Life Science and Biotechnology
Volume 2, Number 2, November 2024
Pages: 58 – 63

Life Science Biotechnology

DOI:10.19184/lsb.v%vi%i.53503

Does the chick of domestic chicken (*Gallus gallus domesticus*) in early development would be able to withstand the injection of crude LPS?

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Abstract

Preventing bacterial infections in poultry farms is a key aspect of effective disease management strategies. Enhancing innate immunity serves as a vital preventive measure in chicken farming. Lipopolysaccharide (LPS), a fundamental component of the bacterial cell wall, has been successfully utilized to boost the innate immune response in susceptible poultry hosts. This study focused on examining the impact of administering crude LPS from *Salmonella typhimurium* to a domestic chicken (*Gallus gallus domesticus*) strain during its early developmental stages up to the pre-maturation phase. Immune responses in the chicks were evaluated by observing changes in body temperature and leukocyte differentiation. The chickens were divided into two groups: control (injected with 0.85% NaCl) and treatment (injected with the LPS). The injections were applied in three different ages 7 days, 24 days, and 41 days. The body temperature and leukocyte differential data were collected following the LPS injection. Results showed a significant difference in body temperature and heterophil at the ages of 24 and 41, indicating an immune response characterized by inflammation and elevated heterophil levels. There was a significant increase in basal temperature during early development in line with chicks' development to maintain their homeostatic system. This study concludes that the chicks demonstrated resilience to the presence of LPS by exhibiting heightened responses. The increasing of heterophils concentration provided strong evidence of their immune capability to overcome bacterial infections.

Keywords: domestic chicken, crude LPS, early development, withstand

Introduction

The Enterobacteriaceae family encompasses *Salmonella* species, which are predominantly motile, gram-negative, flagellated bacilli, with the notable exceptions of *Salmonella pullorum* and *Salmonella gallinarum*, which are non-motile. Avian salmonellosis represents a critical challenge to the poultry industry (Antunes et al., 2016; Hassan et al., 2020). The widespread application of antibiotics in veterinary and human medicine has led to a significant rise in the transmission of antibiotic-resistant Salmonella. Preventing bacterial infections in chicken farms is therefore a crucial component of strategic disease management. In chicken production, strengthening innate immunity is a crucial preventative approach. An essential component of the bacterial cell wall, lipopolysaccharide (LPS) has been used efficiently to enhance the innate immunity of sensitive poultry hosts (Bescucci et al., 2022; Lelono & Surya, 2023; Splichalova et al., 2019). Improving the natural innate immune response in poultry is a key strategy for disease prevention in production systems. Lipopolysaccharide (LPS), a fundamental component of bacterial cell walls, has proven to be an effective agent for boosting the innate immunity of vulnerable poultry populations (Hassan et al., 2020; Yoshimura et al., 2022).

Lipopolysaccharide (LPS) is a crucial structural element of Gram-negative bacteria. It is composed of three main components: lipid A, which anchors in the outer membrane; a core oligosaccharide; and O-antigen units that protrude from the bacterial cell surface in repetitive chains (Chessa et al., 2014; Hoare et al., 2006; Mirzaei et al., 2011). In order to trigger a pro-inflammatory response involving cytokines like interleukin 1β (IL-1β) and interferon-γ (IFN-γ), LPS binds itself to toll-like receptor 4 (TLR4), generating a pattern recognition receptor. The virulence and pathogenicity of various bacterial species identified as endotoxins depend on the molecule LPS (Lin et al., 2017; Shaji et al., 2023; Steimle et al., 2016). Salmonella strains are serotyped according to variations in the content of the LPS O-antigen. Several chemical reagents, namely butanol, ether, hot phenol, and proteinase K, have been used in protocols for the extraction, separation, and purification of LPS. Because of its ability to lyse bacteria and its effect on denaturing proteins, the hot phenol approach is utilized commercially to extract significant quantities of

LPS (Redweik et al., 2020; Sali et al., 2019; Yoshimura et al., 2022).

The hot phenol method, although commonly used, is restricted due to its hazardous nature, requiring the use of a steam hood for safety. In this study, the methanol-chloroform method is employed due to its efficiency in reducing time and costs, as well as its avoidance of phenol usage (Hassan et al., 2020; Mirzaei et al., 2011). This research highlights the importance of using LPS inoculation in chickens as a reliable model for triggering an inflammatory immune response, mimicking bacterial infection without the complexities of live pathogen exposure (Lacroix-Lamandé et al., 2023; Shaji et al., 2023).

This study aimed to investigate the effects of injecting crude LPS of *S. typhimurium* in a domestic chicken strain during its early developmental stages through to the pre-maturation phase. The immune responses of the chicks were assessed by monitoring temperature changes and leukocyte differentiation. The results are expected to offer valuable insights into the physiological adaptation and functioning of the innate and adaptive immune systems in resisting bacterial infections.

