

Effects of dietary phytosterols supplementation on serum parameters, nutrient digestibility and digestive enzyme of white feather broilers

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ABSTRACT

The aim of this study was to evaluate the impacts of dietary phytosterol (PS) addition at different levels on serum parameters, nutrient digestibility and digestive enzyme activities of white feather broilers. A total of 600, 1-day-old male broilers were randomly divided into five groups with six replicates and were fed a basal diet supplemented with 0 (control group), 10, 20, 40, or 80 mg PS/kg for 42 days. The results showed that dietary supplementation with PS at 20 and 40 mg/kg decreased malondialdehyde accumulation and increased glutathione peroxidase concentrations compared with the control group ($p < .05$). Dietary PS at more than or equal to 20 mg/kg level significantly decreased ($p < .05$) the uric acid and aspartate aminotransferase concentrations, but increased serum albumin, immunoglobulin A and immunoglobulin G levels. In addition, compared with the control group, supplementing PS at 40 mg/kg significantly increased the nutrient digestibility of calcium, dry matter and crude protein, improved trypsin and lipase activities of the pancreas as well as lipase and amylase activities of intestine ($p < .05$). In conclusion, dietary PS supplementation, especially at 40 mg/kg, could improve antioxidant capacity, immune function, nutrient digestibility and digestive enzyme activities of white feather broilers, providing insights into its application as a potential feed additive in broiler production.

HIGHLIGHT

- Phytosterols as a new type of feed additives have a positive impact on broilers
- Dietary phytosterols supplementation can improve antioxidant status and immune function in broiler.
- The nutrient digestibility and digestive enzyme activities were improved by dietary addition of phytosterols.

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Introduction

Under large-scale farming conditions, especially with the ban or restriction of antibiotic usage, environmental stressors (such as high temperature) and pathogenic factors (such as *Clostridium perfringens*) are likely to cause adverse consequences for fast-growing broilers, which are related to oxidative stress and immunosuppression (Mujahid et al. 2005; Zhang et al. 2015; Liu et al. 2018). Therefore, it is necessary to find efficient feed additives to improve the antioxidant and immune functions of broilers (Cheng et al. 2020).

With the advent of drug resistance and adverse effects of chemosynthetic drugs, the interest in medicinal herbs and plant extracts/metabolites has augmented, both among general public and researchers

worldwide (Ebrahim et al. 2020; Abo Ghanima et al. 2020; Dhama et al. 2021). Phytosterols (PS) are natural steroid compounds derived from plants, and mainly originate from vegetable oils, plant seeds and legume products, which resemble cholesterol in terms of structure and physiological functions (Moreau et al. 2002; Santas et al. 2013). The most frequent phytosterols in nature are β -sitosterol, campesterol, and stigmasterol (Piironen & Lampi 2003). Since the 1950s, many experimental studies have shown that PS have good prevention and treatment effects on hypercholesterolaemia and cardiovascular diseases (Normén et al. 2000; Jones 2007). In addition, PS have been additionally demonstrated to have such growth regulation, immunomodulatory, anti-inflammatory and anti-diabetic effects (Santas et al. 2013; Xie et al. 2015). Shi

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et al. (2014) observed that long-term use of high-dose phytosterols (up to 800 mg/kg) did not induce any toxicological effects. PS, as one of the efficient functional feed additives with antioxidant and immunomodulatory effects, have received more attention from animal nutritionists (Cheng et al. 2020).

Previous studies have shown that PS can significantly increase antioxidant enzyme activities such as superoxide dismutase and GSH-Px and decrease malondialdehyde concentration, to ameliorate oxidative stress (Baskar et al. 2012; Song et al. 2017). Zhao et al. (2019) reported that PS supplementation improved antioxidant status and meat quality of Partridge Shank chickens, and the recommended dosage is 40 mg/kg. Moreover, a main component of PS (β -sitosterol) has been demonstrated to not only improve antioxidant status but can also improve immune function in broiler by improving intestine IgA, IgG and IgM (Cheng et al. 2020).

Nevertheless, data on the effect of PS on digestive function in poultry is scarce. Therefore, this experiment was conducted to evaluate the impacts of dietary PS addition at different levels on serum parameters, nutrient digestibility and digestive enzyme activities of white feather broilers.

