Improved Low-Temperature Chelation Method for Isolating Rabbit Small Intestinal Villus and Crypt Cells and Evaluation of Isolation Efficiency: Postprint

Authors: Shen Xuemei, Li Jing, Zhang Gang, Cui Hongxiao, Liu Lihui, Yao Junhu, Xu Xiurong

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Abstract

This study investigated the isolation method for rabbit small intestinal villus and crypt cells to provide an in vitro model for in-depth research on small intestinal epithelial structure and function. New Zealand white rabbits were used as experimental material to isolate small intestinal villus and crypt cells under different chelating agent concentrations (5, 10, 15, 20, 25 mmol/L EDTA) and chelation temperatures (4, 25 °C), and the yield, morphology, cell viability, and integrity of the isolated cell populations were examined. The results showed that: 1) The villus and crypt cells isolated under various chelation conditions exhibited intact morphology, with intact genomic DNA and total RNA; 2) Higher chelation temperatures and greater EDTA concentrations yielded higher cell enrichment rates, but also stronger cytotoxic effects; under conditions of 4 °C and 5 mmol/L EDTA, the enrichment rates for villus and crypt cells were 7.75% and 1.01%, respectively, with relative integrity rates of villus and relative viability rates of crypt cells being 91.67% and 93.48%, respectively; under conditions of 25 °C and 25 mmol/L EDTA, the enrichment rates for villus and crypt cells reached as high as 17.89% and 4.99%, respectively, but the relative integrity rates of villus and relative viability rates of crypt cells were only 4.25% and 5.17%; 3) Comprehensive analysis determined that the optimal isolation conditions were 4 °C and 10 mmol/L EDTA; 4) Under optimal conditions, the relative expression levels of lysozyme and -defensin in the isolated crypt enrichments were extremely significantly higher than those in villus enrichments ($P<0.01$), indicating high cell purity; moreover, after 9 h of culture, the relative expression levels of lysozyme and -defensin in crypt cells showed no significant changes ($P>0.05$), indicating that the cells maintained high viability. Therefore, the modified low-temperature chelation method is suitable for efficient isolation

of rabbit small intestinal villus and crypt cells.

Full Text

Separation of Rabbit Intestinal Villus and Crypt Cells by an Improved Low-Temperature Chelating Method and Evaluation of Separation Efficacy

SHEN Xuemei, LI Jing, ZHANG Gang, CUI Hongxiao, LIU Lihui, YAO Junhu, XU Xiurong*

(College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China)

Abstract: This study investigated a method for separating rabbit intestinal villus and crypt cells to provide an in vitro model for 深入研究小肠上皮结构与功能. Using New Zealand white rabbits as experimental material, intestinal villus and crypt cells were isolated under various chelating agent concentrations (5, 10, 15, 20, and 25 mmol/L EDTA) and chelating temperatures (4 °C and 25 °C). The yield, morphology, cell viability, and integrity of the isolated cell populations were evaluated. The results demonstrated: (1) Under all chelating conditions, the separated villus and crypt cells exhibited intact morphology with complete genomic DNA and total RNA; (2) Higher chelating temperatures and EDTA concentrations increased cell enrichment rates but also intensified cytotoxic effects. At 4 °C with 5 mmol/L EDTA, the enrichment rates for villus and crypt cells were 7.75% and 1.01%, respectively, with relative villus integrity and crypt cell viability rates of 91.67% and 93.48%. In contrast, at 25 °C with 25 mmol/L EDTA, enrichment rates reached 17.89% and 4.99%, but relative villus integrity and crypt cell viability rates dropped to only 4.25% and 5.17%; (3) Comprehensive analysis identified 4 °C with 10 mmol/L EDTA as the optimal separation condition; (4) Under these optimal conditions, the relative expression levels of lysozyme and -defensin in crypt enrichments were extremely significantly higher than in villus enrichments ($P<0.01$), indicating high cell purity. Furthermore, after 9 h of culture, the relative expression levels of lysozyme and -defensin in crypt cells showed no significant change ($P>0.05$), demonstrating that the cells maintained high viability. These findings indicate that the improved low-temperature chelating method is suitable for efficient separation of rabbit intestinal villus and crypt cells.

