Protective Effect of L-Theanine Against Hydrogen Peroxide-Induced Apoptosis in Goat Rumen Epithelial Cells: Postprint

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Abstract

This study aimed to investigate the protective effect of L-theanine against hydrogen peroxide (H2O2)-induced apoptosis in goat rumen epithelial cells. Rumen tissues were collected from 42-day-old Xiangdong Black goats for primary cell isolation, culture, and purification. The control group received complete medium, while groups I, II, III, and IV received complete medium supplemented with 800 μmol/L H2O2, 4 mmol/L L-theanine + 800 μmol/L H2O2, 8 mmol/L L-theanine + 800 μmol/L H2O2, and 16 mmol/L L-theanine + 800 μmol/L H2O2, respectively, with three replicates per group. After 12 h of incubation, cell viability was measured by the thiazolyl blue (MTT) assay, cell cycle distribution was analyzed by flow cytometry, and Bax and Bcl-2 gene and protein expression levels were determined by real-time quantitative PCR and Western blotting. The results demonstrated that L-theanine significantly increased the viability of H2O2-damaged rumen epithelial cells ($P<0.05$), significantly elevated the proportion of S-phase cells ($P<0.05$), significantly reduced Bax gene and protein expression levels ($P<0.05$), significantly enhanced Bcl-2 gene and protein expression levels ($P<0.05$), and significantly increased the Bcl-2/Bax expression ratio at both gene and protein levels ($P<0.05$). These results suggest that L-theanine can effectively protect rumen epithelial cells from H2O2-induced damage by inhibiting apoptosis; under in vitro conditions, L-theanine concentrations exceeding 8 mmol/L in the culture medium can effectively exert anti-apoptotic effects.

Full Text

Protection of L-Theanine on Apoptosis of Rumen Epithelial Cells Induced by Hydrogen Peroxide in Goats

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Abstract

This study investigated the protective effects of L-theanine on hydrogen peroxide (H_2O_2)-induced apoptosis in goat rumen epithelial cells. Rumen epithelial tissue was collected from 42-day-old Xiangdong black goats for primary cell isolation, culture, and purification. The control group was cultured in complete medium, while groups I, II, III, and IV were cultured in complete medium supplemented with 800 μmol/L H_2O_2, 4 mmol/L L-theanine + 800 μmol/L H_2O_2, 8 mmol/L L-theanine + 800 μmol/L H_2O_2, and 16 mmol/L L-theanine + 800 μmol/L H_2O_2, respectively. Each group had three replicates. After 12 hours of culture, cell viability was measured by MTT assay, cell cycle distribution was analyzed by flow cytometry, and expression of Bax and Bcl-2 genes and proteins was determined by real-time quantitative PCR and Western blot. The results demonstrated that L-theanine significantly improved the viability of H_2O_2-injured rumen epithelial cells ($P<0.05$), significantly increased the proportion of cells in S phase ($P<0.05$), significantly reduced Bax gene and protein expression ($P<0.05$), significantly increased Bcl-2 gene and protein expression ($P<0.05$), and significantly elevated the Bcl-2/Bax ratio ($P<0.05$). These findings indicate that L-theanine effectively protects rumen epithelial cells from H_2O_2-induced injury by inhibiting apoptosis. Under in vitro conditions, L-theanine concentrations exceeding 8 mmol/L in culture medium exert effective anti-apoptotic effects, offering therapeutic and protective benefits against rumen epithelial cell damage.

Keywords: L-theanine; hydrogen peroxide; rumen epithelial cells; apoptosis

Introduction

Under oxidative stress conditions, livestock produce excessive oxygen free radicals (OFR) that damage biological membranes, impair normal cellular functions, induce lipid peroxidation of membrane phospholipids, cause protein oxidation (affecting receptors and enzymes), and inflict DNA oxidative damage. These oxidative modifications to lipids, proteins, and DNA pose varying degrees of harm to organisms, influencing growth, development, and aging processes. Both acute and chronic stress can induce gastrointestinal oxidative stress through free radical generation. Prolonged stress further increases free radicals in animals, depleting antioxidants such as vitamins and trace elements, compromising immunity, reducing disease resistance, and potentially triggering diseases or even death. Numerous studies have confirmed associations between various types of gastrointestinal ulcers and reactive oxygen species, with hydrogen peroxide (H_2O_2) identified as the primary reactive oxygen species causing oxidative damage to gastric mucosal cells.

