Effects of Glutamine on Hydrogen Peroxide-Induced Oxidative Stress, Injury, and Apoptosis in Human Colon Cancer HT-29 Cells: Postprint

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Abstract

This study aimed to elucidate the antioxidant effects and mechanism of action of glutamine by investigating its influence on hydrogen peroxide (H2O2)-induced oxidative stress injury and apoptosis in human colon cancer HT-29 cells. HT-29 cells were treated with 0 (control group), 0.5, 2.0, or 10.0 mmol/L glutamine in combination with 0.35 mmol/L H2O2 for 12, 24, or 32 h. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content of the cells were measured, quantitative real-time PCR was employed to analyze the relative mRNA expression levels of apoptosis-related genes induced by H2O2, and Annexin V-FITC/PI double staining was used to stain HT-29 cells, with flow cytometry detecting the apoptotic status of the cells. The results showed: 1) After 24 h of treatment, the SOD activity in the 0.5 and 2.0 mmol/L Gln groups was significantly higher than that in the control group ($P<0.05$); after 32 h of treatment, the SOD activity in the 0.5 and 2.0 mmol/L Gln groups was significantly higher than that in the control group ($P<0.05$), while the MDA content was significantly lower than that in the control group ($P<0.05$). 2) After 12 h of treatment, there were no significant differences in the relative mRNA expression levels of Caspase-3 and B-cell lymphoma-2-associated X protein (Bax) among all groups ($P>0.05$). Compared with the control group, the relative mRNA expression level of nuclear factor kappa B (NF- B) in the 0.5 mmol/L Gln group was significantly decreased ($P<0.05$), and the relative mRNA expression levels of B-cell lymphoma-2 (Bcl-2) in the 0.5 and 2.0 mmol/L Gln groups were significantly increased ($P<0.05$). After 24 h of treatment, compared with the control group, the relative mRNA expression levels of Caspase-3, NF- B, and Bax in the 0.5, 2.0, and 10.0 mmol/L Gln groups were all significantly decreased ($P<0.05$), while the relative mRNA expression level of Bcl-2 was significantly increased ($P<0.05$). After 32 h of treatment, compared with the control group, the relative mRNA expression levels of fatty acid synthase (FAS), Caspase-3, NF- B,

and Bax in the 2.0 mmol/L Gln group were all significantly decreased (P<0.05), while the relative mRNA expression level of Bcl-2 was significantly increased (P<0.05); in the 10.0 mmol/L Gln group, the relative mRNA expression levels of FAS, Caspase-3, NF- B, and Bax were all significantly increased (P<0.05). 3) After 24 h of treatment, compared with the control group, glutamine treatment increased the number of viable cells by 5.32%~11.97% and decreased the number of necrotic cells by 6.75%~12.66%. After 32 h of treatment, compared with the control group, glutamine treatment increased the number of viable cells by 1.39%~7.63% and decreased the number of necrotic cells by 3.40%~4.57%. These results demonstrate that glutamine can inhibit oxidative stress responses and reduce H2O2-induced apoptosis in HT-29 cells.

Full Text

Effects of Glutamine on Oxidative Damage and Apoptosis in Human Colon Cancer HT-29 Cells Induced by Hydrogen Peroxide

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Abstract: This study investigated the antioxidant effects of glutamine (Gln) and its underlying mechanisms in hydrogen peroxide (H_2O_2)-induced oxidative stress in human colon cancer HT-29 cells. HT-29 cells were treated with 0 (control), 0.5, 2.0, or 10.0 mmol/L Gln combined with 0.35 mmol/L H_2O_2 for 12, 24, or 32 hours. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured, apoptosis-related gene expression was analyzed by quantitative real-time PCR, and cell apoptosis was assessed by Annexin V-FITC/PI double staining followed by flow cytometry. The results showed: (1) After 24 hours, SOD activity in the 0.5 and 2.0 mmol/L Gln groups was significantly higher than in the control group (P<0.05). After 32 hours, SOD activity in the 0.5 and 2.0 mmol/L Gln groups remained significantly elevated (P<0.05), while MDA content was significantly reduced (P<0.05). (2) At 12 hours, no significant differences in Caspase-3 or Bax mRNA expression were observed among groups (P>0.05). Compared with the control, 0.5 mmol/L Gln significantly decreased NF- B mRNA expression (P<0.05), while 0.5 and 2.0 mmol/L Gln significantly increased Bcl-2 mRNA expression (P<0.05). At 24 hours, 0.5, 2.0, and 10.0 mmol/L Gln significantly decreased Caspase-3, NF- B, and Bax mRNA expression (P<0.05) and significantly increased Bcl-2 mRNA expression (P<0.05). At 32 hours, 2.0 mmol/L Gln significantly decreased FAS, Caspase-3,