Materials and methods

Animals, diets and management

A total of 600, 1-day-old male, white feather broilers with an average initial weight of 45.20 ± 0.30 g and healthy age were allotted to a completely randomised design with 5 treatments for a 42 d feeding trial after individual weighing. Each treatment consisted of 6 replicates (cages) with 20 birds each. During the starter phase, the 20 broilers in each replicate were reared in a single cage ($120 \times 60 \times 50$ cm), whereas during the grower phases, each replicate was housed in 2 cages of this size with 10 birds per cage. Broilers had free access to mashed feed and water throughout the whole period. In a controlled environment with a relative humidity of 45% to 55% and a temperature of 25°C to 34°C, the broilers were maintained on an 18 h light and 6 h dark cycle. In the first week of the experiment, the ambient temperature was maintained at 34°C, then gradually decreased to 25°C after 21 d, and maintained thereafter. Broilers were fed a corn-soybean meal basal diet supplemented with 0 (control group), 10, 20, 40, and 80 mg PS/kg (Zhejiang Delekang Food Co., Ltd., Zhejiang, China), respectively. The dosages of PS were selected according to previous studies (Bo et al. 2015; Zhao et al. 2019).

Table 1. Composition and nutrient level of the basal diet (air-dry basis).

Items	Starter (1 to 21 D)	Grower (22 to 42 D)
Ingredients (%)		
Corn	55.00	60.00
Soybean meal	34.00	29.00
Fish meal	4.00	3.00
Soybean oil	3.00	4.00
Lysine	0.20	0.25
Methionine	0.25	0.20
Threonine	0.05	1.30
Dicalcium phosphate	1.50	1.00
Limestone	1.00	0.25
Premix ^a	1.00	1.00
Total	100.00	100.00
Nutrient composition		
ME ^b (kcal/kg)	3.00	3.10
Crude protein (%)	22.00	20.00
Calcium (%)	1.00	0.90
Available phosphorus (%)	0.67	0.65
Methionine (%)	0.60	0.50
Lysine (%)	1.30	1.25
Methionine + Cystine (%)	0.93	0.80
Threonine (%)	0.86	0.76

^aPremix provided the following per kilogram of diet: vitamin A: 6,000 IU; vitamin D3: 3,000 IU; vitamin E: 30 IU; vitamin B1: 3 mg; vitamin B12: 1 mg; vitamin B2: 6 mg; vitamin K: 2 mg; vitamin B6: 2.5 mg; choline chloride: 1 mg; biotin: 0.2 mg; folic acid: 1 mg; niacin: 30 mg; pantothenic acid: 15 mg; lysine: 2 mg; methionine: 0.5 mg; threonine: 0.8 mg; NaCl: 2.5 mg; Fe: 60 mg; Zn: 70 mg; Mn: 80 mg; Cu: 6 mg; I: 1.1 mg; Se: 0.3 mg.

^bME: Metabolizable Energy, based on calculated values; others were analysed values.

The composition and nutrient content of the corn-soybean meal basal diet (starter diet and grower diet) are presented in Table 1.

Sample collection and processing

At 21 d and 42 d, two birds of moderate weight were randomly picked from each replicate. Blood samples (5 mL) were collected from the brachial vein into a 10 mL anticoagulant-free Vacutainer tube (Greiner Bio-One GmbH, Kremsmunster, Austria). After standing 37°C for 2 h, the serum was separated by centrifugation at 3,000 xg for 15 min at 4°C and stored in a refrigerator at -80°C for further analysis. The broilers were euthanized by cervical dislocation and necropsied immediately. The duodenum contents, the intestine segments of the duodenum, jejunum, and ileum were carefully collected, immediately placed in cryogenic vials, and stored at -80°C.

Serum parameters

The malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), and catalase (CAT) contents in the serum were assayed by using the corresponding commercial assay kits (Nanjing Jiancheng

Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacturer's instructions. The results were normalised against total protein concentration in each sample for intersample comparison.

