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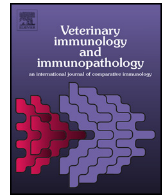

Effects of heat stress on the formation of splenic germinal centres and immunoglobulins in broilers infected by *Clostridium perfringens* type A

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ABSTRACT

Avian necrotic enteritis (NE) induced by *Clostridium perfringens* is a disease that affects mainly the first weeks of poultry's life. The pathogenesis of NE is complex and involves the combination of several factors, such as co-infection with different species of coccidia, immunosuppression and stress. Stress is one of the main limiting factors in poultry production. Although several studies emphasized the effects of stress on immunity, few works analyzed these effects on immunoglobulins and on germinal centres (GCs), which are specialized microenvironments, responsible for generating immune cells with high affinity antibodies and memory B-lymphocytes. Thus, the effects of heat stress associated or not with thioglycolate broth culture medium intake and/or *C. perfringens* infection on corticosterone serum levels, spleen GCs development and immunoglobulin production in broilers were evaluated. Results showed that heat stress, thioglycolate and *C. perfringens* per se increased corticosterone serum levels, although this was not observed in heat stressed and thioglycolate and *C. perfringens*-treated chickens. The serum levels of IgA, IgM and IgY were differently affected by heat stress and/or infection/thioglycolate. Heat stress decreased the duodenal concentrations of sIgA, which was accompanied by a reduction in GCs number in the duodenal lamina propria; a trend to similar findings of sIgA concentrations was observed in the chickens' jejunum. Changes in spleen and Bursa of Fabricius relative weights as well as in spleen morphometry were also noted in heat stressed animals, infected or not. Together, these data suggest that heat stress change GCs formation in chickens infected or not, which that may lead to failures in vaccination protocols as well as in the poultries' host resistance to infectious diseases during periods of exposure to heat stress.
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Abbreviations: Δ_2, nearest neighbour distance in the section plane; Δ_3, nearest neighbour distance in the space; Asp, spleen section area; Agc, area of the germinal centre in the spleen section; BHI, brain heart infusion agar; CP, crude protein; D, germinal centre diameter; ED, experimental day; f, tissue shrinkage factor; GALT, gut associated lymphoid tissue; GC, germinal centres; HE, hematoxilin and eosin; HPA, hypothalamic–pituitary–adrenal; k, correction factor; l, distance of points; Nagc, number of germinal centre per 1 mm^2 of spleen section; Ngc, number of germinal centres per spleen profile; Ngcsp, total number of germinal centre in the spleen; NE, necrotic enteritis; Nvgc, numerical density of the germinal centre in the spleen; PBS, phosphate saline buffer; Pgc, point falling on the germinal centre; Psp, point falling on the spleen section; sIgA, secretory immunoglobulin A; SNS, sympathetic nervous system; Vvgc, volume density of the germinal centre in the spleen; W, spleen weight.

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1. Introduction

Heat stress is one of the most common environmental stressors in the poultry industry. In birds, this condition increases corticosterone and catecholamine serum levels by activating the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS), respectively (Debut et al., 2005; Herman and Cullinan, 1997; Quinteiro-Filho et al., 2010). HPA axis and SNS activation then induce behavioural and immune changes that modulate humoral and other host responses to infections (Minton, 1994).

The spleen is an immune organ that presents several complex compartments, being responsible for the maintenance and production of host immune cells (Jeurissen, 1993). However, due to spleen complexity, refined analyses are required to determine the type of changes that have taken place in splenic compartments, such as in GCs (Jeurissen, 1993; Romppanen and Sorvari, 1981; Romppanen, 1981). Nevertheless, organ relative weights have been reported as being a rapid and relevant way to display immunological impairments, in spite of the fact that the complexity of such organs may not indicate the real immunological or cellular impairment present within the organ (Marsh et al., 1984; Walkden-Brown et al., 2013). Indeed, the spleen is able to contract and thus is directly influenced by the haemodynamic changes imposed by heat stress (Engan and Schagatay, 2015). To better understand the changes that occur in the spleen compartments, such as in GCs, a morphometric analysis with stereological calculation is necessary (Gil and Weibel, 1972).

Circulating and secretory antibodies prevent diseases; the production and maintenance of immunoglobulins rely on lymphoid compartments such as the splenic and mucosal GCs (Jeurissen, 1991). GCs are structures responsible for the maintenance of memory B-lymphocytes; furthermore, at these sites, the antibody isotype class switching takes place, an important component of the maintenance of the humoral response (Arakawa et al., 1996).

