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Glutamine protects cow's ruminal epithelial cells from acid-induced injury *in vitro*

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Abstract: This study was conducted to investigate the effects and mechanisms of glutamine (Gln) on the repair of acid-induced injury in dairy cow ruminal epithelial cells (RECs) *in vitro*. Dairy cow RECs were cultured in a medium with pH of 5.5 for 3 h and subsequently treated with various concentrations of Gln (4, 8, 12, 32 mmol/l) for 12 h. The messenger ribonucleic acid (mRNA) expression levels of occludin (*OCLN*), claudin 1 (*CLDN1*), toll-like receptor 2 (*TLR2*), toll-like receptor 4 (*TLR4*) and genes for inflammatory factors were measured using reverse transcription-quantitative real-time PCR (RT-qPCR). The results showed that cellular activity and *OCLN* expression were significantly highest at 8 mmol/l Gln ($P < 0.05$). *CLDN1* expression was significantly higher at 4 mmol/l Gln compared to the other groups ($P < 0.05$). The relative expression levels of tumour necrosis factor (*TNF*), interleukin 1B (*IL1B*), C-X-C motif chemokine ligand 8 (*CXCL8*), *TLR2* and *TLR4* in the acid treatment group were significantly higher than those in the control group ($P < 0.05$), but they were lower in the Gln-treated groups than in the acid treatment group ($P < 0.05$). These findings demonstrate that Gln promotes the proliferation of RECs, enhances the expression of epithelial cell junction proteins, and inhibits the expression of inflammatory factors and surface receptors. In conclusion, Gln shows a potential for repairing acid-induced injury in RECs.

Key words: bovine; cytokine regulation; gene regulation; RT-qPCR; tight junction integrity

The rumen epithelium in dairy cows serves as a critical barrier within the immune system, preventing harmful substances from entering the bloodstream and ensuring the overall animal health. To meet the high energy requirements of milk production, dairy cows are often fed diets with high carbohydrate content. Large amounts of rapidly fermentable non-structural carbohydrates can lead

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to the accumulation of organic acids in the rumen, resulting in subacute ruminal acidosis (SARA) (Owens et al. 1998; Penner et al. 2011). SARA negatively affects feed intake and milk yield while impairing the digestive and metabolic functions of the rumen. In both subclinical and clinical ruminal acidosis, low pH enhances the accumulation of lactic acid in the rumen, leading to severe damage to the rumen epithelium. When the rumen epithelium is compromised, its permeability increases, allowing harmful substances to enter the blood circulation system and causing a series of diseases such as mastitis, diarrhoea, laminitis, liver abscess and endometrium injury (Steele et al. 2012; Plaizier et al. 2022; Zeng et al. 2023). These issues result in significant economic losses for the dairy industry (Kleen et al. 2003; Plaizier et al. 2008). Garrett et al. (1997) reported that the incidence of SARA in early and mid-lactation dairy cows was between 19% and 26%, leading to an estimated annual economic loss of USD 500 million to USD 1 billion in the U.S. dairy industry (Enemark 2008).

Glutamine (Gln) is a primary energy substance for intestinal epithelial cells (Lacey and Wilmore 1990; Mandir and Goodlad 1999) and it has become a research focus across various disciplines due to its unique and complex physiological functions. Especially under pathological conditions, Gln plays an irreplaceable role in maintaining the structural and functional integrity of the gastrointestinal epithelium (Newsholme 2001). Souba (1991) reported that Gln supplementation can ameliorate intestinal mucosal atrophy and maintain intestinal mucosal integrity. A deficiency in Gln reduces the expression of tight junction proteins and increases the intestinal epithelial barrier permeability, whereas Gln supplementation can prevent these changes (Rhoads et al. 1997). Gln is the first substrate for rapid differentiation of intestinal epithelial cells and immune cells, stimulating cell proliferation and reducing intestinal bacterial and endotoxin translocation. Previous studies on rumen epithelial damage caused by high-concentrate diets primarily focused on correcting low rumen pH. While adding carbonate to diets can neutralise acidic components and temporarily raise pH, this approach does not address the underlying cause. Once carbonate supplementation ceases, rumen microorganisms continue to ferment, organic acids continue to be produced, and ruminal acidosis persists. Owens et al. (1998) found that chronic supplementation of lactic acid bacteria had no effect. Wu et al. (2022) reported that Gln can mediate the mRNA expression of tight junction proteins and cytokines and can improve the barrier function of the ruminal epithelium in Hu lambs fed a high-concentrate finishing diet. However, to our knowledge, the application of Gln to repair ruminal acidosis in dairy cows has not been investigated, therefore the effects on the repair of ruminal acidosis in ruminal epithelial cells (RECs) of dairy cows remain unclear.

