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N-acetylcysteine modulates cyclophosphamide-induced immunosuppression, liver injury, and oxidative stress in miniature pigs

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Abstract

Cyclophosphamide, a cytotoxic anticancer agent, induces immunosuppression and has several adverse effects. N-acetylcysteine alleviates oxidative stress, liver injury, and intestinal tissue damage. The present study examined whether N-acetylcysteine modulates the adverse effects of cyclophosphamide in pigs. Miniature pigs (n = 15) were used as an experimental model to evaluate the effects of N-acetylcysteine treatment on immune reactions, liver injury, and oxidative stress after cyclophosphamide challenge. Corn-soybean meal based dietary treatments were as follows: control diet with either saline injection, cyclophosphamide injection, or 0.5% N-acetylcysteine and cyclophosphamide injection. N-acetylcysteine increased the number of immune cells and decreased TNF-α production after cyclophosphamide injection and decreased TNF-α, IFN-γ, NF-κB, and IL-8 expression and increased IL-10 expression in peripheral blood mononuclear cells. Serum levels of alanine transaminase and aspartate aminotransferase decreased, superoxide dismutase activity increased, and malondialdehyde activity decreased following N-acetylcysteine treatment after cyclophosphamide injection. N-acetylcysteine decreases immunosuppression, liver injury, and oxidative stress in cyclophosphamide-challenged miniature pigs. The present study suggests that N-acetylcysteine has therapeutic application in livestock for modulating immune reactions, liver injury, and oxidative stress.

Keywords: Cyclophosphamide, Miniature pig, N-acetylcysteine, Peripheral blood mononuclear cells

INTRODUCTION

Many factors, such as infection and stress, can induce immunosuppression, resulting in retardation of growth performance, susceptibility to disease, and mortality [1,2]. Immunosuppression in livestock is thus directly related to economic loss. Alleviating adverse immune reactions is necessary for successful livestock production [1,2] Cyclophosphamide (CTX), an alkylating agent, interferes with DNA replication and RNA transcription and is used in cancer chemotherapy [3]. The CTX directly activates immune responses in hematopoietic stem cells and dendritic cells [4,5]. It affects the proliferation of

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Lee SI.
Data curation: Shin S, Lee SI.
Formal analysis: Shin S, Lee SI.
Methodology: Kang KS, Lee SI.
Software: Kang KS.
Validation: Shin S, Lee SI.
Writing - original draft: Kang KS, Shin S, Lee SI.

Ethics approval and consent to participate
The animal care and experimental protocols of the present study were approved by the Animal Care, and Use Committee of Dankook University (IACUC protocol No. DKU-18-035) and all methods were performed in accordance with the relevant guidelines and regulations.

lymphocytes and is extensively used for the treatment of certain tumors, cancers, and autoimmune disorders. Dose-dependent administration of CTX also enhances immune responses. Despite its chemotherapeutic activity, many studies report that CTX treatment induces several adverse effects, including hemorrhagic cystitis in bone marrow transplantation, genotoxicity in human lymphocytes, cardiac toxicity, and pulmonary fibrosis [6–9]. Nevertheless, CTX is widely used as an anticancer agent because it suppresses the immune system [10].

N-acetylcysteine (NAC) is an antioxidant that exerts its effects by directly scavenging reactive oxygen species to protect cells from oxidative damage and by stimulating glutathione synthesis [11,12]. Furthermore, NAC modulates oxidative stress, liver injury, and inflammation of the small intestine [13,14].

The NAC alleviates CTX-induced gonadotoxicity and genotoxicity in experimental animals such as mice and rats [15,16]. However, the effects of dietary supplementation with NAC on CTX-induced immune reactions, liver injury, and oxidative stress have not been investigated in miniature pigs. Miniature pigs are a valuable animal model to investigate the effects of therapeutic agents because of their similarity to humans in terms of anatomical and physiological features and sensitivity to drugs compared with other non-rodent species [17]. The present study thus used miniature pigs to evaluate the effects of dietary supplementation with NAC on CTX-induced immune reactions, including cytokine production and expression of immune-related genes; liver injury; and oxidative stress.

