

IMMUNOSTIMULATORY EFFECT OF IVERMECTIN ON MACROPHAGE ENGULFMENT AND DELAYED TYPE HYPERSENSITIVITY IN BROILERS

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ABSTRACT

Some studies show that ivermectin enhances the immune response in human and animals. An experimental model of broiler chickens was designed and developed using ivermectin to evaluate its immunomodulatory response at different doses. Experiment was done on macrophage engulfment percentage and the skin thickness in the broilers. They were divided into six major groups A through F, having ten broilers in each group. Groups B, C, D, E and F were given ivermectin at the dose rate of 0.15, 0.3, 1, 3 and 5 mg/kg body weights of birds, respectively. Whereas group A was kept as control i.e without ivermectin. Macrophage engulfment percentage, in terms of phagocytosis was significantly high in groups E and F. The mean skin thickness values in broilers of Group F (Highly medicated) was significantly ($P < 0.05$) higher than in broilers of all the groups. Skin sensitivity at 24 hrs. and 48 hrs. was highest in the broilers administered with 5 mg/kg body weight (the highest dose) of ivermectin. Our study indicated that immunostimulatory effect of ivermectin can also be achieved in broilers for the treatment of viral/bacterial disease problems with immunosuppression.

Key words: Ivermectin, Immunomodulatory, Macrophage engulfment assay, Delayed type hypersensitivity, Macrophages, Broilers.

INTRODUCTION

The macrocyclic lactones are chemical derivatives of soil micro-organisms which belong to the genus *Streptomyces*. They consist of two major groups, the avermectins and milbemycins. Commercially used avermectins are ivermectin, abamectin, doramectin, eprino-mectin, and selamectin. Ivermectin is a 16-membered lactone class. It acts as a broad-spectrum antiparasitic drug. Affected organisms are immobilized as drug induced a strong paralysis of the muscles. Studies of *Caenorhabditis elegans* indicate that avermectins bring on paralysis via a group of glutamate-gated chloride channels found only in invertebrates (Arena *et al.*, 1995; Cully *et al.*, 1994; Cully *et al.*, 1996; Dent *et al.*, 1997; Dent, 2006). Glutamate-gated Cl⁻ channels are expressed in pharyngeal muscle cells of these worms, consistent with the effect and marked inhibition of avermectins on the feeding behavior of organisms (Sangster and Gill, 1999). Therefore, ivermectin probably binds glutamate-activated Cl⁻ channels found in nematode nerve or muscle cells, thus induces hyperpolarization by increasing permeability of chloride ions across the cell membrane, leading to parasite paralysis (Ikeda, 2003).

Interestingly, ivermectin having anti-parasitic activity also enhances the immune response in humans and animals (Blakely and Rousseaux., 1991; Savanur *et al.*, 1996; Lopez-Olvera *et al.*, 2006). Although some studies showed its immunopotentiating effect in rabbits (Sajid *et al.*, 2007), but some reports indicated

immunosuppressive effects of ivermectin in lambs, rats and rabbits (Uhlir and Volf, 1992; Stankiewicz *et al.*, 1995).

A few antiparasitics are used to stimulate immunity in various human diseases, including leprosy, Hodgkin's disease, rheumatoid arthritis and in the adjuvant treatment of colorectal cancers. In the animals, simultaneous use of vaccines and immunomodulatory antiparasitic drugs may be useful. Some studies have shown that the properties of ivermectin as an immune stimulator are associated with modified function of T lymphocytes in particular, T-helper lymphocytes in male CD-1 mice (Blakely and Rousseaux, 1991).

The immune system has unique properties of protecting the body against harmful substances by recognizing and responding to the antigens. Vaccination (immunization) is a way to activate an immune response. Effect of vaccination is influenced by immunomodulatory agents and their use may be justified for a specific outcome after vaccination.

The main objective of this study was to investigate the effects of ivermectin on Cellular mediated immune response in broilers.

MATERIALS AND METHODS

The project was designed to study the effect of ivermectin (SIGMA) on macrophages in poultry. In the study, the effect of various doses of ivermectin on the immune status of the broilers was evaluated.

Experimental Birds: A total of sixty day old broiler chicks were purchased from BIG BIRD Hatchery, Lahore, Pakistan. Chicks were vaccinated against infectious diseases. They were reared up to the age of 20 days. The broilers were kept in a confined parasite free environment in the experimental rooms in the Department of Pharmacology & Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan. Commercial feed and water were given *ad libitum* until the end of the experiment. No medication was given to the broilers except the test drug i.e. ivermectin.

