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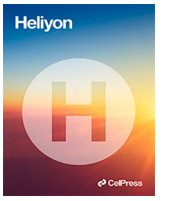

Antagonistic effects of N-acetylcysteine on lead-induced apoptosis and oxidative stress in chicken embryo fibroblast cells

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ABSTRACT

Lead (Pb) is a heavy metal that can have harmful effects on the environment, which has severe cytotoxicity in many animal tissues. N-acetylcysteine (NAC) has antioxidant activity, reducing lead-induced oxidative stress and apoptosis, but its role in chicken cells is unknown. The current study explored the antagonistic effect of NAC on lead-induced apoptosis and oxidative stress in chicken embryo fibroblast (CEF). In this study, CEF was used as a model to measure the cytotoxic effects of lead nitrate at different concentrations, demonstrating a dose-dependent effect on CEF activity. Employing inverted microscopy, the investigation of morphological alterations in CEF cells was conducted. Fluorescence staining methodology enabled the assessment of reactive oxygen species (ROS) levels within CEF cells. Moreover, an enzyme-linked immunosorbent assay was utilized to detect the presence of oxidative damage indicators encompassing superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) activity, malondialdehyde (MDA) content, and total antioxidant capacity (T-AOC) within CEF cells. Furthermore, the determination of the apoptosis rate of CEF cells was accomplished through the utilization of the Hoechst 33258 staining method in combination with the Annexin V-FITC dual staining method. By using RT-qPCR for detection, lead treatment increased expression of pro-apoptotic genes, *caspase-3*, and *caspase-9*, and reduced expression of anti-apoptotic genes, *Bcl-2*, and *BI-1*. Reduced antioxidant capacity was shown by increased ROS and MDA levels in CEF cells after lead treatment. The results showed that NAC inhibited the expression of *caspase-3* and *caspase-9* in lead-treated CEF cells, while NAC had a certain inhibitory effect on the relative expression of *Bcl-2* and *BI-1* mRNA in lead-induced CEF cells. NAC significantly reduced lead-induced oxidative damage and apoptosis. Overall, our results demonstrate a novel protective effect of NAC against lead-induced injury in chicken cells, providing a theoretical basis for future investigations of drugs that are effective in preventing lead poisoning in animals.

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1. Introduction

Lead (Pb) is an environmentally heavy metal with acknowledged toxicity. Lead pollution has become a concern in the wake of industrial development and airborne lead exposure leads to its absorbance by animals and results in poisoning. Lead can be enriched by biological chains due to its accumulation and metabolic difficulties. Poultry industry is an important component of the development of the animal husbandry, and it is vulnerable by factors such as lead pollution in practical production, which will not only lead to the decline in productivity and economic benefits, but also pose a threat to animals and human health. Investigations into the contribution of lead to worldwide environmental contamination have demonstrated various pathogenic effects, including apoptosis [1]. Lead disrupts the delicate oxidative/antioxidant balance in animal cells, thereby leading to the pathogenesis of lead poisoning. An underlying mechanism may involve inhibition of the antioxidant system and stimulation of reactive oxygen species (ROS) production. Increased ROS levels derive from pathologically damaged mitochondria [2] and drive dramatic changes in mitochondrial function, including the collapse of the transmembrane potential, the release of calcium ions, decoupling and mitochondrial swelling. A vicious cycle is established with a further generation of ROS. The adverse effects of lead on animal husbandry involve mechanisms such as oxidative damage and apoptosis [3]. Oxidative stress (OS) refers to an imbalance between oxidants and antioxidants [4], which would normally keep ROS at a consistent level [5]. Abnormal ROS levels, sometimes induced by lead, cause OS and cell damage and may be identified by indicators, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA) and total antioxidative capacity (T-AOC) [6].

Apoptosis is a process of active cell death through the regulation of genes, which can control cell senescence and maintain the relative stability of the environment *in vivo*. Generally considered there are two interconnected pathways of apoptosis: the intrinsic mitochondrial pathway (mediated by caspase-9) and the extrinsic death receptor-mediated pathway (mediated by caspase-8) [7,8]. The mitochondrial pathway is regulated by caspase-9 and Bcl-2 family proteins and lead-induced apoptosis has been reported to affect the expression of pro-apoptotic genes *Bax*, *Bak*, *Bid*, and inhibited the expression of anti-apoptotic genes *Bcl-2*, *Mcl-1*, and *BI-1*(Bax inhibitior-1) [9]. *BI-1* is a newly discovered and evolutionarily conserved inhibitor of apoptosis that is involved in the elimination of cancerous cells. Mitochondrial dysfunction characterizes apoptosis, involving activation of caspase, the release of cytochrome C and disorder of cellular systems [10]. Lead increases *Bax* expression activates the caspase cascade, changes mitochondrial permeability and culminates in apoptosis of the rat adrenal medullary chromaffin tumor cell differentiation line, PC12, via the mitochondrial pathway [11]. High doses of lead-induced apoptosis in rat brain cells result in decreased *Bcl-2* and increased *Bax* expression.

