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Diallyl disulfide attenuates hydrogen peroxide-induced oxidative damage of ovine rumen epithelial cells through the nuclear factor erythroid-2 related factor 2 signaling pathway

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Abstract

This study investigated the impact of diallyl disulfide (DADS) on oxidative stress induced by hydrogen peroxide (H_2O_2) in ovine rumen epithelial cells (RECs). Initially, the effects of DADS were evaluated on cellular reactive oxygen species (ROS) levels, antioxidant capacity in RECs were estimated. Then, RNA-seq analysis was conducted in DADS-treated and untreated cells to analyze the differential gene expression, as well as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Finally, the effects of DADS on Kelch-like ECH associated protein 1/the nuclear factor erythroid 2-related factor 2 (Keap1/Nrf2) signaling pathway in RECs were evaluated. Results showed that DADS remarkably enhanced superoxide dismutase (SOD) activity and total antioxidant capacity (T-AOC) ($P < 0.05$) while reducing ROS and malonaldehyde production ($P < 0.05$) in H_2O_2-treated RECs. Transcriptomic analysis revealed that DADS might influence glutathione synthesis through cysteine and methionine metabolism, thereby affecting the transcription of genes involved in immunity and oxidative stress. The DADS treatment resulted in increased nuclear translocation of Nrf2 and upregulation of mRNA and protein levels of quinone oxidoreductase 1, heme oxygenase 1, and Nrf2. The Nrf2-specific inhibitor nullified the protective effects of DADS on malonaldehyde formation induced by H_2O_2 and decreased T-AOC and SOD activities. In conclusion, DADS demonstrated the ability to alleviate oxidative stress in RECs by promoting antioxidative capacity through the Keap1/Nrf2 signaling pathway.

Introduction

An imbalance between oxidation and antioxidant defense in the body can cause oxidative stress, increasing reactive oxygen species (ROS) levels, damaging intracellular membranes, lipids, and DNA structure, and causing harm to the body (Lin et al. 2022; Valko et al. 2007). Improper animal rearing in nutrition, environment, management, and transportation can lead to oxidative stress in animals (Zhang et al. 2022). The rumen plays a pivotal role in ruminant digestion, significantly influencing their growth, health, and productivity (John et al. 2011). Heat stress in dairy cows leads to increased ROS, oxidative stress, and damage to rumen epithelial cells (RECs) (Guo et al. 2021). Studies revealed that in case of subacute ruminal acidosis-induced oxidative stress, the morphology of RECs changes, and the barrier and immune functions are compromised (Liu et al. 2013; McCann et al. 2016).

Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates cellular redox homeostasis by binding to the antioxidant response elements (AREs) of the genes, facilitating the upregulation of enzyme and antioxidant molecules, such as superoxide dismutase (SOD), heme oxygenase-1 (HO-1), and quinone oxidoreductase 1 (NQO1) (Hochmuth et al. 2011; Xi et al. 2023). The Kelch-like ECH associated protein 1 (Keap1) regulates Nrf2 by inhibiting its activity, but it releases Nrf2 in response to organismal stress. Studies have confirmed that Keap1/Nrf2 antioxidant system possesses defense mechanisms that can alleviate oxidative damage to cells (Moon et al. 2021; Sun et al. 2020).

Diallyl disulfide (DADS) is a lipophilic organosulfur molecule found widely in garlic and other Allium species (Cheng et al. 2020). It possesses various pharmacological properties, including antioxidation (Hosseinzadeh et al. 2017; Zeng et al. 2013), anti-inflammatory (Zhang et al. 2020), and anticancer activities (Xia et al. 2019). Our previous studies have found that

DADS can improve antioxidant capacity, induce antioxidant enzyme activity, and scavenge ROS (Liu et al. 2024; Tang et al. 2024). Silva-Islas et al. (2019) have reported that DADS could prevent oxidative stress in rat brain by inhibiting the Nrf2 signaling pathway (Silva-Islas et al. 2019). However, there is limited research on the effects of DADS on antioxidation in ovine RECs. Thus, this study aimed to investigate whether and how DADS impact the oxidative damage induced by hydrogen peroxide (H_2O_2) in RECs.

Material and methods

Cell culture and treatment

The RECs of Hu sheep were obtained from Dr. Chunlei Yang's laboratory at Zhejiang University of Technology. The cells were kept in Dulbecco's modified Eagle medium/Nutrient Ham's Mixture F12 (Biosharp, Hefei, Anhui, China) which was enriched with 1% antibiotic solution (Invitrogen, Carlsbad, California, USA) and 10% fetal bovine serum (Zeta Life, San Francisco, California, USA). The cells were cultured with 5% CO_2 at 37°C in a cell culture incubator (Thermo Fisher, Waltham, MA, USA). Cells were routinely passaged at a 1:3 split ratio when cell confluency was between 85% and 95%. The RECs in 11–15 passages were used for this study.

The cells were treated with H_2O_2 (200 μM, determined by our previous study), or DADS (Sigma-Aldrich, MO, USA) (150 μM, determined by our previous study) (Zhang et al. 2023) for 2 h, followed by H_2O_2 treatment (Hosseinzadeh et al. 2017; Kim et al. 2005). The cells without H_2O_2 and DADS treatments were served as a control. After 24 h of co-treatment, the cell samples were collected and assayed for ROS content, enzyme activity, gene expression, and protein abundance.

In the experiment to verify that DADS alleviate oxidative damage by mediating the Keap1/Nrf2 signaling pathway, RECs were incubated with or without ML385 (10 μM which was selected in accordance with a prior study by Cao et al. [Cao et al. 2021] and our pilot study) (TargetMol, Shanghai, China), an Nrf2 inhibitor, for 2 h in advance, and then subjected to the treatment with DADS and H_2O_2 as described above.