Experimental Design: The purpose of this study was to evaluate the action of various doses of ivermectin (IVM) on cell mediated immunity (CMI) of broilers. In poultry, macrophage engulfment assay (MEA) and delayed-type hypersensitivity (DTH) were performed to determine CMI against sheep red blood cells (SRBC) and dinitrochlorobenzene (DNCB) respectively. Broilers were divided into six major groups (A through F) having ten chicks in each. Each group was further divided into two sub-groups (1 through 2) having five broilers in each group. Broilers of group A served as a vehicle control without ivermectin while those of groups B, C, D, E and F were treated with ivermectin at the dosage rate of 0.15 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg and 5 mg/kg body weight, respectively. Cellular immunity was determined in all the groups (A1, B1, C1, D1, E1 and F1) through DNCB and MEA in groups (A2, B2, C2, D2, E2 and F2). The skin sensitivity to DNCB was estimated at 24, 48 and 72 hrs. All experimental protocols were carried out with care and guidance on the use of birds for research by the institutional committee.

Effect of Ivermectin on Cellular Mediated Immunity (CMI) in Broiler Chickens: The following experiment was performed to find out the immunomodulatory effect of ivermectin in poultry at various doses as described by Sajid *et al.*, (2006).

Isolation of Peritoneal Exudate Cells (PEC) and culturing of macrophages: For the isolation of peritoneal macrophages (PMs), well known methods of Sugiura *et al.* (2002) in rats and Sandhu *et al.* (2006) in chickens were followed with some modifications.

Briefly, 3% (Wt/Vol) Sephadex granules G-50 (SIGMA, Co. USA) were given two washings and then resuspended in 0.85% normal saline. All the broilers were injected with Sephadex @ 10 ml/kg body weight, intraperitoneally as described by Qureshi *et al.*, (1986) on day 2 of the experiment. 70% alcohol was used to disinfect the skin of all the broilers. On day 5, the broilers were decapitated under ketamine HCl (10 mg/kg; Triattau, Germany). Abdominal cavities of the broilers were flushed with chilled normal saline (pH 7.2) and 0.5 U/ml of heparin through specially prepared harvester.

The macrophages were removed and washed twice with minimal essential media (MEM) as mentioned by Sajid *et al.* (2007). Hemocytometer was used to count the Macrophages obtained from the birds. Macrophage viability was found to be 90 % and their final concentration was adjusted as 2.9×10^4 . About 20 μ l of this concentration was poured on sterilized glass cover slips (13 mm). For the attachment of macrophages, 30 minutes were given. Then the culturing, staining and counting of macrophages on these cover slips was done.

Preparation of Hyper Immune Antiserum: Hyper immune antiserum was prepared by the method described by Muhammad *et al.*, (1994).

Preparation of Sheep Red Blood Cells (SRBCs): The blood of healthy sheep was centrifuged at 1500 rpm for 15 minutes at 4°C. Red blood cells were suspended in 10 ml phosphate buffer saline after removing the upper layer of plasma and buffy coat. Again after centrifugation, phosphate buffer saline was removed. For proper washing of SRBCs, this process was repeated for 3-4 times. Final concentration of these washed SRBCs was adjusted to 0.1 % for further experimentation.

Selection of Broilers: Randomly two broilers from each group (A2, B2, C2, D2, E2 and F2) were taken on day 2nd of the experiment to complete this part of the study.

I. **Macrophage engulfment assay:** Thirty broilers were divided into six major groups (A2 through F2) having five broilers in each. Broilers of group A served as vehicle control without ivermectin while those of groups B, C, D, E and F were treated with ivermectin at dose rates 0.15, 0.3, 1, 3 and 5 mg/kg body weight, respectively. Suspension of 10 μ l of unopsonized SRBC was done over the cover slips after adherence of macrophages. These cover slips were already dipped in cell culture medium. These cover slips were then incubated at 39.6°C in 5 % humidified CO₂ for 45 and 90 minutes.

The purpose of our study was to elaborate whether macrophage functions vary after different doses of ivermectin in broilers. Sandhu *et al.* (2006) and (Qureshi *et al.*, 1986) described a test according to which adherent macrophages could be counted. The engulfment percentage of opsonized and unopsonized SRBCs co-incubation was calculated with some modifications.

