Eckol alleviates intestinal dysfunction during suckling-to-weaning transition via modulation of PDX1 and HBEGF

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Research

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Abstract

Background

Maintaining intestinal health in livestock is critical during weaning. Although intestinal dysfunction during this period can be alleviated by phlorotannins including eckol, the precise mechanisms are not fully understood. We addressed this question by evaluating changes in gene expression and intestinal function after treatment with eckol during the suckling-to-weaning transition. The biological roles of differentially expressed genes in intestinal development were investigated by assessing intestinal wound healing and barrier function and associated signaling pathways, along with oxidative stress levels.

Results

We identified 890 differentially expressed genes in the intestine whose expression was altered by eckol treatment including *pancreatic and duodenal homeobox (PDX) 1*, which directly regulate the expression of *heparin-binding epidermal growth factor-like growth factor (HBEGF)* to preserve intestinal barrier function and promote wound healing via phosphoinositide 3-kinase (PI3K)/AKT and P38 signaling. Additionally, eckol alleviated H₂O₂-induced oxidative stress via PI3K/AKT, P38, and 5' AMP-activated protein kinase signaling, improved growth, and reduced oxidative stress and intestinal permeability in pigs during weaning.

Conclusions

Eckol modulates intestinal barrier function, wound healing, and oxidative stress via PDX/HBEGF and improves growth during the suckling-to-weaning transition, suggesting that it can be used as a feed supplement to preserve intestinal function during this process in pigs and other livestock.

Background

The suckling-to-weaning transition contributes to intestinal dysfunction in livestock, which undermines animal health, growth, and feed intake (1). During this transition, the intestine undergoes marked physiological changes in structure and function including villous atrophy and crypt elongation that decrease its absorptive capacity that influence feeding efficiency (2–4). It is therefore important to alleviate intestinal dysfunction during this process given the direct relationship between animal health and economic productivity.

The intestinal epithelium is composed of a monolayer of columnar epithelial cells sealed by junctional complexes including tight and adherens junctions in close proximity to the apical and lateral sides of the paracellular space (5). It functions as a barrier against harmful substances including pathogenic bacteria and food allergens (6). Intestinal dysfunction induced by the suckling-to-weaning transition disrupts

these junctional complexes, allowing the passage of macromolecules and pathogens through the epithelium into the body, which can influence animal growth and health status (7, 8). Preserving intestinal health minimizes the adverse effects of weaning stress. To this end, nutritional strategies including the optimization of dietary protein or energy content and the use of feed additives have been implemented (9–11). In the pig industry, antibiotics as feed additives are broadly used to enhance intestinal health challenged by weaning stress. Although in-feed antibiotics are used to reduce weaning stress and to enhance growth performance, many alternatives including probiotics, organic acids, and polyphenols are substituted due to a ban on antibiotic use in feedstuffs (12–14). Among these candidates, polyphenols might have the potential to enhance gut intestinal health (15).

Polyphenols are investigated for their potential to promote gut health and regulate the intestinal absorption of nutrients as well as lipid and bone metabolism (16). Phlorotannins are oligomeric polyphenols composed of phloroglucinol units found in brown algae (*Ecklonia* species), including *Eckolonia cava* which is an edible marine brown alga species found in the ocean off the coasts of Japan and Korea. These compounds have anti-diabetic, anti-cancer, anti-oxidant, and anti-bacterial and radio-protective properties, as well as inhibitory effects against the human immunodeficiency virus (17–20). A previous report showed that compounds isolated from the ethanol extract of dried *E. cava* (EEEC) including phloroglucinol, eckol, phlorofucofuroeckol, and dieckol exhibit strong antiviral activity against porcine epidemic diarrhea virus by inhibiting viral entry and/or replication and suppressing adipogenesis through the downregulation of C/EBP α in adipocytes (21, 22). In addition, a previous report suggested that *E. cava* has beneficial effects on growth performance, cecal microflora, and intestinal morphology in weaning pigs (23). However, to our knowledge, there are no studies investigating the effects of these compounds on intestinal function during the suckling-to-weaning transition in pigs, which is the most active period of small intestinal epithelium development.

To this end, we evaluated the effects of EEEC on the small intestine during weaning transition by gene expression profiling. Differentially expressed genes upon EEEC treatment were further analyzed to determine their role in small intestine development.

Methods

Animals and feeding trial

A total of 160 crossbred weaned pigs ([Yorkshire $\times$ Landrace] $\times$ Duroc) with an average body weight of 8.23 ± 0.93 kg were used in a six-week feeding trial. The pigs were sorted into pens ($n = 5$ per pen, eight pens per treatment) and into the following feeding groups: control, corn-soybean meal; T1, control + 0.05% EEEC; and T2, control + 0.1% EEEC. Body weight was recorded at the beginning of the experiment and on days 7, 21, and 42, and feed consumption was recorded for each pen during the experiment to calculate average daily gain (ADG) and average daily feed intake. Blood samples (10 mL) were collected from 10 random pigs per treatment at the end of the feeding trial. Serum was separated by centrifugation at $4000 \times g$ for 30 min at 4 °C, and aliquots were stored at 4 °C for determination of the stress hormones

cortisol, epinephrine, and norepinephrine and oxidative markers such as superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GPx).

