutive tests; 75% of C-WTD-fed mice and 71% of P-WTD-fed mice met this criterion, and, again, this was not significantly different.

3.2. Regression phase

At the end of the regression phase, CD-nourished mice exhibited a reduction in body mass and fat stores in comparison to high-fat-fed mice; however, no differences caused by phytosterol administration were found (Table 2 and Fig. 1A). As expected, both groups of animals returned to a CD exhibited improvement in metabolic parameters (Table 2). Again, phytosterol treatment did not alter fasting plasma glucose (Table 2 and Fig. 1B), insulin concentration (Table 2) or AUC in glucose tolerance and insulin sensitivity tests (Fig. 2). Finally, C-CD– and P-CD–fed animals presented a nonstatistically significant difference in diabetes proportion (37% and 25%, respectively).

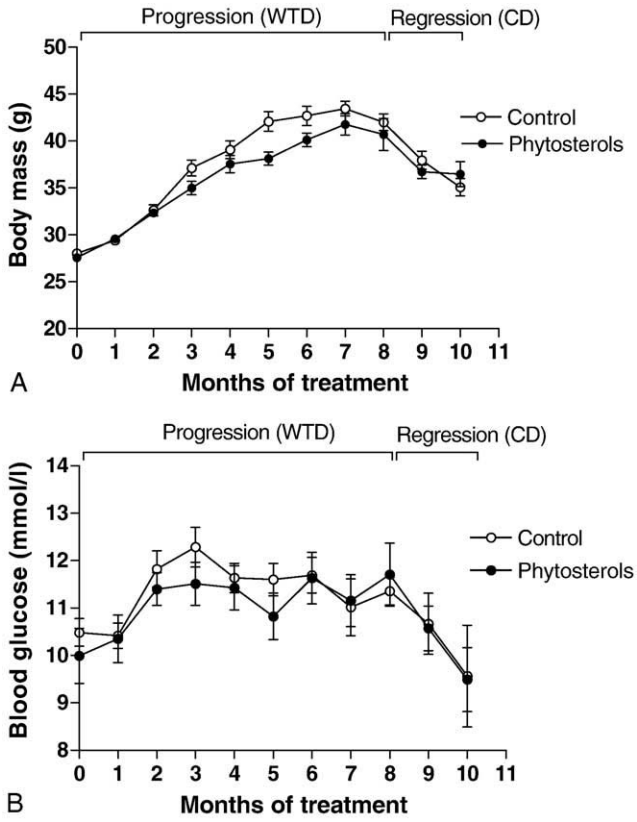

Fig. 1. Effect of phytosterols in a mouse model of diet-induced obesity, insulin resistance, and diabetes. (A) Average monthly weight of phytosterol-treated and nontreated C57BL/6J male mice in the progression (WTD-fed animals) and in regression (CD-fed animals) phases of the study. (B) Time-course concentrations of blood glucose levels in phytosterol-treated C57BL/6J male mice and control littermates in both phases of the study. Data are presented as mean ± SEM. Repeated-measures 2-way analysis of variance was used to compare body mass and blood glucose of the experimental groups over time. In the progression phase, weight and blood glucose were monitored in 24 C-WTD mice and 8 P-WTD mice. In the regression phase, 8 C-CD mice and 8 P-CD mice were analyzed.

4. Discussion

The present study was undertaken to assess the protective effect of chronic phytosterol intake on diabetes development or regression in a mouse model of diet-induced obesity, insulin resistance, and diabetes, taking into account previous reports in the literature on the antidiabetic capacity of these plant compounds [6-9].

