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Effects of Glutamine and Its Dipeptide on Hydrogen Peroxide-Induced Apoptosis and Apoptosis-Related Gene Expression in Goat Rumen Epithelial Cells (Postprint)

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Abstract

This study established a hydrogen peroxide (H2O2)-induced apoptosis model in passaged goat rumen epithelial cells to investigate the effects of glutamine (Gln), glycyl-glutamine (Gly-Gln), and alanyl-glutamine (Ala-Gln) on apoptosis rates and the expression levels of Bcl-2 and Bax genes. Passaged rumen epithelial cells from 60-day-old Xiangdong black goats were cultured with different concentrations of H2O2 [0 (control), 100, 400, 800 µmol/L], and apoptosis was detected by flow cytometry. Cells were divided into five groups: the control group and group 1 received 0 and 800 µmol/L H2O2, respectively; groups 2, 3, and 4 received 800 µmol/L H2O2 plus 17.28 mmol/L Gly-Gln (group 2), 16.0 mmol/L Gln (group 3), or 16.0 mmol/L Ala-Gln (group 4). Apoptosis was assessed by flow cytometry, and Bcl-2 and Bax gene expression was measured by real-time fluorescence quantitative PCR (FQ-PCR). The results showed: 1) Compared with the control, 800 µmol/L H2O2 significantly increased the early apoptosis rate ($P < 0.05$), while the late apoptosis rate initially increased then decreased with rising H2O2 concentrations, though remaining significantly elevated over the control ($P < 0.05$). 2) Compared with the control, group 4 showed significantly increased late apoptosis ($P < 0.05$), and all experimental groups exhibited significantly increased early apoptosis rates ($P < 0.05$). 3) The Bcl-2/Bax ratio was significantly higher in all experimental groups versus the control ($P < 0.05$); compared with group 1, groups 2, 3, and 4 showed significantly increased Bcl-2/Bax ratios ($P < 0.05$), with group 2 being significantly higher than groups 3 and 4 ($P < 0.05$). In conclusion, Gly-Gln demonstrates a protective effect against H2O2-induced early apoptosis in goat rumen epithelial cells.

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Full Text

Effects of Glutamine and Its Dipeptides on Apoptosis and Apoptosis-Related Gene Expression Induced by Hydrogen Peroxide in Goat Rumen Epithelial Cells

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Abstract

This study was conducted to investigate the effects of glutamine (Gln), glycyl-glutamine (Gly-Gln), and alanyl-glutamine (Ala-Gln) on apoptosis rates and the expression of Bcl-2 and Bax genes in apoptotic cells. We established an apoptosis model using hydrogen peroxide (H_2O_2)-induced rumen epithelial cells from passaged cultures of 60-day-old Xiangdong black goats. The cells were treated with different concentrations of H_2O_2 [0 (control), 100, 400, and 800 µmol/L], and apoptosis was detected using flow cytometry.

For the main experiment, passaged rumen epithelial cells were divided into five groups. The control group and group 1 were treated with 0 and 800 µmol/L H_2O_2, respectively. Groups 2, 3, and 4 were all treated with 800 µmol/L H_2O_2, and additionally supplemented with 17.28 mmol/L Gly-Gln (group 2), 16.0 mmol/L Gln (group 3), or 16.0 mmol/L Ala-Gln (group 4). Flow cytometry was used to detect apoptosis rates, while real-time fluorescent quantitative PCR (FQ-PCR) was employed to measure Bcl-2 and Bax gene expression levels.

The results showed that: (1) Compared with the control group, when H_2O_2 concentration increased to 800 µmol/L, the early apoptosis rate increased significantly ($P < 0.05$). The late apoptosis rate initially increased then decreased with rising H_2O_2 concentrations, but remained significantly higher than the control group ($P < 0.05$). (2) Compared with the control group, group 4 showed a significant increase in late apoptosis rate ($P < 0.05$), while all treatment groups exhibited significantly increased early apoptosis rates ($P < 0.05$). (3) Compared with the control group, the Bcl-2/Bax ratio in all treatment groups increased significantly ($P < 0.05$). Compared with group 1, groups 2, 3, and 4 all showed

significantly increased Bcl-2/Bax ratios ($P < 0.05$), with group 2 being significantly higher than groups 3 and 4 ($P < 0.05$).

