hsp70 expression on bursa of Fabricius of embryos submitted to intermittent and mild heat or cold stress and immune response post-hatching in broiler chickens.

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The aim of this work was to evaluate hsp70 expression on bursa of Fabricius of broiler chicken embryos submitted to intermittent mild heat or cold stress, as well as the immune response of birds after hatching. Three treatments were applied: control (setting at 37.5°C), intermittent mild heat (38.4°C) and cold (36.1°C) stress, for 4 hours a day, from embryonic day 13 (ED13) to ED19. Every experimental day, immediately after the stress exposure, bursae from 12 embryos were colleted and pooled in 4 replicates by treatment by day. After hatching, birds were raised under thermo neutral conditions and blood samples were taken to evaluate immune response against Newcastle Disease Virus (NDV). Data were analyzed in a completely randomized design, using SAS® General Linear Models procedure, arranged in a factorial scheme 3 x 7 (temperature x ED). There were no interactions between treatments and ED (P=0.2037). hsp70 presented differences (P=0.0281) between treatments: cold treatment reached the lowest protein level (7.38 ng hsp70·ug of total protein^{-1}) in comparison to control treatment (9.56 ng hsp70·ug of total protein^{-1}); and between age (P=0.0001): the greatest expression occurred at ED17 and ED18 (10.46 and 10.32 ng hsp70·ug of total protein^{-1}, respectively). Treatments by changing temperature during setting did not affect immune function in birds post-hatch (P=0.2365), although it was observed a decay on immune response from day 3 (1.788 S/P ratio) to day 15 (0.146 S/P ratio), probably due to serum neutralization, which was slightly restored at day 21 (0.932 S/P ratio; P<0.0001), characterizing the establishment of adaptive immunity. Thereby, cold stress induced a lower hsp70 expression on embryo's bursa of Fabricius, although the embryonic stress used in this assay wasn't enough to affect the immune response on adult birds.

Keywords: broiler chicken embryos; bursa of Fabricius; heat shock protein; immune response; intermittent and mild heat or cold stress

Introduction

Every organism respond to heat or stressful situations by inducing the synthesis of a protein group named heat shock proteins (hsps). This response is one of the most highly conserved known adaptive mechanisms and occurs in every being. Hsp induction is an emergence, rapid and intense response (PARSELL & LINDQUIST, 1994; KIANG & TSOKOS, 1998), it is essential for cell protection against damages caused by extremely high temperatures, repair or withdrawal of damaged structures and, also, for maintenance of cell structures during stress. These features allow the organisms to rapidly recover its normal functions after stress exposure (NAGAO *et al.*, 1990; MORIMOTO *et al.*, 1994).

Hsps have unique properties capable to generate specific immune responses against infectious agents and seem to be related to antigenic peptides and such association is related to a possible role in antigen presentation and processing, improving immune response (POLLA *et al.*, 1998; ANDERSON & SRIVASTAVA, 2000; WALLIN *et al*., 2002).

Stressing agents may alter immune function by different mechanisms, such as endocrine changes. Stress impact on immune response development depends on nature, intensity and duration of that, and it may lead to modulation of immune response, either by increasing or suppressing it (EL-LETHEY *et al.*, 2003).

The aim of this work was evaluate the mild intermittent heat or cold stress effect during the final third of incubation on hsp70 expression in bursa of Fabricius, as well as on the immune response of birds post-hatch.

Material and methods

Management and treatments during incubation

39 week-old Cobb-500® broiler breeder fertile eggs were used in artificial incubators (IP600, Premium Ecológica Ltda, Belo Horizonte, MG, Brazil). From embryonic day 1 (ED1) to ED12, eggs were kept at 37.5°C, 60% of relative humidity and turning every hour. At ED8, eggs were candled and dead embryos and clear eggs were discarded. Four hundred and fifty eggs with similar weights and divided into three treatments: control (settling temperature at 37.5°C, continuously), intermittent mild heat (38.4°C) and cold (36.1°C) stress, for 4 hours a day. Temperature treatments were applied from ED13 to ED19 and eggs were submitted to higher or lower temperatures for 4 hour each experimental day. Control eggs did not suffer any kind of stress. Heat or cold stress consistent on the conveyance of the eggs belonging to each of these groups to two other incubators adjusted to 38.4°C and 36.1°C, respectively. After 4 hours, heat and cold treatments eggs not used on sampling returned to the incubator kept at 37.5°C until the very next day, when they would be exposed to heat and cold temperatures once more. Thus, temperature treatment started at ED13 and repeated at ED14, ED15, ED16, ED17, ED18 and ED19, and the latest group suffered the treatment every experimental day, totalizing 28 hours of heat or cold cumulated exposure. At ED19, the remaining eggs returned to the control settler to hatch and to obtain hatchlings to be raised and further evaluate immune response.

