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*Original article*

# EFFECT OF LEVAMISOLE ON ACTIVE ANTIBODY TITRES AND HISTOMORPHOMETRIC PARAMETERS OF IMMUNE ORGANS IN BROILER CHICKENS

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**Summary**

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Use of immunomodulators has increased, particularly in poultry production around the world. The study evaluates effect of levamisole HCl (LMS) as an immunostimulatory agent on antibody titres and histomorphometric parameters of caecal tonsils, bursa and thymus of broilers. Sixty chickens were treated with 0 (control), 2, 5, 10, 15 and 25 mg/kg LMS in drinking water for 45 days. Antibody titre against ND, AI, IBD and IB was assayed periodically. At the end of the experiment, histological sections were made from caecal tonsils, bursa and thymus. LMS had no effect on body, caecal tonsil and bursa weights. Treated birds, except those at 15 mg/kg, showed significantly higher antibody titres in some of the sampling occasions. In caecal tonsils, the highest dose had the most remarkable effect. In bursa, the most profound effect of this dose was on number of follicles per plica. Thymus was the least affected organ. In conclusion, LMS administration affected positively active antibody titres and histomorphometric parameters of caecal tonsils, bursa and less profoundly thymus in normal broilers.

**Key words**: antibody, bursa of Fabricius, caecal tonsil, histomorphometry, levamisole, thymus

## INTRODUCTION

Immunomodulators are substances that are able to regulate or modulate immune responses. Immunomodulators may either augment or suppress the immune response, though the term is often used to refer to substances that enhance the immune response (Blecha, 1988). Although they are not routinely applied, the use of

immunomodulators has increased, particularly in poultry production around the world (Porchezhian & Punniamurthy, 2006).

Levamisole HCl (LMS) is a synthetic anthelmintic drug for animals and is widely used in cattle, sheep, goats, swine, and poultry. It is effective against lung worms and gastrointestinal nematodes. This agent is currently being used to treat *Capillaria* infection in poultry (USDA, 1998). The recommended anthelmintic dosage in poultry is 25 mg/kg body weight. The immuno-timulatory effect of LMS, found to enhance the protection of a *Brucella* vaccine in mice, was first reported by Renoux & Renoux (1971) About a decade later, Soppi *et al.* (1979) reported the ability of LMS to modulate immune responses in normal chickens by using thymus dependent and thymus independent antigens. Since then, other researchers have become interested in this feature of the drug and various positive effects of LMS on immune responses of avian species against different antigens have been reported (Habibi *et al.,* 2012; Oladele *et al.,* 2012; Shomali *et al.,* 2012). Although the immunostimulatory properties of LMS in chickens are confirmed by previous studies, its mechanism of action has not yet been fully elucidated.

The aim of the present study was to evaluate the effect of oral administration of different doses of LMS during the rearing period on histomorphometric parameters of three major immune structures of broiler chickens including caecal tonsils, bursa of Fabricius and thymus as well as antibody titres against four important chicken diseases including Newcastle disease, H9N2 avian influenza, infectious bursal disease and infectious bronchitis.

## MATERIALS AND METHODS

### *Birds and experimental design*

All methods used in the study are in compliance with the institutional ethical guidelines for care and use of animals in research and with international guiding principles for biomedical research.

Sixty one-day old chickens (Ross 308) were housed individually in cages. The rearing conditions were the same for all birds and were chosen as suggested by Ross 308 broiler management handbook. The ambient temperature was 33±1 °C during the first week and then the temperature decreased by 2 °C per week and was kept at 24±2 °C from day 22 until the end of the experiment. The lighting programme was 23 hours light and 1 hour of darkness in the first 7 days followed by 19 hours light and 5 hours of darkness until the end of the experiment. Each cage was fitted with an individual feeder and a nipple drinker. Birds had free access to food and tap water during the experimental period. Before allotment of birds in groups, blood samples were collected from jugular vein of 5 randomly selected birds on day one and serum maternal antibody titres against Newcastle disease (ND), H9N2 avian influenza (AI) (both by HI method) as well as infectious bursal disease (IBD) and infectious bronchitis (IB) (both by indirect ELISA test kits, Bio Check®, USA) were assayed. Routine vaccination programmes against ND, IB and AI were followed during the rearing period including live vaccine against ND+H120 on day one via eye drop, B1 via eye drop and oil-emulsified ND+AI vaccine by s.c. route on day 7, H120 via eye drop on day 14 and La Sota via eye drop on day 21.

