This is a “preproof” accepted article for *Animal Nutriomics*.
This version may be subject to change during the production process.
10.1017/anr.2024.13

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

R. Pang[1], Q. Y. Zhang[1], Y. X. Wang[1], X. N. Dong[1], L. Huang[1], Y. Y. Tang[1], C. H. Ren[1], Y. F. Xue[1], Z. J. Zhang[1], W. Zhu*[1]

[1] *College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, P. R. China.*

Short title: Diallyl disulfide attenuates oxidative damage of Ovine rumen epithelial cells

*Correspondence: W. Zhu. Email: zhuwen@ahau.edu.cn.

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 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 and Kyoto Encyclopedia of Genes and Genomes 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 activity and total antioxidant capacity ($P < 0.05$) while reducing reactive oxygen species 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 total antioxidant capacity and superoxide dismutase activities. In conclusion, DADS demonstrated the ability to alleviate oxidative stress in RECs by promoting antioxidative capacity through the Keap1/Nrf2 signaling pathway.

Keywords: rumen epithelial cells; hydrogen peroxide; diallyl disulfide; oxidative stress; Kelch-like ECH associated protein 1/the nuclear factor erythroid 2-related factor 2

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

1. 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 [1, 2]. Improper animal rearing in nutrition, environment, management, and transportation can lead to oxidative stress in animals [3]. The rumen plays a pivotal role in ruminant digestion, significantly influencing their growth, health, and productivity [4]. Heat stress in dairy cows leads to increased ROS, oxidative stress, and damage to rumen epithelial cells (RECs) [5]. 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 [6, 7].

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) [8, 9]. The 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 [10, 11].

Diallyl disulfide (DADS) is a lipophilic organosulfur molecule found widely in garlic and other Allium species [12]. It possesses various pharmacological properties, including antioxidation [13, 14], anti-inflammatory [15], and anticancer activities [16]. Our previous studies have found that DADS can improve antioxidant capacity, induce antioxidant enzyme activity, and scavenge ROS [17, 18]. Silva-Islas et al. (2019) have reported that DADS could prevent oxidative stress in rat brain by

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

inhibiting the Nrf2 signaling pathway [19]. 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.

2. Material and methods

2.1 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 (DMEM/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 μ*M*, determined by our previous study), or DADS (Sigma-Aldrich, MO, USA) (150 μ*M*, determined by our previous study) [20] for 2 h, followed by H_2O_2 treatment [13, 21]. 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 μ*M* which was selected in accordance with a prior study by Cao et al. [22] and our

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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.

2.2 RNA-Seq and differentially expressed genes (DEGs) 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 [23]. 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) [24]. 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 (FPKM) for each gene [25]. The 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 [26].

The Cluster Profiler software was used for conducting pathway analyses, examining Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differentially expressed genes (DEGs) [27].

2.3 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

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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).

2.4 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 [28]. The primers (Table 1) were made by Sangon Biotech (Shanghai, China).

2.5 Western blot analysis

After each treatment, the cells were lysed in RIPA lysis solution (mainly 50mM Tris (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate,

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

0.1% SDS, etc.) containing protein phosphatase inhibitors. The protein concentration was then determined using the bicinchoninic acid (BCA) protein assay kit (Yeasen Biotechnology). The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) 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 [29]. The protein abundance was normalized by the protein abundance of β-actin.

2.6 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.

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

The 4′,6-diamidino-2-phenylindole (DAPI) reagent (Servicebio, Wuhan, Hubei, China) was employed to stain the nuclei, and then a fluorescence microscopy was used to visualize the protein staining.

2.7 Statistical analysis

The data were present as the mean ± standard deviation (SD). The data was statistically analyzed using one-way analysis of variance (ANOVA), 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.

3 Results

3.1 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 ($P < 0.05$).

3.2 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

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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-kappa B signaling pathway, PPAR signaling pathway, among others when compared to the control. Furthermore, significant pathway enrichments in cellular immunity, oxidative stress, and inflammation, such as ECM-receptor interaction, cysteine and methionine metabolism, complement and coagulation cascades, PI3K-Akt signaling pathway, etc.,

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

were observed in DEGs between the DADS + H_2O_2 and H_2O_2 groups.

3.3 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 ($P < 0.05$).

