Effects of Lipoic Acid and Diquat on Growth Performance, Plasma and Jejunal Redox Status, and Jejunal Apoptosis in Finishing Pigs: Postprint

Authors: Bao Weiguang, Gu Xianhong, Hao Yue, Cui Yanjun, Wang Zhanbin

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Abstract

This study was conducted to investigate the effects of lipoic acid (LA) and diquat on growth performance, plasma and jejunal redox status, and jejunal apoptosis in fattening pigs. A 2$\times$2$factorialdesignwasadopted, withintraperitonealinjectionofdiquat(0, 8mg/kgbodywe$
$fourhealthyLargeWhitebarrowswithabodyweightof(70.64\pm$3.61)$ kg were randomly allocated into 4 groups, with 6 replicates per group and 1 pig per replicate. The experimental period lasted 29 days. LA supplementation in the diet of fattening pigs began on day 1 of the experiment and continued until the end; diquat was administered via intraperitoneal injection on day 15, while pigs not receiving diquat stimulation were injected with an equal volume of physiological saline. On day 29 of the experiment, plasma and jejunal samples were collected. Antioxidant indices in plasma and jejunal tissues were measured using assay kits, and Western blotting was performed to determine the relative protein expression levels of heat shock protein 70 (HSP70) and cysteine-aspartic acid protease-3 (caspase-3) in the jejunum. The results showed: 1) Dietary LA supplementation and intraperitoneal diquat injection had a highly significant interaction on jejunal superoxide dismutase (SOD) activity in fattening pigs (P<0.01), but no significant interaction was observed on growth performance, other antioxidant indices in plasma and jejunum, or the relative protein expression levels of jejunal HSP70 and caspase-3 (P>0.05). 2) Intraperitoneal diquat injection highly significantly decreased average daily feed intake (ADFI) and average daily gain (ADG) of fattening pigs, plasma SOD, glutathione peroxidase (GSH-Px) and catalase (CAT) activities, as well as jejunal SOD, manganese superoxide dismutase (MnSOD) activities and total antioxidant capacity (T-AOC) (P<0.01); and highly significantly increased feed to gain ratio (F/G), plasma malondialdehyde (MDA) content, jejunal hydrogen peroxide (H2O2) content, and the relative protein expression levels of jejunal HSP70 and caspase-3 (P<0.01). Dietary LA supplementation significantly increased ADFI (P<0.05) and highly significantly increased ADG (P<0.01) of

fattening pigs, highly significantly increased jejunal MnSOD activity (P<0.01), significantly decreased the relative protein expression level of jejunal HSP70 (P<0.05), and showed a tendency to decrease the relative protein expression level of jejunal caspase-3 (P=0.052). 3) Under diquat challenge, dietary LA supplementation highly significantly increased ADFI and ADG of fattening pigs (P<0.01), significantly increased plasma SOD activity (P<0.05), and highly significantly increased jejunal SOD and MnSOD activities (P<0.01). In conclusion, intraperitoneal diquat injection induced severe oxidative stress in fattening pigs, resulting in decreased growth performance, imbalance in plasma and jejunal redox systems, and triggered jejunal apoptosis. Dietary LA supplementation alleviated diquat-induced oxidative stress in fattening pigs and attenuated jejunal oxidation and apoptosis.

Full Text

1.1 Experimental Animals and Design

A 2$×$2 factorial design was employed with two main factors: intraperitoneal injection of diquat (0 or 8 mg/kg body weight) and dietary supplementation of LA (0 or 800 mg/kg), forming four treatment groups: -Diquat-LA (0 mg/kg diquat + 0 mg/kg LA), -Diquat+LA (0 mg/kg diquat + 800 mg/kg LA), +Diquat-LA (8 mg/kg diquat + 0 mg/kg LA), and +Diquat+LA (8 mg/kg diquat + 800 mg/kg LA). Diquat was purchased from Sigma-Aldrich (USA), and LA from Amresco (USA) with purity $ $99%.

Twenty-four healthy Large White castrated male pigs weighing (70.64$±$3.61) kg were selected and randomly allocated to four groups based on similar body weight, with six replicates per group and one pig per replicate. The experimental period lasted 29 days. Starting from day 1, 800 mg/kg LA was added to the diets of the -Diquat+LA and +Diquat+LA groups until the end of the trial, while the other two groups received no LA supplementation. On day 15, pigs in the +Diquat-LA and +Diquat+LA groups received an intraperitoneal injection of diquat at 8 mg/kg body weight, diluted in 250 mL physiological saline and administered via infusion over approximately 5 minutes, while pigs in the remaining groups received an equal volume of saline.

