In ovo feeding of epidermal growth factor: embryonic expression of intestinal epidermal growth factor receptor and posthatch growth performance and intestinal development in broiler chickens[1]

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ABSTRACT We investigated efficacy of *in ovo* application of epidermal growth factor (**EGF**) on intestinal expression of EGF receptor (**EGFR**) during embryogenesis (experiment 1) and posthatch growth performance and gastrointestinal development in broiler chickens (experiment 2). In experiment 1, 450 fertile Ross 708 eggs were allocated to 3 groups (150 eggs/group): 1) control, 2) 160 μg EGF/kg of egg, and 3) 640 μg of EGF/kg of egg. Eggs were candled for live embryos on day 16 and injected with the respective treatment solutions on day 17 and sampled for jejunal tissue from day 17 to hatch for EGFR analyses. There was no effect of EGF ($P > 0.05$) on EGFR expression on day 17 to 20; however, on day 21, EGF increased ($P < 0.05$) EGFR expression in EGF birds relative to control birds. In experiment 2, 600 fertile Ross 708 eggs were allocated to 5 treatments: 1) intact, no puncture or injection, 2) punched but not injected, 3) control, no EGF, 4) 80 μg of EGF/kg of egg, and 5) 160 μg of EGF/kg of egg. The eggs were incubated and candled for live embryos on D 19, treated, and subsequently transferred to the hatcher. Upon hatching, chicks were weighed, and 90 chicks per treatment placed in cages (15 birds/cage) and allowed free access to a standard antibiotic-free corn-soybean diet for 21 D. Feed intake and body weight were monitored on a weekly basis. Samples of birds were necropsied on D 0, 7, 14, and 21 for measurements of intestinal weight and jejunal histomorphology and excreta samples taken on D 3 to 5 and 17 to 19 for apparent retention of dry matter. There was no EGF effect ($P > 0.05$) on any posthatch response criteria. In conclusion, *in ovo* application of EGF increased EGFR expression but had no effect on posthatch growth performance, DM retention, and intestinal development. The lack of EGF effect on posthatch response was surprising but suggested *in ovo* application of EGF may not be a viable approach.

Key words: broiler, *in ovo* feeding, epidermal growth factor, intestinal development and function

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INTRODUCTION

There is growing evidence that nutritional management during late embryonic stage and early posthatch stage is critical for the enhancement of a functional intestinal tract (Cardeal et al., 2015). The digestive system of a chick undergoes significant changes at this time, both morphologically and physiologically, as the bird transitions from a diet composed of fatty acids from the yolk sac to a carbohydrate-rich diet (Cardeal et al., 2015). Up to 10 D after hatch, studies have shown the small intestine accounts for a large percentage (approximately 7%) of whole body weight (Croom et al., 1999; Wijtten et al., 2012). In addition, it has been estimated that the 21-D incubation period in combination with the 10-D posthatch period make up approximately 50% of the lifespan of a 2.5-kg broiler chicken (Croom et al., 1999; Ferket, 2012). Consequently, great importance is then placed on the development and growth of the intestinal tract in order for digestive capacity and nutrient absorption to be maximized later in life (Croom et al., 1999).

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Epidermal growth factor (**EGF**) is a 53–amino acid single-chain polypeptide found in mammalian colostrum and milk (Jaeger et al., 1990). It is secreted from various sites along the intestinal tract, saliva, and bile secretions; EGF possesses a broad range of bioactivities on the intestinal epithelium, including the stimulation of proliferation, differentiation, and maturation of neonatal intestinal cells (Jaeger et al., 1990; Playford and Wright, 1996). There is extensive literature highlighting the benefits of supplemental exogenous EGF on neonatal or weaned mammals' gut health and function; however, EGF has not been thoroughly assessed for its ability to transition the gastrointestinal tract in broiler chicks and how it is related to intestinal development and ultimately growth performance (James et al., 1987; Barnard et al., 1995). In our previous study, growth performance and indices of gut health and function were assessed in broiler chickens fed a diet with and without exogenous EGF upon challenge with *Eimeria*, a causative agent for coccidiosis (Kim et al., 2017). The results showed that EGF had beneficial effects on prechallenge growth and improved indices of gut function upon *Eimeria* challenge. However, the study lasted for 14 D, and intestinal development was not monitored.