200 macrophages were counted on an average. At the same time, the engulfment percentage of opsonized and unopsonized SRBCs was also calculated as per formula:

$$\text{Engulfing percentage} = \frac{\text{Number of macrophages engulfing SRBCs}}{\text{Number of adherent macrophages}} \times 100$$

II. **Delayed Type Hypersensitivity Test:** Delayed-type hypersensitivity (DTH) reaction was noticed in this test in broilers of all the groups as described by Tiwary and Goel (1985).

Thirty broilers were divided into six major groups (A1 through F1) having five broilers in each. Broilers of group A served as a vehicle control without ivermectin while those of groups B, C, D, E and F were treated with ivermectin at dose rates 0.15, 0.3, 1, 3 and 5 mg/kg body weight, respectively.

For delayed type hypersensitivity reaction with 2, 4-Dinitrochlorobenzene (DNCB), five broilers from each group were taken. For this hypersensitivity reaction described by Tiwary and Goel (1985), two areas of the body of broilers have selected i.e. right flank region for sensitizing dose and left flank for challenge dose. The feathers were plucked carefully without causing the injury to the skin. Digital vernier caliper was used to measure the skin thickness before DNCB application in all the broilers. On day 2 of the experiment, the skin was marked with rounded ring of one-inch diameter by placing the ring on the flank region and marking with permanent marker. One ml of 1% DNCB in acetone was slowly incorporated on the skin in the circle with the help of tuberculin syringe. Immediate drying of the solution was done by continuous blowing on the circle in order to avoid outside flow of solution from the marked area. On day 14th, the same procedure was repeated on the left flank region of the broilers with challenge dose of DNCB after taking skin thickness measurements in each bird. Then after 24 hrs. 48 hrs. and 72 hrs. interval, skin thickness was measured by using vernier caliper (mm).

Statistics: Repeated measure design under randomized complete block design was used for statistical evaluation of all the data (Jerrold, 2007).

RESULTS

A. Effect of ivermectin on macrophage engulfment activity: Groups B, C, D, E and F were given ivermectin at the dose rate of 0.15, 0.3, 1, 3 and 5 mg/kg body weights of broilers, respectively. Whereas group A was kept as control i.e. without ivermectin.

An average of 200 macrophages was counted as adherent and the engulfment percentage of opsonized and unopsonized SRBCs co-incubation was calculated. The phagocytic percentage values in broilers of Groups E and F were significantly ($P < 0.05$) higher than in broilers of all the groups.

At 45 minutes, in the opsonized slides, the mean and standard error of phagocytic percentage of broilers of Group F was 57.00 ± 1.00 while in other groups it ranged between 23.50 ± 0.51 to 39.00 ± 1.00 . This indicated significant difference in the engulfment percentage in group F with all the other groups.

At 90 minutes, in the opsonized slides, the mean and standard error of phagocytic percentage of broilers of Group F was 60.50 ± 1.51 while in other groups it ranged between 28.50 ± 0.51 to 53.00 ± 1.00 . This indicated

significant difference in the engulfment percentage in group F with all the other groups.

At 45 minutes, in the unopsonized slides, the mean and standard error of phagocytic percentage of broilers of Group F was 49.50 ± 1.51 while in other groups it ranged between 13.50 ± 0.51 to 36.00 ± 1.00 . This indicated significant difference in the engulfment percentage in group F with all the other groups.

At 90 minutes, in the unopsonized slides, the mean and standard error of phagocytic percentage of broilers of Group F was 56.50 ± 1.51 while in other groups it ranged between 20.50 ± 1.51 to 45.00 ± 2.01 . This indicated significant difference in the engulfment percentage in group F with all the other groups.

The results indicated significant values in broilers of groups E and F, showing that the immuno stimulatory effect of drug is more in these groups as compared to the others.

Engulfment percentage values indicated that there is significant difference between the values of group A with birds of group D, E and F. Whereas non significant difference was observed between the values of groups A, B and C, indicating that in these groups ivermectin did not show any immuno stimulatory effect (Fig. 1).

B. Effect of ivermectin on skin thickness in the broilers: In the DTH, the effect of ivermectin on skin thickness in broilers was estimated.

The mean skin thickness values in broilers of Group F (Highly medicated) were significantly ($P < 0.05$) higher than in broilers of all the groups. Skin sensitivity at 24hrs. 48 hrs. and 72 hrs. was highest in the broilers administered with 5 mg/kg body weight (the highest dose) of ivermectin.