NF- B, and Bax mRNA expression (P<0.05) and significantly increased Bcl-2 mRNA expression (P<0.05), whereas 10.0 mmol/L Gln significantly increased FAS, Caspase-3, NF- B, and Bax mRNA expression (P<0.05). (3) At 24 hours, Gln treatment increased viable cell numbers by 5.32%–11.97% and decreased necrotic cell numbers by 6.75%–12.66% compared with the control. At 32 hours, Gln increased viable cells by 1.39%–7.63% and decreased necrotic cells by 3.40%–4.57%. These findings demonstrate that glutamine can inhibit oxidative stress and reduce H_2O_2-induced apoptosis in HT-29 cells.

Keywords: glutamine; oxidative damage; cell apoptosis; HT-29 cells; hydrogen peroxide

Introduction

Glutamine (Gln) is a conditionally essential amino acid that promotes protein synthesis and serves as a bioactive substance in intestinal epithelium. It acts as a signaling molecule that regulates signaling pathways and plays a crucial role in maintaining intestinal structural integrity and preventing systemic circulation of harmful luminal microorganisms [1]. Hubert-Buron et al. [2] reported that Gln can reduce inflammatory responses in intestinal cells by limiting the hydrolysis of inhibitor Bα (I Bα). Yan et al. [3] demonstrated that the therapeutic effects of Gln on chemical liver injury are associated not only with its antioxidant and anti-inflammatory properties but also with indirect hepatoprotection through improved intestinal function. Using scanning electron microscopy, Zheng et al. [4] observed that Gln supplementation protected against radiation-induced intestinal damage in rats, showing that while radiation caused disorganized, narrow, and irregular villi with extensive ulceration, Gln-supplemented animals exhibited broad, blunt villi with normal morphology and minimal ulceration.

Oxidative stress results from an imbalance between oxidation and antioxidant systems, leading to the production of damaging oxidative intermediates that cause cellular injury, aging, and malignant diseases such as cancer. Reactive oxygen species act as signaling molecules in cell death pathways, triggering mitochondrial release of pro-apoptotic molecules, activating the cysteinyl aspartate-specific proteinase (Caspase) cascade, and regulating apoptosis. Two major apoptotic pathways have been identified: (1) extracellular signals activating intracellular Caspase family proteins, and (2) mitochondria releasing apoptotic factors that activate Caspases, which then degrade essential cellular proteins to induce apoptosis. Oxidative stress can inhibit intestinal cell growth or cause cell death, while Gln can inhibit apoptosis in small intestinal mucosal cells [5]. Evans et al. [6] showed that Gln dose-dependently inhibited apoptosis in rat colonic epithelial cells, and this effect was specific to Gln, as glutamate (Glu), cysteine (Cys), and glycine (Gly) had no such effect. Hydrogen peroxide (H_2O_2) can oxidize mammalian cells, generating oxygen free radicals that damage cellular components [7]. These free radicals attack DNA double helix structures,

causing structural alterations that impair normal cellular function and may ultimately lead to apoptosis [8]. Previous studies have shown that 0.5, 2.0, and 5.0 mmol/L Gln significantly reduced H_2O_2-induced apoptosis in intestinal porcine epithelial cells (IPEC-1), decreasing dead cell numbers by 14.2%, 54.4%, and 95.4%, respectively [9]. Therefore, this study aimed to investigate the antioxidant function of Gln and elucidate its potential mechanisms by examining its effects on antioxidant capacity, apoptosis-related gene expression, and apoptotic status in H_2O_2-induced oxidative stress in human colon cancer HT-29 cells.

