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Effects of Phytosterols as Food Additives on Adrenal and Reproductive Endocrine Function during Sexual Maturation in Male Japanese Quail (*Coturnix coturnix japonica*)

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Varying amounts of phytosterols (PS) occur naturally in several foods of plant origin. PS, which are structurally and functionally similar to cholesterol, have been shown to reduce plasma total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) levels. Moreover, PS disrupts endocrine function in certain animals. In the present study, we investigated the effects of high doses of PS on adrenal and reproductive endocrine function during sexual maturation in Japanese male quails. Two experiments were conducted; in the first experiment, quail chicks were subjected to long-term chronic feeding of PS (8, 80, and 800 mg/kg body weight [BW]) and the chemicals were gavaged into the crop sac from 7−50 days post-hatching. From the forty-fourth day, half of the animals in each group were subjected to a 6-day adrenocorticotropic hormone (ACTH) challenge for artificial stimulation of the adrenal gland and evaluation of long-term PS effects; in the second experiment, single doses of PS were subcutaneously injected (SC) into adult males (10-weeks-old) to assess the acute direct effect. Results indicated that chronically PS-fed animals showed a better adrenal response to ACTH challenge, and the corticosterone levels were higher ($P<0.05$) than those of the controls. Moreover, corticosterone levels were also high ($P<0.05$) 3 h after SC injection of PS. In contrast, testosterone levels and the testes weights were significantly lower ($P<0.05$) in the groups chronically administered with PS. No differences were observed in the testosterone levels in the acute experiment or luteinizing hormone (LH) levels in either experiment. In conclusion, the differential effects of PS on the adrenal gland and testis might be due to preferential use of different lipoprotein-cholesterol forms for steroid production. In addition, PS might locally perturb testosterone production by its accumulation or delay in testicular maturation.

Key words: adrenal, endocrine, male quail, phytosterol, reproduction

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Introduction

Phytosterols (PS) are plant-derived chemicals, naturally occurring in nuts, legumes, and seeds (Lagarda *et al*., 2006; Liu *et al*., 2012). Except for an extra methyl or ethyl group on C24, the structure of PS is similar to that of cholesterol (Calpe-Berdiel *et al*., 2009; Saeed *et al*., 2015). The most common dietary PS are β-sitosterol, campestrol, and stigmasterol (Yang *et al*., 2004; Lagarda *et al*., 2006; Liu *et al*., 2012). Since 1950, PS have been widely used in human and animal dietary regimens because of their cholesterol-lowering activity and beneficial effects on cardiovascular disease (CVD) (Matvienko *et al*., 2002; Ostlund Jr, 2002; Brufau *et al*., 2008; Elkin and Lorenz, 2009). PS feeding effectively reduces low-density lipoprotein-cholesterol (LDL-C) levels by decreasing intestinal cholesterol absorption without significantly altering high-density lipoprotein-cholesterol (HDL-C) and triglyceride levels (Calpe-Berdiel *et al*., 2009).

PS are plant-based chemicals, and humans and animals cannot endogenously synthesize these substances although cholesterol plays crucial roles in cellular membrane function and steroidogenesis (Yang *et al*., 2004). PS are known to possess endocrine-disrupting activities in different laboratory and aquatic animals (Maclatchy and Vanderkraak, 1995; MacLatchy *et al*., 1997; Moghadasian, 2000). Awad *et al*. (1998) and Singh and Gupta (2016) showed that PS feeding

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significantly reduced testosterone (testosterone plus dihydrotestosterone) levels in male rats. Moreover, β-sitosterol feeding significantly decreased testicular weights and semen quality in rat (Malini and Vanithakumari, 1991; Singh and Gupta, 2016). Goldfish (*Carassius auratus*) exposed to β-sitosterol showed decreased levels of circulating sex steroids and a concomitant decline in gonadal capacity for steroidogenesis (Maclatchy and Vanderkraak, 1995). In the avian model, feeding of PS increased adrenal corticosterone production after adrenocorticotropic hormone (ACTH) stimulation in juvenile male Japanese quail (Liu *et al*., 2012). However, regarding the effects of PS on reproductive endocrine function in avian species during sexual maturation have not been previously reported.

This study investigated the chronic and acute effects of high PS doses on the adrenal and reproductive endocrine function of growing male Japanese quail, and attempted to uncover their mechanism(s) of action.

