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Effects of Glucagon-like Peptide-2 on Morphology, Proliferation and Enzyme Activity of Intestinal Enterocyte Cells of Weaned Piglets *In vitro**

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ABSTRACT : This study was conducted according to the single-factor design principle to investigate *in vitro* the effects of different glucagon-like peptide-2 (GLP-2) concentrations (0, 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L) on the morphology, proliferation and enzyme activity of intestinal enterocyte cells of 28-d-old weaned piglets. These cells were primary cultured in 4 pieces of 24-well cell culture plate. After having been grown for 48 h in culture media with hGLP-2, the ileal enterocyte cells of 28-d-old weaned piglets exhibited the typical characteristics of simple columnar epithelium. Compared with the control groups, the quantities of treated cells significantly increased ($p<0.05$) and their corresponding absorption values in 540 nm (MTT OD) also significantly increased ($p<0.01$). Likewise, lactic acid concentration, total protein content and protein retention significantly increased ($p<0.05$). Na^+, K^+-ATP enzyme activity was more active ($p<0.05$), although the activity of alkaline phosphatase, lactic acid dehydrogenase and creatine phosphokinase in culture media significantly decreased ($p<0.01$). To summarize, the results indicated that GLP-2 *in vitro* is capable of promoting the proliferation of intestinal enterocyte cells of 28-d weaned piglets, restraining their apoptosis and maintaining the integrity of their morphology. (**Key Words :** Glucagon-like Peptide-2, Weaned Piglets, Intestinal Enterocyte Cell, Cell Proliferation, Enzyme Activity)

INTRODUCTION

Studies in the past decades have proven that intestinal hormones play an important role in the activities of digestive enzymes (Koldovsky et al., 1991; Washizawa et al., 2004; Kelly et al., 2005). Especially in recent years, remarkable advances based on studies of glucagon-like peptide-2 (GLP-2) in the pig have deepened our understanding of the effects of intestinal hormones on the structure and function of the gastrointestinal tract.

GLP-2 is a newly-discovered hormone which is uniquely trophic for the intestine. So far, studies of the effects of GLP-2 on experimental subjects such as mice or rats and the effects of GLP-2 on fetal pigs (Benjamin et al., 2000; Martin et al., 2004), and on neonatal piglets (Burrin et al., 2000; Petersen et al., 2003; Stephens et al., 2006) have obtained similar results. These results show that GLP-2 activates relevant protein kinases by being integrated with GLP-2R and then regulates cAMP activities accordingly, to promote the proliferation of intestinal enterocyte cells and to restrain their apoptosis. The studies have proven that GLP-2 affects the height of intestinal villi and their enzymatic activities, stimulates intestinal nutrient absorption, and enhances intestinal epithelial barrier function (Estall and Drucker, 2003). However, it is not clear yet whether GLP-2 has the same effects on the intestinal growth and the intestinal adaptation of weaned piglets. Petersen et al. (2002) and Nielsen et al. (2003) reported that GLP-2 was unable to increase the intestine weight of 31-day-old weaned piglets. Instead, it reduced maltase activity and decreased glucose absorption. Does this mean that the effects of GLP-2 on the intestinal enterocyte cells of weaned piglets are not consistent with the effects of GLP-2 on fetal pigs, neonatal piglets and other experimental subjects, such as mice and rats? Sigalet et al. (2006) showed that GLP-2, independent of enteral feeding, stimulated a classical pattern of intestinal adaptation in the terminal ileum following resection. Therefore, study of the biological function of GLP-2 in a simulated growth environment is beneficial to assess the nutritional function

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of GLP-2 on the gastrointestinal tract.

The objectives of this experiment were to explore the operative properties of GLP-2 function on the intestinal enterocyte cells of weaned piglets by studying the effects of GLP-2 on the morphology, proliferation and enzyme activity of the intestinal enterocyte cells of 28-d-old weaned piglets. The study aimed to provide the experimental basis for further research on how GLP-2 regulates the growth of the intestines of the weaned piglet, and hence deepen our understanding of the growth regularities of the intestinal mucous membrane in fetal, neonatal and weaned piglets.

MATERIALS AND METHODS

Experimental design

The experiment was designed according to the single-factor design principle with a control group and five treatment groups (four replicates per treatment and four wells per replicate). The supplements of GLP-2 in the culture media in the five treatments were 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} and 1×10^{-7} mol/L according to the dose-dependency relationship between GLP-2 and cAMP (Munroe et al., 1999; Velazquez et al., 2003; Estall et al., 2004).