RNA-seq and differentially expressed genes analysis

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the cells. The integrity and quantity of RNA was measured with the Agilent 2100 bioanalyzer. The library construction and RNA-seq was carried out by Novogene Corporation (Beijing, China) using the Illumina HiSeq platform. The fastp software was first used to filter and remove low-quality sequences from raw reads in FASTQ format (Chen et al. 2018). Clean, high-quality data was then used for all subsequent analyses. HISAT2 was employed to align paired-end clean reads to the *Ovis aries* genome (http://ftp.ensembl.org/pub/release104/fasta/ovis_aries_rambouillet/dna/Ovis_aries_rambouillet.Oar_rambouillet_v1.0.dna.toplevel.fa.gz) (Siren et al. 2014). StringTie was utilized for calculating gene expression, and then the gene length and the number of reads mapped to each gene were used to calculate the amounts of fragments per kilobase of transcript per million fragments for each gene (Pertea et al. 2015). The differentially expressed genes (DEGs) were analyzed using DESeq2 among the control, H_2O_2, and DADS + H_2O_2 groups, with a threshold set at a 2-fold change and a Q-value of 0.05 (Anders and Huber 2010).

The Cluster Profiler software was used for conducting pathway analyses, examining GO and KEGG pathways for DEGs (Yu et al. 2012).

Analysis of oxidative and anti-oxidative levels

The production of ROS was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe from Solarbio, Beijing, China. After treatment, cells were exposed to DCFH-DA working solution at 37°C in an incubator for half an hour after being cleaned twice with phosphate buffered saline (PBS). After three PBS rinses, the fluorescence signal was detected using a fluorescence microscope (BD, Franklin Lakes, NJ, USA) and its strength was evaluated using Image J software (National Institutes of Health, Maryland, USA).

To measure antioxidant levels, the cells were broken down by ultrasound after treatment, followed by centrifugation to separate the supernatant. The supernatant was collected for further analysis using enzyme-linked immunosorbent assay (ELISA) kits from Sinobestbio Shanghai, China. These ELISA kits were used to measure various antioxidant-related indicators, such as malondialdehyde (MDA), SOD, total antioxidant capacity (T-AOC), catalase (CAT), and glutathione peroxidase (GPX).

Quantitative real-time polymerase chain reaction (PCR)

The cDNA synthesis was conducted with a reverse transcription kit (Yeasen Biotechnology, Shanghai, China). The ABI 7500 Real-Time PCR System (Applied Biosystems, Singapore) and a SYBR Green Kit (Yeasen Biotechnology) were then used to perform real-time quantitative PCR. Denaturation was done at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA levels of the target genes, with β-actin selected as the reference gene (Livak and Schmittgen 2001). The primers (Table 1) were made by Sangon Biotech (Shanghai, China).

Western blot analysis

After each treatment, the cells were lysed in RIPA lysis solution (mainly 50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], etc.) containing protein phosphatase inhibitors. The protein concentration was then determined using the bicinchoninic acid protein assay kit (Yeasen Biotechnology). The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Primary antibodies against SOD (1:1000, catalog number 306028, ZenBioScience, Chengdu, China), GPX1 (1:1000, ZenBioScience, #R26805), β-actin (1:10000, ZenBioScience, #250136), Nrf2 (1:1000, sc-365949, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Keap1 (1:1000, sc-515432, Santa Cruz), NQO1 (1:1000, sc-393736, Santa Cruz), HO-1 (1:1000, sc-136256, Santa Cruz), and CAT (1:2000, 21260-1-AP, Proteintech Group, Wuhan, Hubei, China) were applied to the membranes overnight at 4°C. Then the membranes incubated with the goat anti-rabbit secondary antibody (1:5000, 511203, ZenBioScience) for 1 hour at room temperature. Finally, the protein bands were visualized using a Bio-Rad gel detection system, and Image J software was utilized to measure the band intensity (Liu et al. 2022). The protein abundance was normalized by the protein abundance of β-actin.

Table 1. Primers used for real-time polymerase chain reaction analysis

Gene	Accession number	Primer sequence (5′–3′)[a]	Product length, bp
SOD2	NM_001280703.1	F:5-ACGTCGCCGAGGAGAAGTACC-3 R:5-GGGGCTCAGATTTGTCCAGAAGATG-3	134
CAT	XM_004016396.5	F:5-AGCCTGCGTCCTGAGTCTCTG-3 R:5-ATCCATATCCGTTCATGTGCCTGTG-3	91
GPX1	XM_004018462.5	F:5-CGCACGGTGTACGCCTTCTC-3 R:5-GCAGGTCATTCATCTGGGTGTAGTC-3	148
NFE2L2	XM_012132956.4	F:5-GCCCAGTCTTCAATGCTCCTTCTC-3 R:5-TTCCTCCCAAACTTGCTCAATGTCC-3	113
HMOX1	XM_027967703.2	F:5-CGGCAGCAAGGCACAAGACTC-3 R:5-GAGGACCCATCGCAGGAGAGG-3	97
NQO1	XM_004015102.5	F:5-CACTCTGCACTTCTGTGGCTTCC-3 R:5-CAGGCGTTTCTTCCATCCTTCCAG-3	112
KEAP1	XM_027969637.2	F:5-GGGCTACGACGGTCACACATTC-3 R:5-ATTCGGGTCACCTCGCTCCAG-3	81
β-actin	NM_001009784.3	F:5-CCATCGGCAATGAGCGGTTCC-3 R:5-CGTGTTGGCGTAGAGGTCCTTG-3	146

SOD2 = superoxide dismutase 2; CAT = catalase; GPX1 = glutathione peroxidase 1; Nrf2 = nuclear-factor-erythroid derived 2 like protein 2; HMOX1 = heme oxygenase 1; NQO1 = quinone oxidoreductase 1, NQO1 = Kelch-like ECH-associated protein.
[a]F = forward; R = reverse.

Immunofluorescence

Following a 20-minute 4% paraformaldehyde fixation, the RECs in each treatment group were rehydrated three times for 5 min each using PBS. After that, the cells were permeabilized for 20 min using 0.5% Triton-X, followed by a PBS wash and a 1-hour blocking step using 1% bovine albumin solution. Subsequently, the cells were exposed to the primary antibody against Nrf2 (1:1000, sc-365949, Santa Cruz) overnight at 4°C, followed by a wash and an hour-long incubation with a fluorescent secondary antibody (1:100, 550036, ZenBioScience) in the dark at room temperature. The 4′,6-diamidino-2-phenylindole reagent (Servicebio, Wuhan, Hubei, China) was employed to stain the nuclei, and then a fluorescence microscopy was used to visualize the protein staining.