Sampling

Immediately after stress, 12 eggs from each treatment were opened e embryos were killed by decapitation. Bursae were taken from these embryos, which were pooled (3 embryos/pool), totalizing 4 replicates by treatment by sampling day. Samples were immediately frozen in liquid nitrogen and kept under - 70°C until processing.

Total protein establishment

Bursae samples were homogenized by using lysis buffer (20 mM Tris-HCl; pH 7,5; 0,9% NaCl; 2 mM β-mercaptoethanol), 5 mL of buffer were used to each tissue gram, for 30 s in a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ, EUA), at 17000 *g*, followed by ice-bath intervals of 30 s. Lysate was centrifuged at 4700 g for 30 minutes at 4°C, the supernatant was transferred to microtubes and stored at -20°C. Protein concentration was determined in triplicates estimated from linear regression of the standard-curve values for each dosage, using bovine serum albumin 99%, fraction V (Sigma, Saint Louis, MO, EUA). Bio-Rad Protein Determination Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) was used, based on BRADFORD (1976) method, according to manufacturer instructions.

Electrophoresis in polyacrylamide gel containing SDS (SDS-PAGE) and Western blotting analysis

Electrophoresis was performed in 9% polyacrylamide gel containing sodium dodecil-sulfate (SDS) (SDS-PAGE, LAEMMLI, 1970), using the Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, EUA) at a constant voltage (100 V). Known quantities of total protein (30 µg) and a reference standard were loaded to the gels (GIVISIEZ *et al.*, 1999). Such reference standard was made from three embryos bursae sampled by ED13, one from each treatment. Before loading, samples kept at -20°C were boiled for 2 minutes and immediately placed on ice.

Western blotting was performed as described by Givisiez et al., 2001. Briefly, after fractionation through SDS-polyacrylamide gels, proteins were electrophoretically transferred to polivinylidene difluoride membranes (45 min at 4°C at 90°V) using Mini Trans-Blot cell (Bio-Rad Laboratories, Hercules, CA, EUA). Non-specific sites were blocked and blots were incubated with monoclonal anti-hsp70 antibody (H-5157, Sigma, Saint Louis, MO) at room temperature in 10 mL of saline phosphate buffer (PBS) with Tween 20 (0,02%) (PBS-T) and 5% non-fat dried milk (1:1000 dilution). After several washes with PBS, blots were then incubated with secondary anti-mouse antibody conjugated to alkaline phosphatase (A-3562, Sigma, Saint Louis, MO). After a new series of washes with PBS, color reaction was developed with nitro-blue tetrazolium (NBT; 50 mg/mL in dimetilformamide) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine (BCIP; 50 mg/mL in dimetilformamide 70%) in 10 mL of alkaline phosphatase buffer (Tris HCl, pH 9,5, 1M; NaCl 2M; $MgCl_2$ 1M). Color signal corresponding to hsp70 was analyzed densitometry at 525 nm (Shimadzu Corporation, Tokyo, Japan). Hsp70 was quantitated according to Givisiez et al., (1999). Briefly, standard curves for hsp70 were constructed using purified hsp70 (H-9776, Sigma, Saint Louis, MO, EUA) and blotted as described. Ratio between samples and reference standard in each membrane was used to determine hsp70 quantity in supernatant. Data were expressed as ng hsp70·µg total protein^{-1}.

Bird management

After hatching, 30 chicks from each treatment were raised for 3 weeks, placed in 3 pens of a climatic chamber with wood shavings. Water and diets were given *ad libitum*. Crude protein and metabolizable energy levels in the starter diet were 21.50% crude protein and 3,200 kcal metabolizable energy·kg^{-1}. Birds were kept at thermoneutral temperature (35°C, 32°C e 28°C during first, second and third week, respectively). At day 7, birds were vaccinated against Newcastle Disease Virus (New Vac-LS, La Sota Tipo B1, Fort Dodge).