N-acetylcysteine (NAC) is a natural amino acid derivative with thiol side chains and is the synthetic precursor of reduced Glutathione (GSH) and L-cysteine derivatives [12]. NAC has been widely used as a clinical therapeutic drug to maintain levels of the intracellular antioxidant, glutathione [13]. NAC levels rise synergistically in response to glutamate and lead and have been shown to prevent apoptosis, alleviate OS, scavenge free radicals and detoxify heavy metals. Furthermore, NAC chelates most heavy metals, facilitating their removal from the body [14]. NAC increased GSH levels in CEF cells, aiding the scavenging of lead-generated ROS. Depletion of GSH impairs its functions in scavenging free radicals, electrophilic toxicity and protein activity regulation leading to impaired cellular defenses against reactive species, cellular damage and death [15]. It inhibited lead-induced apoptosis of human renal proximal tubular epithelial cells [16]; improved OS in rat blood and tissues [17] and reduced ROS levels in rat neuron PC12 cells [18]. Earlier studies have shown that NAC has protective effects on the cytotoxicity of heavy metals such as cadmium and chromium, and has the advantage of reducing the level of cellular oxidative stress and mitigating cytotoxicity [19,20]. It has been reported that selenium (Se) can attenuate the altered expression of Mitogen-activated protein kinase (MAPK) pathway genes induced by lead through modulation of the MARK signaling pathway, thereby reducing apoptosis and necrotic apoptosis in chicken kidney cells [21]. However, NAC is generally used as an effective heavy metal treatment drug due to its advantages of high safety, low price and easy preparation [22]. Furthermore, it has been found that lead can induce lymphocyte necrotic apoptosis through oxidative stress-mediated activation of the MAPK/NF-κB pathway, while (Se) can counteract this apoptosis [23]. It is universally acknowledged that there are four main branch routes of the MAPK [24]. Among them, ERK protein is mainly responsible for cell growth, differentiation and apoptosis [25].

In this study, primary CEF cells were used to detect the cytotoxic effects of lead nitrate at different concentrations, including cell viability, apoptosis, antioxidant capacity, and cellular ROS levels. Simultaneously, the antagonistic effect of NAC on lead-induced cytotoxicity of CEF was preliminarily investigated. Interestingly, we observed a significant decrease in the expression of p-ERK after treatment with NAC. Thus, our research illuminates the theoretical basis for preventing and treating lead poisoning in poultry husbandry.

2. Materials and methods

2.1. The statement of animal welfare

The experiment of CEF cells isolation was approved by the ethics and animal welfare committee of Henan Agricultural University following the national Guide for the Care and Use of Laboratory Animals (Approval No: SYXK-YU-2021-0003).

2.2. Chemicals

Pb $(NO_3)_2$ was purchased from Sigma (10099-74-8, MO, the USA), NAC from GlpBio (GC11786, CA, the USA), DMEM and trypsin from Solarbio (11995, Beijing, China), fetal bovine serum (FBS) from Gibco (CA, the USA), CCK-8 cell viability detection kit from Dongren Chemical Technology Co., Ltd (CK04, Shanghai, China), kits for ROS detection (S0033S), BCA protein concentration

determination (P0012S), total SOD activity detection (S0109), total GSH-Px detection (S0058), CAT detection (S0051), MDA detection (S0131S) and T-AOC detection (S0121) from Biyuntian (Shanghai, China), Hoechst 33258 staining kit from Key GEN Bio TECH (KGA211, Jiangsu, China), annexin V-FITC/PI apoptosis detection kit from Zoman Biotechnology Co., Ltd (ZP327-1, Beijing, China) and FastStart Universal SYBR Green Master (ROX) from Roche (4913914001, Basel, Switzerland).

2.3. Cell culture

CEF cells were prepared from 9-day-old SPF chicken embryos (Boehringer Ingelheim Biotechnology Co., Ltd , Beijing, China) and cultured in DMEM containing 5 % FBS and 1 % penicillin streptomycin antibiotics at 37 °C with 5 % CO_2.

2.4. Cell treatment

The CEF cells were seeded in 96-well plates at a density of 2×10^4 cells/well and stabilized with 1 % FBS maintenance solution for 6 h. After the cell density reached 50–60 %, lead nitrate poisoning was carried out in each group. Pb $(NO_3)_2$ was dissolved in DMEM to final concentrations of 0, 2, 10, 50, 125, 250 and 500 μM. Each group has 3 replicates, and the exposure time was 24 h, 48 h and 72 h respectively. Add 10 μL CCK-8 solution to each well when the exposure ends, the 96 well cell culture plate was incubated in a 37 °C, 5 % CO_2 cell incubator for 2 h, subsequently measured the OD value at 450 nm with a microplate reader. Finally, the OD value was statistically analyzed to obtain the optimal concentration and time of CEF cells treated with lead nitrate. Based to the optimal processing time in the above results, with 0 μM lead nitrate and without NAC as the control group, the NAC of 0, 0.1, 0.25, 0.5, 1, 2, 5 and 10 mM were added as the test group, with 100 μL for each well. As above, the CEF cell viability was calculated after obtaining the OD value, the formula is as follows:

$$\text{CEF cell viability}(\%) = \frac{Test\ group - Blank\ group}{Control\ group - group}\ (OD\ value) \times 100\%$$

The best concentration of NAC effect was obtained by comparing the relative cell viability between groups.

