Selenium Deficiency Causes Inflammatory Injury in the Bursa of Fabricius of Broiler Chickens by Activating the Toll-like Receptor Signaling Pathway

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Abstract
The aim of our study was to observe the effect of selenium (Se) deficiency on inflammatory injury in the bursa of Fabricius of broiler chickens and to determine the role of the Toll-like receptor (TLR)/myeloid differential protein-88 (MyD88)/nuclear factor-κB (NF-κB) signaling pathway during this process. Here, we revealed that severe inflammatory injury occurred in the broiler bursa of Fabricius with Se deficiency via histopathology. Moreover, the ultrastructural pathological results showed that the nuclear, mitochondrial, endoplasmic reticulum and cytomembrane structures were damaged to varying degrees. Additionally, interleukin-2 (IL-2), interleukin-6 (IL-6), and interferon (IFN-γ) mRNA expression was markedly upregulated in the broiler bursa of Fabricius with Se deficiency. Furthermore, TLR, toll-interleukin-1 receptor domain-containing adapter-inducing interferon-β (TRIF), MyD88, and NF-κB mRNA expression was also markedly elevated in the broiler bursa of Fabricius with Se deficiency. The above results suggested that Se deficiency increases the expression of numerous proinflammatory cytokines and is probably due to the activation of the TLR/MyD88/NF-κB signaling pathway, which causes inflammatory injury in the bursa of Fabricius of broiler chickens. Our findings provide a theoretical reference for further studying the underlying mechanism of Se deficiency-induced inflammatory injury in the bursa of Fabricius of broiler chickens.

Keywords Selenium deficiency · Inflammatory injury · Bursa of Fabricius · Toll-like receptor

Introduction
Selenium (Se) is an essential trace element for physiological activities in the body [1, 2]. Organic Se sources rather than inorganic sources are increasingly advocated as Se supplements in livestock and poultry farming [3]. Earlier studies demonstrated that Se plays a vital role in the growth and metabolism of animals [4]. For example, Se and vitamin E deficiency causes white muscle disease and cardiomyopathy in animals [4]. Recently, an increasing number of studies have revealed that Se, selenoprotein, and other Se-containing compounds exert antioxidative functions and inhibit apoptosis and autophagy in multiple organs [1, 5, 6]. Se-containing compounds could also mediate anti-inflammatory functions to maintain the balance between inflammatory and anti-inflammatory responses in organs and tissues [7–9]. Se deficiency could result in severe inflammatory injury in organs. Wu et al. found that Se deficiency causes inflammatory injury of the gastrointestinal tract in broilers [10]. Bi et al. found that Se deficiency facilitates inflammation following S. aureus infection in the mouse mammary gland [7]. It is widely believed that Se is the trace element most closely associated with influenza virus infection [11]. A report showed that early nutritional interventions with selenium, zinc, and vitamin D present the function of raising antiviral resistance against coronavirus disease 2019 (COVID-19) [12]. Therefore, it is necessary to investigate the effect of Se deficiency on organ damage and rational supplementation with Se to improve growth performance and disease resistance in animals.

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Immunity protects the body from a large number of pathogen attacks and is divided into innate immunity and adaptive immunity based on immunological characteristics. Innate immunity is the first barrier to prevent pathogen invasions into the body. For example, the interferon (IFN) signaling pathway exerts broad antiviral functions and is a critical defense system against viruses during the early stage of infection in animals [13]. The body activates the innate immune response to counter pathogenic factors recognized by pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one of the most important PRRs in humans and animals. Scientists have successively identified more than 20 kinds of TLRs, and different kinds of TLRs can recognize different pathogenic invaders as foreign. For example, TLR-1 recognizes lipoproteins of the mycobacterium, TLR-4 recognizes lipopolysaccharides (LPS), and TLR-3 and TLR-7 recognize double-stranded RNA and single-stranded RNA from viruses, respectively [14]. Although the immune response protects the body from viruses, bacteria and other pathogens, excessive or persistent immune responses themselves could cause severe inflammatory injury in organs and further impair organ biological functions. For example, influenza virus infection causes a “cytokine storm” (one of the excessive immune response manifestations) to result in acute lung injury, which contributes to high morbidity and mortality worldwide [15]. Recently, emerging evidence has indicated that Se deficiency can directly or indirectly trigger innate immunity in animals.