Previous studies have shown that heat stress reduces the relative weights of the lymphoid organs and modifies immunoglobulin production in birds (Franci et al., 1996; Quinteiro-Filho et al., 2010). Thus, lymphoid changes induced by stressful conditions might predispose an organism to enteric infections such as salmonellosis (Quinteiro-Filho et al., 2012).

The aim of this study was to analyse the effects of heat stress plus enteric irritation (thioglycolate broth medium) and/or enteric disease (*Clostridium perfringens* infection) on plasma corticosterone levels, serum and intestinal immunoglobulins and the morphometry of spleen GCs in broiler chickens.

2. Materials and methods

2.1. Animals

Male broiler chickens (Cobb®) were housed in isolator chambers of one square meter each (Alesco, São Paulo, Brazil) from the first day post hatching. The animals were kept in a 12/12 h light/dark cycle, with the lights on at 7:00 a.m. The chickens received water ad libitum and specific feeding management (described at the infection protocol). The animals were maintained and used in accordance with the protocol for the Care and Use of Laboratory Animal Resources, School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil (no. 2968/2012).

2.2. Group formation

On experimental day 1 (ED1) until the last experimental day (ED19), a total of 36 broiler chickens were randomly allocated into 6 different groups of 6 animals each: the control group (C): the thioglycolate broth culture medium feed group (T); the *C. perfringens* infected group (I); the control heat stressed group (C/HS35); the thioglycolate broth culture medium feed and heat stressed group (T/HS35) and the *C. perfringens* infected and heat stressed group (I/HS35). The same group formation was proposed by Calefi et al. (2014).

2.3. Heat stress protocol

Chickens of the heat-stressed groups (C/HS35, T/HS35 and I/HS35) were kept in an environmental temperature of $35 \pm 1\,^{\circ}C$ from ED14 to ED19. During this period, the birds of the non-heat stressed groups (N, T and I) were maintained at the recommended temperature for the broiler lineage.

2.4. C. perfringens *inoculum and infection protocol*

A pathogenic strain of *C. perfringens* type A was used (strain CP8.2, genotype α and Tpel toxins). This bacterial strain was kept in our laboratories in glycerol at $-80\,^{\circ}C$. The inoculum was prepared following the procedure described by Cooper and Songer (2010). Briefly, it was prepared by two alternate cultivations through thioglycolate broth culture media (Becton, Dickinson and Company, MD, USA) with 2% yeast extract (Becton, Dickinson and Company) and cooked meat medium (Becton, Dickinson and Company). The bacteria (1×10^{8} cfu/mL) were offered to the chickens in aluminium feeders, and the inoculum was discharged every day.

In the first 7 days of life, the chickens of all groups were fed with a poultry feed containing 24% crude protein (CP) without antimicrobials. From ED8 to ED14, the animals received a high protein concentration diet (28% CP) mixed with fishmeal (55% CP) in a ratio of 1:1 (v/v). From ED15 to ED19 the culture medium containing the pathogenic strain of *C. perfringens* type A (groups I and I/HS35) or the thioglycolate broth culture medium (groups T and T/HS35) were mixed into the feed in the proportion 1:1 (v/v) and given to the chickens (Cooper and Songer, 2010; Calefi et al., 2014). The birds of the control groups (C and C/HS35) were fed with commercial feed only with the same percentage of CP (24% and 28%) that the groups T, T/HS35, I and I/HS35 received. Infection occurs by spontaneous intake of food containing the culture medium plus *C. perfringens*. Birds of each group ingested the total inoculum in a period time of 6 h, after the feed were returned to the isolator chambers.

2.5. Bacterial identification

On ED19, the duodenum, jejunum and ileum of the birds were dissected; their contents were diluted in phosphate buffered saline (PBS, pH 7.2, 0.01 M) and plated afterwards on brain heart infusion medium (BHI) containing 0.5% yeast extract and 5% bovine citrated blood. The diluted content was incubated under anaerobic conditions at $37\,^{\circ}C$ for 48 h. After incubation, bacterial colonies were evaluated by the type of haemolysis they produced in the culture medium and by the colony morphology. Gram staining and breath tests were performed in the isolated colonies to confirm *C. perfringens* morphology.

2.6. Quantification of serum corticosterone

On ED19, blood samples were collected from the brachial veins of the animals of all groups, and the sera were obtained for hormonal quantification. The corticosterone levels were quantified by commercial ELISA kits (Assays Arbor, Michigan, USA) according to the manufacturer's instructions, preceded by standardized dilutions. Serum corticosterone was determined with the aid of a standard curve expressed in picograms of corticosterone per millil-

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