Materials and Methods

1.1 Experimental Materials The HT-29 cell line was obtained from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. RPMI1640 medium, DMEM medium (containing 4,500 mg/L D-glucose and 110 mg/L sodium pyruvate, without L-Gln), 0.25% trypsin cell digestion solution, Gln, and fetal bovine serum were purchased from GIBCO (Gaithersburg, USA). Trizol reagent, agarose, reverse transcriptase, and real-time quantitative PCR kits were obtained from Takara (Dalian, Japan). Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Six-well cell culture plates (Corning, USA) and Annexin V-FITC/PI dual fluorescence apoptosis detection kits were obtained from Lianke Biotechnology Co., Ltd.

1.2 Cell Culture HT-29 cells were routinely cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. Cells were passaged upon reaching 80% confluence, and all experiments were performed using cells in the exponential growth phase.

1.3 Gln and H_2O_2 Treatment of HT-29 Cells HT-29 cells were seeded in six-well plates and grown to 80% confluence. The original medium was removed, and cells were cultured in Gln-free DMEM for 24 hours before treatment with Gln and H_2O_2 to final concentrations of 0 (control), 0.5, 2.0, or 10.0 mmol/L Gln and 0.35 mmol/L H_2O_2. Cells were harvested after 12, 24, or 32 hours for subsequent analyses.

1.4 Biochemical Assays

1.4.1 Cell Lysis After treatment with Gln and H_2O_2 for 12, 24, or 32 hours, the culture medium was discarded and cells were washed three times with ice-cold physiological saline. Cells were scraped into 2.0 mL EP tubes with 0.3 mL ice-cold saline, rinsed with an additional 0.2 mL saline, and lysed by sonication on ice (10-second pulses with 10-second intervals, repeated three times). The lysates were centrifuged at 5,000 r/min for 10 minutes at 4°C, and the supernatants were collected for analysis.

1.4.2 MDA Content Measurement MDA content was determined based on the principle that MDA, a lipid peroxidation degradation product, reacts with thiobarbituric acid to form a red product with maximum absorbance at 532 nm. Procedures followed the kit manufacturer' s instructions.

1.4.3 SOD Activity Measurement SOD activity was measured using a xanthine-xanthine oxidase system to generate superoxide anion radicals, which react with hydroxylamine to form nitrite. In the presence of a chromogenic agent, nitrite produces a purple-red color measurable by spectrophotometry. Samples containing SOD specifically inhibit superoxide anion radicals, reducing nitrite formation and resulting in lower absorbance compared with control tubes, allowing calculation of SOD activity. Procedures followed the kit manufacturer' s instructions.

1.5 Total RNA Extraction and cDNA Preparation Total RNA was extracted using the Trizol method [10] and reverse-transcribed using a Super-Script™ II RTase kit.

1.6 Quantitative Real-Time PCR

1.6.1 Primer Design and Synthesis Primers were designed using Primer Premier 6.0 and Beacon Designer software and synthesized by Shanghai Bio-engineering Co., Ltd. Primer sequences for target genes [fatty acid synthase (FAS), Caspase-3, nuclear factor kappa B (NF- B), B-cell lymphoma-2 (Bcl-2), and Bcl-2-associated X protein (Bax)] and 18S rRNA are listed in Table 1 .

1.6.2 qPCR Amplification System and Conditions Reverse-transcribed cDNA was amplified using SYBR® Premix Ex Taq™ (Perfect Real Time) in a 25 µL reaction volume on a CFX384 real-time PCR system (Bio-Rad, USA). Optimal cycling conditions were: 95°C for 1 minute; 45 cycles of 95°C for 10 seconds and 62°C for 25 seconds; followed by a single-peak melting curve analysis. Each sample was run in triplicate, and the mean Ct value was used for calculating relative mRNA expression.

1.7 Flow Cytometry Analysis HT-29 cells were double-stained with Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry using emission wavelengths of 530 nm (FITC) and 585 nm (PI). Bivariate scatter plots were used for analysis: the lower left quadrant represents viable cells ($FITC^-/PI^-$); upper left, necrotic cells ($FITC^-/PI^+$); lower right, early apoptotic cells ($FITC^+/PI^-$); and upper right, late apoptotic cells ($FITC^+/PI^+$). Total apoptosis rate was calculated as the sum of cells in the upper right and lower right quadrants [11].

