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EFFECT OF DIETARY SUPPLEMENTAL LEVELS OF VITAMIN A ON THE PERFORMANCE AND IMMUNE RESPONSE OF HEAT-STRESSED BROILERS

(With 5 Tables and 4 Figures)

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تأثير إضافة مستويات من فيتامين أ علي الأداء والاستجابة المناعية في بداري التسمين المجهدة حراريا

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في هذه الدراسة تم اختبار تأثير إضافة فيتامين أ المصنع إلى علائق الدواجن من جنس الاربورايكر المرباة تحت درجة حرارة عالية علي كفاءة أداء هذه الطيور وكذلك استجابتها المناعية وذلك من خلال تجربة استمرت ٦ أسابيع. تم فيها تقسيم عدد ١٠٠ كتكوت عمر يوم إلى أربع مجموعات (٢٥ كتكوت/مجموعة). غذيت المجموعة الأولي علي عليقة أساسية مضافا إليها ٠,٥٢ مجم من أسيتات الريتينول /كجم عليقة لتغطية احتياجات الكتاكيت من فيتامين أ طبقا للاحتياجات المقررة بجداول الـ NRC (١٩٩٤) وأعتبرت كمجموعة ضابطة. وغذيت كل من المجموعات الثانية والثالثة والرابعة علي علائق احتوت عشرة وعشرين وثلاثين مرة من الاحتياجات الموصي بها في NRC (٥,٢ و ١٠,٤ و ١٥,٦ مجم اسيتات الريتينول/ كجم) علي التوالي. قدرت المقاييس الخاصة بتقييم أداء النمو وكذلك معدلات استهلاك العليقة وكفاءة التحويل الغذائي، ووجد أن المجموعة الثانية المغذاة علي عليقة بها عشرة أمثال قيمة الاحتياجات (٥,٢ مجم أسيتات الريتينول) تفوقت من حيث زيادة وزن الجسم بمقدار ١,١٨ مرة مثل المجموعة الضابطة مع تحسن ملحوظ في كفاءة التحويل الغذائي بينما سجلت المجموعات المغذاة علي علائق بها عشرون وثلاثون من أمثال الاحتياجات (١٠,٤ و ١٥,٦ مجم أسيتات الريتينول) معدلا أقل في مقدار الزيادة في وزن الجسم. وقد ارتفع تركيز فيتامين أ في مصل دم الطيور تدريجيا مع زيادة كمية فيتامين أ في العلائق. كذلك تم قياس معدل الاستجابة المناعية للطيور عن طريق تقدير كل من قيمة الأجسام المناعية لفيروس مرض النيوكاسل باستخدام اختبار تثبيط التخثر الدموي ودراسة المناعة الباثولوجية للطحال والبرسا وتحسس تزايد الخلايا الليمفاوية من نوع B الإضافة إلى

صورة الدم وقد أظهرت النتائج زيادة أعداد الخلايا الليمفاوية وارتفاع نسبة الأجسام المناعية في اختبار تثبيط التخثر الدموي بالإضافة إلى زيادة أعداد كرات الدم الحمراء والبيضاء في مجموعات الطيور المغذاة علي علائق محتوية عشرة وعشرون أمثال الاحتياجات (٥,٢ و ١٠,٤ مجم اسيتات الرتينول). من هذه الدراسة نستخلص أن زيادة كمية فيتامين أ في العلائق إلى عشرة أمثال الموصي به في NRC له أثر فعال علي معدل الأداء والاستجابة المناعية في بداري التسمين المرباة تحت درجة حرارة عالية.

SUMMARY

The experiment was conducted to evaluate the effect of vitamin A (Retinyl acetate, RA) supplementation of a broiler diet on the performance and immune function of heat-stressed chicks. One hundred, one- day old of Arbor Acre chicks were randomly distributed into 4 equal groups (25 chicks/group). The first group was fed on the basal diet supplemented with 0.52 mg RA/kg diet to cover vitamin A recommendation of NRC (1994), and considered as a control group. The NRC recommendation of vitamin A was folded ten times (5.2 mg RA/kg), twenty times (10.4 mg RA/kg) and thirty times (15.6 mg RA/kg) in the diets of the second, third and fourth groups, respectively. The growth performance, feed intake and feed conversion were assessed. The second group fed on 5.2mg RA-supplemented diet achieved a body weight 1.18 times that of the control group at the end of the experiment, while the chicks of groups 3 & 4 supplemented with 10.4 and 15.6 mg RA scored lowest gain. Supplementation of 5.2 mg RA to the diet of chicks pointed to improved feed conversion compared to the other treated groups including the control one. Increased concentrations of vitamin A in the serum of the experimental broilers increased gradually with increasing dietary RA intake. The immunological assays were achieved by determining the antibody titers to the Newcastle disease virus using hemoagglutination inhibition test (HI), immunopathological studies of the spleen and bursa detecting the B-lymphocyte proliferation in addition to hematological picture. The results indicated that the 5.2 mg RA/kg supplementation (group 2) followed by 10.4 mg RA/kg (group 3) recorded the highest values for HI titers compared to the control. The best activation of cell reactions (B-cells) was pronounced in both groups (groups 2 & 3) supplemented by 5.2 and 10.4 mg RA/kg diet compared to other treated groups. The haematological picture showed significant ($P<0.05$) increase in the total erythrocytic and leucocytic cells count with a marked increase in the percentage of lymphocyte and monocytes in the chicks fed on diets supplemented with

5.2 and 10.4 mg RA/kg as well as control group. The results of the present study suggested that supplementation of vitamin A of ten times than NRC recommendations to diets of broilers reared under heat-stress was beneficial to broiler performance and immune function.