Experimental materials and procedures

The glucagon-like peptide-2 used in this experiment was h[Gly2]-GLP-2, a human GLP-2 analogue. (Purity≥95%; Phoenix Pharmaceuticals Inc., USA). Insulin, CollagenaseXI, Dispasel and MTT were from Sigma Chemical Company (St. Louis, MO, USA). D-glucitol, DMEM (High Glucose) Culture Media, Fetal Bovine Serum, and Transferrin were from Gibco Inc, USA.

All animal procedures were approved and performed under the guideline of the SiChuan Agricultural University Animal Care and Use Committee. The experimental subject, a 28-d-old healthy male weaned piglet (Landrace/Large White), weighing 7.02 kg, was provided by the Experimental Farm, Animal Nutrition Institution of SiChuan Agricultural University. Immediately after slaughter, the ileum of the piglet was removed and fixed in pre-cooled cleaning solution (4°C) in an aseptic state. The methods of digestion and segregation of intestinal enterocyte cells were based on those employed by Evans et al. (1992) and Han et al. (2009). The extracted samples were suspended in the culture media, and mixed evenly for later use.

The extracted intestinal cells, suspended in the culture media, were inoculated onto 4 pieces of 24-well cell culture plates covered with collagen at a concentration of 1×10^5 cells per ml, and statically cultured in the culture box (BB5060UV, Germany) with 5% CO_2 concentration at 37°C for 48 h. Then, the original culture media were replaced with new culture media with the different h[Gly2]-GLP-2 concentrations in each treatment group. After 48-h cultivation, the samples were collected and analyzed.

Cell photograph and sample analysis

After being cultured with *h*GLP-2 for 48 h, the cells from 4 wells in each treatment group were counted. First, the cells were removed from the culture media and washed by cell washing solution 2 or 3 times. Then, 500 μl of digestive juice (300 U/ml XI collagenase and 0.1 mg/ml I neutral protease) was used to digest cells for 30 minutes in each well to form a cell suspension. After being mixed evenly, 20 μl of the cell suspension was taken by pipette and dropped onto a blood-cell counter for cell counting by light microscope (Olympus Imaging Corp, Japan).

During the process of cell cultivation, every 24 h cell morphology was observed and photographed using the transmission phase contrast (PH) method through an A200 inverted microscope (Carl Zeiss Microimaging GmbH, Germany). After being cultured with hGLP-2 for 48 h, the cells from 4 wells in each treatment group were collected for MTT staining. The absorption values (MTT OD) were determined at 540 nm using a Wellscan MK3 enzyme immunoassay instrument (ThermoFisher Scientific Inc., USA), which reflected the number of viable cells and the activity of cell metabolism.

Subsequently cultured with hGLP-2 for an additional 48 h, the culture supernatant was collected from each treatment group to determine the activity of creatine kinase (CK), alkaline phosphatase (AKP), and lactate dehydrogenase (LDH), along with total protein content and lactic acid (LD) content in culture media. At the same time, lysate was collected from each treatment group to determine the protein retention and Na^+, K^+-ATP enzyme activity. The CK, AKP, LDH, LD and Na^+, K^+-ATP enzyme levels were determined using a DV-800 nucleic acid and protein analysis kit (Beckman Coulter Inc., USA). All these collected data were used to evaluate whether cell proliferation, morphology and function were normal (He et al., 1993).

Statistical analysis

All data were subjected to single factor ANOVA procedures using the GLM models. The statistical model included the effects of different concentrations of hGLP-2. Statistical significance of differences was assessed for cell survival, proliferation, apoptosis and integrity by using the least significant difference (LSD) test and the Duncan method. Results were expressed as least-square means and standard error of the mean (SEM). An alpha level of $p<0.05$ was used as the criterion for statistical significance; a level