Serum epinephrine and norepinephrine levels were quantified by ion-exchange purification followed by high-performance liquid chromatography with electrochemical detection. Serum SOD, MDA, and GPx levels were determined by enzyme-linked immunosorbent assay using commercial kits (all from R&D Systems, Minneapolis, MN, USA).

Gene expression profiling

During the feeding trial, five piglets per treatment (control and EEEEC2) were sacrificed on day 14. Intestinal samples were pooled, frozen in liquid nitrogen, and stored at -80°C . Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Library construction was performed using the SENSE 3' mRNA-Seq Library Prep Kit (Lexogen, Wien, Austria) according to the manufacturer's instructions. Briefly, an oligo-dT primer containing an Illumina-compatible sequence at the 5' end was hybridized to 500 ng of total RNA, followed by reverse transcription. After degradation of the RNA template, second-strand synthesis was initiated with a random primer containing an Illumina-compatible linker sequence at the 5' end. The double-stranded library was purified and amplified, and complete adapter sequences required for cluster generation were added. The finished library was purified from PCR components. High-throughput sequencing was performed by single-end 75 sequencing on a NextSeq 500 instrument (Illumina, San Diego, CA, USA).

Quantitative real-time (qRT)-PCR and Western blotting

For qRT-PCR, total RNA (1 μg) was used as a template for cDNA synthesis using the Maxima First-strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). qRT-PCR primers for each target gene were designed using Primer3 (<http://frodo.wi.mit.edu/>; Supplemental Table 5), and the reaction was performed on a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 59°C – 61°C for 30 s, and 72°C for 30 s. Target gene expression levels were normalized to that of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* and were calculated with the $2^{-\Delta\Delta\text{Ct}}$ method

For Western blotting, cultured IPEC-J2 cells subjected to various treatments were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and proteins in each sample (20 μg) were separated by electrophoresis on a 10% polyacrylamide gel for 1 h at 120 V and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA) using a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA). After blocking for 1 h, the membrane was incubated overnight at 4°C with appropriate primary antibodies including anti-HBEGF (Antibodies-online GmbH, Aachen, Germany), anti-PDX1 (Antibodies-online GmbH, Germany), anti-AKT (Cell Signaling Technology, USA), anti-P38 (Cell Signaling Technology, USA), and anti-ZO-1 (Thermo Scientific, Waltham MA, USA). After three washes with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with appropriate secondary antibodies.

Immunoreactivity was visualized with ECL Select Western blotting detection reagent (GE Healthcare, Little Chalfont, UK) and protein bands were imaged using a ChemiDoc imaging system (Bio-Rad). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell culture and treatments

The IPEC-J2 intestinal porcine enterocyte cell line (DSMZ, Braunschweig, Germany) was maintained as previously described (11). Briefly, the cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, 1% insulin-transferrin-selenium-X, and 1% (v/v) penicillin-streptomycin (24). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. IPEC-J2 cells were incubated with various treatments including recombinant HBEGF (Mybiosource, San Diego, CA, USA) LY2944002 (Cell Signaling Technology, Danvers, MA, USA), SB202190 (Cell Signaling Technology, USA), and the AMPK inhibitor (dorsomorphin dihydrochloride):Santa Cruz Biotechnology, Dallas, TX, USA) for different intervals as indicated in the figure legends.

Immunofluorescence

IPEC-J2 cell monolayers on glass coverslips were fixed with 4% paraformaldehyde. After blocking with 2% bovine serum albumin in phosphate-buffered saline, the cells were incubated overnight at 4 °C with primary antibodies against zona occludens (ZO)-1 and occludin diluted 1:100. After washing, fluorophore-conjugated secondary antibody (Alexa Fluor 488) was applied for 1 h at room temperature in the dark. The coverslips were mounted on slides using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and images were acquired using a fluorescence microscope.

Cell proliferation and migration assays

To evaluate proliferation, IPEC-J2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Water-soluble tetrazolium-1 cell proliferation reagent (Roche Applied Science, Indianapolis, IN, USA) was added to each well and the absorbance of the dye at the end of the incubation period was measured at a wavelength of 450 nm with background subtraction at 690 nm using a BioTek Synergy HTTR microplate reader (BioTek Instruments, Winooski, VT, USA).

