Effect of cyclic heat stress on the acute inflammatory response in broilers.

Introduction

Over time, the immune system has continued to develop and evolve due to the intense stress placed upon it by pathogens (Medzhitov & Janeway, 1997). There are two major parts to the immune system, consisting of the innate and adaptive immunity. Innate immunity acts as the body's first line of defense, whereas adaptive immunity is highly specific and protective. One of the innate immune responses developed for dealing with pathogens is the inflammatory response. Inflammation works by accumulating "leukocytes, plasma proteins and fluid derived from blood" to the site of infection to eliminate microbes and repair damaged tissue (Abbas et al., 2018). To recognize infections, the innate immune system has established a method for distinguishing molecular patterns common to groups of pathogens (e.g., lipopolysaccharide (LPS) of Gram-negative bacteria). In doing this, the innate immune system can quickly recognize a pathogen and initiate inflammation (Medzhitov & Janeway, 1997).

This innate immune system is important in the realm of commercial broiler production as chickens are processed at six weeks of ages before their own adaptive immunities have had time to develop (French et al., 2020). To examine the effectiveness of the innate immune system, the pulp of growing feathers (GF) was shown to be an effective minimally invasive skin test to monitor local inflammatory responses to bacterial cell structures, such as LPS (Erf & Ramachandran, 2016). Through the simultaneous injection of multiple GF on a bird, followed by periodic sampling of GF for laboratory analyses, we are able to examine leukocyte infiltration profiles and activities taking place *in vivo*. Because these leukocytes are recruited from the blood, changes in the blood cell profiles may be determined from concurrently sampled blood. Recently, one of the first studies using the "GF and blood dual-window approach" in broilers examined the acute inflammatory response to LPS injected into the pulp (French et al., 2020). For this study, blood and GF were sampled three times – before injection (0 h) and at 6 h and 24 h after LPS injection into GF pulps – to examine the acute phase of the inflammatory response to LPS (French et al., 2020). This study showed extensive recruitment of heterophils and monocytes/macrophages reaching peak levels at 6 and 24 h, respectively. Local cellular activities

included generation of reactive oxygen species (ROS), expression of inflammatory cytokines (e.g., interleukin-1 (IL-1), IL-6, IL-8, IL-10) and anti-oxidant enzyme activity (superoxide dismutase, SOD) (French et al., 2020). In blood, the concentration and proportions of heterophils were greatly elevated at 6 h and returned to baseline levels by 24 h, whereas levels of lymphocytes dropped at 6 h and returned to pre-injection levels by 24 h (French et al., 2020). With the successful adaptation of this two-window approach for use in broilers, the influence of environmental conditions or nutrition on innate immune function in broilers may be investigated.

Heat stress is a common environmental issue associated with broiler production, resulting in decreased feed intake and nutrient efficiency, as well as increased water intake (Ruff et al., 2020). Little is known about the effects of heat-stress on the innate immune system of broilers, other than decreased gut barrier functions – allowing for bacterial translocation (Campbell et al., 2019). Examination of the local and systemic inflammatory response to LPS, similar to the study conducted by French et al. (2020), will provide a pertinent, novel understanding of the impacts of heat stress on the acute inflammatory response of broilers.

We hypothesize that birds subjected to heat stress will exhibit altered inflammatory responses when compared to broilers reared under thermoneutral conditions.

Objectives

The following research objectives guided this study:

1. To establish whether there is a difference in blood- and GF-leukocyte profiles and activities in broilers reared under heat-stress compared to thermoneutral conditions.
2. To examine the local and systemic acute inflammatory response to LPS injected into the pulp of GF in broilers reared under heat-stress compared to thermoneutral conditions.

Materials and Methods

Experimental Animals and Rearing Conditions: For this study, a quantitative design was used in the form of a true experimental methodology. True experimental methodology involves using multiple created groups with similar measured outcomes where the individuals of each group are randomly assigned and not manipulated in any way (Gribbons & Herman, 1996). Newly hatched Cobb 500 broiler chicks will be tagged at hatch and randomly assigned, based upon their tag number, to two different temperature treatment groups (Table 1), thermoneutral or cyclic heat (TN or HS) (Gribbons & Herman, 1996). In total, eight environmental chambers will be used, 4 TN and 4 HS. Each chamber will be evenly split into two pens to produce 8 pens per treatment (16 pens total). This study will be carried out at the UA Poultry Environmental Research Laboratory (PERL) with University of Arkansas Institutional Animal Care and Use Committee (IACUC) approval for all protocols and procedures involving animals used in this trial. The temperature conditions for the broilers during the 42 D growing period for TN and HS conditions will be as described in Table 1.

