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RESEARCH LETTER

Phytosterol additive boosts adrenal response to ACTH in male Japanese quail (*Coturnix coturnix japonica*)

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Abstract To investigate the adrenal effect of a phytosterol (PS) additive, 80 male Japanese quail were divided into four sub-groups and fed 0, 40, 400, and 4,000 ppm of PS, respectively, for 21 days. Subsequently, 50% of the birds from each dosage group were subjected to a 6-day adrenal function test, whereby they were injected with long-lasting adrenocorticotropin (ACTH). The remaining quail in each PS dosage group were raised under normal conditions. The groups receiving 400 and 4000 ppm PS exhibited decreased serum levels of LDL-cholesterol with and without ACTH stimulation ($P < 0.01$). No amount of dose of PS changed serum corticosterone (CORT) under normal conditions ($P > 0.05$). Enhancement of CORT was observed on the 2nd and the 6th days of the ACTH challenge in birds receiving 400 ppm ($P < 0.05$). Average ACTH-induced CORT levels in the 400 ppm group were higher than in the 0 ppm group ($P < 0.01$). Our results demonstrated that PS can boost ACTH-induced CORT levels in male Japanese quail.

Keywords Phytosterol · Corticosterone · Quail · Adrenal

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Introduction

Phytosterol (PS) is a steroid compound widely found in nuts, legumes, and seeds. It is a combination of a steroid nucleus and a 3β-hydroxyl. According to their side chain structures, PSs are divided into various analogs, such as β-sitosterol, campesterol, and stigmasterol [1].

The cholesterol-lowering ability of PS has been well documented. However, the effects of PS on adrenal steroidogenesis remained unclear. The adrenal gland is known to be one of the main organs that accumulate PS [2]. The metabolite of β-sitosterol in a cultured swine adrenal slice is cortisol [3]. Moreover, marathon runners who took capsules containing β-sitosterol maintained a "pre-event cortisol: dehydroepiandrosterone sulfate hormone ratio" [4]. These findings imply that PS is a precursor which undergoes steroidogenesis in the adrenal gland and may affect adrenal function. The effect of a PS-enriched diet on adrenal steroidogenesis attracted our interest. After feeding quail with diets containing 0, 40, 400, and 4,000 ppm PS for 21 days, we monitored ACTH-induced corticosterone (CORT) during a 6-day ACTH challenge and studied the effects of PS on the organs, body weights (BW), and LDL-cholesterol (LDL-C) levels in both normal and ACTH conditions.

Methods

Animal husbandry

At 7-days of age, 80 male quail were selected with BWs of 28 ± 3 g. The quail were assigned to one of four dietary treatments. The basal diet was based on corn, fishmeal, and bran. It was formulated according to the Quail Nutrition Standard (NRC 1994). The main ingredients excluded

soybean to avoid interference from soy sterols. A 90% purified PS mixture (β-sitosterol 43.18%, campesterol 23.62%, and stigmasterol 21.46%) was thoroughly mixed into the basic diet at dosages of 0, 40, 400, and 4,000 ppm.

ACTH challenge

After 21-days of PS feeding, 50% of the birds from each dosage group were selected and subjected to a 6-day ACTH challenge. Meanwhile the remaining quail were raised under normal conditions. All of the birds continued their respective feed treatments during the 6-day period. In the ACTH treatment, each quail received 1 IU/100 g BW long-lasting ACTH peptide (CORTROSYN®Z, Daiichi Sankyo Co. Ltd., Japan) through intramuscular injection at 07:00 every morning. At 09:00 on the 2nd, 4th, and 6th days of the test, bloods were taken by venipuncture. Bloods were taken from quail that did not receive ACTH injections on the 2nd and 6th days of the test to serve as normal coricosterone levels. The serum was collected via centrifuge (3,000 rpm; 10 min). All Quail were euthanized when the ACTH challenge ended.

Biochemical Assay

Serum CORT levels were measured using the double-antibody RIA with ^{125}I-labeled ligand as described previously [5]. The method of precipitating serum levels of LDL-C has been described previously [6]. Cholesterol was measured using Cholesterol Assay Kit (DAOS method). The fresh organs were weighed once euthanization was complete.

Statistics

CORT levels at each day point of testing were analyzed by One-Way ANOVA followed by the Dunnett test to compare the 40, 400, and 4,000 ppm groups with the 0 ppm group (GraphPad Prism Version 5). CORT readings from the 2nd, 4th, and 6th days of the ACTH challenge were pooled and served as the ACTH-induced levels on behalf of each PS dosage group. The CORT data from the 2nd and 6th days obtained from the birds not receiving ACTH injections were pooled and served as normal levels for each PS dosage group. A logarithmic calculation was applied to the pooled CORT levels as observed on the 2nd, 4th, and 6th days before analysis. Hormone levels, organ weights, and LDL-C data were analyzed by two-way ANOVA. The $P < 0.05$ was considered to be significant.