Materials and Methods

Animals models

The fertile domestic chicken (*G. gallus domesticuss*) eggs were supplied by the hatchery company. Those eggs were then artificially incubated at 38°C and 60% of air humidity. Chicks were housed in special cages in mixed composition to eliminate the care factor. We randomly select 8 chicks for treatment and 8 chicks for the control group. The chicks were provided with food and water adlibitum to fulfill their basic requirement for growth.

LPS extraction from *S. typhimurium*

The extraction of LPS from *S. typhimurium* was performed using the method of Mirzaei et al. (2011). The *S typhimurium* bacteria were rejuvenated by growing on slanted Nutrient Agar (NA) media for 24 hours. After rejuvenation, 1 loop of *S. typhimurium* was taken and inoculated into 50 ml of Luria Bertani (LB) media. The bacterial culture in LB media was then placed on a shaker for 6 hours. A total of 50 mL of *S. typhimurium* culture in Luria Bertani media was gradually transferred into 25 Eppendorf tubes with a capacity of 2 mL each and then centrifuged for 10 minutes at 2000 rpm. After centrifugation, the supernatant was discarded. Then, 2 mL of 95% alcohol solution was added to the Eppendorf tubes containing the sediment and resuspended until the bacterial sediment and alcohol were well mixed. After resuspension, the Eppendorf tubes were centrifuged for 10 minutes at 2000 rpm.

The supernatant was removed, and the buffer pellet was rinsed with 2 mL of alcohol solution before being resuspended. This process was repeated three times following the previously outlined method. The supernatant was discarded, and the buffer pellet was resuspended with 1 mL of 10% EDTA and then sonicated for 45 minutes. Then, 1 mL of methanol solution was added. The tube is covered with parafilm and shaken for 2 hours, then centrifuged for 10 minutes at a speed of 2000 rpm. Three distinct layers are produced: methanol at the top, biomass cell lysate in the middle, and chloroform at the bottom. The methanol and chloroform layers are then carefully separated and transferred into new tubes (Mirzaei et al., 2011). Finally, a dilution of 8.4 ml of LPS *S typhimurium* is dissolved into 33.6 ml of 0.85% NaCl solution with a final concentration of 0.2%.

LPS injection and temperature measurement

Sixteen chicks are the animal model in this study, divided into 2 groups, namely the control group consisting of 8 chicks and the treatment group 8 chicks. They were injected at the ages of 7 days, 24 days, and 41 days. The treatment group was injected with crude LPS in 0,85% NaCl solution at doses of 0,1 ml at the first injection (day 7), continued to the second injection at sudah 24 by 0,15 ml and the third injection at 41 days with 0,2 ml respectively. The control group would be injected with 0,85% of NaCl in the same doses and ages. This procedure ensures that the treatment and control receive the same injection (dose and age) and the difference between them is the content of crude LPS. The first injection was applied to the subcutaneous area around the chest. The second and third injections were applied to the peritoneal cavity. The peritoneal injection was applied on an area located 0,25 cm below the tip of the breastbone. The body temperature of the chicks was measured every 60 minutes post-injection for a total of 12 measurements using a digital thermometer (Omron MC 341) with a measurement range of 32-43°C and an accuracy of 0.1°C. Measurements were taken rectally by inserting the thermometer probe 2-3 cm deep for 1 minute.

Preparation of blood smear and leucocyte differentiation

Blood samples from the chicks are taken 14 days after injection from the brachial vein and placed into Eppendorf tubes. A thin blood smear is then prepared with the following steps: blood is taken using a dropper pipette and dropped onto a glass

Life Science Biotechnology
DOI:10.19184/lsb.v%vi%i.53503

slide, then the blood is smeared to spread evenly across the entire surface of the glass slide to form a thin blood smear. The glass slide is then numbered according to the sample number with a paper label while being air-dried. The blood smear is then fixed using methanol for 3-5 minutes. After fixation, the blood can be stained using 3% Giemsa stain. Staining is done by dropping several drops of Giemsa solution onto the blood smear, then left for about ± 30 minutes and rinsed with flowing Aquades until the Giemsa stain is clean. The sample is mounted using enthellan (Wardhana et al., 2023).

Differential percentage of leukocytes especially lymphocytes, heterophils, and monocytes. Lymphocytes have a greater ratio of nucleus to cytoplasm, heterophils have three-lobed nuclei, and monocytes have more nuclei small compared to the cytoplasm. Leukocyte differential was observed using a light microscope with a magnification of 400×. Observations were made in 5 fields of view each with 100 leukocytes cells.

