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Downregulation of TLR4 and 7 mRNA Expression Levels in Broiler's Spleen Caused by Diets Supplemented with Nickel Chloride

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Abstract Toll-like receptors (TLRs) are important immune receptors in discriminating self from nonself and in initiating the innate and adaptive immune response. TLR4 and TLR7 have been proven to be highly expressed in chicken's spleen. Thus, this study was to evaluate the TLR4 and TLR7 messenger RNA (mRNA) expression levels in the spleen of broilers fed diets supplemented with nickel chloride ($NiCl_2$) using the methods of quantitative real-time PCR (qRT-PCR). Two hundred forty-one-day-old avian broilers were equally divided into 4 groups and fed on a corn-soybean basal diet as control diet or the same basal diet supplemented with 300, 600, and 900 mg/kg of $NiCl_2$ for 42 days. Results showed that TLR4 and TLR7 mRNA expression levels in the spleen were lower ($P<0.05$ or $P<0.01$) in the 300, 600, and 900 mg/kg groups than those in the control group. It was concluded that dietary $NiCl_2$ in excess of 300 mg/kg could lower TLR4 and TLR7 mRNA expression levels in the spleen of broilers, implying that $NiCl_2$ could impair the innate and adaptive immunity in spleen by injuring immunocytes and/or decreasing the content of cytokines through TLRs.

Keywords Nickel chloride · TLR4 · TLR7 · mRNA expression · Spleen · Broiler

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Introduction

Nickel (Ni) widely exists in the environment, and is prevalent in human activities [1]. Ni is considered as an essential metal nutrient for proper functioning of animals [2, 3], and plays an important role in growing, multiplication, and glucose and lipid metabolism. Intracellular Ni can change membrane properties and influence oxidation/reduction systems [4–7]. However, previous studies have showed that long-term exposure to Ni (II) can also be toxic to upper respiratory tract, skin, kidney, embryo, and breeding system [8–11]. Ni exposure also has adverse effects on the immune system, including thymic involution, decreased T cell number and natural killer cell activity in the spleen of mice [12], and reduced cell viability and proliferation of Jurkat T cells [13].

Toll-like receptor (TLR) is a family of transmembrane-spanning proteins, which recognize molecules unique to microbes, discriminates self from nonself, and acts fundamentally in initiating the innate immunity and adaptive immunity in vertebrates [14]. Chicken TLRs contain a total of ten members (chTLR1~5, chTLR7, chTLR15, and chTLR21) [15]. TLR4 is the first identified TLR in mammal, and has been involved in the recognition of endogenous or exogenous products of microbes, such as lipopolysaccharide (LPS), heat shock protein (HSP), extracellular matrix components [16, 17]. TLR7, as a member of TLR9 subfamily, which plays an important role in activating antiviral immune responses, has been proven to recognize antiviral compounds and single-stranded viral RNA [18]. TLR4 and TLR7 have been detected in chicken spleen, and are important activators to the immune system [19, 20].

Spleen is mainly composed by B and T lymphocytes, macrophages, and other immune cells, in which both innate and adaptive immune responses can be efficiently mounted, making it a critical immune organ in the body [21]. It has been reported that $NiCl_2$ can accumulate in the spleen of mice,

promote immunosuppression [22, 23], and influence the number and size of giant cells and natural killer (NK) cells activity of splenocyte in mice [24–27]. However, the mechanisms of Ni compounds effect on splenocytes are still unknown [28]. And our study has revealed that $NiCl_2$ could cause oxidative damage and induce apoptosis in the spleen of broiler [29]. In addition, researches have clearly identified that Ni can induce the innate immune response and has interaction with TLRs signal transduction [30, 31].

From abovementioned references, there have been only a few researches on the effects of Ni or Ni compounds on human TLRs and very limited study on chicken TLR4 or TLR7 by using $NiCl_2$. In the present study, the alternations in mRNA expression levels of TLR4 and TLR7 in the spleen of broilers induced by dietary $NiCl_2$ were investigated by quantitative real-time PCR (qRT-PCR), and to provide new experimental evidences for understanding the effect mechanism of $NiCl_2$ or Ni compounds on splenic innate immune responses.

Materials and Methods

Broilers and Diets

Two hundred forty-one-day-old healthy avian broilers were equally divided into 4 groups. Broilers were housed in cages with electrical heaters and provided with water as well as undermentioned experimental diets ad libitum for 42 days.

A corn-soybean basal diet formulated by the National Research Council [32] was the control diet. $NiCl_2$ $6H_2O$ was mixed into the corn-soybean basal diet to produce experimental diets with 300, 600, and 900 mg/kg of $NiCl_2$, respectively.

All experimental procedures involving animals were approved by Sichuan Agricultural University Animal Care and Use Committee.