In ovo injection or feeding is a unique technique that enables the inoculation of exogenous nutrients or additives directly into the amniotic sac of the egg during incubation to facilitate enteric development and metabolism, as well as to increase the nutritional status of the egg (Uni and Ferket, 2004; Yegani and Korver, 2008; Cardeal et al., 2015). By injecting into the amnion, the supplemental solution together with the amniotic fluid can be orally consumed by the embryo before hatch, delivering the nutrients into the intestine of the embryo and thereby stimulating intestinal development (Uni and Ferket, 2004). Furthermore, supplying exogenous nutrients at such a pivotal stage of development is expected to yield many advantages for the bird, such as improving efficiency of nutrient utilization, immune response, muscle development, and reducing posthatch mortality and morbidity (Uni and Ferket, 2004). To further understand the role EGF has in broiler gut development and function, the use of *in ovo* technology was tested as an alternative delivery method for EGF in the present study. It was hypothesized that *in ovo* application of EGF would have beneficial effects on indices of gut health and function during incubation and subsequently impact growth performance and nutrient utilization after hatch. Therefore, the objective was to determine effects of *in ovo* EGF application on embryonic expression of EGF receptors (EGFRs; experiment 1) and on growth performance, gastrointestinal development, and apparent (**AR**) retention of dry matter in broiler chickens (experiment 2).

MATERIALS AND METHODS

The experimental protocol (#3675) was reviewed and approved by the University of Guelph Animal Care Committee, and birds were cared for in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009).

EGF, Production, and Preparation

Porcine EGF expressing *Pichia pastoris* was generated via gene engineering approach as previously described in the study by Levesque et al. (2018). Briefly, an expression vector with an AOX1 promoter was designed for optimal expression of porcine EGF (pJ912-EGF) following codon-optimization method. The EGF-secreting recombinant *P. pastoris* colonies were screened using shake-flask fermentation method in 50 mL of buffered glycerol complex medium (1% yeast extract, 2% peptone, 100-mmol potassium phosphate [pH 6.0], 1.4% yeast nitrogen base [Difco], 0.00004% biotin [Difco]), 1% glycerol (Fisher Scientific) at 30°C under shaking condition (250 rpm). To scale up the fermentation process, EGF was produced in a 3-L Bio-Flo/CelliGen 115 benchtop bioreactor (New Brunswick Scientific, Edison, NJ) with a total working volume of 1 L using basal salt medium (Huynh et al., 2018). Supernatant samples were harvested after 48 h of methanol induction, and EGF concentrations were quantified using a densitometric analysis of Western blot and stored at −80°C until experimentation.

Experiment 1

A total of 450 fertilized broiler (Ross x Ross 708) eggs were procured from a local hatchery (Maple Leaf Foods, New Hamburg, ON, Canada) and set in an incubator under optimal conditions at the hatchery in Arkell Poultry Research Station, Guelph, ON, Canada. Briefly, eggs were incubated at 37.5°C with 55% humidity to day 19 and then transferred to the hatcher set at 36.9°C with 66% humidity (Akbari Moghaddam Kakhki et al., 2020). On day 16 of incubation, eggs containing viable embryos as determined by candling were randomly selected and divided into 3 groups. The experimental treatments were 1) control (fermentation supernatant without EGF), 2) 160 μg of supernatant containing EGF/kg of egg, and 3) 640 μg of supernatant containing EGF/kg of egg. The dosage of EGF was similar to that mentioned in the study by Kim et al. (2017) with an exception that it was based on egg weight. Supernatants were thawed at 4°C overnight and brought to room temperature before being injected. Control eggs were injected with matching volumes of fermentation supernatant of *P. pastoris* without EGF. Dilutions were made using *P. pastoris* supernatant without EGF to achieve 160 and 640 μg of supernatant containing EGF/kg of egg. On day 17 of incubation, all eggs were cleaned with 70% alcohol before a small puncture hole was made at the top of the broad end of the egg. A 1-mL syringe and 2.5-cm, 23-gauge needle was used to inject 0.2 mL of corresponding treatment solutions into the amnion of each egg. The injection holes were then sealed with cellophane tape, and the eggs placed back into the incubator.