Serum immunoglobulins (Ig), including IgA, IgG, and IgM, were measured using the ELISA quantitation kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. According to the instructions of the manufacturer, the per sample was analysed 3 duplicates, and the absorbance was measured at 450 nm. The concentrations of IgA, IgG, and IgM were calculated by using standard curves constructed from the standards run on the plate.

The serum biochemical parameters including total protein (TP), albumin (ALB), globulin (GLO), glucose (GLU), uric acid (UA), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified using the corresponding commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following the manufacturer's instructions.

Apparent nutrient digestibility

Samples were subsequently thawed to room temperature and dried in a forced ventilation oven at 55 °C until constant weight. Feed and diets samples were then ground to a particle size of 0.5 mm. The dry matter (DM) content was obtained by oven drying the samples at 105 °C for 16 h, and crude protein (CP, method 984.13), ash (method 942.05), ether extract (EE, method 920.39), phosphorus (P, method 965.17), and calcium (Ca, method 968.08) contents were analysed by the methods of the AOAC (2007) (AOAC 2007).

Digestive enzymes

Use a homogeniser to thaw and homogenise the pancreas and duodenum contents in 10 volumes of ice-cold PBS (pH 7.0), and then centrifuge at 16,000 × g for 20 min at 4 °C. The supernatant was divided into

aliquots to measure the activities of trypsin, lipase, and amylase. The digestive enzymes activities were measured by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using commercial kits according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA) and Duncan's multiple range test for multiple comparisons using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Results are presented as means with standard error of the mean (SEM). A value of $p < .05$ was considered statistically significant.

Results

Serum antioxidant capacity

The effects of dietary PS supplementation on the antioxidative enzyme activities of serum are shown in Table 2. Compared with the control group, dietary supplementation with PS at 20 and 40 mg/kg decreased MDA accumulation and increased GSH-PX concentrations ($p < .05$). There is no significant difference among all groups in serum SOD, CAT and T-AOC activity ($P > .05$).

Serum immune parameters

The effects of dietary PS supplementation on serum immune parameters are shown in Table 3. The administration of PS at doses of 20 and 40 mg/kg increased ($p < .05$) the IgA and IgG levels compared to the control group, but did not statistically influence ($P > .05$) the IgM.

Serum biochemical parameters

The effects of dietary PS supplementation on serum biochemical parameters are shown in Table 4.

Table 2. Effects of phytosterols supplementation on serum antioxidant capacity of white feather broilers.

Items ^A	PS ^B (mg/kg)					SEM ^C	p Value
	0	10	20	40	80		
SOD (U/mL)	273.29	305.88	329.59	320.73	294.09	13.535	.750
MDA (nmol/mL)	4.62 ^a	3.09 ^{ab}	2.99 ^b	2.96 ^b	4.06 ^a	0.196	.003
GSH-PX (U/mL)	63.10 ^b	70.25 ^b	92.51 ^a	94.26 ^a	92.09 ^a	4.207	.023
CAT (U/mL)	3.48	4.28	4.26	3.16	3.36	0.209	.298
T-AOC (mmol/L)	0.74	0.90	0.91	0.91	0.85	0.033	.495

^ASOD: superoxide dismutase; MDA: malondialdehyde; GSH-Px: glutathione peroxidase; CAT: catalase; T-AOC: total antioxidant capacity.

^BPS: phytosterols.

^CSEM: total standard error of means.

^{a-b}Means with different letters within a row differ significantly ($p < .05$).

Table 3. Effects of phytosterols supplementation on serum immune parameters of white feather broilers.

Items ^A (mg/mL)	PS ^B (mg/kg)					SEM ^C	p Value
	0	10	20	40	80		
IgA	7.53 ^b	10.68 ^{ab}	14.83 ^a	14.69 ^a	12.24 ^a	0.867	.020
IgG	15.31 ^b	22.09 ^a	24.26 ^a	22.38 ^a	21.82 ^a	1.023	.039
IgM	6.66	6.03	7.69	7.56	6.72	0.487	.254

^AIgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M.

^BPS: phytosterols.

^CSEM: total standard error of means.

^{a-b}Means with different letters within a row differ significantly ($P < .05$).

