In Vitro Isolation and Characterization of Broiler Embryo Duodenal Epithelial Cells and Establishment and Evaluation of a Primary Culture Absorption Model: Postprint

Authors: Zhang Shumin, Liao Xiudong, Lü Lin, Zhang Liyang, Luo Xugang

Date: 2018-12-24T00:00:00+00:00

Abstract

This study aimed to evaluate the effects of different seeding densities of duodenal epithelial cells on cell tight junction integrity and cell viability, in order to select the optimal cell seeding density for establishing an absorption model of broiler chicken embryo duodenal epithelial cells in primary culture. A single-factor completely randomized experimental design was employed, consisting of three groups: Group I, Group II, and Group III, with seeding densities of broiler chicken embryo duodenal epithelial cells at 2.90×10 , 6.25×10 , and 8.75×10 cells/mL, respectively, each with 6 replicates, and cultured for 4 days. The results showed: 1) Freshly isolated duodenal epithelial cell clusters were spherical with uniform size, suspended in the culture medium; after adherence, the cell clusters began to extend outward, gradually spreading into sheets, with clear intercellular boundaries, uniform adhesion, and monolayer cobblestone-like morphology. 2) Following alkaline phosphatase staining, the cytoplasm of duodenal epithelial cells in the positive experimental group was stained blue-black, while cells in the negative control group showed no staining. 3) At 48 and 72 h of cell culture, the transepithelial electrical resistance (TEER) values of cells in Groups I and II both met the experimental requirement of >300 $\Omega \cdot cm^2$, and the phenol red permeability rates both met the requirement of <5%; furthermore, the TEER values of Group I cells were significantly higher than those of Group II ($P<0.05$), while the phenol red permeability rates were significantly lower than those of Group II ($P>0.05$). 4) At 24 and 48 h of cell culture, the lactate dehydrogenase (LDH) activity in the culture medium of Group I cells was significantly lower than that of Groups II and III ($P<0.05$). These results indicate that at a cell seeding density of 2.90×10 cells/mL and a culture time of 48 h, the cells exhibited clear intercellular boundaries, good growth status, strong tight junction integrity, and optimal cell viability, thereby demonstrating

the successful establishment of a primary culture model of broiler chicken embryo duodenal epithelial cells for nutrient absorption studies, which provides an excellent experimental model for subsequent investigations into the absorption patterns and molecular mechanisms of duodenal epithelial cells.

Full Text

Isolation and Identification of Duodenal Epithelial Cells from Broiler Embryos In Vitro and Establishment and Evaluation of a Primary Cultured Absorption Model

Zhang Shumin, Liao Xiudong, Lü Lin, Zhang Liyang, Luo Xugang*

Mineral Nutrition Research Division, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

Abstract

This study aimed to evaluate the effects of different inoculation densities of duodenal epithelial cells on tight junction integrity and cell viability, and to determine the optimal cell seeding density for establishing an *in vitro* primary culture absorption model using duodenal epithelial cells from broiler embryos. A single-factor completely randomized design was employed with three groups: Group I, II, and III were seeded at densities of 2.90×10 , 6.25×10 , and 8.75×10 cells/mL, respectively, with six replicates per group. The culture period lasted four days. The results showed: (1) Freshly isolated duodenal epithelial cell clusters were spherical and uniform in size, suspended in culture medium. After adherence, the cell clusters began to spread outward, gradually forming a monolayer with clear boundaries between cells and uniform attachment, exhibiting a typical "cobblestone" appearance. (2) Following alkaline phosphatase staining, the cytoplasm of duodenal epithelial cells in the positive experimental group was stained blue-black, while cells in the negative control group remained unstained. (3) At 48 and 72 h of culture, the transepithelial electrical resistance (TEER) values of Groups I and II exceeded the required threshold of 300 $\Omega\cdot cm^2$, and phenol red permeability rates were below the required 5% threshold. Moreover, TEER values in Group I were significantly higher than those in Group II ($P<0.05$), while phenol red permeability rates were significantly lower ($P<0.05$). (4) At 24 and 48 h of culture, lactate dehydrogenase (LDH) activity in the culture medium of Group I was significantly lower than that of Groups II and III ($P<0.05$). These results indicate that at a seeding density of 2.90×10 cells/mL and a culture duration of 48 h, the cells exhibited clear intercellular boundaries, robust growth, strong tight junctions, and optimal viability. This demonstrates the successful establishment of a primary culture model of duodenal epithelial cells from broiler embryos for nutrient absorption studies, providing an excellent experimental model for subsequent investigations into absorption patterns and molecular mechanisms in duodenal epithelial cells.