MATERIAL AND METHODS

Ethics statement

All experimental procedures were conducted in accordance with the ethical standards set by the Institutional Animal Care and Use Committee (IACUC) of the China Agricultural Research Center. The protocols were approved by the IACUC, ensuring compliance with both national and international guidelines for the care and use of animals in research.

Cell culture and experimental design

Rumen epithelial tissue was collected from adult dairy cows within 10 min post-slaughter at the Yizhong slaughterhouse. The tissue was thoroughly washed with phosphate-buffered saline (PBS) to remove surface contaminants. Subsequently, the tissue was immersed in PBS and transported to the laboratory. To isolate RECs with high viability and strong proliferation capacity, the tissue was digested using 0.1% collagenase I and 0.25% trypsin-EDTA (Thermo Fisher Scientific Inc, Shanghai, China). The isolated cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Hangzhou Sijiqing Biological Engineering Material Co., Ltd, Hangzhou, China), 100 U/ml penicillin and 100 µg/ml streptomycin, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B (Beijing Dingguochangsheng Biotechnology Co., Ltd, Beijing, China). The medium was changed every two days until cells had visibly spread across the bottom of the culture dish. Cells were detached with 0.25% trypsin-EDTA and transferred to new culture dishes. The differential adhesion method was employed repeatedly to purify the RECs, ensuring high purity and vitality.

According to the results of previous studies in our laboratory, the medium pH was adjusted to 5.5 using

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a mixed acid solution containing 5.5 mol/l acetic acid, 2 mol/l propionic acid, 0.9 mol/l butyric acid, and 0.5 mol/l lactic acid. The RECs were cultured in this medium for 3 h to establish a REC injury model (acid injury). Then, the acid-induced injury of RECs was treated with different concentrations of Gln (Gln concentrations of 4, 8, 12, and 32 mmol/l) for 12 hours. Cells cultured in a normal medium were used as the control group (Con). Each treatment was replicated six times to determine cell proliferation and RNA expression.

Determination of cell activity

Cell activity was measured according to the instructions of the CCK-8 Kit (Beijing Dingguochangsheng Biotechnology Co., Ltd, Beijing, China). The optical density (OD) at 450 nm was measured using a microplate reader (Thermo Fisher Scientific Inc., Waltham, USA).

Reverse transcription-quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from RECs using Trizol reagent (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions. The purity and concentration of RNA were assessed using a spectrophotometer (Nanodrop 2000; Thermo Fisher, Waltham, USA) at 260 and 280 nm. The ratios of absorption (260/280 nm) of all samples ranged from 1.8 to 2.0. RNA samples were treated with RNase-free DNase l to carefully remove contaminating genomic DNA. Total RNA was reverse transcribed to cDNA (10 µl reaction system was applied for 500 ng of total RNA) using a Prime Script RT Master Mix Kit (Takara Bio Inc., Shiga, Japan). The reverse transcription reaction system was 10 µl: 5× Prime RT Master Mix 2 µl, RNase free distilled water (dH_2O) was added to a total volume of 10 µl. Then the complementary DNA (cDNA) was stored at –20 °C for further use.

