Selenium Deficiency via the ROS/NLRP3/IL-1β Signaling Pathway Leads to Pyroptosis Injury in Pig Spleen

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Research Article

Keywords: selenium, oxidative stress, pyroptosis, inflammation, spleen

Posted Date: October 24th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2182845/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Biological Trace Element Research on February 14th, 2023. See the published version at https://doi.org/10.1007/s12011-023-03595-2.

Abstract

The aim of the present study was to investigate the effect of selenium (Se) deficiency on the relationship between the pyroptosis and MAPK signaling pathway in spleen injury. A total of 10 two-month-old *Sus scrofa domesticus* specimens were allocated to two groups. The control group was fed a basal diet (0.15-mg/kg Se), and the experimental group was fed a 0.03-mg/kg Se-deficient diet for two months. The pig-spleen histopathological changes were observed with hematoxylin-eosin staining. Frozen sections were prepared to detect the content of ROS in pig-spleen cells. The oxidation stress related indexes were determined using a spectrophotometer. The levels of pyroptosis- and MAPK signaling pathway- related factors were detected via quantitative real-time polymerase chain reaction (qPCR) and western blotting (WB). The results of sections showed that the lymphocytes decreased in number, the spacing of cells widened, and some cells were necrotic in the spleen tissue of pigs fed a low-selenium diet. The content of ROS, malondialdehyde, nitric oxide, H_2O_2 and catalase activity in the low-selenium group was significantly higher than that in the control group. and SOD activity was decreased. The protein-ratio-levels of p-Nrf2 to Nrf2 were decreased. The expression levels of nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3), IL-1β, IL-18, ASC, gasdermin D, and caspase-1, the protein-ratio-levels of p-AKT serine/threonine kinase (p-AKT) to AKT, p-extracellular regulated protein kinases (ERK) to ERK, p-P38 MAPK to p-P38, and p-c-Jun N-terminal kinase (p-JNK) to JNK were significantly increased in the Se-deficient group compared with the control group. These results suggested that Se deficiency can induce oxidant stress, which increases pyroptosis- and inflammation-related factors of pig-spleen injury.

Introduction

An essential micronutrient in organisms, selenium (Se) is widely distributed in various tissues. Excessive or insufficient Se content leads to serious disease [1, 2]. It is related to the occurrence of complications such as arteriosclerosis, hyperlipidemia, and hyperglycemia [3]. In organisms, Se functions mainly in the form of selenoproteins and some antioxidant enzymes. Selenoprotein P is involved in protecting organisms from damaging effects of free radicals and is a good indicator of selenium content in organisms [4]. Selenoprotein W prevents organisms from being overoxidized and participates in muscle metabolism [5]. Selenoprotein S controls the redox balance in cells [6]. Selenium deficiency affects the expression of selenoproteins, and associated with splenic dysfunction and induced oxidative stress, excessive inflammation, abnormal autophagy, and endoplasmic reticulum stress [7]. In summary, the function of Se is to regulate the immune balance of the organism through the antioxidant function of selenium protein. Se deficiency greatly reduces the body's antioxidant capacity and induces oxidative stress.

Oxidative stress is a pathological condition caused by an imbalance between the body's oxidant and antioxidant systems. The content of both H_2O_2 and MDA was changed, as was the related CAT and SOD activity. When ROS increases to a certain point, there will be some damage to an organism's tissues or cells, including adipose-related MDA and nitric oxide (NO)-centered derivatives of RNS. Nrf2/ARE signaling is considered a promising target for the treatment of oxidative-stress-mediated diseases such

as diabetes, fibrosis, neurotoxicity, and cancer [8, 9]. A key factor of oxidative-stress production is ROS content, and ROS production is closely related to mitochondria [10].

Oxidative stress can induce cell pyroptosis. Mitochondria ROS are closely related to the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome. NLRP3 is an important PRR in cytoplasm, which has a three-part carboxy-terminal leucine-rich repeat domain. NLRP3-inflammasome activation is mediated by the related effects of mitochondria and lysosomes, and this process is related mainly to ROS production [11, 12]. A central nucleotide binding domain, which has ATPase activity and mediates self-oligomerization, and an amino-terminal pyrin domain recruit apoptosis-associated speck-like proteins containing CARD (ASC) [13]. Subsequently, the assembled ASC recruits prucaspase-1 through homotypic CARD–CARD domain interaction to form the NLRP3-ASC-caspase-1 protein complex [14]. The NLRP3 inflammasome is activated and induces the self-cleavage and activation of pro-caspase-1, which leads to the maturation of the proinflammatory cytokines interleukin 1β (IL-1β) and interleukin 18 (IL-18). In addition, activated caspase-1 cleaved gas protein D (GSDMD) releases its N-terminal domain, transfers to the cell membrane and forms pores, mediates the release of cell contents, including inflammatory cytokines IL-1β and IL-18, and induces inflammatory cell death, known as pyroptosis [15–17]. NLRP3-inflammasome pyroptosis is a form of inflammatory PCD triggered by pathogen invasion and dependent on caspase activation. GSDMD is a coexecutor of pyroptosis, and there is growing evidence that other members of the gas protein family, including GSDMA3, GSDMB, and GSDME, can be cleaved and induce pyroptosis [16, 18–21].