After blood collection, birds were randomly divided into six equal groups (n=10 each) on day one. Daily water intake of

birds was calculated and birds in different groups were treated with 0 (control group), 2, 5, 10, 15 and 25 mg/kg body weight levamisole (Damloran Razak Pharma Co., Iran) in drinking water from day 1 to 45. Blood samples were collected from wing veins of 5 birds of each group and antibody titre detection against ND, AI, IBD and IB was repeated at the end of second, $4^{th}$, $5^{th}$ and $6^{th}$ week of the experiment.

*Sampling and histological evaluation*

At the end of the treatment period, all birds were weighed and then slaughtered by decapitation. Proximal part of caeca or caecal tonsils, bursa of Fabricius and thymus were immediately removed and weighed except for thymus. Samples were fixed in 10% buffered formalin. Routine histological laboratory methods were used to prepare 6 μm-thick transverse sections stained with H&E and evaluated under light microscope by using a linear graticule. In caecal tonsils, villus height and villus basal width at the crypt-villus junction, as well as nodular unit width and height, follicular width and muscular layer width were measured. The parameters that were evaluated in the bursa included height of plicae, follicular width as well as thickness of follicular cortex and medulla of bursa. In thymus, lobular thickness, lobular cortex and medullar width were measured. Number of follicles in each bursal plica and number of follicles per nodular unit of caecal tonsils were also counted. Arithmetic mean of 15 measurements of each above mentioned parameters per section was calculated.

*Statistical analysis*

Data expressed as mean±SD for histomorphometric parameters and mean±SEM for antibody titres. Data comparisons were performed by one-way ANOVA followed by Tukey's multiple comparison test and differences considered statistically significant at $P<0.05$.

## RESULTS

All birds seemed clinically healthy at the end of experiment and no bird loss or other adverse signs including inappetence or lethargy were observed during the rearing period.

Birds in different groups had statistically the same body weights. Caecal tonsil and bursa of Fabricius weights to body weight ratios were calculated and found not to differ statistically among the groups (data not shown).

*Antibody titres*

On day one of the study, antibody titres of five randomly selected birds against AI, ND, IB and IBD were 6±0.5, 8.2±0.7, 2695±329 and 5904±872 (mean±SEM), respectively. Table 1 summarises antibody titres of different groups against above-mentioned diseases at the end of second, $4^{th}$, $5^{th}$ and $6^{th}$ week of the experiment. As could be deduced from this table, birds treated with LMS at different dosages except for 15 mg/kg showed significantly higher antibody titres in some of the samplings as compared to control group: at the end of the $5^{th}$ week against ND (25 and 5 mg/kg), at the end of the $6^{th}$ week against AI (5 and 10 mg/kg) and at the end of the $4^{th}$ (2 mg/kg) and $5^{th}$ week (25 mg/kg) against IB.

