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Postprint: Characteristics of Cell Cycle Distribution, Proliferation, and Apoptosis in Rumen Epithelial Cells of Liuyang Black Goats

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Abstract

This study aimed to establish an in vitro culture model of rumen epithelial cells from Liuyang black goats and investigate their cell cycle distribution, proliferation, and apoptosis characteristics. Rumen epithelial tissue was collected from 60-day-old Liuyang black goats, and the tissue was digested using 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) digestion method to obtain single rumen epithelial primary cells for in vitro culture. Cell morphology at primary and passage culture stages was observed using an inverted microscope, cell growth activity was detected by cell counting method, passage cells were identified by immunocytochemistry, and cell cycle distribution and apoptosis ratio of goat rumen epithelial passage cells were detected by flow cytometry. The results showed: 1) Goat rumen epithelial primary cells obtained by digestion with 0.25% trypsin + 0.02% EDTA began to adhere and grow at 1 day of culture, started to grow rapidly at 2 days (logarithmic phase), exhibited a typical "wave peak" growth pattern, grew most rapidly at 3-4 days, and showed stable growth rate at 7 days (plateau phase). 2) Identification by immunocytochemistry showed that the cytoplasm was brownish-yellow, indicating positive expression of cytokeratin 19. 3) Annexin V/propidium iodide co-staining showed that the apoptosis ratio increased significantly with prolonged culture time ($P < 0.01$). These results indicate that goat rumen epithelial cells were successfully obtained through the digestion method with 0.25% trypsin + 0.02% EDTA, which can provide a model for future studies on mechanisms and functions related to the rumen of ruminants.

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Full Text

Characteristics of Cell Cycle Distribution, Proliferation, and Apoptosis in Ruminal Epithelial Cells of Liuyang Black Goats

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Abstract

This study aimed to establish an in vitro culture model of ruminal epithelial cells from Liuyang black goats and investigate their cell cycle distribution, proliferation, and apoptosis characteristics. Ruminal epithelial tissue was collected from 60-day-old Liuyang black goats and digested using 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) to obtain individual primary ruminal epithelial cells for in vitro culture. Cell morphology during primary and subculture stages was observed using an inverted microscope, growth activity was assessed by cell counting, subcultured cells were identified via immunocytochemistry, and cell cycle distribution and apoptosis ratios were determined by flow cytometry. The results showed that: (1) Primary ruminal epithelial cells obtained by 0.25% trypsin + 0.02% EDTA digestion began adhering within 1 day of culture, entered rapid logarithmic growth by day 2 with typical "peak-shaped" growth, grew most rapidly at 3–4 days, and reached a stable plateau phase by day 7. (2) Immunocytochemical identification revealed brownish-yellow cytoplasm, indicating positive expression of cytokeratin 19. (3) Annexin V/propidium iodide co-staining demonstrated that the apoptosis ratio increased significantly with prolonged culture time ($P < 0.01$). In conclusion, ruminal epithelial cells from goats were successfully obtained using 0.25% trypsin + 0.02% EDTA digestion, providing a cellular model for future studies on rumen mechanisms and functions in ruminants.

Keywords: Liuyang black goat; ruminal epithelial cell; cycle distribution; apoptosis