The bursa of Fabricius, where B lymphocytes mature, is a vital immune organ in broilers. Thus, the healthy growth and metabolism of the bursa of Fabricius is essential for strengthening antiviral or antibacterial abilities and even for the immune effect of vaccines. Numerous studies have revealed that Se plays a fundamentally important role in regulating the immune function of macrophages, B cells, T cells, natural killer cells, and other immune cells [1, 16]. However, there is little research about the effect of Se deficiency on injury to the bursa of Fabricius and its underlying mechanism during the pathogenic process.

In the present study, we uncovered that Se deficiency markedly elevates enormous pro-inflammatory cytokine mRNA expression, probably dominated by the activation of the TLR/MyD88/NF-κB signaling pathway, thereby causing severe inflammatory injury and multiple organelle damage in the bursa of Fabricius of broilers. Our findings provide a theoretical reference for further studying the underlying mechanism of Se deficiency-induced inflammatory injury in the bursa of Fabricius of broilers.

**Materials and methods**

**Animals and low-Se diets**

One-day-old healthy broilers (clean grade) were purchased from Yinong Poultry Co., Ltd. (Harbin, China). The feeding environment was disinfected during the experiment. The brooding temperature was ensured. All of the broilers ate and drank freely. Research staff received training on animal care and handling. The procedures in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

In the present study, the composition of the difference between the low-Se diet and the control diet (complete formula feed) was only the addition of sodium selenite. The Se content of the low-Se diet was 0.004 mg/kg, while the Se content of the control diet was 0.3 mg/kg. The composition of the low-Se diet used in this experiment is shown in Table 1 [17, 18].

**Animal experiments**

**Experiment 1**

We first observed whether Se deficiency causes inflammatory injury in the broiler bursa of Fabricius. Twelve one-day-old healthy broilers were randomly and averagely divided into a control group (C group) and a low-Se group (L group). Broilers of the C group were fed complete formula feed, and broilers of the L group were fed the low-Se diet. According to our previous studies, we found that Se deficiency could cause severe inflammatory injury in the broiler spleen and muscular stomach on day 55. Thus, we also collected samples to observe inflammatory injury on day 55 [17, 18]. Six broilers per group were anesthetized and sacrificed for pathologic autopsy. Three broiler bursae of Fabricius in each group were fixed in 4% paraformaldehyde for histopathology, and the remaining 3

| Table 1 The composition of the low-Se diet |
|------------------------------------------|
| Composition | Contents (%) |
| Corn a | 63.5 |
| Soybean meal a | 28.0 |
| Wheat meal a | 2.84 |
| NaCl | 0.27 |
| Soybean oil | 1 |
| Choline | 0.07 |
| CaCO₃ | 1.2 |
| CaHPO₄ | 2.3 |
| DL-methionine | 0.18 |
| Lysine | 0.4 |
| Vitamins | 0.04 |
| Minerals | 0.2 |
| Total | 100 |

Note: a Corn, soybean meal and wheat meal were purchased from Longjiang County, a typical Se-deficient area in Heilongjiang Province, China.
broiler bursa of Fabricius in each group were fixed in 2.5% glutaraldehyde solution for ultrastructural pathology.

Experiment 2

Then, we determined whether Se deficiency could elevate proinflammatory cytokine mRNA expression and initiate TLR/MyD88/NF-κB signaling in the bursa of Fabricius. Thirty, one-day-old healthy broilers were randomly and averagely divided into the C group and the L group. The treatments of the C and L groups were the same as those in experiment 1. On days 15, 35, and 55, 5 broilers per group at every point in time were anesthetized and sacrificed for pathologic autopsy. The bursae of Fabricius were collected and stored at −80°C for real-time quantitative PCR (RT-qPCR).