MAPK/MAK/MRK overlapping kinase (MOK) belongs to the MAP-kinase superfamily, which plays an important role in regulating cell growth, division, and differentiation [22]. As a key factor of pyroptosis, caspase-1 plays an important role in caspase-mediated inflammatory injury [23]. Five distinct MAPK signal transduction pathways have been identified in mammalian organisms. Among them, the ERK1/2 signal transduction pathway regulates cell growth and differentiation, and the JNK and P38 signal transduction pathways play important roles in stress responses such as inflammation and apoptosis. Greeberg et al. demonstrated that cell survival or apoptosis is related to different degrees of ERK, JNK, and P38 activation [24]. Cell survival or apoptosis depends on the ERK pathway's activation by growth factors and JNK-P38 pathway's activation by stress homeostasis. Various cytokines promote cell survival and inhibit programmed cell death through the ERK survival pathway, and the JNK and P38 protein kinase pathways are involved in mediating inflammation and apoptosis in response to stress [24, 25]. Moreover, studies have found that under the action of ROS and cytotoxins, JNK kinase is activated and transferred to the nucleus, promoting the expression of pyroptosis-related genes, initiating the formation of pyroptosis, and controlling the development of tumors [26]. Activated JNK can be involved in the release of apoptotic factors (such as cytochrome C from mitochondria), leading to caspase activation and apoptosis [27]. The MAPK pathway is related to the AKT pathway [28]. Spleen the largest immune organ in the human body involved activation and induction of oxidant stress in the Se deficiency have been deeply studied and attracted great attention. In this study, we illuminate the relationships among Se, oxidant stress, and cell pyroptosis in pig spleen.

Materials And Methods

Animals and diets

Two-month-old inbred landrace pigs (*Sus scrofa domesticus*) were provided by the Anyang Fuwang pig farm (Anyang, China) and kept in cages. The animal-operation procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Modern Biotechnology and complied with the State Regulations for the Administration of Experimental Animals. The pigs were divided into two groups based on their initial weight, which was analyzed with SPSS ($n = 5$). The Se-deficient group was fed an Se-deficient diet containing 0.03 mg/kg Se. The control group was fed a basal diet (adding Na_2SeO_3 to an Se-deficient diet, 0.15 mg/kg Se, the powder was supply by Sigma-Aldrich, America). After two months of treatment, the pigs were anesthetized to collect blood and spleen samples.

Histopathology

The spleen-tissue sample was cut into pieces and fixed with a 4% formaldehyde solution for at least 24 h. Pathological paraffin sections (5 μm) were produced using a conventional method and stained with hematoxylin and eosin. The pathological changes were observed under an optical microscope.

Immunofluorescence Of Frozen Sections

The frozen spleen tissues were cut by freezing microtome. The fluorescence quencher, ROS dye solution (SIGMA D7008 1:500) were added on to the prepared frozen spleen tissues section for 5 min. The slides were placed in PBS (PH7.4) and washed three times by shaking on a decolorization shaker, 5 min each time. After the sections had dried slightly, DAPI dye solution (Servicebio G1012) was added to the rings, and the slices were incubated for 10 min at room temperature in the dark. Sealing: The slides were placed in PBS (PH7.4) and washed three times by shaking on a decolorization shaker, 5 min each time. The slices were sealed with an antifluorescence quenching tablet. Microscopic examination and photography: The sections were observed, and images were collected under a fluorescence microscope (DAPI ultraviolet excitation wavelength: 330–380 nm, emission wavelength: 420 nm, blue light; CY3 excitation wavelength: 510–560 nm, emission wavelength: 590 nm, red light).

Determination Of Pig-spleen Oxidant-stress-related Index

The frozen spleen tissues were homogenized in ice-cold saline. The supernatants were collected after the homogenate was centrifuged at 3000 g and 4°C for 10 min. SOD, CAT, H_2O_2, MDA, and NO levels were measured with a spectrophotometer using commercially available assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein concentrations in tissue homogenates were measured with the Bradford protein assay using bovine serum albumin as the standard (Jiancheng Bioengineering Institute, Nanjing, China).

Qpcr Analysis Of Pig-spleen Pyroptosis And Mapk-signal-pathway-related Gene Expressions

Total mRNA was isolated from the frozen spleen tissues using a RNA kit (Tiangen, Beijing, China). Quantitative real-time PCR (qPCR) was carried out for the amplification of cDNA using 2×SYBR Green I PCR Master Mix (Vazyme, Nanjing, China). The PCR procedure consisted of 95°C for 30 s, and 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. The PCR primers used are shown in Table 1. The melting curve and dissociation curve were extrapolated to confirm primer specificity and product purity. The relative abundance of each mRNA was calculated with the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{GAPDH})$ treatment − $(Ct_{Target} - Ct_{GAPDH})$ control.

