Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Oct - Dec 2012/ v.14 / n.4 / 233-304

Effects of Levamisole Hydrochloride on Cellular Immune Response and Flock Performance of Commercial Broilers

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■Keywords

Broilers, Cellular immunity, Delayed wattle reaction, Immunocompromised, levamisole hydrochloride.

Submitted: March/2011
Approved: August/2012

ABSTRACT

Levamisole hydrochloride (Lev.HCl) has been acclaimed to boost immune response particularly in immunocompromised state. Its routine use as an immunomodulator in poultry production is yet to be well embraced, thus its effects of on cellular immunity and flock performance of commercial broilers were evaluated.

One hundred and fifty Anak broiler chicks were separated into two groups of 75 each. Broilers in group 1 were sensitized with 150µg of Staphylococcus aureus antigen each at 4 and 5 weeks, while those in group 2 were not sensitized. Each group was further divided into subgroups A, B, and C. Levamisole hydrochloride (40 mg/kg) was administered orally to 1A and 2A at 45 and 46 days of age and to 1B and 2B at 47 and 48 days of age, while 1C and 2C were not treated. At 47 days of age, 12 broilers from all subgroups were challenged with 75µg of S. aureus antigen each at the right wattle. Wattle thickness was measured till 72 hours post challenge (pc) and delayed wattle reaction (DWR) was determined. Tissues were harvested at 72 hours pc for histopathology. Morbidity, mortality and live weights at 8 weeks of age were recorded. DWR peaked at 4 hours pc in 1A (2.22 ± 0.21 mm) and 1B (2.96 ± 0.21 mm) and 24 hours pc in 1C (3.39 ± 0.34 mm), the difference being significant ($p<0.05$). Inflammatory lesions were observed in wattles of sensitized subgroups and were more severe in 1C. Mortality rates were 4.17% and 29.17% in 1A and 1C respectively. Mean live weights in A and B i.e. 1.57± 0.06 kg and 1.56 ± 0.06 kg respectively, were significantly higher ($p<0.0$) than 1.43 ± 0.08 kg in C. Levamisole enhanced DTH via an early response, improved broiler liveability, and its anti-inflammatory property was confirmed.

INTRODUCTION

Immunomodulators are substances that are able to regulate or modulate immune responses. As the term implies, immunomodulators may either augment or suppress immune response, though the term is often used to refer to substances that enhance the immune response. Other synonyms for immunomodulators include immunostimulants, immunopotentiators and biological response modifiers (Blecha, 1988). The use of immunomodulators has increased, particularly in poultry production around the world (Porchezhian *et al*., 2006), although they are not routinely applied. It is known that the present day poultry are being subjected to a variety of stress factors especially in the tropics, e.g., where there are harsh environmental conditions, poor nutrition and diseases, such as infectious bursal disease (IBD) and chicken infectious anemia (CIA). This makes birds susceptible to several other diseases, such as coccidiosis, salmonellosis, Newcastle disease, staphyloccocosis, Marek's disease, etc., and weakens or suppresses their immune response

(Calnek *et al*., 1997; Oladele *et al*., 2005). Microbes like bacteria, fungi, viruses, protozoa and multi-cellular parasites are prevalent in our environment and can cause disease if they multiply unchecked. The host defense against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity (Abbas *et al*., 2005). The innate immune response is designed to rapidly alert the host of the presence of an invasive microbial pathogen that has breached the integument of multi-cellular eukaryotes (Opal *et al*., 2003). It is able to eradicate microbial pathogens by activation of specific elements of adaptive immune response, i.e. cell mediated and humoral immunity via T and B cells, respectively. The immune system can be overwhelmed by aforementioned factors; hence, the need for routine usage of immunomodulators (Chawak *et al*., 1993; Karnatak *et al*., 1993; Porchezhian and Punniamurthy, 2006).