Table 1. Ingredients composition of the basal diet (%, as fed).

Ingredient	Amount, %
Corn	56.0
Soybean meal	35.0
Soy oil	3.00
Mono calcium phosphate	1.85
Limestone	1.46
Vitamin and mineral premix[1]	1.00
DL-Methionine	0.44
Sodium bicarbonate	0.40
Lysine-HCL	0.35
L-Threonine	0.21
Titanium dioxide	0.20
Salt	0.10
Calculated provisions	
AME, kcal/kg	3,003
Crude protein, %	21.7
Standardized ileal digestible Lys, %	1.28
Standardized ileal digestible Met, %	0.51
Standardized ileal digestible Met + Cys, %	0.98
Standardized ileal digestible Thr, %	0.86
Calcium, %	0.96
Nonphytate P, %	0.48
Sodium, %	0.16
Chloride, %	0.17
Choline, mg/kg	1,325

[1]Vitamin mineral premix provided per kilogram of premix: vitamin A, 880,000 IU; vitamin D3, 330,000 IU; vitamin E, 4,000 IU; vitamin B12, 1,200 mcg; biotin, 22,000 mcg; menadione, 330 mg; thiamine, 400 mg; riboflavin, 800 mg; pantothenic acid, 1,500 mg; pyridoxine, 300 mg; niacin, 5,000 mg; folic acid, 100 mg; choline, 60,000 mg; iron, 6,000 mg; copper, 1,000 mg.

Five eggs from each treatment group (15 eggs in total) were sampled for jejunal tissue samples starting on day 17 of incubation and every day forward until the day of hatch (day 21). Segments of 1-3 cm of jejunum were excised and placed into 2-mL microcentrifuge tubes, labeled accordingly, and filled with 1 mL of RNAlater. The tubes and content were placed on ice and transferred back to the laboratory where they were stored in a −80°C until required for analyses. On the day of hatch, all hatched chicks were weighed and euthanized for intestinal tissue samples.

Experiment 2

A total of 720 fertilized broiler eggs were sourced and set in an incubator as described for experiment 1. At 19 D of incubation, 600 eggs containing viable embryos as determined by candling were randomly selected and divided into 5 groups of 120 eggs per group. The experimental treatments were 1) intact (no punching and no injection), 2) punched but no injection, 3) control (fermentation supernatant without EGF), 4) 80 μg of supernatant containing EGF/kg of egg, and 5) 160 μg of supernatant containing EGF/kg of egg. Injection was as described for experiment 1. The intact group served to parallel routine procedures in commercial hatcheries. Finally, all eggs were placed in multiple hatching trays such that eggs were identified by treatment but balanced across locations within the hatcher.

Upon hatching, a total of ninety unsexed chicks of each treatment group were randomly allocated to cages (15 chicks per pen) in a completely randomized block design to give 6 replicates per treatment. The cages (each measuring 24″ × 20″ × 18″) were housed in an environmentally controlled room at Arkell Poultry Research Station, Guelph, ON. The room temperature was set at 32°C on day 0 and gradually brought down to 27°C by day 21. The lighting program was 23 h of light (20+ LUX) from day 0 to 3, followed by 20 h of light (10-15 LUX) from day 4 onwards. The cages were each equipped with feeders and nipples. The birds had free access to a standard broiler corn-soybean starter diet and drinking water for 21 D. The diet was formulated to meet breeder nutrient specifications (Table 1), was prepared in crumble form, contained TiO_2 as the indigestible marker, and had no antibiotics or antimicrobial growth promoters.