The expression of the target genes was carried out in an optical 96-well plates on CFX96 RT-qPCR in the total volume of 25 µl detection system (Thermo Fisher Scientific Inc., Waltham, USA) using SYBR Primer Script^{TM} RT-PCR Kit (Takara Bio Inc., Shiga, Japan). The PCR protocol was as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles consisting of 94 °C for 30 s, annealing for 20 s, and extension at 72 °C for 15 seconds. The melting curve was recorded at the end of each run. Primers for the target genes were designed using Primer (v5.0) software to ensure that they will specifically bind to the regions of the gene without significant homology to other sequences

Table 1. Primer sequence and annealing temperatures (Ta) for qPCR

Genes	GenBank number	Primer sequence (5' to 3')	Amplicon length (bp)	Ta (°C)
Gene for *GAPDH*	NM_001034034.2	CACCCTCAAGATTGTCAGC ATAAGTCCCTCCACGATGC	99	56
Gene for *CLDN1*	NM_001001854.2	TGCCTTGATGGTGATTGG TTCTGTGCCTCGTCGTCTTC	102	56
Gene for *OCLN*	NM_001082433.2	TATGGAACCTTAATGGGAGC GATATGCCTGACCTTACAACG	221	55
TNF	NM_173966.3	TGACGGGCTTTACCTCATCT TGTTGACCTTGGTCTGGTAGG	122	58
IL1B	NM_174093.1	TCCACGTGGGCTGAATAACC TCGGGCATGGATCAGACAAC	93	60
CXCL8	JN559767.1	CACCTTTCCACCCCAAAT CAACCTTCTGCACCCACTT	141	56
TLR2	NM_174197.2	TTCTACATCTGAAGCAGCCTAT CAACAAAACTTTCATCGGTG	192	55
TLR4	NM_174198.6	ACATCCGCATTGTCTTCCAT ACCTGTTCCAGTTGACACTTAGAG	160	58

CLDN1 = claudin 1; *CXCL8* = C-X-C motif chemokine ligand 8; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *IL1B* = interleukin 1B; *OCLN* = occludin; *TLR2* = toll-like receptor 2; *TLR4* = toll-like receptor 4; *TNF* = tumour necrosis factor

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(Table 1). The primers were synthesised by Beijing Genomics Institute (Shenzhen, China). The specificity of the primers was confirmed using BLAST analysis, and the absence of non-specific products was verified through post-amplification melting curve analysis, indicated by the presence of a single peak.

Statistical analysis

Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) serving as the reference gene. ΔCT = CT (cycle threshold) of target gene – CT of reference gene; ΔΔCT = ΔCT (test group) – ΔCT (control group), relative expression of mRNA = $2^{-\Delta\Delta CT}$. Statistical comparisons of gene expression levels among different groups were analysed using the one-way analyse of variance (ANOVA) followed by Tukey's post-hoc test of SPSS (v.17.0). Significant difference was set at $P < 0.05$.

RESULTS

Effect of Gln on the activity of RECs under low pH

After acid-treated cells were exposed to different concentrations of Gln for 12 h, cell activity was measured. The OD values of the groups treated with different concentrations of Gln were significantly higher than those of the control group ($P < 0.05$). When the Gln concentration was 8 mmol/l, the cell activity was highest, but the OD value decreased as the Gln concentration increased. The OD values of Gln group were higher than those of the control group (Figure 1).