Keywords: duodenal epithelial cells; primary culture; cell isolation; transepithelial electrical resistance

Introduction

The small intestine is a vital digestive organ and the primary site for nutrient digestion and absorption in animals. Intestinal epithelial cells (IECs), as one of the fastest-renewing cell types *in vivo*, serve as mediators between the intestinal internal and external environments and constitute an important component of the body' s immune barrier. They perform critical physiological functions including digestion, absorption, secretion, and immunity. Under appropriate conditions, small intestinal epithelial cells can be isolated and cultured *in vitro*. *In vitro* culture of small intestinal epithelial cells provides a simple and rapid means to study intestinal biological functions, nutrient absorption mechanisms and their regulation, as well as IEC proliferation and differentiation, offering an ideal *in vitro* model for investigating the effects of nutrients and other external factors on intestinal epithelium.

Generally, embryonic tissues survive and grow more readily *in vitro* than adult tissues, making successful cell culture more achievable. Most cells in adult tissues are non-proliferating differentiated cells whose growth is severely inhibited or completely arrested during differentiation. Moreover, the success rate of *in vitro* culture decreases with advancing age of adult tissue, likely related to the differentiation level of embryonic stem cells. Consequently, cell proliferation from adult tissue sources is extremely difficult, and the culture period is shortened. Previous studies have successfully established primary culture models of small intestinal epithelial cells using chicken embryos at various developmental stages: 9-day-old embryos, 17-day-old embryos, 18-day-old embryos, embryos older than 18 days approaching hatching, and 20-day-old embryos. These studies predominantly selected embryos around 18 days of age; therefore, this experiment utilized duodenal epithelial cells from 18-day-old broiler embryos for primary culture.

However, reports on using primary cultured intestinal epithelial cells for nutrient absorption studies are scarce. Numerous studies have employed Caco-2 cell cultures as absorption models and evaluated the tight junction integrity of their monolayers. Previous work in our laboratory by Liu et al. using *in situ* ligated intestinal perfusion revealed that the duodenum is the primary site of phosphorus absorption in broilers, a finding subsequently confirmed by Hu Yixin using natural feeding methods. Building upon these studies, the use of *in vitro* primary cultured duodenal epithelial cells can further elucidate the transport and absorption patterns of phosphorus and their molecular mechanisms in broiler embryos. Therefore, this study adopted methods used for evaluating Caco-2 cell monolayer tight junctions to assess the effects of different inoculation densities and culture durations on tight junction integrity and cell viability, aiming to

identify the optimal seeding density for establishing an *in vitro* primary culture absorption model using duodenal epithelial cells from broiler embryos and ultimately construct a nutrient absorption model for primary cultured duodenal epithelial cells.

Materials and Methods

1.1 Experimental Design A single-factor completely randomized design was employed with three groups. Duodenal epithelial cells were seeded at densities of 2.90×10 (Group I), 6.25×10 (Group II), and 8.75×10 cells/mL (Group III). Each group had six replicates, with each replicate consisting of two culture wells. The culture period lasted four days.

1.3 Reagents and Instruments **Major reagents:** Dulbecco' s phosphate-buffered saline (D-PBS), DMEM/F12 medium, fetal bovine serum, and trypan blue were purchased from Gibco; penicillin-streptomycin and heparin sodium from Sigma; epidermal growth factor and MTT assay kit from Shanghai Fange Biochemical; BCIP/NBT alkaline phosphatase color development kit from Beyotime Biotechnology; streptavidin-biotin complex (SABC) immunohistochemistry kit, DAB color development kit, and 4% paraformaldehyde from Wuhan Boster Bioengineering; lactate dehydrogenase (LDH) assay kit from Nanjing Jiancheng Bioengineering Institute; cytokeratin 18 antibody from Biosynthesis; insulin from Maichen Technology (Beijing); and other reagents were domestic analytical grade.