Materials and Methods

Chemicals

Phytosterol was provided by Tama Biochemical Co., Ltd., Japan, at 97.2% purity (β-sitosterol 42.9%, stigmasterol 23.8 %, campesterol 25.6% and brassicasterol 7.7%). Medium-chain triglyceride (Miglyol 812N) was provided by Mitsuba Trading Co., Ltd, Japan. ACTH (CORTROSYN® Z) was purchased from the Daiichi Sankyo Co. Ltd., Japan.

PS Solution Preparation

The PS suspension was prepared as previously reported to minimize its crystallization and allow for better absorption in the intestinal tract, with minor modifications (von Bonsdorff-Nikander *et al*., 2005). PS and medium-chain triglycerides (MCT) were heated in a vessel with stirring. PS was dissolved at 100℃ until a clear solution was formed. After cooling the solution to 90℃, the vessel was immediately immersed in ice and the suspension was stirred until it reached room temperature (25℃). The suspension was stored in an airtight glass container at 4℃. Before use, the container was kept in warm water (37℃) for 10 min to enable solidification of the solution for easy gavage into the crop sac or for subcutaneous injection (SC).

Experimental Animals and Housing Conditions

Male Japanese quails were used in both experiments. Fertilized quail eggs in our stock were incubated, and newly hatched quails were obtained and housed in cages in a controlled environment (lights on, 5:00−19:00 h; temperature, 24±2℃; humidity, 50±10%; air exchange, 20 times hourly). An additional heater was placed in the cages for the first two weeks to maintain a temperature of approximately 37℃. Chick and adult quails diet was provided by the Quail Cosmos Company, Aichi, Japan. The animals were given free access to food and water *ad libitum*.

Experimental Design

For the chronic PS administration experiment, quail chicks were randomly divided into 5 groups: controls, MCT controls, and PS (8, 80, or 800 mg/kg body weight (BW). Daily single doses of PS and MCT were gavaged by syringe and a plastic needle into the crop sac from 7−50 days of age. At 44 days of age, half of the animals of each group was challenged with a daily intramuscular injection of ACTH (1 IU/100 g of BW) for 6 days consecutively (every morning at 7:00 am) as previously described (Liu *et al*., 2012). Blood was aspirated by jugular venipuncture (using a one-ml tuberculin syringe and 27-gauge needle) on the 2nd, 4th and 6th days of ACTH challenge at 9:00 am. Blood samples were centrifuged at 2,700 *g* for 15 min, and the plasma was stored at −20℃ for the hormone assay. At the end of the experiment, the animals were sacrificed by cervical dislocation under diethyl ether sedation for sampling internal organs. The harvested internal organs were separated from the extra tissues and weighed using a digital balance (ASONE, Corporation, China).

In the acute PS administration experiment, 30 adult male quails (10 weeks of age) were randomly divided into four groups (control and PS [8, 80, or 800 mg/kg BW]). Single doses of MCT and PS were injected subcutaneously at 7:00 am. After injection, blood samples were collected at 3, 6, and 24 h by jugular venipuncture into heparinized plastic tubes and processed for the first experiment. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared at the Tokyo University of Agriculture and Technology.

Testicular Histology

After weighing the testes, the left testis from each animal was quickly fixed in 4% paraformaldehyde (Wako Co., Osaka, Japan). The fixed testis was paraffin embedded, sectioned at 6 μm, and the sections were placed on poly-L-lysine-coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan) for hematoxylin and eosin staining (for details refer to Li *et al*., 2015).

Hormone and Cholesterol Assay

Plasma testosterone and corticosterone were measured using double-antibody radioimmunoassay (RIA) with ^{125}I-labeled radioligand as described previously (Kanesaka *et al*., 1992; Taya *et al*., 1985). Anti-sera against testosterone (GDN250) was provided by Dr. Niswender GD (Colorado State University, Fort Collins, CO. USA); the anti-sera against corticosterone was raised in goat. LH concentrations were measured with RIA (Beltsville, MD, USA) using USDA-cLH-1-3 for iodination and USDA-cLH-K-3 for chicken LH standards. The anti-avian LH (HAC-CH27–01 RBP75, Gunma University, Maebashi, Japan) was used as antisera for LH as described previously (Li *et al*., 2006). The intra- and inter-assay coefficients of variation were 3.6% and 6.1% corticosterone, 4.5% and 9.2% for testosterone, and 6.1% and 5.3% for LH, respectively. The total cholesterol level in quail plasma was measured using Cholesterol E-Test Wako (Wako Pure Chemical Co., Osaka, Japan) according to manufacturer's instruction.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA, USA). Data at each time-point were analyzed using the 1-way analysis of variance (ANOVA) to determine significant differences among the groups, and Tukey's and Dunnett's multiple-comparison tests were used for chronic and acute experiments, respectively. Three-way