Levamisole, an imidazole-thiazole group derivate, is an effective and safe broad spectrum anthelmintic commonly used in veterinary and human medicine (Panigraphy *et al*., 1979). Renoux and Renoux (1971) were the first to report the immunostimulatory capabilities of this drug followed by its use in various disease cases in 1972 when its immunomodulatory effect was discovered in immunosuppressed man and animals (Panigraphy *et al*., 1979; Garszon *et al*., 1992; Holcombe *et al*., 1998). Earlier studies have shown that levamisole is able to enhance both humoral and cellular immune responses in normal chickens (Soppi *et al*., 1979). Its immunomodulatory property was later substantiated in diseased and stressed birds (Giambrone, 1982; Porchezhian *et al*., 2006; Emikpe *et al*., 2010). The mode of action of levamisole is largely unknown, but it has been used to boost immunity in infectious diseases, leprosy and cancer in humans (Kar *et al*., 1986; Mutch *et al*., 1991; Katoch, 1996; Szeto *et al*., 2000). Symoens and Rosenthal (1977) summarized levamisole as a drug that enhances the immune response by restoring phagocyte and T-lymphocyte functions in immunodeficient hosts, but does not increase immune response above normal level in the immunologically competent host. They also described it as a drug found to increase the protective effects of some vaccines and its potential advantage in various chronic and recurrent infections, immunodeficient conditions and neoplastic diseases in man and animals (Panigraphy *et al*., 1979). Although various avian species have been reported to respond to the immunomodulatory effect of levamisole, most of the studies in domestic poultry involved the immunomodulatory effect of the simultaneous administration of levamisole and various vaccine types (Kulharni *et al*., 1973; Porchezhian and Punniamurthy, 2006; Sanda *et al*., 2008). A study conducted by Sanda *et al*. (2008) on the immunomodulatory property of levamisole in cockerels in a tropical environment showed that it was not an efficient immunomodulator; however, another study by Emikpe *et al*. (2010) showed that levamisole enhanced humoral immune response in chemically-immunosuppressed broilers. Further investigation to ascertain the effect of levamisole on immune response is therefore imperative. Its effect on cellular immunity of commercial broilers was evaluated via the assessment of delayed-type hypersensitivity reaction to investigate its immunomodulatory property and possibly provide an insight into this mode of action.

MATERIALS AND METHODS

Experimental Broilers

One hundred and fifty one-day-old Anak broiler chicks were purchased from a commercial hatchery in Ibadan, Nigeria, and were reared in the experimental animal unit of the Department of Veterinary Medicine, University of Ibadan following institutional guidelines. Feed and water were made available to them ad-libitum. The chicks were fed broiler starter from day 0 to 4 weeks and broiler finisher from 4 to 8 weeks of age. The starter feed contained 2873.87 kcal/kg energy, 22.65% protein, 6.12% ash, and 10.35% moisture, while the finisher diet contained 2645.6 kcal/kg energy, 20.35% protein, 7.64% ash, and 9.09% moisture.

Newcastle disease vaccine (NDLS-vac, Tarobina Corporation, Lahore, Pakistan) was administered at days 10 and 31 of age, and infectious bursal disease vaccine (IBD-vac, Tarobina Corporation, Lahore, Pakistan) was administered at days 14 and 28 of age. Mild outbreaks of coccidiosis, chronic respiratory disease and fowl typhoid were treated with Pluricoccin (sulfaquinoxaline, pyrimethamine – Industrial Veterinaria, Barcelona, Spain), tylosin tartrate (Mobedco-Vet, Irbid, Jordan) and norfloxacin (Pantex Holland B.V., Duizel, Holland), respectively.

Sensitization of Broilers for Delayed-Type Hypersensitivity (DTH) reaction

Broiler chickens were divided into two groups of 75 broilers each (Groups 1 and 2) at 29 days of age (Table 1). Each broiler in group 1 was sensitized by administering 150ug *Staphylococcus aureus* antigen mixed 1:1 with PEG to 0.2ml, subcutaneously at the neck region, while broilers in Group 2 were administered 0.2ml PEG

Table 1 – Groups and subgroups of experimental broilers sensitized and challenged with *Staphylococcus aureus* antigen with and without administration of Levamisole hydrochloride

Total	150 commercial broilers (Day-old)																	
Sensitization (29 & 36 days-old)	75 broilers (Group 1) Sensitized							75 broilers (Group 2) Unsensitized Control										
Lev. HCl.	1A (25)* 45 and 46 day-old		1B (25) 47and 48 day-old		1C (25) Control			2A (25) 45 and 46 day-old		2B (25) 47and 48 day-old				2C (25) Control				
Challenge (47 day-old)	12 +	13 -	12 +	13 -	12 +	13 -		12 +	13 -	12 +	13 -		12 +	13 -		12 +	13 -	

*Number of broilers in subgroups; +Challenged; -Unchallenged

only per bird (i.e. unsensitized). On day 36, the above procedure was repeated and on day 40, broilers in each group were further divided into three subgroups of 25 birds each (1A, 1B, 1C and 2A, 2B, 2C).