Major instruments: 150- and 500-mesh cell strainers (Beijing Solarbio Technology); ultrapure water purification system (Milli-Q, Biocell, USA); autoclave (TOMY SS-325); benchtop centrifuge (Eppendorf); 0.22 m filters (Millipore, USA); CO incubator (Thermo, USA); inverted phase-contrast microscope (Olympus, Japan); super-clean workbench (YJ-1450 medical purification workbench, Suzhou Changqiao Purification Equipment); Millicell-ERS voltohmmeter (Millipore, USA); 50 mL centrifuge tubes, 60 mm culture dishes, 6-well plates, and 96-well plates (Corning, USA); and full-wavelength microplate reader (Biotek, USA).

1.4 Digestion and Isolation of Duodenal Epithelial Cells from AA Broiler Embryos Referencing the methods of Guo et al. and Qin et al., 18-day-old AA broiler embryos were obtained. The eggshells were wiped with 75% ethanol, air-dried, and the duodenum was aseptically removed. Intestinal tissue was placed in ice-cold D-PBS containing 2% penicillin-streptomycin. Pancreatic tissue and fascia were removed with forceps, and the tissue was washed 2–3 times with D-PBS containing 2% penicillin-streptomycin. The duodenal tissue was cut into 0.5 cm segments and transferred to a 50 mL centrifuge tube containing 30 mL DMEM/F12 medium. The tube was vigorously shaken several times, the wash solution was aspirated, and this process was repeated 2–3 times. The intestinal segments were then minced into fragments smaller than 1 mm^3,

allowed to settle for several minutes, and washed 3–4 times with DMEM/F12 wash solution until the supernatant became clear.

Ten volumes of 1 mg/mL collagenase I digestion solution were added to the pellet, and digestion was performed at 37°C with slow shaking (80 cycles/min) for 50 min. After digestion, the sample was centrifuged at 1,000×g for 8 min, and the supernatant containing the enzyme solution was discarded. The pellet was resuspended in 10 mL DMEM/F12 wash solution, gently pipetted, centrifuged at 1,000×g for 8 min, and the supernatant was discarded. This washing step was repeated 1–2 times. The pellet was resuspended in 10 mL DMEM/F12 attachment medium, gently pipetted 5–10 times, and filtered sequentially through 150- and 500-mesh cell strainers to remove tissue fragments and fibroblasts. Cell clusters retained on the 500-mesh strainer were rinsed off with DMEM/F12 attachment medium to prepare a cell suspension. Viability of duodenal epithelial cells was assessed using trypan blue staining.

1.5 Viability Assessment of Duodenal Epithelial Cells from AA Broiler Embryos Viability was determined using the trypan blue exclusion method described by Peng et al. Briefly, 200 L of cell suspension was mixed with an equal volume of 0.4% trypan blue solution. One drop of the mixture was placed on a hemocytometer, covered with a coverslip, and observed under an inverted phase-contrast microscope. Unstained cells (viable) and stained cells (non-viable) were counted separately. Viability was calculated as:

Viability (%) = 100 × (number of viable cells) / (number of viable cells + number of non-viable cells)

Cell suspensions with viability 95% were used for subsequent culture.

1.6 Culture of Duodenal Epithelial Cells from AA Broiler Embryos DMEM/F12 medium supplemented with 3% fetal bovine serum was used as the attachment medium. Duodenal epithelial cell clusters were resuspended to prepare a cell suspension. When viability reached 95%, cell density was adjusted according to the experimental design. Then, 1.5 mL of cell suspension was seeded into the upper chamber of Transwell 6-well plates, while 2.6 mL of cell-free DMEM/F12 medium was added to the lower chamber.