In conclusion, Gly-Gln demonstrates protective effects against H_2O_2-induced early apoptosis in goat rumen epithelial cells.

Keywords: Xiangdong black goat; rumen epithelial cell; apoptosis; apoptosis gene

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Introduction

Oxidative stress (OS) is a common physiological condition in weaned livestock and represents a major cause of production losses in the industry [1]. Although intensive livestock production has advanced considerably in recent years, the rapid decrease and subsequent increase in feed intake after weaning leads to reduced then elevated gastrointestinal blood flow, resulting in an "ischemia-reperfusion" phenomenon accompanied by substantial hydrogen peroxide (H_2O_2) production. H_2O_2 generates numerous oxidative free radicals through Fenton and Haber-Weiss reactions, causing damage to gastrointestinal tissue cells [2-5]. Additionally, factors such as excessive dietary polyunsaturated fatty acids, mineral element deficiencies (selenium, zinc, manganese, etc.), environmental temperature fluctuations, ionizing radiation, and extensive use of drugs and vaccines can impair the cellular antioxidant defense system, placing weaned livestock in a state of oxidative stress [6].

H_2O_2 induces oxidative stress in cells, triggering endogenous and/or mitochondrial apoptosis pathways. Pro-apoptotic proteins of the Bcl-2 family, such as Bax and Bak, become activated and cause mitochondrial membrane translocation. Meanwhile, anti-apoptotic Bcl-2 proteins form heterodimers with activated pro-apoptotic proteins, thereby inhibiting mitochondrial membrane damage [7-8]. Furthermore, Bcl-2 can suppress cytochrome C release [9], prevent the formation of complexes between deoxyadenosine triphosphate (dATP) and apoptosis protease activating factor-1 (Apaf-1) in the cytoplasm [10], inhibit caspase-9 activation, and block downstream activation of caspases-3 and -7, ultimately suppressing apoptosis [11].

With the maturation of intensive livestock production and animal health management practices, deeper understanding of practical production challenges has emerged. Therefore, comprehending the concepts, mechanisms, and internal/external factors of oxidative stress enables better application of nutrients such as glutathione (GSH), vitamin E, glutamine (Gln), magnesium (Mg), selenium (Se), vitamin C, and bioactive compounds like dihydromyricetin and tea polyphenols as exogenous antioxidants [12-13].

Appropriate dietary supplementation with Gln, alanyl-glutamine (Ala-Gln), and glycyl-glutamine (Gly-Gln) provides protective effects against oxidative stress damage [12]. However, in industrial feed production, Gln has not been widely

applied due to large required dosages, low absorption efficiency, poor water solubility, instability, and production of harmful substances (pyroglutamic acid and ammonia) [14]. With increasing understanding of dipeptides and gastrointestinal absorption characteristics, Ala-Gln and Gly-Gln have emerged as Gln donor substances and alternatives that overcome these limitations [15]. Moreover, Ala-Gln and Gly-Gln have become research hotspots in human nutrition, with mature studies in total parenteral nutrition (TPN) in clinical medicine, providing theoretical support for their extensive application in feed production [16].

Therefore, this study used H_2O_2 to induce oxidative stress in goat rumen epithelial cells to investigate the effects of Gln, Gly-Gln, and Ala-Gln on apoptosis and Bcl-2/Bax gene expression, providing experimental reference for understanding the relationship between nutritional metabolism and oxidative stress mechanisms in goat rumen epithelial cells.

Materials and Methods

1.1 Goat Rumen Epithelial Cell Culture

Experimental animals consisted of three healthy 60-day-old Xiangdong black goats weighing (6.4 ± 0.8) kg. Animals were euthanized by jugular venous exsanguination, and rumen tissues were collected. After removing contents, tissues were rinsed repeatedly with physiological saline for sample preparation.