3.4 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

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

intracellular GPX and CAT levels in comparison to cells treated with DADS alone ($P < 0.05$).

4 Discussion

Diallyl disulfide, an organic sulfur compound derived from garlic, is known for its powerful antioxidant properties [30]. The rumen, 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 [31, 32]. Therefore, oxidative stress is usually characterized by significant increases in ROS and MDA levels [33, 34]. 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 [3]. 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

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

associated with the regulation of cellular metabolism, redox processes, inflammatory and immune-related biological processes. Thus, the regulation of these biological processes 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 [35]. 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 [36].

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 [37]. The CAT and GPX can also decompose H_2O_2 to protect the body from oxidative damage [38, 39]. 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 [40]. Asdaq et al. (2022) also observed that DADS significantly enhanced SOD and CAT activities in hyperlipidemic rats [41]. Consistent with this earlier research, our study revealed that T-AOC and SOD activities were dramatically reduced when

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

RECs 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 [42,43]. 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 [44], with Nrf2 serving as a pivotal transcription factor in the cellular antioxidant defense system [45]. Under normal conditions, Nrf2 stays in the cytoplasm attachment to Keap1, maintaining the cell in a resting state [46]. 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 [47]. The Keap1 negatively regulates Nrf2 and activates its function [48]. The HO-1 is a cell-protective enzyme with antioxidant capabilities [49]. The NQO1 attenuates the activity of NADPH oxidase, reducing ROS secretion [50, 51]. 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 [52, 53]. In this study, DADS pre-treatment 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.

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

Furthermore, we used ML385, an inhibitor of Nrf2 in the cells [54] to test whether DADS attenuates H_2O_2-induced oxidative damage via the Nrf2-Keap1 signaling pathway in RECs. Indeed, the ML385 disrupted the capacity of DADS to alleviate oxidative damage. Token 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.

5 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.

Statement of conflicting interest

The research presented in this manuscript was not impacted by any known conflicts of interest.

Acknowledgments

The authors thank Dr. Chunlei Yang at Zhejiang University of Technology for providing sheep rumen epithelial cells.

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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.

Funding

This study was supported by the National Natural Science Foundation of China (Grant number: 32002199), The University Synergy Innovation Program of Anhui Province (Grant number: 23108209), Natural Science Foundation of Anhui Province (Grant number: 2008085MC86).

References

[1] Lin L.X., Cao Q.Q., Zhang C.D. et al (2022) Aflatoxin B1 causes oxidative stress and apoptosis in sheep testes associated with disrupting rumen microbiota. ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY, 232, 113225.

[2] Valko M., Leibfritz D., Moncol J. et al (2007) Free radicals and antioxidants in normal physiological functions and human disease. INTERNATIONAL JOURNAL OF BIOCHEMISTRY & CELL BIOLOGY, 39(1), 44-84.

[3] Zhang X., Liu X., Wan F. et al (2022) Protective effect of resveratrol against hydrogen peroxide-induced oxidative stress in bovine skeletal muscle cells. MEAT SCIENCE, 185, 108724.

[4] John L.J., Fromm M., Schulzke J.D. (2011) Epithelial barriers in intestinal inflammation. ANTIOXIDANTS & REDOX SIGNALING, 15(5), 1255-1270.

[5] Guo Z., Gao S., Ding J. et al (2021) Effects of heat stress on the ruminal epithelial barrier of dairy cows revealed by micromorphological observation and transcriptomic analysis. FRONTIERS IN GENETICS, 12, 768209.

[6] Liu J.H., Xu T.T., Liu Y.J. et al (2013) A high-grain diet causes massive disruption of ruminal epithelial tight junctions in goats. AMERICAN JOURNAL OF PHYSIOLOGY-REGULATORY INTEGRATIVE AND COMPARATIVE PHYSIOLOGY, 305(3), R232-R241.

[7] McCann J.C., Luan S., Cardoso F.C. et al (2016) Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. FRONTIERS IN MICROBIOLOGY, 7, 701.

[8] Hochmuth C.E., Biteau B., Bohmann D. et al (2011) Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. CELL STEM CELL, 8(2), 188-199.

[9] Xi H., Hu Z., Han S. et al (2023) FSH-inhibited autophagy protects against oxidative stress in goat Sertoli cells through p62-Nrf2 pathway. THERIOGENOLOGY, 195, 103-114.

