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ORIGINAL PAPER

Development of broiler chickens after treatment with thymulin 5cH: a zoo technical approach

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Modulation of immune response due to thymulin 5cH has been previously observed. The aim of the present study is to evaluate the development of broiler chickens treated with thymulin 5cH by conventional zoo technical indices, phytohemaglutinin induced inflammation test and histomorphometric analysis of lymphoid organs (thymus, Fabricius bursa and spleen). Animals were divided in two groups: (a) test: birds with free access to thymulin 5cH diluted into the drink water and (b) control: birds with free access to water only, from the 1st to the 42nd day of life. All experimental procedures were done in blind. The results show that thymulin 5cH treated group had increased productivity index compared to control (391.45 *versus* 261.93) associated with higher viability in the 7th week ($p = 0.013$), and a possible shunt to B lymphocyte activity. The data suggest that thymulin 5cH could be a viable method to improve productivity in poultry production due to its immune modulation properties. *Homeopathy* (2012) **101**, 68–73.

Keywords: Thymus hormones; Homeopathy; *Gallus gallus*; Poultry; Immune system

Introduction

The lack of animal protein availability to a significant proportion of the world's population has forced the adoption of techniques and procedures in increased meat production, with reduced costs and time. Birds are very efficient in converting vegetable products into protein.[1] Brazil is a major producer and exporter of poultry meat. Broiler hens are bred in confinement, at high concentration, with high level of stress and high probability of infectious diseases. For these reasons, growth promoters and antibiotics are used to prevent disease and improve productivity, reducing the feeding time up to slaughter.[2] Modern technologies, however, involve chemical substances during all phases of production, inducing resistant microorganisms. Some consumption markets, such as Europe, have increased the search for alternative organic products. In veterinary medicine, the use of homeopathic medicines added to water, food or semen represents a potential zoo technical resource, mainly for treatment of animals in big farms.[3–7]

In previous studies, the use of bovine thymus extract in broilers produced modulation of humoral and cell immune response after vaccination against Newcastle disease.[8] Thymulin is an important component of these extracts due to its role in lymphocyte maturation. It is a zinc dependent nonapeptide (GLP–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH) produced by thymus epithelial cells.[9] Several studies have demonstrated the usefulness of thymulin in high and low levels in immunity control in birds.[10–23]

In this work, the aim was to evaluate the effect of treatment with thymulin 5cH on the development of commercial chicken broilers, using the following parameters:

(a) Conventional zoo technical indices (alimentary conversion, weight gain, weight average, mortality, viability and productive efficiency index – PEI).
(b) Intensity of inflammation (local immune cell reaction) induced by phytohemaglutinin.
(c) Gross pathology and histopathology of lymphoid organs, including the thymus, spleen and bursa of Fabricius.

Material and methods

Animals and treatment

The study was performed in the experimental unit of a commercial farm specialized in chicken breeding (*Granja Santana*), in Tietê, São Paulo state, Brazil. One-day old

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Gallus gallus Cobb birds, of both genders, were randomized before the beginning of the study. Each bird was identified by a plastic ring with a number. These rings were changed weekly, according to animal growth. The birds were immunized against Marek and Gumboro diseases (Ceva®, France). 72 birds were maintained in two brick barns, with impermeable flooring and roof. Lateral protections consisted of wire screens and rolling doors. Each barn measured 3 square meters and was equipped with poultry drinkers, manual feeders and gas heating. The chicken barns were disinfected by spray of quaternary ammonia and glutaraldehyde. The floor was covered with a 10 cm layer of *pinus* powder. One-day old chickens were housed at the usual commercial density of 12 birds per square meter.

The birds received food (Flamboyant®, Brazil) and tap water '*ad libitum*'. Food was made from corn and soybean, with protein content adjustment according to the growth phase of birds (from 23 to 18%). No additive or growth promoter was used. All water offered was supplied from a 50 L reservoir which was filled regularly when the water volume reached 10%. The water supply was the same for both barns; water purity and mineral standards were controlled by a governmental agency (Companhia de Saneamento Básico do Estado de São Paulo - SABESP).