MATERIALS AND METHODS

Experimental design, feeding, and CTX challenge

Miniature pigs (n = 15) [MK strain; (Duroc × Yorkshire) × (Pot Valley × Berkshire) × Yucatan] with an average initial body weight of 22.3 ± 0.32 kg were used to evaluate the effects of dietary supplementation with NAC on effects caused by CTX injection in a 4-week trial. Each animal pen was equipped with a one-sided, stainless steel self-feeder and a nipple drinker that allowed access to feed and water *ad libitum*. All diets were formulated to meet or exceed NRC nutrition requirements [18]. The corn-soybean meal based dietary treatments were as follows: T1, control diet + saline injection; T2, control diet + CTX injection; and T3, control diet + 0.5% NAC and CTX injection. For CTX treatment, pigs were injected intraperitoneally with 0.01% CTX (50 mg/kg) or saline solution after a 2-week feeding trial.

Blood sample characteristics and enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected into non-heparinized and K_3-EDTA vacuum tubes (Becton Dickinson Vacutainer Systems) to determine numbers of immune cells, including leukocytes, lymphocytes, and monocytes, after 3 and 4 weeks. Immune cell counts were determined using an automatic blood analyzer (ADVIA120; Bayer, Leverkusen, Germany). ELISA was used to quantify serum concentrations of tumor necrosis factor (TNF)- α (R&D Systems, Minneapolis, MN, USA), alanine transaminase (ALT; Sigma-Aldrich, St. Louis, MO, USA), aspartate aminotransferase (AST; Sigma-Aldrich, St. Louis, MO, USA), superoxide dismutase (SOD; Cohesion Biosciences, USA), malondialdehyde (MDA; Abcam, Cambridge, UK), and glutathione peroxidase (GPx; Cayman Chemical, Ann Arbor, MI, USA).

Peripheral blood mononuclear cell (PBMC) preparation

Blood samples were collected 6 h after injection, and PBMCs were prepared as previously described [19]. Briefly, blood samples were diluted with an equal volume of balanced salt solution and were

mixed with Histopaque solution. After centrifugation at 400×g for 35 min at room temperature, PBMCs were collected from the Histopaque solution-plasma interface.

Quantitative real-time polymerase chain reaction (PCR)

TRIzol reagent (Invitrogen) was used for RNA extraction from PBMCs. For complementary DNA synthesis from total RNA (100 μg), a Maxima First Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) was used. Primer sets were designed using Primer3 software (http://frodo.wi.mit.edu/), and the primer sequences are shown in Table 1. Quantitative real-time PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) with the following conditions: 94℃ for 3 min, followed by 40 cycles at 94℃ for 30 s, 59℃–61℃ for 30 s, and 72℃ for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control, and quantitative real-time PCR data were calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = [threshold cycles (Ct) of the target gene – Ct of *GAPDH*] treatment – [Ct of the target gene – Ct of *GAPDH*] [20].

Statistical analysis

Data were statistically analyzed using analysis of variance and the general linear model (GLM) procedure of statistical analysis software (SAS) program (SAS Institute, Cary, NC, USA), with a completely randomized design. Data are presented as means and standard error of the means. Statistical significance of differences between treatments for immune cells, TNF-α production, inflammatory cytokine-related gene expression, liver function parameters, and oxidative stress markers was analyzed using the GLM in SAS. Duncan's multiple range test was used as a post hoc test to analyze differences between means, and a p-value of < 0.05 was considered statistically significant.

RESULTS

Effects of NAC on immune cells and TNF-α production after CTX challenge

Treatment with CTX decreased immune cell numbers at 3 and 4 weeks compared with that in the control group [T1 group; Fig. 1]. Compared with CTX alone, dietary supplementation with NAC increased the immune cell numbers at 3 and 4 weeks. The effect of dietary supplementation with NAC on TNF-α production after CTX treatment is shown in Fig. 2. CTX treatment increased the levels of TNF-α at 3 and 4 weeks compared with that in the T1 group. Compared with CTX alone (T2 group), dietary supplementation with NAC decreased the levels of TNF-α at 3 weeks.