Table 1. **Effects of PS gavage on body and organ weights in male Japanese quails**

Groups	Animal No	BW (g)	Testes weights (g)		Adrenal weights (mg)	Liver weights (g)
			Right	Left		
Control	10	107.86±2.3	0.92±0.12[a]	1.00±1.15[a]	12.1±1.7	3.58±0.2
MCT	10	107.74±1.9	0.99±0.18[a]	1.09±0.17[b]	15.6±4.1	3.15±0.12
8 mg/kg BW	10	107.00±1.5	0.65±0.12[b]	0.65±0.13[b]	10.5±0.8	3.59±0.22
80 mg/kg BW	10	104.19±2.9	0.69±0.11[b]	0.77±0.13[b]	9.23±1.3	3.19±0.23
800 mg/kg BW	10	107.79±2.4	0.62±0.12[b]	0.53±0.15[b]	10.3±1.8	3.62±0.19

Values represent means±SEM. Different superscript letters within a column denote significant differences among groups ($P<0.05$). Values without superscript letters denote non-significant difference.

ANOVA was used to analyze corticosterone levels affected by PS, ACTH challenge, and the days (2 and 6) after injection in controls, MCT-treated, and PS-treated quails in half of the animals in each group of the chronic dosage experiment (trail version of GraphPad Prism 7). Hormone concentrations in the acute experiment were analyzed using 2-way ANOVA to analyze the changes at 3, 6, and 24 h after SC injection of PS. All results are represented as means± standard error of the mean (SEM). $P<0.05$ was considered to be statistically significant.

Results

Chronic Feeding of PS

As illustrated in Table 1, long-term PS gavage exerted no significant effects on quail body weight or on liver or adrenal gland weight; further, none of the quails died during the experiment. However, testis weight was significantly reduced in animals treated with PS compared to those of intact quails or MCT controls. In addition, cholesterol levels showed a tendency to decrease after long-term PS administration in the doses of 8 mg (208±97 mg/dL), 80 mg (193±41 mg/dL), and 800 mg/kg BW (188±99 mg/dL) compared to the control (211±65 mg/dL) and MCT group (201±33 mg/dL).

Hormone Levels

Corticosterone levels were not different among the normal groups (non-ACTH challenged); however, the PS-treated quails showed higher ACTH-induced corticosterone levels than the controls and the MCT-treated group. After 6 days of ACTH challenge, corticosterone levels were significantly elevated in the groups receiving 80 and 800 mg/kg BW of PS compared to the MCT and control groups ($P<0.01$ and $P<0.05$, respectively). Additionally, corticosterone concentrations were significantly higher in quails injected with ACTH compared to that of the normal control within the same treatment groups ($P<0.001$). A three-way ANOVA was used to determine whether corticosterone levels varied among the groups with PS treatment, ACTH injection, and duration post-ACTH injection. Results indicated that after 6 days of ACTH injection, corticosterone production was significantly enhanced in PS-treated quails were gavaged PS (80 and 800 mg/kg BW) than in the normal and MCT groups (Fig. 1).

In contrast, testosterone levels were significantly reduced after ACTH challenge in the groups receiving 80 ($P<0.05$) and 800 mg/kg BW ($P<0.01$) PS by gavage, compared to the control and MCT group. However, testosterone levels were not significantly different after a 6-day ACTH injection between the normal controls and ACTH-challenged groups except for 800 mg/kg PS ($P<0.01$; Fig. 2). In addition, no significant differences in LH levels were observed among the groups in the absence of ACTH (controls [4.99±0.48 ng/m*l*] and MCT [5.22±0.53 ng/m*l*], and with PS at doses of 8 [5.50±0.75 ng/m*l*], 80 [6.84±0.55 ng/m*l*], or 800 mg/kg BW [4.52±0.65 ng/m*l*]), or with ACTH injections (controls [5.12 ±1.09 ng/m*l*] and MCT [5.74±1.00 ng/m*l*], and with PS at doses of 8 [4.60±0.82 ng/m*l*], 80 [4.76±1.46 ng/m*l*], or 800 mg/kg BW [4.37±0.14 ng/m*l*]).