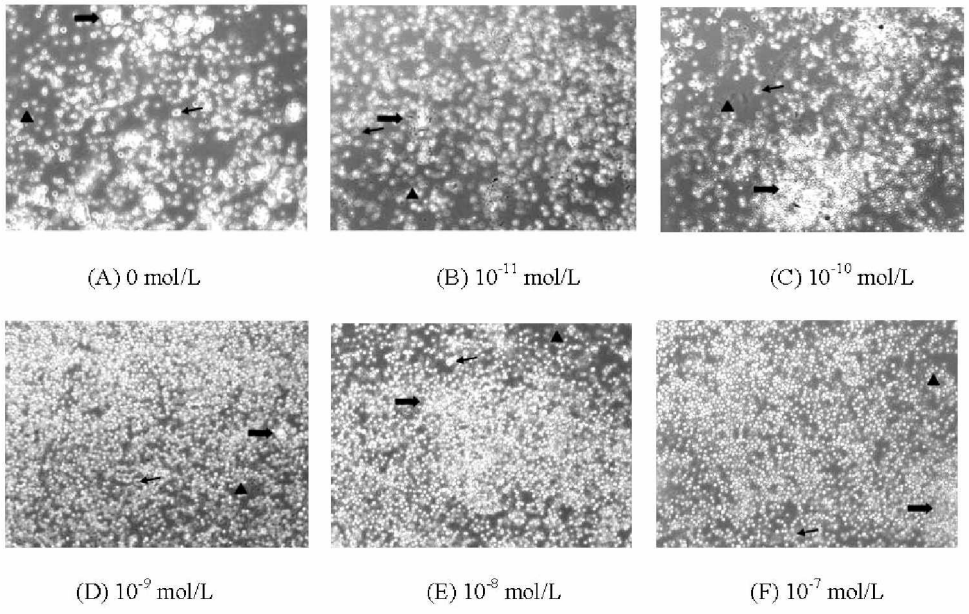

Figure 1. Effects of different hGLP-2 concentration on the proliferation and morphous of the ileal mucous membrane enterocyte cell of the weaned piglet. ⟶ irregular cell, ➡ exfoliated cells, ▲ apoptotic cell. In Figure 1, there are pictures of cells, photographed through a A200 inverted microscope by the method of transmission phase contrast (PH) (×200), when the cells were cultured in culture media with different GLP-2 concentrations for 47 h. From these pictures, compared with the controls, the number of cells increased, the form and structure of the cells were regular and well developed with clear edges, and the number of apoptotic and impaired cells decreased. In D and E, cells grew into pavement-like shape which had the typical characteristics of simple columnar epithelium.

of $p<0.01$ was used as the criterion for extreme significance.

RESULTS AND DISCUSSION

Observation of cell morphology

The effects of hGLP-2 in different concentrations on weaned piglet ileal mucous membrane enterocyte cell proliferation and morphology after supplements of hGLP-2 for 47 h are depicted in Figure 1. Compared with the control, the cell density in treatment groups increased, which implied that the number of cells increased; the form and structure of the cells were regular and well developed with clear edges; the number of apoptotic cells and impaired cells decreased in the culture media with hGLP-2. In the treatments with higher supplement of GLP-2, cells grew into pavement-like shape characteristics of simple columnar epithelium, which may account for the change in the height of intestinal villi and the depth of crypt during weaning (Kim et al., 2007).

Effects of GLP-2 on cell survival and proliferation

Effects of the culture media with different GLP-2 concentrations on cell proliferation of the intestinal enterocyte cells of weaned piglets are shown in Table 1. The results showed that after being cultured in hGLP-2 culture media for 48 h, the quantity of cells in the 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7} treatment groups was significantly greater than in the control group($p<0.01$); there was less difference between the treatment groups with hGLP-2 concentrations of 1×10^{-8} and 1×10^{-7}. The absorption values (MTT OD) were significantly higher than in the control group ($p<0.01$), and greatest for the culture media with a GLP-2 concentration of 1×10^{-7} mol/L; the concentration of protein retained in each treatment group was significantly higher