Detection of the Splenic TLR4 and TLR7 mRNA Expression Levels by qRT-PCR

The method was described by Huang et al. 2013 [29]. At the 14, 28, and 42 days of age, 5 broilers in each group were humanely killed, and spleens were removed and stored in liquid nitrogen immediately. Adding liquid nitrogen, the spleens were ground into homogenized powder with pestle. Total RNA was extracted from the powder of spleen by RNAiso Plus (9108/9109, Takara, Japan). The mRNA was then reverse transcribed into complementary DNA (cDNA) using PrimeScript™ RT reagent kit with gDNA Eraser (RR047A, Takara, Japan). The cDNA was used as a template for qRT-PCR analysis. Sequences of primer were obtained from GenBank and NCBI. Primers were designed using Primer 5 and synthesized by BGI Tech (Shenzhen, China; Table 1).

Table 1 Nucleotides used as primers in qRT-PCR of mRNA expression in the spleen

Primer		Sequence(5′ → 3′)	Accession number
TLR-4	F	AGTCTGAAATTGCTGAGCTCAAAT	AY064697
	R	GCGACGTTAAGCCATGGAAG	
TLR-7	F	TCAGAGGTGGCTGCACAC	NM001011688
	R	CAACAGTGCATTTGACGTCCTT	
β-Actin	F	TGCTGTGTTCCCATCTATCG	L08165
	R	TTGGTGACAATACCGTGTTCA	

For qRT-PCR reactions, 25-μL mixture was made by using SYBR® Premix Ex Taq™II (DRR820A, Takara, Japan), containing 12.5 μL Tli RNaseH Plus, 1.0 μL of forward and 1.0 μL of reverse primer, 8.5 μL RNAase-free water, and 2 μL cDNA. Reaction conditions were set to 3 min at 95 °C for 1 cycle, followed by 44 cycles of 30 s at Tm of a specific primer pair, followed by 1 cycle of 10 s at 95 °C, 72 °C for 10 s, and 10 s at 95 °C, using Thermal Cycler (C1000, Bio-Rad, USA). Actin was used as an internal control gene. Results were analyzed with the method of $2^{-\Delta\Delta CT}$.

Statistical Analysis

The significance of difference among four groups was analyzed by variance analysis, and results were presented as means ± standard deviation ($\overline{X}$ ± SD). The analysis was performed using one-way analysis of variance (ANOVA) test of SPSS 16.0 for windows. A value of $P<0.05$ was considered significant.

Results

Changes of TLR4 mRNA Expression Levels in the Spleen

As showed in Fig. 1, TLR4 mRNA expression levels were significantly lower ($P<0.01$) in the 300 mg/kg group at 42 days of age, and were significantly lower ($P<0.05$ or $P<0.01$) in the 600 and 900 mg/kg groups from 14 to 42 days of age than those in the control group.

Melting curve analysis of TLR4 was showed in Fig. 2. The Tm of TLR4 was 81 °C. At the Tm, the PCR products produced a melting curve with a single peak. The TLR4 mRNA expression levels detected by RT-PCR analysis were confirmed by real-time PCR analysis.

Changes of TLR7 mRNA Expression Levels in the Spleen

Changes of TLR7 mRNA expression levels were showed in Fig. 3. The splenic TLR7 mRNA expression levels were

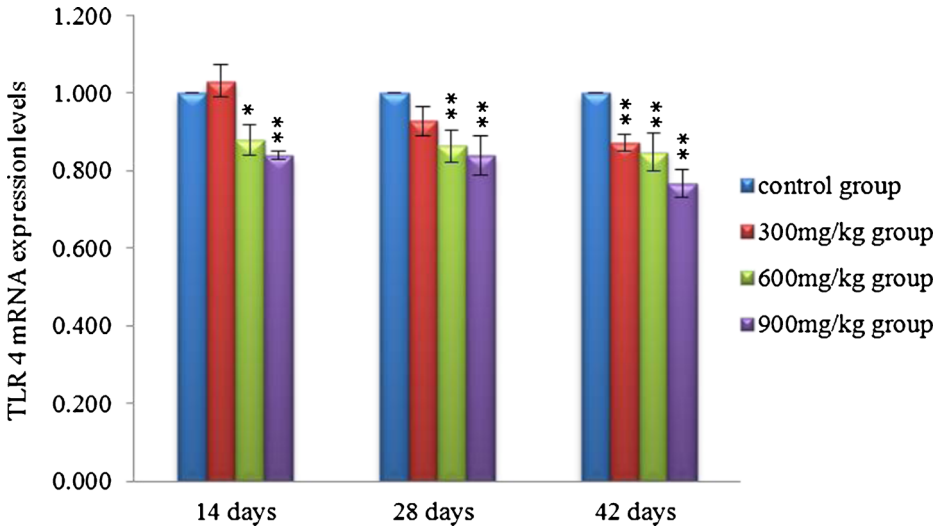

Fig. 1 Changes of TLR4 mRNA expression levels in the spleen. Data are presented with the means ± standard deviation (n=5). *P<0.05, **P<0.01, compared with the control group. Data were analyzed by variance (ANOVA) test of SPSS 16.0 software

significantly decreased (P<0.05) in the 300 mg/kg group at 28 and 42 days of age, and were significantly decreased (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups from 14 to 42 days of age in comparison with those of the control group.