Table 4. Effects of phytosterols supplementation on serum biochemical parameters of white feather broilers.

Items ^A	PS ^B (mg/kg)					SEM ^C	p Value
	0	10	20	40	80		
TP (g/L)	33.44	34.53	35.48	34.71	35.96	0.768	.895
ALB (g/L)	12.47 ^b	12.77 ^{ab}	14.09 ^a	14.95 ^a	13.61 ^{ab}	0.278	.013
GLO (g/L)	20.98	21.75	21.39	19.76	22.35	0.791	.903
GLU (mmol/L)	12.44	11.73	13.51	13.18	13.51	0.320	.343
UA (μ mol/L)	103.27 ^a	95.00 ^{ab}	87.82 ^b	86.03 ^b	91.27 ^b	5.166	.026
ALT (U/gprot)	7.00	6.84	6.97	6.73	6.89	0.504	.824
AST (U/gprot)	36.25 ^a	29.33 ^b	24.36 ^b	26.15 ^b	27.38 ^b	1.277	.014

^ATP: Total protein; ALB: albumin; GLO: globulin; GLU: glucose; UA: uric acid; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

^BPS: phytosterols.

^CSEM: total standard error of means.

^{a-b}Means with different letters within a row differ significantly ($p < .05$).

Table 5. Effects of phytosterols supplementation on apparent nutrient digestibility of white feather broilers.

Items ^A (%)	PS ^B (mg/kg)					SEM ^C	p Value
	0	10	20	40	80		
DM	72.92 ^b	74.24 ^{ab}	75.67 ^{ab}	77.28 ^a	76.84 ^a	2.768	.025
CP	72.07 ^b	76.08 ^{ab}	78.31 ^a	79.85 ^a	78.05 ^a	2.278	.013
CF	24.48	23.84	22.68	22.43	23.48	0.791	.903
EE	69.13	69.59	70.05	72.81	67.35	2.320	.543
Ca	44.36 ^b	43.82 ^b	52.52 ^a	49.45 ^a	47.52 ^{ab}	1.304	.024
P	43.28	42.94	44.99	42.41	44.26	1.277	.614

^ADM: dry matter; CP: crude protein; CF: crude fibre; EE: ether extract; Ca: calcium; P: phosphorus.

^BPS: phytosterols;

^CSEM: total standard error of means.

^{a-b}Means with different letters within a row differ significantly ($p < .05$).

Compared with the control group, PS at dosages of 20 and 40 mg/kg increased the concentration of serum ALB ($p < .05$), and decreased the UA and AST concentrations. No significant differences in TP, GLO, GLU and ALT were found among the treatment group and the control group ($P > .05$).

Apparent nutrient digestibility

The effects of dietary PS supplementation on apparent nutrient digestibility are shown in Table 5. The DM and CP digestibility were increased ($p < .05$) with dietary supplementation of PS (40 mg/kg). And dietary supplement PS (20 mg/kg) increased Ca digestibility ($p < .05$). Statistically significant differences were not observed regarding the CF, EE and P digestibility ($P > .05$).

Digestive enzymes activities

The effects of dietary PS supplementation on digestive enzyme activities in the pancreas and intestine of broilers are shown in Table 6. Broilers receiving PS administration at a dosage of 40 mg/kg increased ($p < .05$) pancreas trypsin and lipase activities, whereas no differences ($P > .05$) were found in the contents of the amylase among all groups. PS supplementation also increased intestine lipase and amylase activities, but the trypsin activities showed no differences ($P > .05$) among the groups.

Discussion

The use of natural products containing bioactive components showed a promising approach in poultry industry (Alagawany et al. 2020; Abd El-Hack et al.

Table 6. Effects of phytosterols supplementation on digestive enzyme activities of white feather broilers.