2.5. Nuclear morphology

Apoptotic morphological changes in nuclear chromatin were detected by staining with the DNA-binding fluorescent dye, Hoechst 33258. CEF cells in logarithmic growth were digested with 0.25 % trypsin-EDTA and seeded onto a 6-well plate at a density of 4×10^5 cells/well. Cells were stabilized with 1 % FBS-containing maintenance solution for 6 h and incubated with 0, 2, 10 and 50 μM lead nitrate or 50 μM lead nitrate +2 mM NAC for 48 h. Cells were washed twice with PBS and fixed with 1 mL of 4 % formaldehyde solution for 10 min at 4 °C. Fixative was removed, cells washed twice with PBS, 100 μL Hoechst 33258 working solution added and cells stained at room temperature for 10 min, rinsed with water and air dried. CEF cells were observed and photographed under a fluorescence microscope with UV light at 340 nm.

2.6. Flow cytometry for annexin V-FITC/PI staining

Cells were treated as above, digested with 0.25 % trypsin-EDTA and centrifuged at 400 g for 5 min. The supernatant was discarded, and cells were collected and washed twice with PBS for counting. CEF cells (5×10^5) were resuspended, centrifuged at 400 g for 5 min, supernatant discarded and 500 μL of 1 × Binding Buffer (10 × Binding Buffer diluted with ddH_2O 1:9) added. 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI) were added with mixing and incubated at room temperature for 15 min in the dark. Apoptosis was measured by flow cytometry within 1 h alongside a control without Annexin V-FITC and PI. At 488 nm, Annexin V-FITC was detected as green fluorescence by the FITC channel (FL-1, 530 nm) and red fluorescence by PI channel (FL-2, 585 nm). At least 30,000 cells were counted for each sample.

2.7. Determination of intracellular ROS

Cells were treated as above, harvested with 0.25 % trypsin-EDTA, centrifuged at 400 g for 5 min and washed 2–3 times with serum-free cell culture medium. Cells were resuspended in a serum-free cell culture medium, DCFH-DA staining solution added to a final concentration of 10 μM with mixing and incubated at 37 °C for 20 min in the dark before washing 3 times with serum-free cell culture medium. Subsequently, place it under a fluorescence microscope for observation and photography.

2.8. Detection of cell antioxidant capacity by SOD, GSH-Px, CAT, MDA and T-AOC

After grouping and incubating the cultured CEF cells with toxins, the culture medium is collected in centrifuge tubes at the desired time. The cells are washed twice with pre-chilled PBS at 4 °C, and the cells adhered to the walls are scraped off using a cell scraper and collected in the centrifuge tubes. The tubes are then centrifuged at 400 g for 10 min, and the supernatant is discarded. Please note that all steps should be performed on ice or at 4 °C. Then determine the concentration of total protein using the BCA assay kit. Levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA) and total antioxidants (T-AOC) were determined spectrophotometrically according to the instructions provided in the assay kit.

2.9. RT-qPCR analysis

Cells (4×10^5/well) were cultured in 6-well plates and total RNA was extracted using Trizol solution, reverse transcribed by PrimeScriptTMRT Reagent Kit with gDNA Eraser (Takara) and quantitatively assessed by FastStart Universal SYBR Green Master (ROX), according to the manufacturers' instructions. PCR was performed on a real-time instrument with 95 °C pre denaturation for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 1 min. Primer sequences are shown in Table 1. We evaluated four housekeeping genes, *GAPDH*, *Actin*, *Tubulin* and *OVO* by using GeNorm software. The results showed that the stability of the internal reference gene reference of *GAPDH* was very high in different groups, so we selected *GAPDH* as the internal reference gene for RT-qPCR.

2.10. Western blot analysis

The extracted total protein was loaded onto SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane after electrophoresis separation. Western blotting was performed by sealing the membrane in 5 % skim milk for 2 h and incubating overnight with a diluted primary antibody against ERK (1:500, ABclonal, A16686), p-ERK (1:500, ABclonal, AP0485), MEK (1:2000, ABclonal, A4868), p-MEK (1:2000, ABclonal, AP1359). After washing with TBST for three times, it was incubated with the secondary antibody and then washed again in TBST for three times. Finally, the nitrocellulose membrane was exposed on Amersham Image 680 using an enhanced chemiluminescence assay reagent from Bioman Technology Co. Ltd (Beijing, China).

2.11. Statistical analysis

All experimental data are expressed as mean ± standard deviation of triplicate determinations. Prism 8 (GraphPad software, San Diego, the USA) was used for *t*-test and analysis of variance, *p* values equal to or less than 0.05 were considered significant, $p < 0.01$ was considered as being great significant.

3. Results

3.1. Effects of different concentrations of lead nitrate and NAC on cell viability

Lead significantly decreased CEF viability measured by CCK-8 in a time- and concentration-dependent manner with different rates of decline at each time point (Fig. 1A). When the treatment time was 48 h, the dose effect of each lead nitrate treatment group tended to be stable, and when the final concentration of lead nitrate is 0, 2, 10, 50 μM, the dose effect was relatively more stable, and the percentage of CEF cell activity decreased regularly, with a significant difference ($p < 0.01$).