Table 1. Experiment set-up including temperature, diet and light schedules[1].

Days	TN Temperature, °C	HS Temperature, °C 8 am - 10 PM	HS Temperature, °C 10 pm - 8 am
1 to 3	32	32	32
4 to 6	31	35	31
7 to 10	29	35	29
11 to 14	26	35	26
15 to 42	24	35	24

[1]Twenty three birds will be placed into each pen on wood shavings litter with a stocking density of 10 birds/m^2
Temperature scheduling subject to change based upon welfare and happiness of broilers during trial early on.
Temperature conditions based upon normal industry settings (TN) and industry settings experiencing environmental heat stress (HS).
Diet will consist of Rochell starter D 0-10, Rochell Grower D 11-28, and Rochell finisher D 28-42 for all treatments.
Lighting schedule of 24 h of light D 0-1; 23 h of light with 1 h of dark D 2-7; 20 h of light with 4 h of dark D 8-14; and 18 h of light with 6 h of dark D 15-42 for all treatments.

Experimental Induction of the Inflammatory Response: There will be four treatment groups each consisting of 8 broilers based on injection and temperature conditions: LPS-TN, phosphate-buffered-saline (PBS)-TN, LPS-HS, and PBS-HS. Two broilers will be randomly selected from each pen with one for LPS and one for PBS (vehicle) injection. When the broilers are 37 days of age, 6 GF from each breast tract will be injected with 10 µL of LPS (100 µg/mL of PBS) or 10 µL of PBS (French et al., 2020).

Pulp Sample Collection and Processing: GF (6) will be collected before (0 h) and at 6 and 24 h post GF pulp injection (French et al., 2020). Two of the GF will be used to prepare pulp cell suspensions for immunofluorescent staining and cell population analysis by flow cytometry (French et al., 2020). Cell populations will be identified using fluorescently labeled (FITC or PE) mouse monoclonal antibodies (mAb) for chicken leukocyte markers. Suspensions will be dual labeled for total leukocytes and macrophages using mAb CD45-PE and KUL01-FITC, respectively. A second dual labeling will be used for B and T cell determination using Bu-1-FITC and CD3-PE, respectively (French et al., 2020). Data will be expressed as percent of a leukocyte population in the pulp suspension. Using the pulp cell suspension, ROS generation shall be determined using a kinetic fluorescence assay using 2',7'-dicholorfluorescine-diacetate. (Rath et al., 1998). The remaining 4 GF will be placed in aluminum foil pouches and flash frozen in liquid nitrogen and stored at -80 °C for later cytokine analysis (IL-1, IL-6, IL-8 and IL-10) (French et al., 2020). The frozen GF will be equilibrated to room temperature to perform RNA isolation and synthesized into cDNA. cDNA will then be used in qPCR to analyze the relative expression of cytokine genes.

Blood Sample Collection and Processing: At each time point, 1 mL of blood will be collected from the wing vein using heparinized 3 mL syringes with 25-gauge x 1-inch needles (French et al., 2020). The blood will be used for preparation of Wright-stained blood smears to determine the proportions of lymphocytes, heterophils, monocytes, basophils, and eosinophils by microscopic evaluation of at least 300 white blood cells (WBC) per blood smear. Blood will also be used to isolate plasma to measure acute phase protein (Alpha-1-Acid Glycoprotein (AGP)) and inflammatory cytokine interleukin-1β by ELISA.

Statistical Analyses: Three-way repeated measures analysis of variance will be used to determine the effects of temperature (TN & HS), time (0, 6, & 24 h) and treatment (LPS & PBS) and their interactions followed by Tukey's multiple means comparison as appropriate. Statistical significance will be considered at $P \leq 0.05$.

Project Schedule

Late August 2022 through Mid-September– Reserving space within the PERL to perform the experiment and gaining approval from IACUC.

Mid-September 2022 through October – Performing necessary feed calculations for feed to be ordered from University of Arkansas Division of Agriculture (UADA) feed mill. Completing order forms for birds required for research project and UADA-IACUC forms. Begin growing birds mid-October.

November 2022 – Continue with research project, growing birds until the end of November, including daily animal care (i.e., feeding, checking waterlines, and manual thermostat operation to ensure proper temperature conditions); injection and sampling on D 37, 38 and 39 and running time-sensitive analyses. End trial end of November.

December 2022 through Mid-February – Complete analysis of acquired flow cytometry and ROS data and continue data collection from ELISA, differential leukocyte counts, and qPCR. Perform statistical analyses of the data and compiling results to be evaluated.

Mid-February 2022 through April – Finish writing Thesis, submit research project for publication and defend Thesis.

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