When primary goat rumen epithelial cells reached 80-90% confluence, DMEM/F12 complete medium [containing 5% fetal bovine serum (FBS), 10% penicillin-streptomycin, 0.1 mg/mL gentamicin, and 2.5 µg/mL amphotericin B] was discarded. Cells were washed 1-2 times with phosphate-buffered saline (PBS), then 1 mL of 0.25% trypsin + 0.02% EDTA digestion solution was added. Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 2-3 min. When cells began to round and brighten under an inverted microscope, digestion was immediately terminated with DMEM/F12 complete medium. Adherent cells were pipetted into suspension, transferred to 15 mL centrifuge tubes, and centrifuged at 94 × g for 5 min at 4 °C. After discarding supernatant, cells were resuspended in 1 mL DMEM/F12 complete medium and passaged at a 1:2 ratio. Following 30 min incubation at 37 °C in a 5% CO_2 atmosphere, the cell-containing DMEM/F12 medium was transferred to culture dishes for continued culture. This process was repeated once for purification.

1.2 Reagents and Equipment

Cell culture reagents including fetal bovine serum (FBS), DMEM/F12 basal medium, 0.25% trypsin + 0.02% EDTA, and penicillin were purchased from Gibco. Gentamicin/amphotericin B solution was from Thermo, and Gln, Gly-Gln, and Ala-Gln were from Abcam.

PCR-related reagents: Reverse transcription kit from Beijing CoWin Biotech; EDTA, Tris, DEPC, and EB from Sigma; Trizol from Invitrogen; Taq polymerase, DL2000 DNA marker, and dNTPs from Genstar; primers from Nanjing GenScript; SYBR Green PCR Mix from Invitrogen; and routine chemical reagents from Beijing Chemical Reagent Company.

Equipment included a desktop refrigerated centrifuge (Eppendorf), real-time fluorescent quantitative PCR (FQ-PCR) instrument (Thermo), electrophoresis apparatus (Bio-Rad), horizontal agarose electrophoresis tank (Beijing Liuyi Instrument Factory), precision pH meter (Leici Instrument Factory), and electric glass homogenizer (Ningbo Xinzhi Biotechnology).

1.3 Solutions and Preparation

Primary solutions included: reduced 5× SDS loading buffer, electrophoresis buffer, transfer buffer, TBS buffer, TBST buffer, 5× TBE solution, RNase A stock solution (10 mg/mL), and agarose gel. H_2O_2 dilution: H_2O_2 was diluted with sterile, pyrogen-free water to final concentrations of 100, 400, and 800 µmol/L.

1.4 Experimental Design

1.4.1 H_2O_2 Induction of Apoptosis in Rumen Epithelial Cells Passaged cells at logarithmic growth phase (4th passage) were digested and seeded at 1×10^6 cells/well in 6-well plates. After 12 h of culture, medium was aspirated and cells were washed twice with PBS. Two milliliters of fresh medium without PBS were added, and cells were divided into four groups treated with 0 (control), 100, 400, and 800 µmol/L H_2O_2 for 24 h.

1.4.2 Effects of Gln and Its Dipeptides on Bcl-2 and Bax Expression Cell selection and pretreatment were identical to section 1.4.1. Cells were divided into five groups: control group and group 1 received 0 and 800 µmol/L H_2O_2, respectively. Groups 2, 3, and 4 all received 800 µmol/L H_2O_2, supplemented with 17.28 mmol/L Gly-Gln (group 2), 16.0 mmol/L Gln (group 3), or 16.0 mmol/L Ala-Gln (group 4).

1.5 Detection Methods

1.5.1 Flow Cytometry Detection of Apoptosis (Annexin V/PI Double Staining) Passaged goat rumen epithelial cells were cultured in DMEM/F12 containing 5% FBS. After 24 h of culture, cells were collected for apoptosis detection with three replicates per group. The procedure was as follows: (1) Cells were digested with EDTA-free trypsin and collected; (2) Cells were washed twice with PBS, centrifuged at 376 × g for 5 min each time, and approximately 1×10^5 to 5×10^5 cells were collected; (3) 500 µL of binding buffer was added to resuspend cells; (4) 5 µL of apoptosis detection reagent (Annexin V-FITC apoptosis detection kit) was added, mixed, followed by 5 µL PI, and mixed

again; (5) Cells were incubated at room temperature in the dark for 5-15 min; (6) Samples were analyzed within 1 h using a BD FACSCalibur flow cytometer.