Table 1. List of primers

Gene symbol	Description	Accession No.	Forward (5' → 3')	Reverse (5' → 3')
TNF-α	Tumor necrosis factor alpha	NM_214022	TCTCCTTCCTCCTGGTCGCA	TCCCTCGGCTTTGACATTGG
IL1beta1	Interleukin-1 beta1	NM_214055	CCGAAGCTGACAGAAGGGGA	AGTGGATGGGGCCTGAGGAT
IL-8	Interleukin-8	NM_213867	GGCTGTTGCCTTCTTGGCAG	TTTGGGGTGGAAAGGTGTGG
IFN-γ	Interferon gamma	NM_213948	GGCCATTCAAAGGAGCATGG	GATGGCTTTGCGCTGGATCT
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001048232	GACAACATCTCCTTGGCGGG	TCTGCTCCTGCTGCTTTGAGG
IL-4	Interleukin-4	NM_214123	TCCACGGACACAAGTGCGAC	TGTTTGCCATGCTGCTCAGG
IL-6	Interleukin-6	NM_214399	AGCCCACCAGGAACGAAAGA	AGCCATCACCAGAAGCAGCC
IL-10	Interleukin-10	NM_214041	CATCCACTTCCCAACCAGCC	CTCCCCATCACTCTCTGCCTTC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001206359	AATGGGGTGATGCTGGTGCT	GGCAGAAGGGGCAGAGATGA

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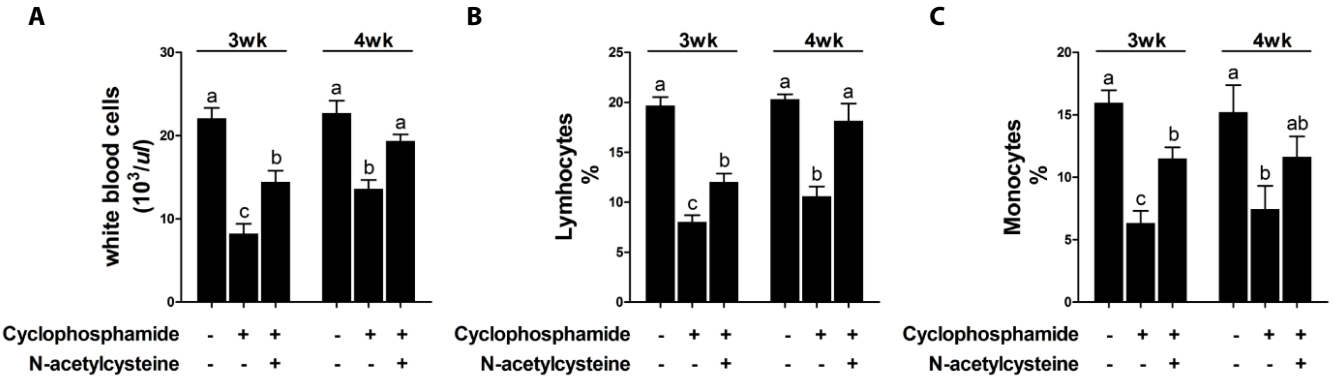

Fig. 1. Effects of dietary supplementation with N-acetylcysteine (NAC) on numbers of immune cells. White blood cells (A), lymphocytes (B), and monocytes (C) were collected from the blood of cyclophosphamide-induced immunosuppressed miniature pigs. Immune cell numbers were determined using an automatic blood analyzer (n = 5). Error bars indicate the standard error of the mean (SEM). A *p*-value of < 0.05 was considered statistically significant. [a–c]Indicate significant differences between treatments as determined by Duncan's multiple range tests.