The treatment consisted of adding thymulin 5cH or vehicle (70% ethanol) to the respective reservoir, blind, using the codes 'A' or 'B'. These codes were broken only after statistical analysis. Thymulin 5cH was prepared in distilled water on the day of use, according to the Brazilian Homeopathic pharmacopeia (2nd Edition), from the commercial Thymulin 4cH (Boiron®, France). According to information obtained from the supplier, the Thymulin 4cH is prepared from 98.66% of pure peptide solution, without coupled zinc. Additional chemical analysis (CBO analyses − Brazil) revealed that no Zinc was present in the 5cH vial. Thus, the mother solution was the pre-thymulin peptide. Control was prepared by the same method from the same vehicle. The entire content of the flasks (20 mL) was added to the respective reservoirs every time they were filled. The final expected concentration was 4×10^{-14} M, according to the technical information supplied by the manufacturer.

Zoo technical indices

During the experiment, birds were weighed and food consumption was calculated at 1, 7, 14, 21, 28, 35, 42 days, and just before slaughter (46 days). Weight gain, feed conversion, mean weight, mortality and viability were calculated for both groups. The PEI was calculated by the following formula:[24]

$$\text{PEI} = [(\text{weight gain} \times \text{viability}/\text{age at slaughter} \times \text{feed conversion})] \times 100$$

The PEI calculation was performed twice by two different observers.

Local cellular immunity evaluation

At the age of 45 days, the end of experimental period, local cell immunity was evaluated using the *cutaneous basophilic response method*,[25–27] using a subcutaneous injection of phytohemaglutinin (Cultilab®, Brazil). The inter-digital skin test (between the 3rd and 4th right digits) was performed by measuring just before the injection of 0.1 mL of sterile 1% phytohemaglutinin. The inter-digital membrane thickness of the corresponding part of the left foot was also measured and inoculated with 0.1 mL of sterile NaCl 0.9% solution. A digital micrometer (Mitutoyo®, Brazil) was used for these measurements. After 12 h, another inter-digital skin test measurement was made and the difference between the last and the first measurement was calculated, considering each foot side separately. The difference between the right foot thickness and the left foot was considered as the basophilic specific cell reaction for each animal.

Necropsy and anatomopathologic examination of lymphoid organs

At 46 days of age, birds were killed by cervical dislocation and were submitted to necropsy for macroscopic evaluation of lymphoid organs. From each bird, thymus, spleen and Bursa of Fabricius bursa were analyzed considering their general appearance, size and color. Representative fragments were harvested, labelled and fixed in 10% formaldehyde for about 72 h, for subsequent histopathological examination. Slides were viewed with an optical microscope (1000×) and digital photomicrographs were taken with additional magnification of 4.8×. Histometric evaluation was performed using the software Image Tool 3.0 for Windows. To evaluate the inter-digital skin inflammation, the number of inflammatory cells per 5 randomly chosen fields was counted, per slide. For the lymphoid organs, the diameters of lymphoid follicles were measured in spleen and Bursa of Fabricius. Five randomly chosen follicles per slide were analyzed. Thymus was analyzed in a magnification of 400× for qualitative − descriptive evaluation, since the limits of its structures − medulla and cortex − were not defined enough to allow quantitative analysis.

Statistical analysis

The methods used were Student '*t*' test for parametric variables and Mann−Whitney test for non-parametric variables. Viability was analyzed by a contingency table and the Fisher's test was used in this case. The values of $p \leq 0.05$ were considered significant. All statistical analysis was done using the software INSTAT for Windows 3.

Bioethics

This study was approved by the Bioethical committee of Universidade Paulista (UNIP) − CEP/ICS/UNIP protocol 006/07 − and was conducted according to the Brazilian laws for animal use in scientific procedures.

Results

Weight gain

The females from the group treated with thymulin 5cH had a higher weight gain than females in the control group,

considering the whole experimental period (from 1 to 46 days old). On the other hand, males from thymulin 5cH treated group had lower weight gain in relation to control, except in the last week before slaughter, when the ratio was inverted (Figure 1A and B).

