Pakistan J. Zool., pp 1-7, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20210909020909

Effects of Recombinant Porcine Epidermal Growth Factor on Diarrhea, Growth Performance and Intestinal Development in Healthy and Piglets with Enteritis

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Article Information
Received 09 September 2021
Revised 25 June 2022
Accepted 23 August 2022
Available online 23 May 2024
(early access)

Authors' Contribution
Wenjun XU, Wei Zhou and Bingbing Xia: Conception; Wenjun XU, Fenghua Li and Yanan Wang: Performed the experiment; Wenjun XU, Fenghua Li and Yanan: Analysis and manuscript preparation; Wenjun XU: data analyses and manuscript preparation; Jun Zhao: Analysis.

Key words
Piglets, Porcine transmissible gastroenteritis virus, Epidermal growth factor, Intestinal tract, Growth performance

ABSTRACT

Studies had shown that epidermal growth factor could promote intestinal development and improve growth performance of early weaned piglets. There was still insufficient data on that EGF could alleviate intestinal damage caused and reduce the degree of diarrhea by viral infection. In this study, twenty-four 10-day-old piglets divided into four groups. Group 1and Group 2 were oral administration of $2\times10^{5.5}TCID^{50}$ for TGEV challenge on day 4. rEGF solution (400μg/day/pig) was orally administered for 14 days from day 4 of the trial in Group 3 and Group 4. The pigs were euthanized by electric shock on day 17. The experiment results show that compared to group 1, average time of relieving diarrhea had a tendency to improve by rEGF treatment in group 2 (P=0.08). The symptoms of enteritis were significantly improved. ADG of second week in group 2 had tendency to increase(P=0.07) compare with group 1 and group 2 had higher final BW. ADG of the first week and second week, ADG of the first week and ADG of second week in group 4 were greater than in group 3($P < 0.05$). The length of villi in the jejunum and ileum ($P < 0.05$) was longer in the rEGF treatment group 4 than in control groups 3.The VH/CD of the jejunum of group 4 treated with rEGF significantly improved also ($P < 0.05$). In summary, the results indicated that oral administration of recombinant rEGF alleviate intestinal damage caused by TGEV challenge, promotes the health of the intestine and growth rate in piglets.

INTRODUCTION

Early weaning of piglets is one of the significant technologies in large-scale and intensive production. Early weaning of piglets can not only maximize the reproductive performance and increase the number of litters per year of sows, but also reduce the transmission of pathogens from sows to piglets (Whiting and Pasma, 2008).

The key stages of intestinal growth and development of piglets were lactation and weaning. Challenges of diets and environment caused weaning stress, which promotes to growth disorders, intestinal diseases, diarrhea, digestion and malabsorption. The nutrients that have not been digested and absorbed would promote the growth of intestinal microbes, so early weaned piglets were susceptible to pathogenic microorganisms (Lackeyram *et al*., 2010; Lallès *et al*., 2004; Smith *et al*., 2010), such as transmissible gastroenteritis virus.

Porcine transmissible gastroenteritis was caused by transmissible gastroenteritis virus which was one of the typical coronaviruses (Laude *et al*., 1990). The incubation period of the virus is very short, about 12 to 18 h. Within 24 h of exposure to the pathogen, the villi of the ileum and jejunum were atrophied and the intestinal epithelial cells were damaged. Piglets were susceptible to infection within 2 weeks. The symptoms of infected piglets such as yellow

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0030-9923/2024/0001-0001 $ 9.00/0

watery diarrhea, vomiting, rapid weight loss and death was showed (Enjuanes *et al*., 1995; Wu *et al*., 2020).

Epidermal growth factor (EGF) is a single-chain polypeptide active substance which was isolated and purified from mouse mandibular glands by Cohen (1962). Amino acid sequence analysis revealed that EGF contains 53 amino acid residues. EGF was released into emulsion, saliva, urine, intestinal fluid, blood and amniotic fluid which was mainly secreted by lactating breasts, mandibular glands, kidneys, duodenal Brunner glands, pancreas, and placenta (Zeng and Harris, 2014). As a mitogen, it plays an important role in regulating cell growth, survival, migration, apoptosis, proliferation and differentiation (Jeong *et al*., 2013). EGF could promote the secretion of interleukin-13 and keratinocyte growth factor, and promote the expression of intestinal immune globulin and the proliferation of lymphocytes (Bedford *et al*., 2015).