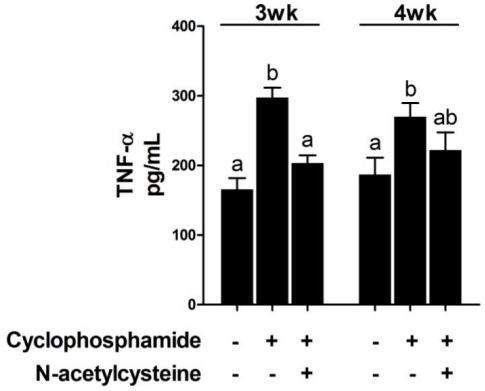

Fig. 2. Effects of dietary supplementation with N-acetylcysteine (NAC) on serum TNF-α levels in the serum of cyclophosphamide-induced immunosuppressed miniature pigs (n = 5). Error bars indicate the standard error of the mean (SEM). A *p*-value of < 0.05 was considered statistically significant. [a,b]Indicate significant differences between treatments as determined by Duncan's multiple range tests.

Effects of NAC on the expression of immune-related genes in PBMCs after CTX challenge

CTX increased *NF-κB*, *IFN-γ*, *TNF- α* , *IL-8*, and *IL-1beta1* expression and decreased *IL-10* and IL-4 expression compared with the results in the control group (Fig. 3). Dietary supplementation with NAC decreased *NF-κB*, *IFN-γ*, *TNF- α* , and *IL-8* expression and increased *IL-10* expression.

Effects of NAC on liver function parameters after CTX challenge

CTX increased the serum AST and ALT levels at 3 and 4 weeks compared with the results in the control group (Fig. 4). Dietary supplementation of NAC after CTX challenge decreased the serum AST and ALT levels.

Effects of NAC on oxidative stress markers after CTX challenge

CTX challenge decreased the serum SOD and GPx levels and increased the serum MDA levels at 3 and 4 weeks compared with the results in the control group (Fig. 5). Dietary supplementation with NAC following treatment with CTX increased the serum SOD and GPx levels and decreased