Key words: *Vitamin A, performance, immune response, heat stress, broilers.*

INTRODUCTION

Animals are subject to stress from a number of sources, among them management/husbandry practice, nutrition and environment. For poultry in tropical countries, environmental temperature is one of the major stressor because this can range from 30-43°C for prolonged periods. Routine management practice (medication and vaccination) also added to stress (Rao *et al.,* 2003). The interaction between feed intake, environmental temperature, and performance is of utmost importance in formulation of poultry diets for selection of economically optimal combination of nutrition and environmental temperature. High environmental temperatures have deleterious effects, reducing the performance of poultry. A decreased rate of growth was reported in broilers reared at high environmental temperatures (Donkoh, 1989). This negative effect of heat stress on growth rate and production is speculated to be due primarily to reduced feed intake and subsequently body weight gain and feed efficiency (Hurwitz *et al.,* 1980).

Several methods are available to alleviate the negative effects of high environmental temperature on the performance of poultry. Since it is expensive to cool animal buildings, such methods are mostly focused on the dietary manipulation. In this respect, vitamin C, vitamin E and vitamin A are used in the poultry diet because of their anti-stress effects and also because their synthesis is reduced during the heat stress (Sykes, 1978; Hornig *et al.,* 1984; Sahin *et al.,* 1999, 2001$_{a \& b}$ and Naziroglu *et al.,* 2000). Factors like disease, environmental stress due to temperature and/or humidity may increase the requirement of vitamin A, which is relatively unstable under tropical conditions (Christensen, 1983). More recently new functions of vitamin A have been discovered such as its effect on immune response, cell differentiation, proliferation and morphogenesis by means of modulating gene expression (Boliag, 1996 and Gerster, 1996). Immune responsiveness increases as an animal's immune system matures and the immune system of chicks becomes competent at about three weeks of age (Lee and Bacon, 1983).

The NRC (1994) recommended 1500 IU/kg diet of vitamin A for broilers, but higher levels than this are commonly used. Disease and heat stress reduced performance of broilers, a situation that was partially offset by the utilization of enhanced vitamin fortification over NRC and industry average levels (Ferket and Qureshi, 1992). On the other hand, Raza *et al.* (1997) found that sufficient vitamin A in the diet maintained the epithelium of gastro-intestinal tract which in turn helped in maximum absorption of nutrients, resulting in improved weight gain, feed consumption and feed conversion. There is strong evidence that vitamin A enhances the immune response in poultry as tested with model antigens, with vaccination against major pathogens or with infection of birds and the antibody contents depends on the dose of vitamin A in the diet (Leutskaya and Fais, 1977).

The present study was conducted to determine the effects of supplementation of dietary vitamin A levels on performance and immune response of broiler chickens reared under heat-stress.

MATERIALS and METHODS

1- Birds:

One hundred (one day old) of Arbor Acre chicks were obtained from a local hatchery. The chicks were nearly of a uniform weight, averaging 30g, where they were randomly distributed into four equal groups (25 chicks/each). The chicks were floor reared in a hygienic room of four compartments, bedded with wheat straw. Fresh and clean water was supplied ad libitum throughout the experimental period which lasted for 6 weeks. The experiment was carried out at the Department of Animal and Clinical Nutrition, Fac. of Vet. Medicine, Assiut University on July and August which are two of the hot summer months in Upper Egypt. The birds were subjected to heat stress, during which the maximum daytime temperature and relative humidity were 39 °C and 69% and the maximum nighttime temperature and relative humidity were 34 and 67%.

The chicks were individually weighed and food consumption was recorded every week. During the experiment, the chicks of the four groups were equally cared for, and clinical signs of significant importance, if any, were recorded.

Birds were vaccinated against Newcastle using Hitchner B1 strain in drinking water at the age of 5 days, while Lasota vaccine was used at the age of 17, 27 and 35 days. At the age of 4 and 6 weeks, five

chicks were randomly chosen from each group for slaughtering and immunity studies.

2- Diets:

Two basal diets, starter and grower-finisher were formulated. The starter diet was fed for the first 3 weeks, while the grower-finisher one for the last 3 weeks of the experimental period. The physical and the calculated chemical compositions of the basal diets are illustrated in Table (1). Vitamin A (Retinol equivalents: 1 IU vit.A = 0.344 μg retinyl acetate, NRC, 1994) was added separately as retinyl acetate, RA (Hoffmann La Roche Company, Egyptian agency). The first group was fed on the basal diet supplemented with 0.52 mg RA/kg, to satisfy the needs recommended and stated by the NRC (1994) for chicks and considered as a control group. The second, third and fourth groups were fed on the basal diet supplemented with ten (5.2 mg RA/kg), twenty (10.4 mg RA/kg) and thirty times (15.6 mg RA/kg), respectively of the NRC recommendation for vitamin A. The basal diet was formulated using NRC (1994) guideline and contained 20-23% protein and 3200 kcal/kg metabolizable energy.

