

Effects of Different Culture Methods on Growth and Keratin 18 Expression in Goat Rumen Epithelial Cells (Postprint)

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Abstract

This study aimed to investigate the effects of different culture methods on the growth and cytokeratin 18 (CK18) expression of goat rumen epithelial cells. Rumen epithelial tissues were collected from 42-day-old goats and cultured in vitro using enzymatic digestion and tissue explant methods. Cell morphology at primary and passage culture stages was observed under optical microscope, the growth curve of passage 5 goat rumen epithelial cells was detected, and cell immunofluorescence was used to identify goat rumen epithelial cells. The results showed that: 1) Primary cultured goat rumen epithelial cells obtained by digestion with 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid began to adhere and grow at 2 d, cells started to increase significantly at 5 d, and reached maximum cell number at 10 d. 2) Primary cultured goat rumen epithelial cells obtained by tissue explant method began to “migrate out” from the tissue explants at 4 d, cells started to increase significantly at 8 d, and reached maximum cell number at 14 d. 3) Immunofluorescence staining showed that CK18 was positively expressed in the cytoplasm of cells obtained by both methods, and cell purity of the latter was significantly higher than that of the former. 4) CK18 expression level in cells obtained by tissue explant method was significantly higher than that by enzymatic digestion method ($P < 0.05$). In conclusion, compared with enzymatic digestion method, tissue explant method could successfully obtain goat rumen epithelial cells with higher purity.

Full Text

Effects of Different Culture Methods on Growth and Keratin 18 Expression in Goat Ruminal Epithelial Cells

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Abstract

This study investigated the effects of different culture methods on the growth and cytokeratin 18 (CK18) expression of goat ruminal epithelial cells. Ruminal epithelial tissue was collected from 42-day-old goats and cultured *in vitro* using either enzymatic digestion or tissue explant methods. Cell morphology during primary and subculture stages was observed under optical microscopy, growth curves were established for passage 5 cells, and immunofluorescence staining was performed for cell identification. The results showed that: (1) Primary ruminal epithelial cells obtained by 0.25% trypsin + 0.02% EDTA digestion began adherent growth on day 2, showed significant proliferation on day 5, and reached maximum cell numbers on day 10. (2) Cells obtained via the tissue explant method began migrating from tissue fragments on day 4, showed marked increase on day 8, and peaked on day 14. (3) Immunofluorescence revealed CK18-positive expression in the cytoplasm of cells from both methods, with significantly higher purity in the tissue explant method. (4) CK18 expression was significantly higher in cells from the tissue explant method compared to enzymatic digestion ($P < 0.05$). In conclusion, the tissue explant method successfully yielded goat ruminal epithelial cells with higher purity compared to enzymatic digestion.

Keywords: goat; rumen; epithelial cell; primary culture; isolation; CK18

The rumen is the most important site for digestive metabolism and nutrient absorption in ruminants. The ruminal wall comprises four layers: mucosa, submucosa, muscularis, and serosa. The ruminal epithelium is stratified, consisting from mucosal to serosal surface of four layers: the keratinized cell layer, granular cell layer, spinous cell layer, and basal layer. Ruminal epithelium plays crucial physiological roles including nutrient absorption and transport, volatile fatty acid metabolism, and protection of the ruminal wall, making its growth, development, and physiological functions important research topics.

In recent years, increasing attention has been devoted to ruminal epithelium research, with applications in studying pathogenesis, pathology, and nutrition. For example, Stumpff et al. [?] found that ruminal epithelial cells maintained viability when exposed to high concentrations of *Bacillus thuringiensis* toxin Cry1Ab, independent of specific membrane receptor insertion. Oba et al. [?] reported that replacing corn starch with sugars affected ruminal fermentation and metabolic regulation of intracellular pH, with sucrose showing better effects than lactose. Wang et al. [?] demonstrated that *Bacillus subtilis* could induce -defensin-1 gene expression in sheep ruminal epithelial cells. However, com-

parative studies on ruminal epithelial cell culture methods remain unreported. Most studies have employed enzymatic digestion for isolation [?], with few using the tissue explant method, and no comparative research has been published. Therefore, this study aimed to establish a method for obtaining higher purity ruminal epithelial cells to provide a technical foundation for studying rumen-related functions in ruminants.