Therefore, in the subsequent tests, 48 h was selected as the time of exposure treating CEF cells with lead nitrate, and 2, 10 and 50 were selected the best concentration of lead nitrate to treat CEF cells, which were low, medium and high levels respectively in the lead nitrate treatment group. CEF cells were treated with 0, 0.1, 0.25, 0.5, 1, 2, 5 and 10 mM lead nitrate plus NAC for 48 h and peak cell viability was shown at 2 mM NAC ($p < 0.05$; Fig. 1B) and decreased with further increases in NAC concentration ($p < 0.05$ or $p <$ 0 0.01; Fig. 1B). 2 mM NAC was selected as the most protective concentration for subsequent experiments.

CEF cells were treated with 0, 2, 10, 50 μM lead nitrate and 50 μM lead nitrate + 2 mM NAC for 48 h, which showed good adhesion and confluence (Fig. 1C). The increase of lead concentration promoted cell apoptosis and necrosis (Fig. 1D–F), while NAC reversed this effect (Fig. 1G).

3.2. NAC inhibited lead-induced ROS production

Changes in ROS levels in lead-treated CEF cells were measured (Fig. 2A) as control group showed little fluorescence. After treatment with different concentrations of lead nitrate for 48 h, the fluorescence intensity in (Fig. 2B–D) gradually increased with the increase of lead nitrate dose, indicating that the content of ROS in CEF cells was increasing. NAC treatment reduced fluorescence intensity in lead-treated CEF cells, indicating an inhibitory effect on intracellular ROS production (Fig. 2E–F).

Table 1
Primer sequences for target genes and *GAPDH* reference gene.

Genes	Accession number	Primer	Sequences (5′-3′)	Product size (bp)
GAPDH	NM_204305.1	Forward	GTCAACGGATTTGGCCGTAT	70
		Reverse	CCACTTGGACTTTGCCAGAGA	
caspase-3	NM_204725.1	Forward	TGCAGAAGTCTAGCAGGGAAACCC	246
		Reverse	AAGTTTCCTGGCGTGTTCCTTCAG	
caspase-9	AY057940.1	Forward	CGAAGGAGCAAGCACGAC	129
		Reverse	CGCAGCCCTCATCTAGCAT	
Bcl-2	NM_205339.2	Forward	GGACAACGGAGGATGGGATG	141
		Reverse	CCAAGATAAGCGCCAAGAGTG	
BI-1	XM_015290060.2	Forward	CGACTGGGATGACAGGAAAG	124
		Reverse	GGAGCGCACAGGTGAGACA	

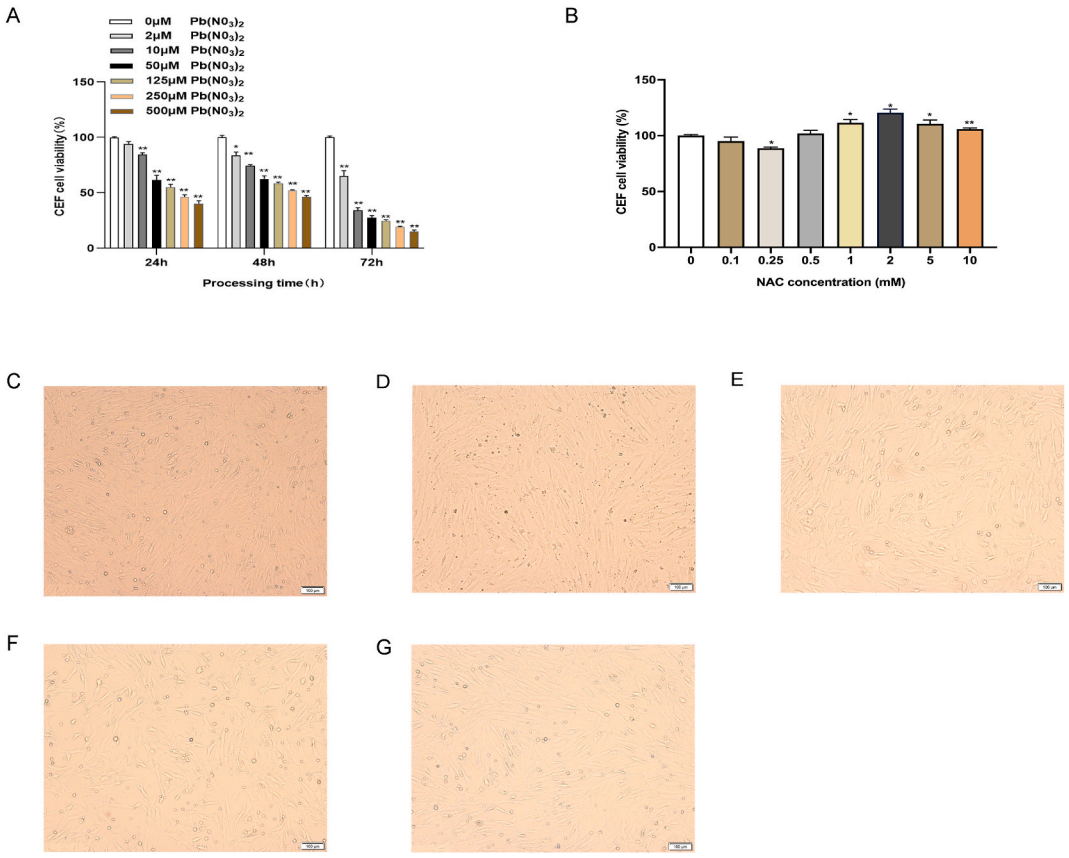

Fig. 1. Cellular activity and morphological changes of CEF cells cultured for 48 h. A: Effect of different lead nitrate concentrations on CEF cells viability at different time points; B: Effect of different NAC concentrations on CEF cells viability; C: 0 μM lead nitrate; D: 2 μM lead nitrate; E: 10 μM lead nitrate; F: 50 μM lead nitrate; G: 50 μM lead nitrate +2 mM NAC (100 × magnification); C-G were captured using a standard inverted fluorescence microscope. Three replicates for each set of experiments; data expressed the mean ± standard deviation by GraphPad Prism 9. $*p < 0.05$, $**p < 0.01$, compared to the control group.