Data analysis

The data were analyzed first for normality before further testing. The differences in temperature increase after LPS injection between the control and treatment groups were tested by independent t-test. The differences in temperature increase between the two groups in the first, second, and third of the three different injection times were analyzed by a mixed model with treatment, the period of injection, repeated measurement, and the interaction between the period of injection and repeated measurement as a fixed factor and the identity of chicks in both group as a random factor. The difference in the chicks' body temperature increase between the first, second, and third injection of each group (treatment vs control) was analyzed by independent t-test. There were 5 different leucocyte types were analyzed in the same way. All data were analyzed using IBM SPSS 21 Materials and Methods should emphasize the procedures and data analysis.

Results and Discussion

There were significant differences in the chicks' body temperature between the three different periods of injection (F=113.49, p=0.000), but no difference between control and treatment if all sequences were analyzed in one model (F= 2.359, p=0.096). However, there were significant differences between control and treatment in the second and third injections.

The temperature responses of the chick after LPS injection

In this study, we assessed whether the age of chicks would be able to withdraw the negative effect of the LPS injection. Here, we investigate the immunity development of the domestic chick during an early age when innate immunity remains the role as the main factor and continues to the adaptive immunity when the chicks start to develop their own ability. The capacity to demonstrate a clear response following injection begins at a later age during the second and third injections, as illustrated in Figure 1 and detailed in Table 1. We found evidence that during early age (before ten days old), the chick was unable to show different responses to LPS injection.

Table 1. The statistical differences in chick body temperature (°C) between LPS injection in three different periods and the age of chicks

	Control	Treatment	t value	p-value
First	40.57 ± 0.05	40.60 ± 0.05	-0.370	0.721
Second	40.88 ± 0.05	41.08 ± 0.09	2.033	0.043
Third	40.28 ± 0.03	41.44 ± 0.04	-3.410	0.001

Note: * ($p \leq 0.05$)

Here we also found that the basal body temperature increased significantly with the age of the chicks. This was the process of the development in the heat produced and the ability of the body to maintain body temperature. The low ability of chicks to balance their normal body temperature was the reason why mother usually protect their young by transferring their body heat. In artificial rearing, chicks need the support of an external heat source to maintain temperature. The injection of LPS could increase the body temperature of chicks from a normal body temperature as an early indicator of the immune system response on days 7, 24, and 41. Moreover, the injection of LPS increases differential leukocytes especially heterophils on days 7 and 24.

The simple indicator of the avian immune response to bacterial infection is the changes in the body temperature of the chicks. The crude LPS of *S typhimurium* can produce various effects, one of which is a change in body temperature. The administration of LPS intraperitoneally could stimulate an acute inflammatory response (Bescucci et al., 2022; Chen et al., 2018), and cause inflammation, leading to fever (Hassan et al., 2020). Inflammation promotes the restoration of cellular functions and the innate and adaptive immune

system tissues responsible for defense against pathogens. LPS *S typhimurium* can produce various effects such as changes in body temperature (Hassan et al., 2020; Steimle et al., 2016; Yoshimura et al., 2022). The increase in body temperature in chicks infected with LPS *S typhimurium* indicates that the immune response is active as a reaction to infection. This increase in temperature can accelerate the body's metabolism to strengthen the immune system in chicks. In addition, the increase in body temperature can also suppress the transcription of the SP 1 gene in Salmonella, which plays a key role in host cell invasion (Bescucci et al., 2022; Troxell et al., 2015).

Table 2. The statistical analysis of leucocyte differentiation at the first and second injection between two group of the chicks 2 weeks after each injection.

	First injection			Second injection		
	control	treatment		control	treatment	
Leukocyte	(mean±S.E)	(mean±S.E)	t value	(mean±S.E)	(mean±S.E)	t value
Heterofil	18.95 ± 1.05	26.22 ± 2.04	-3.38*	27.25 ± 1.69	36.78 ± 3.93	-2.61*
Eosinofil	18.45 ± 1.54	11.16 ± 1.16	-1.32	13.72 ± 1.61	13.73 ± 3.61	-0.00
Monosit	9.04 ± 1.43	8.16 ± 1.33	0.43	21.47 ± 2.00	19.51 ± 2.97	0.55
Basofil	0.37 ± 0.17	1.05 ± 0.29	-2.06	1.21 ± 0.32	1.35 ± 0.41	-0.24
Limfosit	24.91 ± 1.75	26.05 ± 2.65	-0.37	36.33 ± 2.26	28.61 ± 3.61	1.88

Note: * ($p \leq 0.05$)