[10] Moon S., Jhun J., Ryu J. et al (2021) The anti-arthritis effect of sulforaphane, an activator of Nrf2, is associated with inhibition of both B cell differentiation and the production of inflammatory cytokines. PLOS ONE, 16(2), 12.

[11] Sun Y., Dong H., Zhan A. et al (2020) Protection of teprenone against hypoxia and reoxygenation stress in stomach and intestine of Lateolabrax maculatus. FISH PHYSIOLOGY AND BIOCHEMISTRY, 46(2), 575-584.

[12] Cheng F., Ali M., Liu C. et al (2020) Garlic allelochemical diallyl disulfide alleviates autotoxicity in the root exudates caused by long-term continuous cropping of tomato. JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, 68(42), 11684-11693.

[13] Hosseinzadeh A., Jafari D., Kamarul T. et al (2017) Evaluating the protective effects and mechanisms of diallyl disulfide on interlukin-1beta-induced oxidative stress and mitochondrial apoptotic signaling pathways in cultured chondrocytes. JOURNAL OF CELLULAR BIOCHEMISTRY, 118(7), 1879-1888.

[14] Zeng T., Zhang C., Song F. et al (2013) The activation of HO-1 / Nrf-2 contributes to the protective effects of diallyl disulfide (DADS) against ethanol-induced oxidative stress. BIOCHIMICA ET BIOPHYSICA ACTA-GENERAL SUBJECTS, 1830(10), 12.

[15] Zhang H., Shang C., Tian Z. et al (2020) Diallyl disulfide suppresses inflammatory and oxidative machineries following carrageenan injection-induced paw edema in mice. MEDIATORS OF INFLAMMATION, 2020, 8508906.

[16] Xia L., Lin J., Su J. et al (2019) Diallyl disulfide inhibits colon cancer metastasis by suppressing Rac1-mediated epithelial-mesenchymal transition. ONCOTARGETS AND THERAPY, 12, 5713-5728.

[17] Tang Y.T., Huang L., Sun X.Y., et al (2024) Effects of allicin on growth

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

performance, antioxidant profile, and microbiota compared to monensin of growing goats. ANIMAL SCIENCE JOURNAL, 95(1), e13917.

[18] Liu T.W., Pang R., Huang L., et al (2024) Effects of allicin addition on growth performance, rumen microbiome, and ruminal epithelial proteome of high-grain-fed goats. ANIMAL FEED SCIENCE AND TECHNOLOGY, 310, 115944.

[19] Silva-Islas C.A., Chanez-Cardenas M.E., Barrera-Oviedo D. et al (2019) Diallyl trisulfide protects rat brain tissue against the damage induced by ischemia-reperfusion through the Nrf2 pathway. ANTIOXIDANTS, 8(9).

[20] Zhang Q.Y., Huang L., Wang Y.M. et al (2023) Alleviation effect of diallyl disulfide on oxidative stress damage in Hu sheep rumen epithelial cells induced by hydrogen peroxide. CHINESE JOURNAL OF ANIMAL NUTRITION, 35(12), 8036-8045.

[21] Kim J.G., Koh S.H., Lee Y.J. et al (2005) Differential effects of diallyl disulfide on neuronal cells depend on its concentration. TOXICOLOGY, 211(1-2), 86-96.

[22] Cao L., Zhao J., Ma L. et al (2021) Lycopene attenuates zearalenone-induced oxidative damage of piglet sertoli cells through the nuclear factor erythroid-2 related factor 2 signaling pathway. ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY, 225, 112737.

[23] Chen S., Zhou Y., Chen Y. et al (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. BIOINFORMATICS, 34(17), i884-i890.

[24] Siren J., Valimaki N., Makinen V. (2014) Indexing graphs for path queries with applications in genome research. IEEE-ACM TRANSACTIONS ON COMPUTATIONAL BIOLOGY AND BIOINFORMATICS, 11(2), 375-388.

[25] Pertea M., Pertea G.M., Antonescu C.M. et al (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. NATURE BIOTECHNOLOGY, 33(3), 290-295.

[26] Anders S., Huber W. (2010) Differential expression analysis for sequence count data. GENOME BIOLOGY, 11(10), R106.