Current literature indicates that multiple factors can induce oxidative stress and subsequent digestive tract injury, including heavy metals (arsenic, lead, mercury), mycotoxins, pesticides, excessive dietary energy and protein, high iron and copper levels, oxidized fats, trauma, and antioxidant deficiencies (selenium, vitamins A, E, C, zeaxanthin, etc.). Such damage affects nutrient digestion and absorption. Young animals experiencing dietary and environmental changes are particularly susceptible to oxidative stress. Post-trauma stress can cause intestinal ischemia, generating abundant superoxide radicals accompanied by xanthine and hypoxanthine accumulation in mucosa, which are subsequently converted to more toxic hydroxyl radicals. Studies have demonstrated that ischemia-reperfusion affects intestinal permeability, electrolyte secretion, and mucus release, often leading to enteritis.

L-theanine, a flavor compound in green tea, is a natural amino acid widely applied in the food industry. As a primary active component in tea plants, L-theanine is transported via amino acid transport systems and exerts protective effects against brain and liver tissue damage while demonstrating unique mechanisms and targets in immune cells, nerve cells, and cancer cells.

H_2O_2, a crucial reactive oxygen species component, can induce apoptosis in many cell types through oxidative stress injury. Research has shown that L-theanine possesses antioxidant activity and protects nerve cells from apoptosis induced by various stimuli. Investigating whether L-theanine can inhibit rumen epithelial cell apoptosis and counteract oxidative stress-induced damage holds significant importance for ruminant gastrointestinal development. The protective mechanism of L-theanine is associated with its antioxidant activity and apoptosis inhibition. The Bcl family plays a critical role in mammalian apopto-

sis, primarily functioning upstream of irreversible cell damage at the mitochondrial level. This family comprises two categories: anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax. Bax can form heterodimers with Bcl-2, antagonizing Bcl-2 function and regulating apoptosis by activating downstream genes. This study employed an H_2O_2-stimulated rumen epithelial cell oxidative stress model to investigate the protective effects of L-theanine against H_2O_2-induced rumen epithelial cell injury, providing a theoretical basis for elucidating the protective mechanism of L-theanine in rumen epithelium.

Materials and Methods

1.1 Reagents Fetal bovine serum, DMEM/F12 medium, 0.25% trypsin + 0.02% EDTA, and penicillin-streptomycin were purchased from Gibco (USA). Amphotericin B and gentamicin were from Thermo Fisher Scientific (R-015-10). L-theanine (CAS: 3081-61-6, purity $ $99%) and MTT were from Sigma (USA). Primary antibodies against Bax and β-actin were from Cell Signaling Technology (USA); Bcl-2 primary antibody was from Signaling Antibody Biotechnology (USA); β-actin primary antibody was from Bioworld (Nanjing, China). Secondary antibodies were from Calbiochem (USA). Cell cycle detection kits were from Nanjing KeyGen Biotech. Agarose was from Shanghai Macklin Biochemical. Reverse transcription kits were from Beijing CoWin Biosciences. DEPC and EB reagents were from Beijing SBS Genetech. TRIZOL was from Invitrogen (USA). Taq polymerase, DNA molecular weight markers (DL2000), and dNTPs were from MBI Fermentas (USA). Primers were synthesized by Shanghai Sangon Biotech. SYBR Green PCR Mix was from TaKaRa (Japan). Routine chemical reagents were from Beijing Chemical Reagents Company.

1.2 Experimental Procedures

1.2.1 Goat Rumen Epithelial Cell Isolation and Grouping Four healthy Xiangdong black goats aged 42 days (body weight 6.4$\pm$0.8kg) were selected as experimental animals. After euth
$F12$ medium containing $1,000 U/mL$ penicillin$-$streptomycin and $125 \mu g/mL$ gentamicin at 4°C before transfer
5 times with phosphate$-$buffered saline (PBS) containing $500 U/mL$ penicillin$-$
streptomycin, minced thoroughly, and digested with 0.25×10^{7}$ cells/mL and cultured in a CO_2 incubator at 37°C with 5% CO_2.