Testicular Histology

Histological examination of quail testes in the different groups revealed structural differences. The seminiferous tubules and lumen of testes were enlarged in the animals of the control and MCT group (Fig. 3A and 3B, respectively). Moreover, typical spermatogenesis and the presence of multi-nuclear spermatocytes indicated functional maturity of the testes. However, testes of quails treated with different doses of PS (8, 80, 800 mg/kg BW) showed a reduction in seminiferous tubule size (Fig. 3C, 3D, and 3E, respectively). Therefore, higher number of seminiferous tubules were visible in one microscopic field of view. In addition, the lumen of the seminiferous tubules was not formed, and active spermatozoa were not observed.

Acute Effects of PS

Quails in the groups receiving 8 or 80 mg/kg PS had significantly higher ($P<0.05$) corticosterone levels 3 h after PS injection. However, the corticosterone levels were decreased and were maintained at a basal level. Except for quails injected with 8 and 800 mg/kg BW PS, no differences were observed in corticosterone levels within the same groups in terms of PS treatment and various time-points. Corticosterone levels were significantly reduced 6 h after injection of 8 and 80 mg/kg BW PS compared to that observed 3 h post-injection ($P<0.001$; Fig. 4). A slight reduction in testosterone level was observed at each time-point after PS injection compared to the controls. Moreover, no differences were identified in testosterone levels within the same groups in terms of PS treatment and various time-points (Fig. 5). LH levels did not change significantly among the groups at

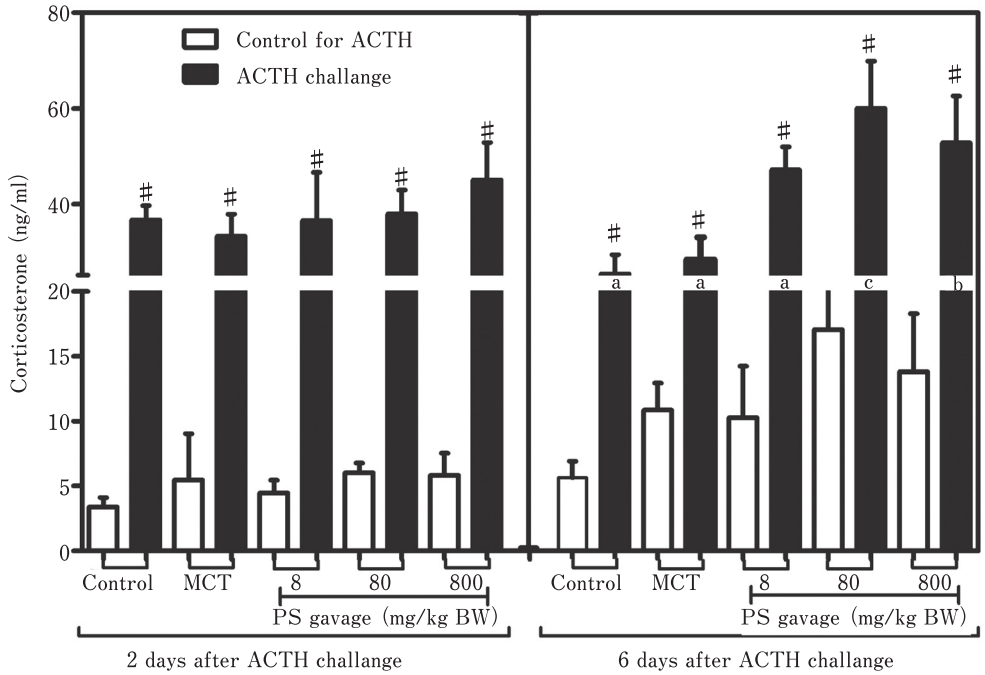

Fig. 1. **Corticosterone concentrations after 2 and 6 days of ACTH challenge in the normal controls (white bars), and ACTH-challenged (black bars) male Japanese quails after chronic gavage of PS.** Hash-marks denote significant differences in corticosterone levels between quails in the same treatment groups challenged by ACTH or kept as normal control (non-ACTH injection; $P<0.001$). Different letters indicate significance differences among groups injected ACTH ($P<0.05$).