1.5.2 Effects of Gln and Its Dipeptides on Bcl-2 and Bax Gene Expression

1.5.2.1 Total RNA Extraction **Preparation**: All instruments and consumables for RNA extraction were soaked overnight in 1% DEPC water, then packaged in newspaper and sterilized by autoclaving at 121 °C for 60 min.

Trizol Extraction: (1) 1 mL Trizol was added to cells, pipetted thoroughly, and lysed at room temperature for 3 min; 0.2 volumes of chloroform were added, shaken, and left at room temperature for 3-5 min. (2) Samples were centrifuged at 12,000 × g for 15 min at low temperature; the aqueous phase was collected, mixed with equal volume isopropanol, and stored at -20 °C for 20 min. (3) Samples were centrifuged at 12,000 × g for 15 min at low temperature; supernatant was discarded, and 1 mL of 75% ethanol (prepared with sterile 1% DEPC water) was added to the pellet, mixed by vortexing. (4) Samples were centrifuged at 12,000 × g for 5 min; supernatant was discarded, and pellets were air-dried for 5-10 min. Total RNA was dissolved in 40 µL sterile 1% DEPC water. (5) RNA concentration was measured using a UV spectrophotometer: 2 µL of total RNA solution was diluted to 100 µL with nuclease-free water, and absorbance was measured at 260 and 280 nm to calculate concentration and purity (target: 1.8-2.0).

Concentration (ng/µL) = A_{260} × dilution factor × 40
Purity = A_{260}/A_{280}

Agarose Gel Electrophoresis: 1% denaturing agarose gel was heated until dissolved, cooled to 60 °C, mixed with 0.5 µL EB (10 mg/mL), and poured into an electrophoresis tank pretreated with sterile 1% DEPC water. Two microliters of total RNA were mixed with loading buffer at a 1:5 ratio and electrophoresed at 170 V constant voltage until bromophenol blue migrated to 2/3 of gel length. Gels were visualized under a gel imaging system.

Reverse Transcription PCR: The 30 µL reaction system contained: dNTP (2.5 mmol/L) 6 µL, Primer Mix 3 µL, total RNA 6 µL, 5× RT Buffer 6 µL, DTT (0.1 mmol/L) 3 µL, HiFiScript (200 U/µL) 1.5 µL, and RNase-free water 4.5 µL.

1.5.2.2 FQ-PCR **Primer Design**: Target gene sequences were obtained from NCBI, and primers were designed using Primer 5 software and synthesized by Nanjing GenScript. Primer parameters are shown in Table 1 .

Table 1 Primer parameters

Genes	NCBI Accession No.	Primer sequences (5’ -3’)	Product length (bp)
Bcl-2	JN036559	F: GATGACCGAGTAC-CTGAACCGR: GACAGCCAGGA-GAAATCAAACA	
Bax	JN036558	F: AGTGGCGGCT-GAAATGTTR: GACAGCCAGGA-GAAATCAAACA	
GAPDH		F: TCCACGGCACAGT-CAAGGR: TCAGCACCAGCAT-CACCC	

FQ-PCR System: The 30 µL reaction contained: template (RT product) 1 µL, forward and reverse primers (10 µmol/L) 0.5 µL each, water 13 µL, and 2× SYBR Green PCR Master Mix 15 µL. Each sample had three replicates, with 10 µL per well.

Amplification Program: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of 95 °C for 5 s, 60 °C for 30 s.

1.6 Statistical Analysis

Apoptosis data were collected using a BD FACSCalibur flow cytometer and analyzed with Flowjo software. Experimental data were analyzed using SAS 9.2, with significance defined as $P < 0.05$ and $P > 0.05$ indicating no significant difference.