Statistical analysis

The data were present as the mean ± standard deviation (SD). The data was statistically analyzed using one-way analysis of variance, followed by the Duncan post hoc test with the statistical program SPSS 22.0. The statistical difference was declared if the *P*-value was less than 0.05.

Results

DADS attenuated H_2O_2-induced oxidative damage in RECs

As Fig. 1 illustrates, the amounts of intracellular ROS and MDA were higher in RECs treated with H_2O_2 ($P < 0.05$). In RECs exposed to H_2O_2, the DADS pretreatment significantly decreased the ROS and MDA production ($P < 0.05$).

Treatment of the cells with H_2O_2 dramatically reduced SOD and T-AOC activities compared to the control group ($P < 0.05$). However, pretreatment of DADS with H_2O_2 significantly elevated T-AOC and SOD levels compared to the group treated with H_2O_2 alone ($P < 0.05$). Additionally, the H_2O_2 group exhibited higher GPX and CAT levels than the control group ($P < 0.05$). The cells treated with DADS and H_2O_2 decreased CAT and GPX levels compared to the H_2O_2 group ($P < 0.05$).

Transcriptomic analysis

RNA-seq analysis was carried out in RECs with or without H_2O_2 and DADS treatment to understand how DADS mitigates oxidative damage induced by H_2O_2. As shown in Fig. 2, cells treated with the H_2O_2 had 22 DEGs (6 upregulated and 16 downregulated), compared to the control group. Pretreatment of the cells with DADS resulted in 9 downregulated genes and 25 upregulated genes in comparison to the cells treated with H_2O_2 only.

The GO analysis of DEGs revealed significant enrichment in biological process (BP) between the H_2O_2 and control groups, including regulation of inflammatory response, inflammatory response, and positive regulation of inflammatory response, etc. In terms of cellular composition (CC), there was notable enrichment in extracellular space, cytoplasmic ribonucleoprotein granule, and extracellular matrix, etc. Additionally, significant enrichment in molecular function (MF) included AU-rich element binding, mRNA 3′-UTR AU-rich region binding, and mRNA 3′-UTR binding, etc. GO term analyses of DEGs between the DADS + H_2O_2 and H_2O_2 groups showed significantly enriched BP including epithelial cell development, serine family amino acid biosynthetic process, and serine family amino acid metabolic process, etc. In terms of CC, there was notable enrichment in extracellular matrix, proteinaceous extracellular matrix, and basement membrane, etc. Furthermore, significant enrichment in MF included protease binding, protein C-terminus binding, intracellular calcium activated chloride channel activity, etc.

Functional annotation of DEGs using KEGG pathway enrichment analysis revealed that the H_2O_2 group exhibited significant alterations in the following pathways: IL-17 signaling pathway, complement and coagulation cascades, NF-κ B signaling pathway, PPAR signaling pathway, among others when compared to the control. Furthermore, significant pathway enrichments in

