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 Bcl-2 and Bax gene expression levels in apoptotic cells. Passaged rumen epithelial cells from 60-day-old Xiangdong black goats were cultured with different concentrations [0 (control group), 100, 400, 800 μmol/L] of H2O2, and apoptosis was detected by flow cytometry. The passaged rumen epithelial cells were divided into five groups: the control group and group 1 were supplemented with 0 and 800 μmol/L H2O2, respectively; groups 2, 3, and 4 were all supplemented with 800 μmol/L H2O2 along with 17.28 mmol/L Gly-Gln (group 2), 16.0 mmol/L Gln (group 3), and 16.0 mmol/L Ala-Gln (group 4), respectively. Apoptosis was detected by flow cytometry, and Bcl-2 and Bax gene expression levels were determined by real-time fluorescent quantitative PCR (FQ-PCR). The results showed: 1) Compared with the control group, when the H2O2 concentration increased to 800 μmol/L, the early apoptosis rate increased significantly ($P < 0.05$), while the late apoptosis rate exhibited an initial increase followed by a decrease with increasing H2O2 concentration, but both were significantly increased relative to the control group ($P < 0.05$). 2) Compared with the control group, the late apoptosis rate in group 4 increased significantly ($P < 0.05$), and the early apoptosis rates in all treatment groups increased significantly ($P < 0.05$). 3) Compared with the control group, the Bcl-2/Bax ratio in all treatment groups increased significantly ($P < 0.05$); compared with group 1, the Bcl-2/Bax ratio in groups 2, 3, and 4 increased significantly ($P < 0.05$), and group 2 was significantly higher than groups 3 and 4 ($P < 0.05$). In conclusion, Gly-Gln exerts a certain protective effect against H2O2-induced early apoptosis

in goat rumen epithelial cells.

Full Text

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

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Abstract: This study established an apoptosis model for subcultured goat ruminal epithelial cells induced by hydrogen peroxide (H_2O_2) 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. Subcultured ruminal epithelial cells from 60-day-old Xiangdong black goats were cultured with different concentrations [0 (control), 100, 400, and 800 μmol/L] of H_2O_2, and apoptosis was detected by flow cytometry. The 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 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 detected by flow cytometry, and Bcl-2 and Bax gene expression levels were measured by real-time fluorescent quantitative PCR (FQ-PCR). The results showed that: (1) Compared with the control group, the early apoptosis rate increased significantly when H_2O_2 concentration reached 800 μmol/L ($P < 0.05$), while 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, the late apoptosis rate in group 4 increased significantly ($P < 0.05$), and the early apoptosis rates in all treatment groups increased significantly ($P < 0.05$). (3) Compared with the control group, the Bcl-2/Bax ratio in all treatment groups increased significantly ($P < 0.05$); compared with group 1, the Bcl-2/Bax ratio in groups 2, 3, and 4 increased significantly ($P < 0.05$), with group 2 being significantly higher than groups 3 and 4 ($P < 0.05$). In conclusion, Gly-Gln exerts a protective effect against early apoptosis induced by H_2O_2 in goat ruminal epithelial cells.

Keywords: Xiangdong black goat; ruminal epithelial cell; apoptosis; apoptosis

gene

Oxidative stress (OS) is a common physiological condition in weaned livestock and represents a major cause of production losses in the industry. Although intensive livestock production has advanced considerably in recent years, the rapid post-weaning decline and subsequent recovery in feed intake leads to substantial fluctuations in gastrointestinal blood flow, creating an "ischemia-reperfusion" phenomenon accompanied by large amounts of hydrogen peroxide (H_2O_2) production. Through Fenton and Haber-Weiss reactions, H_2O_2 generates numerous oxygen free radicals that damage gastrointestinal tissue cells. 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 cellular antioxidant defense systems, placing weaned livestock in a state of oxidative stress.

H_2O_2 induces oxidative stress in cells, triggering the intrinsic or mitochondrial apoptosis pathway. Pro-apoptotic proteins of the Bcl-2 family, such as Bax and Bak, become activated and translocate to the mitochondrial membrane, while anti-apoptotic Bcl-2 proteins form heterodimers with these activated pro-apoptotic proteins to inhibit mitochondrial membrane damage. Furthermore, Bcl-2 can suppress cytochrome C release and prevent the formation of complexes between deoxyadenosine triphosphate (dATP) and apoptosis protease activating factor-1 (Apaf-1) in the cytoplasm, thereby inhibiting caspase-9 activation and subsequent downstream activation of caspases-3 and -7, ultimately preventing apoptosis.