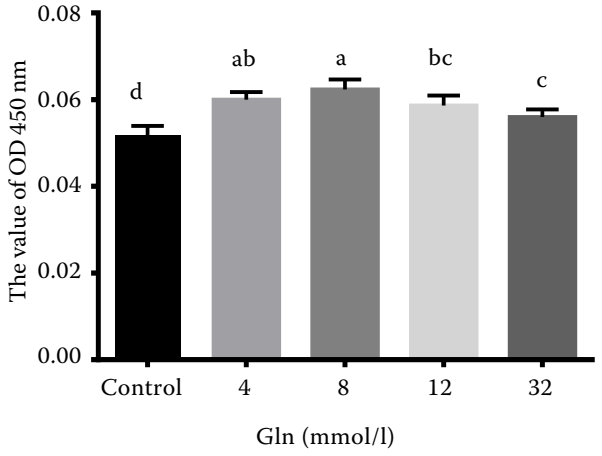

Figure 1. The cell activity after treatment with different concentrations of Gln

[a–d]means differing significantly at level $P < 0.05$

Gln = glutamine; OD = optical density

Effects of Gln on occludin (*OCLN*) and claudin 1 (*CLDN1*) mRNA expression

The 8 mmol/l concentration of Gln led to the highest expression of *OCLN*. When the concentration was 8 and 12 mmol/l, the expression level was significantly higher than that of the control group ($P < 0.05$). However, when Gln concentration reached 32 mmol/l, the gene expression was significantly lower than in the acid treatment group ($P < 0.05$). The expression of *CLDN1* gene in the acid-treated group was higher than in the control group. When the Gln concentration was 4 mmol/l, the expression level was significantly higher than in the other groups ($P < 0.05$). With the increase of Gln concentration,

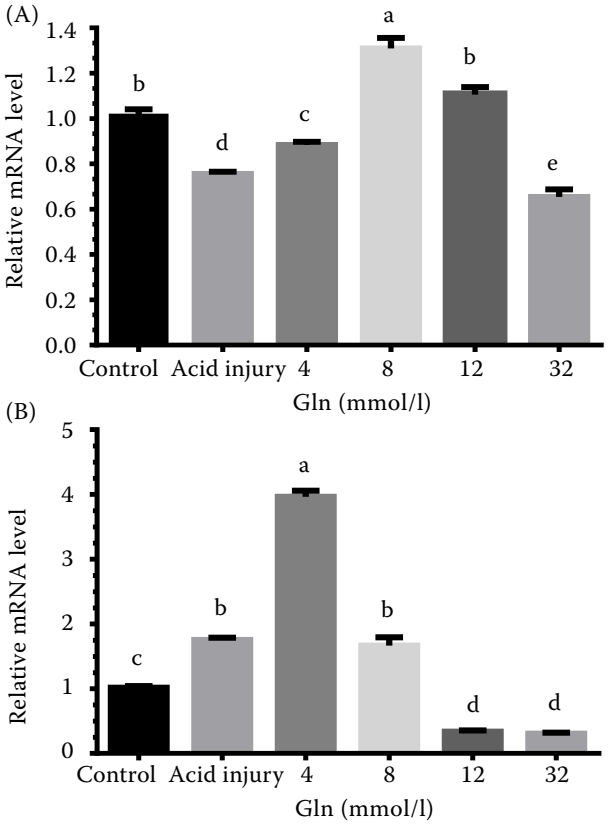

Figure 2. The mRNA level of relative expression of (A) *OCLN* and (B) *CLDN1*

[a–e]means differing significantly at level $P < 0.05$

CLDN1 = claudin 1 Gln = glutamine; *OCLN* = occludin

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the expression level gradually decreased. When the Gln concentration was 12 and 32 mmol/l, the expression level was significantly lower than that of the acid treatment group ($P < 0.05$) (Figure 2).

Effects of Gln on the mRNA expression of inflammatory factor genes

The relative expression levels of tumour necrosis factor (*TNF*), interleukin 1B (*IL1B*), and C-X-C motif chemokine ligand 8 (*CXCL8*) after acid treatment were higher than those in the control group, and acid-injured cells were repaired with different concentrations of Gln. The mRNA levels of *TNF*, *IL1B*, and *CXCL8* were significantly lower than after the acid treatment alone ($P < 0.05$). The mRNA level of *TNF* was the lowest when the concentration of Gln was 8, 12 and 32 mmol/l, but there was no significant difference between the three concentrations ($P > 0.05$). The mRNA level of *IL1B* was the lowest when the concentration of Gln was 8 mmol/l, moreover, it was significantly different from the levels at the other concentrations ($P < 0.05$). When the concentration of Gln was 12 mmol/l, the mRNA level of *CXCL8* was the lowest, and it was also significantly different from the expression at the other treatment concentrations ($P < 0.05$) (Figure 3).