When primary goat rumen epithelial cells reached 80-90% confluence, medium was discarded and cells were washed 1-2 times with PBS before adding 1 mL of 0.25% trypsin + 0.02% EDTA digestion solution. After 2-3 minutes incubation at 37°C with 5% CO_2, cells were observed under an inverted microscope. When cells became bright and rounded, digestion was immediately terminated with complete medium. Adherent 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 removing supernatant, cells were resuspended in 1 mL complete medium and passaged at a 1:2 ratio. Following 30 minutes incubation at 37°C with

5% CO_2, cell-containing medium was transferred to culture dishes for continued culture, with the purification step repeated once. Purified goat rumen epithelial cells were subsequently cultured in DMEM/F12 medium containing 5% fetal bovine serum. When cell density reached 60-70%, cells were divided into five groups: control group with complete medium only, and groups I, II, III, and IV supplemented with 800 μmol/L H_2O_2, 4 mmol/L L-theanine + 800 μmol/L H_2O_2, 8 mmol/L L-theanine + 800 μmol/L H_2O_2, and 16 mmol/L L-theanine + 800 μmol/L H_2O_2, respectively. Each group had three replicates.

1.2.2 MTT Assay for Cell Viability Purified goat rumen epithelial cells were seeded in 96-well plates at 1$×10^{6}$ cells/mL. When cell density reached 60-70%, cells were divided into six groups: control with complete medium, positive control with 8 mmol/L L-theanine, and groups I, II, III, and IV supplemented with 800 μmol/L H_2O_2, 4 mmol/L L-theanine + 800 μmol/L H_2O_2, 8 mmol/L L-theanine + 800 μmol/L H_2O_2, and 16 mmol/L L-theanine + 800 μmol/L H_2O_2, respectively. Each group had 12 replicates. L-theanine was added first for 1-hour pre-incubation, followed by H_2O_2 addition according to experimental design. After 12 hours of culture, medium was aspirated from 96-well plates and cells were washed three times with sterile PBS. Each well received 20 L of 5 mg/mL MTT solution and incubation continued at 37°C for 4 hours, after which supernatant was removed. Then 150 L of dimethyl sulfoxide (DMSO) was added to each well, followed by 10 minutes of shaking. Absorbance (OD) values were measured at 570 nm using a microplate reader. Cell survival rate (%) was calculated relative to the control group using the formula:

Cell survival rate (%) = 100 × (OD_{570} nm of positive control or treatment group) / (OD_{570} nm of control group)

1.2.3 Flow Cytometry Analysis of Cell Cycle and Apoptosis Purified goat rumen epithelial cells were seeded in 6-well plates at 1$×10^{6}$ cells/mL. When cell density reached 60-70%, cells were divided into five groups: control with complete medium, and groups I, II, III, and IV supplemented with 800 μmol/L H_2O_2, 4 mmol/L L-theanine + 800 μmol/L H_2O_2, 8 mmol/L L-theanine + 800 μmol/L H_2O_2, and 16 mmol/L L-theanine + 800 μmol/L H_2O_2, respectively. Each group had six replicates. L-theanine was added first for 1-hour pre-incubation, followed by H_2O_2. After 12 hours of culture, cell cycle distribution was detected by flow cytometry using propidium iodide (PI) staining.

1.2.4 Apoptosis Gene Expression in Rumen Epithelial Cells Cell grouping and treatment were identical to section 1.2.3. After 12 hours of culture, total RNA was extracted from each group using the TRIZOL one-step method, with masks and disposable gloves worn throughout the procedure. DNase I treatment of total RNA samples and reverse transcription were performed according to referenced protocols and kit instructions.

1.2.5 Primer Design and Synthesis Based on sequencing results of novel genes, primers were designed using molecular biology software (Primer Premier 5, Primer 3.0, Oligo 6.71) and specificity was analyzed using BLAST. Real-time quantitative PCR was employed to detect tissue-specific expression. Primer sequences are shown in and were synthesized by Shanghai Sangon Biotech.

TABLE:1 Primer information

Gene	GenBank Accession No.	Primer Sequence (5' -3')	Product Size (bp)
β-actin	NM_{001314342}.1	CTGGCACCACACCTTC-TACAGGGTCATCTTCTCACGGTTG	
Bcl-2	XM_{005696234}	GCTACGACACGGAGTT-CCACCCAGTTGATGCCGCTCT	
Bax	XM_{013971446}.2	TGAAGCGCATTGGAGA-TGGGCCTTGAGCACCAGTTT	

1.2.6 Western Blot Analysis Cell grouping and treatment were identical to section 1.2.3. Total protein extraction and Western blot procedures were performed according to referenced protocols.