Previous studies have shown that EGF supplementation in piglet diets has also been shown to facilitate intestinal recovery from rotavirus infection (Donovan *et al*., 1994). Exogenous EGF supplementation could increases body weight gain, gain to feed ratio, villus height and crypt depth and improve the nutrient absorption capacity, feed utilization rate and growth performance (Bedford *et al*., 2015; Wang *et al*., 2015). Despite the benefits of EGF on early weaned piglets, there still are controversies regarding the effect of EGF on virus-infected piglets. In this study, an enteritis model was established by TGEV infection. The effects of EGF on growth performance, diarrhea and intestinal development of piglets with enteritis and normal piglets were explored.

MATERIALS AND METHODS

Ethics statement

Animal experiments and experimental protocols have been approved by the Research Ethics Committee of Anhui Medical University.

Drugs

Recombinant epidermal growth factor (rEGF) were produced by Wuhu Interferon Biological Products Industry Research Institute Co. Ltd (Application Number 202010611379.5).

Virus

Porcine transmissible gastroenteritis virus (TGEV) SC-T clinical isolate, isolated and gifted by Sichuan Agricultural University, and experimentally identified by the China Veterinary Drug Administration.

Tested animals

Twenty four piglets (10-day-old Landrace piglets) were selected and purchased from the Anhui Huajie Agriculture and Animal Husbandry Co. The piglets are kept in a single fence in a breeding fence for regular feeding, and no drugs were added to the feed during the breeding. Sera from pigs were confirmed to be negative for TGEV antibodies using an enzyme-linked immunosorbent assay. They were fasting for 12 h before the test and free drinking.

Animal experiments with piglets

Twenty four 10-days-old piglets from a TGEV-free sow were adaptively fed fresh liquid milk diluted in warm water every 4 h. The piglets randomly divided into four groups for 4d. The first group (Group 1) was a positive control group, without oral administration of rEGF therapy, oral administration of $2\times10^{5.5}TCID^{50}$ for TGEV challenge on day 1. The second group (Group 2) had oral administration of $2\times10^{5.5}$ $TCID^{50}$ for TGEV challenge. The test method was based on Xia *et al*.(2018) after slight modifification. 72 h after oral administration of TGEV, rEGF solution (400μg/day/pig) was orally administered for 14 days from day 4 of the trial with a flexible tube feeding needle. The third group (Group 3) was a negative control group with oral administration of normal saline for 14 days from day 4 of the trial, without TGEV challenge. The fourth group (Group 4) had oral administration of rEGF solution (400μg/day/pig) for 14 days from day 4 of the trial.

After inhaling the virus solution, each piglet was fed with 30 mL 10% glucose solution. The piglets were fed 3 h after the end of the virus challenge. After the TGEV challenge, the piglet excrement was observed and recorded every day, and the weight was measured on day 4, day 10 and day 17. After the experiment, the pigs were euthanized by electric shock, and the piglet tissue samples were taken for visual inspection and H & E staining for pathological analysis.

Artificial feeding

The piglets were fed with formulated milk at 6:00~24:00 every day from day 1 to day 3, once every 3 h, seven times a day. The supplementary feeding included: (1) supplementary nutrition: oral vitamin B was added into artificial milk, once a day, 0.2 mL/pig; (2) energy maintenance: When piglets have no appetite or refuse to eat, they are given oral nutrition cream for pigs with the oral dose of 1 cm / kg/time. All piglets were fed equally. The piglets were fed with liquid feedstuff from day 4 to day 17,three times a day. All piglets were fed equally.

Observation of clinical signs

After the virus challenge, the pigs were visually inspected and monitored for about 7 days to check the severity of diarrhea. The severity of diarrhea was scored every day. The scoring criteria for virus-induced infectious transmissible gastroenteritis in piglets were expressed as 0~4. The symptoms including soft stool, but the stool was still in shape, no vomiting, accompanied with mental depression were expressed as 0. The symptoms including stool appeared paste, not forming, but the feces and water have not yet been separated; piglets showed mild diarrhea were expressed as 1. The symptoms including stool was liquid, The feces are separated from the water; piglets showed severe diarrhea with vomiting were expressed as 2. The symptoms including stool was liquid, piglets showed severe diarrhea with mental depression were expressed as 3 Death were expressed as 4. When piglets' clinical manifestations achieve more than 2 (including 2), the piglets were considered to have infectious gastroenteritis symptoms and diarrhea.