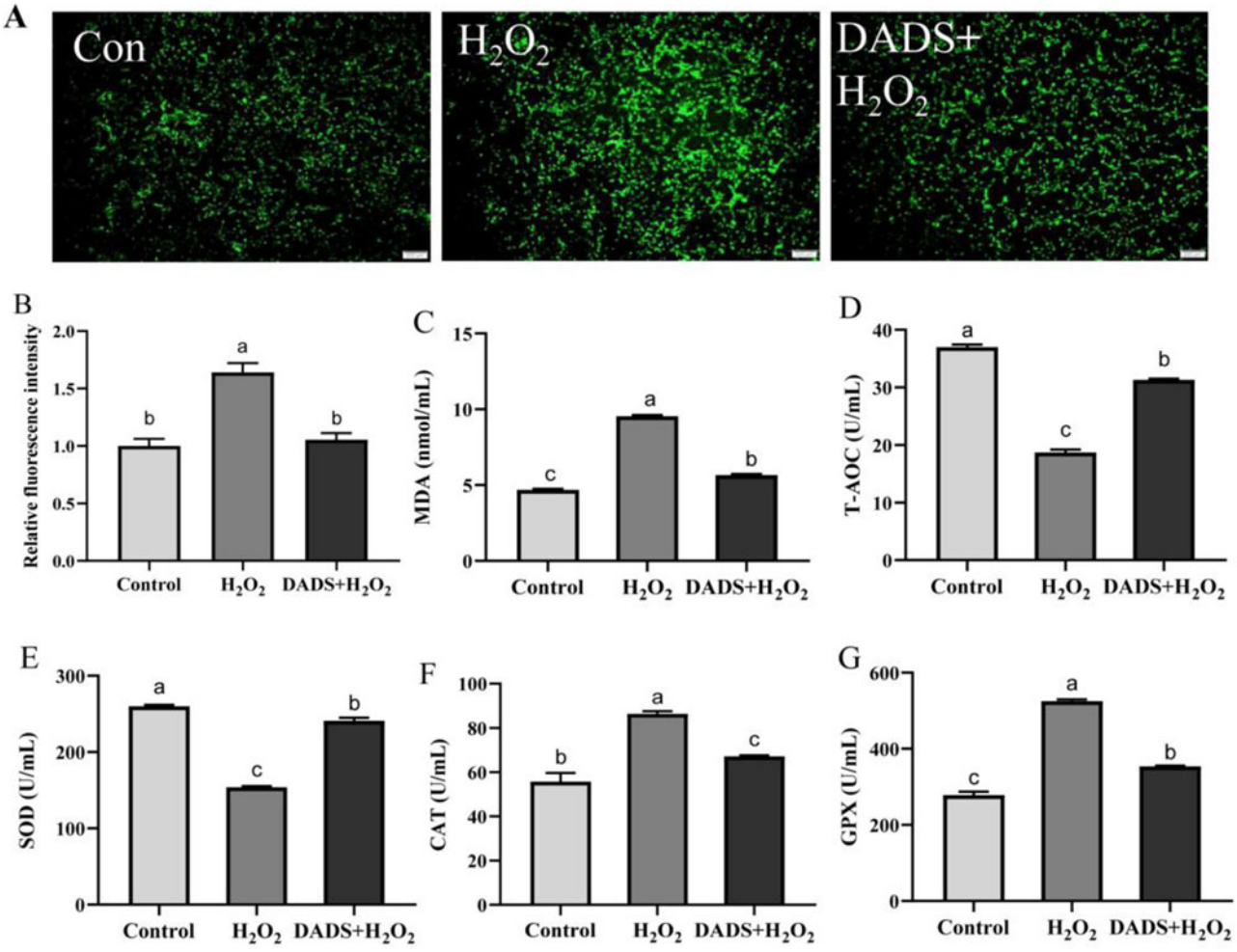

Figure 1. Effects of DADS on H_2O_2-induced oxidative stress in RECs. The RECs underwent a 2-hour pretreatment with or without 150 μM DADS and a 24-hour treatment with or without 200 μM H_2O_2. (A) The staining of ROS in RECs treated with H_2O_2 or DADS (Scale bar: 200 μM), (B) the relative fluorescence intensity of ROS assessed by Image J software. (C-G) Effect of DADS pretreatment on (C) MDA content, (D) T-AOC, (E) SOD, (F) CAT, and (G) GPX enzyme activities in RECs. The results are shown as the mean ± SD ($n = 3$), with different letters (a–c) designating significant differences ($P < 0.05$). CAT = catalase; DADS = diallyl disulfide; GPX = glutathione; H_2O_2 = hydrogen peroxide; MDA = malondialdehyde; RECs = rumen epithelial cells; ROS = reactive oxygen species; SOD = superoxide dismutase; T-AOC = total antioxidant capacity.

cellular immunity, oxidative stress, and inflammation, such as ECM-receptor interaction, cysteine and methionine metabolism, complement and coagulation cascades, PI3K-Akt signaling pathway, etc., were observed in DEGs between the DADS + H_2O_2 and H_2O_2 groups.

DADS activated the Keap1/Nrf2 signaling pathway

We assessed the impact of DADS on Keap1/Nrf2 signaling pathway by analyzing the intracellular distribution of Nrf2 and relative mRNA expression and protein abundance of Nrf2, Keap1, HO-1, and NQO1. As depicted in Fig. 3, the treatment with H_2O_2 significantly inhibited the nuclear translocation of Nrf2, and reduced the mRNA expressions and protein abundances of Nrf2, HO-1, NQO1, whereas increased Keap1 mRNA expression and protein abundance ($P < 0.05$). In contrast, compared to the H_2O_2 group, DADS pretreatment and sulforaphane (SFN) treatment (as a positive control) significantly enhanced the nuclear translocation of Nrf2, increased the mRNA expressions and protein abundances of Nrf2, NQO1, HO-1, and decreased Keapl mRNA expression and protein abundance ($P < 0.05$).

Inhibition of Keap1/Nrf2 attenuated the antioxidant effects of DADS in H_2O_2-induced oxidative damage in RECs

We treated the RECs with the ML385 to inhibit the Nrf2 signaling pathway to provide further confirmation of the role of Nrf2 pathway in mediating the antioxidant effects of DADS. In Fig. 4, it's evident that the ML385 group exhibited significantly higher mRNA expressions and protein abundance of Keap1 ($P < 0.05$) and significantly lower mRNA expressions and protein abundances of Nrf2, NQO1, HO-1 compared to the DADS group.

Figure 5 shows that there was a substantial increase in intracellular MDA content and a decrease in intracellular T-AOC and SOD ($P < 0.05$) in ML385 group compared to H_2O_2 group. Cells co-treated with ML385 and H_2O_2 exhibited increased intracellular GPX and CAT levels in comparison to cells treated with DADS alone ($P < 0.05$).

Discussion

DADS, an organic sulfur compound derived from garlic, is known for its powerful antioxidant properties (Wu et al. 2002). The rumen,