Table 1
Gene-special primers used for qPCR.

Target gene	Accession number	Primer	Primer sequence (5′-3′)
GSDMD	XM_021090506.1	Forward	5' GCCCCTTCTACTTCCATGACAC 3'
		Reverse	5' GTCACCACGAACACATCGTTCC 3'
ASC	AB 873106.1	Forward	5' CCTTCCCCTGAAAGCAGACAAC 3'
		Reverse	5' GTACTGCTCTTCCGTCAGCACC 3'
Caspase-1	NM_214162.1	Forward	5' GCAGAAGCAAGCGTGACAGC 3'
		Reverse	5' TGGCATCAAAAGCTGCACATAC 3'
NLRP3	NM_001256770.2	Forward	5' TGTGCTTTATTCCCCTGGTCTG 3'
		Reverse	5' TCTTGAGATTGCAACAAAGTGG 3'
IL-1β	NM_214055.1	Forward	5' GATGAAAGATAACACGCCCACC 3'
		Reverse	5' TCTGCTTGAGAGGTGCTGATGTAC 3'
IL-18	NM_213997.1	Forward	5' TTTGAGGATATGCCTGATTCTG 3'
		Reverse	5' GCACAGAGATGGTTACTGCCAG 3'
GAPDH	NM_001206359.1	Forward	5' ACCAGGGCTGCTTTTAACTCTG 3'
		Reverse	5' TTCTCCATGGTCGTGAAGACAC 3'

Western-blotting Analysis Of Pig-spleen Pyroptosis And Mapk-signal-pathway-related Protein Expression

For protein-expression analysis, the extraction and isolation of proteins were performed using a Cytoplasmic and Nuclear Protein Extraction Kit (Beyotime, Nanjing, China) according to the

manufacturer's instructions. The protein concentration was determined using a BCA assay kit (Beyotime, Nanjing, China). Equal amounts of protein extracts were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions in concentrate protein gel 5% (pH = 6.8) and separating protein gel 12% (pH = 8.8). The separated proteins were transferred to PVDF membranes using tank transfer for 2 h at 200 mA in tris–glycine buffer with 15% methanol. The membranes were blocked with 5% skim milk for 3 h and incubated for 12 h with corresponding antibodies for 2 h at 37°C. The secondary antibodies (IgG/HRP) were incubated for 2 h at 37°C. The antibody information is shown in Table 2. The images of the blots were visualized using ECL (Genshare, Xi'an, China).

Table 2
Antibodies used in the present study.

Antibodies	KD	Dilution ratio	Resource	Account number
p-P38 MAPK	42 kDa	1:500	Bioss	bs-5476R
P38 MAPK	41 kDa	1:1000	Bioss	bs-28027R
ERK1/2	42 kDa	1:1,000	Bioss	bs-2637R
p-ERK	41kDa	1:1,000	Bioss	bs-3016R
JNK	42 kDa	1:1000	Bioss	bs-10562R
p-JNK	50 kDa	1:500	Bioss	bs-17591R
AKT	56 kDa	1:500	Bioss	bs-0115R
p-AKT	56 kDa	1:500	Bioss	bs-4089R
NLRP3	118 kDa	1:300	Proteintech,	19771-1-AP
ASC	22 kDa	1:500	Bioss	bs-6741R
Caspase-1	48 kDa	1:500	Beyotime	AF1681
GSDMD	53 kDa	1:2,000	Proteintech	20770-1-AP
IL-1β	32 kDa	1:500	Bioss	bs-0812R
IL-18	22 kDa	1:500	Bioss	bs-4988R
Nrf2	68 kDa	1:500	Beyotime	AF7623
p-Nrf2	68 kDa	1:500	Bioss	bs-2013R
GAPDH	36 kDa	1:5,000	Proteintech	10494-1-AP
Goat Anti-Mouse IgG H&L / HRP		1:10,000	Bioss	bs-0296G-HRP
Goat Anti-Rabbit IgG H&L / HRP antibody		1:10,000	Bioss	bs-0295G-HRP

Statistical Analysis

The results were presented in terms of at least five measurements, duplicated for each set and having a coefficient of variation below 5%. One-way ANOVA followed by Duncan's multiple range test ($p < 0.05$) with SPSS 20.0 (SPSS Inc, Chicago, IL, USA) was applied for the mean values compared.