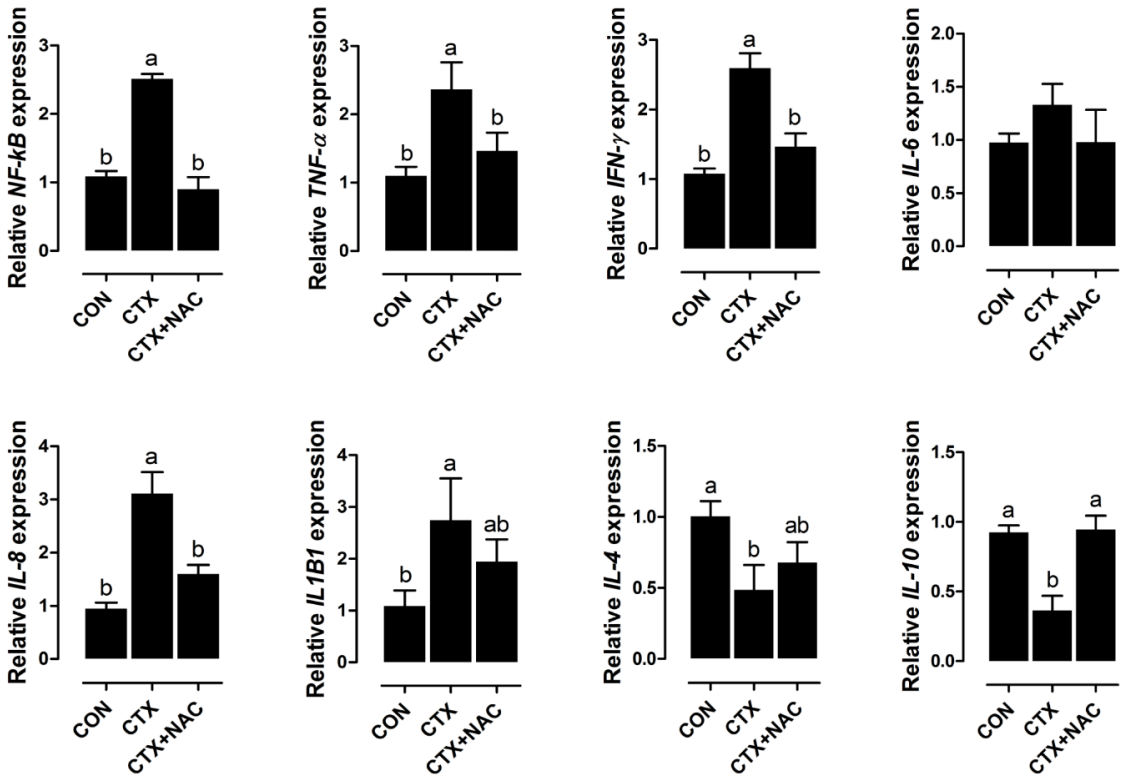

Fig. 3. Effects of dietary supplementation with N-acetylcysteine (NAC) on the relative expression of cytokine genes, including *NF-κB, TNF-α, IFN-γ, IL-6, IL-8, IL-1beta1, IL-4*, and *IL-10*, in peripheral blood mononuclear cells from cyclophosphamide-induced immunosuppressed miniature pigs. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) data were calculated using the $2^{-\Delta\Delta Ct}$ method (n = 5). Error bars indicate the standard error of the mean (SEM). A *p*-value of < 0.05 was considered statistically significant. [a,b]Indicate significant differences between treatments as determined by Duncan's multiple range tests.

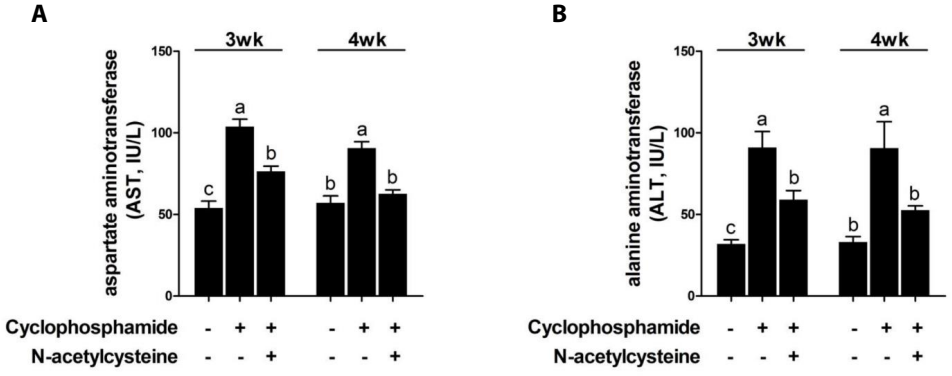

Fig. 4. Effects of dietary supplementation with N-acetylcysteine (NAC) on liver injury markers. Serum AST (A) and ALT (B) levels of cyclophosphamide-induced immunosuppressed miniature pigs were determined using enzyme-linked immunosorbent assay (ELISA; n = 5). Error bars indicate the standard error of the mean (SEM). A *p*-value of < 0.05 was considered as statistically significant. [a–c]Indicate significant differences between treatments as determined by Duncan's multiple range tests.

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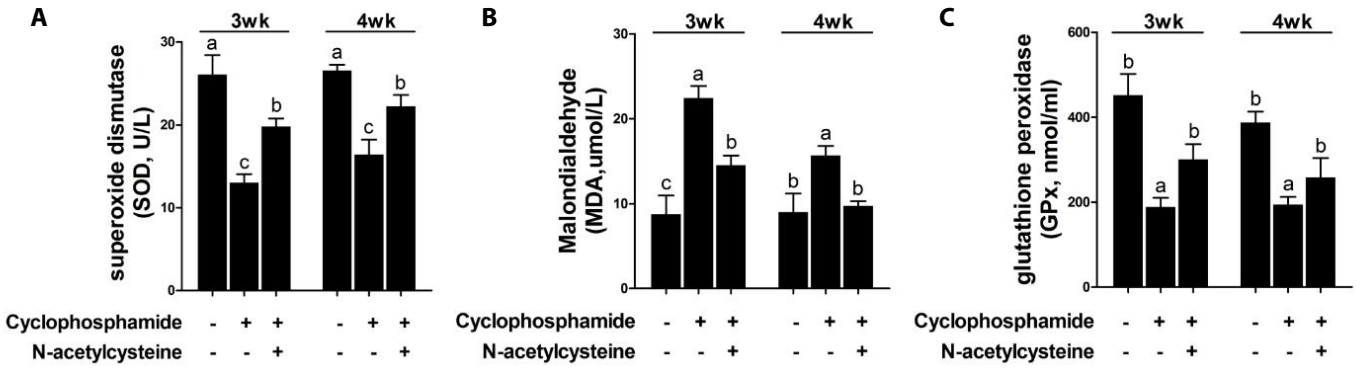