Results

2.1 Apoptosis Rate of Rumen Epithelial Cells Induced by H_2O_2

As shown in Table 2 , compared with the control group, early apoptosis rate increased significantly when H_2O_2 concentration reached 800 µmol/L ($P < 0.05$). Late apoptosis rate initially increased then decreased with rising H_2O_2 concentrations, but all treatment groups showed significant increases compared with the control group ($P < 0.05$). These results indicate that H_2O_2 can exacerbate apoptosis in goat rumen epithelial cells.

Table 2 Apoptosis rate of ruminal epithelial cells with apoptosis induced by H_2O_2 ($n = 3$), %

Items	H_2O_2 concentration (μmol/L)	Late apoptosis	Early apoptosis	P-value
	0 (Control)	1.60	5.03	<0.01
	100	8.35	5.38	<0.01
	400	9.33	3.76	<0.01
	800	4.19	10.57	<0.01

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

Figure 1 [Figure 1: see original paper] shows the flow cytometry results of H_2O_2-induced apoptosis in rumen epithelial cells.

2.2 Effects of Gln and Its Dipeptides on Apoptosis Rate

Table 3 shows that compared with the control group, group 4 exhibited a significant increase in late apoptosis rate ($P < 0.05$). Groups 1 and 3 showed increased late apoptosis, while group 2 decreased, but differences were not significant ($P > 0.05$). All treatment groups showed significantly increased early apoptosis rates compared with the control group ($P < 0.05$), with group 2 being the lowest among treatment groups. These results suggest that Gly-Gln can alleviate H_2O_2-induced early apoptosis in goat rumen epithelial cells.

Table 3 Effects of Gln and its dipeptides on apoptosis ratio of ruminal epithelial cells with apoptosis induced by H_2O_2 ($n = 3$), %

Items	Late apoptosis	Early apoptosis
Control group	4.10	2.82
Group 1	4.19	10.57
Group 2	2.09	18.17
Group 3	5.71	35.09
Group 4	17.88	11.37
P-value	<0.01	<0.01

Figure 2 [Figure 2: see original paper] presents the flow cytometry results showing how Gln and its dipeptides affect H_2O_2-induced apoptosis in rumen epithelial cells.

2.3 FQ-PCR Detection Results

2.3.1 Total RNA Extraction Total RNA extraction results are shown in Figure 3 [Figure 3: see original paper]. The extracted total RNA showed clear bands and was suitable for FQ-PCR analysis.

Figure 3 [Figure 3: see original paper] Electrophoretogram of partial total RNA samples.

2.3.2 FQ-PCR Results Table 4 shows that compared with the control group, Bax expression increased significantly while Bcl-2 expression decreased significantly in treatment groups ($P < 0.05$). Compared with group 1, Bax expression in the control group and groups 2, 3, and 4 decreased significantly ($P < 0.05$). Bcl-2 expression in groups 2 and 4 increased significantly ($P < 0.05$). The Bcl-2/Bax ratio in groups 2, 3, and 4 increased significantly compared with group 1 ($P < 0.05$), with group 2 being significantly higher than groups 3 and 4 ($P < 0.05$). All treatment groups had significantly lower Bcl-2/Bax ratios than the control group ($P < 0.05$).

Table 4 Effects of Gln and its dipeptides on Bcl-2 and Bax gene expressions in H_2O_2-induced apoptotic rumen epithelial cells (n = 3)

Items	Bcl-2	Bax	Bcl-2/Bax
Control group	0.95	1.04	0.28
Group 1	1.09	3.56	0.33
Group 2	1.82	1.64	0.39
Group 3	2.62	1.82	0.45
Group 4	1.64	1.82	0.18
SEM	0.18	0.04	0.03
P-value	<0.01	<0.01	<0.01

Discussion

3.1 Effects of Gln and Its Dipeptides on H_2O_2-Induced Apoptosis Rates

High oxygen concentrations directly damage rumen epithelial cells, promoting apoptosis or exacerbating gastric diseases. Numerous oxidative free radicals constitute a primary cause of cellular damage, with ischemia-reperfusion, drug metabolism, and heavy metal poisoning inducing substantial free radical production [17]. H_2O_2, as a major component of oxidative free radicals, has been widely used in research [18-20]. H_2O_2 causes cellular damage through multiple mechanisms, including mitochondrial injury and ATP depletion, oxidation of intracellular proteins and lipids, DNA damage, and induction of apoptosis [21].