For the migration assay, IPEC-J2 cells were cultured with various compounds (eckol, heparin-binding epidermal growth factor-like growth factor [HBEGF], and inhibitors) in 60-mm culture dishes until they reached confluence. A straight scratch was made with a P200 pipette tip across the bottom of the dish and photographs of migrating cells were acquired at different times.

Transepithelial electrical resistance (TEER) and intestinal permeability

Confluent monolayers of IPEC-J2 cells were cultured in 24-well transwell chambers (polycarbonate membrane, filter pore size = 0.4 µm, area = 0.33 cm²; Costar) under various treatment conditions for 24 h. The cells were washed twice and incubated with lipopolysaccharide (LPS; 1 µg/µL) for 1 h. TEER was

measured using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). TEER values were calculated by subtracting the blank filter (90 Ω) and multiplying by the surface area of the filter. All measurements were performed for a minimum of three wells.

IPEC-J2 cells growing in a confluent monolayer ($\geq 1 \text{ k}\Omega \text{ cm}^2$) were treated with various compounds for 24 h. The cells were washed twice and incubated with LPS for 1 h. After two additional washes, the permeability assay was initiated by adding 500 μL of culture media containing 1 mg/ml fluorescein isothiocyanate–dextran (FD)-4 (Sigma-Aldrich, St. Louis, MO, USA) to the apical chamber, while the basolateral chamber was filled with 1.5 ml of culture medium. The FD-4 was allowed to permeate overnight (18 h at 37 °C and 5% CO_2) from the apical to the basolateral chamber, and 100 μL of the basolateral chamber media was transferred to a 96-well plate to measure the amount of permeated FD-4 using a fluorometer (excitation, 490 nm; emission, 520 nm).

Cellular reactive oxygen species (ROS) detection

IPEC-J2 cells were seeded in a clear-bottomed 96-well black plate at a density of 1×10^4 cells per well. After treatment with eckol and inhibitors with or without H_2O_2 , the cells were stained with 2.5 μM 2',7'-dichlorofluorescein (DCF) diacetate and fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, with a fluorometer. Values are expressed as fold increase relative to control cells.

Vector construction, gene silencing, and luciferase assay

For *pancreatic and duodenal homeobox (PDX) 1* knockdown, IPEC-J2 cells were transfected with specific small interfering (si)RNAs (Supplemental Table 6) using RNAiMAX (Invitrogen) according to manufacturer's instructions. After 24 h, total RNA was extracted and analyzed by qRT-PCR.

For the promoter assay, upstream sequences of the *HBEGF* gene and the – 1000 upstream sequence containing deletions of the *PDX1*-binding site synthesized by Bioneer (Daejeon, Korea) were sub-cloned into the pGL3-Basic vector. Each vector containing a different length of the *HBEGF* upstream sequence was transfected into IPEC-J2 cells. After 4 h, cells were analyzed with a luciferase assay kit (Promega, Madison, WI, USA). Briefly, cells were re-suspended in 100 μL of diluted Passive Lysis Buffer, and the extracts were centrifuged at $13,000 \times g$ for 5 min; firefly and *Renilla* luciferase activities in the supernatant were measured using a GLOMAX 20/20 luminometer (Promega). Measured values were normalized to *Renilla* activity.

Statistical analysis

Data were analyzed by evaluating differences among treatments with Duncan's multiple range tests using the general linear model function of SAS software (Systat, Cary, NC, USA). Results are expressed as the mean $\pm$ standard error ($n \geq 3$, where n is the number of replicates). A p -value < 0.05 was considered statistically significant.

Results

Identification and validation of differentially expressed genes (DEGs)

We used high-throughput sequencing to identify genes that were differentially expressed in response to EEEEC by comparing the gene expression profiles of small intestinal tissue with or without EEEEC treatment for 14 days. Of the 890 annotated DEGs, 639 were upregulated and 251 were downregulated (Fig. 1A).

A Gene Ontology (GO) enrichment analysis showed that the DEGs were related to calcium, heme, iron, heparin, carbohydrate, and lipid binding as well as chemokine, transporter, serine-type endopeptidase inhibitor, and hormone activities (Supplemental Table 1 and Fig. 1B). Genes within the cellular component category were mainly associated with the extracellular region, integral component of plasma membrane, cell surface, and cell terms (Supplemental Table 2), whereas biological process terms included inflammatory, immune, and innate immune response, oxidative-reduction process, cell surface receptor signaling pathway, cell adhesion, cytokine-mediated pathway, response to lipopolysaccharide, and chemokine-mediated signaling pathway (Supplemental Table 3). The Kyoto encyclopedia of genes and genomes pathway analysis showed that the proteins were mainly associated with the following categories: cytokine-cytokine receptor, neuroactive ligand-receptor, and extracellular matrix-receptor interactions, peroxisome proliferator-activated receptor γ , chemokine, Janus kinase-signal transducer and activator of transcription (STAT), tumor necrosis factor, Toll-like receptor, and transforming growth factor (TGF)- β signaling; inflammatory bowel disease (IBD), retinol metabolism (Supplemental Table 4 and Fig. 1C).