At 15th day (24 hrs. postchallenge), the mean and standard error of skin thickness of broilers of Group F was 3.12 ± 0.47 mm while in other groups it ranged between 0.91 ± 2.26 mm to 2.62 ± 0.17 mm. This indicated prolonged effect of drug on skin thickness in group F.

At 16th day (48 hrs. postchallenge), the mean and standard error of skin thickness of broilers of Group F was 3.44 ± 0.56 mm while in other groups it ranged between 0.87 ± 0.25 mm to 2.17 ± 0.23 mm. This is also indicative of prolonged effect of drug in group F as compared to other groups.

At 17th day (72 hrs. postchallenge), the mean and standard error of skin thickness of broilers of Group F was 2.14 ± 0.17 mm while in other groups it ranged between 0.81 ± 0.25 mm to 1.55 ± 0.23 mm. This value is more as compared to the other groups but less as compared to the previous values in the same group, indicating that the effect of drug is decreased but still present in group F as compared to the other groups (Table No. 3).

There was significant difference between the values of all the groups on all the days i.e 24, 48 and 72 hours (Fig. 2).

DISCUSSION

To our knowledge, no data is available showing the ivermectin stimulated macrophage engulfment activity in broilers/birds. The results showed significant increase in the engulfment percentage at 3 and 5mg/kg body weight doses of ivermectin. This may be due to the immunostimulatory effect of ivermectin or more expression of surface receptors for engulfment. After opsonized SRBC co-incubation, overall macrophage engulfment increased (Fig 1).

This was due to the fact that phagocytosis is facilitated by antibody mediated opsonization. The communication between macrophages and B-cell is continuing through cytokine signaling as well as cell-cell contact involved in antigen presentation.

Our study indicated that in addition to effectively control parasites, immunostimulatory effect of ivermectin can also be achieved in broilers. The mechanism involved in this effect is still unknown. However, Holcombe *et al.*, (1998), indicated that immunostimulatory effect of levamisole is due to its potentiating activity of interleukin-2 which is a T-lymphocyte growth factor that controls T-lymphocyte functions.

The inflammatory response against DNCB was much higher at 5 mg/kg of ivermectin after 24 and even after 72 hours of sensitization. The response of DNCB remained elevated in broilers of this dose of ivermectin after 24hrs. and 72 hrs. may be due to its immunopotentiating action, because the resolution of inflammation is directly proportional to the quantity and action of cells of the immune system. The reason for sustained skin thickness during 24-72 hours in the highest dose of DNCB inoculation may be that, heterophils have proteases and hydrolases which would cause damage to the extracellular matrix, promote chemotaxis, the apoptosis of down-regulated and switched off heterophils might have increased inflammation in that area.

The mean skin thickness values in broilers of Group F (Highly medicated) was significantly ($P<0.05$) higher than in birds of all the groups. Skin sensitivity at 24 and 48 hrs. was highest in the broilers administered with 5 mg/kg body weight (the highest dose) of ivermectin.

At 16th day (48 hrs.) postchallenge, the broilers of Group F showed prolonged effect of drug on skin thickness.

At 17th day (72 hrs.) postchallenge, the skin thickness was more as compared to the other groups but less as compared to the previous values in the same

group, indicating that the effect of drug was decreased but still present as compared to the other groups (Fig. 2)

Our results are in accordance with Tiwary and Goel 1985. They reported that there is slow development of cutaneous hypersensitivity, which reaches to its maximum expression after 24 hrs. of challenge and decreases after that. There was classical delayed type hypersensitivity skin reaction. Histopathologically it was characterized by congestion, oedema, mononuclear and heterophilic cell infiltration in the dermal layer.

Dose-dependent pharmacokinetics of ivermectin was observed in humans, dogs and red deer (Krishna and Klotz, 1993). Development of contact sensitivity is a measure of cellular immunity to a new antigen to which it has not been formerly exposed (Stites, 1978). Delayed-type hypersensitivity reaction was the result of DNCB.