Viability

In the last week before the slaughter, 9/36 birds from control group died (mortality 25%), eight males (22.2%) and one female (2.7%). In the control group, 3 birds had clinical signs of arthritis, two males and one female. The histopathological analysis of affected joint revealed inflammatory mononuclear cell infiltrate at the tendon insertion, typical of viral arthritis lesions. In the group treated with thymulin 5cH, only one male bird died (2.7%), without previous clinical signs (Table 1). No clinical sign of arthritis was observed in thymulin 5cH treated animals.

Macroscopic and microscopic evaluation of lymphoid organs

No gross alteration was seen in thymus, spleen or Bursa of Fabricius, in relation to color, size and consistency patterns. No anatomic and histological alteration was observed in the thymus of both groups. The control group had an increase of inter-digital skin thickness of 68.54%, and 107.97 ± 32.22 inflammatory cells per field. The thymulin 5cH treated group had a mean 61.28% increase of inter-digital skin thickness (p = 0.03), the number of inflammatory cells per field was 81.60 ± 14.90 (p = 0.005) (Table 1).

Thymulin 5cH treated birds had greater average of diameter of germinative follicular center in the spleen (p = 0.0001), compared to controls (Table 1). The microscopic analysis also revealed higher cellularity in spleen follicles of treated birds (Figure 2A and B). However, no significant difference in follicle diameter in the Bursa of Fabricius between groups was seen (Table 1).

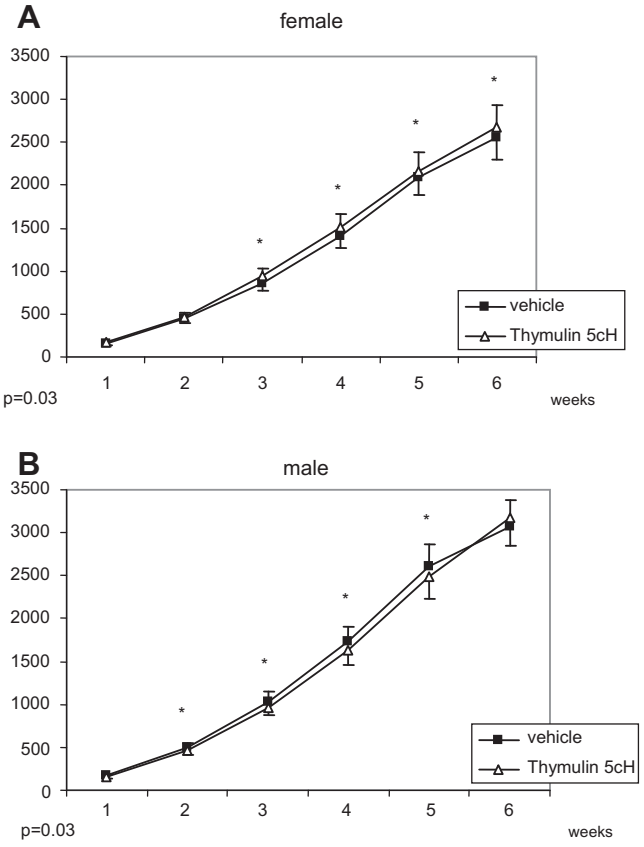

Figure 1 Cumulative weekly weight gain (grams) in females (A) and males (B) of control group and group treated with thymulin 5cH in drinking water (*ad libitum*), from the first day of life up to slaughter. *p = 0.03, in relation to control. Mann–Whitney test.

Table 1 Parameters of productivity and lymphoid response in broilers administered thymulin 5cH, *ad libitum*, in drinking water from the first day of life up to slaughter *versus* controls. Levels of significance in relation to control: (a) p = 0.013, Fisher test; (b) p = 0.03, Mann–Whitney; (c) p = 0.005, Student '*t*' test; (d) p = 0.0001, Student '*t*' test. Values inferior to p = 0.05 were considered statistically significant

Parameter	*Control*	*Thymulin 5cH*
PEI	261.93	391.45
Viability	75%	97.3% (a)
Inter-digital skin growth (*)	68.54%	61.28% (b)
Inter-digital number of basophiles per field (*)	107.97 ± 32.22	81.60 ± 14.90 (c)
Average of follicle diameter (spleen) #	383.14 ± 37.89	470.44 ± 52.02 (d)
Average of follicle diameter (Bursa of Fabrícius) #	684.67 ± 144.30	766.38 ± 131.67

(*) Subcutaneous tissue inoculated with phytohemaglutinin.
(#) Number of pixels.