Keywords: crypt; villus; intestinal epithelial cells; chelating agents; rabbits

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The small intestine serves as a critical site for digestion and absorption, with its functions closely related to intestinal epithelial cells. The intestinal lumen is lined with a simple columnar epithelium that folds systematically into villi and crypts. The leaf-like structures protruding into the intestinal lumen are villi, while the finger-like invaginations into the lamina propria form crypts [**?**].

Villi and crypts differ in cellular composition and physiological functions, and their isolated cultures can be used to investigate distinct biological questions. Villus culture is valuable for studying intestinal nutrient absorption and transport [**?**], whereas crypt culture is essential for investigating intestinal stem cell physiology and Paneth cell immune functions [**?**]. Current methods for isolating small intestinal villus and crypt cells primarily include mechanical separation, chelation, and enzymatic digestion [**?**], with chelation being the most widely applied approach. However, conventional chelation methods suffer from prolonged separation times, poor separation of villi and crypts, and low cell viability.

Pothier et al. [**?**] used a salt solution containing 1.5 mmol/L ethylenediaminetetraacetic acid (EDTA) to incubate intestinal tissue at 37 °C, but the resulting villus and crypt cells were heavily contaminated with each other, yielding low enrichment rates. Bjerknes et al. [**?**] increased EDTA concentration to 30 mmol/L, which shortened separation time and improved enrichment rates but caused substantial villus damage. Ayabe et al. [**?**] reduced the separation temperature to room temperature, which improved crypt integrity but failed to enrich villus cells. Flint et al. [**?**] replaced EDTA with dithiothreitol (DTT) and further lowered the temperature to 4 °C, obtaining more intact crypt cell clusters but with significant villus damage. Fuller et al. [**?**] found that reducing EDTA concentration to 3 mmol/L combined with 2% sorbitol effectively decreased cellular damage but required longer separation times. These studies all used mice or rats as experimental material, and no reports exist on separating rabbit small intestinal villus and crypt cells. Moreover, different researchers have employed inconsistent chelating agent types, concentrations, and temperatures, and none have simultaneously achieved ideal results for enrichment rate, integrity, purity, and separation time. This study comprehensively analyzed the advantages and limitations of previous methods to investigate how different combinations of chelating agent concentration and temperature affect the separation of rabbit intestinal villus and crypt cells, aiming to identify optimal conditions that provide high enrichment rates, good cell viability, and short separation times for subsequent studies on rabbit small intestinal cell functions and nutrient absorption mechanisms.

1.1 Experimental Design

Healthy 14-day-old New Zealand white rabbits served as experimental material. A 2×5 completely randomized design was employed with two temperature levels: low temperature (LT) at 4 °C and room temperature (RT) at 25 °C; and five EDTA concentrations: 5, 10, 15, 20, and 25 mmol/L. Each treatment included three replicates, with each replicate using 5 g of small intestinal tissue.

1.2 Preparation of Chelating Agent

D-Hanks solution was prepared according to reference [**?**], supplemented with 0.5 mmol/L DTT, 100 U/mL penicillin, and 100 µg/mL streptomycin. The chelating agent contained: 1.5 mmol/L KCl, 96 mmol/L NaCl, 8 mmol/L KH PO , 5

mmol/L Na HPO , 44 mmol/L sucrose, 55 mmol/L sorbitol, and 5 mmol/L ethylene glycol-bis(-aminoethyl ether)-N,N,N' ,N' -tetraacetic acid (EGTA), with EDTA added at the five experimental concentrations.

1.3 Isolation of Small Intestinal Villus and Crypt Cells

Following euthanasia by air injection into the ear marginal vein, rabbits were immersed in 75% ethanol for 3 min. The entire small intestine was removed via laparotomy and flushed three times with ice-cold D-Hanks solution (containing DTT and antibiotics). The intestinal segments were everted and cut into 1–2 cm pieces, mucus was washed away, and 5 g of intestinal tissue was allocated to each treatment. Each treatment received 50 volumes (all subsequent steps used 50 volumes) of chelating agent with the corresponding EDTA concentration. LT and RT groups were processed at their respective temperatures: (1) Shaking at 150 r/min for 15 min followed by manual inversion shaking (~60 times/min) for 2 min, after which the suspension was discarded and fresh chelating agent was added. Shaking continued under the same conditions for 5–15 min until pinhead-sized cell clusters became visible in the suspension, which was then discarded; (2) Chelating agent was added to the remaining intestinal segments, manually inverted for 3 min, and the suspension centrifuged at 150×g for 3 min. The pellet represented the villus cell clusters; (3) Fresh chelating agent was added to the remaining segments, shaken at 150 r/min for 5 min, and the suspension discarded. This step was repeated three times to remove most villi from the intestinal wall; (4) Fresh chelating agent was added, and the suspension was gently inverted manually. The suspension was collected every 2 min, fresh chelating agent was added, and this process was repeated four times. The collected suspension was placed on ice for 2 min to sediment, the lower layer containing villus cell clusters was removed, and the suspension was centrifuged at 300×g for 5 min to remove single cells. The resulting pellet represented the crypt cell clusters.