Results

As illustrated in Fig. 1a, on the 2nd and 6th days of the adrenal function test, CORT levels in each dosage group were found to be comparable under normal conditions ($P > 0.05$). Figure 1b describes the CORT levels during ACTH stimulation. Quail receiving 400 ppm PS exhibited higher ACTH-induced CORT levels on the 2nd and 6th days compared with quail in the 0 ppm group ($P < 0.05$).

The effects of PS on CORT levels, LDL-C, adrenal, thymus, and BWs under normal conditions and under the ACTH challenge are shown in Table 1. ACTH markedly increased CORT levels ($P < 0.001$). The effects of PS on CORT were considered very significant ($P = 0.004$). Interaction between ACTH and PS was found to affect serum CORT levels ($P = 0.0055$). PS boosted ACTH-induced CORT levels particularly at the dose of 400 ppm ($P < 0.01$). Serum LDL-C concentrations decreased consistently between the 400 and 4,000 ppm PS groups in both normal and ACTH conditions ($P = 0.002$). ACTH treatment also reduced serum LDL-C ($P = 0.032$). ACTH inhibited the thymus ($P < 0.001$) and final BWs ($P < 0.001$). Although PS intake did not affect these parameters significantly ($P > 0.05$), BW

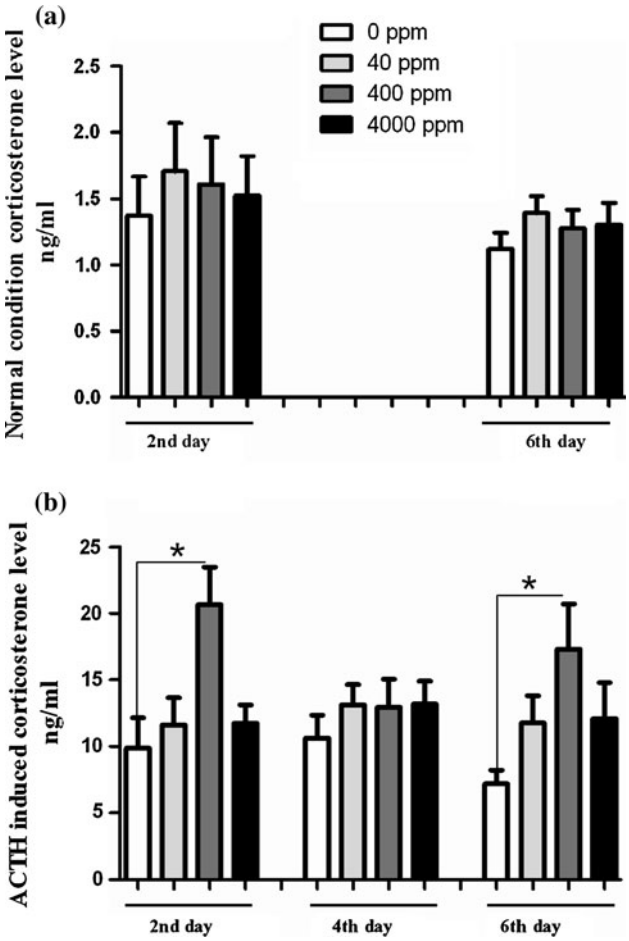

Fig. 1 CORT levels on day 6 of the adrenal function test in normal (**a**) or ACTH condition (**b**). Data were compared within day point. Columns highlighted with *stars* represent the significantly different values obtained when compared to those from the corresponding 0 ppm group ($P < 0.05$)

Table 1 Effects of PS additive on CORT, LDL-C, BW, thymus, and adrenal in male Japanese quail with and without ACTH stimulation

Items	Supplemented PS feed additive								*P* value			
	0 ppm		40 ppm		400 ppm		4,000 ppm		SEM	PS[a]	ACTH	Interaction
	Normal	ACTH	Normal	ACTH	Normal	ACTH	Normal	ACTH				
CORT[b] (ng/ml)	Lg1.20	Lg9.55	Lg1.41	Lg12.17	Lg1.34	Lg16.98	Lg1.32	Lg12.35	0.0049	0.004	<0.001	0.0055
LDL-C[c] (mg/dl)	55.23	46.13	41.55	32.61	37.07	33.28	38.08	35.59	3.11	0.002	0.032	0.6632
BW[d] (g)	89.60	86.51	87.95	77.31	90.55	80.91	92.35	74.51	3.09	0.25	<0.001	0.1191
Adrenal (g)	0.0072	0.0082	0.0084	0.0988	0.0082	0.0987	0.0081	0.0115	0.0009	0.092	0.0017	0.4486
Thymus (g)	0.063	0.035	0.072	0.029	0.065	0.03	0.07	0.003	0.0044	0.95	<0.001	0.3897

Values of each term were analyzed by two-way ANOVA. Except normal CORT ($n = 20$) and ACTH induced CORT ($n = 30$) which were analyzed using a Logarithmic calculation before analysis, values of other items were expressed as means ($n = 10$)

[a] *PS* phytosterol

[b] *CORT* pooled corticosterone

[c] *LDL-C* LDL-cholesterol

[d] *BW* finish body weight

and thymus weights in the 40, 400, and 4,000 ppm groups tended to be lower than in the 0 ppm group under the ACTH challenge. ACTH stimulation increased adrenal weight ($P = 0.007$). Although the effects of PS on adrenal weights were not very significant ($P = 0.092$), adrenal weight tended to increase along with PS dosages after ACTH treatment, particularly in the 4,000 ppm group where significant increases in adrenal weight were observed under the ACTH treatment ($P < 0.05$).