Elicitation of Delayed Wattle Reaction

Levamisole hydrochloride (Lev.HCl) manufactured by Pantex Holland B.V., Duizel, Holland, was administered in the drinking water at a dosage of 40 mg/kg body weight to broilers in subgroups 1A and 2A at 45 and 46 days of age (i.e., 24 and 48 hours before being challenged for DTH at 47 days of age). Subgroups 1B and 2B were also administered Lev.HCl at 47 and 48 days of age (i.e., simultaneous with challenge at 47 days), while subgroups 1C and 2C did not receive Lev.HCl.

At 47 days of age, 12 broilers per subgroup were selected and challenged with 0.2 ml (75µg) of S. aureus antigen mixed with PBS, subcutaneously at the right wattles, while the left wattles were inoculated with 0.2 ml PBS only to serve as control for each bird. The thickness of both wattles at challenge sites was measured to the nearest 0.01mm using a digital vernier caliper at 0, 4, 12, 24, 48 and 72 hours post challenge (pc). Difference in wattle thickness in each bird was referred to as Delayed Wattle Reaction (DWR) and was calculated by subtracting the thickness of the left wattle from the thickness of the right wattle.

Histopathology

At 72 hours pc, representative birds from each subgroup were euthanized in CO_2 chamber. Their wattles were excised and each was fixed in 10 ml of 10% formalin solution. Tissue sections were cut at 5µm, stained with hematoxylin-eosin and evaluated by light microscopy (American Registry of Pathology, 1968).

Statistical analysis

Mean (± SEM) DWR values were compared between subgroups 1A and 2A, 1B and 2B, 1C and 2C using independent Student t-tests. Analysis of variance (ANOVA) and least significant difference (LSD) method of multiple comparisons were used to compare mean values between all subgroups.

RESULTS

Mean DWR values were significantly higher ($p<0.05$) from 4 to 72 hours pc in subgroups 1A and 1B which were administered Lev.HCl compared with their unsensitized subgroups (Figures 1 and 2). Also in group C the sensitized subgroup 1C had significantly higher ($p<0.05$) mean DWR values from 12 to 72 hours pc than the unsensitized subgroup 2C (Figure 3).

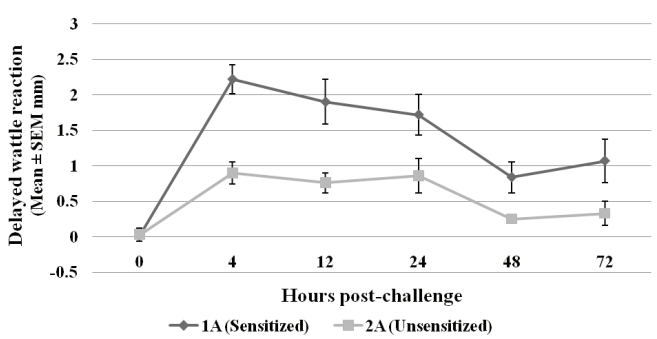

Figure 1 – Delayed wattle reaction in broilers challenged with S. aureus antigen 24 – 48 hours post-levamisole hydrochloride administration.

Peak mean DWR values of 2.22 + 0.21mm and 2.96 ± 0.21mm were obtained at 4 hours pc in subgroups 1A and 1B respectively, which were administered Lev. HCl compared with 3.39 ± 0.34mm at 24 hours pc in control subgroup 1C. However, it was observed that

peak DWR value in subgroup 1B that was administered Lev.HCl simultaneously with challenge was higher than that of subgroup 1A that was administered Lev.HCl 24 to 48 hours before challenge (Figure 4).