1.1 Samples and Reagents

Four 42-day-old Xiangdong black goats weighing (5.9 ± 0.8) kg were used. Animals were euthanized by jugular venous exsanguination, viscera were removed, and the rumen was isolated. After removing contents, the rumen was rinsed repeatedly with sterile physiological saline before sample collection.

Fetal bovine serum, DMEM/F12, trypsin, and penicillin-streptomycin were purchased from Gibco. Cytokeratin 18 (CK18) epithelial cell-specific primary antibody was from Abcam. Bicinchoninic acid (BCA) protein assay kit, immunostaining fixative, 4',6-diamidino-2-phenylindole (DAPI) staining solution, antifade mounting medium, SDS-PAGE loading buffer ($5\times$), SDS-PAGE gel preparation kit, Western blot electrophoresis buffer, Western blot transfer buffer, Western blot wash buffer, Western blot primary and secondary antibody diluents, and BeyoECL Plus (ultrasensitive ECL chemiluminescence kit) were from Beyotime Biotechnology. Gentamicin and amphotericin B were from BBI. Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies. Fluorescent-labeled donkey anti-mouse IgG secondary antibody [DyLight 488] was from Earthox. Triton X-100 and bovine serum albumin (BSA) were from Amresco. 100 mm culture dishes were from Corning.

1.2 Enzymatic Digestion Method for Ruminal Epithelial Cell Isolation

Ruminal tissue was collected, contents removed, and rinsed repeatedly with sterile physiological saline. The ruminal epithelium was bluntly dissected and washed with solution containing 500 g/mL penicillin-streptomycin, 5 g/mL amphotericin B, and gentamicin, then placed in DMEM/F12 with the same antibiotics for transport to the cell culture room. The tissue was washed 2-3 times with phosphate-buffered saline (PBS), minced into approximately 1 mm^3 fragments (appearing paste-like macroscopically), and washed once each with PBS and DMEM/F12. After removing supernatant, three volumes of digestion solution [0.25% trypsin:0.02% EDTA (1:1)] were added and digested at 37°C with agitation for 5 min.

Digestion was performed five times, with centrifugation at 1,000 rpm for 5 min after each round. The first two supernatants were discarded as they contained mostly keratinocytes. The remaining three digestions were pooled, and DMEM/F12 complete medium [containing 5% fetal bovine serum, 1% penicillin-streptomycin, 0.1 mg/mL gentamicin, 2.5 g/mL amphotericin B, 5 ng/mL epidermal growth factor (EGF), 1 g/mL insulin, 1 g/mL apo-transferrin, and 3.4

nmol/L sodium selenite] was added to resuspend cells. The suspension was filtered, and the filtrate was centrifuged at 1,000 rpm for 5 min. The pellet was washed with PBS and centrifuged again at 1,000 rpm for 5 min. Cells were seeded at 1×10^6 cells/mL in CO incubator (37°C, 5% CO₂).

1.3 Tissue Explant Method for Ruminal Epithelial Cell Culture

Minced epithelial tissue was washed once each with PBS and DMEM/F12. Tissue fragments were evenly distributed on the bottom of culture dishes. Dishes were inverted in CO incubator (37°C, 5% CO₂) for 2 h to prevent floating. After 2 mL DMEM/F12 complete medium was added, dishes were placed upright. The following day, medium was supplemented to 10 mL total in each 100 mm dish.

1.4 Cell Subculture

When cells reached 85% confluence, medium was removed and cells were washed with PBS (tissue fragments were gently dislodged with pipette tips in explant cultures). Cells were digested with 1 mL digestion solution at 37°C until they began rounding, then digestion was terminated with serum-containing medium. Detached cells were pipetted into suspension, centrifuged at 1,000 rpm for 5 min, and resuspended in fresh medium. To remove fibroblasts, differential adhesion was performed by plating resuspended cells in new dishes for 30 min at 37°C, then transferring the medium to another new dish for another 30 min. After two rounds of differential adhesion, cells were passaged at a 1:3 ratio.

1.5 Morphological Observation

Cell morphology and growth status were observed using a Leica DMI 3000B inverted microscope during primary and subculture stages.

