



Effect of Dietary Stevia on Immune Response of Growing Pigs Challenged with *Escherichia coli* Lipopolysaccharide

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Abstract – This experiment was conducted to investigate the effect of Stevia (*R.bertoni*) on growing pigs' blood immune responses after an *E.coli* lipopolysaccharide (LPS) challenge. Twenty four growing pigs (22.1± 0.5 kg of BW) were used to determine the effect of Stevia on Red Blood Cells (RBC), White blood Cells (WBC), Lymphocyte, Cortisol, tumor necrosis factor- α (TNF- α) concentration and body temperature. Animals were randomly allotted to 1 of 4 treatments: 1) CON (basal diet+ saline), 2) ADD (CON+ 0.30% Stevia+ saline), 3) CON-LPS (CON+LPS), and 4) ADD-LPS (ADD+ LPS). At the end of the feeding period, 24 pigs were selected from the CON and ADD-LPS treatments, and 6 pigs received LPS and the other 6 pigs received an equivalent amount of saline. The data of blood immune system and rectal temperature were collected at 0, 2, 4, 6, 8, and 12 h after challenge. LPS challenge depressed lymphocyte concentration at 4 and 12 h ($P<0.05$) in CON-LPS and ADD-LPS group. LPS injection increased rectal temperature at 2 and 4 h post-challenge ($P<0.05$). TNF- α concentration increased significantly at 2 and 12 h post-challenge ($P<0.05$). In conclusion, supplementation of Stevia in growing pigs improves blood immunity and alleviates the inflammatory responses in LPS challenge.

Keywords – Blood Immune System, *E.coli*, Growing Pigs, Lipopolysaccharide Challenge, Stevia.

I. INTRODUCTION

The use of botanicals and dietary supplements derived from natural substances as an adjunct for their purported medical benefits has become increasingly common around the world in animal and human diet [1]. On the other hand the trend of replacing chemical and inorganic substances with natural and organic ones in the diet of commercial animals is rising and scientists

A perennial shrub, Stevia *Rebaudiana*, and its extracts are used as a natural sweetener and have been shown to possess antimicrobial and antifungal properties. However, little information exists on the effective use of Stevia on immune system when it was challenged. [2]. Stevia *Rebaudiana* is one of 154 members of genus Stevia and one of only 2 species that produce sweet glycoside. Its extracts are used as food additives by Japanese and Brazilians and as a non-caloric sweetener [3]. Its extracts (steviol and stevioside) can be used as the blood glucose-lowering substances [4, 12].

The effect of Stevia and its extracts on different animals have been studied very limitedly. The aim of this study is to evaluate the effect of Stevia on time-depending responses of blood immune parameters in LPS-stimulated growing pigs.

II. MATERIALS AND METHODS

Growing pigs used in this study were approved by the Animal Care and Use Committee of Dankook University.

A. Animals, Diets, and Facility

A total of 24 growing pigs with an average initial body weight of 22.1± 0.5 kg were used in this study. The feeding period of this study was 14 days. Growing pigs were allocated to 1 of 4 treatments and each treatment was consisted of 6 replications. Dietary treatments were: 1: CON (basal diet + saline injection), 2: ADD (CON + 0.30% Stevia + saline injection), 3: CON-LPS (CON + LPS injection), and 4: ADD-LPS (ADD + LPS injection). Treatments were arranged into a 2 × 2 factorial, with the main effects being Stevia (0 or 0.3%) and lipopolysaccharide (LPS) challenge (0 or 50 µg/kg of BW). Pigs were housed individually in metabolism cages (1.8 × 0.7 m) in an environmentally controlled room (24 to 26°C) and allowed free access to feed and water during experimental period. Feed formula and the chemical composition are presented in table I. No vaccines or antibiotics were administered to these pigs before or throughout the study. The LPS (*E. coli*: serotype O55:B5; Sigma Chemical Co., St. Louis, MO) was injected (50 µg/kg of BW) at the start of the challenge trial. The LPS dosage was based on the results of previous studies (Wright et al., 2000). The LPS solution (1 mg/mL) was made by diluting LPS with sterile saline. The control pigs were injected with an equal amount of sterile saline solution.