1.7 Alkaline Phosphatase Staining Identification of Duodenal Epithelial Cells Following the method of Hong et al., alkaline phosphatase staining was performed on primary cultured duodenal epithelial cells after 48 h of attachment. One 6-well plate was selected, culture medium was aspirated, and wells were washed twice with phosphate-buffered saline (PBS) to remove non-adherent cells and debris, followed by fixation with 4% paraformaldehyde for 30 min. After two PBS washes to remove fixative, cells were ready for identification. Three wells served as the positive experimental group and three as the negative control group. Staining was performed according to the BCIP/NBT alkaline phosphatase color development kit instructions.

1.8 Sample Collection and Preparation At 24, 48, 72, and 96 h of culture, 0.5 mL of culture medium from the upper chamber was immediately collected into sterile 1.5 mL centrifuge tubes. Media from two wells were pooled to constitute one replicate, with six replicates collected total. Samples were centrifuged at 3,500 r/min for 10 min at 4°C, and the supernatant was stored at -20°C for LDH activity determination. Remaining culture medium was aspirated from both upper and lower chambers, and phenol red permeability was measured using phenol red-containing and phenol red-free media. Samples were stored at -20°C for phenol red permeability analysis. Transepithelial electrical resistance (TEER) values were measured for each well using a Millicell-ERS voltohmmeter. Samples were collected continuously for four days.

1.9 Sample Analysis At 24, 48, 72, and 96 h of culture, 0.5 mL of medium from upper and lower chambers was transferred to test tubes. Five milliliters of 1 mol/L NaOH solution was added for color development, mixed well, and absorbance (OD) was measured at 560 nm using 1 mol/L NaOH as blank. Phenol red permeability was calculated as:

Phenol red permeability (%) = 100 × OD_lower / (OD_upper + OD_lower)

TEER values were calculated as:

TEER value ($\Omega \cdot cm^2$) = (value of cell growth well - value of blank well) × membrane area

where the membrane area of Transwell 6-well plate inserts is 4.67 cm^2.

Frozen culture medium samples from six replicates were thawed, and LDH activity was determined according to the kit instructions from Nanjing Jiancheng Bioengineering Institute.

1.10 Statistical Analysis Data were analyzed using the General Linear Model (GLM) procedure in SAS 9.0 software for one-way ANOVA, with each replicate sample as an experimental unit. Significant differences identified by ANOVA were further analyzed using the Least Significant Difference (LSD) method to compare means. Differences were considered significant at $P<0.05$.

Results

2.1 Isolation and Observation of Primary Cultured Duodenal Epithelial Cells from AA Broiler Embryos Microscopic observation revealed numerous duodenal epithelial cell clusters and minimal single epithelial cells. Freshly isolated duodenal epithelial cell clusters were spherical and uniform in size, suspended in culture medium [Figure 1: see original paper]-A. Intercellular spaces were larger in Group I, moderately spaced in Group II, and markedly reduced in Group III. Viability assessment via trypan blue staining showed all three groups achieved >95% viability, indicating that the isolated duodenal epithelial cells were suitable for primary culture model establishment.

2.2 Morphological Observation of Primary Cultured Duodenal Epithelial Cells from AA Broiler Embryos Cell growth status and morphological characteristics were observed every 24 h under an inverted microscope. At 24 h, duodenal epithelial cell clusters had settled to the plate bottom and begun attaching, with attached clusters spreading outward to form sheets. Group I showed good attachment and spreading with relatively flat cell surfaces, while Group II exhibited some incompletely spread clusters, and Group III displayed numerous unspread clusters [Figure 1: see original paper]-B. At 48 h, attached cells in Groups I and II showed clear boundaries, tight arrangement, and monolayer distribution, predominantly cobblestone-shaped, oval, and tightly packed, displaying characteristic "cobblestone" growth. Group III cells showed blurred boundaries with numerous non-adherent dead cell clusters attached [Figure 1: see original paper]-C. At 72 h, Groups I and II maintained relatively clear boundaries and tight arrangement with persistent "cobblestone" morphology, whereas Group III cells appeared blurred with obvious detachment [Figure 1: see original paper]-D. At 96 h, all groups showed some cell detachment and blurred morphology, with proliferation essentially ceased. Groups I and II exhibited blurred boundaries and partial detachment, while Group III showed marked detachment leaving numerous vacuoles [Figure 1: see original paper]-E. These results indicate that Groups I and II at 48 and 72 h had clear boundaries, good growth status, and uniform attachment, meeting the conditions for experimental treatment.