Items	PS ^A (mg/kg)					SEM ^B	<i>p</i> Value
	0	10	20	40	80		
Pancreas							
Trypsin (U/mgprot)	703.03 ^c	712.86 ^c	802.15 ^a	820.65 ^a	760.51 ^b	26.999	.042
Lipase (U/gprot)	2089.77 ^b	2410.49 ^a	2426.18 ^a	2631.92 ^a	2212.09 ^{ab}	55.000	.005
Amylase (U/mgprot)	6.47	5.61	7.05	7.72	7.77	0.284	.062
Intestine							
Trypsin (U/mgprot)	482.82	473.82	517.08	528.31	508.82	31.956	.986
Lipase (U/gprot)	10.14 ^b	12.34 ^a	13.14 ^a	12.99 ^a	11.94 ^{ab}	0.726	.043
Amylase (U/mgprot)	2.09 ^b	2.42 ^a	2.37 ^a	2.60 ^a	2.02 ^b	0.163	.025

^APS: phytosterols.

^BSEM: total standard error of means.

^{a-c}Means with different letters within a row differ significantly ($p < .05$).

2020; Bilal et al. 2021). PS as a new type of feed additives have been applied in animal production, which has growth regulation functions and promotes animal health (Fernandes and Cabral 2007). The present study aimed to evaluate the effects of PS supplementation on serum parameters, nutrient digestibility and digestive enzyme activities of white feather broilers.

Antioxidant capacity is usually evaluated by determining the activity of antioxidant enzymes (Elwan et al. 2021). Oxidative stress refers to metabolic and radical substances or so-called reactive (oxy-gen, nitrogen, or chlorine) species (Götz et al. 1994). Free radical overproduction can be quenched by nonenzymatic (like GSH-Px) and enzymatic antioxidant systems (like SOD, CAT). MDA is an end product of lipid peroxidation in which carbon-carbon double bond of lipid is attacked by a free radical, and serves as an indicator to reflect lipid peroxidation (Liu et al. 2018). Previous studies have shown that PS can improve antioxidant enzymes activities in vitro studies (Vivancos and Moreno 2005) and in vivo studies (Summanen et al. 2010). It has been reported that PS chemically acts as an antioxidant, a modest radical scavenger (Yoshida and Niki 2003). In the current study, dietary PS supplementation increased serum GSH-Px and SOD activities, whereas it decreased its MDA accumulation of white feather broilers. This result accords well with the finding of Zhao et al. (2019), who has indicated that dietary PS supplementation increased serum GSH-Px activity and hepatic GSH-Px and SOD activities of Partridge Shank chickens at 21 d. Furthermore, Cheng et al. (2020) reported that dietary β -sitosterol supplementation increased intestinal GSH and CAT activities, and reduced MDA content in broilers. Dietary PS can improve antioxidant status of animals by activating nonenzymatic and enzymatic antioxidant system.

Serum immunoglobulins are important indicators of an animal's immune status (Wang et al. 2017). IgA, IgG and IgM are three major classes of immunoglobulin in broiler, protecting the immune system and

maintaining the health status of the body (Wang et al. 2021). IgA is the main form of antibodies in body secretions and plays an important role in defense against many pathogens (Jiang et al. 2018). The IgG, secreted by B cells, directly contributes to an immune response including neutralisation of toxins and viruses (Song et al. 2018). The main effect of IgM is that when the body is violated by the pathogen, it is combined with the complement to dissolve the pathogenic bacteria, and finally the immune effect is achieved (Han et al. 2018). In this study, we observed that dietary supplement PS increased the IgA and IgG levels compared to the control group, but did not influence the IgM significantly. Cheng et al. (2020) also demonstrated that dietary β -sitosterol addition at higher than or equal to 60 mg/kg level increased jejunal IgG content and its dosage higher than or equal to 80 mg/kg level improved ileal SIgA content.

Biochemical indicators in the blood can be used to display the health status of birds (Reda et al. 2020). Serum total proteins consist of albumin and globulin, and their content can effectively reflect protein metabolism, feed conversion rate and growth of animals (Xie et al. 2010). In this study, we find that dietary supplementation of PS can increase the concentrations of serum ALB. Wang (2014) reported that the addition of PS significantly increased the TP and ALB concentrations of 21-day-old broilers. PS can promote protein deposition in broilers in vivo, improve feed conversion rate and promote growth. Serum ALT and AST are very low under normal conditions (Zhu et al. 2014). When liver damages or an increase in the permeability of liver cells are present, ALT and AST are released into the blood to increase their activity and their activity level is considered to be a specific indicator of liver injury or damage (Toghyani et al. 2011; Zhu et al. 2014; Króliczewska et al. 2017). ALT is mainly distributed in the liver, followed by skeletal muscles, kidney, myocardial tissue, etc. AST is mainly distributed in myocardial cell, liver tissue, etc. ALT is a more specific