Breeding sampling and serology

Blood samples were taken by jugular vein puncture at day 3, 7, 15 and 21, of six birds per treatment, randomly. Blood were kept at 37°C for one hour, then it was centrifuged at 3500 g for 30 min and serum obtained was transferred to microtubes and stored at -20°C until serology analysis.

Anti-Newcastle antibody presence in serum was evaluated by ELISA against Newcastle Disease Virus antigen, using a commercial kit (FlockCheck, IDEXX Laboratories, Westbrooke, EUA). Results were expressed as S/P ratio.

Statistical analysis

Hsp70 and S/P ratio were analyzed in a completely randomized design, in a 3 x 7 (three incubation temperature x seven embryonic days) arrangement. The analysis of variance was performed by using General Linear Models procedure of the SAS® (LITTELL et al., 2002), and differences between means were evaluated by Turkey's test ($P \leq 0.05$). Polynomial regressions of the embryonic days and age post-hatch were calculated for each treatment.

Results and discussion

There was no interaction between embryonic day and mild temperature treatments to hsp70 levels (P=0.2037). Mild cold stress induced to a lower hsp70 expression (P=0.0281) (*Figure 1*) in comparison to control treatment, and heat exposure causes a similar hsp70 expression to the other treatment.

Although there are no records so far of a quantitative study of hsp70 expression in the bursa of Fabricius, KAWAZOE et al. (1999) demonstrated that this organ expresses the referred protein in a constitutive and induced by shifting up temperature manner at ED12. Cold stress for 4 to 6 hours at days ED13, ED16 and ED19 carried to a lower hsp70 expression in lungs at ED13 and ED19, and in heart at ED13, while the same exposure range to heat stress induced to a higher expression of this protein in heart at ED 13 and ED19 and in lungs at ED19 (LEANDRO *et al*., 2004). No matter what kind of stress was applied, hsp70 concentrations up to 5 times higher than the other tissues (heart, liver, breast muscle and lungs) were obtained. GIVISIEZ *et al.* (2001) found similar results for hsp70 expression in embryos brain at ED19 and ED20, when studying the heat or cold chronic embryonic stress effects.

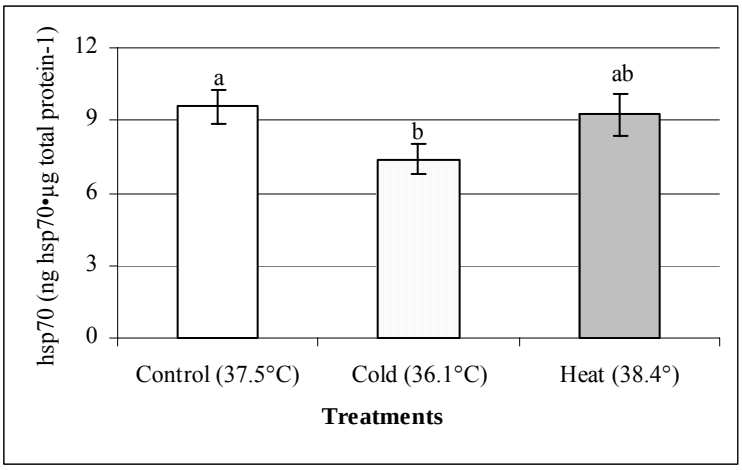

Figure 1 hsp70 levels means ± standard errors in bursa of Fabricius of broiler chickens embryos submitted to mild intermittent embryonic stress, from ED13 to ED19 (*P*=0,0281).

There was a raise (*P*<0.0001) in hsp70 expression between the beginning and the end of temperature treatments (*Figure 2*). Polynomial regression analysis for embryonic day indicates that hsp70 expression increased through days (Y_{hsp70} = -0.2794 (embryonic day)^2 + 9.6421 (embryonic day) – 72.698; R^2 = 0.99), and the highest levels of this protein were reached at ED17, followed by a decay on remaining days. This expression pattern differs from the one observed by LEANDRO *et al*. (2004), whom described higher hsp70 levels in precocious stages of embryonic development.