Introduction

The rumen serves as a crucial digestive organ in ruminants, and understanding its epithelial morphology and function is essential for maintaining digestive system stability and promoting animal health. The rumen wall consists of three layers from the lumen outward: the serosal layer, muscular layer, and mucosal layer [1]. The rumen epithelium, most closely associated with absorption and metabolic functions, comprises four strata from the serosa to the mucosa: the stratum corneum, stratum basale, stratum spinosum, and stratum granulosum [2]. Ruminal epithelial cells include basal cells, granular cells, spinous and granular layer cells, and highly differentiated corneocytes [3]. The key to successful in vitro isolation and culture of ruminal epithelial cells (REC) lies in obtaining sufficient quantities of relatively pure, viable cells from the intermediate spinous and granular layers while removing corneocytes and other contaminating cells. Weekes [4] used papain to dissociate papillary tissue and obtained viable epithelial cells. Gálfi et al. [5] first applied sequential trypsin digestion to study ruminal epithelial ketogenesis, removing the tough stratum corneum before obtaining optimal spinous and granular layer cells for in vitro nutritional metabolism studies. They later used the same method to successfully isolate and culture ovine ruminal epithelial cells, though the 8-hour isolation process limited metabolic research applications [6]. Klotz et al. [7] optimized digestion buffer and trypsin concentrations to obtain large numbers of morphologically normal ruminal epithelial cells. Stumpff et al. [8] improved sampling methods by designing a mold to restrict trypsin action to ruminal papillae, yielding purer epithelial cells. Fan et al. [9] established an in vitro isolation and culture method for reindeer ruminal epithelial cells using 0.25% trypsin + 0.02% EDTA digestion at 37°C with continuous shaking for 3–4 hours, obtaining viable primary cells that could be passaged once.

Although previous studies have investigated ruminal epithelial cells, most have focused only on primary cells through the first passage, with limited reports on subsequent experimental research. Therefore, this study aimed to establish an effective and rapid method for obtaining goat ruminal epithelial cells suitable for successful primary culture, and to conduct preliminary investigations of their cell cycle distribution and apoptosis ratios, providing technical support and theoretical reference for future studies on rumen mechanisms and functions in ruminants.

Materials and Methods

Experimental Animals and Reagents Three healthy 60-day-old Liuyang black goats weighing (6.4 $\pm$ 0.8) kg were used. Animals were euthanized by jugular exsanguination, and rumen tissue was harvested, emptied of contents, and rinsed repeatedly with physiological saline for sample collection. Fetal bovine serum (FBS), Dulbecco' s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), 0.25% trypsin + 0.02% EDTA, and penicillin-streptomycin were purchased from Gibco; gentamicin/amphotericin B solution from Thermo (R-

015-10); Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories; hematoxylin and phosphate buffer saline (PBS, pH 7.2–7.6) from Wellbio; two-step detection kit and DAB kit from Zhongshan Jinqiao; cytokeratin-19 (CK19) antibody from Abcam; and routine chemical reagents from Shanghai Guoyao Biological.

Cell Isolation and Culture Procedures

Ruminal Epithelial Cell Isolation and Primary Culture After weighing, goats were euthanized by carotid artery exsanguination. Abdominal wool was shaved with a scalpel, the skin disinfected with benzalkonium bromide, and the animal positioned on the operating table. The abdominal region was wiped three times with iodine and once with 75% ethanol before incision. The rumen was removed, rinsed with 4°C physiological saline to remove digesta particles, and transferred to a sterile laminar flow hood. Rumen epithelial tissue was bluntly dissected, washed with a solution containing 2% penicillin-streptomycin, 1% amphotericin B, and 1% gentamicin, and transported to the cell culture room in DMEM/F12 containing the same antibiotics.

The epithelial tissue was washed 4–5 times with PBS, and connective tissue, fat, and visible ductal tissue were trimmed away. Tissue blocks were minced finely (~1 mm^3, appearing paste-like to the naked eye) and washed once each with PBS and DMEM/F12. After discarding the supernatant, three volumes of 0.25% trypsin + 0.02% EDTA digestion solution were added, and the tissue was digested at 37°C with air bath shaking for 10 minutes. Following digestion, samples were centrifuged at 1,000 r/min for 5 minutes, and the supernatant was collected (with vigorous pipetting to promote single-cell dissociation). This process was repeated 3–5 times. The collected digest was resuspended in DMEM/F12 complete medium (containing 5% FBS, 10% penicillin-streptomycin, 0.1 mg/mL gentamicin, and 2.5 g/mL amphotericin B), filtered through a 100 m mesh, and centrifuged at 1,000 r/min for 5 minutes. The supernatant was discarded, and if a black cell layer or keratinized cells were present (typically from the first two digestions in adult animals), they were discarded. The pellet was resuspended in PBS, centrifuged again at 1,000 r/min for 5 minutes, and resuspended in DMEM/F12 medium at a concentration of 1×10^7 cells/mL for plating in culture dishes. After 30–60 minutes of incubation in a CO_2 incubator (37°C, 5% CO_2), the supernatant was removed under microscopic observation, centrifuged at 1,000 r/min for 5 minutes, and the pellet was resuspended in fresh DMEM/F12 complete medium for continued culture.