1.6 Immunofluorescence Identification

Culture medium was carefully removed and cells were washed twice with PBS. Immunostaining fixative (4% paraformaldehyde) was added for 10 min at room temperature. After fixation, cells were washed twice with PBS, permeabilized with 0.1% Triton X-100 for 7-10 min, and washed again. Blocking was performed with 5% BSA for 1-2 h at room temperature. Primary antibody (diluted 1:200), which is epithelium-specific and suitable for goat samples, was added and incubated overnight at 4°C. After three PBS washes, secondary antibody was added and incubated for 1 h at room temperature in darkness, followed by three PBS washes. DAPI staining solution (500 L) was added for 1-3 min at room temperature, washed three times with PBS, and 500 L antifade mounting medium was added before fluorescence microscopy imaging.

Cell purity was calculated as: (number of green-fluorescent cells / number of blue-fluorescent cells) \times 100%.

1.7 Growth Activity Assay of Passaged Cells

Passage 5 cells from both methods in logarithmic growth phase were seeded in 96-well plates at 5×10^3 cells/well (four replicates per method). After 24 h culture, CCK-8 reagent was added and incubated for 4 h, then absorbance at 450 nm (OD₄₅₀) was measured. Growth curves were plotted with culture time on the x-axis and OD₄₅₀ on the y-axis.

1.8 Western Blot Analysis of CK18 Expression

When cells reached >85% confluence, they were washed with PBS and lysed with 400 μ L protein lysis buffer on ice for 30 min with vortexing every 10 min. Lysates were centrifuged at $12,000 \times g$ for 5 min at 4°C, and supernatants were collected. Protein concentration was determined using BCA assay. Loading buffer (5 \times) was added to achieve uniform concentration.

Electrophoresis was performed using 10% separating gel and 5% stacking gel at 80 V for stacking and 120 V for separation until bromophenol blue reached the gel bottom. Proteins were transferred at 200 mA for 1.5 h, then blocked with 5% BSA for 2 h on a shaker. Primary antibody was incubated overnight at 4°C, followed by secondary antibody for 1 h on a shaker. Bands were visualized using chemiluminescence reagent.

1.9 Statistical Analysis

Western blot bands were analyzed using Gel-Pro Analyzer 4 for grayscale values, which were then subjected to t-test analysis using SAS 8.2 software. Differences were considered significant at $P < 0.05$.

2.1 Morphology of Enzymatically Digested Ruminal Epithelial Cells

Inverted microscopy observation revealed that primary ruminal epithelial cells obtained by enzymatic digestion began adherent growth on day 2, with cells starting to cluster sporadically [Figure 1: see original paper]-A. By day 5, cell numbers increased significantly, though fibroblasts were present [Figure 1: see original paper]-B. After 10 days, most cells exhibited a cobblestone morphology [Figure 1: see original paper]-C, forming a confluent monolayer with maximum cell numbers nearly covering the entire dish. Passage 5 cells showed no obvious morphological changes compared to primary cells [Figure 1: see original paper]-D.

2.2 Morphology of Tissue Explant Cultured Ruminal Epithelial Cells

Inverted microscopy of tissue explant cultures showed ruminal epithelial cells beginning to migrate from tissue fragments on day 4 [Figure 2: see original paper]-A. Cell numbers increased markedly after day 8, with cells growing in clusters [Figure 2: see original paper]-B. By day 14, cells covered the entire dish, with some areas forming multiple layers [Figure 2: see original paper]-C.

Passage 5 cells maintained similar morphology to primary cells [Figure 2: see original paper]-D.

2.3 Identification of Goat Ruminal Epithelial Cells

Immunofluorescence identification of passage 5 cells using CK18 antibody was performed under inverted fluorescence microscopy. DAPI staining revealed blue nuclei [Figure 3: see original paper]-A, B, while cytoplasm showed positive CK18 expression [Figure 3: see original paper]-C, D. Negative controls showed no fluorescence [Figure 3: see original paper]-G, H, confirming the epithelial nature of cultured cells. The enzymatic digestion method yielded approximately 92% CK18-positive cells [Figure 3: see original paper]-E, while the tissue explant method achieved approximately 98% purity [Figure 3: see original paper]-F, demonstrating higher purity with the latter approach.

2.4 Growth Curve of Goat Ruminal Epithelial Cells

As shown in [Figure 4: see original paper], growth curves of passage 5 cells from both methods exhibited typical S-shaped patterns, including lag phase, logarithmic growth phase, and plateau phase. Days 1-2 represented the lag phase with slow growth, followed by logarithmic growth after day 2 and plateau phase after day 7 when growth slowed and cell numbers stabilized, consistent with normal *in vitro* cell growth patterns [?]. The tissue explant method demonstrated higher proliferative activity than enzymatic digestion.