3.3. NAC inhibited lead-induced antioxidant changes

In previous studies, lead is acknowledged to induce oxidant stress and reduce the activities of antioxidant enzymes, such as SOD, GSH-Px, SOD, GSH-Px and CAT [1,26]. Lead treatment produced a dose-dependent decrease in intracellular antioxidant enzyme activity, the higher the concentration of lead nitrate, the lower the activity of antioxidant enzyme in CEF cells., while the addition of NAC increases the antioxidant capacity of cells (Fig. 3A–C). MDA content increased with lead treatment and T-AOC decreased (Fig. 3D–E). However, the condition of 50 μM lead nitrate +2 mM NAC significantly decreased MDA content ($p < 0.05$) compared with 50 μM lead nitrate and T-AOC increased. A dose-effect relationship was shown between lead concentration and MDA content in CEF cells and NAC reduced lead-induced MDA increases.

3.4. NAC reduced CEF cells apoptosis

It can be observed that CEF cells exhibit weak fluorescence and fewer apoptotic cells from Fig. S1A. In Figs. S1B–D, CEF cells were treated with different concentrations of lead nitrate. As the concentration of lead nitrate increases, the number of apoptotic cells gradually increases, and the density of CEF cells decreases. Therefore, it can be inferred that more apoptotic CEF cells were found after lead treatment compared with negative controls (Figs. S1B–D) and NAC significantly decreased the number of apoptotic cells (Figs. S1E–F). Flow cytometry analysis confirmed a dose-dependent increase in apoptosis with lead treatment and that NAC treatment reduced apoptosis resulting from 50 μM lead nitrate (Fig. 4A–F). Although the apoptosis rate of CEF cells in the 50 μM lead nitrate +2 mM NAC group (Fig. 4F) was significantly higher than that in the 0 μM lead nitrate group (Fig. 4A), the apoptosis rate was lower than that in the 50 μM lead nitrate group (Fig. 4D). Thus, lead-induced apoptosis may be ameliorated by NAC.

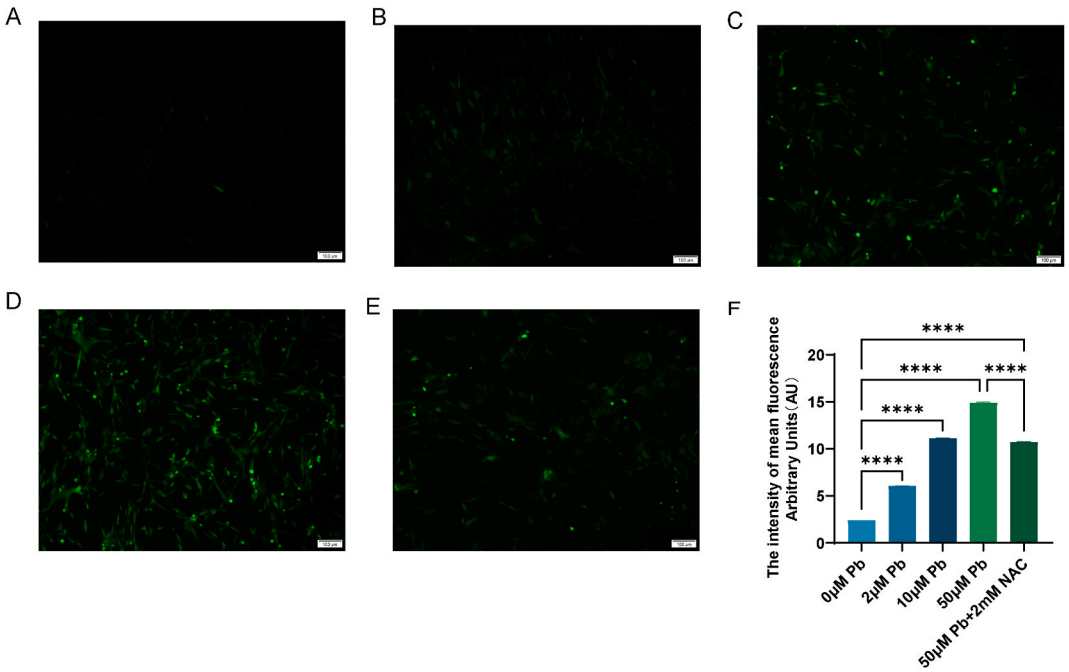

Fig. 2. Changes of ROS fluorescence intensity in CEF cells cultured for 48 h. A: 0 μM lead nitrate; B: 2 μM lead nitrate; C: 10 μM lead nitrate; D: 50 μM lead nitrate; E: 50 μM lead nitrate +2 mM NAC (100 × magnification); F: The quantification of ROS fluorescence intensity by Image J software; $^{****}p < 0.0001$, compared to the control group.