Gln and its dipeptides are non-essential amino acids with specific pharmacological properties and no toxic side effects [22]. They provide nitrogen sources for amino acid and protein synthesis in rapidly dividing tissues such as gastrointestinal epithelial and immune cells [23-24], playing crucial roles in maintaining mucosal cell integrity and functional stability [25]. Research has shown that

Gln and its dipeptides can reduce peroxidative damage during disease or stress states, likely by decreasing apoptosis under oxidative stress, enhancing antioxidant enzyme defense, increasing heat shock protein expression, and inducing autophagy [26]. Although Gln and its dipeptides have been research hotspots in surgical studies over the past two decades and have achieved widespread clinical application [27-31], their effects on H_2O_2-induced apoptosis in goat rumen epithelial cells remain poorly documented.

Reducing H_2O_2-induced apoptosis rates is crucial for alleviating oxidative stress damage and improving physiological function. This study detected apoptosis rates in passaged goat rumen epithelial cells, and flow cytometry results demonstrated that Gly-Gln supplementation alleviated H_2O_2-induced oxidative stress damage. The underlying mechanisms may include: (1) As a Gln alternative, Gly-Gln offers advantages including lower required dosage, higher absorption efficiency, better water solubility, greater stability, and reduced production of harmful substances (pyroglutamic acid and ammonia), which may help increase and maintain GSH content in goat rumen epithelial tissues, thereby exerting stronger antioxidant and anti-apoptotic effects [32-33]. (2) Gly-Gln may more effectively inhibit reactive oxygen species translocation compared to Gln and Ala-Gln. Since reactive oxygen species directly or indirectly induce apoptosis in goat rumen epithelial cells, Gly-Gln can partially mitigate H_2O_2-induced apoptosis. However, the specific mechanisms by which Gln, Ala-Gln, and Gly-Gln alleviate oxidative stress in goat rumen epithelial cells require further investigation.

3.2 Effects of Gln and Its Dipeptides on Bcl-2 and Bax Gene Expression

Research indicates that the ratio of pro-apoptotic (Bax) to anti-apoptotic (Bcl-2) genes in the Bcl-2 family determines the opening degree of mitochondrial permeability transition pores (MPTP), forming a regulatory hub for apoptosis. Consequently, the Bcl-2/Bax ratio is considered a "rheostat" that controls cell death [34]. Under internal and external stimuli, the balance between Bcl-2 and Bax determines cell fate, with the Bcl-2/Bax ratio being a critical factor in apoptosis initiation and progression [35].

Based on cell culture experiments, this study employed FQ-PCR to examine how Gln and its dipeptides affect Bcl-2 and Bax expression in H_2O_2-induced apoptotic goat rumen epithelial cells. The results demonstrate that H_2O_2 can induce apoptosis in these cells, while Gly-Gln reduces pro-apoptotic Bax expression and increases anti-apoptotic Bcl-2 expression, consistent with apoptosis rate findings. However, the mechanisms underlying how Gln, Gly-Gln, and Ala-Gln influence Bcl-2 and Bax expression remain unclear and warrant further investigation. These effects may be attributed to increased GSH content [36] or enhanced cell membrane stability that reduces H_2O_2-induced peroxidative damage [37]. The significant increase in Bcl-2 expression observed with Gly-Gln and Ala-Gln supplementation may represent a compensatory response. When

extensive membrane structures are damaged and cannot maintain Bcl-2 protein structure, the compensatory response may be insufficient. Gly-Gln and Ala-Gln supplementation may reduce membrane damage, stabilize membrane-associated Bcl-2 protein structure, and promote compensatory responses.

In conclusion, Gly-Gln demonstrates protective effects against H_2O_2-induced early apoptosis in goat rumen epithelial cells.

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

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