After the allocation of cages, 10 chicks of unallocated chicks per treatment were sacrificed, weighed, and dissected for the gizzard, small intestine, and ceca empty weights. Segments (∼3 cm) of mid-jejunum were taken and placed in buffered formalin for histomorphology analysis in accordance with the study by Kim et al. (2017). The same sampling was repeated on day 7, 14, and 21 after hatch using 5 birds per cage for the respective days. Excreta samples for AR of dry matter were collected on day 3 to 5 and day 17 to 19 after hatch and stored at -20°C until required for analyses. Body weight and feed intake was monitored weekly for calculation of body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR).

Sample Processing and Laboratory Analyses

Jejunal tissue samples from experiment 1 were used to measure expression of EGFR. The total RNA was isolated from 50 mg of homogenized jejunal samples using TRIzol reagent (Life Technologies Inc.) according to the manufacturer's instructions. The integrity of RNA was checked by 1% agarose gel electrophoresis with bands stained with SYBR Safe gel stain (Life Technologies Inc.). The concentration and quality (A260:A280 ratio) of extracted RNA sample was measured and analysed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). The RNA samples were stored at −80°C until further analyses. A total of 1 µg of RNA was used to synthesize the first-strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) according to the manufacturer's instructions. The primers for real-time PCR analysis was designed with Primer-Blast (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA) (Table 2). Real-time PCR was carried out using SYBR Green Supermix (Bio-Rad) on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). One microliter of cDNA was added to a total volume of 25 µL containing 12.5 µL of SYBR Green mix and 1 µM each of forward and reverse primers. We used the following conditions:

Table 2. Forward and reverse primers for quantitative PCR.

Genes	Sequence (5′-3′)	Genbank ID
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	FP: ACTGTCAAGGCTGAGAACGG RP: CACCTGCATCTGCCCATTTG	NM_204305
Epidermal Growth Factor Receptor (EGFR)	FP: TCCTATCCATAAATGCCACAAACA RP: AAGGCATCCCCTAGAAATGCA	NM_396494

Abbreviations: FP, forward primer; RP, reverse primer.

3 min at 95°C, then 40 cycles of 20 s at 95°C, 30 s at 60°C, and 30 s at 72°C. At the end of each cycle, the fluorescence was monitored for 10 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. A melting curve program was conducted to confirm the specificity of each product and the size of products. Real-time reverse transcription quantitative PCR analyses were all performed in duplicate for each sample. We used chicken glyceraldehyde 3-phosphate dehydrogenase as the internal control to relatively quantify the expression of the target gene used in the real-time reverse transcription quantitative PCR for all the samples. Relative mRNA abundance was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Fixed jejunal tissues from experiment 2 were embedded in paraffin wax, sectioned (5 μm), and stained with hematoxylin and eosin for morphological examinations. In each cross-sectioned tissue, at least 4–5 complete villous-crypt structures were examined under a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). Villous height and crypt depth were measured using a calibrated micrometer (Kim et al., 2017). The excreta sample was thawed, pooled by cage, and subsequently freeze dried. Samples of the basal diet and freeze-dried excreta samples were finely ground using a coffee grinder. All samples were analyzed for DM and titanium dioxide. Dry matter determination was carried out according to standard procedures (AOAC Intenational, 2005, method 930.15). Titanium content was measured on a UV spectrophotometer following the method of Short et al. (1996).

Table 3. Effects of *in ovo* application of epidermal growth factor (EGF) on expression of EGF receptor from day 17 (E17) of incubation through to hatching (E21).

Incubation, day	EGF[1], μg	Relative expression
E17	0	1.02[b]
E17	160	1.91[b]
E17	640	1.43[b]
E18	0	1.01[b]
E18	160	0.90[b]
E18	640	0.54[b]
E19	0	1.06[b]
E19	160	1.38[b]
E19	640	1.62[b]
E20	0	1.00[b]
E20	160	1.87[b]
E20	640	0.91[b]
E21	0	1.07[b]
E21	160	4.43[a]
E21	640	5.64[a]
SEM		0.517
Main effect of incubation day		
E17		1.46[b]
E18		0.82[b]
E19		1.35[b]
E20		1.26[b]
E21		3.71[a]
SEM		0.299
Main effect of EGF[1], μg		
0		1.03[b]
160		2.10[a]
640		2.03[a]
SEM		0.23
P value		
Sampling day		<0.01
EGF		0.01
Sampling*EGF		0.002

Means assigned different letters (a, b) within a response criterion are significantly different, $P < 0.05$; n = 5.