B. Blood Sampling and Assay

For the serum profile, blood samples (10 mL) from each of those 24 pigs were collected via anterior vena cava puncture at 0, 2, 4, 6, 8, and 12 h after the administration of LPS. At the time of collection, blood samples were collected into both non-heparinized tubes (5 mL) and vacuum tubes (5 mL) containing K₃EDTA (Becton, Dickinson and Co., Franklin Lakes, NJ) to obtain serum and whole blood, respectively. After collection, serum samples were centrifuged (2,000 × g) for 30 min at 4°C. The white blood cells (WBC), red blood cells (RBC) counts, and lymphocyte concentration in the whole blood were determined using an automatic blood analyzer (Advia 120, Bayer Corp., Tarrytown, NY).

One-half of the whole blood samples were subsequently centrifuged (3,000 × g) for 15 min at 4°C, and the plasma was harvested. Thereafter, the samples were frozen and stored at -20°C until further analysis. Immediately after

each blood collection, plasma tumor necrosis factor- α (TNF- α) and IGF-1 concentrations were evaluated using commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN). Rectal temperature was also determined using a digital electronic thermometer.

C. Preparation of Blood for Lymphocyte Subpopulation Assay

A sample (3 mL) of whole blood was added into 3 mL of acid citrate dextrose solution (25 mM citric acid, 51.7 mM sodium acetate, and 1.6 mM d-glucose). The blood solution was then centrifuged (1,500 \times g) for 10 min at 4°C, after which the WBC were collected with a sterile Pasteur pipette and placed on the surface of a Hypaque Ficoll (Histopaque1.803; Sigma Chemical Co) and centrifuged (500 \times g) for 30 min at 4°C.

Table I: Diet composition for growing pigs (as-fed basis)

Ingredients, %	Content
Corn	55.3
Soybean meal	33.4
Molasses	2.50
Animal fat	5.3
Difluorinated Phosphate	1.9
Limestone	0.8
L-lysine HCl	0.17
Trace mineral premix ¹	0.10
Vitamin premix ²	0.12
Salt	0.20
DL-Methionine	0.03
Choline chloride	0.03
Chemical composition	
ME (kcal/kg)	3,360
Crude protein (%)	20.00
Lysine (%)	1.30
Calcium (%)	0.90
Phosphorus (%)	0.80

¹ Provided per kg of complete diet: 12.5 mg Mn, 179 mg Zn, 140 mg Cu, 0.5 mg I and 0.4 mg Se.

² Provided per kg of complete diet: 20,000 IU of vitamin A; 4,000 IU of vitamin D₃; 80 IU of vitamin E; 16 mg of vitamin K₃; 4 mg of thiamine, 20 mg of riboflavin; 6 mg of pyridoxine; 0.08 mg of vitamin B₁₂; 120 mg of niacin; 50 mg of Ca-pantothenate; 2 mg of folic acid and 0.08 mg of biotin.

The lymphocytes were obtained from the interface between the Ficoll and plasma, after which the cell suspensions were washed 3 times and re-suspended in a Ca⁺ and Mg⁺ free PBS (11.3 mM sodium phosphate, 3.8 mM potassium phosphate, 125 mM sodium chloride, 100 units of penicillin/mL, and 100 μ g of streptomycin/mL).

D. Statistical Analysis

The data were analyzed as a 2 \times 2 factorial arrangement of treatments (Stevia and LPS) using the repeated statement of the MIXED procedure of SAS, and using SAS GLM when time as not part of the model [5]. The individual pig was considered as the experimental unit. When Stevia and LPS interactions were detected, mean separations by LSD were used to determine treatment differences. The variability of all the data was expressed as the pooled SEM, and the established level of significance was P < 0.05.

III. RESULTS

A. Blood Immune System

Injection of LPS and Stevia + Saline (treatment ADD-LPS), increased the count of RBC (P<0.05) at 2 hours after injection (Table II).

WBC count decreased significantly (P< 0.05) 2 hours after injection in LPS challenge (Table II).

CON-LPS grouped showed a significant (P< 0.05) increase at the percentage of Lymphocytes at the time of injection. But there wasn't any difference 2 hours after injection.