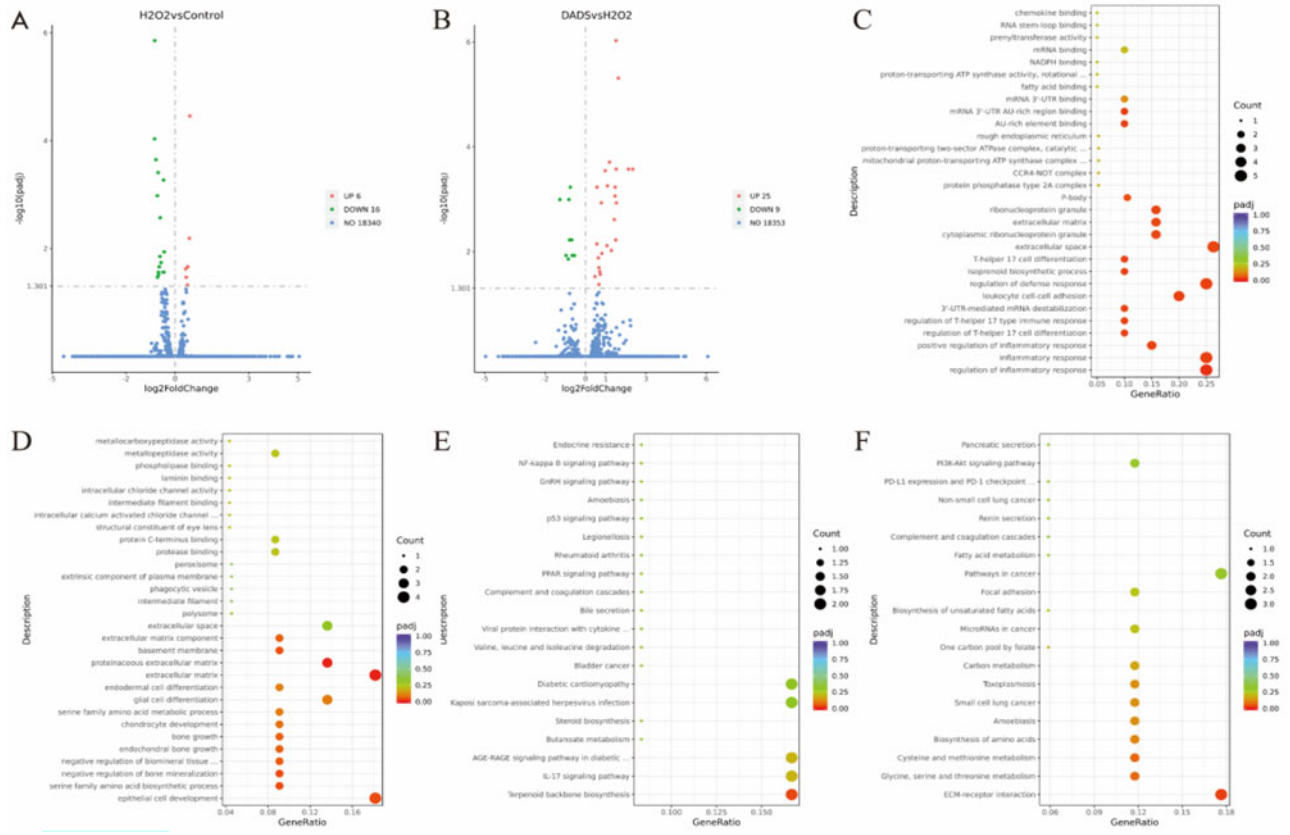

Figure 2. Transcriptomic analysis of DADS treatment in RECs with oxidative stress induced by H_2O_2. The RECs received a 2-hour pretreatment with or without 150 μM DADS, followed by a 24-hour treatment with or without 200 μM H_2O_2. (A–B) The volcano plot shows the results of significant differences in gene expression (Red, green, and blue indicate significantly upregulated, downregulated, and non-differentially expressed genes, respectively. The number marked represents the number of genes). (A) H_2O_2 group vs. control group; (B) DADS + H_2O_2 group vs. H_2O_2 group. (C-D) GO enrichment analyses of DEGs identified in: (C) H_2O_2 group vs. Control group and (D) DADS + H_2O_2 group vs. H_2O_2 group. The first ten, middle ten and last ten correspond to the enrichment in terms of molecular function, cellular component and biological process, respectively. (E-F) KEGG enrichment analysis diagram of DEGs in: (E) H_2O_2 group vs. Control group and (F) DADS + H_2O_2 group vs. H_2O_2 group. DADS = diallyl disulfide; DEG = differentially expressed genes; GO = Gene Ontology; H_2O_2 = hydrogen peroxide; KEGG = Kyoto Encyclopedia of Genes and Genomes; RECs = rumen epithelial cells.

an essential digestive organ for ruminants, plays a significant role in overall health. However, there is limited research on whether DADS can mitigate oxidative damage in ovine RECs. This study aimed to investigate the impact of DADS treatment on the antioxidant capacity of RECs and the underlying molecular mechanisms.

Normally, the body maintains a dynamic equilibrium between the generation and elimination of ROS under various circumstances. However, during oxidative stress, the generation of ROS exceeds the body's clearance capacity, leading to the accumulation of ROS, which damages lipid biomembranes and generates MDA, resulting in cell injury (Ho et al. 2013; Yang et al. 2021). Therefore, oxidative stress is usually characterized by significant increases in ROS and MDA levels (Bernabucci et al. 2002; Cui et al. 2023). Previous research has reported that H_2O_2 induces excessive ROS and MDA production in bovine skeletal muscle cells, and resveratrol can mitigate oxidative damage to cells by quenching ROS and MDA (Zhang et al. 2022). In line with these findings, we noted that H_2O_2 increased the ROS and MDA levels in RECs, while DADS addition decreased the ROS and MDA levels, suggesting that DADS can alleviate H_2O_2-induced oxidative stress.

Subsequently, RNA-seq technology was used to analyze the gene expression spectrum of RECs treated with DADS and H_2O_2. The results revealed numerous DEGs among the treatments. To understand the pathways and biological effects of these DEGs, GO analysis was conducted. It was shown that these DEGs are primarily associated with the regulation of cellular metabolism, redox processes, inflammatory, and immune-related BPs. Thus, the regulation of these BPs is crucial for DADS in protecting RECs from oxidative damage.

Pathway enrichment analysis was then conducted on genes regulated by DADS using the KEGG database. The results revealed enrichment in complement and coagulation cascades and PI3K-Akt signaling pathway, which are linked to cellular immunity and metabolism. These pathways are intimately associated with DADS-mediated regulation of oxidative stress. Most importantly, cysteine and methionine metabolism, which involves the synthesis of GSH, a vital antioxidant, was significantly enriched. The GSH can directly neutralize free radicals, alleviating oxidative stress-induced damage to cells (Zhong et al. 2022). The enrichment of cysteine and methionine metabolism suggests that the promotion of GSH synthesis may be a mechanism of DADS action. This observation was consistent with previous research that showed pumpkin polysaccharides could enhance the antioxidant capacity of mouse liver tissue by activating the synthesis of cysteine and methionine pathways and increasing GSH production (Yang et al. 2024).