As intensive livestock production and animal health management practices mature, deeper understanding of practical production problems has emerged. Consequently, investigating 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 antioxidant substances.

Dietary supplementation with appropriate amounts of Gln, alanyl-glutamine (Ala-Gln), and glycyl-glutamine (Gly-Gln) provides protective effects against oxidative stress damage. However, Gln has not been widely applied in practical production due to its high required dosage, low absorption rate, poor water solubility, instability, and tendency to produce harmful substances (pyroglutamic acid and ammonia) during feed processing. As understanding of dipeptides and gastrointestinal absorption characteristics improves, Ala-Gln and Gly-Gln have emerged as Gln donors and alternatives that overcome these limitations. Moreover, Ala-Gln and Gly-Gln have become research hotspots in human nutrition, with mature studies in clinical total parenteral nutrition (TPN), providing theoretical support for their broad application in feed production.

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

1.1 Main Reagents and Equipment

Cell culture reagents including fetal bovine serum (FBS), Dulbecco' s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), 0.25% trypsin + 0.02% EDTA, and penicillin were purchased from Gibco. Gentamicin/amphotericin B solution was obtained from Thermo. Gln, Gly-Gln, and Ala-Gln were acquired 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 dNTP 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 from Eppendorf, real-time fluorescent quantitative PCR (FQ-PCR) instrument from Thermo, electrophoresis apparatus from Bio-Rad, horizontal agarose electrophoresis tank from Beijing Liuyi Instrument Factory, precision pH meter from Leici Instrument Factory, and electric glass homogenizer from Ningbo Xinzhi Biotechnology.

1.2 Main Solutions and Preparation

Main 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 was prepared by diluting H_2O_2 with sterile, mold-free water to final concentrations of 100, 400, and 800 μmol/L.

1.3 Goat Ruminal Epithelial Subculture Cells

Three healthy 60-day-old Xiangdong black goats weighing (6.4 ± 0.8) kg were used. Animals were euthanized by jugular exsanguination, and rumen tissues were collected, emptied, and rinsed repeatedly with physiological saline for sample preparation.

When primary goat ruminal epithelial cells reached 80-90% confluence, DMEM/F12 complete medium [containing 5% FBS, 10% double antibiotics, 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 digestion solution containing 0.25% trypsin + 0.02% EDTA was added. After 2-3 min incubation at 37°C with 5% CO_2, cells were observed under an inverted microscope. When cells began to brighten and round up, 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. The supernatant was discarded, and cells were resuspended in 1 mL DMEM/F12 complete medium for subculturing at a 1:2 ratio. After 30 min incubation at 37°C with 5% CO_2, the cell-containing DMEM/F12 medium was transferred to culture dishes for continued culture. This purification step was repeated once.

1.4.1 Apoptosis Rate of H_2O_2-Induced Ruminal Epithelial Cells

Passage 4 cells in logarithmic growth phase were digested and seeded in 6-well plates at 1$×10^{6}$ cells/well. After 12 h of culture, the 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 Gene Expression in H_2O_2-Induced Apoptotic Ruminal Epithelial Cells

Cell selection and pretreatment were identical to section 1.4.1. 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 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).

1.5.1 Detection of Ruminal Epithelial Cell Apoptosis Rate by Flow Cytometry (Annexin V/PI Double Staining)

Subcultured goat ruminal epithelial cells were cultured in DMEM/F12 containing 5% FBS. After 24 h, 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 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 [AV-fluorescein isothiocyanate (FITC) apoptosis detection kit] was added, mixed, followed by 5 μL of propidium iodide (PI), and mixed again; (5) Cells were incubated at room temperature in the dark for 5-15 min; (6) Samples were analyzed on a BD FACSCalibur flow cytometer within 1 h.