Table II: Effect of Stevia supplementation on blood immune system in growing pigs¹

Items		CON	ADD	CON-LPS	ADD-LPS	SE ²
RBC, 10⁶/μL	0 h	6.27	6.27	5.90	6.37	0.42
	2 h	6.25 ^b	6.33 ^{ab}	6.72 ^{ab}	7.19 ^a	0.27
	4 h	6.04	6.18	5.53	5.85	0.74
	12 h	5.60	5.99	6.11	5.69	0.44
WBC, 10³/μL	0 h	24.25	18.70	24.37	17.28	3.43
	2 h	22.71 ^a	17.17 ^b	7.21 ^c	5.22 ^c	1.69
	4 h	22.62 ^a	18.46 ^{ab}	13.23 ^b	16.20 ^{ab}	1.78
	12 h	25.22	19.42	16.31	15.19	3.78
Lymphocyte, %	0 h	58.88 ^{ab}	57.78 ^b	71.93 ^a	55.58 ^b	4.20
	2 h	59.93	51.10	65.05	70.05	6.00
	4 h	58.20 ^a	57.95 ^a	36.97 ^b	20.05 ^b	5.91
	12 h	47.60 ^{ab}	50.33 ^a	44.23 ^{ab}	30.75 ^b	4.69
Cortisol, μg/mL	0 h	6.50 ^c	15.15 ^c	37.45 ^a	28.45 ^b	2.47
	2 h	13.83 ^c	7.53 ^c	73.70 ^b	95.53 ^a	6.57
	4 h	13.40 ^b	19.13 ^b	51.65 ^{ab}	68.10 ^a	14.10
	12 h	19.03	22.18	35.10	45.10	8.85

TNF-α, $\mu\text{g/mL}$	0 h	0.10	0.12	0.10	0.11	0.02
	2 h	0.08 ^b	0.12 ^b	20.65 ^a	20.02 ^a	5.87
	4 h	0.09	0.12	0.98	3.77	1.17
	12 h	0.08 ^b	0.24 ^{ab}	0.39 ^{ab}	0.80 ^a	0.20
Haptoglobin, mg/mL	0 h	0.36	0.40	0.42	0.32	0.13
	2 h	0.25	0.41	0.28	0.18	0.14
	4 h	0.27	0.25	0.34	0.19	0.10
	12 h	0.29	0.34	0.64	0.31	0.13

¹Abbreviation: CON, basal diet; ADD, CON + 0.3% Stevia; CON-LPS, CON + LPS Inj.; ADD-LPS, CON + 0.3% Stevia + Inj.

²Standard error. abc Means in the same row with different superscripts differ ($P < 0.05$)

The lowest percentage of Lymphocyte was observed in treatment ADD-LPS 4 h after injection.

The amount of Cortisol in treatment CON-LPS was significantly ($P < 0.05$) higher than other treatments at the time of injection but after 2 hours of injection its amount in treatment ADD-LPS increased dramatically ($P < 0.05$). However 4 and 12 hours after injection the amount of Cortisol decreased gradually in all treatments but treatment ADD-LPS had higher level of Cortisol than other treatments (Table II).

At the time 0 and 4 hours after injection there wasn't any significant difference on the amount of TNF- α between treatment but 4 hours after injection, the pigs receiving LPS in CON-LPS treatment and LPS+ Stevia in ADD-LPS treatment indicated a remarkable ($P < 0.05$) increase in the level of TNF- α . Its amount reduced dramatically after 4 and 12 hours of injection (Table II).

The data represented in figure 1 show that the level of IGF-1 is falling by passing the time. The pigs receiving Stevia, LPS injection or Stevia+ LPS injection had lower levels of IGF-1 compared to CON treatment. But comparing to the level of IGF-1 at 0 h the lowest levels of IGF-1 belong to CON-LPS which didn't receive Stevia (Fig. 1). Any significant effect wasn't observed on the amount of Haptoglobin (Table II).

