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EFFECTS OF EGG YOLK IMMUNOGLOBULIN ON GROWTH PERFORMANCE, DIARRHEA SCORE, DIARRHEA INCIDENCE AND SERUM ANTIBODY TITER IN PRE- AND POST-WEANED PIGS

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A total of 8 Landrace × Yorkshire multiparous (n = 3) sows and their piglets (n = 80, 10 piglets/sow) were used in this 42-d trial to evaluate the effects of egg yolk immunoglobulin (IgY) on growth performance, diarrhea score, diarrhea incidence, and serum antibody titer in pre-weaned period as well as effects on growth performance in post-weaned period in piglets. The experiment included 2 phases (phase 1: pre-weaned period for 21 d, and phase 2: post-weaned period for 21 d). In phase 1, 40 piglets did not orally receive IgY (NC), while another 40 piglets received 2 ml IgY/pig (PC) on d 3. From d 10 to 21, piglets were fed with creep feed. Piglets received IgY had lower ($P<0.05$) diarrhea incidence and diarrhea score compared with pigs that did not receive IgY. On d 7 and 14, piglets serum antibody titer to *Escherichia coli* and porcine epidemic diarrhea virus in PC treatment was increased ($P<0.05$) compared with those in NC treatment. In conclusion, orally received IgY increased serum antibody titer in piglets, and protected piglets from diarrhea and enterotoxigenicity in pre-weaned period, however, had no effects on growth performance in pre-and post-weaned periods.

Key words: Blood antibody titer, diarrhea incidence, growth performance, IgY, piglets

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrheal disease, mortality, and enteric colibacillosis in neonatal and weaned pigs (Yokoyama et al. 1992, Nagy and Fekete 1999). Porcine epidemic diarrhea virus (PEDV) is the etiological agent of entero-pathogenic diarrhea, which causes high mortality rates in neonatal pigs (DeBouck and Pensaert 1980).

Piglets are born without immunoglobulins, so immunoprotection for newborn piglets mainly consists of passive immunity through colostral immunoglobulins from the immunized dam (Roth 1999). However, during the transition from colostrum to mature milk, immunoglobulin concentrations markedly decreased (Mavromichalis 2006). Oral administration of antibodies derived from serum and colostrum has been very successful. However, it is prohibitively expensive (Kuhlman et al. 1988). The egg yolk immunoglobulin (IgY) from immunized chickens is a convenient source of specific antibodies on a large-scale (Chernysheva et al. 2004). Oral administration of specific IgY presents a promising approach for the passive immunization of animals and humans suffering from enteric disease (Mine and Kovacs-Nolan 2002). Specific IgY also provides passive protection against ETEC infection in neonatal and early-weaned pigs (2-week of age) (Yokoyama et al. 1992, Marquardt et al. 1999). The immunoprophylactic effects of IgY against PEDV have been documented, and IgY reduces diarrhea incidence and mortality of piglets after challenge exposures (Kweon et al. 2000). However, the activity of IgY may be reduced or destroyed by gastric conditions, particularly because of the low pH (Shimizu et al. 1988).

The present study was based on the hypothesis that IgY could influence the serum antibody concentration and thus benefit pre- and post-weaned pigs by protecting piglets from diarrhea. Therefore,

the aim of this study was to evaluate the effects of IgY on growth performance, diarrhea score, diarrhea incidence, and serum antibody titer in the pre-weaning period, as well as the effects on growth performance in post-weaned pigs.

MATERIALS AND METHODS

The animal care and use protocol was approved by the Animal Care and Use Committee of Dankook University.

Preparation of Fimbriae and Virus

The fimbriae of *Escherichia coli* (*E. coli*) F4 (K88), F5 (K99) and F6 (987P) were obtained as previously described (Jin et al. 1998). Routinely, the bacteria were cultivated at 37°C for 36 h with tryptic soy broth (TSB, Difco) separately. ETEC was recycled and put in the phosphoric acid buffer saline (PBS, pH = 7.2) and then boiled for 30 min at 60°C for inactivation. For the isolation of F4, F5 and F6 fimbriae, the solution of ETEC was centrifugated for 10 min to homogenize, and after centrifugation, 2.5% citric acid was added to the supernatant (pH = 4), and the supernatant was stirred for 30 min in the room temperature and then the supernatant placed for 2 h at 4 °C and centrifugated at 14,000 × g for 20 min, and then the deposit was again added into the PBS and treated as mentioned before for 3 times, and at last ETEC fimbrial antigens were gained.