2.5 CK18 Expression in Cells from Two Culture Methods

Western blot analysis showed significantly higher CK18 expression in cells isolated by tissue explant method compared to enzymatic digestion ($P < 0.05$) [Figure 5: see original paper].

Common primary culture methods include enzymatic digestion and tissue explant approaches [?]. In 1980, Gálfi et al. [?] first cultured bovine ruminal epithelial cells using minced ruminal papillae and serial enzymatic digestion, establishing an *in vitro* culture system. However, this method yielded low-purity cells, limiting experimental applications. The tissue explant method is the simplest approach for primary culture. Without enzymatic treatment, it yields highly viable and proliferative cells, providing a foundation for cell line establishment [?].

DMEM/F12 basal medium, suitable for most epithelial tissues, was used in this study. The major difference from other goat ruminal epithelial cell culture protocols was supplementation with EGF, insulin, transferrin, and selenium to promote growth and enable faster experimental readiness. Gao et al. [?] added EGF when culturing avian amniotic epithelial cells, while Ma et al. [?] supplemented insulin, transferrin, and selenium for porcine oral mucosal epithelial cell culture.

Enzymatic digestion used 0.25% trypsin + 0.02% EDTA. According to Sun et al. [?], this condition yields ideal cell isolation and proliferative activity for epithelial cells. Digestion time varies among epithelial cell types—human epithelial cells require longer digestion [?], while Beijing fatty chicken amniotic epithelial cells require 5 min [?]. This study also used 5 min for goat ruminal epithelial cells based on preliminary experiments showing optimal viability at this duration; 15 min digestion resulted in poor adherent growth. To improve purity, the first two digestions were discarded as they contained mostly keratinocytes, retaining only the last three digestions.

Regarding cell viability, tissue explant method yields significantly more active cells than enzymatic digestion, as confirmed by Shen [?] in comparative studies of human dental pulp cell culture methods. Prolonged or high-concentration enzymatic digestion inactivates many cells and may damage surface proteins, increasing membrane permeability, reducing viability, and slowing growth [?]. For cell purity, although tissue explant primary cultures may contain more fibroblasts than enzymatic digestion [?], the former allows selective transfer of epithelial cell-rich areas to new dishes during passage 2, essentially eliminating fibroblasts and greatly increasing purity. This aligns with findings by Hao et al. [?] in improved human umbilical cord mesenchymal stem cell culture methods. The tissue explant method also shows higher success rates, possibly because enzymatic digestion is difficult to control precisely [?]. Tissue digestion speed depends on multiple factors including fragment size, agitation frequency, collagenase type, enzyme activity, and concentration [?]. However, enzymatic digestion offers shorter culture cycles [?], enabling rapid acquisition of large cell numbers with primary culture completed 4-5 days faster than tissue explant method.

Cytokeratins constitute the epithelial cell cytoskeleton and represent specific epithelial antigens with multiple phenotypes. This study used CK18 as an epithelium-specific primary antibody for immunofluorescence identification. Positive cytoplasmic staining with green fluorescence confirmed the epithelial nature of cultured cells, with higher purity in tissue explant-derived cells [?]. This is consistent with Zhou et al. [?] who identified porcine small intestinal mucosal epithelial cells by CK18 expression, and Li et al. [?] who used CK18 immunofluorescence to identify bovine mammary epithelial cells.

Since fibroblast contamination is inevitable in primary culture, differential adhesion was employed based on fibroblasts' faster adhesion than ruminal epithelial cells [?]. After digestion, cells were cultured for 30 min, then supernatant transferred to a new dish for another 30 min static culture. Repeating this step twice improved ruminal epithelial cell purity, with epithelial cells eventually outcompeting fibroblasts during subsequent passages.

In conclusion, both tissue explant and enzymatic digestion methods successfully cultured goat ruminal epithelial cells. However, tissue explant method yielded higher purity, growth activity, and CK18 expression. For experiments requiring high-purity, high-viability cells, tissue explant method is safer and more reliable;

for rapid acquisition of large cell numbers, enzymatic digestion is more suitable.

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