*Histomorphometric parameters of caecal tonsils*

Although many parameters were affected with doses as low as 5 mg/kg, the highest dose i.e. 25 mg/kg had the most profound effect on majority of histomorphometric

**Table 1.** Antibody titres (mean±SEM) against Newcastle disease (ND), H9N2 avian influenza (AI) (log 2 for both), infectious bronchitis (IB), and infectious bursal disease (IBD) of different groups at the end of second, 4th, 5th and 6th weeks of the experiment.

| | Week | Antibody titres | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | LMS 2 mg/kg | LMS 5 mg/kg | LMS 10 mg/kg | LMS 15 mg/kg | LMS 25 mg/kg |
| ND | 2 | 5.4±0.5$^{a}$ | 5.6±0.2$^{a}$ | 5.3±0.8$^{a}$ | 6±0.4$^{a}$ | 5.6±0.3$^{a}$ | 5.8±0.4$^{a}$ |
| | 4 | 4.8±0.2$^{a}$ | 5.6±0.2$^{a}$ | 5.2±0.4$^{a}$ | 4.4±0.2$^{a}$ | 4.7±0.4$^{a}$ | 5.6±0.2$^{a}$ |
| | 5 | 3.8±0.4$^{a,b,d,e}$ | 5.4±0.4$^{b,c,f}$ | 6.6±0.3$^{c,f}$ | 2.6±0.2$^{d,e}$ | 3±0.4$^{e,f}$ | 5.8±0.5$^{f}$ |
| | 6 | 5.2±0.5$^{a,b,c}$ | 7±0.6$^{b}$ | 6±0.7$^{b}$ | 2.7±0.2$^{c}$ | 7±0.6$^{b}$ | 5.7±0.6$^{b}$ |
| AI | 2 | 2.8±0.2$^{a,b,c,d}$ | 3.2±0.2$^{b,d}$ | 3.5±0.5$^{b}$ | 2.8±0.2$^{b,c,d}$ | 1.8±0.4$^{c,d}$ | 2±0.3$^{d}$ |
| | 4 | 0.2±0.2$^{a}$ | 0.2±0.2$^{a}$ | 0.4±0.2$^{a}$ | 0.2±0.2$^{a}$ | 0.2±0.2$^{a}$ | 0±0$^{a}$ |
| | 5 | 1±1$^{a}$ | 1±0.6$^{a}$ | 1.4±0.6$^{a}$ | 0.2±0.2$^{a}$ | 0±0$^{a}$ | 0.2±0.2$^{a}$ |
| | 6 | 0.5±0.5$^{a,b,e,f}$ | 0.7±0.4$^{b,e,f}$ | 3.2±0.2$^{c,d,f}$ | 5.3±0.8$^{d}$ | 0.5±0.5$^{e}$ | 0±0$^{f}$ |
| IB | 2 | 332±40$^{a,b,c,d,e,f}$ | 278±43$^{b,d,e,f}$ | 204±54$^{d,f}$ | 430±48$^{e,c,f}$ | 286±22$^{f}$ | 505±62$^{c}$ |
| | 4 | 520±56$^{a}$ | 1391±184$^{b}$ | 539±49$^{a}$ | 498±88$^{a}$ | 526±77$^{a}$ | 290±39$^{a}$ |
| | 5 | 151±15$^{a}$ | 283±35$^{a}$ | 408±101$^{a,b}$ | 250±121$^{a}$ | 259±7$^{a}$ | 850±223$^{b}$ |
| | 6 | 277±10$^{a,b,c}$ | 438±21$^{b,c}$ | 679±29$^{b}$ | 221±36$^{c}$ | 329±42$^{b,c}$ | 325±111$^{b,c}$ |
| IBD | 2 | 521±79$^{a}$ | 467±42$^{a}$ | 750±71$^{a}$ | 1136±231$^{a}$ | 352±69$^{a}$ | 660±108$^{a}$ |
| | 4 | 60±10$^{a,b}$ | 101±18$^{a}$ | 90±6$^{a}$ | 56±5$^{a,b}$ | 21±8$^{b}$ | 34±8$^{b}$ |
| | 5 | 40±3$^{a,b}$ | 59±13$^{a}$ | 59±11$^{a}$ | 35±11$^{a,b}$ | 19±2$^{b}$ | 44±7$^{a,b}$ |
| | 6 | 51±6$^{a}$ | 18±5$^{a}$ | 30±4$^{a}$ | 51±10$^{a}$ | 19±5$^{a}$ | 30±7$^{a}$ |

Values with a common superscript letter in a row, have no significant difference (P>0.05).

parameters of caecal tonsils as compared to control group. Villi became shorter and more flattened while nodular units became narrower and taller in LMS treated groups. The width of follicles increased; moreover each nodular unit had higher numbers of follicles. Muscular layer width significantly decreased in birds treated with 5 mg/kg or higher doses of LMS as compared to control group. Data are summarised in Table 2.