Effects of Gln on *TLR2* and *TLR4* mRNA expression

The expression levels of the genes toll-like receptor 2 (*TLR2*) and toll-like receptor 4 (*TLR4*) were lower in Gln-treated groups compared to the acid-treated group. The most significant decrease in *TLR2* and *TLR4* expression was observed at the Gln concentration of 32 mmol/l ($P < 0.05$) (Figure 4).

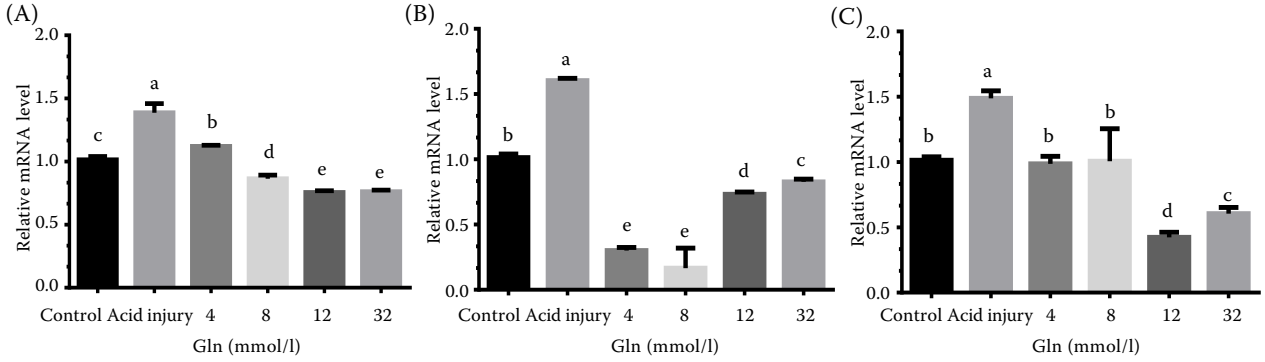

Figure 3. The mRNA level of relative expression of (A) *TNF*, (B) *IL1B*, and (C) *CXCL8*

[a–e]Means differing significantly at level $P < 0.05$

CXCL8 = C-X-C motif chemokine ligand 8; Gln = glutamine; *IL1B* = interleukin 1B; *TNF* = tumour necrosis factor

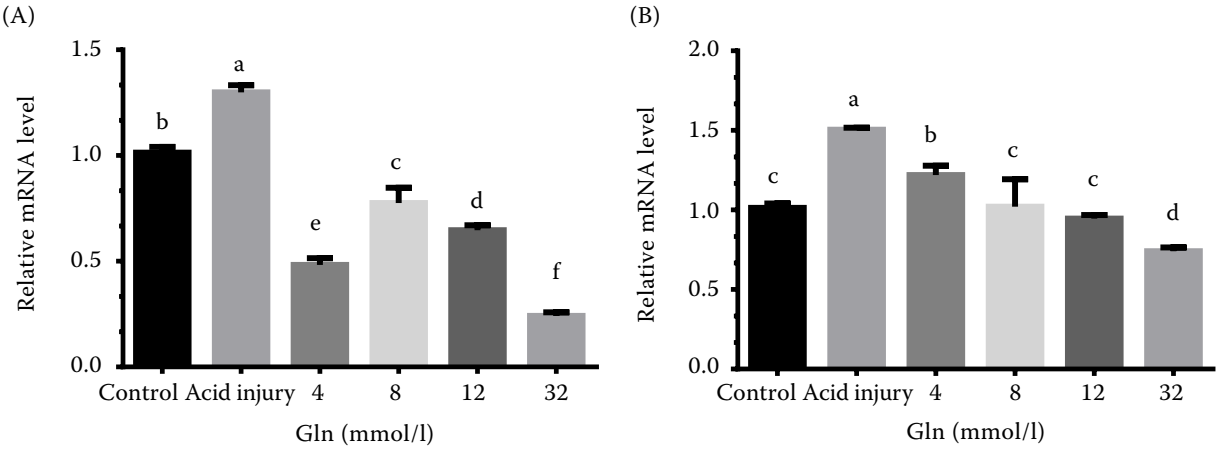

Figure 4. The mRNA level of relative expression of *TLR2* and *TLR4*

[a–f]Means differing significantly at level $P < 0.05$

Gln = glutamine; *TLR2* = toll-like receptor 2; *TLR4* = toll-like receptor 4

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DISCUSSION

Gln serves as the primary energy source for the small intestine mucosa and plays a crucial role in providing nitrogen and carbon for protein and nucleic acids synthesis, thereby promoting the proliferation of intestinal mucosal cells (Tuhacek et al. 2004).