[1]Epidermal growth factor, μg/kg egg, was injected into amniotic sac of eggs on day 19 of incubation.

Calculations and Statistical Analysis

The target gene expression was normalized with that of a selected reference gene, and relative gene expression was determined by using R = 2 (Ct(reference)-Ct(test)) (Kleta et al., 2004). The average of Ct values of 2 reference genes (glyceraldehyde 3-phosphate dehydrogenase and β-actin) was used for the normalization of the expression of target genes. The gizzard, small intestine, and ceca weights were reported as mg/g BW. The AR of DM was calculated according to the study by Kiarie et al. (2014). Data were subjected to MIXED procedures (SAS Inst. Inc., Cary, NC). For experiment 1, effects of EGF, sampling day, and associated interactions were included in the model. For experiment 2, the data were analyzed with treatment as a fixed factor with contrast statements for effects of punching, injection, and EGF. Where effects of EGF was observed, coefficients for linear and quadratic effects were generated using interactive matrix language procedures of SAS. An α level of $P \leq 0.05$ was used as the criterion for statistical significance.

RESULTS

Experiment 1

There was an interaction ($P = 0.002$) between EGF and sampling day effect EGFR expression (Table 3) such that the embryos receiving EGF had a much higher expression level than the control birds on the day of hatch. However, the EGF response was quadratic ($P < 0.05$), and there was no difference ($P > 0.05$) in EGFR of 160 and 640 μg of EGF groups. Generally, effects of EGF on EGFR were only seen at hatch (Table 3).

Table 4. Growth performance and apparent retention of dry matter (AR of DM) in broiler chickens hatched from eggs subjected to *in ovo* application of epidermal growth factor (EGF) on day 19 of incubation.

	Treatments[1]						*P* values[2]		
	A	B	C	D	E				
EGF, μg	-	-	0	80	160	SEM	Punched	Injected	EGF
Hatch BW, g/bird	44.8	47.2	46.4	46.2	45.4	0.53	0.02	0.88	0.44
Body weight gain, g/bird									
Day 0–7	121	112	115	112	118	2.86	0.61	0.86	0.88
Day 8–14	272	269	263	262	264	4.77	0.21	0.08	0.26
Day 15–21	379	436	415	445	444	27.8	0.10	0.27	0.18
Day 0–21	773	817	794	822	825	27.1	0.18	0.64	0.22
Feed intake, g/bird									
Day 0–7	141	136	136	140	139	2.83	0.83	0.90	0.71
Day 8–14	348	352	338	343	336	8.98	0.12	0.21	0.65
Day 15–21	549	634	624	603	582	20.8	0.12	0.51	0.85
Day 0–21	1,038	1,122	1,098	1,085	1,057	24.9	0.46	0.93	0.78
Feed conversion, g/g									
Day 0–7	1.164	1.221	1.186	1.212	1.186	0.03	0.71	0.91	0.62
Day 8–14	1.277	1.306	1.284	1.308	1.273	0.03	0.79	0.92	0.75
Day 15–21	1.306	1.443	1.503	1.351	1.317	0.05	0.08	0.27	0.29
Day 0–21	1.276	1.367	1.383	1.317	1.283	0.03	0.29	0.54	0.18
AR of DM, %									
Day 3–5	70.3	70.7	70.7	70.1	70.4	0.12	0.73	0.79	0.46
Day 17–19	74.2	74.9	74.4	75.0	74.2	0.17	0.54	0.94	0.90

[1]A, intact (not punched and no injected); B, punched but not injected, and C to E, punched and injected with either EGF fermentation broth or EGF at 80 or 160 μg/kg of egg into amniotic sac.
[2]Preplanned comparison, punched, treatment 1 *vs.* 2; injected, treatment 2 *vs.* 3 to 5; EGF, treatments 3, 4 and 5. n = 6.