Histopathology

The fixed bursae of Fabricius from the C and L groups were embedded in paraffin and sectioned for histopathological experiments. Sections were stained with hematoxylin and eosin (H&E) for microscopy. The results were confirmed and described by an experienced and qualified pathologist.

Transmission electron microscopy test

The fixed bursae of Fabricius from the C and L groups were dehydrated, saturated, embedded, and sectioned for ultrastructural pathology experiments. Sections were stained by uranyl acetate and lead citrate staining. The results were described by experienced and qualified research staff.

RT-qPCR

Total RNA in the bursa of Fabricius was extracted by TRizol reagent (TransGen, ET101-01, China). Next, cDNA was synthesized using the Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa, RR037A, Japan) and detected by RT-qPCR with Tli RNase H Plus (TaKaRa, SYBR Premix Ex Taq II, Japan). β-actin gene was used as the loading control gene. The relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 2.

Data analysis

GraphPad Prism 6.0 was used to perform statistical analysis. Data are presented as the mean ± SD. The statistical significance between two groups was analyzed using one-way ANOVA followed by Student’s t test. A value of $P < 0.05$ was considered significant.

Table 2 Primer sequences for RT-qPCR

| Gene name | Sequence (5'-3') |
|-----------|-----------------|
| β-actin   | F: CGTGACTGACTGACTACCT R: TGATCGAATACGATGCCAC |
| IFN-γ     | F: GCTGGGGTGACCTATTATTGTAGAG R: TTCTTCAGCCCATGGAAGTTG |
| IL-2      | F: TTCTGCACTCCTCTTGATGTC R: GCGTGCTACTGACTCATTGAG |
| IL-6      | F: ATGGTGTAATAATCCCATGAAG R: CCTCAGCTTCTTCCATCA |
| TNF-α     | F: CAGATGGGAAGGGAAGTACC R: AGAACATCAACGCAGGAAG |
| MyD88     | F: CCAGCAGAGGACGAGAAGGACG R: TGAGCAGAGGACGAGAAGGACG |
| TRIF      | F: CCAATCTGCCTTCTGCTG R: GTTGGATATGCGGTGCTG |
| NF-κB     | F: TCACGCAGACCTAGACCAT R: GCAGATAGCAATTCAGATG |
| TLR1      | F: GGCTCGACAGAGAAATTTGTT R: TGATTTCATACGGCTGGTCA |
| TLR2      | F: GGTTGCCAGAAAGGCTACATC R: GGTTGCGAGATAAAGGACTTC |
| TLR3      | F: CTGGGGAATCTGCGTCTCCT R: TGCAACTTCACCAAGACGAG |
| TLR4      | F: GATTTGGCAGCTCCGCTCTCA R: CCTCCTGCAGATATGGGAG |
| TLR5      | F: TGCACTTCATTTCCACTGC R: GACAGCAGAGATTTGAGTG |
| TLR7      | F: TGCAATTGATGATGCTGTTG R: AACAAGCTCCTCCATTG |
| TLR15     | F: CTCCATCGTCAGCATCAGCACTT |
| TLR21     | F: TGCACTTCAGGAGACGAGAG R: GGAGGACAGTGCAGAAGTGT |

Note: F, forward; R, reverse; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; TRIF, toll-interleukin-1 receptor domain-containing adapter-inducing interferon-β; MyD88, myeloid differential protein-88; NF-κB, nuclear factor-κB; TLR, Toll-like receptor

Results

Se deficiency causes inflammatory injury in the broiler bursa of Fabricius

In the present study, histopathological results showed that no pathological change in the bursa of Fabricius of the C group was observed (Fig. 1A). However, the whole tissue structure of the bursa of Fabricius was seriously destroyed and presented incomplete and disheveled in the L group. The lymph
nodes were atrophied, and epithelial mucosae presented serrated atrophy in the L group. Multiple and severe hemorrhages obviously occurred in the lymph nodes and mucosal lamina propria of the L group. The interstitial space in the lymph nodes was enlarged in the L group, implying that edema occurred or the number of lymphocytes was reduced (Fig. 1B). The destroyed tissue structure, severe hemorrhage, and edema indicated that Se deficiency causes severe inflammatory injury in the broiler bursa of Fabricius.