2.3 Alkaline Phosphatase Staining Identification of Duodenal Epithelial Cells Using Group I cells as an example, alkaline phosphatase staining was performed after 48 h of attachment following the method of Hong et al. [Figure 2: see original paper] shows that the cytoplasm of duodenal epithelial cells in the positive experimental group stained blue-black (staining rate >95%), while fibroblasts, blood cells, and other contaminating cells remained unstained. The negative control group showed no staining in duodenal epithelial cells. These results confirm that the obtained cells were fully differentiated and functionally intact duodenal epithelial cells.

2.4 TEER Values of Duodenal Epithelial Cells TEER values were measured at 24, 48, 72, and 96 h of culture [Figure 3: see original paper]. From the perspective of same cell density across different culture times, TEER values in all three groups differed significantly over time ($P<0.05$). Groups I and II showed similar trends, with Group I TEER values at 48 and 72 h significantly higher than at 24 and 96 h ($P<0.05$) and remaining constant between 48 and 72 h ($P>0.05$). Group II TEER values at 48 h were significantly higher than at 24 and 96 h ($P<0.05$). Group III TEER values peaked at 24 h and decreased significantly at 48, 72, and 96 h ($P<0.05$).

From the perspective of different cell densities at the same culture time, Group I TEER values at 24 h were significantly lower than Groups II and III ($P<0.05$). At 48, 72, and 96 h, Group I TEER values were significantly higher than

Group II, which were significantly higher than Group III ($P<0.05$). Numerically, Groups I and II reached maximum TEER values at 48 h that persisted through 72 h, with Group I values exceeding those of Group II.

2.5 Phenol Red Permeability of Duodenal Epithelial Cells Phenol red permeability was measured at 24, 48, 72, and 96 h of culture [Figure 4: see original paper]. From the same cell density perspective, phenol red permeability differed significantly over time in all groups ($P<0.05$). Groups I and II showed similar trends, with permeability decreasing significantly from 24 to 72 h ($P<0.05$) and increasing significantly at 96 h ($P<0.05$). Group III showed low permeability at 24 and 48 h, increasing significantly at 72 and 96 h ($P<0.05$).

From the different cell density perspective at the same culture time, Groups I and II showed significantly lower phenol red permeability than Group III at 48, 72, and 96 h ($P<0.05$).

2.6 LDH Activity in Duodenal Epithelial Cell Culture Medium LDH activity in culture medium was measured at 24, 48, 72, and 96 h [Figure 5: see original paper]. From the same cell density perspective, LDH activity differed significantly over time in all groups ($P<0.05$). Group I showed no significant changes from 24 to 72 h ($P>0.05$) but increased significantly at 96 h ($P<0.05$). Group II maintained low activity at 24 and 48 h, increasing significantly at 72 and 96 h ($P<0.05$). Group III showed progressive significant increases from 24 to 72 h ($P<0.05$), decreasing significantly at 96 h ($P<0.05$).

From the different cell density perspective, LDH activity differed significantly among groups at 24, 48, and 72 h ($P<0.05$). At 24 and 48 h, Group I activity was significantly lower than Group II, which was significantly lower than Group III ($P<0.05$). At 72 h, Group I was significantly lower than Group III, which was significantly lower than Group II ($P<0.05$). At 96 h, Groups I and II showed significantly higher activity than Group III ($P<0.05$). Numerically, Group I maintained low LDH activity at 24, 48, and 72 h, with a marked increase only at 96 h.