1.5.2 Effects of Gln and Its Dipeptides on Bcl-2 and Bax Gene Expression in H_2O_2-Induced Apoptotic Ruminal Epithelial Cells

1.5.2.1 Total RNA Extraction **Preparation:** All instruments and consumables for RNA extraction were soaked overnight in freshly prepared 1% DEPC water, wrapped in newspaper, and autoclaved at 121°C for 60 min.

Trizol extraction of total RNA: (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, vortexed, 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 an equal volume of isopropanol, and stored at -20°C for 20 min; (3) Samples were centrifuged at 12,000×g for 15 min at low temperature, the supernatant was discarded, and 1 mL of 75% ethanol (prepared with sterile 1% DEPC water) was added to the pellet, vortexed, and mixed; (4) Samples were centrifuged at 12,000×g for 5 min at low temperature, the supernatant was discarded, and the pellet was air-dried for 5-10 min; 40 µL of sterile 1% DEPC water was added to dissolve the pellet to obtain total RNA solution; (5) Total RNA concentration was measured using a UV spectrophotometer: 2 µL of total RNA solution was diluted to 100 µL with nuclease-free water, absorbance was measured at 260 and 280 nm, and concentration and purity (required to reach 1.8-2.0) were calculated using the following formulas:

RNA concentration (ng/µL) = A_{260} × dilution factor × 40
RNA purity = A_{260}/A_{280}

Agarose gel electrophoresis of RNA: 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 pre-treated with sterile 1% DEPC water. Two microliters of extracted total RNA were mixed with loading buffer at a 1:5 ratio and electrophoresed at 170 V constant voltage until the bromophenol blue front migrated to 2/3 of the gel length. The gel was visualized under a gel imaging system.

Reverse transcription PCR: The reverse transcription PCR system (30 µL) 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 searched in NCBI, 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-CTGAACCG; R: GACAGCCAGGA-GAAATCAAACA	-

Genes	NCBI accession No.	Primer sequences (5' -3')	Product length/bp
GAPDH	JN036558	F: AGTGGCGGCT-GAAATGTT; R: TCAGCACCAGCAT-CACCC	-

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

FQ-PCR 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 statistical significance defined at $P < 0.05$ and non-significance at $P > 0.05$.

2.1 Apoptosis Rate of H_2O_2-Induced Ruminal Epithelial Cells

As shown in Table 2 , compared with the control group, the early apoptosis rate increased significantly when H_2O_2 concentration reached 800 µmol/L ($P < 0.05$). The 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 induce aggravated apoptosis in goat ruminal 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)	1.60d	5.03b	<0.01
	100	8.35b	5.38b	<0.01
	400	9.33a	3.76c	<0.01
	800	4.19c	10.57a	<0.01

Values in the same row with different small letter superscripts mean significant difference ($P < 0.05$), while with the same small letter superscripts mean no significant difference ($P > 0.05$). The same as below.

Figure 1 [**Figure 1: see original paper**] FCM results of ruminal epithelium cells with apoptosis induced by H_2O_2

2.2 Effects of Gln and Its Dipeptides on Apoptosis Rate of H_2O_2-Induced Ruminal Epithelial Cells

As shown in Table 3 , compared with the control group, the late apoptosis rate in group 4 increased significantly ($P < 0.05$), while groups 1 and 3 showed increases and group 2 showed a decrease, though these differences were not significant ($P > 0.05$). Compared with the control group, early apoptosis rates in all treatment groups increased significantly ($P < 0.05$), with group 2 showing the lowest rate among treatment groups. These results demonstrate that Gly-Gln can alleviate early apoptosis caused by H_2O_2 in goat ruminal 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	P-value
Control group	4.10bc	2.82d	<0.01
Group 1	4.19bc	10.57c	<0.01
Group 2	2.09c	18.17b	<0.01
Group 3	5.71b	35.09a	<0.01
Group 4	17.88a	11.37c	<0.01
SEM	0.89	0.99	

Figure 2 [**Figure 2: see original paper**] FCM results of Gln and its dipeptides affected ruminal epithelial cells with apoptosis induced by H_2O_2

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**] The electrophoretogram of partial total RNA

2.3.2 FQ-PCR Detection Results

As shown in Table 4 , 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 ($P < 0.05$); and the Bcl-2/Bax ratio in groups 2, 3, and 4 increased significantly ($P < 0.05$), with group 2 being significantly higher

than groups 3 and 4 ($P < 0.05$). The treatment groups showed significantly lower ratios than the control group ($P < 0.05$).