In a study done by Sajid *et al* 2007, on rabbits, the cell mediated immunity against dinitrochlorobenzene (DNCB) was determined by delayed-type hypersensitivity. The skin sensitivity to DNCB at 24hrs. and 48hrs. was highest ($P> 0.05$) in rabbits administered with 600 μ g/kg b.w. An immunopotentiating effect of ivermectin at higher doses was due to graded dose immune response. This is in accordance with the mean skin thickness values in broilers of Group F (Highly medicated) in our study which is significantly ($P<0.05$) higher than in broilers of all the groups. Skin sensitivity at 24 hrs. and 48 hrs. was highest in the broilers administered with 5 mg/kg body weight (the highest dose) of ivermectin.

Similar results were shown by Munir *et al* 2009. They reported that skin thickness and lymphoproliferation of salinomycin medicated chicks were significantly greater ($P< 0.05$) than those of levamisole, cyclophosphamide and cyclosporine treated chicks. They concluded that against hydropericardium syndrome virus and Newcastle disease, salinomycin had beneficial effects on the cell mediated immune responses of broiler chicks.

The results of this study contradict with the findings of Kadian *et al* 1988. They observed that 0.3 ppm dietary aflatoxin B 1 significantly suppressed the Cell mediated immunity of chickens as the delayed type hypersensitivity response occur at 15, 30 and 45 days of age of aflatoxin B 1 fed chickens to Dinitrofluorobenzene. The results showed immunoenhancement in ivermectin medicated broilers of Groups E and F. Macrophage engulfment percentage, in terms of phagocytosis was significantly high in groups E and F.

Our results are in agreement with the findings of various scientists (Blakley and Rousseaux, 1991; Savanur *et al.*, 1996; Lopez-Olvera *et al.*, 2006) regarding the potentiating effect of ivermectin on immune system of humans and other animal species.

Uhlir and Volf, 1992 and Stankiewicz *et al.*, 1995 demonstrated the immunosuppressive effect of ivermectin in rabbits, rats and lambs but it was observed on therapeutic doses of ivermectin. These findings are not in accordance with the findings of our study as we have used high doses in addition to therapeutic doses of ivermectin in broilers and observed immuno stimulatory effect of this drug on these doses only.

Krishna and Klotz (1993) described that with the increase in dose, there is linear increase in blood plasma in different animal species. So due to variable plasma concentration, the immune response to antigen may also vary. This study is in full agreement with our results.

Further research in this area may be helpful in finding out the facts about its immuno stimulatory mechanism in birds and to boost poultry industry and health status.

Acknowledgement: The authors would like to thank Dr. M. Shahbaz Yousaf, Assistant Professor, Department of Physiology, University of Veterinary and Animal Sciences, Lahore, Pakistan for taking interest in the completion of this manuscript.

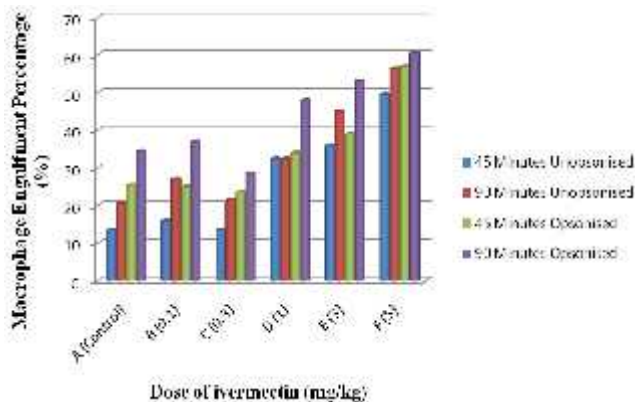


Fig.1. Graphical presentation showing macrophage engulfment percentage in ivermectin medicated and nonmedicated groups of Broilers

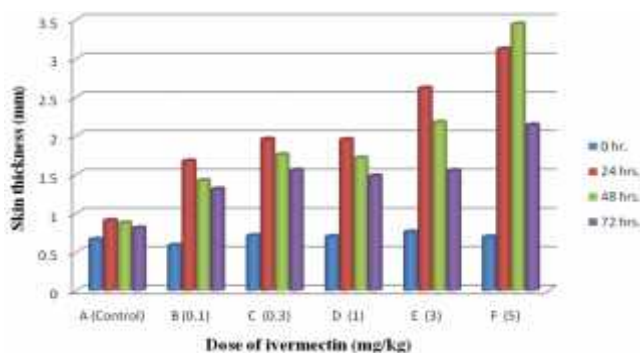


Fig. 2. Graphical presentation showing values of Skin Thickness (mm) in ivermectin medicated and nonmedicated groups of Broilers (Time wise)

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