The influence of a non-feed removal molting program on certain parameters of cell mediated and humoral immune response of the laying birds during 2nd and 3rd production cycles were recorded. Birds at the end of their 1st production cycle were induced to molt through the supplementation of high dietary zinc oxide (3000 mg/Kg) for their 2nd and 3rd production cycles. Macrophage engulfment (%) and engulfment/cell were significantly increased after the use of opsonized sheep red blood cells (SRBC) for co-incubation during 2nd production cycle. Lipopolysaccharides (LPS) greatly enhanced the production of nitric oxide, as compared to the SRBC stimulation during the 2nd production cycle. Zinc feeding strengthened the antibody response against Newcastle disease virus (NDV) and egg drop syndrome virus (EDS) at the peak of the 2nd and 3rd production cycles, but decreased at 5% production stages. NDV also provoked a better antibody response when compared to EDS. Zn concentration in plasma showed a positive correlation with an increase in egg production. Our results validate the strengthened immune response during the 2nd production cycle after zinc-induced molting.

Key words: antibody titers, laying hen, molting, peritoneal macrophages, zinc

the in-vitro phagocytic activity of abdominal exudates cells in young turkeys (Kidd et al., 1994).

With the use of zinc in the molting of birds, studies are deficient in examining the effects of zinc-induced molting on MO function, nitric oxide production, enhancement of acquired immunity and T-cell functioning during the 2nd and 3rd production cycles.

Materials and Methods

A flock of 200 Single Comb White Leghorn hens (Gallus domesticus) at 67 weeks of age and averaging 1.4 kg weight were procured from a commercial farm and delivered to the Department of Physiology and Pharmacology, University of Agriculture, Faisalabad, Pakistan. Upon arrival, the birds were group-weighed and randomly allocated to the groups. The birds were acclimated for 4 weeks during which they were fed normal layer bird feed. The birds were subjected to molting by supplementation of zinc oxide at the dose of 330 mg/Kg feed with a slight decrease in lighting schedule. The lighting schedule was 16 hL (hour light), and the temperature set point was 30±2°C. At the beginning of the second week, the birds were subjected to molting by supplementation of ZnO at the dose of 330 mg/Kg feed with a slight decrease in lighting schedule 12hL : 16hL (Table-1), and offered 35 g/bird feed on a daily basis. After the molting period, the lighting was increased gradually to 14hL : 16hL, and the birds were fed ground corn rations, during which they resumed laying. Throughout the experimental period, the birds had free access to water through nipple drinkers. Sampling was carried out after the cervical dislocation of seven randomly selected birds from each replicate at the end of the 1st production cycle (Control), again at 5% production, their peak production and the end of production stages during 2nd production cycle. At that stage, the birds were again molted by using the above-mentioned ZnO supplemented feed, after which sampling was done at the 5% and peak production stages of the 3rd production cycle. All the experimental protocols complied with the guidelines on the care and use of animals for research by the institutional committee.

Abdominal Exudate Cell Collection

Abdominal exudate cells (AEC) were collected as described by Qureshi et al. (1986) with some modifications. The abdominal cavity was briefly cleaned with alcohol and a 3% suspension of pre-swollen sephadex G-50 wt/vol (Sigma, Co. USA) was prepared in 0.85% normal saline and injected intra-peritoneal at the dose of 1mL/100g body weight. AEC were collected aseptically by washing the abdominal cavity with a sterilized normal saline (0.85%) combined with 0.5 U/mL of heparin. The AEC suspension was kept on ice in autoclaved, siliconized test tubes for settling of non-phagocytosed sephadex granules, while the supernatant was poured into another siliconized test tube and centrifuged (350 x g, 10 minutes). The MØ pallet was washed twice with RPMI-1640 (Sigma, Co. USA) without phenol red but with low endotoxins, and was later supplemented with heat inactivated 5% fetal calf serum and antibiotics. The MØ number 2.3x10^5/mL was maintained in RPMI-1640 by screening with trypan blue for their viability.