**Se deficiency causes organelle damage in the broiler bursa of Fabricius**

In this study, the ultrastructural pathological results showed that no damaged organelles in the bursa of Fabricius of the C group were observed (Fig. 2A). However, in the L group, lymphocyte membranes were destroyed, the shaped nuclei were irregularly present, and nuclear membranes were damaged. Obviously, mitochondria were swelled, and some of their structures disappeared and were even vacuolized in the L group. Moreover, the endoplasmic reticulum was vacuolar and fractured. We also found that autophagic vacuoles might be produced in the L group (Fig. 2B). The foregoing results indicated that Se deficiency causes multiple organelle damage in the broiler bursa of Fabricius.

**Se deficiency markedly elevates proinflammatory cytokine mRNA expression in the broiler bursa of Fabricius**

In the present study, compared to the C group, *IL-6* mRNA expression was elevated in the L group and was especially markedly elevated on day 15 (*P* < 0.0001) (Fig. 3A). Meanwhile, *IL-2* mRNA expression was markedly elevated in the L group on day 15 (*P* < 0.0001) (Fig. 3B). Additionally, *IFN-γ* mRNA expression was increased in the L group and was especially markedly elevated on days 15 and 35 (*P* < 0.0001) (Fig. 3C). Compared to the C group, *TNF-α* mRNA expression was increased in the L group, with no difference (Fig. 3D). The foregoing data indicated that Se deficiency significantly initiates the mRNA expression of multiple proinflammatory cytokines in the broiler bursa of Fabricius, especially in the early stage of Se deficiency (on day 15).

**Se deficiency activates the TLR/MyD88/NF-κB signaling pathway in the broiler bursa of Fabricius**

Then, whether Se deficiency activated TLR signaling was determined. Compared to the C group, *TLR-1* mRNA expression was elevated in the L group on days 15 and 35, and was especially markedly elevated on day
Fig. 2 Se deficiency causes multiple organelle damage in the bursa of Fabricius. (A) The ultrastructural pathology of the bursa of Fabricius in the C group. (B) The ultrastructural pathology of the bursa of Fabricius in the L group. The destroyed membrane of the lymphocytes (purple arrows). The irregularly shaped nuclei (deep pink arrows). Mitochondria were swollen, their structures disappeared and they showed vacuolization (green arrows). The vacuolar and fractured endoplasmic reticulum (blue arrows). The damaged nuclear membranes (red arrows) and autophagosomes (yellow arrow). The results were similar in 3 biological replicates per group. C, control. L, low selenium

Fig. 3 Se deficiency markedly elevates proinflammatory cytokine mRNA expression in the bursa of Fabricius. (A) The mRNA expression of IL-6 in the C and L groups (n = 5). (B) The mRNA expression of IL-2 in the C and L groups (n = 5). (C) The mRNA expression of IFN-γ in the C and L groups (n = 5). (D) The mRNA expression of TNF-α in the C and L groups (n = 5). IL-6, interleukin-6. IL-2, interleukin-2. IFN-γ, interferon-γ. TNF-α, tumor necrosis factor-α. C, control. L, low selenium. **** = P < 0.0001. NS, nonsignificant
35 ($P < 0.05$) (Fig. 4A); TLR-2 mRNA expression was elevated in the L group with no difference ($P > 0.05$) (Fig. 4B); both TLR-3 and TLR-4 mRNA expressions were markedly elevated in the L group on day 15 ($P < 0.0001$ and $P < 0.01$) (Fig. 4C and D); TLR-5 mRNA expression was markedly elevated in the L group on day 55 ($P < 0.05$) (Fig. 4E); TLR-7 mRNA expression was elevated in the L group on day 15 with no difference ($P > 0.05$), but markedly declined on day 35 ($P < 0.05$) (Fig. 4F); TLR-15 mRNA expression was markedly elevated in the L group from days 15 to 55 and was especially markedly elevated on day 15 ($P < 0.001$) (Fig. 4G), and TLR-21 mRNA expression was markedly elevated in the L group on day 35 ($P < 0.01$) (Fig. 4H).