We verified the expression of the top 10 DEGs in the small intestine with or without EEEEC treatment by qRT-PCR (Fig. 1D) and confirmed that *lectin, galactoside-binding, soluble 13* ($p < 0.01$), *pheromaxin C subunit* ($p < 0.01$), *PDX1* ($p < 0.01$), *solute carrier family 22 member A7* ($p < 0.01$), *adenylate cyclase activating polypeptide 1* ($p < 0.01$), *resistin* ($P < 0.05$), *HBEGF* ($p < 0.05$), *RNA-binding protein 7* ($p < 0.06$), *Spalt-like transcription factor 1* ($p < 0.05$), and *A disintegrin and metalloproteinase with thrombospondin motifs 20* ($p < 0.05$) were upregulated in the EEEEC treatment group relative to expression in the untreated group (Fig. 1D).

Eckol induces PDX1 and HBEGF expression

We next evaluated the toxicity of EEEEC, eckol, dieckol, and phlorofucofuroeckol in IPEC-J2 cells with a cell viability assay. Pre-treatment for 24 h with 50 μ M EEEEC, 100 μ M eckol and dieckol, or 200 μ M phlorofucofuroeckol reduced cell viability (Fig. 2A). Based on these results, 10 μ M EEEEC, 50 μ M eckol and dieckol, and 100 μ M phlorofucofuroeckol were deemed safe for subsequent experiments. *HBEGF* expression was induced by treatment with 10 μ M EEEEC ($p < 0.01$) and 50 μ M eckol ($p < 0.05$) (Fig. 2B). These results suggest that eckol is the main component of EEEEC that mediates differential gene expression. Indeed, incubation with varying concentrations of eckol (10 μ M, 20 μ M, 50 μ M, 100 μ M, and

200 μ M) for 24 h resulted in a concentration-dependent increase in *PDX1* (Fig. 3A and 3B) and *HBEGF* (Fig. 3C and 3D) mRNA and protein level.

Eckol-mediated induction of PDX1 regulates HBEGF expression

We further investigated the activation of PDX1 which directly regulates HBEGF expression in intestinal epithelial cells in response to eckol treatment. We cloned porcine *HBEGF* gene promoter sequences of different lengths into the firefly luciferase plasmid to evaluate the transcriptional activity of PDX1 in response to eckol treatment. Luciferase activity was stimulated in the presence of the – 2000, – 1500, and – 1000 sequences relative to that of the control (Fig. 4A). Three regions (– 954, – 790, and – 767) of the PDX1 binding sequence (TAAT) were identified between – 1000 and – 500 (Fig. 4B). Deletion of the PDX1 binding sequence upstream of – 790 reduced luciferase activity relative to control levels (Fig. 4C), suggesting that it is essential for the basal transcriptional activity of the *HBEGF* promoter.

We next examined whether *PDX1* knockdown affects *HBEGF* expression. Three different siRNA sequences against porcine *PDX1* were confirmed to reduce *PDX1* expression in IPEC-J2 cells as compared to that with a non-specific siRNA with no homology to porcine sequences, with knockdown efficiencies of $13.32\% \pm 14.96\%$, $78.47\% \pm 16.99\%$ ($p < 0.05$), and $5.35\% \pm 23.06\%$ for siRNA-1, siRNA-2, and siRNA-3, respectively (Fig. 4D). According to these results, we used PDX1-siRNA-2 for subsequent experiments. *PDX1* knockdown reduced *HBEGF* expression after eckol treatment (Fig. 4E). Taken together, our results indicate that PDX1 directly regulates *HBEGF* expression by binding to the promoter region upstream of – 790.

Eckol Enhances Intestinal Barrier Function And Promotes Wound Healing

To investigate the mechanism by which eckol modulates HBEGF expression and function in the intestine, we examined the activation of phosphoinositide 3-kinase (PI3K)/AKT (Fig. 5A) and P38 (Fig. 5B) signaling pathways. Treatment of IPEC-J2 cells with eckol and HBEGF increased AKT phosphorylation, whereas treatment with the PI3K inhibitor LY294002 decreased AKT phosphorylation following treatment with eckol and HBEGF, as compared to that in untreated controls. Eckol and HBEGF treatment also increased P38 phosphorylation, which was reversed by the application of the P38 mitogen-activated protein kinase (MAPK) inhibitor SB202190.