To further investigate how DADS scavenges ROS, we examined the activities of antioxidant enzymes in RECs treated by DADS. Free radicals can be scavenged by SOD, which catalyzes the disproportionation H_2O_2 to O_2 and H_2O (McCord and Edeas 2005). The CAT and GPX can also decompose H_2O_2 to protect the

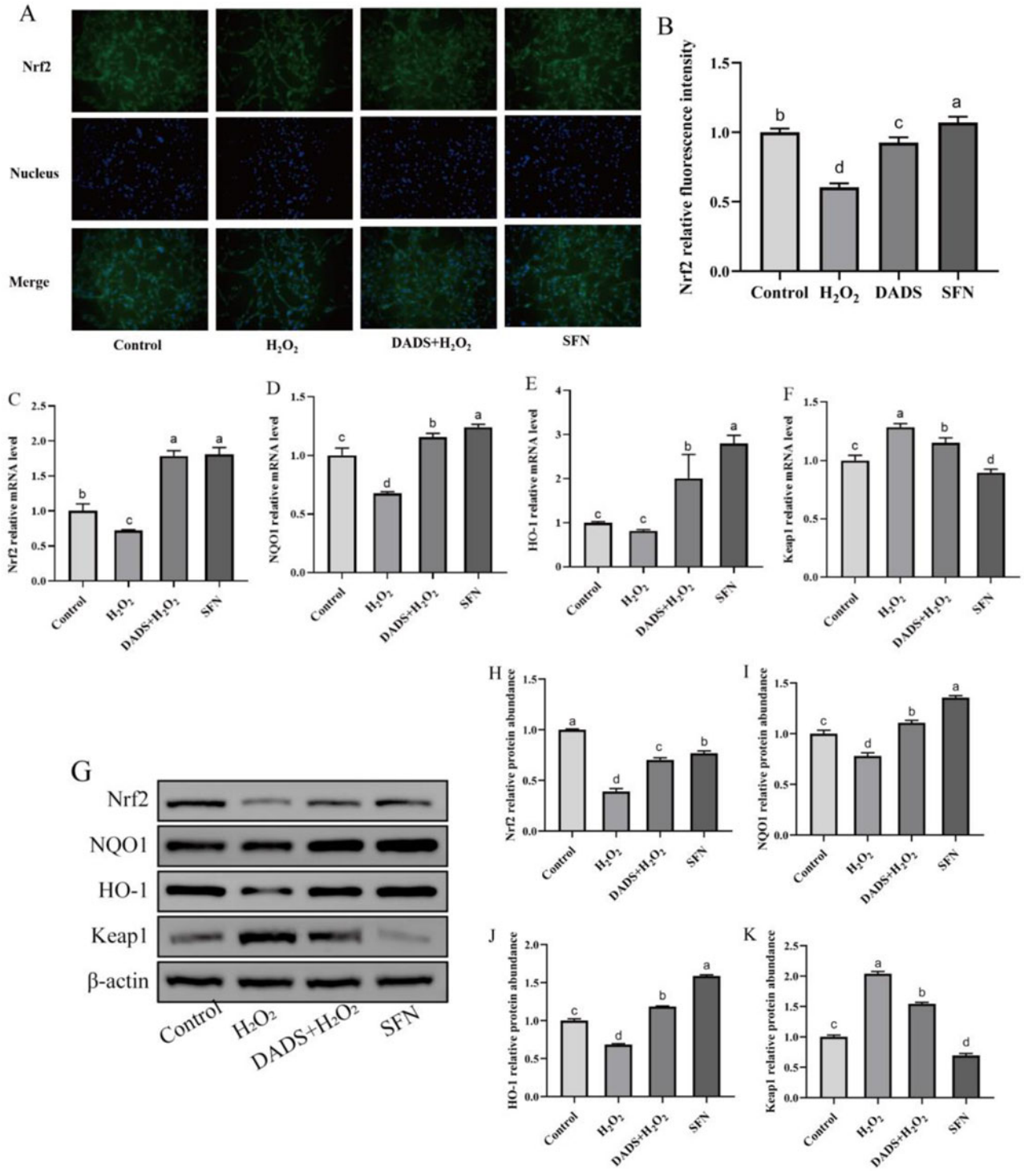

Figure 3. Effects of DADS on the Nrf2 signaling pathway in RECs with induced oxidative stress. The RECs underwent a 2-hour pretreatment with or without 150 μM DADS and a 24-hour treatment with or without 200 μM H_2O_2. (A) Immunofluorescence staining of Nrf2. (B) Quantitative Nrf2 fluorescence intensity. (C-F) Effects of DADS pretreatment on (C) Nrf2, (D) NQO1, (E) HO-1, and (F) Keap1 mRNA expression. (G–K) Effects of DADS pretreatment on the protein abundance of (H) Nrf2, (I) NQO1, (J) HO-1, and (K) Keap1 in RECs (G: representative western blots of these proteins). The results are shown as the mean ± SD (n = 3), with different letters (a-d) designating significant differences ($P < 0.05$). DADS = diallyl disulfide; H_2O_2 = hydrogen peroxide; HO-1 = heme oxygenase 1; Keap1 = Kelch-like ECH-associated protein; NQO1 = quinone oxidoreductase 1; Nrf2 = nuclear factor-erythroid 2-related factor 2; RECs = rumen epithelial cells; SFN = sulforaphane, positive control.

body from oxidative damage (Pan et al. 2018; Shao et al. 2019). It was reported that DADS could alleviate oxidative–antioxidative imbalance in a rat model of pulmonary emphysema by increasing the activities of GPX, SOD, and T-AOC (Liu et al. 2018). Asdaq et al. (2022) also observed that DADS significantly enhanced SOD and CAT activities in hyperlipidemic rats (Asdaq et al. 2022). Consistent with this earlier research, our study revealed that T-AOC and SOD activities were dramatically reduced when RECs