Results

Histopathological observation of the spleen

Spleen histopathology after L-Se treatment is illustrated in Fig. 1. In the control group, the amount of white pulp, the morphology of red pulp, and the number of lymphocytes were normal. The lymphocytes in the white pulp were closely arranged, and the connective tissue in the red pulp was closely arranged and exhibited normal cell spacing (Fig. 1 NC). In the L-Se treatment group, the amount of white pulp and number of lymphocytes in the spleen were lower, and the lymphocytes in the white pulp were loosely arranged or it was no white pulp structure in the spleen (blue arrow). The red pulp was extensively and moderately edematous, with loose connective tissue and wide cell spacing (black arrow), few cells in the red pulp, and more necrotic cell debris (red arrows), and the number of lymphocytes was severely reduced (Fig. 1L-Se). The results indicated that Se deficiency induced spleen injury.

Selenium-deficiency-induced Oxidative Stress In Pig Spleen

The content of MDA, H_2O_2, and NO, the activity of CAT and SOD, and the expression of Nrf2 were tested (see Fig. 2). In the Se-deficient group, the content of MDA, H_2O_2, and NO was significantly increased (Fig. 2A–C) and the activity of SOD was significantly decreased (Fig. 2E) compared with those in the control group ($p < 0.05$, $n = 5$). By contrast, the activity of CAT was significantly increased after Se-deficiency treatment ($p < 0.05$, $n = 5$, Fig. 2D). The protein expression of phosphorylated Nrf2 was significantly increased in the Se-deficient group ($p < 0.05$, $n = 5$, Fig. 2F). The fluorescence results for frozen sections showed that the ROS content in the L-Se group's spleen cells was significantly higher than that in the control group ($p < 0.05$, $n = 5$, Fig. 2G). The results indicated that Se deficiency induced oxidant stress in pig spleen.

Selenium-deficiency-induced Pyroptosis In Pig Spleen

To evaluate the effect of selenium deficiency on pyroptosis in pig-spleen cells, the NLRP3, ASC, caspase-1, GSDMD, IL-1β, and IL-18 were determined with quantitative real-time PCR (Fig. 3A) and WB (Fig. 3B). The mRNA-expression levels of NLRP3, ASC, caspase-1, GSDMD, IL-1β, and IL-18 genes were significantly increased in the Se-deficient group compared with those in the control group ($p < 0.05$, $n = 5$). The results of WB were similar to those of qPCR, and the relative average value of GSDMD expression in the low-

selenium group was higher than that in control group (Fig. 3B, n = 3). The results indicated that Se deficiency induced pyroptosis in pig spleen.

Selenium-deficiency Activity Mapk Signaling Pathway In Pig Spleen

The effects of selenium deficiency on JNK, p-JNK, P38, p-P38, ERK1/2, p-ERK1/2 AKT, and p-AKT are shown in Fig. 4. Selenium deficiency significantly enhanced the phosphorylation proportion of related proteins in the treatment group compared with the control group ($p < 0.05$, $n = 3$). The protein-ratio levels of p-AKT1 to AKT, p-ERK to ERK, p-P38 to P38, and P-JNK to JNK in L-Se group were increased 4.5 fold, 2.3 fold, 3.1 fold, and 1.28 fold, respectively, compared with those in the control group. The results showed that Se deficiency can activate the MAPK pathway in pig spleen.

Discussion

Selenium-deficiency-induced spleen injury, destructed spleen's antioxidant capacities. In the Se-deficient pathological tissue sections we observed, the number of cells in the white pulp and red pulp was significantly reduced, and the connective tissue was loosely arranged and exhibited decisively necrotic cells. Selenium is a component of GSH structure and has antioxidant capacities. Studies have found that oxidative stress caused by selenium deficiency can affect spleen function [22]. The most intuitive manifestation of oxidative stress is the increase of ROS. To detect ROS levels in the spleen, this experiment tested typical nitrogen-containing compounds, ROS levels and detected the loss caused by oxidative stress. The results showed that Se deficiency clearly increased the content of MDA and ROS. This indicates that in the presence of O_2, biofilms are attacked and decomposed by free radicals and their active derivatives. The increase of NO content also indicates the increase of free radicals in the body. When oxidative stress occurs, the oxidative system in the body is strong and activates the antioxidant system. The results also showed that Se deficiency increased H_2O_2 content. CAT, responsible for catalyzing the decomposition of H_2O_2, was significantly increased, but Se deficiency induced a decrease in SOD, suggesting that Se deficiency disturbed the oxidative balance and induced oxidative stress. Nrf2 is a key factor in the regulation of oxidative stress and a defense mechanism of the body that is activated when oxidative stress occurs [29]. It can regulate related inflammatory factors through the MAPK signaling pathway. The content of p-Nrf2 is significantly reduced after H_2O_2 treatment [30]. As Fig. 2 shows, under the selenium-deficient condition, the expression of p-Nrf2 and the activity of SOD were significantly decreased, those induced the content of NO, H_2O_2, ROS and MDA were significantly increased, the above results indicate Se deficiency induced spleen oxidative stress.