1.2 Experimental Diets and Management

A corn-soybean meal basal diet (powder form) was formulated according to the nutrient requirements specified in NRC (2012) and the Chinese Feeding Standard for Swine (NY/T 65-2004). The composition and nutrient levels of the basal diet are presented in Table 1 . To ensure diet quality, the total weekly feed intake was estimated based on daily consumption, and diets were mixed once weekly according to the experimental design by adding 0 or 800 mg/kg LA to the basal diet to produce two experimental diets.

The experiment was conducted at the experimental pig farm of the Institute

of Animal Sciences, Chinese Academy of Agricultural Sciences. Before the trial, pens were thoroughly cleaned and disinfected twice with 2–3% NaOH and Baidusha disinfectant, followed by fumigation for 24 hours (potassium permanganate:formaldehyde = 1:2, V/V) and ventilation for 5 days. Baidusha was applied again on the day of pig transfer. Pigs were housed individually in metabolism cages (160 cm × 90 cm × 120 cm) and allowed a one-week adaptation period before the formal experiment. Throughout the trial, dedicated personnel managed and fed the pigs according to standard operating procedures, minimizing unrelated stress factors. Environmental temperature was maintained at 15–20°C with relative humidity of 40–60%.

1.3 Sample Collection

On day 29, blood samples were collected from the anterior vena cava using heparinized tubes, centrifuged at 3,000 rpm for 10 minutes after standing for 10–15 minutes, and plasma was stored at -20°C. Pigs were stunned with an electric stunner (110 V, 5 A) and exsanguinated via carotid artery suspension, with the entire process completed within 30 seconds to minimize animal suffering. Immediately after loss of corneal reflex, the abdominal cavity was opened along the midline, viscera were removed, and a 1–2 cm segment of the mid-jejunum was excised. After rinsing with chilled physiological saline to remove digesta, the sample was placed in a centrifuge tube, snap-frozen in liquid nitrogen, and stored at -80°C.

1.4.1 Growth Performance

Body weight was measured at the start of the formal experiment, before diquat injection, and before slaughter. Feed intake was recorded daily, and residual feed in troughs was measured to calculate individual daily feed intake. Average daily gain (ADG), average daily feed intake (ADFI), and feed-to-gain ratio (F/G) were calculated. Since diquat was injected on day 15, growth performance data for days 1–14 were not determined for the +Diquat-LA and +Diquat+LA groups.

1.4.2 Plasma Antioxidant Indices

Plasma superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities, as well as malondialdehyde (MDA) content, were measured using colorimetric assay kits from Nanjing Jiancheng Bioengineering Institute.

1.4.3 Jejunum Antioxidant Indices

Frozen jejunum samples were ground into powder in a mortar under liquid nitrogen. The powder was accurately weighed and mixed with physiological saline at a 1:9 ratio (m/V) to prepare 10% tissue homogenates, which were centrifuged at 3,000 rpm for 15 minutes at 4°C. The supernatant was collected and diluted

to 1% homogenate for protein concentration determination using a Coomassie brilliant blue kit (Nanjing Jiancheng Bioengineering Institute). Jejunum SOD, manganese superoxide dismutase (MnSOD) activity, total antioxidant capacity (T-AOC), and hydrogen peroxide (H_2O_2) content were measured using kits from the same institute.

1.4.4 Jejunum Apoptosis Protein Expression

Western blotting was performed to determine protein expression levels of heat shock protein 70 (HSP70) and caspase-3. Jejunum tissue (100 mg) was lysed using tissue lysis buffer, and proteins were extracted according to the kit instructions (Nanjing Jiancheng Bioengineering Institute). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 minutes, then transferred to nitrocellulose (NC) membranes using wet transfer (350 mA, 90 minutes). Membranes were blocked in 3% bovine serum albumin-Tris-buffered saline with Tween 20 (BSA-TBST) for 30 minutes at room temperature with gentle shaking. Primary antibodies (HSP70 mouse monoclonal antibody, TDY 062, 1:2000; caspase-3 rabbit polyclonal antibody, TDY 411, 1:2000) diluted in 3% BSA-TBST were incubated for 10 minutes at room temperature, followed by overnight incubation at 4°C. The next day, membranes were incubated at room temperature for 30 minutes, then washed five times with TBST for 3 minutes each. Secondary antibody [horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) HRP, 1:10000] diluted in 5% skim milk-TBST was incubated for 40 minutes at room temperature with gentle shaking, followed by six TBST washes (3 minutes each). Enhanced chemiluminescence (ECL) reagent was applied for 3–5 minutes, and membranes were exposed for 10 seconds to 5 minutes (adjusted based on signal intensity), developed for 2 minutes, and fixed. Image Master Total Lab V 1.10 software was used for densitometric analysis, with HSP70/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and caspase-3/GAPDH ratios representing relative protein expression levels.