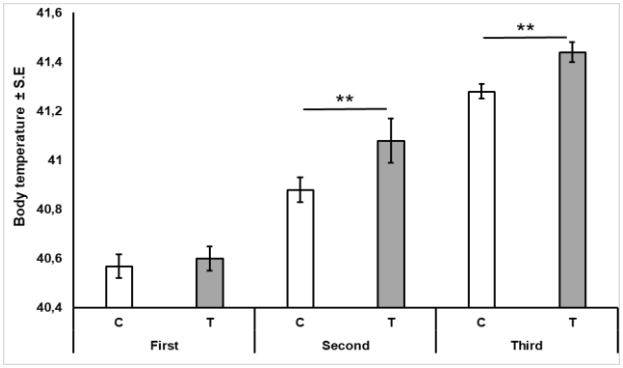

Figure 1. Body temperature of chicks in three different injection series of *S.tiphymurium* lipopolysaccharide

Leukocytes differentiation

We found a significant increase in the heterophil concentration between the treatment vs control group during the first and second injections (Table 2). This indicates how the chicks respond by producing a key component of leukocytes, essential for addressing bacterial infections. Since the LPS injection simulated a bacterial infection, the findings highlight the progression of the innate to adaptive immune system as the chicks mature. Five different types of leukocytes were analyzed in this study, each playing a distinct role in the avian immune system, as illustrated in Figure 2.

Leukocytes (white blood cells) in domestic chickens are differentiated into granulocytes (heterophils, basophils, eosinophils) and agranulocytes (lymphocytes and monocytes). Each type of leukocyte has its function, concentration, and differential leukocyte count in the immune system (Feng et al., 2019; Hassan et al., 2020; Shaji et al., 2023). Lymphocytes, which are one of the differentials of leukocytes, work specifically as adaptive immunity and can be differentiated into B Lymphocytes (B Cells) and T Lymphocytes (T Cells). Heterophils are a type of polymorphonuclear leukocyte that is dominant in poultry, playing an important role in the innate immune response, especially in dealing with inflammation (Shaji et al., 2023; Zhang et al., 2023). Domestic chickens have different forms of differential leukocytes and have different percentages depending on the environment, health status, and age (Odunitan-Wayas et al., 2018).

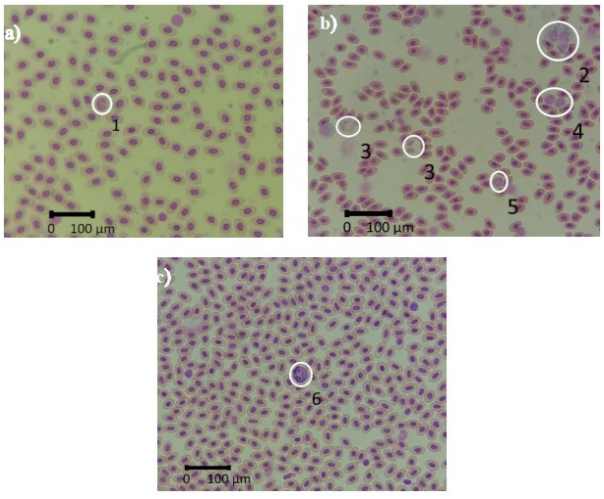

Figure 2. The leucocyte differentiation consists of five different types: heterophil (1), monocytes (2), eosinophil (3), lymphocyte (4) and heterophil (5).

The increase in the number of heterophiles indicates the stimulation of the innate immune response, which is important for clearing pathogens through phagocytosis and antimicrobial mechanisms (Huang et al., 2017; Munyaka et al.,

Life Science Biotechnology
DOI:10.19184/lsb.v%vi%i.53503

2012; Yoshimura et al., 2022). The correlation between the decrease in lymphocytes and the increase in heterophiles can occur due to an increase in corticosterone or stress hormones in chicks. The LPS *S. typhimurium* causes degenerative changes in the morphology of lymphocytes and destroys them. Conversely, corticosterone promotes the redistribution of lymphocytes to other parts of the body in response to stress signals. In chickens, heterophils serve as a crucial element of the innate immune system, functioning analogously to neutrophils in mammals (Bowen et al., 2009; Munyaka et al., 2012; Redmond et al., 2009).

This study concludes that the chicks of domestic chickens (*G. gallus domesticus*) demonstrated resilience to the presence of LPS by exhibiting heightened responses during early development. Their temperature response immediately following injection, along with an increased concentration of heterophils, provided strong evidence of their immune capability to overcome bacterial infections.

Acknowledgments

This study was supported by RISPRO BRIN Batch IV funding in 2024 (No. 094/UN25.3.1/LT/2024). We extend our heartfelt gratitude to Efi Fajriah Eka Dewi, Rizky Surya Wijaya, Anis Noviani, and Wardza Izza Wulandari for their assistance and valuable contributions to chemical preparation, animal care, blood sampling, data analysis, and discussions.

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