1.4 Determination of Villus and Crypt Cell Enrichment Rates, Villus Relative Integrity Rate, and Crypt Cell Relative Viability Rate

Morphology and purity of villus and crypt cell clusters were observed under 4× optical microscopy. The criterion required no more than three crypt cell clusters in a single 4× field of villus enrichment, and no more than three villus fragments within crypt cell clusters [**?**]. The weight of enriched villus and crypt cells was measured for each treatment, with enrichment rate calculated as the percentage of cell weight relative to 5 g of small intestinal tissue. After diluting villus cell clusters to the same concentration, the number of broken villus fragments was counted in a single 4× field, with 30 fields counted per sample. Assuming equal total villus numbers per field, villus integrity rate was calculated as (1 - number of villus fragments/total villus number) for one 4× field. The relative integrity rate for each treatment was then determined by setting one treatment' s villus integrity rate as 100%. Crypt cell relative viability rate was determined using

trypan blue staining to count dead cells on individual crypt cell clusters, with 30 clusters counted per treatment, and calculated using the same algorithm as for villus relative integrity rate.

1.5 Extraction and Electrophoresis of Genomic DNA and Total RNA

Genomic DNA and total RNA were extracted from separated villus and crypt cells following kit protocols to assess nucleic acid integrity. Samples were analyzed by 1% agarose gel electrophoresis and imaged using a Biorad imaging system.

1.6 Culture of Crypt Cells

Enriched crypt cells were washed three times with DMEM/F-12 medium, and residual chelating agent was removed by low-speed centrifugation. Equal amounts of cells were seeded in 6-well culture plates and incubated at 37 °C with 5% CO in a humidified atmosphere. Cells were collected at 1, 3, 5, 7, and 9 h, washed three times with phosphate-buffered saline (PBS), and total RNA was extracted for reverse transcription and real-time quantitative PCR analysis of crypt cell-related gene expression changes.

1.7 Real-Time Quantitative PCR for Gene Expression Analysis

Real-time quantitative PCR was performed to detect expression levels of lysozyme (LYZ) and -defensin (DEFEN), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene [**?**]. Primers were designed based on conserved sequences of rabbit LYZ and DEFEN genes from GenBank. Primer sequences for each gene are listed in Table 1 . The reaction mixture contained: 10 µL SYBR Premix Ex Taq™ II (2×), 1 µL PCR forward primer (10 µmol/L), 1 µL PCR reverse primer (10 µmol/L), 1 µL cDNA, and ddH O to a final volume of 20 µL. The amplification program consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

1.8 Statistical Analysis

Data were analyzed using SPSS 20.0 software and presented as mean ± standard error. Differences were considered significant at $P<0.05$ and extremely significant at $P<0.01$.

2.1 Morphology and Purity of Villus and Crypt Cell Clusters

Relatively intact villus and crypt cell clusters were obtained under all EDTA concentrations and temperatures. During the procedure, the time required for pinhead-sized cell clusters to appear in step 2 (villus elution) was shorter in RT

groups (5–9 min) than in LT groups (7–15 min), with higher EDTA concentrations requiring less time. In step 4 (crypt sedimentation), the LT+5 mmol/L EDTA group showed extremely low crypt yield with substantial villus contamination, yielding only approximately 5 mg of crypt cell clusters after purification of the 2-min eluate—far less than the LT+10 mmol/L EDTA group. Under 4× optical microscopy, cell clusters from LT conditions exhibited more intact morphological structure than those from RT conditions, though with lower yields. The LT+5 mmol/L EDTA group showed the most intact cell cluster structure but the lowest cell concentration (Fig. 1 [Figure 1: see original paper]-A, B). As temperature and EDTA concentration increased, cell cluster structural integrity gradually decreased while cell concentration increased. Under RT+25 mmol/L EDTA conditions, despite the highest enrichment rate, villus enrichments contained numerous crypts, and crypt enrichments contained substantial villus fragments (Fig. 1-E, F). Visual assessment indicated that separation effects were superior under LT+10–15 mmol/L EDTA conditions (Fig. 1-C, D).