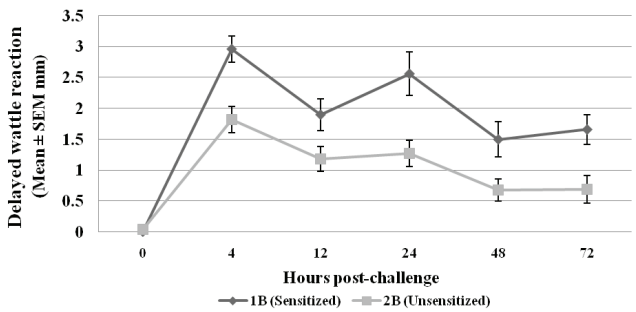

Figure 2 – Delayed wattle reaction in broilers challenged with S. aureus antigen simultaneously with levamisole hydrochloride administration.

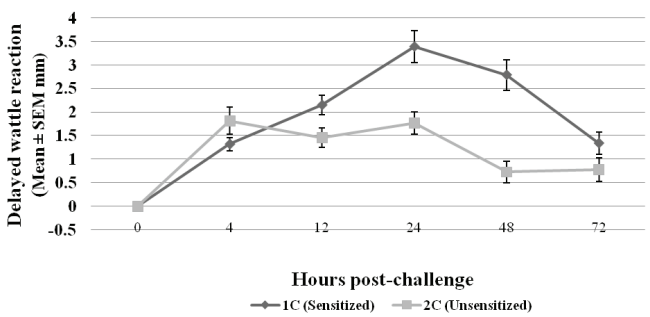

Figure 3 – Delayed wattle reaction in broilers challenged with *S. aureus* antigen without levamisole hydrochloride administration.

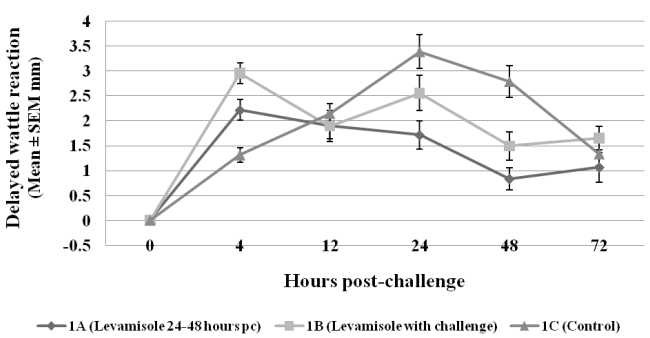

Figure 4 – Delayed wattle reaction in sensitized broilers challenged with *S. aureus* antigenand administered levamisole hydrochloride.

Comparing the three sensitized subgroups, at 4 hours pc (Figure 4), mean DWR value in subgroup 1B was significantly higher ($p<0.05$) than that of subgroups 1A and 1C while that of subgroup 1A was significantly higher ($p<0.05$) than that of subgroup 1C. Mean DWR values of subgroup 1C were significantly higher ($p<0.05$) than those of subgroup 1A at 24 and 48 hours pc and subgroup 1B at 48 hours pc only. Amongst the unsensitized subgroups, 2A that was administered Lev.HCl 24 to 48 hours before challenge generally had the lowest DWR values which were statistically significant ($p<0.05$) at 4, 12 and 24 hours pc.

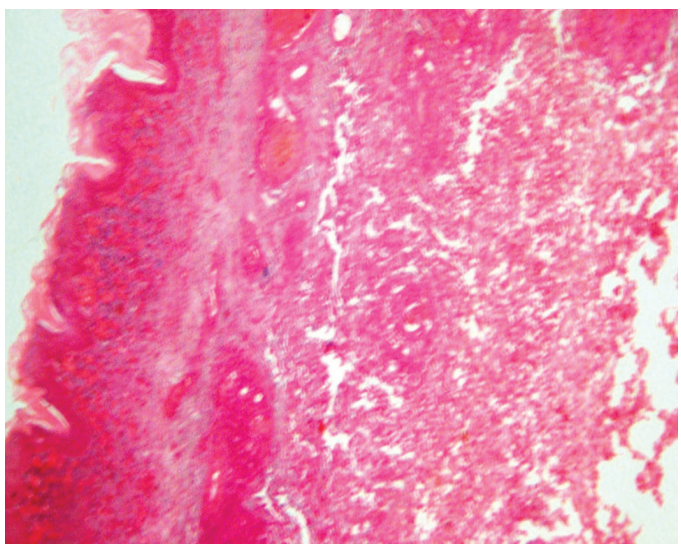

Figure 5 – Photomicrograph of the right wattle of sensitized broiler administered Lev. HCl before challenge (Subgroup 1A) showing edema, massive cellular infiltration in the tissue. (H&E stain x100).