Preparation of Hyperimmune Sera

A hyper immune serum against SRBC was raised in broilers as previously described by Muhammad et al. (1994). Briefly, the SRBC were washed and a

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Stage of Molting</th>
<th>Feed</th>
<th>Water</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>Pre-molt</td>
<td>(120 g/bird/day)</td>
<td>Adlibitum</td>
<td>16 hr</td>
</tr>
<tr>
<td>68-69</td>
<td>Molting period with ZnO</td>
<td>(35 g/bird/day feed)</td>
<td>-do-</td>
<td>12 hr</td>
</tr>
<tr>
<td>70-73</td>
<td>Without ZnO</td>
<td>(50 g/bird/day feed)</td>
<td>-do-</td>
<td>12 hr</td>
</tr>
<tr>
<td>74*</td>
<td>Production</td>
<td>(70 g/bird/day feed)</td>
<td>-do-</td>
<td>14 hr</td>
</tr>
<tr>
<td>75 to Onward</td>
<td>Production</td>
<td>(120 g/bird/day feed)</td>
<td>-do-</td>
<td>16 hr</td>
</tr>
</tbody>
</table>

* At 74th week 8% of total birds started laying eggs. The time elapsed between laying two eggs was 2-3 days.
ND = New Castle Disease
EDS = Egg Drop Syndrome
5% suspension was prepared in phosphate buffer saline (PBS). The suspension was then mixed with an equal volume of Alsever’s solution (Sigma Co. USA) and stored at 4°C. On the first day, the SRBC suspension (0.25 mL) was injected subcutaneously under the wing of 5 broiler birds. The dose (0.5 mL) was repeated seven days later and a final dose (0.5 mL) was injected after fifteen days of the first inoculation. After seven days of the last inoculation, blood was collected and the serum was separated aseptically (centrifugation at 400 x g for 10 minutes) and kept in sterile tubes for further use at -20°C. Anti-SRBC antibodies in the sera were monitored by hemagglutination assay. The SRBC were opsinized as described by Qureshi and Miller (1991). Briefly, a 5% solution of heat inactivated antisera was coated with subagglutination concentration to SRBC. Following washing in PBS, the cells were adjusted to a 2.5% concentration in RPMI-1640 medium. A 2.5% solution of unopsonized SRBCs was separately made in RPMI-1640 medium.

**In vitro Phagocytosis Assay**

One milliliter of 2.3 x 10⁶ MØ suspension was placed in two separate sets of Perti dishes per bird (1 for unopsonized and 1 for opsonized) containing four cover slips each, and then incubated in RPMI-1640 with a 5% heat-inactivated fetal calf serum and antibiotics (100 U/mL penicillin and 50 μg/mL streptomycin), at 39°C with 5% CO₂ and 95% fresh air. After 2-h of incubation all the coverslips containing adherent MØ (indicating their livability) were gently gassed and then shifted to the fresh culture medium. The phagocytic ability of adherent MØ was determined using an in vitro 2.5% solution of unopsonized and opsonized SRBC inoculation into the culture medium for the study of engulfment % and engulfment/cell capacity of MØ. Cover slips were removed from the culture medium after 45 and 90 minutes of incubation, respectively. After incubation, the cover slips were removed and washed with 0.85% normal saline for removal of any free RBC. They were then fixed in methanol for 10 minutes, stained with Dip Quick (Bio. Whittaker, USA), and mounted on clean glass slides, after which 200 cells/cover slip were counted for morphological criteria and an engulfing percentage was calculated as follows:

\[ \text{Engulfing\%} = \frac{\text{Total cell engulfing in an area}}{\text{Total cell adherent in that area}} \times 100 \]

Moreover, all the coverslips were examined and the MØ adherent factor was calculated separately for each bird.

**Nitric Oxide (NO) Production Assay**

Nitrites (NO₃⁻) and nitrate (NO₂⁻) are stable decomposing byproducts of NO₂ and measurement of these products is widely used as an index of the amount of NO produced by cultured cells (Tracey, 1992). To estimate the concentration of NO, one of its principal reactive products, NO₃ was measured in the culture medium in which MØ were incubated by a colorimetric-based assay (Green et al., 1982). Briefly, the macrophage culture supernatant was transferred to 96 wells flat bottom plates, combined with Griess reagent [1% sulphanilamide (Sigma, Co. USA) and 1% naphthylethylene-diamine dihydrochloride (Merck, Co. Germany)] and incubated for 15 minutes at room temperature. The change in color was indicative of nitrate, and a nitrite presence was quantified by reading the plates at A₅₂₀ on an ELISA plate reader. An average of two measurements per sample was used in the final analysis. A standard curve for NO was determined by dissolving sodium nitrite in RPMI-1640 (without phenol red). NO concentration was determined with reference to a standard curve, generated with sodium nitrite (10–100 μmol/mL).