Oxidative stress may induce pyroptosis. During oxidative stress, ROS accumulates in the body, the content of H_2O_2 and other oxidants increases, and the balance of oxidation and antioxidation in organisms shifts, thus causing damage to cells or tissues. In this state, the NLRP3 inflammasome was increased, caspase-1 underwent greater activation, and GSDMD was effectively cleaved [31]. With increased H_2O_2 content, caspase-1, which affects pyroptosis, is also significantly increased, which can be

manifested as the release of IL-1β, IL-18, or other inflammatory factors [22]. Our study found that low selenium can also cause pyroptosis. As Fig. 3 shows, the content of related pyroptosis proteins ASC, NLRP3, caspase-1, GSDMD, IL-1β and IL-18 were increased significantly in the low-selenium treatment compared with the control group. The occurrence of pyroptosis under the condition of low selenium was confirmed. The activation of the NLRP3 inflammasome is governed by GSTO1-promoted ASC deglutathionylation in macrophages [32]. The expression of ASC, NLRP3, and caspase-1 increased in the low-selenium group. ASC is involved in NLRP3 activation. NLRP3 can bind to ASC protein, which in turn binds to pro-caspase-1 to form the NLRP3 inflammasome. Thus, when inflammation is severe, the ASC in neutrophils also increases. Caspase-1 regulates cell inflammation by promoting the processing and release of inflammatory factors. When activated by the inflammatory body, caspase-1 excises IL-1β precursors and IL-18 precursors to make them active, and caspase-1 promotes the release of these processed and mature inflammatory factors. The experimental results clearly showed an increased expression of IL-1β and IL-18 inflammatory factors. When cells are in an inflammatory environment, the feedback leads to cell death.

Oxidative stress is closely related to inflammation, and various signaling pathways and signaling factors are interlinked in the body. The process of pyroptosis is regulated by the MAPK pathway. MAKP-signal-pathway-related proteins were further identified using WB. The content of Nrf2, an antioxidant protein associated with the inflammatory response, was significantly reduced, and elevated AKT may participate in the inflammatory response via the PI3K/AKT pathway. The levels of P38 and P-JNK proteins, which are associated with the inflammatory response, were significantly increased. H_2O_2 can activate the p-ErK1/2 protein, and our results showed that its content was also significantly increased (Fig. 4). The expression level of the GSDMD gene, which is a key protein of cell pyroptosis was significantly increased.

To summarize, we examined five indicators of oxidative stress in pig spleen and identified the expression of 11 inflammatory proteins and factors. We found that Se deficiency may increase the expression level of free radicals, pyroptosis, and inflammation. The results indicate that selenium deficiency may be involved in the pyroptosis of porcine spleen cells by regulating the ROS/NLRP3/IL-1β signaling pathway.

Declarations

Declarations

The institution of Animal Protection and Utilization Committee at Anyang Institute of Technology approved this experiment.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lichao Song, Zhihui Jiang, Xingwang Zhang and Yuwei Song. The first draft of the manuscript was written by Lichao Song, Zhihui Jiang and Guodong Wang, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Natural Science Foundation of China (32102744) and the Natural Science Foundation of Henan Province (202300410008). We thank Scribendi's (https://www.scribendi.com/) employee for editing the English text of a draft of this manuscript.