1.5 Data Processing and Analysis

Data were analyzed using two-way ANOVA with interaction in SAS 9.2 software. t-tests were performed to compare LA-supplemented and non-supplemented groups under diquat-challenged or non-challenged conditions. All data are expressed as "mean ± standard deviation." $P<0.05$ was considered statistically significant, and $P<0.01$ was considered highly significant.

2.1 Effects of LA and Diquat on Growth Performance of Finishing Pigs

As shown in Table 2 , dietary LA supplementation and intraperitoneal diquat injection showed no significant interaction effects on ADFI, ADG, or F/G of

finishing pigs (P>0.05). During days 1–14, dietary LA had no significant effects on ADFI, ADG, or F/G (P>0.05). During days 15–28, diquat injection highly significantly affected ADFI, ADG, and F/G (P<0.01), decreasing ADFI (3,093.5 g/d vs. 2,238.5 g/d, -27.64%) and ADG (1,080.5 g/d vs. 562.5 g/d, -47.94%) while increasing F/G (2.895 vs. 4.065, +40.41%). Dietary LA significantly increased ADFI (2,451 g/d vs. 2,881 g/d, +17.54%; P<0.05) and highly significantly increased ADG (765.5 g/d vs. 877.5 g/d, +14.63%; P<0.01), but did not significantly affect F/G (P>0.05). Compared with the +Diquat-LA group, the +Diquat+LA group showed highly significant increases in ADFI (1,900 g/d vs. 2,577 g/d, +35.63%) and ADG (477 g/d vs. 648 g/d, +35.85%) (P<0.01), while no significant differences were observed between the -Diquat-LA and -Diquat+LA groups (P>0.05). These results indicate that dietary LA alleviated the diquat-induced decline in ADFI and ADG, while having minimal effects on growth performance under non-stressed conditions.

2.2 Effects of LA and Diquat on Plasma Antioxidant Indices of Finishing Pigs

As shown in Table 3 , dietary LA and intraperitoneal diquat injection showed no significant interaction effects on plasma antioxidant indices (P>0.05). Diquat injection highly significantly decreased plasma SOD (39.73 U/mL vs. 22.50 U/mL, -43.37%), GSH-Px (597.85 U/mL vs. 461.78 U/mL, -22.76%), and CAT activities (11.59 U/mL vs. 5.86 U/mL, -49.44%), while highly significantly increasing plasma MDA content (1.855 nmol/mL vs. 2.81 nmol/mL, +51.48%) (P<0.01). Dietary LA had no significant effects on plasma SOD, GSH-Px, CAT activities, or MDA content (P>0.05). Compared with the +Diquat-LA group, the +Diquat+LA group showed a significant increase in plasma SOD activity (20.87 U/mL vs. 24.13 U/mL, +15.62%; P<0.05), while no significant differences were observed between the -Diquat-LA and -Diquat+LA groups (P>0.05). These findings suggest that dietary LA alleviated diquat-induced decreases in plasma SOD activity and partially improved the redox status under oxidative stress, while having minimal effects on plasma redox status under non-stressed conditions.

2.3 Effects of LA and Diquat on Jejunum Antioxidant Indices of Finishing Pigs

As shown in Table 4 , dietary LA and intraperitoneal diquat injection showed a highly significant interaction effect on jejunum SOD activity (P<0.01), but no significant interactions on MnSOD activity, T-AOC, or H_2O_2 content (P>0.05). Diquat injection highly significantly decreased jejunum SOD (183.20 mg/g prot vs. 131.45 mg/g prot, -28.25%), MnSOD activities (40.76 mg/g prot vs. 26.22 mg/g prot, -35.67%), and T-AOC (2.53 mg/g prot vs. 1.61 mg/g prot, -36.24%), while highly significantly increasing H_2O_2 content (3.47 mmol/g prot vs. 4.87 mmol/g prot, +40.55%) (P<0.01). Dietary LA highly significantly increased jejunum MnSOD activity (31.74 mg/g prot vs. 35.24 mg/g prot, +11.01%;