*Histomorphometric parameters of bursa of Fabricius*

LMS at the dosage of 2 mg/kg had no appreciable effects on histomorphometric parameters of bursa of Fabricius except for follicular medullar thickness. As observed in caecal tonsils, the most prominent effect of LMS was related to its highest dose (25 mg/kg) which was the only dosage of the drug that significantly increased thickness of follicular cortex as compared to control group. The most profound effect of this dose was on number of follicles per plica which increased three times compared to that of control group. The effect of the 3 intermediate doses i.e. 5, 10 and 15 mg/kg on most of the studied parameters was statistically the same. Data are shown in Table 3.

*Histomorphometric parameters of thymus*

Thymus was the least affected organ by different dosages of LMS (Table 4). The only LMS dosage that resulted in significant increase in two histomorphometric parameters of thymus including lobular thickness and lobular cortical width was 25 mg/kg.

**Table 2.** Histomorphometric parameters (mean±SD) of caecal tonsils of birds in different groups at the end of the experiment.

| | Villus height (mm) | Villus width (mm) | Nodular unit width (mm) | Nodular unit height (mm) | Follicular width (mm) | Follicle number per nodular unit | Muscular layer width (mm) |
|---|---|---|---|---|---|---|---|
| Control | 0.22±0.04$^{a}$ | 0.03±0.01$^{a}$ | 0.32±0.03$^{a}$ | 0.56±0.02$^{a}$ | 0.05±0.00$^{a}$ | 4.40±0.55$^{a}$ | 0.17±0.01$^{a}$ |
| LMS 2 mg/kg | 0.07±0.01$^{b}$ | 0.05±0.01$^{b,a}$ | 0.29±0.05$^{a,c}$ | 0.57±0.08$^{a}$ | 0.07±0.01$^{a,b}$ | 5.80±0.84$^{b,a}$ | 0.13±0.0$^{a}$ |
| LMS 5 mg/kg | 0.18±0.02$^{b,a}$ | 0.05±0.01$^{b,a}$ | 0.24±0.05$^{b,c}$ | 0.75±0.04$^{b}$ | 0.08±0.00$^{b,c}$ | 6.40±0.55$^{b}$ | 0.12±0.01$^{b}$ |
| LMS 10 mg/kg | 0.18±0.04$^{a,b}$ | 0.06±0.01$^{b}$ | 0.20±0.03$^{b}$ | 0.76±0.07$^{b}$ | 0.07±0.01$^{b,c}$ | 5.80±1.30$^{b,a}$ | 0.12±0.01$^{b}$ |
| LMS 15 mg/kg | 0.13±0.02$^{b}$ | 0.06±0.01$^{b}$ | 0.22±0.03$^{b}$ | 0.77±0.15$^{b,c}$ | 0.08±0.02$^{b,c}$ | 6.20±1.30$^{b,a}$ | 0.10±0.02$^{b}$ |
| LMS 25 mg/kg | 0.14±0.03$^{b}$ | 0.06±0.01$^{b}$ | 0.23±0.02$^{b,c}$ | 0.92±0.03$^{c}$ | 0.09±0.01$^{c}$ | 7.00±0.22$^{b}$ | 0.10±0.02$^{b}$ |

Values with a common superscript letter in a column, have no significant difference (P>0.05).