We next examined the effects of eckol and HBEGF on intestinal barrier function by evaluating TEER, permeability, and tight junction protein expression in IPEC-J2 cells. Treatment with LPS for 1 h decreased TEER relative to that in untreated control cells, an effect that was abolished in the presence of eckol and HBEGF (Fig. 5C). In contrast, application of LY294002 and SB202190 reduced TEER in LPS-challenged cells. Exposure to LPS for 1 h also reduced permeability to FD-4, which was reversed by eckol and HBEGF treatment (Fig. 5D) and exacerbated by the application of LY294002 and SB202190.

Immunocytochemical and Western blot analyses showed that ZO-1 expression was downregulated

relative to control levels in IPEC-J2 cells treated with LPS for 1 h and increased in the presence of eckol and HBEGF, which was abrogated by treatment with LY294002 and SB202190 (Fig. 5E and 5F).

Intestinal wound healing depends on a precise balance among migration, proliferation, and differentiation of epithelial cells adjacent to the wound. Here we investigated the effects of eckol and HBEGF on intestinal wound healing using IPEC-J2 cells, by assessing cell proliferation and migration. LPS treatment reduced cell growth, but adding eckol and HBEGF to the culture media stimulated cell proliferation (Fig. 6A). Similarly, eckol and HBEGF enhanced the migratory capacity of LPS-challenged cells, an effect that was abrogated by the application of LY294002 and SB202190 (Fig. 6B).

To determine the effects of eckol and HBEGF on intestinal epithelial cell differentiation, we analyzed the expression of wound healing-related genes such as *matrix metalloproteinase (MMP)2*, *MMP9*, and *Rho family GTPase (RND)3* in IPEC-J2 cells with or without eckol and HBEGF treatment (Fig. 6C). The levels of *MMP2*, *MMP9*, and *RND3* were downregulated relative to that in untreated control cells by LPS treatment, but were increased in the presence of eckol and HBEGF.

Eckol Protects Against Oxidative Stress In The Intestine

To investigate the effects of eckol on intestinal oxidative stress, we analyzed *heme oxygenase (HO)-1* and *manganese superoxide dismutase (MnSOD)* expression and ROS production in IPEC-J2 cells. H_2O_2 treatment for 1 h reduced *HO-1* levels as compared to that in untreated cells; this was abolished in the presence of eckol and LY294002 had a similar effect (Fig. 7A). Cells treated with H_2O_2 showed higher DCF fluorescence relative to that in the untreated control; this was also reversed by eckol and SB202190 treatment each also increased DCF fluorescence (Fig. 7B). Thus, eckol alleviates H_2O_2 -induced oxidative stress via PI3K/AKT signaling.

MnSOD expression was decreased and DCF fluorescence was increased in IPEC-J2 cells exposed to H_2O_2 as compared to that in control cells; eckol treatment restored the levels of *MnSOD* and reduced DCF fluorescence, but 5' AMP-activated protein kinase (AMPK) inhibitor treatment reversed these effects (Fig. 7C and 7D). These results indicate that eckol alleviates H_2O_2 -induced oxidative stress via AMPK signaling.

EEEC Supplementation Improves Intestinal Function In Pigs During Weaning

To investigate the *in vivo* effects of EEEEC, we evaluated growth, and serum levels of stress markers in weaned pigs administered EEEEC as a dietary supplement. Average daily gain (ADG) was higher in pigs fed 0.05% and 0.1% EEEEC than in those animals fed the control diet during phase I (days 1–7) and phase II (days 7–21) ($p < 0.05$; Fig. 8A). At the end of the feeding trial, (day 42), cortisol levels were lower in pigs fed 0.05% ($p < 0.05$) and 0.1% ($p < 0.05$) EEEEC, and epinephrine and norepinephrine levels were reduced in

pigs fed 0.1% EEEC ($P < 0.05$; Fig. 8B). Pigs in the 0.05% and 0.1% EEEC groups also had lower serum SOD and glutathione peroxidase (GPx) levels and higher serum malondialdehyde (MDA) levels than control animals on days 7 and 21 ($p < 0.05$; Fig. 8C). Finally, serum FD-4 fluorescence was lower on day 7 in pigs fed 0.1% EEEC ($p < 0.05$) and on day 21 in those animals fed 0.05% or 0.1% EEEC ($p < 0.05$) relative to that in control animals (Fig. 8D). Thus, EEEC supplementation improves growth performance and reduces stress and intestinal permeability in pigs during weaning.

Discussion

The suckling-to-weaning transition is associated with various stressors such as maternal separation, mixing stress, transportation/relocation, and dietary changes. These stressors collectively contribute to intestinal dysfunction including intestinal villus atrophy, crypt hyperplasia, and increased intestinal permeability, which results in reduced feed intake, growth retardation, and susceptibility to diseases. This period therefore represents a major bottleneck in livestock production (1, 3). To minimize intestinal dysfunction during the transition, nutritional strategies including the manipulation of dietary protein and non-starch polysaccharide content and the addition of antibiotics, zinc, probiotics, prebiotics, and polyphenols to the diet have been applied to pig production (15, 25). Phlorotannins such as eckol, dieckol, and phlorofucofuroeckol found in brown algae have protective effects against oxidative stress-induced mitochondrial dysfunction and radiation-induced intestinal injury (26, 27). In this study we investigated the mechanism of action of EEEC in intestinal development and its effects during the suckling-to-weaning transition.