2.2 Effects of Separation Conditions on Villus Cell Enrichment Rate and Villus Relative Integrity Rate

As shown in Fig. 2 [Figure 2: see original paper], villus cell enrichment rates in RT groups were extremely significantly higher than those in LT groups at all EDTA concentrations ($P<0.01$), with enrichment rates increasing with EDTA concentration under both temperatures. When EDTA concentration was 15 mmol/L, villus relative integrity rates in LT groups were extremely significantly higher than in RT groups ($P<0.01$), decreasing as EDTA concentration increased. At concentrations 20 mmol/L, no significant difference in villus relative integrity rate was observed between RT and LT groups ($P>0.05$).

2.3 Effects of Separation Conditions on Crypt Cell Enrichment Rate and Relative Viability Rate

As shown in Fig. 3 [Figure 3: see original paper], crypt cell enrichment rates in RT groups were extremely significantly higher than those in LT groups at all EDTA concentrations ($P<0.01$), with enrichment rates increasing with EDTA concentration under both temperatures. When EDTA concentration was 10 mmol/L, crypt cell relative viability rates in LT groups were extremely significantly higher than in RT groups ($P<0.01$), gradually decreasing as EDTA concentration increased. At concentrations 15 mmol/L, both LT and RT groups exhibited extremely low crypt cell relative viability rates with no significant difference between groups ($P>0.05$). The LT+5–10 mmol/L EDTA and RT+5 mmol/L EDTA groups showed higher crypt cell relative viability rates, though the LT+5 mmol/L EDTA group had an extremely low crypt cell enrichment rate of only $(1.01\pm0.02)\%$.

2.4 Integrity Assessment of Genomic DNA and Total RNA from Villus and Crypt Cells

Genomic DNA and total RNA were extracted from enriched cell clusters and analyzed by 1% agarose gel electrophoresis to assess integrity, with results shown in Fig. 4 [Figure 4: see original paper] and Fig. 5 [Figure 5: see original paper]. Under all treatment conditions, cell genomic DNA remained intact without diffuse trailing, and total RNA showed distinct 28S, 18S, and 5S bands, indicating that cells remained viable during the separation process.

2.5 Differential Gene Expression in Villus and Crypt Cell Clusters

Based on the aforementioned results, villus and crypt cells separated under the optimal condition of LT+10 mmol/L EDTA were selected for detection of DEFEN and LYZ relative expression levels. As shown in Table 2 , the relative expression levels of DEFEN and LYZ in crypt enrichments were extremely significantly higher than those in villus enrichments ($P<0.01$).

2.6 Changes in Related Gene Expression During Crypt Cell Culture

Crypt cells isolated under LT+10 mmol/L EDTA conditions were cultured for 1, 3, 5, 7, and 9 h, and DEFEN and LYZ relative expression levels were measured (Fig. 6 [Figure 6: see original paper]). No significant changes in DEFEN and LYZ relative expression levels were observed during the 1–9 h period ($P>0.05$), though expression levels at 1 h showed a trend toward being lower than at 3–9 h ($0.05<P<0.10$).

In vitro culture of small intestinal epithelial cells represents an important tool for studying intestinal physiological functions. Compared with cell lines, primary cultured cells more closely resemble the physiological state *in vivo* and can better reflect authentic intestinal characteristics [**?**]. To more deeply investigate intestinal stem cell physiology and Paneth cell immune functions, various villus and crypt cell isolation methods have been reported, with EDTA and DTT being commonly used chelating agents [**?**]. EDTA chelates and reduces calcium and magnesium ions in tissue fluid, disrupting intercellular tight junctions and enabling longitudinal separation of intestinal villi and crypts under mechanical force [**?**]. DTT disrupts intermolecular disulfide bonds, rapidly removing mucus and adherent bacteria to achieve cell separation [**?**]. Chelating agents exert certain toxic effects on cells, with toxicity depending on agent type, concentration, temperature, and exposure time [**?**]. Separation conditions must be adjusted according to animal age, physiological status, intestinal segment size, and tight junction status.