Observation and evaluation of the intestinal villi length and crypt depth of the tested animals

After the experiment, three piglets with a weight close to the average weight were randomly selected from each group to be sacrificed. The abdominal cavity of piglets was cut along the midline of the abdomen, and the duodenum (6 cm away from the pylorus of the stomach), ileum, and jejunum were cut out about 2 cm. The extraintestinal chyme was washed with distilled water, and then the remaining water was absorbed by filter paper. After ligating one end of the intestine, it was fixed in 4% paraformaldehyde solution for more than 1 day (pH value 7.4, 4°C). Then, after routine dehydration, paraffin embedding, sectioning, and hematoxylin-eosin (H & E) staining, the changes in the morphological structure of intestinal villi in each group were observed and compared in detail under a light microscope. Four sections were taken from each intestinal tube, and 2–5 visual fields were selected from each section. For each field of view, the straight and well-stretched villi were chosen. The villi length and crypt depth were measured by Image Pro Plus pathological image analysis system under 400 times microscope, and the villus-height-to-crypt-depth ratio (VH/CD) was calculated.

Statistical analysis

The experimental data are all expressed in the form of the mean ± SD and SPSS 13.0 software is used for analysis and processing. Differences between groups were analyzed using a one-way ANOVA variance test and unpaired two-tailed Student's t-test. $P<0.05$ was considered as a significant difference.

RESULTS

Changes in clinical symptoms of piglets

After the TGEV challenge, piglets in group 1 and group 2 showed anorexia symptoms after TGEV inoculation, and typical clinical signs such as diarrhea and vomiting appeared about 12 h later. During the whole experiment, one piglet died in group 1 on day 6 and no deaths occurred in the other groups. Piglets of group 1 and group 2 were accompanied by varying degrees of diarrhea on day 3 of the experiment. From day 4 today 10, compared to group 1, average time of relieving diarrhea had a tendency to improve by rEGF treatment in group 2 ($P=0.08$) (Table I).

Growth performance of early-weaned piglets

The effects of rEGF treatments on growth performance of early-weaned piglets are shown in Table II. Group 2 and group 4 had higher final BW compared with group 1 and group 3, respectively. Overall ADG (from 4 to 17 d), ADG of the first week and second week in group 4 were greater than in group 3($P<0.05$). ADG of second week in group 2 had tendency to increase ($P=0.07$) compared with group 1. There was no significant difference in final weight between the groups.

Histopathological examination of piglets

All the piglets were dissected after the experiment. The necropsy showed that the main pathological changes of the piglets in group 1 and group 2 were enteritis. The small intestine of the piglets in group 1 can be seen to be dilated and filled with fluid and gas, and the wall of the small intestine becomes thinner. The symptoms of enteritis in group 2 were significantly improved by rEGF treatment. The necropsy showed that there were no obvious pathological changes in group 4 and just only slight flatulence in group 3 (Fig. 1).

Through observation under a microscope for H & E staining, the small intestinal mucosal layer of the piglet in group 1 is atrophied, ruptured and blunt villi of intestinal wall become thin, and intestinal mucosal congestion, intestinal dilatation and infiltration of inflammatory cells lymphocytes.

The intestinal inflammation in group 2 was significantly improved. The small intestinal villi did not show noticeable pathological changes such as small intestinal villi atrophy, rupture become blunt.

The villi of the small intestine in group 4 were slightly ruptured. The piglets of group 3 showed intact villi and intestinal wall (Figs. 2 and 3).

Table I. Effect of rEGF on the incidence of TGEV-infected pigs.

Groups	Diarrhea frequency (%)	Diarrhea rate (%)	Diarrhea index	Mortality (%)	Average time of relieving diarrhea (h)
Group 1	7.67±3.14	60.50±16.73	1.12±0.38	16.67	79.2±17.06
Group 2	6.67±4.36	52.67±11.98	1.06±0.36	0	64±8.39

Group 1, positive control group, without oral administration of rEGF but with oral administration of 2×105.5TCID50 for TGEV challenge on day 1.
Group 2, oral administration of 2×105.5 TCID50 for TGEV challenge, 72 h after that rEGF was orally administered for 14 days (400μg/day/pig).
Group 3, negative control group with oral administration of normal saline for 14 days without TGEV challenge.
Group 4, oral administration of rEFG (400μg/day/pig) for 14 days.

Table II. Effects of diets supplemented with rEGF on growth performance of piglets.

	Group 1	Group 2	Group 3	Group 4
Day 4 weight (kg)	2.70±0.21	2.73±0.19	3.00±0.14	3.00±0.15
Final weight (kg)	4.56±0.29	4.75±0.20	4.60±0.29	5.02±0.30
ADG (g/day)				
4~10d	88.71±19.54	98.93±13.22	63.93±16.55	95.00±13.03*
11~17d	171.00±28.65	198.21±16.55	163.21±12.46	186.96±12.33*
4~17d	123.86±10.91	143.57±13.30	113.57±17.72	141.55±13.83*

For details of groups, see Table I.