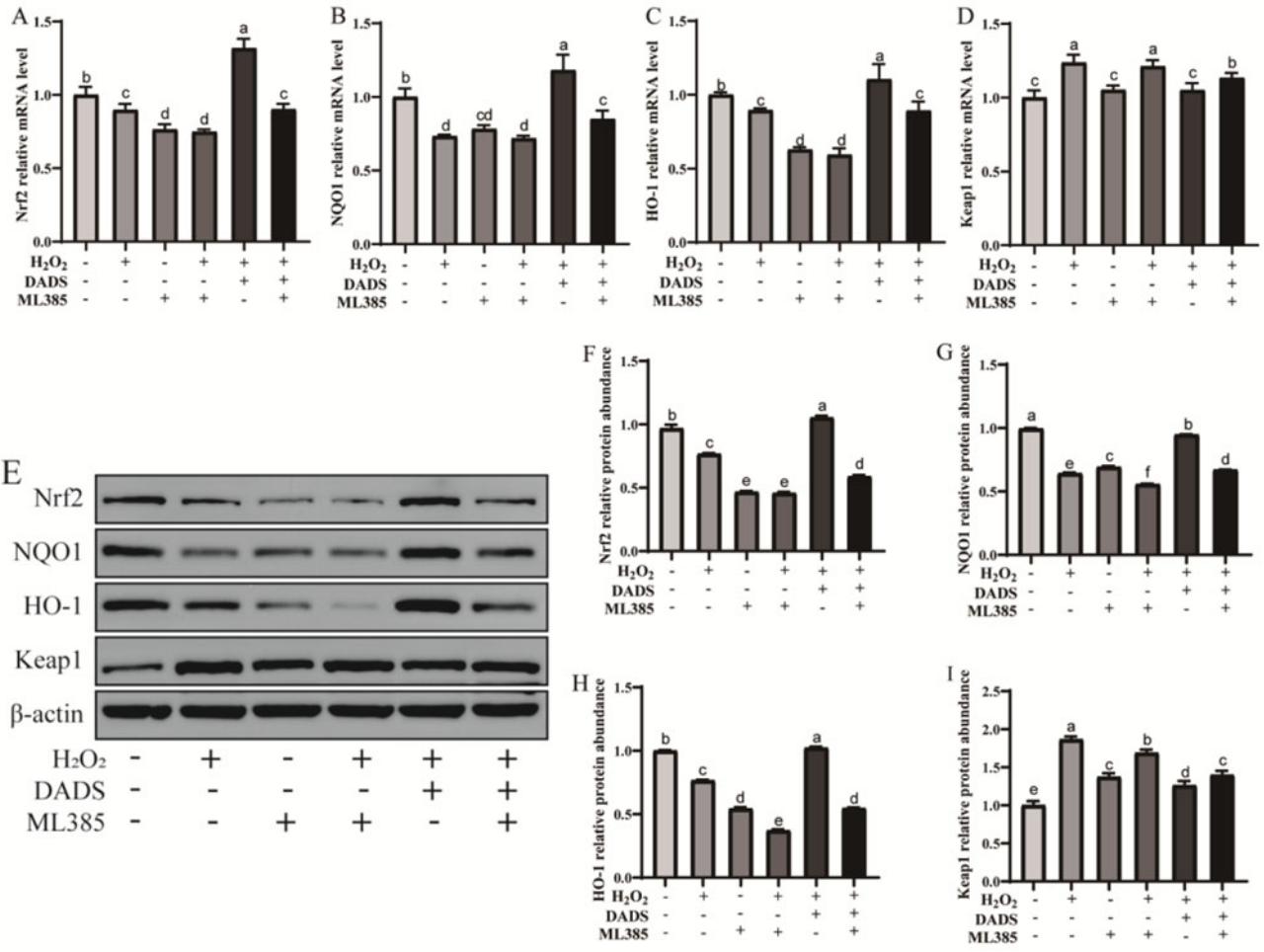

Figure 4. Effects of inhibition of Nrf2 signaling pathway on DADS's action on Nrf2 signaling pathway in RECs. The RECs underwent 2-hour pretreatment with or without 10 μM ML385 and 150 μM DADS and a 24-hour treatment with or without 200 μM H_2O_2. (A-D) Effects of DADS pretreatment on the mRNA expression of (A) Nrf2、(B) NQO1、(C) HO-1, and (D) Keap1 after inhibition of Nrf2 signaling pathway. (E-I) Effects of DADS pretreatment on the protein abundance of (F) Nrf2、(G) NQO1、(H) HO-1, and (I) Keap1 after inhibition of Nrf2 signaling pathway (E: representative western blots). The results are shown as the mean ± SD ($n = 3$), with letters (a–d) designating significant differences ($P < 0.05$). DADS = diallyl disulfide; H_2O_2 = hydrogen peroxide; HO-1 = heme oxygenase 1; Keap1 = Kelch-like ECH-associated protein; NQO1 = quinone oxidoreductase 1; Nrf2 = nuclear factor-erythroid 2-related factor 2; RECs = rumen epithelial cells.

were exposed to H_2O_2, and DADS pretreatment significantly alleviated these activities. However, the CAT and GPX activities were significantly enhanced after H_2O_2-induced oxidative stress and were reduced after DADS treatment unexpectedly. Similar results were also reported by previous studies (Leung et al. 2020; Ouyang et al. 2022). Considering the functions of these enzymes in decomposing H_2O_2, it can be inferred that the overexpression of CAT and GPX in our study may be the response to the increased level of exogenous H_2O_2 addition.

The Keap1/Nrf2 signaling pathway play an important role in regulating oxidative stress (Yang et al. 2016), with Nrf2 serving as a pivotal transcription factor in the cellular antioxidant defense system (Shaw and Chattopadhyay 2020). Under normal conditions, Nrf2 stays in the cytoplasm attachment to Keap1, maintaining the cell in a resting state (Kang et al. 2004). In the presence of oxidative stress, the Nrf2 separates from Keap1 and moves to the nucleus. There, Nrf2 binds to AREs of genes to start the transcription of downstream antioxidant genes, such as NQO1and HO-1. This activation helps to alleviate oxidative–antioxidative imbalance (Magesh et al. 2012). The Keap1 negatively regulates Nrf2 and activates its function (Piao et al. 2022). The HO-1 is a cell-protective enzyme with antioxidant capabilities (Maines and Panahian 2001). The NQO1 attenuates the activity of NADPH oxidase, reducing ROS secretion (Ma et al. 2021; Mizunoe et al. 2018). Many studies have reported that antioxidants protect cells from oxidative harm by activating the Keap1/Nrf2/ARE antioxidant pathway, which inhibits excessive ROS production and lipid peroxidation within cells (You et al. 2021; Zhu et al. 2022). In this study, DADS pretreatment significantly facilitated Nrf2 translocation to the nucleus and enhanced Nrf2, NQO1, and HO-1 gene expressions and protein abundances. Simultaneously, DADS diminished the mRNA expression and protein abundance of Keap1. To further support these results, SFN, a commonly used Nrf2 activator, was employed as a positive control. Indeed, our study showed that DADS acted like SFN. Furthermore, we used ML385, an inhibitor of Nrf2 in the cells (Zhou et al. 2021) to test whether DADS attenuates H_2O_2-induced oxidative damage via the Nrf2–Keap1 signaling pathway in RECs. Indeed, the ML385 disrupted the