A strain of attenuated PEDV was plaque-purified once at the passage level of 81 and further cloned through limiting dilution method three times in Vero cells (CCL81, USA) (Kweon et al. 1999). Vero cells were regularly maintained in alpha-MEM supplemented with 5% fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 unit/ml) and amphotericin (100 unit/ml). For the preparation of the antigen, the virus-inoculated cells were maintained in alpha-MEM with 0.02% yeast extract, 0.3% tryptose phosphate broth and 1-2 µg of trypsin. The infected cells and culture supernatant were frozen-thawed once and harvested after centrifugation at 6,000 × g for 15 min. The collected supernatant was then concentrated with polyethylene glycol (PEG, M.W. 6000, Serva, Germany). The PEG-treated viral solution was resuspended at 1/200 of original volume with TEN buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH = 7.4).

Immunization of Chickens

Hyline brown layers, 10 weeks of age, were immunized with concentrated ETEC fimbrial and PEDV antigens as described above. Primary immunizations were conducted by intramuscular injection with 1 ml (0.5 mg/ml) of antigens emulsified with an equal volume of Freund's complete adjuvant (**FCA**). The second and third immunizations were conducted at a 2-week interval after the first inoculation. On the day of inoculation, blood was collect from wing vein, and the serum was obtained by centrifugation at 4°C for 20 min. IgY was extracted from the egg yolk by using 0.1% λ carrageenan solution (Sigma Chemical, Type IV, St Louis, USA) and concentrated by the procedures described by Jesenius et al. (1981). The titers of IgY were initially screened by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, IgY diluted to 1:1,000 in PBST containing 10% skim milk was added to the microwell coated with antigen (ETEC fimbrial or PEDV) and incubated for 2 h at 37°C. After incubation, 100 µl of anti-chicken IgG conjugated with horseradish peroxidase (KPL, Maryland, USA), which was diluted (1:5,000) in PBST containing 10% skim milk, was added to each well for 2 h at 37°C. The plates were washed three times with PBST and added to 2,2-azino-3 ethyl-benzthiazoline-6-sulfnate substrate solution (KPL, Maryland, USA). After 30 min incubation at room temperature, the reaction was stopped by adding 0.1% sodium dodecyl sulfate solution and optical density was measured at 405 nm.

Preparation of Egg Yolk Antibodies

The IgY was prepared as Marquardt et al. (1999) reported. Briefly, egg was broken, egg white was discarded, and yolk was rolled on filter paper to remove residues of egg white. The yolks were then transferred into a funnel fitted with filter paper that was opened at the bottom of the funnel, the yolk membranes were ruptured, and yolks were collected in a measuring cylinder leaving the yolk membrane attached to the paper. The yolks were diluted (1:9, v/v) with acidified

double distilled water to obtain a final pH of 5.0, frozen at -20°C overnight, defrosted and centrifuged at 10,000 × g for 30 min at 15°C. The supernatant was filtered twice with filter paper. The IgY powder was obtained by freeze-dried without purification. The titer of the IgY was tested by indirect ELISA (Jin et al. 1998).

Farrowing Facilities and Management

Eight Landrace × Yorshire breed sows were used in this experiment and the parity of the sows was 3. During gestation, sows were fed the same gestation diet until 7 d before parturition. On d 107 of gestation, sows were moved into farrowing crates in an environmentally regulated farrowing room. Farrowing crates (2.1 × 0.6 m) contained an area for new born pigs each side after birth, and the temperature in the farrowing house was maintained at a minimum of 18°C. Supplemental heat was provided for pigs using heat lamps and zone cooling directed to the heads of sows (snout cooling) was provided in hot summer weather. Each farrowing stall had a drinker and a feeder. Piglets were treated according to routine management practices that included teeth clipping, tail docking, ear notching, and subcutaneous iron dextran injections (50 mg/pig) within 24 h. During lactation, sows were given the same lactation diets ad libitum. Both gestation and lactation diets (Table 1) met or exceeded the nutrient requirements of sows for gestation and lactation according to the NRC (1998).