1.3 Statistical Analysis Cell cycle data were analyzed using the GLM model in SAS 8.2. Contrast statements were applied for pairwise comparisons between groups. LSMEANS method was used to calculate mean values. Orthogonal polynomial contrasts were employed to examine linear or quadratic effects on P-values. Prior to orthogonal polynomial analysis, coefficients were corrected using the IML procedure in SAS 9.2. Differences were considered significant at $P<0.05$.

Results

2.1 Effects of L-Theanine on Viability of H_2O_2-Injured Rumen Epithelial Cells As shown in , compared with the control group, cell viability in group I was significantly reduced ($P<0.05$). Groups III and IV showed viabilities of (65.28$±9.10)±7.39)*{2}O*{2}$ significantly decreased rumen epithelial cell viability, while L-theanine at concentrations above 8 mmol/L could antagonize this effect.

TABLE:2 Effects of L-theanine on survival rate (SR) of rumen epithelial cells injured by H_2O_2

Item	Control	Positive Control	Group I	Group II	Group III	Group IV	P-value
Survival Rate (%)	98.02$±3.40^{a}$	96.55±2.85^{a}	51.70±2.50^{d}	53.39±3.53^{d}	67.14±2.40^{c}	74.66±$2.25	

Values in the same row with the same or no letter superscripts indicate no significant difference (P>0.05), while different letters indicate significant difference (P<0.05). The same applies below.

2.2 Effects of L-Theanine on Cell Cycle of H_2O_2-Induced Rumen Epithelial Cells As shown in [Figure 1: see original paper] and , compared with the control group, group I exhibited a significantly increased proportion of cells in G_0/G_1 phase (P<0.05), a significantly decreased proportion in S phase (P<0.05), and a significantly increased proportion in G_2 phase (P<0.05), indicating that H_2O_2 arrested the cell cycle at S phase. In contrast, groups II, III, and IV showed significantly increased G_0/G_1 phase proportions and significantly decreased S phase proportions compared with the control group, suggesting that L-theanine + H_2O_2 arrested cells at G_0/G_1 phase. These findings demonstrate that under H_2O_2-induced oxidative stress, L-theanine can induce both G_0/G_1 and S phase arrest, ultimately alleviating apoptosis.

TABLE:3 Effects of L-theanine on proportions of cells at different cell cycles of rumen epithelial cells injured by H_2O_2

Item	Control	Group I	Group II	Group III	Group IV	P-value
G_0/G_1 phase (%)	69.46$±3.36^{d}$	86.57±3.36^{a}	80.31±1.45^{b}	72.79±1.81^{c}	72.35±4.87^{c}	$2.09 0.001\|\|Sphase(±4.22^{a}\|8.98±2.31^{c}\|9.92±1.86^{c}\|16.57±3.36^{b}\|20.74±4.12^{b}\| < 0.001\|\|G_{2}$ phase (%)

2.3 Effects of L-Theanine on Apoptosis Gene Expression in H_2O_2-Induced Rumen Epithelial Cells As shown in , compared with the control group, Bax gene expression increased while Bcl-2 gene expression decreased in treatment groups, with significant changes in groups I and II (P<0.05). Compared with group I, Bax gene expression was significantly lower in the control group and groups II, III, and IV (P<0.05), while Bcl-2 gene expression was significantly higher in the control group and group IV (P<0.05). The Bcl-2/Bax ratio was significantly reduced in groups I, II, III, and IV compared with the control group (P<0.05).

TABLE:4 Effect of L-theanine on expression levels of apoptosis genes of ruminal epithelial cells injured by H_2O_2

Item	Control	Group I	Group II	Group III	Group IV	P-value
Bax (%)	0.92$±0.19^{c}\|1.53±0.16^{b}\|1.12±0.06^{bc}\|1.03±0.10^{c}\|0.26±0.02^{a}| < 0.001||*Bcl−*					
	2(±0.15^{a}|0.13±0.01^{c}|0.15±0.03^{bc}|0.19±0.03^{abc}|0.22±0.02^{ab}| < 0.001||*Bcl−*					
	2/*Bax*|0.28±0.01^{a}|0.04±0.02^{e}|0.09±0.03^{d}|0.16±0.02^{c}|0.21±$0.04					

2.4 Effects of L-Theanine on Apoptosis Protein Expression in H_2O_2-Induced Rumen Epithelial Cells As shown in [Figure 2: see original paper] and , compared with the control group, group I exhibited significantly decreased Bcl-2 protein expression (P<0.05), increased Bax protein expression (P<0.05), and significantly reduced Bcl-2/Bax ratio (P<0.05), indicating that H_2O_2 stimulation downregulated Bcl-2 and upregulated Bax, thereby decreasing the Bcl-2/Bax ratio. In contrast, groups II, III, and IV showed significantly increased Bcl-2 protein expression (P<0.05) and significantly decreased Bax protein expression (P<0.05) compared with group I, with groups III and IV demonstrating significantly elevated Bcl-2/Bax ratios (P<0.05). These results suggest that L-theanine alleviates H_2O_2-induced alterations in Bcl-2 and Bax protein expression.