Effects on cell proliferation

L-glutamine is known to enhance the proliferation of intestinal epithelial cells. Deng et al. (2023) demonstrated that supplementation of Gln significantly increased the proliferation of intestinal cells, as indicated by a higher positive rate of proliferating cell nuclear antigen (PCNA). In our study, the OD values were higher in all Gln-treated groups compared to the control. Notably, when the concentration of Gln was 8 mmol/l, the cell activity was highest, although the cell viability was not directly proportional to the Gln concentration. This suggests that Gln supplementation promotes the proliferation of RECs, with lower doses being more effective than the application of higher doses.

Effects on *OCLN* and *CLDN1* mRNA expression

Tight junction proteins occur extensively between epithelial cells and endothelial cells, playing a role in maintaining the integrity of the intercellular structure and ensuring the proper function. The OCLN and CLDN1 belong to the key integral membrane proteins that constitute the tight junctions, which are essential for maintaining the epithelial and endothelial barrier function. And their lack will lead to the disorder of the tight junction structure and function, to the increase in cell permeability and to the outbreak of diseases, such as tumour, stroke and inflammation (Al-Sadi et al. 2011; Oshima et al. 2012). The expression of *CLDN1* and claudin-4 genes was significantly increased and the expression of *OCLN* gene was decreased when RECs were cultured *in vitro* by low pH treatment and lipopolysaccharide (LPS) treatment. Goswami et al. (2016) reported that Gln deficiency could lead to the disruption of epithelial integrity in rats. Bidula and Schelenz (2016) found that acid stimulation could disrupt the epithelial barrier by altering the expression of tight junction proteins. Moreover, Gln was shown to maintain gastrointestinal mucosal integrity (Yoshinari et al. 2001). In our experiments, the mRNA expression of *OCLN* in RECs of dairy cows was decreased in the acid-treated group, and the mRNA expression of *CLDN1* was increased, indicating that the tight junction structure was destroyed by lower pH. This observation was consistent with the previously published studies (Swierk et al. 2023). Moreover, when the acid-induced injury of RECs was repaired with different concentrations of Gln, we found that the expression of *OCLN* first rose and then decreased, and the effect was the highest when the concentration of the Gln was 8 mmol/l. Similarly, the expression of *CLDN1* also first rose and then decreased. When the concentration of Gln was 12 or 32 mmol/l, the expression of *CLDN1* was lower, and even below the control. These results suggest that adding Gln can repair the tight junction structure in cow RECs damaged by the acid.

Effects of Gln on the mRNA expression of inflammatory factor genes

Inflammatory responses play an important role in the mechanism of gastric mucosal injury. *TNF*, *CXCL8*, and *IL1B* are important inflammatory mediators that induce gastric mucosal injury, and they are usually used to determine the degree of gastric mucosal inflammation. *TNF* can activate myosin binding protein kinase and can destroy the tight junction structure and function, which results in tight junctional disorder of the intestinal epithelium, increased permeability of the paracellular pathway, and severe damage to the epithelial mucosal barrier (Yoshinari et al. 2001). *TNF* increases paracellular permeability by activating long myosin light chain kinase (MLCK) transcription, expression, enzymatic activity, and recruitment to the actomyosin ring (Cunningham and Turner 2012). MLCK activation triggers the perijunctional actomyosin ring contraction, leading to molecular reorganisation of the tight junction structure, including OCLN endocytosis (He et al. 2020). *TNF* could induce the expression of *IL1B* and *IL6*. *TNF* was one of the strongest inflammation factors, and it had enhanced effects of other inflammatory cytokines. Gln could inhibit inflammatory reactions in septic mice and reduce their mortality. Chuang