**Table 3.** Histomorphometric parameters (mean±SD) of bursa of Fabricius of birds in different groups at the end of the experiment

| | Height of plicae (mm) | Follicular width (mm) | Follicular cortical thickness (mm) | Follicular medullar thickness (mm) | Number of follicles per plica |
|---|---|---|---|---|---|
| Control | 0.30±0.03$^{a}$ | 0.06±0.01$^{a}$ | 0.02±0.00$^{a}$ | 0.04±0.01$^{a}$ | 11.33±5.11$^{a}$ |
| LMS 2 mg/kg | 0.38±0.10$^{a}$ | 0.07±0.03$^{a}$ | 0.02±0.00$^{a}$ | 0.06±0.03$^{b}$ | 14.17±2.32$^{a}$ |
| LMS 5 mg/kg | 0.69±0.09$^{b}$ | 0.09±0.02$^{b}$ | 0.03±0.01$^{a}$ | 0.08±0.02$^{b}$ | 16.50±2.07$^{b}$ |
| LMS 10 mg/kg | 0.62±0.06$^{b}$ | 0.09±0.01$^{b}$ | 0.03±0.01$^{a}$ | 0.09±0.01$^{b}$ | 22.00±3.16$^{c}$ |
| LMS 15 mg/kg | 0.70±0.10$^{b}$ | 0.12±0.00$^{b}$ | 0.02±0.01$^{a}$ | 0.08±0.00$^{b}$ | 29.50±2.66$^{d}$ |
| LMS 25 mg/kg | 1.00±0.08$^{d}$ | 0.15±0.02$^{c}$ | 0.04±0.01$^{b}$ | 0.11±0.02$^{c}$ | 31.33±2.50$^{d}$ |

Values with a common superscript letter in a column, have no significant difference (P>0.05).

**Table 4.** Histomorphometric parameters (mean±SD) of thymus of birds in different groups at the end of the experiment.

| | Lobular thickness (mm) | Lobular cortical width (mm) | Lobular medullar width (mm) |
|---|---|---|---|
| Control | 0.23±0.03[a] | 0.13±0.02 [a] | 0.10±0.02 [a] |
| LMS 2 mg/kg | 0.26±0.05[a] | 0.15±0.03 [a] | 0.11±0.02 [a] |
| LMS 5 mg/kg | 0.26±0.03[a] | 0.15±0.03 [a] | 0.11±0.02 [a] |
| LMS 10 mg/kg | 0.25±0.02[a] | 0.14±0.01 [a] | 0.11±0.01 [a] |
| LMS 15 mg/kg | 0.26±0.03[a] | 0.15±0.03 [a] | 0.11±0.01 [a] |
| LMS 25 mg/kg | 0.39±0.04[b] | 0.27±0.04[b] | 0.12±0.01 [a] |

Values with a common superscript letter in a column, have no significant difference (P>0.05).

## DISCUSSION

Traditionally, the lymphoid system of chickens is divided into central or primary lymphoid tissue which consists of thymus and bursa of Fabricius, and peripheral or secondary lymphoid tissue which includes several immune structures; most notably the spleen and all mucosa-associated lymphoid tissues (Schat *et al.,* 2014). Intestinal mucosal immune stimulation has attracted much interest as a means of generating protective immunity against mucosal and systemic pathogens (Revolledo *et al.,* 2006). Kitagawa *et al.* (1998) found that 45.7% of lymph nodules are accumulated in caecal tonsils of adult chickens, which makes it the largest lymphoid organ of the avian gut-associated lymphoid tissue. Therefore, caecal tonsils are involved as an immune structure in some important infectious diseases of poultry including ND, AI etc.

It is believed that the interdigitating meshwork of villi at the caecal entrance acts as a filter. In our study the villi became shorter and more flattened in all LMS-treated groups as compared to control. This possibly can set the scene for higher exposure of immune structures of caecal tonsils to potential antigens in the gut. On the other hand, thinning of muscular layer in LMS-treated groups, with possibly lower strength for peristaltic movements, may help in longer presence of microorganisms and other antigens in close proximity of immune structures in caecal tonsils. The resultant higher stimulation of immune structures may lead to prominent increase in follicular width as well as number of follicles per nodular unit observed in our study, although this needs to be confirmed by more sophisticated methods including evaluation of cytokine profile as well as possible changes in B and/or T cells populations. Higher doses had statistically similar or even lower effects on above mentioned parameters.