Melting curve analysis of TLR7 was showed in Fig. 4. The Tm of TLR7 was 83 °C. At the Tm, the PCR products produced a melting curve with a single peak. The TLR7 mRNA expression levels detected by RT-PCR analysis were confirmed by real-time PCR analysis.

Discussion

Spleen, as an important peripheral immune organ, contains three parts as follows: the red pulp, the white pulp, and the marginal zone. The red pulp is efficient in blood filtering, iron recycling, blood-borne bacterial removing, and producing antibody. The white pulp, which is similar to lymph node, can be divided into T and B cell compartments which are strictly involved in the adaptive immunity. However, the marginal zone is involved in both innate and adaptive immunity through its specific macrophages and B cells [21]. In the spleen, the innate immune defense is efficient in trapping blood-borne pathogens and antigens, expressing specific receptors (such as TLRs) in the resident marginal zone, and the adaptive immune response in producing antibodies by B lymphocytes and plasmocytes, and generating cytokines by helper and cytotoxic T lymphocytes in the red and/or white pulp [21].

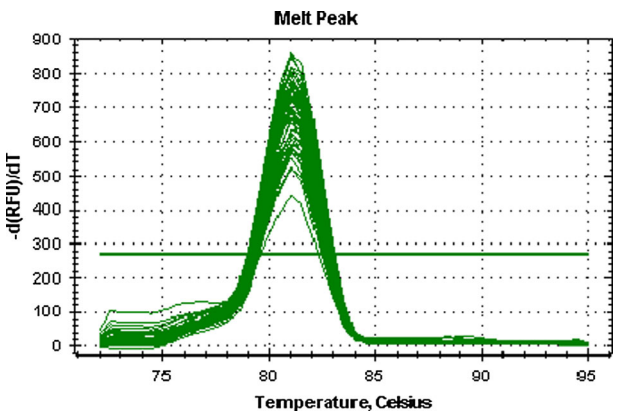

Fig. 2 Melting curve analysis of TLR4 gene transcripts detected by real-time PCR. cDNA samples were amplified in real-time PCR with specific set of primers and melting curve analysis was performed to confirm the identity of the PCR products

TLRs play a fundamental role in initiating the innate and adaptive immune responses in vertebrates [14]. Applying to their roles, TLRs mRNA are highly expressed in tissues which are important in the defense or immune function such as lungs, gastrointestinal tracts, peripheral blood leukocytes, and spleens [33]. The innate immune system recognizes pathogens by discriminating certain conserved structure of the microbes (such as LPS, a major component of the outer membrane of gram-negative bacteria) [34]. Bacterial ligands are mainly recognized by TLRs on the cell surface, and during the process, MyD88 is reported to be an essential compound for TLRs [35–37]. At the meantime, immune cells and cytokines, which are involved in the adaptive immune system, have also been critically affected by the process of TLRs responding to bacterial ligands [35, 38, 39]. All these indicate that TLRs are very important in both the innate and adaptive immunity, and also in the interaction between the innate and adaptive immune systems. Thus, the changed TLR mRNA expression levels may cause impact on the immune response of the spleen. Previous reports have described that TLR4 and TLR7 are important activators of the immune system [40–42]. The mice without TLR4 and TLR7 have lower or vacant ability in responding to ligands or pathogens [35, 43], and activation of TLR4 and TLR7 could influence immune cells on differentiation, responding to pathogens and cellular