Discussion

Common cell purification methods include differential adhesion, mechanical scraping, and cell straining. Differential adhesion exploits the faster attachment of fibroblasts compared to intestinal epithelial cells. Most fibroblasts complete attachment within 1–2 h, while most intestinal epithelial cells fail to attach or attach unstably during this period, detaching with gentle agitation. Collecting the non-adherent cell suspension containing numerous intestinal epithelial cell clusters and reseeding onto new plates can enrich the epithelial cell population. Many studies have used this method to remove fibroblasts and obtain intestinal epithelial cell clusters. However, this approach results in loss of some attached intestinal epithelial cell clusters, reducing their density in the suspension, and fibroblasts cannot be completely eliminated. In primary culture, epithelial and

fibroblastic cells often grow in mixed, patchy distributions on the culture surface. Mechanical scraping can remove unwanted fibroblast areas while preserving epithelial regions, but this method often causes contamination and uneven cell distribution.

Sequential filtration through 150–500-mesh cell strainers can effectively remove large undigested tissue fragments (using 150-mesh) and smaller-diameter blood cells, fibroblasts, and single intestinal epithelial cells (using 500-mesh), while retaining larger intestinal epithelial cell clusters on the 500-mesh strainer. These retained clusters are rinsed off with culture medium to prepare a cell suspension for seeding. This method effectively eliminates large numbers of fibroblasts and poorly adherent single epithelial cells, leaving mostly intestinal epithelial cell clusters in suspension. This study employed sequential 150–500-mesh filtration to remove fibroblasts and obtain numerous duodenal epithelial cell clusters. Morphological observation and alkaline phosphatase staining confirmed minimal contamination from fibroblasts, blood cells, or other cell types, indicating thorough purification.

Current methods for identifying small intestinal epithelial cells include morphological assessment, alkaline phosphatase staining, and immunocytochemistry. In this study, duodenal epithelial cells proliferated rapidly, forming colonies within 2–3 days that grew as monolayers with a "cobblestone" pattern, clear boundaries, and flattened polygonal, cobblestone, or oval shapes—characteristic features of primary cultured intestinal epithelial cells. However, microscopic observation alone can only identify cell-like morphology, requiring further confirmation. Alkaline phosphatase is a marker enzyme of small intestinal epithelial cell microvilli, concentrated on the cell surface and involved in intracellular and extracellular digestion. Successfully stained cells are confirmed as duodenal epithelial cells. Hong et al., Yang et al., and Jia et al. successfully identified duodenal epithelial cells using alkaline phosphatase staining.

Tight junction integrity of Caco-2 cell monolayers is typically evaluated using TEER values and apparent permeability coefficients of markers. TEER measurement is a simple yet authoritative evaluation method, with TEER values strongly correlating with tight junction formation. When TEER exceeds a certain level (typically $>300\ \Omega \cdot cm^2$), it indicates good tight junction integrity, with higher values reflecting stronger connections. Phenol red, a water-soluble small molecule not metabolized by intestinal mucosa or transported across cell membranes, can indirectly reflect paracellular permeability and serve as a tight junction marker. Therefore, this study used phenol red as a probe to evaluate tight junction integrity in duodenal epithelial cells cultured on Transwell inserts, with phenol red permeability $<5\%$ serving as the monolayer formation criterion. At 24, 48, and 72 h, Groups I and II exceeded the $300\ \Omega \cdot cm^2$ TEER threshold and the 5% phenol red permeability threshold. Both groups reached maximum TEER at 48 h that persisted through 72 h, with Group I showing higher TEER and lower phenol red permeability than Group II, indicating stronger tight junction integrity in Group I.

LDH is a stably expressed intracellular enzyme that leaks out when cell membranes are damaged; thus, LDH activity in culture medium reflects the degree of cell injury. Qin et al. used LDH activity in primary cultured cardiomyocyte medium as a marker of heat stress-induced injury. Similarly, this study measured LDH activity to indirectly assess membrane integrity and cell viability. Group I maintained low LDH activity at 24, 48, and 72 h, with a significant increase only at 96 h, while the other groups showed marked elevation by 72 h, indicating that Group I cells exhibited the best growth status and optimal viability at 24, 48, and 72 h.