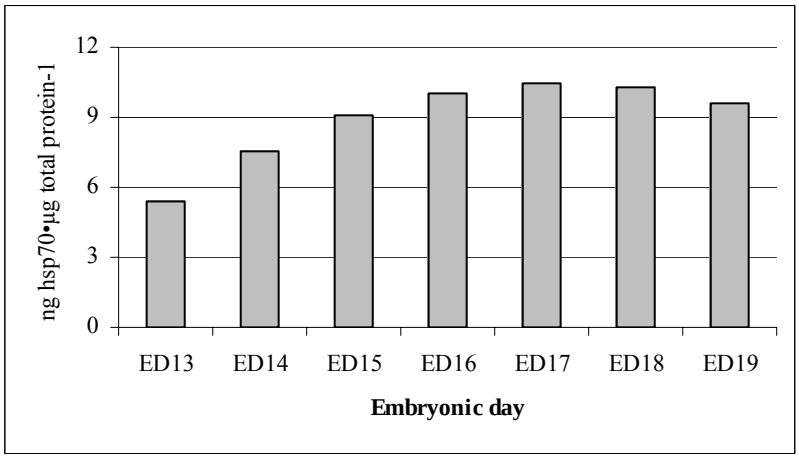

Figure 2 Hsp70 expression adjusted means in bursa of Fabricius of broiler chickens embryos submitted to mild intermittent embryonic stress, from ED13 to ED19 to embryonic day effect (*P*<0,0001).

No interaction was found between treatments and ED for S/P ratio (*P*=0.2086). Treatment effects on S/P ratio were not observed (*P*=0.2365), although birds belonging to cold or heat treated groups during embryonic period showed a slightly improvement when compared to control treatment. There was a gradual decrease (*P*<0.0001) from day 3 to day 15, followed by a sudden recovery at day 21 (*Figure 3*), described by the equation: $Y_{S/P}$ = 0.0123 (age)^2 – 0.3526 (age) + 2.8573; R^2 =0.9229.

S/P ratio for age effect indicates a decrease on innate immunity as age increases up to day 15, and at day 21 it is observed an immunity recovery, most likely due to vaccination performed at day 7. The decrease noticed between days 7 to 15 is possibly due to the competition of the vaccine virus by maternal antibodies and also the initiation of adaptive immune response.

Short length cold stress in different layer hens lines 5 week-old induced to an increase on phagocytic activity as well as innate humoral immunity, without changing adaptive humoral immunity, though (HANGALAPURA *et al*., 2003). However, in a further study, a longer exposition of birds to low temperature did not affect immune response (HANGALAPURA *et al*., 2004b).

Using cyclic and heat stress o layer hens, MASHALY *et al*. (2004) verified a significant decrease humoral immune response in the heat stressed group and attributed that to a series of events, which

initiates with the increase of inflammatory cytokines production , these stimulates corticotrophin releasing hormone (CRH) secretion, that stimulates corticosterone synthesis, culminating in antibodies production depression.

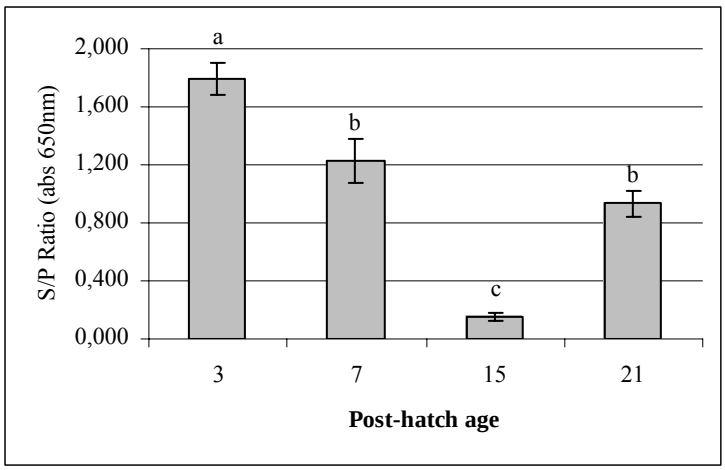

Figure 3 S/P ratio means ± standard error (absorbance at 650 nm) in serum of broiler chickens embryos submitted to mild intermittent embryonic stress, from ED13 to ED19, raised in thermoneutral temperature and vaccinated at day 7 against Newcastle Disease Virus, to age effect (*P*<0,0001).

It was observed that the conditions adopted in this study haven't been stressful enough to incite an evident immune response on birds post-hatch, and that antibodies levels at day 21 returned to elevated values of S/P ratio. Thus, stress conditions during incubation do not seem to exert deleterious effects on birds' immune response.

The results suggest that cold mild intermittent stress induced a lower hsp70 expression in comparison to control treatment, whilst heat or cold stress were not enough to alter immune response in birds post-hatch.

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