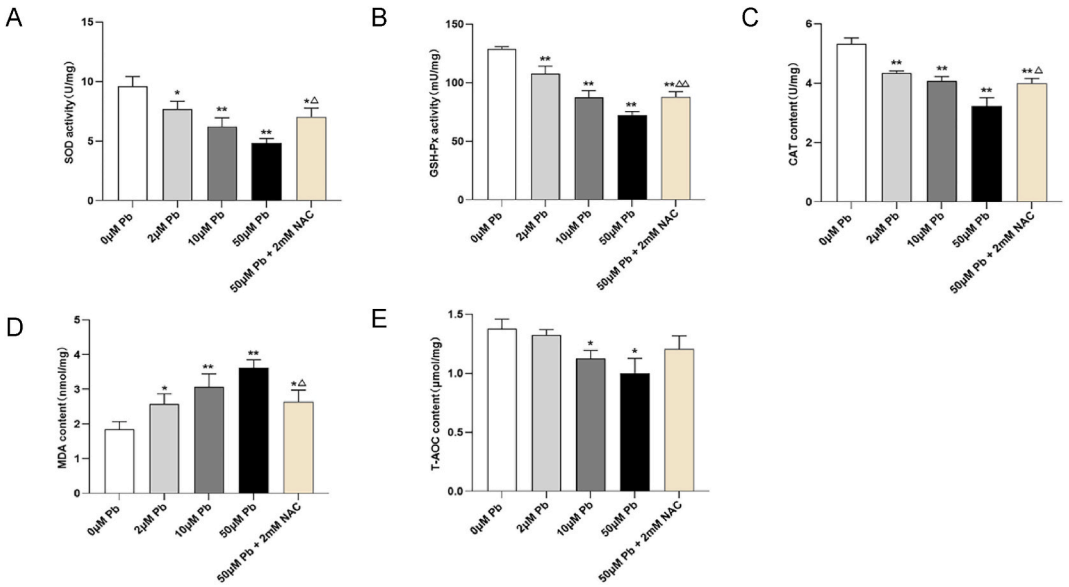

Fig. 3. Effect of lead nitrate and NAC on activities of antioxidant enzymes, SOD, GSH-Px, CAT, MDA and T-AOC in CEF cells. CEF cells were treated with 0, 2, 10 and 50 μM lead nitrate and 2 mM NAC for 48 h and SOD, GSH-Px, CAT, MDA and T-AOC were detected by a microplate reader; A–E: The SOD activity, GSH-Px activity, CAT content, MDA content and T-AOC content of the 0, 2, 10 and 50 μM lead nitrate and 2 mM NAC groups; $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the control group, $\Delta\ p < 0.05$, compared to the 50 μM Pb group; $\Delta\Delta\ p < 0.01$, compared to the 50 μM Pb group.

3.5. The effect of NAC on expression of apoptosis-related genes and MAPK pathway-associated proteins in lead-stimulated CEF cells

CEF cells were treated with 0, 2, 10 or 50 μM lead nitrate and 50 μM lead nitrate +2 mM NAC for 48 h. Expression of *caspase-3* and *caspase-9* in 2, 10 and 50 μM lead nitrate-treated cells increased significantly compared with 0 μM lead nitrate ($p < 0.05$; Fig. 5A–B), demonstrating a dose-dependent positive effect on *caspase-3* and *caspase-9* mRNA. Levels of *caspase-3* mRNA were significantly