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et al. (2014) found that Gln could reduce *IL1B*, *IL6*, *TNF*, *IL10* levels to reduce inflammatory reactions and to boost immunity levels. In this study, we investigated the impact of Gln on the mRNA levels of *IL1B*, *CXCL8*, and *TNF* in dairy cow RECs following the acid treatment. The results indicated that mRNA levels of these cytokines were significantly elevated in the acid treatment group compared to the control group, suggesting damage to the RECs. Upon treatment with varying concentrations of Gln, we observed a significant reduction in mRNA levels of *IL1B*, *CXCL8*, and *TNF* compared to the acid treatment group ($P < 0.05$). Specifically, *IL1B* and *CXCL8* mRNA levels were lower across all Gln concentrations than in the control group. Furthermore, at Gln concentrations of 8, 12, and 32 mmol/l, *TNF* mRNA levels were also reduced compared to the control group. These findings suggest that Gln can mitigate inflammation by reducing the expression of inflammatory cytokines in RECs.

Effects on *TLR2* and *TLR4* mRNA expression

Toll-like receptor (TLR) is known as the "gateway" protein that initiates the inflammatory response, belonging to the family of animal pattern recognition receptors. It could not only specifically identify pathogen-associated molecular patterns but also specifically recognise damage-associated molecular patterns. These patterns transduce the signals of pathogenesis-related molecular stimuli from the extracellular environment through membrane-associated transmembrane proteins. This process activates the downstream signalling pathways, such as nuclear factor κB (NF-κB) or MAPK by myeloid differentiation factor My D88. This activation promotes the synthesis and secretion of inflammatory factors and chemokines, thereby initiating and inducing the inflammatory response (Kawai and Akira 2010; Park et al. 2014). The TLR2 and TLR4 proteins could identify endotoxin. Tanaka et al. (2016) showed that the ability of TLR4 to combine with endotoxin was very low, primarily binding to the complex of LPS-LPB-CD14, and subsequently activating TLR4 and intracellular inflammatory pathways, and promoting the synthesis and release of inflammatory mediators such as IL1, IL6 and TNF through signal transduction. The reported results showed that the relative expression level of *TLR2* and *TLR4* mRNA was higher in the acid-treated group than in the control, while when Gln was added to repair RECs, they were lower than in the acid-treated group. The addition of Gln significantly reduced the relative expression level of *TLR2* and *TLR4* mRNA after acid treatment ($P < 0.05$), it may reduce the expression of cellular receptor proteins, but further studies are needed to confirm the changes in the protein level. Its possible mechanism is that adding Gln leads to a decrease in the relative expression levels of *TLR2* and *TLR4* mRNA, which might reduce the activation of intracellular inflammatory pathways. This leads to decreasing the binding of TLR4 to endotoxin and to reducing the inflammatory response induced by endotoxin, which is reflected by decreased relative expression levels of *TNF*, *IL1B* and *CXCL8* mRNA. The ability of inflammatory cytokines to increase cell membrane permeability through downregulation of OCLN and upregulation of *CLDN1* was supposed to be weakened, and the structure and function of the RECs tight junction could be restored.

CONCLUSION

The present study examined the effect of Gln on the repair of ruminal epithelial cells (RECs) injury in dairy cows by assessing cell viability and mRNA levels of intercellular junction proteins, inflammatory factors, and cell surface receptors. In summary, Gln was found to promote RECs proliferation, reduce mRNA expression of ruminal epithelial receptor proteins and inflammatory factors, and enhance mRNA expression of *OCLN*. However, the repair effects of Gln were not directly proportional to the concentration. On the gene level, these findings indicate a repairing effect of Gln on RECs injury. Future research should investigate the mechanisms at a protein level to provide a clearer understanding of how Gln mitigates acid-induced damage in dairy cow RECs.

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Conflict of interest

The authors declare no conflict of interest.

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