Table 1. Effects of different hGLP-2 concentration on the survival and proliferation of the ileal mucous membrane enterocyte cell of weaned piglets

hGLP-2 (mol/L)	0 (control)	1×10^{-11}	1×10^{-10}	1×10^{-9}	1×10^{-8}	1×10^{-7}
Cell quantity (10^6 cells/ml)	1.25 ± 0.05^{aA}	1.36 ± 0.08^{bA}	1.40 ± 0.07^{bA}	1.67 ± 0.05^{cB}	1.85 ± 0.06^{dC}	1.91 ± 0.12^{dC}
MTT OD value	0.251 ± 0.016^{aA}	0.338 ± 0.012^{bB}	0.361 ± 0.013^{eB}	0.484 ± 0.012^{dC}	0.590 ± 0.011^{eD}	0.643 ± 0.015^{fE}
Lactic acid (mmol/L)	0.625 ± 0.011^{aA}	0.692 ± 0.021^{bA}	0.931 ± 0.011^{cB}	1.065 ± 0.021^{dC}	1.135 ± 0.026^{eD}	1.143 ± 0.016^{eD}
Protein retention (mg/ml)	0.601 ± 0.062^{aA}	0.748 ± 0.083^{bA}	1.056 ± 0.075^{cB}	1.354 ± 0.038^{dC}	1.349 ± 0.022^{dC}	1.812 ± 0.061^{eD}
Total protein content (mg/ml)	1.207 ± 0.075^{aA}	1.321 ± 0.021^{aA}	1.663 ± 0.057^{bB}	1.990 ± 0.109^{cC}	2.135 ± 0.042^{dCD}	2.267 ± 0.091^{eD}

In the same row, values with different small superscripts differ significantly ($p<0.05$); different capital letter superscripts indicate extremely significant difference ($p<0.01$).

Table 2. Effects of different hGLP-2 concentration on the apoptosis and the integrality of ileal mucous membrane enterocyte cell of weaned piglet

hGLP-2 (mol/L)	0 (control)	1×10^{-11}	1×10^{-10}	1×10^{-9}	1×10^{-8}	1×10^{-7}
AKP activity[a] (U/g·prot)	1.460 ±0.066 ^{dC}	0.780 ±0.050 ^{cB}	0.694 ±0.033 ^{bB}	0.702 ±0.030 ^{bB}	0.417 ±0.056 ^{aA}	0.478 ±0.030 ^{aA}
LDH activity[b] (U/L)	205.1 ±9.050^{aA}	169.9 ±4.608^{bB}	162.9 ±7.981^{bB}	133.7 ±6.280^{cC}	118.7 ±1.742^{dD}	105.6 ±3.017^{eD}
CK activity[c] (U/ml)	1.323 ±0.093^{aA}	0.168 ±0.010^{bB}	0.124 ±0.013^{bcBC}	0.077 ±0.011^{cCD}	0.019 ±0.003^{dD}	0.018 ±0.008^{dD}
Na^+, K^+-ATP activity[d] (U/mg·pro)	0.0535 ±0.0101^{aA}	0.0865 ±0.0079^{bcABC}	0.0783 ±0.0061^{bAB}	0.1077 ±0.0054^{cdBC}	0.0942 ±0.0105^{bcBC}	0.1186 ±0.0262^{dC}

In the same row, values with different small superscripts differ significantly ($p<0.05$); different capital letter superscripts indicate extremely significant difference ($p<0.01$).

[a] One unit will transfer 0.01 mol/L di-sodium phenyl phosphate to 1mg phenol at 37°C for 15 min; [b] One unit will transfer 1.0 μmole of pyruvate at pH 7.5 to lactic acid per minute at 25°C; [c] One unit will transfer 1.0 μmole of phosphate from creatine phosphate to ADP per minute at 37°C; [d] One unit will transfer ATP from 1 mg protein to 1.0 μmole phosphate per hour at 37°C.

than in the control group ($p<0.05$), and at its highest in the culture media with a GLP-2 concentration of 1×10^{-7} mol/L; the total protein content and lactic acid (LD) content in treatment group media were significantly higher than in the control group ($p<0.05$), and there was less difference between the treatment groups with hGLP-2 concentrations of 1×10^{-8} and 1×10^{-7} mol/L.

The results of this experiment showed that cell quantity, and especially living cell quantity, significantly increased and the activity of cell metabolism grew more intense with increased levels of GLP-2. The cell quantity and the total protein content corresponded to the state of cell proliferation. The protein retention and lactic acid (LD) content in medium and the absorption values (MTT OD) correlated with the activity of cell metabolism and the number of living cells, and also indirectly reflected the state of cell proliferation.