Our transcriptome analysis identified 639 and 251 genes that were up- and downregulated, respectively, in response to EEEC treatment. These DEGs were enriched in various GO categories; the expression patterns of the top 10 genes were confirmed in the small intestine. Consistent with our results, previous studies demonstrated that polyphenols alter the expression of genes related to Wnt signaling, chemokine and cytokine activities, bone morphogenetic protein, TGF- β signaling, apoptosis, adipogenesis, cytokine and inflammatory responses, and proliferation (28, 29). To investigate the role of individual EEEC components, we analyzed *PDX1* and *HBEGF* expression in intestinal epithelial cells treated with each phlorotannin. The fact that eckol induced the expression of *PDX1* and *HBEGF* to a greater extent than dieckol and phlorofucofuroeckol indicated that eckol is the main active compound in EEEC.

PDX1 is a homeodomain transcription factor and key regulator of genes involved in pancreatic development and intestinal differentiation (30, 31), and along with (sex determining region Y)-box 2 and caudal type homeobox 2, it regulates the differentiation of enterocytes, Brunner's gland cells, and entero-endocrine cells in the proximal intestine by modulating genes related to lipid metabolism and iron absorption (32–34). We found here that *PDX1*, induced by eckol, activate the expression of *HBEGF*, which is implicated in wound healing, heart development, and gut protection (35–37). *HBEGF* protects against hypoxia, ischemia/reperfusion injury, and necrotizing enterocolitis in the intestine by suppressing pro-inflammatory cytokine-induced apoptosis and ROS production (38–40). In addition to *PDX1*, *HBEGF* expression is regulated by nuclear factor- κ B, specificity protein 1, and myogenic differentiation

transcription factors in diverse biological processes such as inflammation, homeostasis, and development (41–43). In the present study, the PDX1 binding site (C/TTAATG) was detected in the upstream region (– 954, – 790, and – 767) of the porcine *HBEGF* gene. A deletion analysis confirmed that PDX1 directly binds the sequence upstream of – 790 to regulate the basal transcriptional activity of *HBEGF* in small intestinal epithelial cells.

The intestinal epithelium restricts the paracellular penetration of potentially toxic substances that can cause intestinal inflammation and injury by means of tight and adherens junction complexes; it also supports wound repair by stimulating epithelial cell migration, proliferation, and differentiation (44–46). We found that HBEGF treatment increases TEER and decreases FD-4 penetration resulting from LPS-induced intestinal barrier dysfunction by increasing the expression of the tight junction proteins ZO-1 and occludin. In agreement with our results, earlier studies reported that *HBEGF* knockout mice have shorter villi and exhibit increased mucosal permeability and perturbed intestinal wound healing, which was reversed by HBEGF overexpression (47, 48). These findings suggest that HBEGF serves as a prophylactic or therapeutic agent for the prevention or treatment of intestinal diseases such as hypo-perfusion injury and peritonitis-induced sepsis. Extracellular signal-regulated kinase (ERK)1/2 MAPK, PI3K/AKT, nucleotide-binding oligomerization domain-containing protein 1/2, c-Jun N-terminal kinase (JNK)1/2, and STAT signaling pathways are involved in intestinal barrier function and wound healing (11, 45, 49). The present study reported that HBEGF ameliorates LPS-induced intestinal dysfunction via PI3K/AKT and P38 signaling pathways. In a previous report, HBEGF enhanced wound repair following intestinal ischemia/reperfusion via PI3K/AKT and MAPK/ERK kinase/ERK1/2 activation (50). Our results also provide evidence that HBEGF preserves the barrier function of intestinal cells exposed to LPS and promotes wound healing via P38 and PI3K/AKT signaling.

The present study confirmed that eckol extracted from EEEEC reduces oxidative stress in intestinal epithelial cells. Oxidative stress caused by ROS or reactive nitrogen species production is linked to various intestinal disorders such as IBD, gastroduodenal ulcers, and colon cancer (51, 52). Polyphenols protect against oxidative stress by eliminating free radicals (53). Oxidative stress is attenuated by eckol via the upregulation of transcription factors including nuclear respiratory factor (NRF) and Forkhead box O3a, which directly regulate the ROS-scavenging enzymes HO-1 and MnSOD via NRF2/JNK, ERK, PI3K/AKT, and AMPK signaling pathways (26, 54, 55). Consistent with these findings, we showed that eckol alleviates H₂O₂-induced oxidative stress by stimulating the expression of *HO-1* and *MnSOD* via PI3K/AKT, P38, and AMPK signaling pathways. Based on these findings, we propose that eckol can serve as a therapeutic agent to alleviate oxidative stress.