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Figures

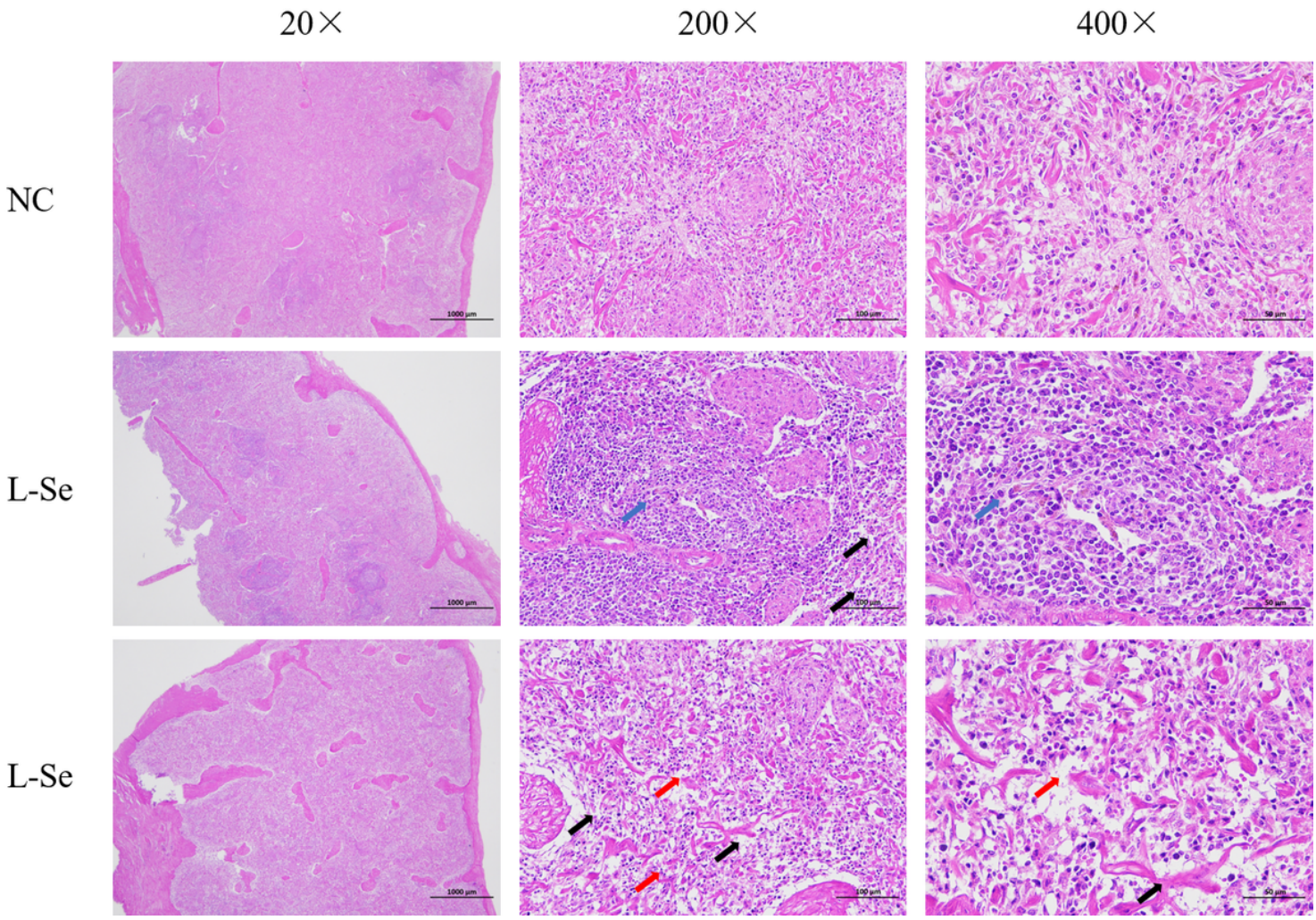

Figure 1

Pig-spleen histopathology. Lymphocytes in the white pulp were loosely arranged (blue arrow) and edematous, with loose connective tissue and wide cell spacing (black arrow) and necrotic cell debris (red arrows). NC, control group; L-Se, Se-deficient group; *n* =5.