Experiment 2

Chicks hatched from eggs that were punched but not injected were heavier ($P = 0.02$) at hatch than chicks from intact eggs (Table 4). There was no effect ($P > 0.05$) of EGF seen on BWG, FI, or FCR (Table 4). There was no effect ($P > 0.05$) of punching or injection seen on these parameters either. When overall growth performance data were examined from day 0 to 21, there was no effect ($P > 0.05$) of EGF, punching, or injection on BWG, FI, and FCR (Table 4). The AR of DM was examined from day 3 to 5 after hatch and day 17 to 19 after hatch (Table 4). For AR of DM from day 3 to 5, there was no EGF, punching, or injection effect ($P > 0.05$) (Table 4). Similarly, no effect ($P > 0.05$) of EGF, punching, or injection was seen on AR of DM from day 17 to 19 (Table 4). There was an effect of punching ($P = 0.03$) seen on day 0 ceca weights such that punching decreased the relative ceca weight in the punched group when compared to the intact group (Table 5). However, there was no ($P > 0.05$) effect of punching on the gizzard or small intestine on day 0. There was no ($P > 0.05$) effect of EGF or injection on day 0 for gizzard, small intestine, or ceca weights. Similar to day 0, there was no effect ($P > 0.05$) of punching, injection, or EGF seen on the gizzard, small intestine, or ceca on day 7, 14, and 21 (Table 5). There was no ($P > 0.05$) effect of punching, injection, or EGF on jejunal histomorphology (villi height to crypt depth ratio) on day 0, 7, 14, or 21 (Table 5).

DISCUSSION

Numerous studies have been conducted to evaluate the efficacy of *in ovo* feeding in poultry, ranging from feeding carbohydrates, proteins, amino acids, vitamins, and/or other biologics (Uni and Ferket, 2004; Roto et al., 2016). To our knowledge, there are no studies available that indicated the presence and activity of intestinal EGFR during embryogenesis in broiler chickens. Although there was no difference in EGFR expression between control and the EGF-treated groups on day 17, 18, 19, or 20 of incubation, there was an increase in expression in the EGF-treated groups on day 21 compared to the control group. In a study by Adamson and Warshaw (1982), mouse embryonic tissues subjected to smaller (10-20 ng/mL) amounts of EGF showed a slight reduction in binding capacity 3 h after *in vivo* injection, however, binding rose again to match that of control after 14 h, whereas larger (200-500 ng/mL) amounts of EGF showed a much more drastic reduction in binding capacity after *in vivo* injection, and the response was sustained for a longer period of time. It has been suggested that during embryonic development, EGFR is exposed to a certain level of EGF which allows for optimal ligand and receptor binding, and thus leading to a mitogenic response (Adamson and Warshaw, 1982). Given that tumorigenesis is commonly associated with the overexpression of EGFR, it is likely that an overstimulation of EGF can lead to the downregulation of EGFR in an effort to

Table 5. Gastrointestinal weight (mg/g BW) and jejunal histomorphology of broiler chickens hatched from eggs subjected to *in ovo* application of epidermal growth factor (EGF) on day 19 of incubation.