PEI

The PEI in the last week was 261.93 for the control group and 391.45 for the thymulin 5cH treated group (Table 1).

Discussion

The breeding of broilers at high population density is a widespread practice around the world with the aim of reducing production costs. The impact of this practice upon animal welfare is great, not only because of animal density itself, but due to the high concentration of fecal matter and ammonia and competition for space. The chronic stress can produce an negative effect on profitability and quality of products offered for consumers.[28]

Ferket[29] concluded that approximately 70% of reduction in performance of chickens under immune stress is due to reduction of food consumption and 30% due to the lack of optimal absorption of nutrients. The excess of light exposure, mainly during the first week, also causes immunological distress. In 2000, Molinero *et al.*[30] showed that birds continuously exposed to light have decreased of thymic peptide concentration, including thymulin. The excess of blood corticosteroids also is an important immune suppressive factor, causing inhibition of bone growth.[31] Even after the correction of corticosteroid levels, the number of NK cells in the blood is maintained low for a certain period.[32]

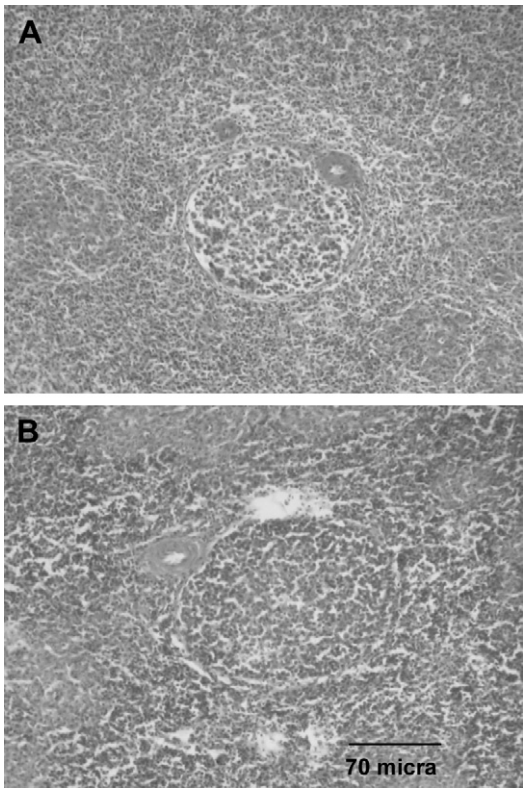

Figure 2 Photomicrography of spleen lymphoid follicle in a control broiler (A) and in a broiler treated with thymulin 5cH in the drinking water (*ad libitum*) from the first day of life up to slaughter (B). Observe differences in cellularity of both follicles.

In the last week before slaughter the 25% of mortality of males in control group was associated with specific arthritis symptoms, such as ventral decumbency, difficulty standing and extreme difficulty of locomotion. Histopathological analysis of joints from sick birds revealed lesions compatible with reovirus infection, such as mononuclear inflammatory infiltrate in the tendon attachment region. There was a history of avian reovirus at the farm. Birds treated with thymulin 5cH remained healthy, without clinical signs or histopathological changes. This improvement of pre-slaughter viability and welfare had a positive impact on PEI (261.93, in the control group *versus* 391.45, in thymulin group), which represents a useful productivity gain for farmers. Viral diseases that induce immune depression in broilers are bursal infectious disease, avian infectious anemia, reovirus disease, Marek disease, lymphoid leucosis and reticuloendotheliosis.[33–35] Reovirus is particularly widely disseminated throughout the world. Its structural characteristics permits its survival in the environment, in which its transmission can be horizontal as well as vertical.[36] These viruses are causes of arthritis or synovitis, mainly in leg articulations. Sick birds have difficulty of locomotion, with impact on feed conversion, weight gain and viability.[37]