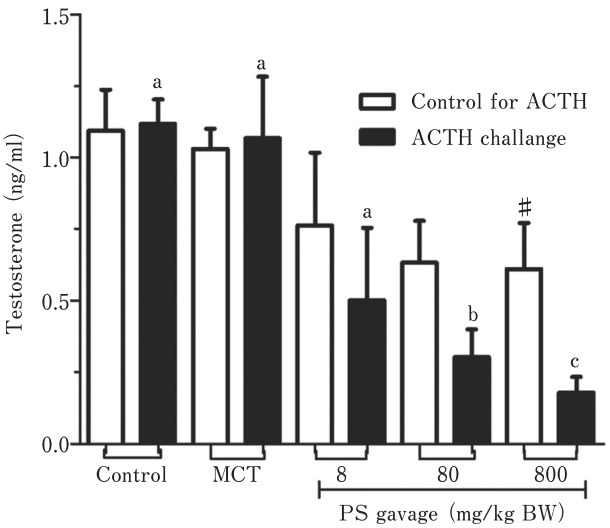

Fig. 2. **Plasma testosterone levels in normal controls (white bars) and ACTH- challenged (black bars) male Japanese quails after long-term PS gavage.** Different letters denote significant differences among groups challenged by ACTH ($P<0.05$) and the hash mark represents significant difference in testosterone level between quails in the same treatment groups injected ACTH or kept as normal control (non-ACTH challenge; $P<0.01$).

each time-point: 3 h (control [6.58±1.28 ng/m*l*] after PS administration at doses of 8 [7.16±1.17 ng/m*l*], 80 [6.95±1.79 ng/m*l*] and 800 mg/kg BW [6.52±0.95 ng/m*l*]), 6 h (control [6.58±1.33 ng/m*l*] and after PS administration at doses of 8 [4.63±0.97 ng/m*l*], 80 [5.69±1.23 ng/m*l*], and 800 mg/kg BW [5.37±0.88 ng/m*l*]), and 24 h (control [5.80±0.46 ng/m*l*] and after PS administration at doses of 8 [4.41±0.60 ng/m*l*], 80 [5.27±0.73 ng/m*l*] and 800 mg/kg BW [4.61±0.75 ng/m*l*]). Cholesterol levels did not differ significantly among controls (188±92 mg/dL) and PS-treated animals at doses of 8 mg (180±64 mg/dL), 80 mg (179±13 mg/dL) or 800 mg/kg BW (175±81 mg/dL).

Discussion

In this study, we investigated the effects of high PS doses on adrenal and reproductive endocrine function of male quails during sexual maturation. No treatment-related deaths and clinical signs of toxicity were observed throughout the study period. Moreover, quails treated with PS effectively responded to an ACTH challenge and produced high corticosterone levels relative to controls. However, testosterone levels and testicular weights were significantly reduced after long-term PS feeding compared to that in the controls.

Chronic feeding of PS significantly reduced testicular weight and testosterone production in growing male quails.