	Treatments[1]						*P* values[2]		
	A	B	C	D	E				
EGF, μg	-	-	0	80	160	SEM	Punched	Injected	EGF
Gizzard									
Day 0	68.1	65.3	69.6	66.6	69.2	0.81	0.86	0.41	0.93
Day 7	35.6	35.8	36.3	38.3	36.4	0.47	0.38	0.21	0.16
Day 14	22.7	23.7	24.1	23.3	22.7	2.83	0.41	0.86	0.48
Day 21	17.1	16.8	17.2	16.4	16.7	0.15	0.48	0.59	0.23
Small intestine									
Day 0	39.7	44.2	39.9	42.7	44.0	3.48	0.39	0.42	0.14
Day 7	71.0	65.4	69.7	71.2	68.3	1.05	0.31	0.40	0.57
Day 14	40.2	37.6	44.7	42.0	39.5	1.21	0.71	0.08	0.95
Day 21	29.9	31.6	31.3	32.3	31.2	0.4	0.16	0.38	0.40
Ceca									
Day 0	11.7	6.9	10.4	9.3	9.3	0.78	0.03	0.71	0.72
Day 7	5.9	5.5	5.4	5.4	5.3	0.11	0.08	0.18	0.32
Day 14	3.3	3.0	3.0	3.0	3.2	0.07	0.14	0.46	0.92
Day 21	3.4	3.1	3.2	3.1	3.7	0.11	0.54	0.80	0.46
Jejunal VH:CD[3]									
Day 0	5.2	5.4	5.4	5.0	4.7	0.14	0.85	0.21	0.68
Day 7	3.7	3.7	3.4	4.0	3.8	0.10	0.73	0.94	0.85
Day 14	5.1	5.0	5.2	5.2	5.0	0.04	0.86	0.67	0.48
Day 21	4.7	5.1	4.4	5.0	5.4	0.18	0.34	0.33	0.12

[1]A, intact (not punched and no injected); B, punched but not injected; and C to E, punched and injected with either EGF fermentation broth or EGF at 80 or 160 μg/kg of egg into amniotic sac

[2]Preplanned comparison, punched, treatment 1 *vs.* 2 to 5; injected, treatment 2 *vs.* 3 to 5; EGF, treatments 3, 4, and 5.

[3]Villi height to crypt depth ratio, n = 6.

maintain homeostatic balance (Adamson and Warshaw, 1982; Hudson et al., 1989). In this manner, it may indicate why we did not see a difference in EGFR expression between treatment groups until the last day of incubation when the number of differentiated cells in the embryo is greatest, thus having a greater ability to bind EGF (Adamson and Warshaw, 1982). However, further analyses are required to understand the differences in domains between species-specific EGFR and how these differences play a role in binding affinity, biological responsiveness, the expression pattern, and activity of EGFR during and after embryonic development.

We hypothesized that EGF, a potent mitogenic and antiapoptotic peptide, would stimulate intestinal tract development and thus have beneficial effects on growth performance in broilers. The mitogenic response of EGF is transduced by the interaction between EGF and EGFR, a transmembrane glycoprotein with an extracellular ligand-binding region and cytoplasmic domain with intrinsic protein tyrosine kinase activity (Lax et al., 1988, 1989). Ligand binding results in the autophosphorylation of the intracellular tyrosine kinase domain, followed by phosphorylation of various cellular proteins and activation of signal transduction pathways involved in DNA synthesis and cell proliferation (Lax et al., 1988; Playford and Wright, 1996; Wong and Wright, 1999). The complexity and specificity of this signal-response system between EGF and EGFR allows for signals to be relayed with precision, efficiency, and speed, ensuring that undesirable crosstalk of different signaling pathways is avoided and unnecessary cell proliferation is controlled (Alberts et al., 2002). Despite demonstration of presence of EGFR in experiment 1, we did not observe EGF effects on gastrointestinal weights and jejunal histomorphology. The lack of EGF effect was surprising, particularly because EGF increased expression of EGFR at hatch and improved BWG in our previous study (Kim et al., 2017). Perhaps a possible explanation for this contrast is the continuous supplementation of EGF in the previous study compared to the single supplementation given in this present study. Suggesting repeated exposure to EGF may have increased the likelihood of observing a response and alluding to the possibility that EGF may be effective if applied in starter feed with or without a combination with *in ovo* application.