We also investigated the *in vivo* effects of EEEEC on growth, serum stress hormone, and oxidative stress levels in pigs during weaning. Dietary EEEEC supplementation improved growth performance, reduced the levels of stress hormones (cortisol, epinephrine, and norepinephrine) and anti-oxidants (SOD and GPx). The positive effect of eckol on growth might be attributed to its ability to improve intestinal dysfunction and reduce oxidative stress. Growth during the suckling-to-weaning transition is directly related to the total market days of the animal; for example, the number of days to market was approximately 6–10

days shorter for piglets with a higher ADG (227 g/day) during the first week after weaning than for those animals with a lower ADG (150 g/day) (1). In the present study, EEEEC as a feed additive supplement improved ADG at phase I (1–7 days) and II (7–21 days), but not phase III (21–42 days). Based on this result, it is possible that EEEEC is effective during phase I and II development which is associated with rapid intestinal changes; however, it is not effective at phase III when intestinal development has returned to normal. Thus, further study is required to determine if EEEEC supplementation is directly related to a decrease in total market days with respect to pig production.

Conclusions

In conclusion, the present study demonstrates that eckol enhances intestinal function during the suckling-to-weaning transition in pigs by improving intestinal barrier function and wound healing and reducing oxidative stress via PDX-induced HBEGF expression (Fig. 9). Our results indicate that eckol as a feed additive improves the overall health and growth of livestock by preventing intestinal dysfunction during a critical developmental period, which can increase livestock marketability.

List Of Abbreviations

ADG, average daily gain; AMPK, AMP-activated protein kinase; CDH1, cadherin 1; DAPI, 4',6-diamidino-2-phenylindole; DEG, Differentially expressed genes; EEEEC, ethanol extract of dried *E. cava*; GO, gene ontology; GPx, glutathione peroxidase; HBEGF, heparin-binding epidermal growth factor-like growth factor; HO-1, heme oxygenase-1; IBD, inflammatory bowel disease; MDA, malondialdehyde; MMP, matrix metalloproteinase; PDX1, pancreatic and duodenal homeobox 1; PI3K/AKT, phosphoinositide 3-kinase (PI3K)/protein kinase B; qRT-PCR, Quantitative real-time polymerase chain reaction; RND3, Rho family GTPase 3; ROS, reactive oxygen species; SOD, superoxide dismutase; STAT, Janus kinase-signal transducer and activator of transcription; TEER, Transepithelial/transendothelial electrical resistance; TGF- β , transforming growth factor- β ; ZO-1, zona occludens-1

Declarations

Ethics approval and consent to participate:

The animal care and experimental protocols were approved by the Animal Care, and Use Committee of Dankook University and all methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: The authors declare no competing interests.

Author' contributions: S.I.L. designed the study, performed the experiments, analyzed and interpreted the data, and wrote the manuscript, I.H.K. supervised the study, interpreted the data, and wrote the manuscript.

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Figures

Figure 1

Gene expression profiling of the small intestine of pigs treated with ethanol extracted of dried *E. cava* (EEEC). (A) Venn diagram of genes up- or downregulated at least 2 fold relative to control levels after EEEEC treatment. (B) GO terms assigned to biological processes, cellular components, and molecular functions ($p < 0.01$). (C) Kyoto Encyclopedia of Genes and Genomes pathway analysis of the same gene sets ($p < 0.01$). (D) Quantitative analysis of the top 10 differentially expressed genes (DEGs; $n = 3$). Relative expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars indicate standard error of triplicate analyses. * $p < 0.05$, ** $p < 0.01$.

A **B** **Figure 2**

The toxicity of ethanol extracted of dried *E. cava* (EEEC), eckol, dieckol, and phlorofucofuroeckol (A) The toxicity of EEEEC, eckol, dieckol, and phlorofucofuroeckol was evaluated by performing a cell viability assay using IPEC-J2 cells incubated with the indicated concentrations of each compound for 24 h. (B) HBEGF expression in cells treated with EEEEC, eckol, dieckol, and phlorofucofuroeckol. In qRT-PCR analysis, target gene expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars indicate standard error of triplicate analyses. * p < 0.05 and ** p < 0.01.