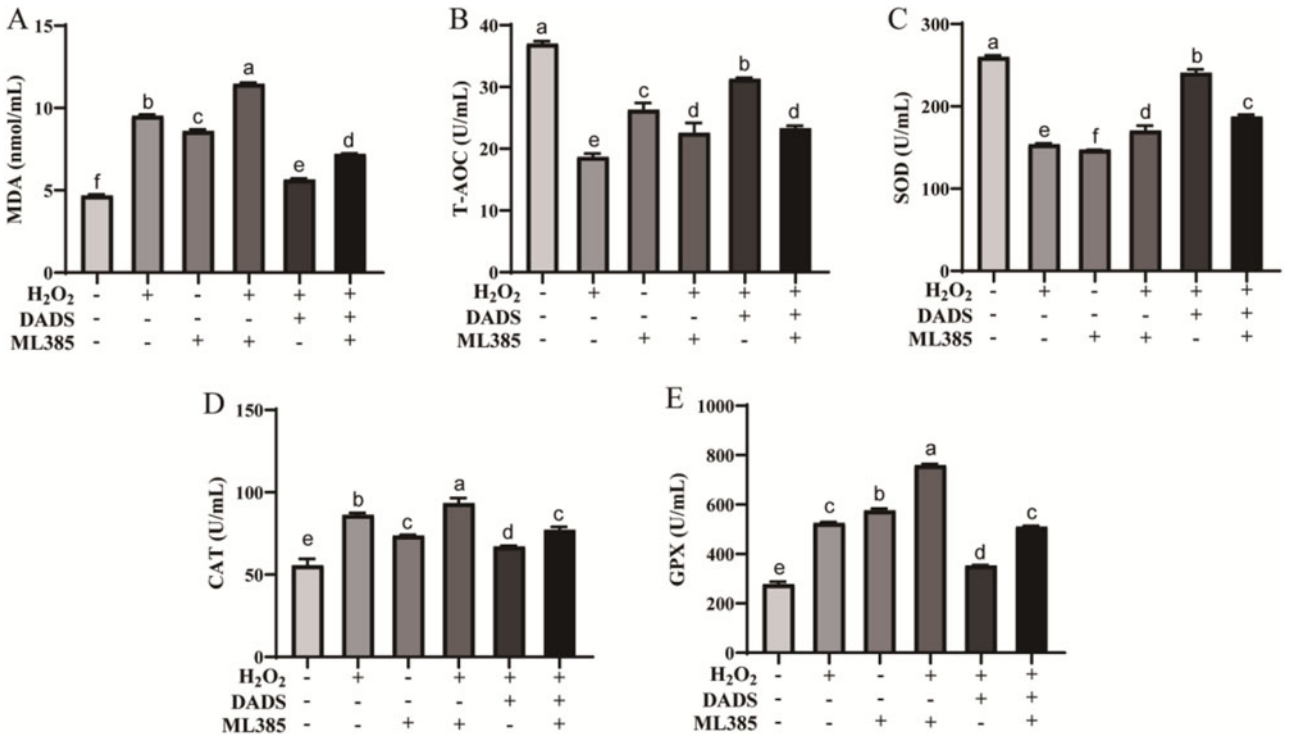

Figure 5. Effects of DADS on MDA content and antioxidant enzyme activity in H_2O_2-treated RECs after inhibition of Nrf2 signaling pathway. The RECs underwent 2-hour pretreatment with or without 10 μM ML385 and 150 μM DADS and a 24-hour treatment with or without 200 μM H_2O_2. Effects of DADS pretreatment on (A) MDA content and the enzyme activities of (B) T-AOC, (C) SOD, (D) CAT, and (E) GPX. The results are shown as the mean ± SD ($n = 3$), with distinct letters (a–d) designating significant differences ($P < 0.05$). CAT = catalase; DADS = diallyl disulfide; GPX = glutathione peroxidase; H_2O_2 = hydrogen peroxide; MDA = malondialdehyde; RECs = rumen epithelial cells; ROS = reactive oxygen species; SOD = superoxide dismutase; T-AOC = total antioxidant capacity.

capacity of DADS to alleviate oxidative damage. Taken together, our data demonstrated that DADS induced Nrf2 to dissociate and translocate into the nucleus, elevated the gene and protein expressions associated with the Keap1/Nrf2 signaling pathway (Nrf2, HO-1, and NQO1), and downregulated the levels of the Keap1 mRNA and protein.

Conclusion

In conclusion, DADS alleviated oxidative stress in H_2O_2-treated ovine RECs via activating cysteine and methionine metabolism to promote glutathione synthesis and enhancing the SOD and T-AOC activities. The DADS also activates the Keap1/Nrf2 signaling pathway by promoting the nuclear translocation of Nrf2 and increase the gene expression and protein abundance of downstream antioxidant factors (NQO1 and HO-1). This study offers supporting evidence of the potential use of DADS in antioxidative stress in ruminants.

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Author contributions. Tang Yingying designed and carried out this experiment; Pang Rui performed the experiment, analyzed the data and wrote the first draft of the manuscript; Zhang Qingyue, Wang Yuxin, Dong Xiaona, and Huang Li performed the experimental work and analyzed the data; Ren Chunhuan, Xue Yanfeng, and Zhang Zijun have edited the manuscript. Zhu Wen was involved in conducting this study and was responsible for obtaining funding, project management, and manuscript editing. The final version of the manuscript was read and approved by each co-author.

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