The experimental results showed that GLP-2 promoted the *in vitro* proliferation of intestinal enterocyte cells of 28-d-old weaned piglets. This result accorded with the reports of Benjamin et al. (2000) and Martin et al. (2004), but differed somewhat from Petersen et al. (2002) and Sangild et al. (2007) who reported that GLP-2 had limited efficacy in promoting proliferation of intestinal enterocyte cells of piglets. Some possible reasons for differences in experimental results can be inferred: i) the effective dosage of GLP-2 on piglets at different stages of development is uncertain (Petersen et al., 2001; Burrin et al., 2003; Pedersen et al., 2008); ii) there were differences between dipeptidylpe-peptidase IV (DPP-IV) activity in weaned piglets and in culture media (Hartmann et al., 2000); iii) morphological and functional changes of intestines resulting from intestinal adaptation in weaned piglets do not match with GLP-2 secretion level and its operating effects (Petersen et al., 2002; Cottrell et al., 2006); iv) GLP-2R activity and abundance are different in piglets at different stages of development (Munroe et al., 1999; Estall et al., 2004; Pedersen et al., 2008). Further studies need to be conducted to establish the decisive factor or factors.

Effects of GLP-2 on cell apoptosis and integrality

Effects of different GLP-2 concentrations on enzyme activity related to the integrity and morphology of ileal enterocyte cells of weaned piglets are shown in Table 2. When the cells were grown in GLP-2 culture media for 48 h, the activities of AKP, LDH and CK in culture media significantly decreased ($p<0.01$) with significant differences among treatment groups ($p<0.05$). The activities of AKP, CK and LDH gradually decreased with the increase of GLP-2 concentrations in culture media; the culture media with GLP-2 concentrations of 1×10^{-7} mol/L showed the strongest effect. The value of CK, AKP and LDH indicated the integrity of cell membranes and the impairment of cell morphology (He et al., 1993). Our results showed that GLP-2 reduced cell damage and protected the integrity of cell structure. This result was basically identical to the report by Petersen et al. (2001, 2002) and consistent with Estall et al. (2004).

Na^+, K^+-ATP enzyme activity in each treatment group was significantly more active than in the control group. With the increase of Na^+, K^+-ATP enzyme activity in lysate, caspase-3 activity decreased and hence inhibited cell apoptosis. So, our results showed GLP-2 inhibited apoptosis of intestinal enterocyte cells of weaned piglets. Boushey et al. (2001) also reported that culture media with h[Gly-2] GLP-2 reduced chemotherapy-associated mortality and enhanced cell survival rate of enterocyte cells of the mid-ileum and colon. Likewise, Burrin et al. (2000) reported that GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis, which accorded with the results of this experiment. Sigalet et al. (2006) showed that glucagon-like peptide-2 induced a specific pattern of adaptation in remnant jejunum. Therefore,

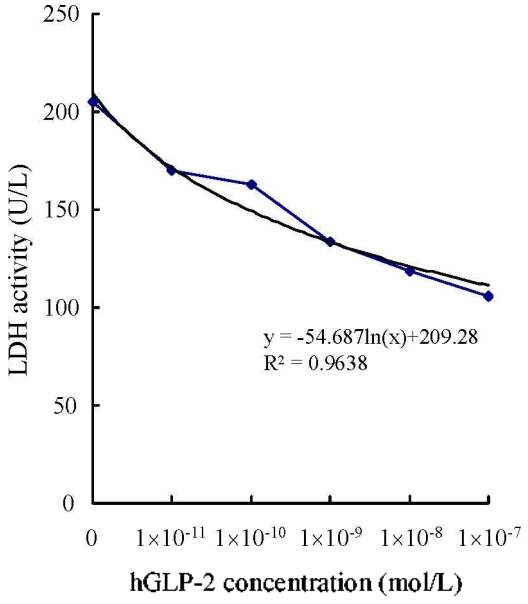

Figure 2. The relationship between LDH activity and hGLP-2 concentration.

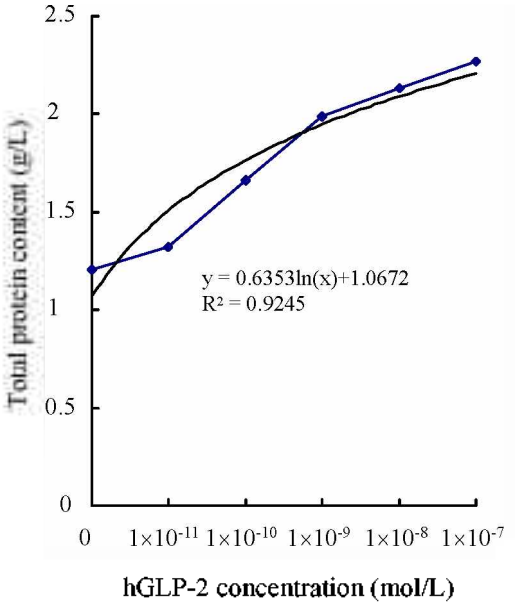

Figure 3. The relationship between total protein level and hGLP-2 concentration.