The bursa of Fabricius plays a central role in the development of the antibody-producing B-lymphocyte lineage in birds. Since the bursal lumen is connected to the gut lumen by the bursal duct, this provides a mechanism by which the development of bursal B cells after hatch occurs in the presence of materials derived from the gut (Ratcliffe, 2006). It is in the follicular

cortex that most cell division occurs after hatch (Ratcliffe, 2006); moreover most peripheral B cells are derived from the follicular cortex (Paramithiotis & Ratcliffe, 1994). In our study the obvious increase that was observed in follicular cortex thickness by the highest tested dose of LMS, may be due to higher cell proliferation and/or lower cell migration from cortex to periphery. Regarding the fact that only about 5% of the bursal cells generated each day immigrate to the periphery (Lassila, 1989) and there is clearly no major migration of cells from the cortex to the medulla (Paramithiotis & Ratcliffe, 1994), it is more probable that higher cell proliferation is the cause behind the increased follicular cortex thickness in our study especially when considering higher follicular width and thicker follicular medulla. On the other hand, there are significant levels of B cell death in the bursa, especially after hatch (Motyka & Reynolds, 1991; Paramithiotis *et al.,* 1995) and we cannot exclude the fact that the increase in above parameters may be due to inhibition of apoptosis of B-lymphocytes by LMS in treated birds.

The subcapsular zone of the thymic cortex is the major site of cell proliferation. During T cell maturation, the cells migrate towards the cortico-medullary border where the thymocytes are selected before their entrance into medulla and circulation via the medullary post-capillaries (Schat *et al.,* 2014). In our study, the lobular thickness of the thymus increased by the highest dose of LMS, which was primarily due to the increase in lobular cortical thickness. We cannot precisely differentiate whether this increase was due to higher proliferation of cells in cortex or lower migration to medulla. Although the first suggestion seems more plausible, since the medullary part seemed quite normal with the proper density of cells and its thickness even increased slightly. The thymus was the organ least affected by LMS. One explanation may be the presence of blood-thymus barrier that can limit the penetration of the drug.

In the present study, the antibody titre against ND, AI, IB and IBD was assayed as a criterion for immunostimulating properties of LMS. The results showed that LMS can enhance antibody titres against ND, AI and IB but not IBD in some of the samplings. It should be mentioned that birds were not vaccinated against IBD and circulating maternal antibodies declined progressively which, consistently with other studies, may describe why LMS was ineffective to enhance IBD antibody titres. We previously reported that administration of 150 mg/kg/day of LMS by oral gavage for 10 consecutive days post B1 vaccination to chickens resulted in antibody titres significantly higher than those in control group on the 7$^{th}$ day post booster vaccination with La Sota vaccine (Mosleh *et al.,* 2013). Habibi *et al*. (2012) observed that chickens that received 30 mg/kg of LMS in drinking water for 2 days after different vaccinations against ND, had increased antibody levels and significant results were seen in chickens vaccinated with Avinew (28$^{th}$ day) and La Sota (28$^{th}$ and 42$^{nd}$ days). With regard to findings of the current study, it seems that LMS had positive effects on antibody titres not only against ND but also AI and IB, which may be the result of positive effects of this agent on micro structures of lymphatic tissues.

In conclusion, administration of LMS during the rearing period of broilers can positively affect active antibody titres and histomorphometric parameters of caecal tonsils, bursa of Fabricius and less profoundly thymus in healthy broilers. These

positive changes in histological features of lymphoid organs may at least partly describe the mechanism of immunostimulatory effect of levamisole in broilers.

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