[27] Yu G., Wang L., Han Y. et al (2012) clusterProfiler : an R package for comparing biological themes among gene clusters. OMICS-A JOURNAL OF INTEGRATIVE BIOLOGY, 16(5), 284-287.

[28] Livak K.J., Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. METHODS, 25(4), 402-408.

[29] Liu S., Mao X., Ge L. et al (2022) Phenethyl isothiocyanate as an anti-nutritional factor attenuates deoxynivalenol-induced IPEC-J2 cell injury through inhibiting ROS-mediated autophagy. ANIMAL NUTRITION, 8(1), 300-309.

[30] Wu C.C., Sheen L.Y., Chen H.W. et al (2002) Differential effects of garlic oil

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

and its three major organosulfur components on the hepatic detoxification system in rats. JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, 50(2), 378-383.

[31] Yang M., Kuang M., Wang G. et al (2021) Choline attenuates heat stress-induced oxidative injury and apoptosis in bovine mammary epithelial cells by modulating PERK/Nrf-2 signaling pathway. MOLECULAR IMMUNOLOGY, 135, 388-397.

[32] Ho E., Karimi G.K., Liu C.C. et al (2013) Biological markers of oxidative stress: Applications to cardiovascular research and practice. REDOX BIOLOGY, 1(1), 483-491.

[33] Bernabucci U., Ronchi B., Lacetera N. et al (2002) Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. JOURNAL OF DAIRY SCIENCE, 85(9), 2173-2179.

[34] Cui L., Guo J., Wang Z. et al (2023) Meloxicam inhibited oxidative stress and inflammatory response of LPS-stimulated bovine endometrial epithelial cells through Nrf2 and NF-kappaB pathways. INTERNATIONAL IMMUNOPHARMACOLOGY, 116, 109822.

[35] Zhong Z.H., Li Z.C., Li H. et al (2022) Glutathione metabolism in Cryptocaryon irritans involved in defense against oxidative stress induced by zinc ions. PARASITES & VECTORS, 15(1), 318.

[36] Yang Z., Chen S.D., Sun W.X. (2024) Study on the mechanisms by which pumpkin polysaccharides regulate abnormal glucose and lipid metabolism in diabetic mice under oxidative stress. INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES, 270(Pt 2), 132249.

AQUATIC TOXICOLOGY, 224, 105516.

[37] McCord J.M., Edeas M.A. (2005) SOD, oxidative stress and human pathologies: a brief history and a future vision. BIOMEDICINE & PHARMACOTHERAPY, 59(4), 139-142.

[38] Shao Y., Chen Z., Wu L. (2019) Oxidative stress effects of soluble sulfide on human hepatocyte cell line LO2. INTERNATIONAL JOURNAL OF ENVIRONMENTAL RESEARCH AND PUBLIC HEALTH, 16(9).

[39] Pan Y.F., Jia X.T., Song E.F. et al (2018) Astragaloside IV protects against abeta1-42-induced oxidative stress, neuroinflammation and cognitive impairment in rats. CHINESE MEDICAL SCIENCES JOURNAL, 33(1), 29-37.

[40] Liu Y., Li A., Feng X. et al (2018) Pharmacological investigation of the anti-inflammation and anti-oxidation activities of diallyl disulfide in a rat emphysema model induced by cigarette smoke extract. NUTRIENTS, 10(1), 16.

[41] Asdaq S.M.B., Yasmin F., Alsalman A.J. et al (2022) Obviation of dyslipidemia by garlic oil and its organosulfur compound, diallyl disulphide, in experimental

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

animals. SAUDI JOURNAL OF BIOLOGICAL SCIENCES, 29(4), 6.

[42] Leung W.S., Kuo W.W., Ju D.T. et al (2020) Protective effects of diallyl trisulfide (DATS) against doxorubicin-induced inflammation and oxidative stress in the brain of rats. FREE RADICAL BIOLOGY AND MEDICINE, 160, 141-148.

[43] Ouyang J., Zhou H., Li Q. et al (2022) Tryptophan alleviates acute heat stress-induced impairment of antioxidant status and mitochondrial function in broilers. FRONTIERS IN VETERINARY SCIENCE, 9, 863156.