Oliver and Marsh[14] observed that thymulin in pharmacological concentrations increases NK cell activity in response to bronchitis virus infection. Other work demonstrates the usefulness of pharmacological thymulin in avian immune control,[11–13] suggesting its utilization as a zoo technical resources in stress – immune depressed animals. However, few studies about homeopathic thymulin preparation are available in the literature. In this study, thymulin 5cH treated birds had higher weight gain in females, compared to control. Although in much higher dilutions, the results are in agreement with the findings described by Barbour *et al.* in 2001,[10] in which chickens treated with small amounts of thymulin (10 ng) presented higher titer of antibodies against infections bronchitis virus and Newcastle disease virus. The action of high diluted homeopathic preparations of thymulin and its immune modulation effects in laboratory rodents were studied in the 1980s,[17,18] but little has been published since. The present work contributes to this neglected but promising field.

Although Thymulin 5cH was prepared from a pure peptide solution (98.66%), as related by the supplier, an additional chemical analysis of Thymulin 5cH, Thymulin 5cH incubated in Chelex 100® (SIGMA)[38] and the vehicle itself was performed by a certified laboratory (data not shown). It revealed the presence of only small traces of Zinc in all flasks, 0.05 μg/mL, even in that treated with Chelex 100®. This represents less than 0.001 ppm (w/v) after the final dilution in the drinking water: much less than the 40 ppm necessary to induce some pharmacological immune modulation in broilers, as a trace mineral itself.[39] Thus, the difference of effects between both groups observed herein cannot be attributed to this mild and equal inorganic zinc contamination of samples; instead, the very small amounts of pre-thymulin peptide present in water – if one consider the possibility of molecular non-homeopathic activity – could be linked to the zinc present in diet, at the moment of feeding by the experimental birds. To better define which mechanism is involved in these findings, further experiments using larger number of animals and higher homeopathic pre-thymulin dilutions will be the next step.

The inter-digital hemaglutinin test was used to evaluate the T-cell response associated with basophilic cutaneous reaction. It is important to consider that the kinetics and dynamics of basophils in chickens are completely different from those of mammals, these cells have phagocytic properties not found in rodents.[40] This is a classical method to evaluate the activity of T lymphocyte in chickens,[25–27,40,41] since specific monoclonal antibodies subsets against chicken immune cells are available in the market, impairing the use of more specific methods of immune cell counting such as flow cytometry and immuno-histochemistry. The subcutaneous injection of phytohemaglutinin leads to the increase of local skin thickness, peaking at 12 h post challenge. Basophils migrate to the site, following chemotaxis induced by T-cell cytokines and inducing vascular reactivity, through secretion of histamine and serotonin. Thus, the intensity of cutaneous hypersensitivity reaction is an indirect and specific tool to measure T-cell activity.[42]

The reduction of inter-digital skin thickness in thymulin 5cH treated birds was accompanied by reduction of cell migration in the inoculation site. This result agrees with that obtained by Bastide *et al.*,[17,43] in which high diluted

thymulin decreased thymus-dependent lymphocyte response in new zeland black mice (NZB). In addition, the anti-inflammatory effect of pharmacological thymulin is also known.[44–46] No qualitative alteration in thymus histology could be seen in treated animals, but the germinative center diameter in spleen follicles was statistically bigger in treated birds. In previous studies, the treatment of mice with thymulin 5cH was able to modulate cellularity of lymphoid follicles stimulated by Bacillus Calmette-Guérin (BCG).[47] Although the germinative centers of spleen follicles are composed mainly of B cells, their development is T-cell dependent.[48,49] The Bursal follicles diameters were not significantly different between groups, although a clear tendency of increase was seen in treated group. On the other hand, the presence of phagocytes in cutaneous and joint lesions can represent an important focus in antigen presentation, which can impact the systemic immune reaction as a whole.[50] Further studies performed in mice using more specific immunological methods, such as flow cytometry, could explain better this mechanism.

These data, taken together, suggest that thymulin 5cH offered in water to broiler chickens is able to improve zoo technical parameters (PEI), quality of health and viability, mainly during the last week before slaughter. These effects are probably related to immune modulation of anti-virus response, but further specific studies are needed.

Conflict of interest

There is no conflict of interest regarding this study.

Acknowledgements

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