Subculture of Ruminal Epithelial Cells When primary cells reached 80–90% confluence, the medium was removed and cells were washed 1–2 times with PBS before adding 1 mL of 0.25% trypsin + 0.02% EDTA digestion solution. Cells were incubated at 37°C with 5% CO_2 for 2–3 minutes until they became bright and rounded under inverted microscopy, at which point digestion was terminated with serum-containing medium. Detached cells were pipetted into suspension, transferred to 15 mL centrifuge tubes, and centrifuged at 1,000

r/min for 5 minutes at 4°C. After discarding the supernatant, cells were resuspended in 1 mL medium and passaged at a 1:2 split ratio. Following 30 minutes of incubation in a 37°C, 5% CO_2 incubator, the cell-containing medium was transferred to new culture dishes. This purification step was repeated once.

Morphological Observation Morphology and growth status of primary and subcultured ruminal epithelial cells were observed using a Leica DMI3000B inverted microscope.

Immunocytochemical Identification Epithelial cells were identified by detecting the characteristic intermediate filament protein cytokeratin. Logarithmically growing subcultured cells were digested with 37°C 0.25% trypsin + 0.02% EDTA and seeded onto coverslips in 6-well plates. At 80–90% confluence (3 days), coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, air-dried for 5 minutes, and rinsed three times with PBS (3 minutes each). Endogenous peroxidase activity was blocked with 3% H_2O_2 (2 drops or 100 L per well) for 10 minutes at room temperature, followed by three PBS washes. After blocking with serum for 20 minutes, CK19 antibody (2 drops or 100 L per well) was applied and incubated at 37°C for 60 minutes, followed by three PBS washes. Samples were placed in a humidified chamber at 4°C overnight, then rewarmed at 37°C for 45 minutes. After five PBS washes (5 minutes each), anti-rabbit/mouse immunoglobulin G-horseradish peroxidase polymer (50–100 L per well) was applied at 37°C for 30 minutes, followed by three PBS washes. Freshly prepared DAB solution (50–100 drops or 300–400 L per well) was added and monitored microscopically for 1–5 minutes. After washing with distilled water for 10 minutes, samples were counterstained with hematoxylin for 5–10 minutes, differentiated with 0.25% hydrochloric acid alcohol for 5–10 seconds, blued with PBS for 20 minutes, dehydrated through graded alcohols (60–100%, 5 minutes each), cleared in xylene for 10 minutes (repeated twice), and mounted with neutral resin for microscopic observation.

Growth Activity Assay Logarithmically growing subcultured cells were digested and seeded in 96-well plates at 3×10^3 cells per well in 100 L volume. After incubation at 37°C with 5% CO_2, cells were assayed on days 1–9, with cell-free medium as control. CCK-8 solution (10 L per well) was added without generating bubbles (which affect optical density readings), followed by 4 hours of incubation. Absorbance at 450 nm (OD_{450}) was measured using a microplate reader, and growth curves were plotted with culture time on the x-axis and OD_{450} on the y-axis.

Cell Cycle Analysis Cell cycle distribution in subcultured ruminal epithelial cells at different culture times was analyzed by flow cytometry. Cells were cultured in DMEM/F12 with 5% FBS, and passaged cells cultured for 2, 4, and 8 days were collected in triplicate. After trypsinization and PBS washing, single-cell suspensions were prepared at 1×10^6 cells/mL. Cells were fixed by

resuspending in 1 mL cold PBS, centrifuging at 800 r/min for 5 minutes, discarding the supernatant, gently resuspending in 400 L PBS, and adding 1.2 mL ice-cold 100% ethanol dropwise to a final concentration of 75% for overnight fixation at 4°C. Fixed cells were centrifuged at 800 r/min for 5 minutes, washed twice with cold PBS, and stained with 150 L propidium iodide (PI) working solution for 30 minutes at 4°C in the dark. Samples were analyzed by flow cytometry using a 488 nm argon laser for excitation and a 630 nm bandpass filter for detection. Forward scatter/side scatter (FSC/SSC) dot plots were used to collect 10,000 cells, with gating to exclude doublets and debris. Cell cycle percentages were analyzed from PI fluorescence histograms.