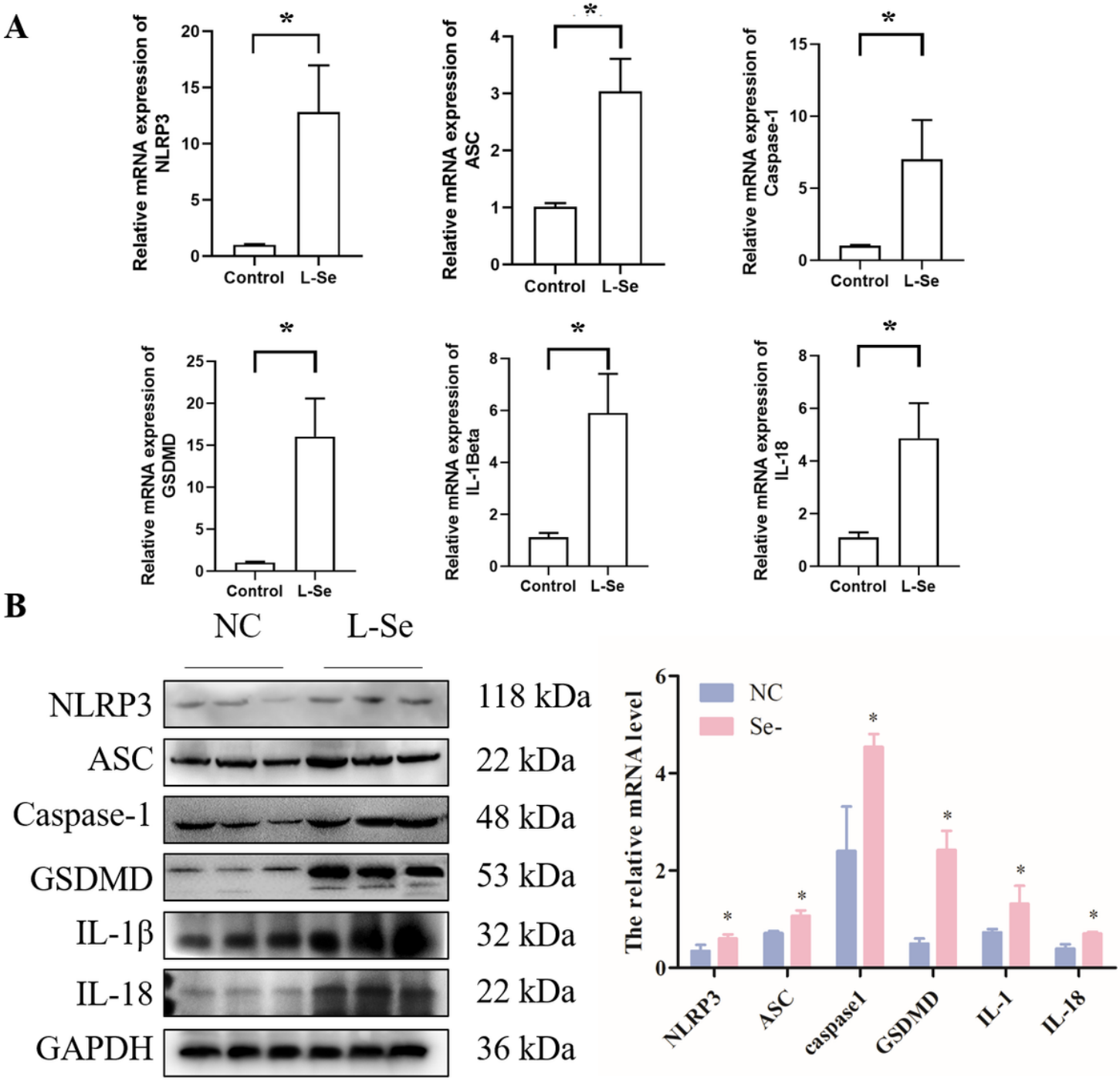

Figure 2

Selenium-deficiency-induced oxidative stress in pig spleen. A–C, content of MDA, H_2O_2, and NO, respectively; D–E, activity of CAT and SOD, respectively; F, protein-level ratio of p-Nrf and Nrf2; G, ROS levels; *$p < 0.05$, $n = 5$.