TABLE:5 Effects of L-theanine on expression levels of apoptosis proteins of ruminal epithelial cells injured by H_2O_2

Item	Control	Group I	Group II	Group III	Group IV	P-value
Bax (%)	0.15$±0.01^{e}\|0.69±0.02^{a}\|0.45±0.02^{b}\|0.34±0.02^{c}\|0.27±0.02^{d}| < 0.001||*Bcl−*					
	2(±0.02^{a}|0.11±0.01^{e}|0.18±0.01^{d}|0.24±0.03^{c}|0.39±0.02^{b}| < 0.001||*Bcl−*					
	2/*Bax*|3.12±0.06^{a}|0.16±0.01^{e}|0.40±0.05^{d}|0.72±0.07^{c}|1.44±$0.15					

Discussion

3.1 Effects of L-Theanine on Apoptosis Rate in H_2O_2-Induced Goat Rumen Epithelial Cells High oxygen concentrations directly damage rumen epithelial cells, promoting apoptosis or exacerbating gastric diseases. Excessive oxygen free radicals constitute a major cause of cellular injury, generated through ischemia-reperfusion, drug metabolism, metal poisoning, and other stimuli. H_2O_2, as a principal component of oxygen free radicals, has been extensively studied and can cause cellular damage through multiple mechanisms including mitochondrial injury, ATP depletion, protein and lipid oxidation, DNA damage, and induction of apoptosis.

Reducing H_2O_2-induced apoptosis is crucial for mitigating oxidative stress damage and improving physiological function. Our results demonstrate that L-theanine supplementation alleviates H_2O_2-induced oxidative stress injury in goat rumen epithelial cells. The underlying mechanism may involve L-theanine' s role as a glutamine derivative that metabolizes to glutamate, participating in glutathione (GSH) synthesis. Additionally, L-theanine can maintain intracellular antioxidant enzyme activity, reducing GSH consumption while increasing GSH production, thereby stabilizing intracellular GSH content and maintaining redox balance to protect against oxidative stress damage.

3.2 Effects of L-Theanine on Apoptosis Gene and Protein Expression in H_2O_2-Induced Goat Rumen Epithelial Cells In the mitochondrial apoptosis regulatory system, Bcl family proteins—including anti-apoptotic members (Bcl-2, Bcl-xL) and pro-apoptotic members (Bax, Bad, Bid)—regulate apoptosis. Bcl-2 protein, located on mitochondrial membranes, modulates release of pro-apoptotic cytochrome c by binding to voltage-dependent anion channels and can also bind Bax to prevent its insertion into mitochondrial membranes, thereby maintaining mitochondrial membrane potential. Bax is a pro-apoptotic protein, and research indicates that the Bcl-2/Bax ratio determines mitochondrial permeability transition pore (MPTP) opening, forming a central hub for apoptosis regulation. Consequently, the Bcl-2/Bax ratio has been described as a "rheostat" controlling cell death. Under various stimuli, the balance between Bcl-2 and Bax determines cell fate, with the Bcl-2/Bax ratio serving as a critical factor in apoptosis initiation and progression, thus reflecting the degree of cellular damage. Studies show that H_2O_2 induces increased Bax expression and decreased Bcl-2 expression, reducing the Bcl-2/Bax ratio. L-theanine can reverse these H_2O_2-induced changes in Bax and Bcl-2 protein expression in rumen epithelial cells, increasing the Bcl-2/Bax ratio and thereby protecting against H_2O_2-induced apoptosis.

Conclusion

1. L-theanine effectively protects rumen epithelial cells from H_2O_2-induced injury by inhibiting apoptosis, promoting Bcl-2 protein expression, suppressing Bax protein expression, and inhibiting the elevation of Bax/Bcl-2 ratio, which represents an important pathway for its anti-apoptotic effects.
2. Under in vitro conditions, L-theanine concentrations exceeding 8 mmol/L in culture medium effectively exert anti-apoptotic activity.

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