results of this experiment indicate that GLP-2 is capable of maintaining the integrity of cell morphology and inhibiting cell apoptosis. And Yang et al.(2007) reported when weaned piglets maintained a higher feed intake, there were no change in the height of intestinal villi and the depth of crypt even if they fed on soy-protein-based diets. So, further experimental corroboration is also needed to determine whether results obtained from our cell models can be applied to weaned piglets *in situ*.

Dose-dependence in activating cell survival and proliferation

Burrin et al. (2005) reported that GLP-2 dose-dependently activates intestinal cell survival and proliferation in neonatal piglets, but does the dose-dependent relationship apply to the *in vitro* intestinal cells of 28-d-old weaned piglets?

The experimental results showed that GLP-2 within the concentration range of 10^{-11}-10^{-7} mol/L promoted the proliferation of the intestinal enterocyte cells of weaned piglets and maintained the integrity of cell morphology. Figure 2 shows the regression curves for the relationship between LDH activity and GLP-2 concentration, and Figure 3 shows the relationship between total protein level and GLP-2 concentration. Within the concentration range of GLP-2 used in this experiment, there exists a definite dose-dependent relationship between GLP-2 concentration level and the proliferation of intestinal enterocyte cells of weaned piglets. There is also a direct correlation between GLP-2 concentration level and maintenance of the integrity of cell morphology. According to the statistical analysis, there were smaller differences in the number of cells, lactic acid (LD) in medium, CK activity and AKP activity as GLP-2 concentration increased in the range of 1×10^{-8} to 1×10^{-7} mol/L. Thus it is inferred that there exists an effective GLP-2 dosage between the concentration of 1×10^{-8} and 1×10^{-7} mol/L which will have an optimal effect on the intestinal enterocyte cells of weaned piglets. With GLP-2 concentrations below the 1×10^{-8} dosage, the effects of GLP-2 increase with the increase of GLP-2 concentration; with the GLP-2 concentration above this dosage, the effects of GLP-2 stabilize. This ratiocination on the effective dosage of GLP-2 was consistent with the dose-effect of GLP-2 on the cAMP activity reported by Munroe et al. (1999) and the dose-effect of GLP-2 on GLP-2 receptor reported by Petersen et al. (2001).

Na^+, K^+-ATP enzyme activity in all treatment groups was more active than in the control groups, but its effects did not increase with the increase of GLP-2 dosage, which indicated that there existed differences between the effects of GLP-2 on inhibiting cell apoptosis and on promoting cell proliferation. Burrin et al. (2003) reported that when piglets suffered nutritional stress, the differences between pathological dosage and physiological dosage of GLP-2 on the intestines of piglets resulted in different effects of GLP-2 on intestinal growth, development and adaptation of piglets at different stages of development. All the above observations suggest that appropriate dosages of supplements of GLP-2 are one of the key parameters influencing results.

Furthermore, physiological and biochemical parameters related to the proliferation, apoptosis, structure and function

of enterocyte cells vary significantly when piglets are at different developmental stages or under pathological conditions (e.g. weaning stress). So, it is not easy to accurately assess the biological action of GLP-2 outside carefully controlled conditions. The specific physiological and pathological conditions of the weaned piglet need to be taken into consideration to determine the optimal dosage of GLP-2 to maximize its effect on their enterocyte cells.

IMPLICATION

In summary, we isolated highly purified ileal enterocyte cells of a 28-d-old weaned piglet, and evaluated the *in vitro* effects of GLP-2 at different levels on the survival, proliferation and apoptosis of intestinal enterocyte cells in a primary culture. The results show that GLP-2 is capable *in vitro* of promoting the proliferation of intestinal enterocyte cells of a 28-d-old weaned piglet, inhibiting their apoptosis and maintaining the integrity of their morphology. However, further study is needed to confirm whether the effects obtained from the cell model are applicable to weaned piglets *in situ*.

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