Another possibility for the lack of response is that the supplemented EGF may have been interpreted as a threat in the sterile egg environment. As an oviparous animal, the embryo depends on an environment isolated from that of their mother and must rely on nonspecific defense mechanisms for protection against invading antigens and pathogens (Board and Fuller, 1974). It has been suggested that a defense system is established through a combination of physical protection by the shell and shell membranes and numerous biochemical molecules in the albumen to defend the embryo against microbial colonization (Board, 1970; Board and Fuller, 1974). In the present study, we injected the eggs with EGF that was generated through recombination technology with the porcine EGF gene. Although both pigs and chickens are identified to possess EGFR, it is

possible the homology between the pig EGF and chicken EGF is diverse enough for the pig EGF to be partially or wholly unrecognized by chicken EGFR. A comparison of domain III, the major ligand-binding region of the EGFR, of human and chicken EGFR reported a 75% sequence similarity (Lax et al., 1988). However, the affinity for binding EGF to EGFR may be influenced by amino acids that make up domain III of human EGFR, in addition to domains I and II (Lax et al., 1989). Even if EGFR expression was noted in experiment 1, perhaps it may have been immature, offering an explanation as to why an EGF effect was not seen in the present study. It is also plausible that an immune response was elicited against the foreign material that was introduced into the hostile environment of the egg (Board and Fuller, 1974; Kang et al., 2010). Even before introducing the EGF, we were unsure of what consequences would arise from compromising the integrity of the egg when a hole was punched at the injection site. However, punching had no negative effects as we saw no differences between treatments on growth performance and nutrient retention. There was an effect of punching on hatch body weight; however, this effect was not evident in subsequent growth, suggesting it was transient.

The essence of *in ovo* technology is capitalization of amniotic fluid that is naturally consumed by the late-term embryo, thereby providing a plausible EGF delivery into the intestine (Noy and Uni, 2010). In addition to previously presented arguments, the lack of response of EGF on intestinal tract in the present study raises the question as to whether the injected EGF was degraded by proteases in the amniotic fluid before delivery into the developing gut, or if EGF was subjected to degradation by pepsin in the stomach or pancreatic proteases in the small intestine (Playford et al., 1993, 1995; Playford and Wright, 1996). On day 11 of embryonic development (ED11), the secondary seroamniotic suture, which is the location where the head and tail folds of the amnion meet on ED4 to enclose the amniotic sac, ruptures and leaves an opening for the contents of the albumen to pass gradually into the amniotic cavity for supplemental nutrients (Baintner and Fehér, 1974; Yoshizaki et al., 2002). This sequential transfer of albumen proteins through the amniotic sac to the embryo and the presence of protease activity in the amniotic fluid suggests that the fluid may possess an additional role of digestion beyond just protection against mechanical stress and dehydration (Da Silva et al., 2017). Interestingly, it is known that the albumen contains many protease inhibitors, such as ovoinhibitor, ovomucoid, cystatin, and ovostatin, to prevent early proteolytic degradation of albumen proteins (Saxena and Tayyab, 1997; Da Silva et al., 2017). In this context, it stands to reason that little to no protease activity would be detected in the amniotic fluid. However, in a study by Da Silva et al. (2017), lower trypsin-like activity was detected in the amniotic fluid from ED8 to 11 than from ED12 to 16 while no trypsin-like activity was seen in albumen, suggesting that albumen protease inhibitors may not effectively inhibit protease activity within the amniotic fluid. Although we did not collect the amniotic fluid to assess protease activity in the experiment 1, the lack of EGF effect on all parameters measured could be linked to the possibility of proteolytic breakdown of EGF in the amniotic fluid before consumption. Conversely, if the supplemented EGF had managed to evade proteolytic degradation in the amniotic fluid, there are potential challenges of degradation in the stomach and small intestine. Playford et al. (1993) showed that human EGF was susceptible to pepsin digestion particularly in fasted rats and not in fed rats, as food proteins compete for the substrates of luminal proteases. In consideration of either circumstance, the concentration of EGF injected into each egg may not accurately reflect what was consumed by the embryo.

In conclusion, the results of the present study suggest that *in ovo* injection of porcine EGF into the amniotic sac did stimulate EGFR expression but did not improve growth performance, gastrointestinal development, and nutrient retention, thus overall suggesting that *in ovo* application of EGF may not be a viable strategy in broiler chickens.

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