[44] Yang X., Yao W., Shi H. et al (2016) Paeoniflorin protects Schwann cells against high glucose induced oxidative injury by activating Nrf2/ARE pathway and inhibiting apoptosis. JOURNAL OF ETHNOPHARMACOLOGY, 185, 361-369.

[45] Shaw P., Chattopadhyay A. (2020) Nrf2-ARE signaling in cellular protection: Mechanism of action and the regulatory mechanisms. JOURNAL OF CELLULAR PHYSIOLOGY, 235(4), 3119-3130.

[46] Kang M.I., Kobayashi A., Wakabayashi N. et al (2004) Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 101(7), 6.

[47] Magesh S., Chen Y., Hu L. (2012) Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. MEDICINAL RESEARCH REVIEWS, 32(4), 687-726.

[48] Piao F., Gao B., Yuan X. et al (2022) Taurine ameliorates oxidative stress in spinal cords of diabetic rats via Keap1-Nrf2 signaling. ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, 1370, 235-242.

[49] Maines M.D., Panahian N. (2001) The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions? ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, 502, 249-272.

[50] Ma Y., Zhang Y., Zhang H. et al (2021) Thiamine alleviates high-concentrate-diet-induced oxidative stress, apoptosis, and protects the rumen epithelial barrier function in goats. FRONTIERS IN VETERINARY SCIENCE, 8, 663698.

[51] Mizunoe Y., Kobayashi M., Sudo Y. et al (2018) Trehalose protects against oxidative stress by regulating the Keap1-Nrf2 and autophagy pathways. REDOX BIOLOGY, 15, 115-124.

[52] You L., Peng H., Liu J. et al (2021) Catalpol protects ARPE-19 cells against oxidative stress via activation of the Keap1/Nrf2/ARE pathway. CELLS, 10(10).

[53] Zhu W., Tang H., Cao L. et al (2022) Epigallocatechin-3-O-gallate ameliorates

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

oxidative stress-induced chondrocyte dysfunction and exerts chondroprotective effects via the Keap1/Nrf2/ARE signaling pathway. CHEMICAL BIOLOGY & DRUG DESIGN, 100(1), 108-120.

[54] Zhou Y., Tan Z., Huang H. et al (2021) Baicalein pre-treatment alleviates hepatic ischemia/reperfusion injury in mice by regulating the Nrf2/ARE pathway. EXPERIMENTAL AND THERAPEUTIC MEDICINE, 22(6), 1380.

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

Gene	Accession number	Primer sequence (5′ to 3′) [1]	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

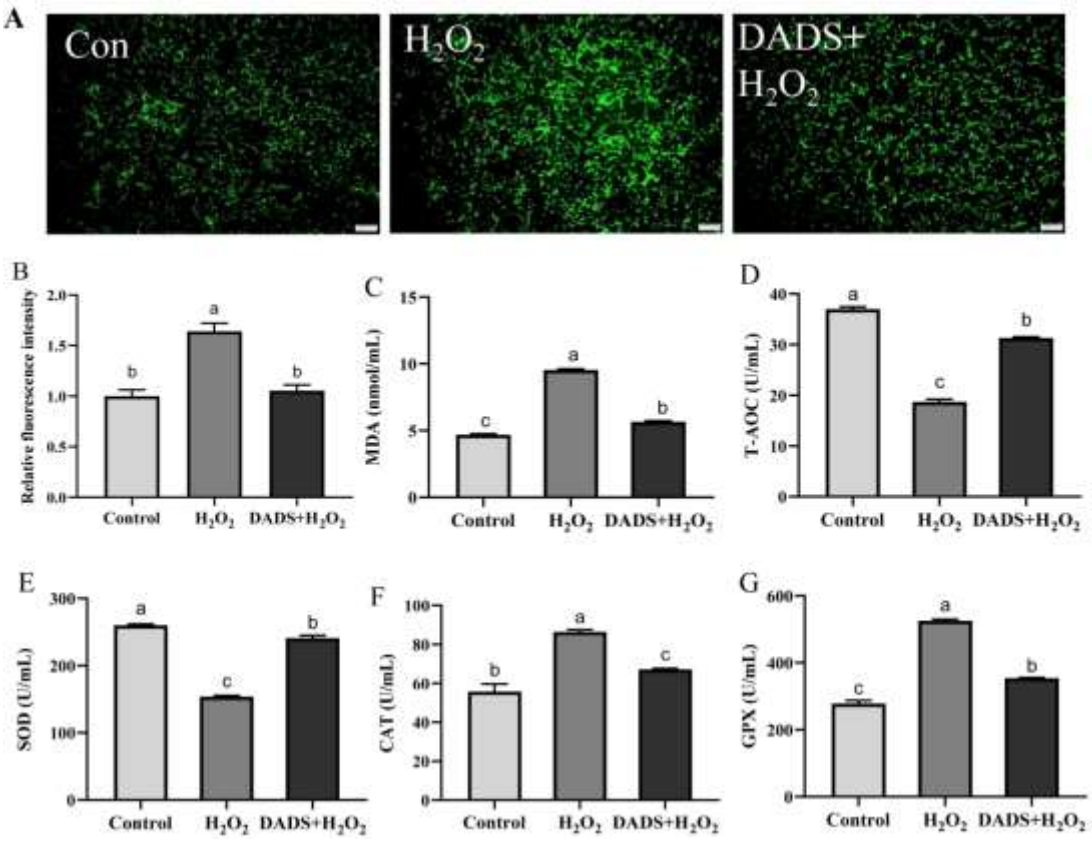
SOD2 = superoxide dismutase 2; CAT = catalase; GPX1 = glutathione peroxidase 1; Nrf2 = nuclear-factor-erythroid derived 2 like protein 2; HMOX1 = Heme oxygenase 1; NQO1 = Quinone oxidoreductase 1，NQO1 = Kelch-like ECH-associated protein