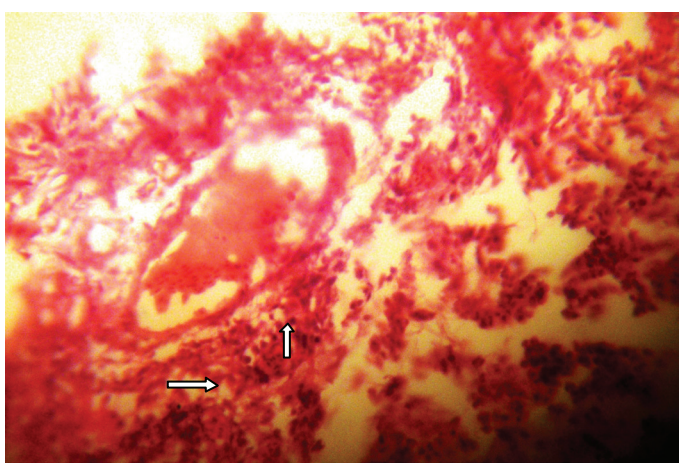

Figure 6 – Photomicrograph of the right wattle of sensitized broiler administered Lev. HCl before challenge (Subgroup 1A) showing edema, massive infiltration of tissue, with mononuclear cells including macrophages (arrows) and lymphocytes (H&E stain x400).

Tissue sections from the right wattles of sensitized broiler in subgroups 1A, 1B and 1C generally showed edema, congested blood vessels as well as infiltration of dermis and subcutaneous tissues with mononuclear cells, particularly lymphocytes and macrophages (Figures 5 and 6). Tissue infiltration was more severe in subgroup 1C. Left wattles of sensitized and both wattles of unsensitized broilers showed mild to no histopathologic lesions (Figure 7).

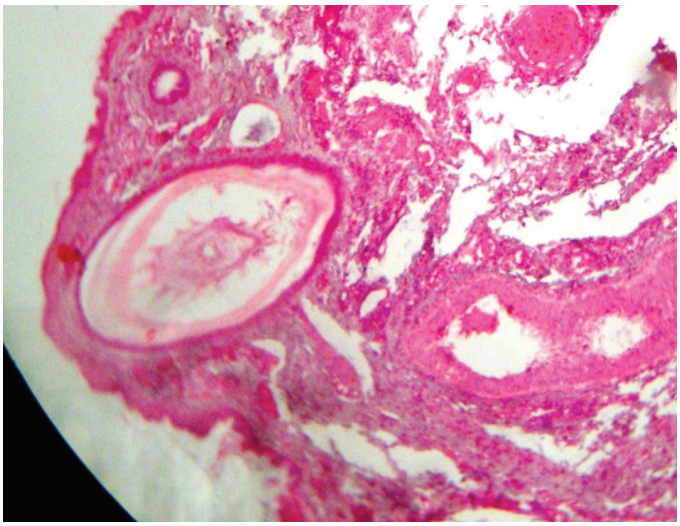

Figure 7 – Photomicrograph of the right wattle of unsensitized broiler administered Lev. HCl simultaneously with challenge (Subgroup 2B) showing milder inflammatory reaction with congested blood vessels. (H&E stain x100).

DISCUSSION

DWR is commonly used to evaluate in vivo DTH reactions in chickens and is shown to be a valid method of assessing T-cell mediated immunity (Watabe and Glick 1983; Dharsana and Spadbrow, 1985).