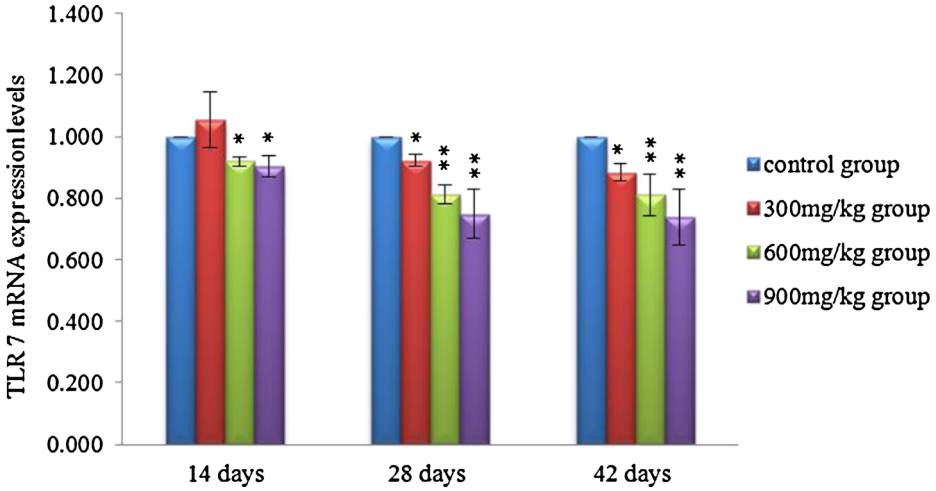

Fig. 3 Changes of TLR7 mRNA expression levels in the spleen. Data are presented with the means ± standard deviation (n=5), *P<0.05, **P<0.01, compared with the control group. Data were analyzed by variance (ANOVA) test of SPSS 16.0 software

activity [43, 44]. Furthermore, the combination of TLR4 and TLR7 could enhance association among immune cells and causes sequential production of regulatory and proinflammatory cytokines by naive $CD4^+$ T cells [45, 46]. TLRs (including TLR4 and TLR7) could induce the production of various cytokines, such as interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFNs) [18, 43, 47, 48]. In turn, cytokines could also influence TLRs, for example IFN-γ could enhance TLR4 expression on the surface of human monocytes and macrophages [49]. From above discussion, it is apparent that TLR4 and TLR7 have great impact on immunocytes and cytokines, and play critical roles in the innate and adaptive immunity. There is no doubt that the downregulation in TLR4 and/or TLR7 mRNA expression levels may cause damage on immune organs or may cause negative impact on the immune system. Consistently, in the present study, the downregulation of TLR4 and TLR7 mRNA expression levels in the 300, 600, and 900 mg/kg groups have caused impairment on splenocytes and the splenic immune function of broilers.

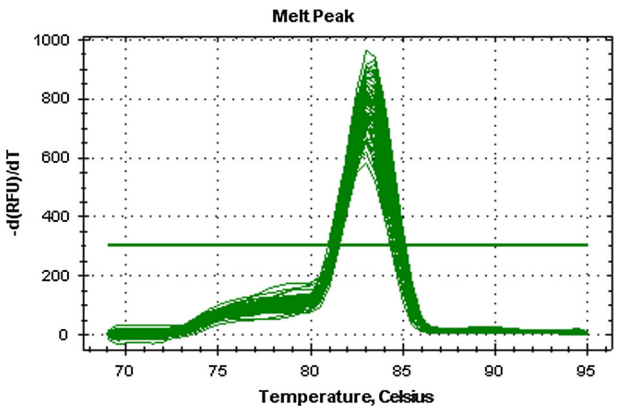

Fig. 4 Melting curve analysis of TLR7 gene transcripts detected by real-time PCR. cDNA samples were amplified in real-time PCR with specific set of primers and melting curve analysis was performed to confirm the identity of the PCR products

Ni is a chemical hapten, which can activate the innate and adaptive immune system, regulate skin barrier function and cellular stress response including redox balance and inflammation (caused by both innate and adaptive immunity) [50, 51]. Ni has also been proved to be an inorganic activator of the TLRs system [52]. Also, it has bee reported that Ni^{2+}-mediated allergy requires both Ni^{2+}-bound antigens and additional signals including TLRs family [53]. In addition, researches have also revealed that Ni can interact with TLRs by directly activating proinflammatory intracellular signal transduction involved in the stimulation of transcription factor nuclear factor-B (NF-κB) (a regulator of the immune, inflammatory, stress, proliferative, and apoptotic responses of a cell to a very large number of different stimuli) [31, 53, 54]. It has been identified that TLR4 acts as a receptor and directly binds to Ni^{2+} in human [52], and is capable of mediating Ni^{2+}-induced proinflammatory responses when introduced into human embryonic kidney cells [53]. From abovementioned discussions, it is obvious that the downregulation of TLR4 and TLR7 mRNA expression levels is closely related to $NiCl_2$ added in the diets in the present research.

Conclusions

According to the results of the present study and the above discussions, it was concluded that dietary $NiCl_2$ in excess of 300 mg/kg could lower TLR4 and TLR7 mRNA expression levels in the spleen of broilers, implying that $NiCl_2$ could impair the innate and adaptive immunity in spleen by injuring immunocytes and/or decreasing the content of cytokines through TLRs.

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Conflict of Interest The authors declare no conflict of interest.

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