[1] F = forward; R = reverse.

https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

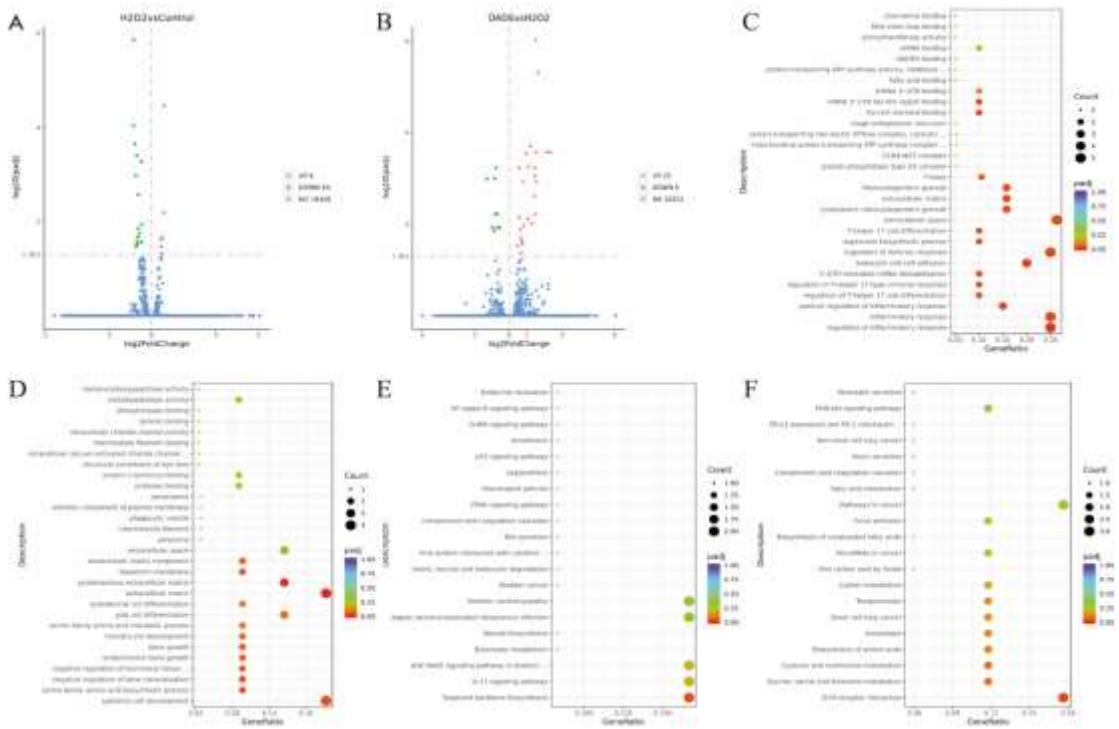
Figure legends

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 μ*M* DADS and a 24-hour treatment with or without 200 μ*M* H_2O_2. (A) The staining of ROS in RECs treated with H_2O_2 or DADS (Scale bar: 200 μ*M*), (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.