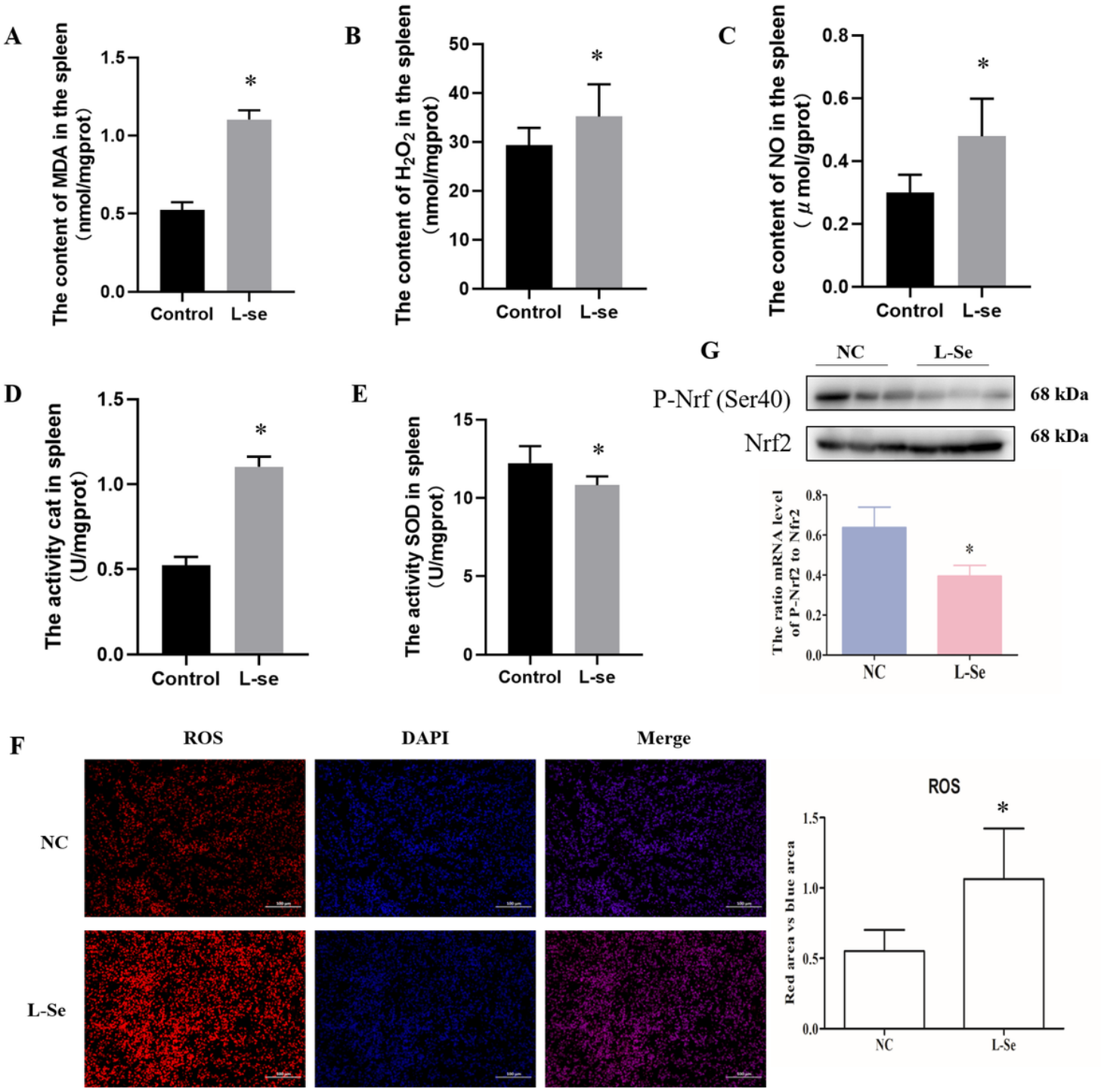

Figure 3

Selenium-deficiency-induced pyroptosis in pig spleen. A, mRNA levels of pyroptosis-related factors; B, protein levels of pyroptosis-related factors; *$p < 0.05$, $n = 3$.

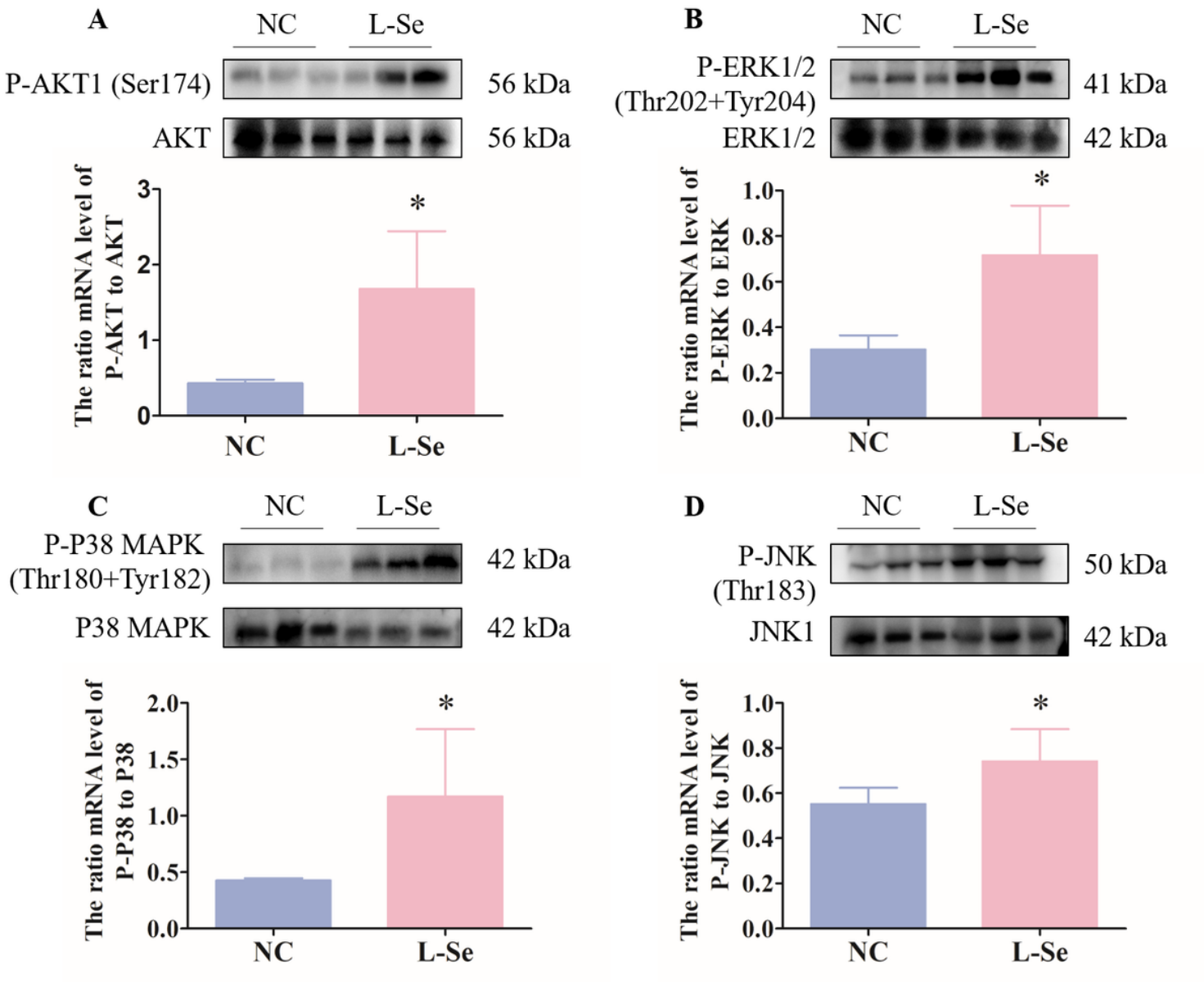

Figure 4

Selenium-deficiency increased MAPK signaling pathway related factors levels. A–D, protein-ratio levels of P-AKT to AKT, P-ERK1/2 to ERK1/2, P-P38 to P38, and P-JNK to JNK, respectively; *p< 0.05, n = 3.