Discussion

Thymus weight and growth are negatively correlated to CORT level in Japanese quail [7]. Thymus weight and BW were probably inhibited by PS in an indirect way. Specifically, PS significantly boosted ACTH-induced CORT, thereby suppressing thymus and muscular developments. Although no significant effects of PS on thymus and BW were detected, a downward trend of these two parameters in PS groups after ACTH challenge implied that doses of 40, 400, and 4,000 ppm suppressed the thymus and muscular growth more severely than that was seen in the 0 ppm group. The differences in inhibition on the thymus and BWs coincide with the differences of ACTH-induced CORT levels between the PS and non-PS groups.

LDL-C was consistently reduced by feeding 400 and 4,000 ppm PS in our experiment. However, unlike statins, PS did not inhibit CORT genesis. The CORT levels observed in the 40–4,000 ppm PS groups were no less than those observed in the 0 ppm group with or without ACTH challenge. Circulating LDL-C is the precursor of the steroid hormone—the extreme inhibition of LDL-C would possibly lead to insufficient steroid hormone. For example, 40–80 mg/day Simvastatin and 40 mg/day Lovastatin reduced the testosterone level in hypercholesterolemia men [8]. However, PS-treated quail in our experiment did not show an insufficiency in steroidogenesis despite declined levels of LDL-C. Similarly, in familial hypercholesterolemia patients LDL receptor of which was reduced by 50%, the "increase rate and plateau concentrations of cortisol" during ACTH challenge appeared as normal [10, 11]. Cortisol production from the adrenals positively correlates with the endogenous cholesterol synthesis rate [9]. PS prevents cholesterol absorption mainly through "competitively displacing cholesterol from micelles" or "precipitating marginally soluble cholesterol into non-absorbable state" [1]. Since PS mildly inhibits exogenous cholesterol absorption and preserves endogenous cholesterol synthesis, we suppose endogenous LDL-C remains sufficient for corticoid synthesis after PS inhibition.

PS probably supplements the steroid precursors. Like cholesterol, campesterol and β-sitosterol also peaked in the rat adrenal fraction assay as observed by gas–liquid

chromatographic mass spectrometric [12]. In guinea pigs, β-sitosterol supplied the tetracyclic nuclease for cortisol production when supplied by either feed or intravenous injection. They also speculated β-sitosterol was transformed to cortisol by the same enzymatic system involved in steroidogenesis [13]. The absorption rate of β-sitosterol is 1.9% in male rats [2]; and more β-sitosterol accumulated in the adrenal gland than cholesterol after intraperitoneal injection in rats [14]. We suppose when PS intake reaches a certain level, PS probably serves as the precursor to compensate for the loss of steroid when the declined LDL-C cannot meet the requirement of steroidogenesis. In our experiments, The ACTH-induced CORT on the 6th day was generally lower than on the 2nd day. The adrenal response to ACTH showed a downward trend which indicated that the adrenal function was declining or the steroid precursor was approaching insufficiency during the ACTH challenge days. In this extreme situation, the effects of PS supplementation were still potent enough to increase steroidogenesis. In summary, the PS precursor boosted CORT genesis, when a large vacancy of steroid precursors existed under the ACTH challenge.

Interestingly, the 4,000 ppm feed did not significantly boost CORT levels beyond those that had already been achieved by the 400 ppm feed. We suppose the effects of PS on adrenal function are dose dependent and do not increase in a linear manner. Data are limited to explain the mechanism; however, similar findings were observed in the field vole (*Microtus agrestis*) [15]. Plasma estradiol and testosterone concentrations in male voles were higher due to their PS supplement being set at 5 mg/kg per day. Those authors speculate that PS precursors possibly increased sex steroid synthesis. However, no significant elevation happened in sex steroid levels when the dose reached 50 mg/kg. In the phytosterolemia patients, whose ATP-binding Cassette sub-family G (ABCG) 5/8 gut transport protein mutated, plasma PS was ∼200–500 times normal. A raised ACTH and poor cortisol response to ACTH appeared in the patient [16]. This suggested that overloading with PS can disrupt adrenal function. We speculate that PS affects adrenal steroidogenesis in a dose-dependent manner.

Conclusion

A PS additive, particularly at a dose of 400 ppm, enhances CORT genesis under ACTH stimulation in male Japanese quail. PS influences adrenal function in a dose-dependent manner. We suppose PS can compensate for LDL-C loss by serving as the precursor of steroid hormone when large amounts of precursors are needed.

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