indicator of liver damage than AST. In the present study, we found that dietary supplement PS can decrease AST concentration. The addition of phytosterols will not damage liver cells, so the reduction of ALT is not significant. It may be because it is a natural steroid compound extracted from plants, so it is beneficial to animals. Serum urea level is an indicator that reflects the status of protein metabolism, renal function and nutrition of the body. UA is mainly generated by protein and nucleic acid degradation and is the main form of ammonia excretion in chickens (Xie et al. 2010). In our experiment, the UA level in the serum of Mahua broilers significantly decreased in the treatment groups, thus showing that PS improves protein synthesis in broilers and increases the efficiency of nitrogen use.

There are few reports about the effects of PS supplementation on digestion performance of white feather broilers. Cheng et al. (2017) reported that PS supplementation can significantly improve dry matter, organic matter and crude protein digestibility, indicating that adding PS can improve the digestion function of finishing pigs. In this study, we also find that dietary PS supplementation increased DM, CP and Ca digestibility. Digestive enzymes are special proteins that promote the degradation of food in the digestive tract, including proteases, lipases, and amylases (Ding et al. 2020). The level of digestive enzyme activity in an animal's intestine reflects its digestive ability and determines the efficiency of feed utilisation (Lindemann et al. 1986). Increased activity of intestinal digestive enzymes in birds may be an indicator for improving nutrient digestibility and increasing the productive performance (Alagawany et al. 2021). Previous study observed that adding a mixture of phytosterols, soyasaponins, and isoflavones to the experimental diet of Atlantic salmon had an effect on the activities of Maltase and Trypsin (Gu et al. 2015). In the present study, a similar effect is exerted by dietary addition of PS, which could significantly increase the activities of trypsin, lipase, and amylase of broilers. Cholesterol is an insoluble substance that needs to be emulsified with the help of intestinal bile acids to form spherical micelles before it can be absorbed by intestinal cells. The structure of PS is similar to cholesterol, so it can compete with cholesterol for the formation of spherical micelles, thereby reducing the absorption of cholesterol and increasing the reverse transport and excretion of cholesterol (Arienne et al. 2003; He et al. 2018). Therefore, PS supplementation will significantly increase intestine lipase activity, but the increase in intestine trypsin activity is not significant. Previous

study has shown that PS has the effect of promoting protein synthesis to improve growth performance (Zhao et al. 2019). In our study, we also found that the digestive enzyme activities in the pancreas higher than in the intestine. The pancreas contains two parts, the exocrine part and the endocrine part. The exocrine glands are composed of acini and ducts. The acinar secretes pancreatic juice, and the ducts are the channels through which pancreatic juice is discharged. Pancreatic juice contains trypsin, lipase, amylase, etc. The pancreatic juice is discharged into the intestine through the pancreatic duct, and the activity of digestive enzymes is reduced. Therefore, the digestive enzyme activity in the pancreas is higher than that in the intestine. These results indicate that PS supplementation induced the expression of digestive enzymes, which effectively increase the digestibility of starch and protein of diet and lead to improved broilers performance. The increased nutrient digestibility and digestive enzyme activities by PS inclusion in this study suggested that dietary PS addition is of great significance for improving the digestion performance of broilers.

Conclusions

The results of the present study indicate that dietary PS supplementation can improve serum antioxidant capacity, serum immune parameters, serum biochemical parameters, nutrient digestibility and digestive enzyme activities of white feather broilers. Furthermore, a level of 40 mg PS/kg addition is recommended in the diet of white feather broilers.

Ethical approval

All animal works in this experiment were conducted by following the Chinese Guidelines for Animal Welfare and approved by the Zhejiang University Institutional Animal Care and Use Committee (No. ZJU2013105002) (Hangzhou, China).

Disclosure statement

No potential conflict of interest was reported by the authors.

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