Animals, Experimental Diets, Procedure and Design

On the parturition day (d 0), body weight (BW) of newborn piglets was weighed. Litter number of each sow was over 12 and adjusted to 10. Ten piglets of each sow which were healthy and close to the average litter BW were chosen. All piglets were given creep feed from d 10 to 21. All piglets (n = 80) were allotted to 1 of 2 treatments. Treatments included: 1) NC, piglets received no IgY; 2) PC, piglets orally received 2 ml IgY/pig orally on d 3 after farrowing.

Individual piglet BW was determined on d 1, 5 and 21 and then average daily gain (ADG) was calculated. All piglets were given creep feed (Table 2) from d 10 to 21. Food disappearance from each litter was recorded every day and then the average daily feed intake (ADFI) was also calculated. Blood samples were collected via jugular venipuncture from 5 piglets per litter on d 1, 7 and 14. Blood samples were collected into both– nonheparinized and K_3EDTA vacuum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Serum antibody titer against *E. coli* F4, F5 and porcine PEDV was analyzed by indirect ELISA as described by Jin et al. (1998). The appearance of diarrhea in piglets was observed and recorded 3 times per day during the experiment. To assess the severity of diarrhea, faeces from each pen were scored according to the method of Hart and Dobb (1988). Scores were 0, normal, firm feces; 1, possible slight diarrhea; 2, definitely unformed, moderately fluid faeces; or 3, very watery and frothy diarrhea. A cumulative diarrhea score per diet and day was then calculated (Montagne et al. 2004). Diarrhea incidence was calculated according to the formula reported by Sun et al. (2008): diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × 21 d) × 100, and "number of pigs with diarrhea" was the total number of pigs with diarrhea observed each day.

After weanling, piglets were moved to the weaned pig house and piglets from same litter were raised in the same pen (4 pens per treatment and 10 pigs in each pen). Individual BW of weaned pigs was determined on d 42, and ADFI was also calculated. All diets were formulated to meet or exceed the nutrient requirements recommended by NRC (1998) (Table 2).

Statistical Analysis

All Data were subjected to ANOVA by using the General Linear Models procedure of SAS (SAS Ins. Inc., Cary, NC). The pen (pro-weaning period) or litter (pre-weaning period) was considered the experiment unit. Differences among treatments were evaluated by T-test option of SAS. Variability in the data is expressed as the standard error of mean (SEM) and a probability level of $P < 0.05$ was considered to be statistically significant.

Table 1. Composition of sow diets (as-fed basis).

Item	Gestation diet	Lactation diet
Ingredient, g/kg		
Maize	571.0	511.2
Soybean meal, 460g/kg CP	106.5	246.1
Wheat bran	120.0	40.0
Rapeseed meal	37.0	25.0
Rice bran	60.0	50.0
Tallow	35.9	60.5
Molasses	36.0	35.0
Dicalcium phosphate	15.2	16.4
Limestone	9.9	7.6
Salt	6.0	5.0
Lys, 980 g/kg	0.5	1.2
Vitamin premix[a]	1.0	1.0
Mineral premix[b]	1.0	1.0
Calculated composition		
Metabolism energy, kcal/kg	3190	3440
Analyzed composition, g/kg		
Crude protein	131.1	171.3
Crude fat	68.9	91.2
Lysine	6.4	10.1
Calcium	8.7	8.5
Phosphorus	7.6	7.3

[a] Provided per kilogram of complete diet: vitamin A, 100,000 IU; vitamin D_3, 2,000 IU; vitamin E, 48 IU; vitamin K_3, 1.5 mg; riboflavin, 6 mg; niacin, 40 mg, D-pantothenic, 17mg; biotin, 0.2 mg; folic acid, 2 mg; choline, 166 mg; vitamin B_6, 2 mg; and vitamin B_{12}, 28 µg.