**Humoral Immunity**

Antibody titers against Newcastle Disease virus (NDV) and Egg Drop Syndrome virus (EDS) were measured using the methods (Raj et al., 2004; King, 2001) respectively. The birds were briefly immunized with NDV (La Sota strain) and EDS vaccines (76, virus strain). One week before molting, blood was collected from the brachial vein and the sera were inactivated at 56°C for 30 minutes. Antibody titers were then measured through a hemagglutination inhibition test, using standard concentrations of chicken RBC (4HAU) microtitration technique. For the conversion of logarithmic mean titers to geometric mean titers (GMT), the following formula was used (Thrusfield, 1999).

\[ \text{GMT} = (\text{Anti} \sum \log/n) \]
Concentration of Zn in Plasma

For the collection of plasma, blood was drawn by a wing vein puncture from six healthy chickens during each production phase. Plasma was then stored in ependorf at $-4^\circ$C for zinc estimation. Plasma and feed samples were subjected to wet digestion following the method of Richards (1968). After wet digestion, zinc concentration was measured by atomic absorption spectrophotometer (Perkin Elmer-405).

Statistical Analysis

The design used was Two Factorial Completely Randomized Design (CRD). The experiment data was analyzed using the mixed model procedure of SAS software (SAS, 1995). Differences among the means were determined using the Duncan Multiple Range Test (Duncan, 1955).

Results

MØ thus obtained by peritoneal cavity lavage was kept in RPMI-1640 medium for the assessment of their functional properties. The adherence properties of MØ remained non-significant at all production stages during the 2nd and 3rd production cycles (Fig. 1). Meanwhile, engulfing% of MØ increased significantly when co-incubated with opsonized SRBC as compared to unopsonized SRBC treatment. MØ engulfment% increased during all stages of the 2nd production cycle, as compared to the end of the 1st production, but later decreased significantly during the 3rd production cycle (Fig. 2). That decreased engulfment was appreciated in both the unopsonized and opsonized SRBC treated groups. Overall means of engulfment % was increased after 90 minutes of incubation, rather than 45 minutes after both kinds of treatments (Unopsonized and Opsonized SRBC co-incubation), but the highest value was observed at the peak of the 2nd production cycle that decreased during the 3rd production cycle with the increase in the bird’s age (Fig. 3). The numbers of SRBC engulfed were highest at the end of the second production cycle, and remained high overall among the opsonized SRBC treated group, except at the end of the first production cycle. The engulfed SRBC number remained lower and non-significant among all unopsonized SRBC co-incubated groups (Fig. 4). NO production was significantly high when MØ were treated with both LPS and SRBC for 90 minutes. Also, a gradual increase towards the 2nd production cycle and then a decrease in nitric oxide production was observed after all the treatments, as the birds aged towards their 3rd production cycles (Fig. 5). Humoral antibody response against NDV was found at higher levels than the EDS virus vaccine. Results showed that NDV antibody titer decreased from GMT (19.7) to GMT (9.8) at 5% production stage during the 2nd production cycle. Later, a graded increase was observed to GMT (32.2) at peak production stage and then declined

![Fig. 1. Macrophages adherence to glass cover slips at different stages of production after zinc induced molting. Bars are mean ± SE (n = 7). Prod = Production](image-url)
further at the end of production during the 2nd production cycle GMT (29.4), and decreased further during the 3rd production cycle, while an abrupt increase in GMT (32.0) was observed at the peak of the 3rd production cycle. Immune response against the EDS virus vaccine also showed a slight decrease...
in the level of GMT antibodies from the end of the 1st production (8.4) to the 5% production stage (5.3) during the 2nd production cycle. At the peak production stage, the GMT antibody titer enhanced to (19.7), and later a decrease in antibody response was recorded in a similar pattern, as was observed in the case of NDV detailed in (Fig. 6). The plasma concentration of zinc was highest at the peak of the 2nd production cycle, which started increasing with the increase in egg production, but declined at the end of production. The lowest concentration of zinc was at the end of the 1st production cycle, when compared to all the stages of the 2nd and 3rd production cycles (Fig. 7).
Discussion