Based on the foregoing results of the activation of TLR signaling during the period of Se deficiency, we then determined whether the MyD88/NF-κB signaling pathway (the downstream signaling of TLR) was activated. MyD88 mRNA expression was elevated in the L group compared to the C group and was especially markedly elevated on day 35 ($P < 0.0001$) (Fig. 5A). TRIF mRNA expression was elevated in the L group compared to the C group and was especially markedly elevated on day 35 ($P < 0.05$) (Fig. 5B). Compared to the C group, NF-κB mRNA expression was markedly elevated in the L group from days 15 to 55 ($P < 0.01, P < 0.001$) (Fig. 5C). The foregoing data indicated that the TLR/MyD88/NF-κB signaling pathway was activated in the broiler bursa of Fabricius with Se deficiency.

![Fig. 4 Se deficiency initiates TLR signaling in the bursa of Fabricius.](image)
Numerous studies have revealed that a diet with Se deficiency can cause inflammatory damage to broiler organs, such as the intestine, kidneys, and spleen [6, 10, 17, 19]. In the present study, we revealed that severe inflammatory injury and multiple organelle damage obviously occurred in the broiler bursa of Fabricius of the L group via histopathology and ultrastructural pathology. The biological functions of the endoplasmic reticulum are the synthesis, modification and transportation of proteins, and the biosynthesis of lipids. The biological functions of the damaged endoplasmic reticulum might be intercepted in the broiler bursa of Fabricius in the later stage of Se deficiency. Mitochondria are the main sources of energy for aerobic respiration. Numerous studies found that the abnormal aerobic respiratory chain in mitochondria could lead to $O_2^-$ accumulation, further resulting in an imbalance between oxidative and antioxidative responses [20, 21]. In the present study, Se deficiency caused swelling in mitochondria, strongly suggesting that the aerobic respiratory chain in mitochondria might be destroyed and induce $O_2^-$ accumulation, which gives rise to excessive activation of the oxidative response. The imbalance between oxidative and antioxidative responses could induce inflammatory responses, which further contribute to organ damage.

Cytokines, which are divided into proinflammatory and anti-inflammatory cytokines based on their functions in the immune response, effectively regulate inflammatory response networks in humans and animals. IL-6 and TNF-α are two kinds of critical proinflammatory cytokines in the early stage of innate immunity [15]. In the present study, we determined that Se deficiency increased IL-6 and TNF-α mRNA expression on day 15. IL-2 has been regarded as the key cytokine for the maturation of B lymphocytes abundantly existing in the bursa of Fabricius [14]. We also found that IL-2 mRNA expression was significantly increased in the L group on day 15, which could also induce an inflammatory response in the bursa of Fabricius. IFN-γ mediates macrophages to produce hydrogen peroxide and nitric oxide to prevent pathogens from invading the body [22]. In this study, IFN-γ mRNA expression was significantly increased in the L group on days 15 and 35. The foregoing results revealed that enormous pro-inflammatory cytokine production was significantly increased during the early period of Se deficiency, which brings about excessive inflammation in the broiler bursa of Fabricius.