P<0.01) but had no significant effects on SOD activity, T-AOC, or H_2O_2 content (P>0.05). Compared with the +Diquat-LA group, the +Diquat+LA group showed highly significant increases in jejunum SOD (120.39 mg/g prot vs. 142.50 mg/g prot, +18.37%) and MnSOD activities (23.42 mg/g prot vs. 29.01 mg/g prot, +23.87%) (P<0.01), while no significant differences were observed between the -Diquat-LA and -Diquat+LA groups (P>0.05). These results indicate that dietary LA alleviated diquat-induced decreases in jejunum SOD and MnSOD activities and partially improved the redox status under oxidative stress, while having minimal effects on jejunum redox status under non-stressed conditions.

2.4 Effects of LA and Diquat on Jejunum Apoptosis Protein Expression of Finishing Pigs

As shown in Figure 1 [Figure 1: see original paper], intraperitoneal diquat injection increased the relative protein expression levels of HSP70 and caspase-3 in jejunum, while dietary LA decreased their expression. As shown in Table 5 , dietary LA and diquat injection showed no significant interaction effects on HSP70 or caspase-3 protein expression (P>0.05). Diquat injection highly significantly increased HSP70 (0.555 vs. 0.830, +49.55%) and caspase-3 (0.425 vs. 0.760, +78.82%) protein expression (P<0.01), while dietary LA decreased HSP70 (0.710 vs. 0.675, -4.93%; P<0.05) and caspase-3 (0.615 vs. 0.570, -7.32%; P=0.052) expression. These findings suggest that dietary LA can inhibit jejunum cell apoptosis to some extent, regardless of stress status.

Previous studies have shown that intraperitoneal injection of 12 mg diquat/kg body weight induced oxidative stress in weaned piglets, and a single injection of 8 mg diquat/kg induced oxidative stress in growing pigs with effects lasting 28 days. In this study, intraperitoneal injection of 8 mg diquat/kg caused restlessness, sham chewing, vomiting, and anorexia in all pigs, with feed intake gradually recovering after approximately one week. Pigs injected with saline showed no abnormal behavior. These results are consistent with previous findings and establish a foundation for oxidative stress models in large animals. Although no studies have reported LA' s alleviation of diquat-induced oxidative stress, research has demonstrated LA' s effectiveness in treating paraquat poisoning in rats. Paraquat, an organic heterocyclic contact herbicide, shares similar structure and function with diquat.

3.1 Effects of LA and Diquat on Growth Performance and Antioxidant Indices of Finishing Pigs

The results demonstrate that diquat-induced oxidative stress significantly decreased ADFI and ADG while increasing F/G, indicating substantial adverse effects on growth performance. These findings align with studies by Zheng et al., Lv et al., and Zhao et al. in weaned piglets, confirming the successful establishment of an oxidative stress model in finishing pigs. Dietary LA significantly increased ADFI and highly significantly increased ADG, particularly

under stress conditions, suggesting that LA supplementation alleviated oxidative stress in finishing pigs, though the underlying mechanisms require further investigation.

Diquat generates reactive oxygen species (ROS) by capturing electrons from molecular oxygen, while LA, with its dithiolane ring structure, exhibits significant electrophilicity and radical-scavenging capacity, reducing ROS production. Following diquat administration, ROS levels surge, depleting antioxidant enzymes such as SOD, GSH-Px, and CAT and severely disrupting redox homeostasis. Xu et al. reported that diquat significantly decreased serum GSH-Px and SOD activities and increased MDA content in growing pigs on days 7, 14, 21, and 28, with a decreasing trend in CAT activity. In this study, the diquat-challenged group showed significantly lower plasma SOD, GSH-Px, and CAT activities and higher MDA content compared to the control group. Under stress conditions, LA supplementation significantly increased plasma SOD activity, indicating that diquat induced oxidative stress in finishing pigs and that LA alleviated this stress by scavenging excess ROS.