Table 4 Effects of Gln and its dipeptides on Bcl-2 and Bax gene expressions of ruminal epithelial cells with apoptosis induced by H_2O_2 (n=3)

Items	Bcl-2	Bax	Bcl-2/Bax	SEM	P-value
Control group	0.95d	1.04a	0.28d	0.18	<0.01
Group 1	1.09a	3.56a	0.33b	0.04	<0.01
Group 2	1.82c	1.64c	0.59b	0.03	<0.01
Group 3	2.62b	1.82c	0.39cd	0.03	<0.01
Group 4	1.09a	2.62b	0.45c	0.03	<0.01

3.1 Effects of Gln and Its Dipeptides on Apoptosis Rate of H_2O_2-Induced Apoptotic Cells

High oxygen concentrations directly damage ruminal epithelial cells, promoting apoptosis or exacerbating gastric diseases. Numerous oxidative free radicals constitute a major cause of cellular damage, with ischemia-reperfusion, drug metabolism, and heavy metal poisoning inducing massive free radical production. H_2O_2, as a primary component of oxidative free radicals, has been widely used in research. 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.

Gln and its dipeptides are non-essential amino acids with special pharmacological properties and no toxic side effects. They provide nitrogen sources for amino acid and protein synthesis in rapidly dividing tissues such as gastrointestinal epithelial and immune cells, playing crucial roles in maintaining mucosal cell integrity and functional stability. Research has shown that Gln and its dipeptides can reduce oxidative damage during disease or stress states, possibly by decreasing apoptosis under oxidative stress, enhancing antioxidant enzyme defense, increasing heat shock protein expression, and inducing autophagy. Although Gln and its dipeptides have been research hotspots in surgical studies over the past two decades and have gained extensive clinical application, their effects on H_2O_2-induced apoptosis in goat ruminal epithelial cells remain poorly documented.

Reducing apoptosis rates in H_2O_2-induced cells is crucial for alleviating oxidative stress damage and improving organismal function. This study detected apoptosis rates in subcultured goat ruminal 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 with advantages including lower required dosage, higher absorption rate, better water solubility, greater stability, and reduced production

of harmful substances (pyroglutamic acid and ammonia), Gly-Gln more effectively increases and maintains GSH content in goat ruminal epithelial tissues, conferring stronger antioxidant and anti-apoptotic effects. (2) Gly-Gln may inhibit reactive oxygen species translocation more effectively than Gln and Ala-Gln, thereby mitigating H_2O_2-induced apoptosis since reactive oxygen species directly or indirectly trigger cell death. However, the specific mechanisms by which Gln, Ala-Gln, and Gly-Gln alleviate oxidative stress in goat ruminal epithelial cells require further investigation.

3.2 Effects of Gln and Its Dipeptides on Bcl-2 and Bax Gene Expression in H_2O_2-Induced Apoptotic Goat Ruminal Epithelial Cells

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. 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 severity.

This study examined apoptosis mechanisms and utilized FQ-PCR to detect Bcl-2 and Bax expression in H_2O_2-induced apoptotic goat ruminal epithelial cells treated with Gln and its dipeptides. Results showed that H_2O_2 induced apoptosis in goat ruminal epithelial cells, while Gly-Gln decreased pro-apoptotic Bax expression and increased anti-apoptotic Bcl-2 expression, consistent with apoptosis rate findings. However, the mechanisms by which Gln, Gly-Gln, and Ala-Gln affect Bcl-2 and Bax expression remain unclear and require further study. These effects may be attributed to increased GSH content or enhanced cell membrane stability that reduces H_2O_2-induced oxidative damage. The significant increase in Bcl-2 expression in H_2O_2-induced cells treated with Gly-Gln and Ala-Gln dipeptides may represent a compensatory response. When extensive membrane structures are damaged and cannot maintain Bcl-2 protein structure, the compensatory response becomes insufficient; supplementation with Gly-Gln and Ala-Gln dipeptides reduces membrane damage and helps stabilize Bcl-2 protein structure on membranes, promoting compensatory responses.

In conclusion, Gly-Gln exerts a protective effect against early apoptosis induced by H_2O_2 in goat ruminal epithelial cells.

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