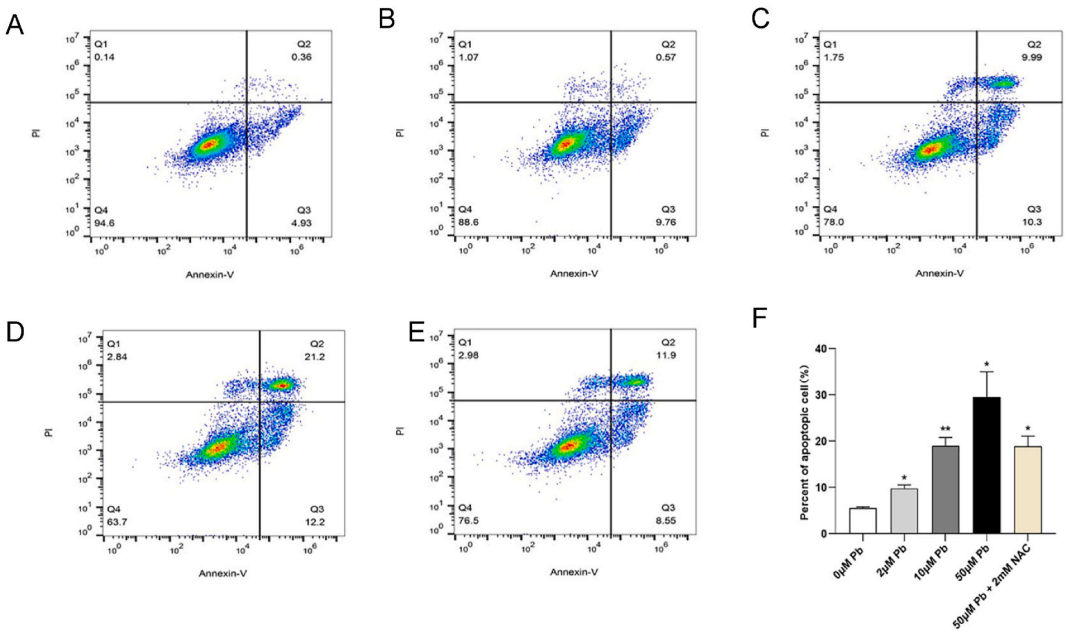

Fig. 4. Effect of lead nitrate and NAC on CEF cells apoptosis. A: 0 μM lead nitrate; B: 2 μM lead nitrate; C: 10 μM lead nitrate; D: 50 μM lead nitrate; E: 50 μM lead nitrate +2 mM NAC; F: The quantification of the percentage of apoptotic cells; $*p < 0.05$, $**p < 0.01$, compared to the control group.

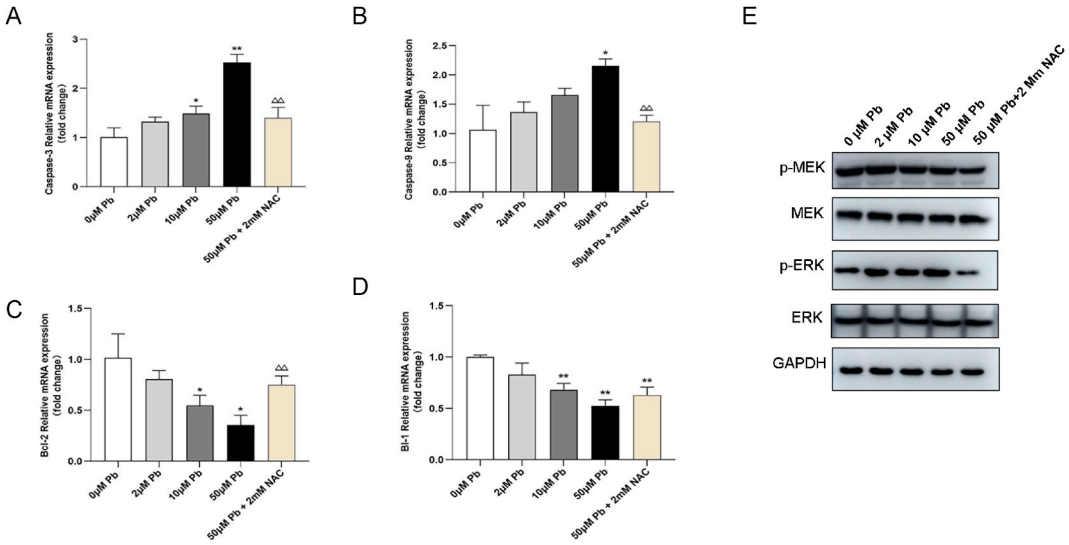

Fig. 5. Effect of NAC on lead-induced apoptosis related genes and proteins of CEF cells. A–D: The *caspase-3*, *caspase-9, Bcl-2*, *BI-1* mRNA expression level of the 0, 2, 10 and 50 μM lead nitrate and 2 mM NAC groups; E: The MAPK signaling pathway-related proteins expression in the 0, 2, 10 and 50 μM lead nitrate and 2 mM NAC treated groups; $*p < 0.05$, $**p < 0.01$, compared to the control group, $\Delta\Delta\, p < 0.01$, compared to the 50 μM Pb group.

reduced in the 2 mMNAC+50 μM lead nitrate group compared with the 50 μM lead-nitrate group ($p < 0.01$; Fig. 5A -B). These results indicated an inhibitory effect of NAC on *caspase-3* and *caspase-9* expression in lead-stimulated CEF cells. Relative expression of apoptosis-suppressing genes, *Bcl-2* and *BI-1*, decreased with increasing lead concentration ($p < 0.05$). Relative expression of *Bcl-2* was significantly higher in the 50 μM lead nitrate +2 mM NAC group compared with the 50 μM lead nitrate group ($p < 0.01$), although differences were not significant for *BI-1* mRNA ($p > 0.05$). Thus, there is some evidence for an inhibitory effect of NAC on *Bcl-2* and *BI-1* mRNA levels in lead-induced CEF cells (Fig. 5C–D) which further supports the role of NAC in reversing lead-induced apoptosis. In addition, expression of several proteins associated with the MAPK signaling pathway were also detected the results, indicated that there are no differences of ERK, MEK, p-MEK expression but significant lower expression of p-ERK after NAC treatment (Fig. 5E).