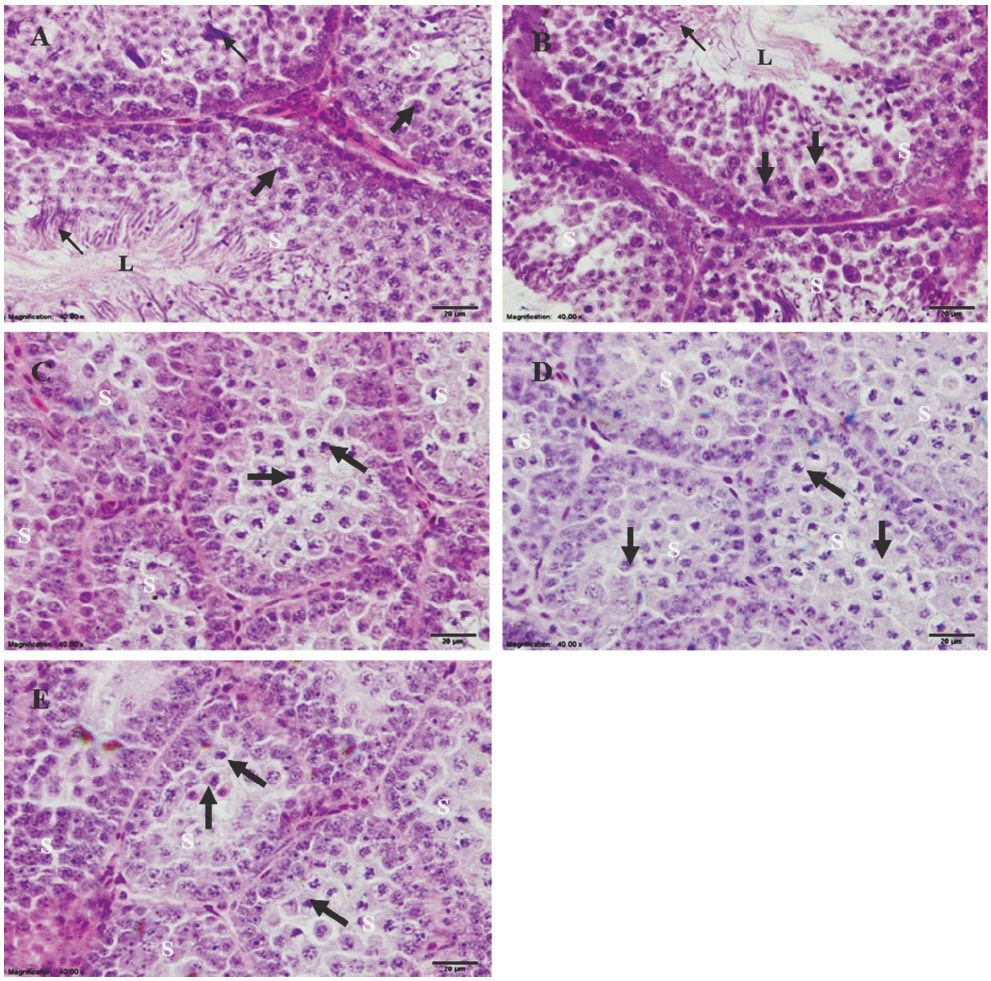

Fig. 3. **Transverse sections of quail testes in the different groups; control (A), MCT (B), and PS at doses of 8 mg (C), 80 mg (D), and 800 mg/kg BW (E).** The different letters represent the seminiferous tubules (S) and lumen (L). Arrows denote spermatozoa (thin arrows) and spermatocytes (thick arrows). Objective magnification, ×40.

Similar results were previously reported by Singh and Gupta (2016) for the male albino rat, as PS feeding reduced gonadal weight and testosterone production (Awad *et al*., 1998; Singh and Gupta, 2016). Moreover, intraperitoneal injections of β-sitosterol significantly decreased testosterone and 11-ketotestosterone levels in male and testosterone and 17β-estradiol levels in female goldfish (Maclatchy and Vanderkraak, 1995). Maclatchy and Vanderkraak (1995) also reported that pregnenolone levels were significantly reduced in goldfish exposed to β-sitosterol. The male Japanese quail usually attains maturity between 50 and 60 days of age, and testicular weights and testosterone levels rapidly increase between 25 and 60 days under normal conditions (Ottinger and Brinkley, 1979). In the present study, histological examination revealed that testes in the PS-treated animals were not mature, and that lumens of seminiferous tubules were not formed. Long-term PS feeding during growth presumably slowed testicular maturation and reduced testosterone because of the low cholesterol levels.

Compared to the controls, corticosterone production was higher after chronic and acute exposure to PS. Animals effectively responded to ACTH challenge and produced high amounts of corticosterone. Comparable results were previously reported for juvenile male Japanese quails (Liu *et al*., 2012). Except for one study in humans (Mushtaq *et al*., 2007), evidence regarding PS induced adrenal insufficiency is lacking. Liu *et al*. (2012) suggested that as PS reduced circulating cholesterol (LDL-C) levels, adrenal glands likely used PS as precursors for steroidogenesis; alternatively, endogenous LDL-C was sufficient for adrenal corticosterone production. Adrenal glands and gonads may preferentially use different sources of cholesterol (lipoproteins) for steroidogenesis. Human and rodent adrenals utilize HDL-C (Yang *et al*., 2004; Bochem *et al*., 2013); in contrast, testi-