Table III. Effects of diets supplemented with rEGF on intestinal development of piglets.

	Group 1	Group 2	Group 3	Group 4
Jejunum				
Villus height (μm)	491.72±32.93	503.48±32.31	500.31±83.48	569.91±55.71*
Crypt depth (μm)	152.76±14.60	158.29±10.59	173.09±26.70	151.18±12.49
VH/CD	3.31±0.19	3.28±0.27	2.89±0.30	3.76±0.52*
Ileum				
Villus height (μm)	439.43±53.94	433.55±51.20	437.48±14.01	482.86±33.89*
Crypt depth (μm)	127.11±29.70	130.65±3.47	141.27±29.22	148.57±16.70
VH/CD	3.68±0.83	3.46±0.40	3.29±0.61	3.36±0.49

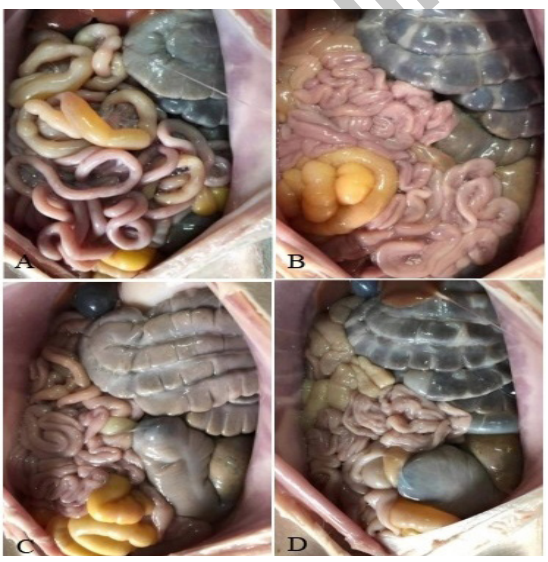

Fig. 1. Tissue observation of intestine necropsy of piglets in each group at the end of the experiment. A, group1; B, group 2; C, group 3; D, group 4.

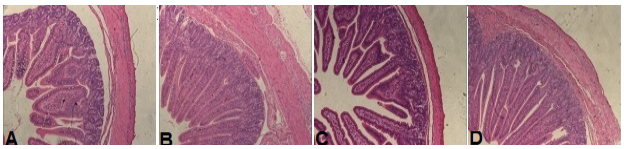

Fig. 2. Histopathological observation of the jejunum of piglets (H & E staining, ×200). A, group1; B, group 2; C, group 3; D, group 4.

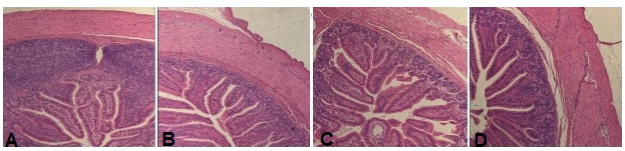

Fig. 3. Histopathological observation of the ileum of piglets (H & E staining, ×200). A, group1; B, group 2; C, group 3; D, group 4.

Intestinal development

As shown in Table III, the length of villi in the jejunum and ileum ($P < 0.05$) was longer in the rEGF

treatment group 4 than in control groups 3. The VH/CD of the jejunum of group 4 treated with rEGF significantly improved also ($P < 0.05$).

The results showed that compared with group 1, the length of villi, the depth of crypt and the VH/CD of ileum and jejunum in group 2 were not significantly different.

DISCUSSION

EGF which was a mitotic peptide was one of the most abundant milk-derived growth factors. It involves many physiological processes, including cell proliferation and differentiation, small intestine maturation, and maintenance of epithelial cell homeostasis (Tang *et al*., 2016). EGF concentration is 124 μg/L in sow milk (Cheung *et al*., 2009). Deficiency of milk-derived growth factors such as EGF is usually accompanied by abnormal intestinal epithelial growth, villi atrophy and related morbidity, intestinal barrier damage, and inflammatory bowel disease (Smith *et al*., 2010). In this experiment, it was found that the piglets had slight flatulence in the intestines and slight breakage of the intestinal villi in group 3. Exogenous supplementation of rEGF improved the symptoms of intestinal pathology in group 4.