[b] Provided per kilogram of complete diet: Fe (as $FeSO_4 \cdot 7H_2O$), 90 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 15 mg; Zn (as $ZnSO_4$), 50 mg; Mn (as MnO_2), 54 mg; I (as KI), 0.99 mg; and Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.25 mg.

RESULTS

No differences ($P > 0.05$) were observed on average BW, ADG, and ADFI between treatment groups (Table 3). Piglets received IgY had lower ($P < 0.05$) diarrhea incidence and diarrhea score compare with pigs did not receive IgY. On d 7 and 14, serum antibody titer to *E. coli* F4, F5 and PEDV in PC group was increased ($P < 0.05$) compare with pigs in NC group in pre-weanling pigs (Table 4).

DISCUSSION

We hypothesized that the ADG of weanling pigs could be improved by IgY is not supported by the present results. In agreement with our results, Marquardt et al. (1999) observed that IgY did not improve the ADG of 14 to 18-d-old piglets in a field experiment. However, other researchers reported body weight gain was improved by administration of anti-F4 hyper immune egg yolk antibodies in weaned piglets (Marquardt et al. 1999) and neonatal piglets (Wiedemann et al. 1991, Yokoyama et al. 1992). It is suggested that IgY could be proteolytically degraded during passage (Harmsen et al. 2005), restricting the amount that actually reaches the jejunum (Chernysheva et al. 2004). Furthermore, in our study, it was also confirmed by the serum antibody titer. In some instances, the oral administration of IgY in the pre-weanling period might delay the immune response of piglets towards the pathogen. In that case, we suggest that price and the health situation of piglets in the farm should be considered when using IgY products.

Serum antibody titer is the indicator of humoral immunity (Masic et al. 2010). Our results also showed that serum antibody titers in the IgY treatments were higher than that in CON treatment on d 7 and 14. Furthermore, we observed that serum antibody titer in CON treatment on d 1, 7 and 14 decreased, while oral administration

of IgY depressed the decreasing of serum antibody titer. Moreover, data of diarrhea incidence and diarrhea score also confirmed that the significant increase of antibody titer observed on d 7 after treatment with IgY, which did not reflect severe infection of these pathogens, and a subsequent production and release of antibody.

Table 2. Composition of pre-and post-weaned pig diets (as-fed basis)

Items	Pre-weaned period	Post-weaned period
Ingredient, g/kg		
Extruded maize	121.5	357.2
Extruded oat	100.0	
Biscuit meal		50.0
Soybean meal, 440 g/kg CP	80.0	200.0
Fermented soybean meal	78.0	82.0
Fish meal	50.0	40.0
Soy oil	41.5	48.0
Lactose	100.0	60.0
Whey	165.0	100.0
Milk product	130.0	20.0
Monocalcium phosphate	12.5	10.0
Sugar	40.0	20.0
Plasma powder	65.0	
L-Lys·HCl, 780 g/kg	1.2	2.5
DL-Met, 500 g/kg	2.6	1.5
L-Thr, 890 g/kg	7.7	0.8
Choline chloride, 250 g/kg	2.0	1.0
Vitamin premix[a]	1.0	1.0
Trace mineral premix[b]	2.0	2.0
Limestone		2.0
Salt		2.0
Calculated composition		
Metabolism energy, MJ/kg	14.8	14.8
Analyzed composition, g/kg		
Crude protein	221.2	209.7
Lysine	15.7	14.1
Methionine	6.0	4.9
Calcium	8.0	7.8
Phosphorus	7.6	7.6

[a] Provided per kg of complete diet: vitamin A, 11,025 IU; vitamin D_3, 1,103 IU; vitamin E, 44 IU; vitamin K, 4.4 mg; riboflavin, 8.3 mg; niacin, 50 mg; thiamine, 4 mg; d-pantothenic, 29 mg; choline, 166 mg; and vitamin B_{12}, 33 μg.

[b] Provided per kg of complete diet: Fe (as $FeSO_4 \cdot 7H_2O$), 80 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 12 mg; Zn (as $ZnSO_4$), 85 mg; Mn (as MnO_2), 8 mg; I (as KI), 0.28 mg; and Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.15 mg.