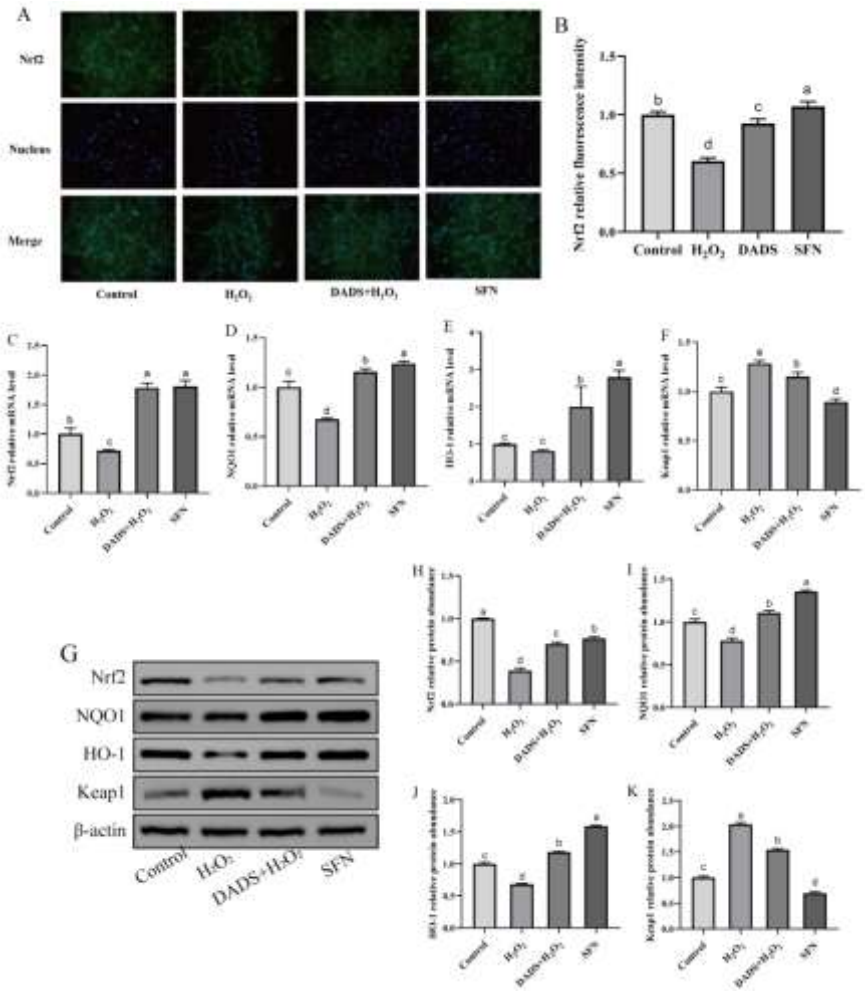
https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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 μ*M* DADS, followed by a 24-hour treatment with or without 200 μ*M* 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.