Several cell types are responsible for antigen presentation to T cells, including Mϕ, B-lymphocytes, dendritic cells, and Langerhans cells. One important antigen-presenting cell is the macrophages. Mϕ are ubiquitously located within the body and are involved in the initiation of immune responses against microbial invaders and other malignancies by nature of their phagocytic, cytotoxic, and antigen-presenting capabilities (Adams and Hamilton, 1984). The ability of Mϕ to present antigen is crucial to immune function, and when this ability is compromised it results in an increased risk for morbidity and mortality due to infection (Cozen and Janeway, 1988; Polyak et al., 1997). Chicken Mϕ
express surface receptors for the Fc (Duncan and McArthur, 1978) and for C₃b (Dietert et al., 1991). Lower M₁ engulfment during the third production cycle may be due to the fact that, with the increase in age, innate and adaptive immune functions decline (Plowden et al., 2004). The capability of M₁ to engulf unopsonized and opsonized SRBC was unaffected during the second production, but further declines during the third production, possibly due to down regulation of the surface receptors. Our results are augmented by (De La Fuente et al., 2000; Tasat et al., 2003) that phagocytosis by M₁ is inversely proportional to an increase in age. Conventional (fasting) molting methods induce food deprival stress (Holt 1992), but molting with high levels of supplemented zinc reduces the uptake of feed (Johnson and Brake, 1992), and thus induces less stress on the birds. The overall response of M₁ from the second production cycle revealed much better results when compared to the end of the first and third production cycles. These results may be due to immunopotentiating effects of dietary zinc. Zinc induced molting ends up in egg production of 75.30%, egg weight 66.94 g and overall 1% mortality during 2nd production cycle. However, during 3rd production cycle, egg production was 71.10%, egg weight 56.68 g and over all mortality was 2.5% in zinc treated birds. The stress during molting results in more shedding of Salmonella enteritidis in feces (Holt, 2003), but Salmonella may not be present in every flock (Gama et al., 2003). Even upon examination of eggs from birds that were artificially infected with S. enteritidis showed no relationship between fecal carriage of S. enteritidis and the presence of bacterium in egg contents (Holt, 2003; Humphrey et al., 1991). Cumulative evidence shows that nitric oxide production is involved in the induction of T-cell immunosuppression during infection and killing of microorganisms (Nascimento et al., 2002). Our results indicated that nitric oxide production was significantly higher after 1.5 hours incubation with LPS, rather than without stimulation and SRBC stimulated M₁. This increase in nitric oxide production may be due to more responsiveness of M₁ to LPS. Furthermore, differences in M₁ nitric oxide production were associated with resistance or susceptibility to microorganisms (Tsat et al., 2003). Chicken INF-β, but not type-I INF, induces nitric oxide synthesis (Lowenthal et al., 1995). However, turkey INF-I acts with LPS to induce nitric oxide production from bone marrow M₁ (Suresh et al., 1995). The immune response evidently reflected immunosuppressive effects of the molting process at the 5% production stage of 2nd or 3rd production cycle. The stress due to molting leads to elevated levels of circulatory adrenal corticoids (Akram et al., 2002), which may also be the cause of an impaired immune response at that stage. Whereas, at peak production stages, the zinc-induced molting process was found efficient as it showed immunopotentiating effects, specifically on the NDV and EDS virus antibody responses in chickens. These results are inconsistent with previous report by Bartlett and Smith (2003) that diets supplemented with zinc tend to improve the ability of the birds to produce antibodies. The increase in antibody titers against NDV and EDS after zinc-induced molting may be due to the immunopotentiating effects of zinc. Some other findings state that the progeny from unsupplemented hens had reduced titers to sheep red blood cells, compared to those receiving the 10 and 20-ppm hens had reduced titers to sheep red blood cells, compared to those 10 and 20-ppm zinc treatments (Stahl et al., 1984). The difference in antibody production between NDV and EDS may be due to different kinds of invading antigens that have provoked different amounts of specific antibodies against them. The increase in zinc concentration during the 2nd and 3rd production was due to accumulation of zinc residues in bone and liver (Emmert and Baker, 1995), which was subsequently released for use during a period of zinc deficiency. With the onset of production, zinc concentration increased (Bakst and Richards, 1985) and maximum concentration was observed at peak production. Also we did not observe any toxic effects in our birds those were given zinc supplemented diet for induced molting.

Appropriate reactivity by innate and acquired immune systems that anticipate infection challenges may ensure disease resistance and, ultimately, longer survival and increased production. Genetic differences may also affect physiological and immunological responses in birds kept under severe feed restriction. Feed restriction in birds was found to affect the innate immunity, while the acquired immune response was not changed specifically against Mycobacterium butyricum and keyhole limpet hemocyanin type antigens (Hangalapura et al., 2005).

In conclusion, zinc induced molting may be the
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