The result that enormous pro-inflammatory cytokine mRNA expression was obviously upregulated during the early process of Se deficiency prompted us to investigate whether Se deficiency induces the enormous pro-inflammatory cytokine production with high strength that is involved in the activation of the TLR/MyD88/NF-κB signaling pathway. To prove this hypothesis, we measured the expression of 8 fundamental important TLR mRNAs in the broiler bursa of Fabricius during the inflammatory response. We found that TLR-1, TLR-3, TLR-4, and TLR-15 mRNA expressions were markedly increased in the broiler bursa of Fabricius during the early period of Se deficiency. The results of this study were similar to those of our previous study in which Se deficiency caused excessive inflammation in the broiler spleen via the TLR-4/NF-κB signaling pathway [17]. Gao et al. found that Se deficiency facilitates inflammation following S. aureus infection by regulating TLR-2-related pathways in the mouse mammary gland, suggesting that TLR signaling also plays a vital role in the process of Se deficiency-induced inflammation [23]. The downstream TLR signaling pathway is the MyD88-mediated signaling pathway, containing MyD88 protein-mediated (classic MyD88) and TRIF protein-mediated (non-classic MyD88) signaling pathways [24]. In the present study, on day 15, MyD88 and TRIF mRNA expression was not different between the C and L groups. However, MyD88 mRNA expression in the L group was much higher than that in the C group. The intragroup error in vivo is always larger than that in vitro. Thus, it is most likely that MyD88 signaling activated downstream signaling on day 15. We uncovered that MyD88 and TRIF mRNA expressions were markedly increased in the L group. We also found that NF-κB mRNA expression was significantly increased in the broiler bursa of Fabricius with Se deficiency. The foregoing results obviously indicated that Se deficiency activates the TLR/MyD88/NF-κB signaling pathway.
Glutathione peroxidase (GPX) is an enzyme with antioxidant activity that neutralizes reactive oxygen species. GPX has been considered a Se-containing enzyme, and its activity is obviously decreased in many organs and tissues by feeding a Se-deficient diet, resulting in a lower antioxidant capacity [25–27]. The imbalance of oxidative and antioxidative responses could induce heat shock protein (HSP) production with high strength in animals. Emerging studies have indicated that endogenous HSP can also activate the TLR signaling pathway in humans and animals [28, 29]. For example, Hsp70 elicits rapid calcium flux, binds with high affinity to the plasma membrane, activates NF-κB and upregulates the expression of TNF-α in human monocytes [30]. Considering the published studies and findings in this study, we speculate that Se deficiency activates the TLR signaling pathway, and is probably due to the increased expression of HSPs in the bursa of Fabricius, which is our future subject as well.

It is believed that biological functions are strongly associated with organ and tissue structures and that they interact with each other. In the present study, Se deficiency initiated an excessive inflammatory response at 15 days, and persistent and excessive inflammation caused severe damage and destruction in the broiler bursa of Fabricius at 55 days. Because of the biological function impaired by damaged organ and tissue structures, proinflammatory cytokine expression was not significantly different at 55 days. We uncovered that Se deficiency obviously initiates the inflammatory response in the bursa of Fabricius, but the excessive inflammatory response causes severe injury to the broiler bursa of Fabricius, which decreases the immune response level in the later stage of Se deficiency. This is one of the reasons that Se deficiency impairs the maturation of the broiler bursa of Fabricius. This fact may be even more damaging for laying hens because of their longer growth period. Elucidation of the mechanism by which Se deficiency induced inflammatory injury in the broiler bursa of Fabricius from the perspective of initiating the TLR signaling pathway will help us to study further the underlying mechanism of Se deficiency-induced inflammatory injury in the bursa of Fabricius and to add Se to feed rationally during the process of livestock and poultry farming.

Indeed, there is a limitation in our present study. Given the considerable technical challenge of lacking antibodies, especially against chickens, we have not yet determined the protein level changes of proinflammatory cytokines and the TLR/MyD88/NF-κB signaling pathway in the broiler bursa of Fabricius. Additionally, this issue is a major dilemma in other studies on chickens, which urgently needs to be resolved in future studies.

Taken together, Se deficiency could markedly elevate enormous proinflammatory cytokine production by triggering the TLR/MyD88/NF-κB signaling pathway, which causes an excessive inflammatory response and thereby leads to severe inflammatory injury in the broiler bursa of Fabricius (Fig. 6).

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**Author contribution** Yu Bai, Ruili Zhang and Qing Liu designed the research, performed most of the experiments, analyzed the data and drafted the manuscript. Rong Guo, Guangxing Li, Bin Sun, Di Zhang and Yang Chen helped perform the experiments. Xiaodan Huang modified the manuscript. All authors approved the final manuscript.

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