The epithelium of the small intestine and the colon is the site for pathogen proliferation. Infection of the enterocytes causes vacuolization and finally destruction, which leads villous atrophy and watery diarrhea due to malabsorption (Walsh et al. 2002). Presently, the diarrhea incidence and diarrhea score were lowered by the oral administration of IgY. Similarly, Marquardt et al. (1999) found that consuming 1.5 g of IgY per day per piglet was sufficient to prevent the diarrhea induced by infection with 10^{10} ETEC, and the addition of 0.2% IgY in the diet prevented piglets from diarrhea in a commercial farm. It has been proposed that IgYmediates protection against ETEC by preventing fimbriae-mediated attachment of ETTC to its receptors on the intestinal epithelial surfaces (Marquardt et al. 1997). Kweon et al. (2000) found that total survival rate of piglets was higher from IgY treated groups (49.24%)

compared with control group (33.71%) under the challenge of PEDV. In contrast, Harmsen et al. (2005) reported that llama single-domain antibody fragments effectively inhibited in vitro ETEC adhesion to intestinal brush borders but poorly protected piglets against diarrhea. Different results might be due to the dose and natural characteristics of the different antibodies. Besides, in the study of Harmsen et al. (2005), the antibody fragments might have been proteolytically degraded during passage through the gastrointestinal tract. Furthermore, IgY can mediate its disease-protecting effects by blocking the release of toxins by the bacteria through an undefined mechanism (Li et al. 2008).

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Table 3. Effects of egg yolk immunoglobulin on growth performance and the appearance of diarrhea in pre-and post-weaned pigs[a]

Items	NC[b]	PC[b]	SEM[c]	P-value
BW, kg				
Initial	1.43	1.42	0.07	0.521
21 d	5.87	5.94	0.12	0.663
42 d	13.26	13.50	0.25	0.746
ADG, g				
d 0 to 21	211	215	10	0.437
d 22 to 42	352	360	16	0.412
d 0 to 42	282	288	9	0.504
ADFI, g				
d 10 to 21, pre-weaned period	16	16	2	0.325
d 22 to 42, post-weaned period	478	472	11	0.447
Diarrhea incidence[d], %	13.2[x]	8.0[y]		0.012
Diarrhea score[d]	17.0[x]	14.9[y]		0.023

[a] Data are means of 4 replicates of 10 pigs per pen/liter.
[b] Abbreviation: 1) NC: piglets received no egg yolk immunoglobulin (IgY) ; 2) PC, piglets received 2 ml/pig IgY orally on day 3.
[c] Standard error means.
[d] Means are different by a chi-square contingency test.
[x,y] Means in the same row with different superscripts differs ($P<0.05$).

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Table 4. Effects of egg yolk immunoglobulin on serum antibody titer of *Escherichia coli* and porcine epidemic diarrhea virus (PEDV) in pre-weaned pigs[c]

Items, %	NC[d]	PC[d]	SEM[e]	P-value
Escherichia coli F4				
d 1	5.88	6.16	0.42	0.546
d 7	0.38[b]	17.56[a]	2.13	<0.001
d 14	0.00[b]	7.40[a]	1.47	<0.001
Escherichia coli F5				
d 1	3.91	3.73	0.34	0.453
d 7	0.70[b]	8.90[a]	1.78	<0.001
d 14	0.00[b]	3.84[a]	1.01	<0.001
PEDV				
d 1	6.22	7.58	0.65	0.478
d 7	0.00[b]	8.10[a]	2.01	<0.001
d 14	0.00[b]	0.58[a]	0.12	<0.001

[a,b] Means in the same row with different superscripts differs (P<0.05).

[c] Data are means of 4 replicates of 5 pigs per liter

[d] Abbreviation: 1) NC: piglets received no egg yolk immunoglobulin (IgY) ; 2) PC, piglets received 2 ml·pig^{-1} IgY orally on day 3.

[e] Standard error means.

CONCLUSION

Oral administration of IgY can increase serum antibody titer, and protect pre-weanling pigs from diarrhea, but only poorly improve growth performance in pre- and pro-weanling pigs.

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