Based on the mechanisms of each component, we modified the frequently used chelating agent formulation from Flint et al. [**?**] by adding 5 mmol/L EGTA in

addition to EDTA and incorporating 0.5 mmol/L DTT during intestinal segment washing to rapidly remove mucus. During separation, after eluting villus and crypt cells from small intestinal tissue using chelating agents, the chelating agent must be quickly removed to minimize exposure time and cytotoxicity. Purification of enrichments must account for the different sedimentation coefficients of villi and crypts. When centrifuging to purify villi, rotation speed and time must be determined based on the experimental animal size, requiring centrifugal force to be sufficiently low ($<300\times g$) to prevent structural damage to villi and avoid pelleting crypt cells with villus precipitates [**?**]. For larger animals, natural sedimentation can be used to obtain high-purity villi. During crypt elution, trace amounts of villi inevitably contaminate the preparation, necessitating determination of appropriate natural sedimentation time to purify crypt cells. Generally, a reasonable sedimentation time is when pinhead-sized cell clusters (villi) have completely settled to the bottom of the supernatant [**?**].

Our results demonstrate that although cell genomic DNA and total RNA remained intact under all separation conditions, significant differences in separation efficacy existed among conditions. Higher temperatures and greater chelating agent concentrations yielded more enrichments but compromised cell cluster purity, villus integrity, and crypt cell viability, even producing cytotoxic effects. First, while high EDTA concentration improved enrichment rates, it was the primary factor reducing crypt cell viability and villus integrity. This study showed that when EDTA concentration exceeded 15 mmol/L, its damaging effects on villus integrity and crypt cell viability far exceeded those of temperature. Since cell viability and tissue integrity are primary considerations for subsequent culture, EDTA concentration should not exceed 15 mmol/L during separation. Second, higher chelating temperatures increased enrichment rates but simultaneously reduced villus integrity and crypt cell viability. For villi, higher temperatures produced more villus fragments, with RT groups showing extremely significantly lower villus integrity than LT groups even at the lowest EDTA concentration. Therefore, low temperature should be selected for separation. For crypts, both LT groups with EDTA concentrations below 10 mmol/L and the RT+5 mmol/L EDTA group showed good cell viability rates, though the LT+5 mmol/L EDTA group had an extremely low crypt enrichment rate of only $(1.01\pm0.02)\%$, insufficient for subsequent experiments. Considering separation efficacy for both villus and crypt cells, LT+10 mmol/L EDTA was selected as the optimal condition.

In small intestinal epithelial cells, DEFEN and LYZ are predominantly expressed in Paneth cells of the crypts. Therefore, differential expression of these two genes in enrichments can effectively indicate cell purity and separation efficacy. Our results showed that relative expression levels of DEFEN and LYZ in isolated crypt enrichments were extremely significantly higher than in villus enrichments, demonstrating high purity of the separated villus and crypt cells and achieving the expected separation effect. We conducted short-term culture of crypt cells isolated under LT+10 mmol/L EDTA conditions to assess suitability for subsequent research. During 3–9 h of culture, no significant changes in DEFEN and

LYZ relative expression levels were observed, indicating stable cellular physiological status during culture. At 1 h, expression levels of LYZ and DEFEN showed a trend toward being lower than at 3–9 h, possibly due to nutrient deficiency and cellular starvation and stress caused by low temperature during separation, but expression recovered during subsequent culture. These results demonstrate that small intestinal villus and crypt cells isolated under LT+10 mmol/L EDTA conditions meet the requirements for subsequent studies.

Conclusions

1. Significant differences in separation efficacy for rabbit small intestinal villus and crypt cells were observed across the two temperatures and five EDTA concentrations. Low-temperature chelation yielded lower cell enrichment rates but higher cell viability, while higher EDTA concentrations increased cell enrichment rates but correspondingly decreased cell viability.
2. The optimal separation condition in this study was LT+10 mmol/L EDTA, which provided relatively high enrichment rates and purity for both villus and crypt cells, with intact morphology and high cell viability.
3. Crypt cells cultured at 37 °C with 5% CO in serum-free growth medium maintained high viability after 9 h, indicating that the separated villus and crypt cells can be used for studies on rabbit small intestinal physiological characteristics.

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