The significantly higher ($p<0.05$) DWR values obtained for all three sensitized subgroups compared with their unsensitized counterparts was as expected in such experiments (Zhu *et al*., 1999) and demonstrates its validity. It was observed that subgroups 1A and 1B that were administered Lev. HCl had earlier DWR peak, i.e at 4 hours pc, whereas peak reaction in the control subgroup 1C was observed at 24 hours pc, as previously reported in earlier studies (Taubler, 1968; Cotter *et al*., 1987; Zhu *et al*., 1999a; 1999b). While Spitznagel (1977) and Zhu *et al*. (1999), in separate studies, argued that wattle swelling at 4 hours pc in sensitive and unsensitized birds after challenge may be simply due to antibody-mediated immediate reaction rather than a cell-mediated delayed reaction, the absence of appreciable wattle swelling at 4 hours pc in control subgroup 1C suggests that the swelling observed in subgroups 1A and 1B was cell mediated. The earlier peak reaction observed in this study in subgroups 1A and 1B (sensitized broilers) shows that levamisole was able to enhance cellular immune response in these groups resulting in faster response. At different times, Schuerman (1975) and Thrower (1983) had earlier found that Lev.HCl enhances DTH. This study therefore agrees with the independent studies of those authors,particularly with the latter's findings that Lev. HCl activates T-cell mediated immune response. The clinical implication of early cellular response is that microbial organisms invading the body can be promptly arrested, thereby preventing systemic invasion resulting in disease. Although both subgroups 1A and 1B were administered Lev. HCl, the significantly higher ($p<0.05$) peak DWR obtained in subgroup 1B shows that the time lag between administration of levamisole and elicitation of challenge determines the intensity of levamisole-enhanced cellular immune reaction. However, this is likely to be dependent on availability of adequate concentration of levamisole in plasma. It had earlier been reported that total and unchanged levamisole was found to be present in plasma, urine and feaces of mammals for up to 72 hours (Kouassi *et al*., 1986; Heykants *et al*., 1990). Nevertheless, peak DWR was significantly higher ($p<0.05$) in the control subgroup that was not administered Lev.HCl than those of subgroups 1A and 1B. This is an indication of a less intense response in subgroups 1A and 1B, which could be due to the anti-inflammatory attribute that had been associated with levamisole (Dayrens *et al*., 1983).

In the unsensitized subgroups, intensity of reaction was highest in control subgroup 2C while subgroup 2A had the least. The unsensitized subgroups simulate broilers whose immune system had not been compromised. The result of this study therefore shows that Lev.HCl was unable to enhance cellular immune response in immunocompetent broilers as earlier reported (Symoens & Rosenthal, 1977; Singh and Dhawedkar, 1993), rather, an anti-inflammatory effect was also observed in these subgroups as reported by Thrower (1983) and Panacri, (2009).

The histopathologic examination of swollen wattles showing edema, congested blood vessels and cellular infiltrations by mononuclear cells particularly lymphocytes and macrophages is a characteristic feature of DTH reaction (Anderson, 1971; Klessius *et al*.,1977; Stites, 1994). The more severe histopathologic lesions observed in subgroup 1C compared with subgroups 1A and 1B is consistent with the degree of DWR observed and a confirmation of the anti-inflammatory property of Lev.HCl (Thrower *et al*., 1983; Panacri *et al*., 2009).

It was observed that broilers in group B were healthier, with no clinical disease or mortality. Morbidity commenced in group C at 47 days of age and was observed in group A at 49 days. The resulting effect was significantly higher ($p<0.05$) mean live weights in groups A (1.57 ± 0.06 kg) and B (1.56 ± 0.06 kg), which were administered Lev. HCl compared with

control group C, in agreement with earlier reports (Giambrone *et al*. 1985; Padmavathi *et al*., 1988).

In conclusion, this study showed that oral administration of Lev.HCl resulted in earlier DWR in sensitized/immunocompromised broilers, while there was no such influence in unsensitized/ immunocompetent broilers. Thus, enhancement of cellular immunity by levamisole is probably due to its ability to initiate prompt cellular reaction. In addition, the anti-inflammatory effect of levamisole was evident in both sensitized and unsensitized subgroups. Increased liveability was also attributed to oral administration of levamisole. Therefore, levamisole can be used routinely to enhance immune response and boost productivity in broilers especially in the face of constant challenges to the immune system as those present in tropical environments.

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