Fig. 5. Effects of dietary supplementation with N-acetylcysteine (NAC) on oxidative stress markers. Serum SOD (A), MDA (B), and GPx (C) level of cyclophosphamide-induced immunosuppressed miniature pigs were determined using ELISA (n = 5). Error bars indicate the standard error of the mean (SEM). A *p*-value of < 0.05 was considered statistically significant. [a–c]Indicate significant differences between treatments as determined by Duncan's multiple range tests.

the serum MDA levels at 3 and 4 weeks.

DISCUSSION

CTX is a commercially available agent used to treat immune disorders after organ transplantation [1]. It is widely accepted that CTX-induced immunosuppression reduces cellular immunity, oxidative stress, as well as daily weight gain and feed intake in livestock [1,21]. NAC, traditionally used as an antioxidant, modulates oxidative stress-mediated liver injury and inflammation of the small intestine [13,14]. Miniature pigs as an experimental model for humans have advantages of similar sensitivity to drugs compared with other non-rodent species and similar anatomical and physiological features [17,22,23]. Dietary supplementation with NAC increased immune cell numbers and decreased the level of TNF-α production compared with CTX treatment alone. Consistent with our findings, previous studies reported that other dietary agents also modulate lymphocytes, IL-2, and IFN-γ in CTX-induced immunosuppressed pigs [21]. The present study used PBMCs, which comprise lymphocytes, monocytes, and macrophages, as representative immune cells. In molecular nutrition and nutrigenomics, these cells are good targets because they directly reflect gene expression levels following chemical treatments [24].

The immune system is divided into an innate immune response that consists of cellular defenses against invading pathogens and an adaptive immune response that consists of memory cells for the elimination of pathogens [25]. In both innate and adaptive immune responses, NF-κB is activated by toll-like receptors and directly binds to upstream sequences of inflammatory cytokines, including TNF-α, a marker of immune system activation [26]. The present study examined the effects of dietary supplementation with NAC on serum cytokines in CTX-induced immunosuppressed pigs. Dietary supplementation with NAC decreased TNF-α production, decreased *NF-κB*, *IFN-γ*, *TNF-*α, and *IL-8* expression and increased *IL-10* expression in PBMCs from CTX-induced pigs. Thus, NAC may influence immune-related gene expression via the inactivation of *NF-κB* after CTX-induced immunosuppression in miniature pigs.

The present study revealed that dietary NAC decreased levels of ALT and AST as markers of liver function and modulated levels of SOD, MDA, and GPx as markers of oxidative stress after CTX-induced immunosuppression. NAC is well known to alleviate oxidative stress, liver toxicity, and heart disorders [27]. In previous reports, dietary supplementation with NAC moderated liver

injury, anti-oxidative capacity, and energy metabolism [14,28]. The present study used CTX as an inducer of liver injury and oxidative stress. CTX is widely used to induce hepatotoxicity and oxidative stress in the liver, with a concomitant increase in serum levels of liver markers, including ALT and AST [29,30]. Abnormal increases in ALT and AST levels indicate CTX-induced cellular damage to hepatic cells [29,30]. NAC might thus be used as a therapeutic agent for modulation of hepatic injury and oxidative stress after CTX-induced immunosuppression in miniature pigs.

In conclusion, dietary supplementation with NAC modulates immune cell populations and TNF-α levels in serum after CTX-induced immunosuppression in miniature pigs. The present study also confirms that dietary NAC decreases the expression of *NF-κB*, *IFN-γ*, *TNF-α*, and *IL-8* and increases *IL-10* expression in PBMCs. Moreover, dietary NAC decreases serum levels of the liver function markers ALT and AST and modulates levels of the oxidative stress markers SOD, MDA, and GPx. These results suggests that NAC can be used as a therapeutic agent to modulate immune reaction, liver injury, and oxidative stress in livestock.

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