1.8 Statistical Analysis All data were analyzed using SPSS v16.0 software and expressed as means ± standard error. Differences were considered significant at $P<0.05$.

Results

2.1 Effects of Gln on H_2O_2-Induced Oxidative Status Changes As shown in Figure 1 [Figure 1: see original paper], no significant differences in SOD activity or MDA content were observed among groups at 12 hours ($P>0.05$). After 24 hours, SOD activity in the control group was significantly lower than in the 0.5 and 2.0 mmol/L Gln groups ($P<0.05$), while MDA content showed no significant differences among groups ($P>0.05$).

At 32 hours, SOD activity in the 0.5, 2.0, and 10.0 mmol/L Gln groups increased by 46.40% ($P<0.05$), 67.99% ($P<0.05$), and 19.71% ($P>0.05$), respectively, compared with the control group. Correspondingly, MDA content decreased by 17.79% ($P<0.05$), 37.66% ($P<0.05$), and 13.09% ($P>0.05$), respectively.

2.2 Effects of Gln on H_2O_2-Induced Apoptosis-Related Gene Expression As shown in Figure 2 [Figure 2: see original paper], treatment with 0.35 mmol/L H_2O_2 for 12, 24, and 32 hours consistently increased pro-apoptotic gene expression (FAS, Caspase-3, NF- B, Bax) in a pattern of initial decrease followed by increase, while the anti-apoptotic gene Bcl-2 showed the opposite trend.

At 12 hours, no significant differences in Caspase-3 or Bax mRNA expression were observed among groups ($P>0.05$). Compared with the control, 0.5 mmol/L Gln significantly decreased NF- B mRNA expression ($P<0.05$), while 0.5 and 2.0 mmol/L Gln significantly increased Bcl-2 mRNA expression ($P<0.05$).

At 24 hours, 0.5, 2.0, and 10.0 mmol/L Gln significantly decreased Caspase-3, NF- B, and Bax mRNA expression ($P<0.05$) and significantly increased Bcl-2 mRNA expression ($P<0.05$) compared with the control. Specifically, 2.0 mmol/L Gln decreased FAS and Caspase-3 mRNA expression by 62.27% and 71.13% ($P<0.05$), respectively, while 0.5 mmol/L Gln decreased NF- B and Bax mRNA expression by 64.85% and 65.05% ($P<0.05$), respectively. Bcl-2 mRNA expression increased by 1,404.65%, 1,559.21%, and 623.76% in the 0.5, 2.0, and 10.0 mmol/L Gln groups, respectively ($P<0.05$).

At 32 hours, 2.0 mmol/L Gln significantly decreased FAS, Caspase-3, NF- B, and Bax mRNA expression by 63.55%, 45.11%, 41.49%, and 30.22% ($P<0.05$), respectively, and increased Bcl-2 mRNA expression by 44.30% ($P<0.05$). In contrast, 10.0 mmol/L Gln significantly increased FAS, Caspase-3, NF- B, and Bax mRNA expression by 31.78%, 25.83%, 34.77%, and 20.35% ($P<0.05$), respectively, with no significant change in Bcl-2 mRNA expression ($P>0.05$).

2.3 Flow Cytometry Analysis of Apoptosis Flow cytometry analysis following Annexin V-FITC/PI double staining revealed that at 24 hours, viable

cell percentages were 71.37%, 76.69%, 83.34%, and 78.30% in the control, 0.5, 2.0, and 10.0 mmol/L Gln groups, respectively, while necrotic cell percentages were 12.78%, 6.03%, 0.12%, and 1.31%. Gln treatment increased viable cells by 5.32%–11.97% and decreased necrotic cells by 6.75%–12.66%.

At 32 hours, viable cell percentages were 61.73%, 66.70%, 70.36%, and 63.12%, while necrotic cell percentages were 21.61%, 17.57%, 17.04%, and 18.21% in the control, 0.5, 2.0, and 10.0 mmol/L Gln groups, respectively. Gln treatment increased viable cells by 1.39%–7.63% and decreased necrotic cells by 3.40%–4.57%.