Apoptosis Ratio Determination Subcultured ruminal epithelial cells were cultured in DMEM/F12 with 5% FBS, and cells cultured for 2, 4, and 8 days were collected in triplicate. After trypsinization without EDTA and two PBS washes (centrifuged at 2,000 r/min for 5 minutes), 1×10^5 to 5×10^5 cells were resuspended in 500 L binding buffer. Annexin V-FITC (5 L) and PI (5 L) from the apoptosis detection kit were added, mixed, and incubated at room temperature in the dark for 5–15 minutes before flow cytometric analysis within 1 hour.

Statistical Analysis Immunocytochemistry images were captured digitally and analyzed using Image-Pro-Plus (IPP) software. Flow cytometry data were collected using a BD FACSCalibur and analyzed with FlowJo software. Statistical analysis was performed using SAS 9.2, with significance defined as $P < 0.01$.

Results

Morphology of Primary and Subcultured Cells After the fourth centrifugation following 0.25% trypsin + 0.02% EDTA digestion, abundant white cell pellets were observed, though cell numbers decreased by the sixth digestion as tissue became sticky. Microscopic examination revealed numerous single, uniformly sized cells with good refractivity [Figure 2: see original paper]-A. After adjusting cell density and 2 days of culture, most cells had adhered and begun growing in island-like distributions with good refractivity, round morphology, and small size [Figure 2: see original paper]-B. By day 4, cells proliferated rapidly, exhibiting typical cobblestone morphology and entering logarithmic growth phase [Figure 2: see original paper]-C. After 3–4 passages of purification, relatively pure ruminal epithelial cells with characteristic cobblestone morphology were obtained [Figure 2: see original paper]-D.

Immunocytochemical Identification Immunocytochemical staining revealed brownish-yellow cytoplasm, indicating positive CK19 expression [Figure 3: see original paper], confirming the epithelial nature of subcultured cells.

Growth Activity of Subcultured Cells Growth activity measurements at different time points showed a typical S-shaped curve [Figure 4: see original paper]. The proliferation process included a lag phase (days 1-2) with slow growth, a logarithmic phase (days 3-7) with rapid growth, a plateau phase (days 7-8) with stable proliferation, and a decline phase after day 8 with reduced proliferative activity.

Cell Cycle Distribution Flow cytometry analysis revealed the cell cycle distribution in subcultured cells at different culture times [Figure 4: see original paper]. The cell cycle comprises G_0/G_1, S, and G_2/M phases, with an apoptosis peak (sub-G_0 phase) preceding G_0/G_1, confirming apoptotic cells. The absence of a debris peak indicated proper gating, and coefficients of variation (CV) below 10% ensured reliable results. Statistical analysis using SAS 9.2 (Table 1) showed that as culture time increased from 2 to 8 days, G_0/G_1 phase cells decreased significantly ($P < 0.01$) while S and G_2/M phase cells increased significantly.

Apoptosis Ratio Annexin V/PI double staining detected apoptosis ratios in subcultured cells at different culture times [Figure 5: see original paper]. Data collected using a BD FACSCalibur flow cytometer and analyzed with Cell FlowJo software revealed percentages of early (LR), middle (UR), and late/necrotic (UL) apoptotic cells. The total apoptosis ratio was calculated as LR + UR + UL. Statistical analysis (Table 2) showed that the apoptosis ratio at day 8 was significantly higher than at days 2 and 4 ($P < 0.01$), indicating that apoptosis increased with culture duration, particularly by day 8.