4. Discussion

Lead exists widely in the environment and enters the body through various pathways, which can cause multi-organ and multi-system damage to biological organism. It cannot be degraded and exhibits a time-dependent accumulation in the atmosphere, water, food and organisms living in contaminated areas [27]. CEF cells cultured in vitro with 0, 2, 10, 50, 125, 250 and 500 μM lead nitrate for 24, 48 and 72 h showed a dose- and time-dependent reduction in viability, although decreases in survival rates were different at each time point (Fig. 1A). On exposure to lead nitrate for 24 h, the survival rate suddenly increased between 10 μM and 50 μM while decreases in survival rate in other regions remained relatively stable. Declining CEF survival over 48 h lead treatment was relatively stable and decline trends between each concentration gradient were similar, indicating the dependence of CEF viability on lead concentration. Therefore, a 48 h treatment with low, medium and high lead concentration was selected for further examination. After 72 h of treatment, CEF viability declined sharply even at low lead concentrations and an increase in lead exposure produced no significant change. As previously described, Liu [28] et al. who reported results of CEF culture at 2.5, 12.5 and 62.5 μM chromium trichloride, used a 48 h time period. Cell damage is dose-dependent. Zhang [29] et al. reported that 8 h treatment with 150 μM hexavalent chromium reduced DF-1 cell viability by half. Thus, it indicates that CEF cells have different tolerance to different heavy metals, usually heavy metals at the μM level can cause changes in CEF cells activity. Lead toxicity varies by species, organ, tissue, and cell type. NAC is an antioxidant showing rapid dilatation in cells which provides cysteine for intracellular GSH synthesis [30]. Few *in vivo* or in vitro studies on NAC treatment of lead poisoning in avian species have been conducted. CCK-8 assays of CEF cells cultured 0, 0.1, 0.25, 0.5, 1, 2, 5 and 10 mM NAC showed a peak of viability protection at 2 mM NAC (Fig. 1B). The current study attempts to fill the gaps in knowledge regarding the inhibition of lead-induced CEF damage by NAC and identified an optimal concentration of 2 mM NAC. Lead toxicity is known to be connected with oxidative damage, alterations in activities of SOD, GSH-Px, CAT and other anti-oxidant enzymes, reduced scavenging of ROS and oxidative damage. The current study found significantly increased ROS levels in CEF cells, decreased SOD, GSH-Px and CAT activities, decreased T-AOC and increased MDA content with lead treatment. These findings are consistent with previously reported trends in anti-oxidant capacity [31–33]. SOD, GSH-PX, CAT activities and T-AOC were all elevated in the NAC-protected group (Fig. 3) and intracellular MDA was lower, illustrating that NAC reduced the production or level of oxygen free radicals. Consequently, the protective effect of NAC appears to be mediated by its antioxidant properties.

Lead is a common environmental contaminant that accumulates through biological chains [30]. Therefore, evaluating apoptosis during lead-induced cytotoxicity is an important issue. Lead has been reported to induce apoptosis of mouse TM_3 cells through the Fas death receptor pathway [34] and the PPAR γ*/Caspase-3/*PARP signaling pathway [35]. It may also increase histone acetylation and induce apoptosis in rat vascular and cardiac tissues [36]. However, a variety of literature focuses on mammalian and cancer cells and there is a lack of research on lead-induced apoptosis in avian cells. The current study addressed CEF apoptosis in response to lead giving insights into avian toxicology. Caspase-9 and Bcl-2 are key proteins in mitochondrial apoptosis [37]. Damaged mitochondria release cytochrome C which forms a complex with Apaf-1 and activates *caspase-9* and *caspase-3* [38]. The mitochondrial membrane and endoplasmic reticulum protein, *Bcl-2,* regulates apoptosis as a homodimer or heterodimer with *Bax* and induces caspase release [39]. The current study showed that decreased CEF viability ($p < 0.05$) was accompanied by upregulation of the pro-apoptotic genes, *caspase-3* and *caspase-9,* and downregulation of *Bcl-2* and *BI-1* (Fig. 5). These data suggest that lead decreases the mRNA expression levels of *Bcl-2* in CEF cells, activating downstream apoptosis by cutting *caspase-9*. Furthermore, NAC inhibited this trend. Thus, the inhibition of the mitochondrial apoptosis pathway may be caused by upregulation of *Bcl-2*.

In addition to mitochondrial apoptosis, the endoplasmic reticulum pathway and the death receptor pathway also lead to cell death [40]. The endoplasmic reticulum pathway activates caspase-12 and downstream caspase-3 to inhibit the anti-apoptotic protein, Bcl-2, and reduce Bcl-2/Bax heterodimers, promoting apoptosis [41–44]. The death receptor pathway involves caspase-8-stimulated activation of caspase-3 by self-cleavage, inducing apoptosis [45]. We believe that lead induces CEF apoptosis was probably due to the mitochondrial pathway. However, it is far from enough to verify the expression of several related genes only from the mitochondrial pathway. Further exploration of the deeper mechanism of lead-induced CEF apoptosis mitochondrial pathway is needed. Similarly, the mechanisms of endoplasmic reticulum pathway and death receptor pathway is required to be further explored for fully illuminating the mechanisms involved.

5. Conclusion

In conclusion, lead inhibited CEF viability, weakening the antioxidant capacity, inducing oxidative damage and apoptosis. The mitochondrial pathway is central to lead-induced apoptosis. Damaging effects of lead on CEF cells showed a dose-response relationship and NAC had an antagonistic effect. Whether the death receptor pathway and endoplasmic reticulum pathway also play a role in the apoptosis process remains to be further investigated.

Data availability

All data generated or analyzed during this study are included in this submitted manuscript.

CRediT authorship contribution statement

Lele Wang: Data curation, Investigation, Methodology, Writing – original draft. **Yijie Xu:** Data curation, Investigation, Methodology, Writing – original draft. **Xuyang Zhao:** Data curation, Formal analysis, Validation. **Xiaojing Zhu:** Data curation, Formal

analysis, Validation. **Xiuyuan He:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Aijun Sun:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Guoqing Zhuang:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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