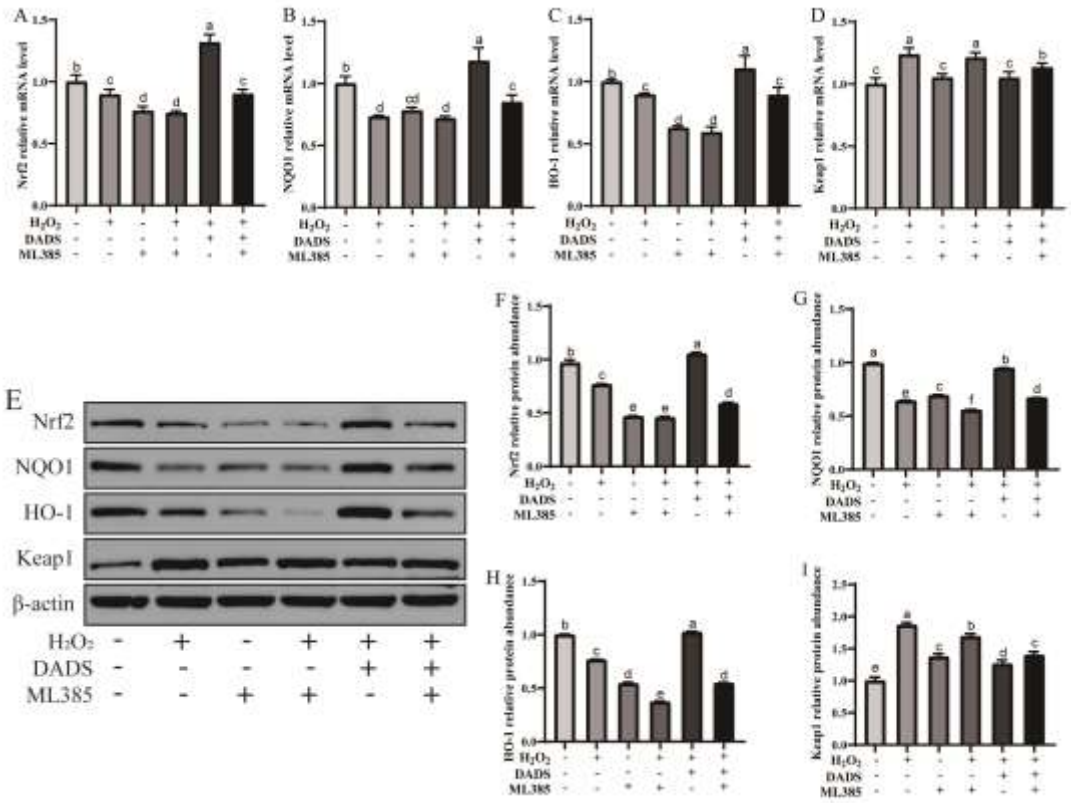
https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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 μ*M* DADS and a 24-hour treatment with or without 200 μ*M* 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.



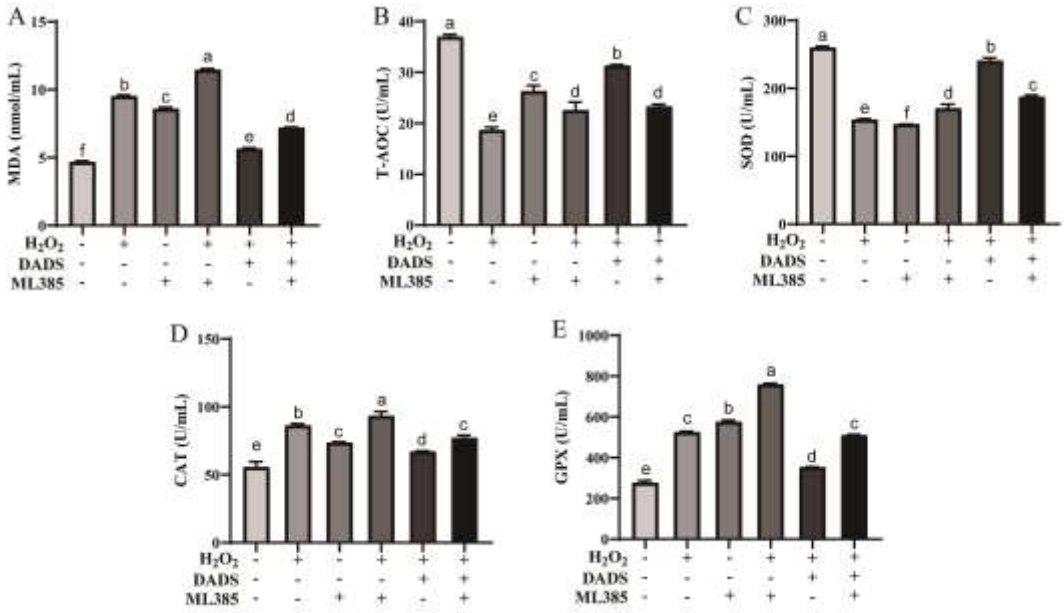
https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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 μ*M* ML385 and 150 μ*M* DADS and a 24-hour treatment with or without 200 μ*M* 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.



https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press

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 μ*M* ML385 and 150 μ*M* DADS and a 24-hour treatment with or without 200 μ*M* 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.



https://doi.org/10.1017/anr.2024.13 Published online by Cambridge University Press