Discussion

In Vitro Isolation and Culture of Ruminal Epithelial Cells In vitro culture systems are generally classified as primary or subculture methods [10]. Primary culture techniques include enzymatic digestion (high yield, good morphological differentiation, large tissue requirement) and tissue block culture (prone to detachment, low success rate, long culture time, small tissue requirement) [11]. Sun et al. [12] investigated primary culture methods for goat ruminal epithelial cells (RNC) and found that 2.50% trypsin + 0.02% EDTA digestion yielded optimal cell isolation and proliferation for Liuyang black goat ruminal epithelium. Accordingly, we employed 10-minute sequential digestion with 2.50% trypsin + 0.02% EDTA and 100 m filtration to obtain numerous viable single cells. Additionally, incorporating 2% penicillin-streptomycin, 1% gentamicin, and amphotericin B in tissue wash and primary culture media enabled successful passaging beyond four generations with normal growth activity and morphology.

Primary cultures often become contaminated with fibroblasts that grow faster than epithelial cells [13]. Purification exploits fibroblast sensitivity to trypsin-EDTA, which causes earlier detachment and stronger adhesion [14]. Common purification methods include differential digestion, differential adhesion, scrap-

ing, cloning rings, and limited dilution in 96-well plates [15-16]. Inooka et al. [17] used differential digestion with trypsin pretreatment for approximately 2 hours to separate epithelial cells from fibroblasts and successfully culture ruminal epithelial cells. Our study employed differential adhesion: when primary cells reached 80-90% confluence, the medium was removed, cells were washed 2-3 times with PBS containing 2% penicillin-streptomycin, and digested with 0.25% trypsin-EDTA. The resuspended cells were incubated for 30 minutes at 37°C with 5% CO_2, then transferred to new dishes. Repeating this process 1-2 times for 2-3 passages yielded relatively pure (80%) ruminal epithelial cells.

Apoptosis Ratio and Cell Cycle Distribution in Subcultured Cells Advances in cell and molecular biology have improved understanding of apoptosis mechanisms and detection techniques, including DNA agarose gel electrophoresis, electrochemical methods, Western blot, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and reverse transcription-polymerase chain reaction (RT-PCR) [18-20]. Flow cytometry enables rapid quantitative detection of apoptotic cells based on biological and biochemical changes in nuclei, organelles, and membranes, identifying cells at early, middle, and late apoptosis stages.

Our study used Annexin V/PI co-staining to detect apoptosis ratios in subcultured ruminal epithelial cells at different culture times, finding that apoptosis increased progressively, most markedly by day 8. This elevation may relate to mitochondrial polarity damage, as Tan et al. [21] demonstrated a positive correlation between apoptosis ratio and mitochondrial polarity impairment using rhodamine 123 and PI co-staining. Mitochondrial free radical accumulation causes swelling and non-specific pore formation in the inner membrane, releasing pro-apoptotic factors into the cytoplasm [22]. Additionally, flow cytometry analysis of cell cycle distribution showed that proliferative phase (S and G_2/M) cells increased significantly while quiescent phase (G_0/G_1) cells decreased from days 2 to 8, indicating that extended culture did not severely impair the G_0/G_1 to proliferative phase transition. This suggests that DNA polymerase, RNA polymerase, gene regulatory proteins, and genetic material remained undamaged over time. However, apoptosis increased with culture duration, negatively correlating with G_0/G_1 arrest, warranting further mechanistic investigation.

Conclusions

1. Sequential digestion with 0.25% trypsin + 0.02% EDTA combined with differential adhesion yielded numerous, relatively pure ruminal epithelial cells from Liuyang black goats with normal morphology and strong viability.

2. Immunocytochemical identification confirmed the epithelial origin of subcultured cells through brownish-yellow cytoplasmic staining, indicating positive CK19 expression.

3. Cell counting assays demonstrated that subcultured ruminal epithelial cells exhibited a typical S-shaped growth curve.
4. Annexin V/PI co-staining revealed that apoptosis ratios increased significantly with prolonged culture time, peaking at day 8.

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