Figure 3

Eckol induces PDX1 and HBEGF expression. PDX1 mRNA (A) and protein (B) expression in cells treated with various concentrations of eckol (0 μM , 10 μM , 20 μM , 50 μM , 100 μM , and 200 μM). HBEGF mRNA (C) and protein (D) expression in cells treated with various concentrations of eckol. Protein band intensity in the Western blot analysis was quantified using the threshold function of ImageJ software. For qRT-PCR analysis, target gene expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars indicate the standard error of triplicate analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4

Eckol-induced PDX1 regulates HBEGF expression. (A) Activity of HBEGF upstream region. IPEC-J2 cells were transfected with DNA sequences of different length (-2000, -1500, -1000, and -500) comprising the upstream region of HBEGF (n = 3). (B) Upstream sequence of the putative core region of the HBEGF promoter. Sequence numbering is relative to the transcription start site. Putative PDX1 binding sites (TAAT) are boxed and labeled above (upstream of -954, -790, and -767). (C) Deletion assay of putative PDX1 binding sites. Sequences in which the binding sites (upstream of -954, -790, and -767) were deleted were transfected into IPEC-J2 cells (n = 3). (D) PDX1 knockdown assay. siRNA-mediated suppression of PDX1 in IPEC-J2 cells was confirmed by qRT-PCR. (E) Relative expression of HBEGF in PDX1-silenced IPEC-J2 cells treated with Eckol. Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase and the relative expression level was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars indicate standard error of triplicate analyses. Lowercase

letters (a, b, and c) indicate significant differences among treatments based on Duncan's multiple range test.

Figure 5

Effects of eckol on intestinal barrier function. Activation of PI3K/AKT (A) and P38 (B) signaling pathways in IPEC-J2 cells treated with eckol and HBEGF for 2 h under LPS challenge. The phosphorylation of AKT (Ser473) and P38 (Thr180/Tyr182) was evaluated by Western blotting. Protective effects of eckol and HBEGF on intestinal barrier function in IPEC-J2 cells under LPS challenge, as determined by measuring TEER (C) and the permeability of fluorescein isothiocyanate-FD-4 (D) (n = 3). ZO-1 expression upon treatment with eckol and HBEGF under LPS challenge was evaluated by immunofluorescence analysis (E) and Western blotting (F). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). IPEC-J2 cells were treated with eckol and HBEGF under LPS challenge in the absence or presence of PI3K/AKT (LY2944002) and P38 (SB202190) inhibitors. Error bars indicate standard error of triplicate analyses. *p < 0.05, **p < 0.01, ***p < 0.001. Lowercase letters (a, b, and c) indicate significant differences among treatments based on Duncan's multiple range test.

Figure 6

Eckol promotes intestinal wound healing through HBEGF. (A) Cell proliferation was assessed at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h with the water-soluble tetrazolium-1 assay (n = 3) (B) Migration was assessed at 0, 6 h, and 12 h with a wound-healing assay (n = 3) (C) Relative expression levels of cell migration-related genes was analyzed by qRT-PCR in IPEC-J2 cells treated with eckol and HBEGF under LPS challenge in the absence or presence of PI3K/AKT (LY2944002) and P38 (SB202190) inhibitors. Error bars indicate standard error of triplicate analyses. Lowercase letters (a, b, c, and d) indicate significant differences (P < 0.05) among treatments based on Duncan's multiple range test.

Figure 7

Eckol protects against H₂O₂-induced oxidative stress in IPEC-J2 cells. Relative expression levels of HO-1 (A) and MnSOD (C) were evaluated by qRT-PCR (n = 3). Relative ratio of cellular ROS was determined based on DCF fluorescence in the absence or presence of PI3K/AKT (LY2944002), P38 (SB202190) (B), or AMPK (D) inhibitor (n = 3) in cells treated with eckol exposed to H₂O₂-induced oxidative stress. Lowercase letters (a, b, c, and d) indicate significant differences (P < 0.05) among treatments based on Duncan's multiple range test.

Figure 8

In vivo feeding trial during the suckling-to-weaning transition in pigs. The pigs were sorted into pens ($n = 5$ per pen, eight pens per treatment) and the following feeding groups: control, corn-soybean meal; T1, control + 0.05% EEEEC; T2, control + 0.1% EEEEC. (A) Dietary ethanol extract of dried *E. cava* (EEEC) supplementation improved average daily gain (ADG) in pigs during three phases of development (days 1–7, 7–21, and 21–42). Serum levels of the stress-related hormones cortisol, epinephrine, and

norepinephrine (B) and the oxidative stress markers SOD, MDA, and GPx (C) were determined by enzyme-linked immunosorbent assay (n = 10). Error bars indicate the standard error of triplicate analyses. *p < 0.05.

Figure 9

Schematic illustration of the effect of eckol on intestinal development during the suckling-to-weaning transition. Eckol induces the expression of PDX1, a transcription factor that directly regulates the expression of HBEGF, leading to enhanced intestinal barrier function, the acceleration of wound healing, and the suppression of oxidative stress via various intracellular signaling pathways.

Supplementary Files

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