The intestine is highly susceptible to oxidative damage. Intraperitoneally injected diquat first passes through the intestinal mucosa, where excess oxygen radicals can damage DNA, lipids, proteins, and other macromolecules. These radicals can be eliminated by antioxidant systems comprising non-enzymatic components and antioxidant enzymes. In the intestinal environment, independent redox systems exist in mitochondria, nuclei, and endoplasmic reticulum, and disruption of any system can cause intestinal mucosal oxidative stress, leading to intestinal lesions. Song et al. reported that oxidative stress significantly decreased intestinal SOD and GSH-Px activities and increased MDA content in weaned piglets. Chen et al. found that glutathione supplementation increased SOD and CAT activities in intestinal epithelial cells, possibly by scavenging free radicals and reducing their ability to inactivate antioxidant enzymes. These studies demonstrate that oxidative stress reduces antioxidant enzyme activities, while nutrient supplementation can partially restore them. In this study, the diquat-challenged group showed significantly lower jejunum SOD and MnSOD activities and T-AOC, with significantly higher H_2O_2 content. Under oxidative stress, dietary LA significantly increased MnSOD activity. SOD includes two isoforms: copper-zinc SOD (CuZn-SOD) and MnSOD, with MnSOD being the mitochondrial antioxidant enzyme that scavenges free radicals. These results suggest that diquat-induced oxidative stress may have caused organelle damage. LA' s unique chemical structure confers both water and lipid solubility, facilitating its penetration through cell membranes into organelles. This structure also enables LA to scavenge excess free radicals, inhibit lipid peroxidation, and reduce DNA oxidative damage. Therefore, dietary LA substantially improves the antioxidant defense system, likely by maintaining redox balance in organelles to repair jejunal oxidative damage, though the exact repair mechanisms remain unclear.

3.2 Effects of LA and Diquat on Jejunum Cell Apoptosis in Finishing Pigs

Under oxidative stress, ROS can induce apoptosis through multiple signaling pathways. Mitochondria release cytochrome c upon receiving apoptotic signals, which is regulated by B-cell lymphoma-2 (Bcl-2) family proteins and participates in the death receptor pathway to activate caspase-3 and induce apoptosis. When toll-like receptor 4 (TLR4) binds to its ligand lipopolysaccharide (LPS), it activates the NF- B pathway, which promotes expression of pro-apoptotic Bcl-2 family members, increases mitochondrial membrane permeability, facilitates cytochrome c release, and stimulates expression of interleukin-1β and tumor necrosis factor-α (TNF-α), leading to high caspase-3 expression and apoptosis. Regardless of the pathway, caspase-3 activation ultimately leads to apoptosis. In this study, diquat-challenged pigs showed significantly higher caspase-3 expression, suggesting that oxidative stress induced jejunum cell apoptosis, though the specific pathway requires further investigation.

Heat shock proteins (HSPs) play important roles in cell growth regulation and apoptosis. Research indicates that HSPs modulate apoptosis signaling through the Fas death receptor pathway, c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway, and caspase pathway, partially dependent on their molecular chaperone functions that control cell fate. HSP70 contains a highly conserved nucleic acid sequence at its C-terminus, and deletion or mutation of this sequence abolishes substrate binding, suggesting its potential involvement in HSP70-mediated caspase-3 inhibition. Studies have shown that low and high concentrations of H_2O_2 induce caspase-dependent and -independent apoptosis, respectively, with HSP70 inhibiting both types. In this study, diquat-induced oxidative stress dramatically increased HSP70 expression, but caspase-3 expression did not decrease correspondingly, suggesting that HSP70 may act at early stages of apoptosis, while caspase-3' s involvement in HSP70-mediated regulation remains uncertain.

Research has shown that LA can regulate NF- B activation, block HIV replication, and affect C-fos expression. Bojunga et al. reported that LA intervention inhibits apoptosis, downregulates caspase activity, and increases expression of the anti-apoptotic factor Bcl-2, thereby exerting protective effects. In this study, dietary LA inhibited HSP70 and caspase-3 expression, with more pronounced effects under diquat-induced oxidative stress. LA may have reduced HSP70 expression by scavenging excess free radicals and alleviating stress, while modulating NF- B activation to inhibit the caspase-3 pathway and prevent jejunum cell apoptosis.

Intraperitoneal diquat injection decreased growth performance, disrupted plasma and jejunum redox status, and induced jejunum cell apoptosis in finishing pigs. Dietary LA supplementation after diquat-induced oxidative stress improved growth performance, enhanced plasma and jejunum redox status, and partially alleviated jejunum cell apoptosis.

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