Conclusion

Based on comprehensive evaluation of four indicators—cell morphology, TEER values, phenol red permeability, and LDH activity—the optimal conditions were determined to be a seeding density of 2.90×10 cells/mL with 48 h of culture. Under these conditions, cells showed good growth status, clear intercellular boundaries, strong tight junction integrity, and optimal viability. Therefore, the nutrient absorption model using primary cultured duodenal epithelial cells from broiler embryos was successfully established, and this seeding density (2.90×10 cells/mL) can be used for subsequent studies on absorption patterns and molecular mechanisms in duodenal epithelial cells.

References

[1] Oswald I P. Role of intestinal epithelial cells in the innate immune defence of the pig intestine[J]. Veterinary Research, 2006, 37(3): 359-368.

[2] Hong Z M, Jia Y J, Qu M R, et al. Isolation and primary culture of chick embryo small intestinal epithelial cells[J]. Acta Agriculturae Universitatis Jiangxiensis, 2011, 33(6): 1164-1170.

[3] Zhang Z R. Culture cytology and cell culture technology[M]. Shanghai: Shanghai Scientific and Technical Publishers, 2004: 10-11.

[4] Li Y, Peng C Y, Liang R W, et al. Isolation, culture and identification of chicken small intestinal epithelial cells[J]. China Animal Husbandry and Veterinary Medicine, 2011, 38(2): 68-72.

[5] Guo S, Li C, Liu D, et al. Inflammatory responses to a *Clostridium perfringens* type A strain and -toxin in primary intestinal epithelial cells of chicken embryos[J]. Avian Pathology, 2015, 44(2): 81-91.

[6] Ma Y L, Xu Z R, Guo T, et al. Isolation and primary culture method of chicken intestinal epithelial cells[J]. Chinese Journal of Veterinary Science, 2007, 27(1): 74-76, 80.

[7] Gu S P, Wang M X, Zhao S F, et al. Study on purification methods for chicken embryo cecal epithelial cells *in vitro*[J]. Journal of Shanxi Agricultural University (Natural Science Edition), 2011, 31(4): 369-372.

[8] Derache C, Esnault E, Bousergent C, et al. Differential modulation of -defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chicken inbred lines[J]. Developmental and Comparative Immunology, 2009, 33(9): 959-966.

[9] Jia G D, Zhao Y B, Liu G Q, et al. Improved study on primary culture method of chicken intestinal epithelial cells[J]. China Poultry, 2012, 34(19): 25-28, 32.

[10] Jiang X H, Jia Y T, Yuan Y, et al. Application of Caco-2 cell model in studying absorption processes of oral drugs[J]. Chinese Pharmaceutical Journal, 2002, 37(5): 325-327.

[11] Lu Z L, Feng Y, Xu D S, et al. Application of Caco-2 cell model in studying absorption and mechanisms of traditional Chinese medicine[J]. Chinese Traditional and Herbal Drugs, 2006, 37(4): 616-619.

[12] Li X L, Xie J J, Lu L, et al. Kinetics of manganese transport and gene expressions of manganese transport carriers in Caco-2 cell monolayers[J]. Biometals, 2013, 26(6): 941-953.

[13] Tang J M, Lü L, Zhang L Y, et al. Effects of different seeding densities on differentiation and depolarization evaluation indices of Caco-2 cell absorption model[J]. Journal of Hebei Normal University of Science and Technology, 2016, 30(3): 66-70.

[14] Sun X, Luo Y P. Establishment and evaluation of Caco-2 cell monolayer model[J]. Drug Evaluation, 2015, 12(14): 33-35, 39.

[15] Xu L F, Zhang X J, Lin N, et al. Establishment of intestinal mucosal barrier model using Caco-2 cells and its application in permeability studies[J]. International Journal of Pediatrics, 2016, 43(3): 239-243.

[16] Liu S B, Li S F, Lu L, et al. Estimation of standardized phosphorus retention for corn, soybean meal, and corn-soybean meal diets in broilers[J]. Poultry Science, 2012, 91(8): 1879-1885.

[17] Hu Y X. Effects of dietary non-phytate phosphorus levels on phosphorus absorption in small intestine and expression of related phosphorus transporter genes in broilers[D]. Master' s thesis. Beijing: Chinese Academy of Agricultural Sciences, 2016.