Discussion

MDA is one of the most important products of membrane lipid peroxidation, and its content reflects the degree of membrane damage, indirectly indicating impairment to membrane systems. SOD plays a critical role in scavenging free radicals, preventing oxidative damage, and maintaining cellular structure. In this study, we established an oxidative damage model by stimulating HT-29 cells with H_2O_2 and found that Gln increased SOD activity. The H_2O_2-induced control group showed high MDA levels, while treatment with 0.5 and 2.0 mmol/L Gln significantly reduced MDA content, indicating that Gln enhances antioxidant capacity and protects against increased membrane permeability caused by oxidative stress [12].

Apoptosis-related genes FAS, Caspase-3, NF- B, and Bax, along with the anti-apoptotic gene Bcl-2, function antagonistically in oxidative stress-induced apoptosis [6-7]. Our results demonstrate that 0.5 and 2.0 mmol/L Gln decreased expression of pro-apoptotic genes FAS, Caspase-3, NF- B, and Bax while increasing anti-apoptotic Bcl-2 expression in H_2O_2-stressed HT-29 cells.

Haynes et al. [13] noted that due to its chemical properties, Gln cannot directly alter H_2O_2, and intestinal cell apoptosis requires an extended period (e.g., 12 hours) to affect gene expression and signal transduction rather than occurring upon immediate H_2O_2 contact. This delay occurs because H_2O_2 diffusion into cells is limited and occurs via aquaporin homologs [14]. Previous studies have reported that H_2O_2 induces apoptosis by activating oxidative stress regulators Caspase-3 and NF- B [15]. Caspase-3 is a key executioner in the apoptotic pathway, normally present as an inactive zymogen in the cytoplasm. Activation by extracellular apoptotic signals converts it to active Caspase-3, which inactivates critical cytoplasmic, nuclear, and cytoskeletal proteins, leading to apoptosis. Intracellular H_2O_2 activates the FAS gene and increases cytochrome C release from mitochondria, activating Caspase-3 via Caspase-8 and Caspase-9 pathways and causing DNA fragmentation [16]. Additionally, H_2O_2 activates NF- B, increasing expression of “death”-related genes while suppressing “survival”-related genes [17]. Bcl-2 and its homologs prevent mitochondrial membrane damage and release of pro-apoptotic factors like cytochrome C, whereas Bax promotes these events; the Bcl-2/Bax ratio typically serves as an indicator of programmed cell

death [18]. Our findings show that Gln increases the Bcl-2/Bax ratio in oxidatively stressed HT-29 cells, suggesting that Gln inhibits oxidative stress-induced apoptosis through the mitochondrial pathway.

The mechanism by which Gln promotes Bcl-2 expression may involve increased mitochondrial membrane stability or enhanced expression of heat shock protein 70 (HSP70), which subsequently upregulates Bcl-2 [19].

Interestingly, when HT-29 cells were co-cultured with H_2O_2 and high-concentration Gln (10 mmol/L) for 32 hours, pro-apoptotic gene expression (FAS, Caspase-3, NF- B, Bax) increased by 31.78%, 25.83%, 34.77%, and 20.35% compared with the control. Flow cytometry analysis confirmed these results, showing apoptotic cell numbers similar to the control and higher than in the 0.5 and 2.0 mmol/L Gln groups. This indicates that Gln' s anti-apoptotic effects are concentration-dependent. Within a certain range, Gln effectively inhibits oxidative stress-induced apoptosis, but beyond this range, apoptosis rates show no direct linear relationship with Gln concentration, consistent with previous findings [20-21]. This phenomenon may occur because Gln is metabolized by glutaminase to produce glutamate and ammonia (NH_3) [13]. During prolonged culture with high Gln concentrations, NH_3 accumulates to levels that can damage cells.

Conclusion

Glutamine effectively increases SOD activity, decreases MDA content, downregulates pro-apoptotic genes (FAS, Caspase-3, NF- B, Bax), upregulates the anti-apoptotic gene Bcl-2, and reduces oxidative damage and apoptosis in H_2O_2-induced oxidative stress in HT-29 cells.

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