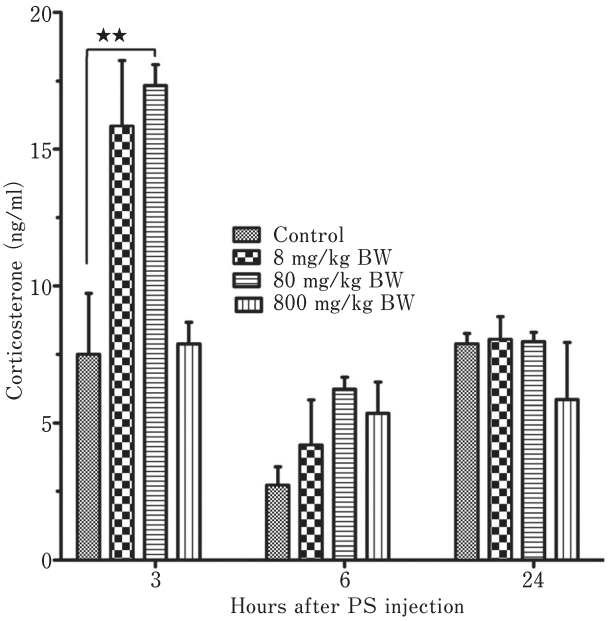

Fig. 4. **Corticosterone levels in male quails after 3, 6, and 24 h of acute exposure to PS.** The asterisks denote significant differences from controls ($P<0.05$).

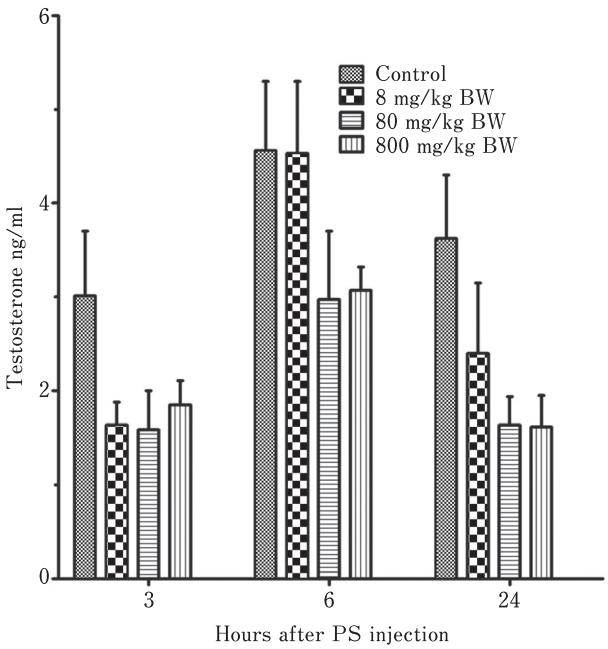

Fig. 5. **Testosterone levels after 3, 6, and 24 h of SC injection of PS in male quails.**

cular steroidogenesis mostly depends upon a continuous supply of cholesterol derived from either *de-novo* synthesis or from uptake of circulating LDL-C level (Dobs *et al*., 2000). Conversely, PS effectively reduced LDL-C levels, while HDL-C and triglyceride levels were not significantly altered (Moghadasian and Frohlich, 1999; Calpe-Berdiel *et al*., 2009). Therefore, the differential impacts of PS on lipoprotein-cholesterol absorption might contribute to the various effects observed on adrenal and gonadal steroidogenesis. Furthermore, after long-term administration by gavage, PS might accumulate in the testes and perturb testosterone production by suppressing genes responsible for cholesterol trafficking, as previously reported in fish (Gilman *et al*., 2003; Leusch and MacLatchy, 2003; Sharpe *et al*., 2007).

Acute and chronic exposure to PS did not change LH levels among groups. Yanmin and Tian (2008) previously demonstrated that feeding PS significantly increased estradiol (E2) and decreased LH in the laying hen. Sriraman *et al*. (2015) found that PS exhibited estrogenic activity in MCF-7 cells *in vitro*. However, *in vivo* estrogenic activity of PS on laboratory animals has not been clearly confirmed. In male Japanese quails, LH secretion occurs in an episodic manner, and thus we recommend that this be pursued in further studies.

In conclusion, our results suggest that PS feeding might induce local effects on the adrenal gland and on gonadal functions. The controversial effects of PS on adrenal and gonadal steroidogenesis might cause preferential utilization of lipoprotein-cholesterol as a steroid precursor. Moreover, chronic feeding of PS might ultimately delay sexual maturation and reduce testosterone production.

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