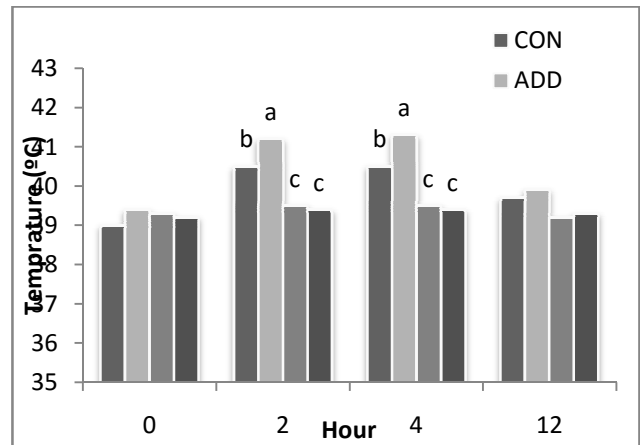


Fig.II. Effect of Stevia supplementation on rectum temperature in growing pigs

B. Rectal Temperature

There wasn't any significant effect on rectal temperature at 0 and 12 hours after injection in all experimental treatments, but after 2 and 4 hours of injection of Stevia in treatment ADD, and LPS in treatment CON-LPS, the rectal temperature increased significantly ($P < 0.05$, Fig. II).

IV. DISCUSSION

It has been reported that using Stevia may result in reducing metabolic disorders. It has some antioxidant, anti-inflammatory, immune- modulatory activities [3, 13]. It has been also reported that the positive effect of stevioside in type 2 diabetic patients led to the hypothesization that supplementation of test meal stevioside causes a reduction in postprandial blood glucose levels [6].

Another researcher reported that stevioside at 1 mM significantly suppressed LPS-induced release of TNF- α [7]. It has been also reported that LPS challenge increased TNF- α concentration at 2 and 4 hours after LPS injection [8].

Wang et al (2011) the LPS increased the WBC counts and decreased lymphocyte percentage of growing pigs at 6 and 8 hours. These results are in consistent with the result of current study [9].

Pigs challenged with LPS had greater TNF- α concentration which is consistent with findings of previous researchers [8]. It is commonly believed that an

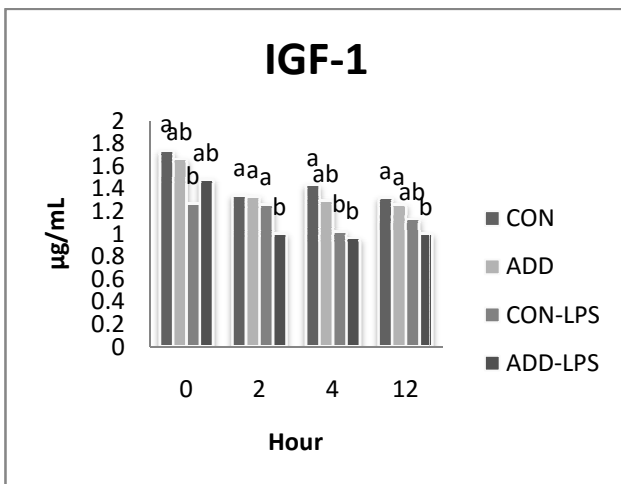


Fig.I. Effect of Stevia supplementation on blood IGF-1 in growing pigs

inflammatory response is mediated through increased production of pro-inflammatory cytokines [10; 11]. During the challenge phase, IGF-I and TNF- α concentrations and rectal temperature were increased in LPS treatments. These factors indicate that the LPS challenge stimulated the defense system of the pigs and, therefore, presented a typical inflammation response. The data of LPS treatments in current study confirm the result of previous researchers' results [3; 9], and that injection of Stevia alleviated inflammation responses. Boonkaewwan et al (2006) suggested that stevioside attenuates the synthesis of inflammatory mediators in LPS-stimulated cells [7]. Our finding alongside previous studies showed that methanolic extracts of Stevia may not only be a good dietary source of natural antioxidants, but also that they protect against the inflammatory responses in LPS challenge.

Rectal temperature was increased by LPS challenge at 2, 4, 6, and 8 hours after injection and it might be due to the inflammatory responses.

V. CONCLUSION

In conclusion, the result of this study suggested that the pig immune systems were stimulated by the LPS challenge, whereas typical inflammation response by injection of Stevia alleviated LPS challenge inflammatory with a time-depend responses. Further studies are warranted to shed more light on this important issue.

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