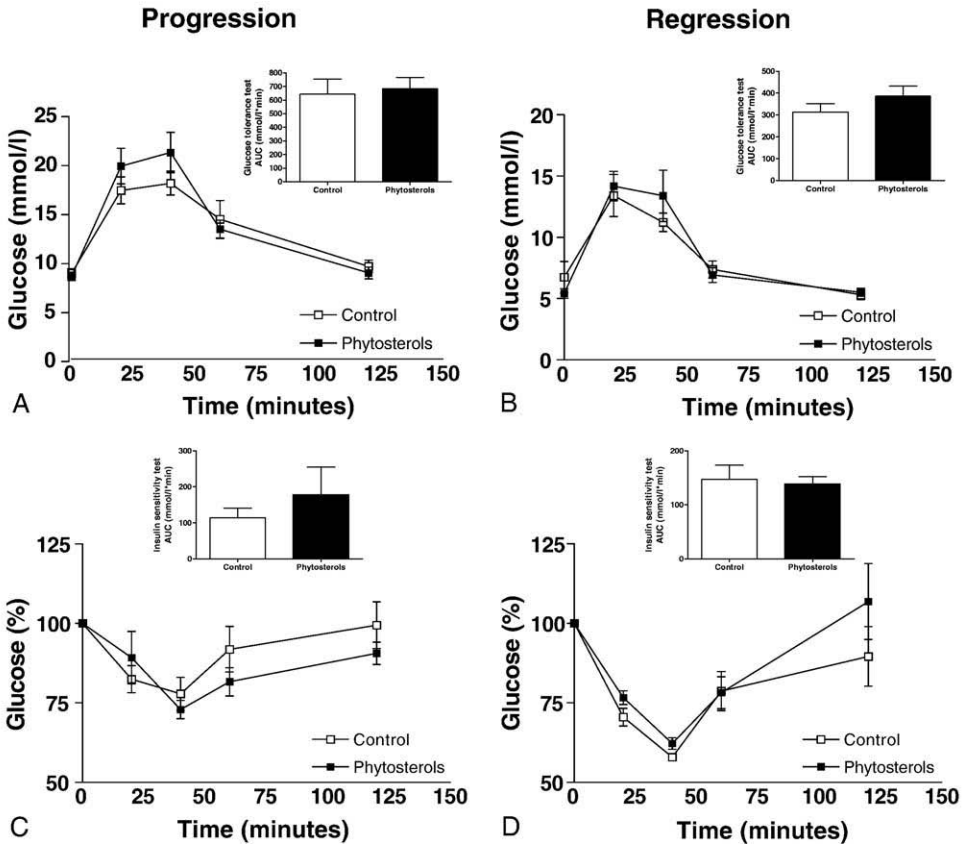

Fig. 2. Effect of phytosterols on glucose metabolism after an intraperitoneal glucose tolerance test (A, B) and insulin sensitivity test (C, D) in fasted mice at the end of the progression (A, C) and regression (B, D) phases. In the insulin sensitivity test, the initial glucose concentration was set at an arbitrary 100%. Each point represents the mean ± SEM of 7 to 8 mice. A to D insets, the AUC of glucose in glucose tolerance and insulin sensitivity tests. Unpaired *t* test was used for comparison of the experimental groups.

Daily food consumption and body mass gain did not change because of the dietary treatment. This contrasts with a previously reported effect of a modified form of phytostanol (FM-VP4) on weight gain in a dietary-induced mouse model of obesity [16,17]. However, most studies on the hypocholesterolemic action of phytosterols in mice did not report any weight loss properties [3,18-20], suggesting that the putative antiobesity effect of FM-VP4 may be specific to this plant stanol compound.

In previous studies, the hypocholesterolemic effect of phytosterols was described in severely hyperlipidemic mice with high plasma non–HDL cholesterol concentrations [3,18,21]. However, phytosterol treatment did not lower plasma cholesterol in normolipidemic C57BL/6J mice, in whom HDL is the dominant lipoprotein species, despite inducing a reduction in intestinal cholesterol absorption [3,22]. In the present study, a reduction in plasma total cholesterol, due to HDL cholesterol fraction, was observed at the end of the progression phase in mice fed a phytosterol supplement. These results suggest that long-term depletion of dietary cholesterol eventually affects the HDL pool, the major lipoprotein fraction in these mice.

No modifications in glucose clearance, insulin responsiveness, and diabetic animal proportion were detected among groups in either the progression or regression phases in our study. These results are inconsistent with the hypoglycemic action of 5 phytosterols extracted from aloe vera described in diabetic db/db mice after 28 days of treatment [6]. Again, a feasible explanation for the different results between that study and ours could be the different plant sterols used or that their derivatives might have singular properties. The structures of antihyperglycemic phytosterols derived from aloe vera were 4-monomethyl and dimethyl sterols [6]. Intestinal phytosterol absorption is known to be very low [23,24], but β-sitosterol is even less absorbed than other phytosterols [23-25]. It is thus worth noting that the major compound used in the present study was β-sitosterol, and Tanaka et al did not find blood glucose level reductions in db/db mice given sitosterol [6]. In contrast, an intravenous extract of stigmasterol derivatives from cashew improved glucose tolerance in healthy dogs [8].

It is also possible that the different origin of the obesity/diabetes (leptin-receptor defective vs diet-induced obesity) could differ in the response of mice to phytosterols. In fact, the administration of phytosterols from aloe vera did not change blood glucose levels in healthy C57BL/6 mice [6]. Further, a physiologic context, that is, absence of obesity/diabetes, can also influence phytosterol effects. The

synthetic phytostanol FM-VP4 improved glucose tolerance in fat type 2 diabetic rats after a 30-day phytostanol treatment, but this did not occur in lean rats [7].

Therefore, the antidiabetic effect of phytosterols reported previously could be highly influenced by several factors such as the nature of the plant sterol and animal model used. Further studies are required to determine the molecular basis of the differential effects of phytosterols on different animal models of type 2 diabetes mellitus.

In conclusion, we were unable to substantiate a protective effect of phytosterol intake (mainly composed by β-sitosterol, campesterol, and stigmasterol) on diabetes development or regression in a mouse model of dietary-induced obesity, insulin resistance, and diabetes mellitus.

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