[18] Qin S Z, Liao X D, Lu L, et al. Manganese enhances the expression of manganese superoxide dismutase in cultured primary chick embryonic myocardial cells[J]. Journal of Integrative Agriculture, 2017, 16(9): 2038-2046.

[19] Peng B, Wu J J, Li Y, et al. Methodological study on trypan blue exclusion test for adherent cells[J]. Acta Laser Biology Sinica, 2011, 20(2): 269-273.

[20] SAS Institute Inc. SAS/user' s guide: statistics. Version 9.0[M]. Cary, NC: SAS Institute Inc., 2003.

[21] Yang L, Ren C C, Li M J, et al. Preliminary study on differentiation of human amniotic mesenchymal cells into endometrial epithelial cells *in vitro*[C]//Proceedings of 2015 Henan Provincial Annual Meeting of Obstetrics and Gynecology. Zhengzhou: Henan Medical Association, Henan Physicians Association, 2015.

[22] Wu Z T, Fan R Y, Li S R, et al. Comparison of two methods for collecting intestinal exfoliated cells[C]//Proceedings of the 4th China Cancer Congress and 5th Cross-Strait Cancer Conference. Tianjin: Chinese Anti-Cancer Association, 2006.

[23] Zhan K, Zuo X X, Gong X X, et al. Isolation, culture, and identification of chick embryo small intestinal epithelial cells *in vitro*[J]. Chinese Journal of Animal Nutrition, 2015, 27(7): 2150-2156.

[24] Wang X Y, Liu F, Jiang D W, et al. Primary culture and identification of chicken intestinal epithelial cells[J]. Heilongjiang Animal Science and Veterinary Medicine, 2015(21): 10-12.

[25] Perreault N, Beaulieu J F. Use of the dissociating enzyme thermolysin to generate viable human normal intestinal epithelial cultures[J]. Experimental Cell Research, 1996, 224(2): 354-364.

[26] Yang W P, Gao F, Xu H T, et al. Study on primary culture of chicken intestinal epithelial cells *in vitro*[J]. Acta Agriculturae Jiangxiensis, 2007, 19(5): 113-115, 118.

[27] Incecayir T, Tsume Y, Amidon G L. Comparison of the permeability of metoprolol and labetalol in rat, mouse, and Caco-2 cells: use as a reference standard for BCS classification[J]. Molecular Pharmaceutics, 2013, 10(3): 958-966.

[28] Mao X Q, Jia X F. Establishment and evaluation of Caco-2 cell monolayer model[J]. Sichuan Medical Journal, 2013, 34(1): 7-10.

[29] Sun M J, Sheng X, Hu Y Q. Establishment and validation of Caco-2 cell monolayer model[J]. Chinese Pharmaceutical Journal, 2006, 41(18): 1431-1434.

[30] Jia X Y, Zhao X L, Wei M J. Evaluation of Caco-2 cell models at different seeding densities[J]. Chinese Journal of Medicinal Guide, 2010, 12(2): 289-290.

[31] Mohrmann I, Mohrmann M, Biber J, et al. Sodium-dependent transport of Pi by an established intestinal epithelial (CaCo-2)[J]. American Journal of Physiology, 1986, 250(3 Pt 1): G323-G330.

[32] Song X L, Qian L J, Li F Z. Study on injury effects of heat stress on neonatal rat cardiomyocytes[J]. Chinese Journal of Applied Physiology, 2000, 16(3): 227-230.

[33] Cheng Y B, Wang Y J, Zhang S C, et al. Response of porcine hepatocytes in primary culture to plasma from severe viral hepatitis patients[J]. World Journal of Gastroenterology, 2005, 11(48): 7585-7590.

[34] Liu Y L, Dang Y N, Duan Y L, et al. Primary culture of chicken hepatocytes and effects of folic acid on expression of lipid metabolism-related genes[J]. Scientia Agricultura Sinica, 2017, 50(21): 4205-4211.

Note: Figure translations are in progress. See original paper for figures.