TGEV is the pathogen of swine intestinal infectious diseases. It occured frequently in newborn pigs under two weeks of age. Piglets infected with TGEV could cause a variety of symptoms, including intestinal inflammation, atrophy of villi, diarrhea, malabsorption, and growth retardation (Saif *et al*., 2010; Pensaert and Martelli, 2016). In this experiment, it was found that after the TGEV challenge, piglets showed anorexia symptoms and typical clinical signs such as diarrhea and vomiting appeared. The necropsy showed that the main pathological changes of the piglets were enteritis. The small intestine could be seen to rupture and blunt villi of villi, and the intestinal wall becomes thin, and intestinal mucosal congestion, intestinal dilatation and infiltration of inflammatory cells lymphocytes. Other studies had found similar symptoms (Xia *et al*., 2018; Gao *et al*., 2021).

EGF is heat-stable, acid-stable and resistant to protease digestion (Tang *et al*., 2016). This characteristics allowed EGF to reach the small intestine through the harsh environment of the stomach, where EGF could bind to its receptors and exert numerous nutritional effects. Studies have found that about 70% of EGF can reach the middle of the small intestine with intact protein and exert its effect on promoting cell proliferation (Goldman *et al*., 1978; Bedford *et al*., 2015). This experiment results show that exogenous rEGF supplementation can alleviate intestinal inflammation and improve atrophy, rupture and blunt of villi by TGEV challenge. Exogenous rEGF supplementation could alleviates diarrhea and trend to improves growth performance of piglets. The research had shown that supplementation of EGF can promotes recovery from atrophic enteritis in PEDV-infected piglets (Jung *et al*., 2008). Previous studies have shown that EGF supplementation in piglet diets has also been shown to facilitate intestinal recovery from rotavirus infection (Donovan *et al*., 1994).

The results of our study indicate that exogenous rEGF supplementation could promote intestinal development, improve the growth performance of piglets. Previous studies also found EGF can improve intestinal morphology, up-regulate intestinal structural integrity protein, increase the expression of nutrient transporter, and increase the activities of digestive enzymes, so as to promote intestinal development and nutrient digestibility of early weaned piglets (Xu *et al*., 2015; Wang *et al*., 2019).

EGF plays a biological activity by binding to EGFR, participating in small intestinal maturation and maintenance of epithelial cell homeostasis in the small intestine. EGF could induce autosphosphorylation of RTK, and then activate a variety of signal transduction pathways to regulate intestinal development, TJS expression and mucin secretion (Duh *et al*., 2000; Tang *et al*., 2016). The recent studies had shown that adding EGF to diet can promote the proliferation of intestinal epithelial cells. The increase in the number of ki67-positive cells as a reliable cell proliferation marker confirms this points (Smith *et al*., 2010). The experiments had shown that EGF stimulates the increase of Ki67 protein and mRNA expression (Zhao *et al*., 2017). Cell proliferation is one of the foundations of intestinal growth and development of weaned piglets (Cheng *et al*., 2017). The results of this study found that EGF increase the length of villi and VH/CD and improve intestinal damage, which may be related to the enhanced proliferation of intestinal epithelial cells. The recent study found that dietary EGF stimulated goblet, enteroendocrine and Paneth cell differentiation in piglets. EGF improved piglet intestinal morphology through stimulating the proliferation and differ entiation of enterocytes (Wang *et al*., 2019, 2020).

EGF could promote the absorption of Na+-dependent glutamine, which could increase the transport activity, mRNA expression and protein expression of the glutamine carrier ASCT2 in intestinal epithelial cells (Ray *et al*., 2005). EGF also could increase the concentration of SGLT-1 on the brush border and promote the transport of glucose in the intestinal lumen to epithelial cells (Yang *et al*., 2011). This also explained that exogenous rEGF supplementation could promote the growth performance of weaned piglets in this experiment. Therefore, EGF could be used as a functional dietary component to promote

healthy intestinal morphology and digestive function.

In summary, the results obtained from the in vivo study indicated that oral administration of recombinant rEGF alleviate intestinal damage caused by TGEV challenge, promotes the health of the intestine and growth rate in piglets. The rEGF can be used as adjuvant therapy for piglet viral diarrhea or weaning stress.

DECLARATIONS

Acknowledgements

We thanked these institutions for their support of this experiment. This experiment was supported by the programs from the 2019 Wuhu Science and Technology Plan Project (Grant No. 2019yf44), Natural Science Foundation of Anhui Province (Grant No. 1808085MC75), Natural Science Foundation of Zhejiang Province (Grant No. LY19C010006).

Statement of conflict of interest

The authors have declared no conflict of interest.

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