3- Determination of vitamin A:

The estimation of vitamin A in the blood serum was carried after Carr and Price (1926).

4- Immunological Assays:

4- 1. Serum:

At the end of the experiment, a blood specimen was collected from each of the slaughtered chicks in the four groups. The blood samples were allowed to clot at ambient temperature, centrifuged for 10 minutes at 3000 rpm, and serum was separated. The serum samples were kept frozen at –20°C until immunity parameters were measured.

4- 2. Blood picture:

Both total (Lucas and Jamoz, 1961) and differential blood cell counts were determined. Differential counts were made by screening Giemsa-stained slides (Hudson and Hay, 1976).

4- 3. Haemoagglutination inhibition test (HI):

It was applied as a rapid mean for measuring the immune response of the birds to Newcastle vaccination. The antibody titers in response to NDV were assessed by ten-two fold serial dilutions method

for antisera against Lasota strain of NDV as described by Beard and Wilkes (1973).

5- Immunopathological examination:

The organs having immunological importance were removed from the bird carcasses in the different groups (spleen and bursa of fabricious). The organs were stored in Zinker's formal solution for immunopathological examination. Several sections from all the samples were prepared and stained with heamatoxlin and eosin for routine histological examination according to that cited by Bancroft and Stevens (1977). B-lymphocytes were detected by using Alkaline phosphatase reaction (Gomori method, 1952).

6- Statistical analysis:

Statistical analysis of the collected data was carried out according to procedures of completely random design, SAS (1995).

RESULTS and DISCUSSION

1- Performance of chicks:

The performance of the chicks in the different groups as reacting to the vitamin A supplementation was evaluated through the body weight development, feed intake and efficiency of conversion during the experimental period. Tables (2 & 3) summarized the results.

There were significant differences ($P<0.05$) between different treated groups in the mean values of body weight during the experimental period. The chicks of second group fed on 5.2mg RA-supplemented diet (ten times the recommended level) achieved a body weight (2100g) 1.18 times that of the control (1786g) group at the end of the experiment, while the chicks of group 4 supplemented with 15.6 mg RA scored lowest body gain 1500g (84% of the control). Sahin *et al.* (2001) and Kucuk *et al.* (2003) reported that vitamin A supplementation (15,000 IU retinol/kg diet) to the diet of broiler under heat-stress resulted in an improved live weight gain and feed efficiency.

Making consideration for the average initial weight of the chicks in each of the four groups, it was found that groups 2 and the control surpassed 70 and 60 times, respectively, while groups 3 & 4 were the lowest (50 times). Eventually, it could be concluded that high levels of vit.A (10.4 and 15.6 mg RA/kg) has a bad effect on body weight. This agreed with that reported by Vahl and van Klooster (1987) who found that excessive intake of vit.A (63 mg RE/kg) had a negative effect on the

body weight of broilers. Sklan (1983) found that high levels of dietary vit.A reduced overall absorption of vit.A and enhanced the duodenal flow of retinyl glucuronide.

It seems that the improving effect of vit.A on body gain as a reflection of increased food consumption and/or improved feed conversion (Table, 3).

Calculating the amount of food consumed by the chicks of different groups, the utmost consumption was achieved by the second group supplemented with 5.2 mg RA (3893 g/chick) compared to other groups. The chicks of group 2 fed on diet supplemented with ten times of the recommended level had improved feed conversion (1.88 kg feed/kg gain) compared to other groups as it reaches 2.17, 2.36 and 2.53 for the control and groups 3 and 4, respectively.

For a conclusive recommendation, it could be stated, as far as it is tested, that supplementation the diets of chicks with vit.A caused improvement in live weight gain and feed intake, if it did not exceed the recommended level by more than ten times, probably alleviating the negative effects of heat stress on broilers under heat stress. Increasing the added amount will not add extra-effect but on the contrary, it has a bad effect as found by Vahl and van Klooster (1987).

2- Serum vitamin A levels:

There was significant ($P<0.05$) differences in the concentration levels of vitamin A in the serum of the experimental groups (Table, 4). The concentration of the vitamin A was increased by 13.51%, 102.7% and 143.24% in the serum of chick groups 2, 3 and 4, respectively than the control group. The vitamin level was gradually increased in the serum of the experimental broilers as the dietary vit.A increased. This result agreed with that found by Sklan *et al.* (1995) who found that plasma vitamin A was increased as the dietary intake of vitamin A increased in turkey diet from 0 to 13.2 μg/g retinol equivalents. It was observed that serum vitamin A level significantly decreased during heat stress in laying hens (Sahin *et al.*, 1999).

3- Immunological Assays:

Regarding the immune response to NDV, according to Beard and Wilkes (1973), the HI test is generally considered to be reliable, economical and rapid means of measuring the immune response of poultry to Newcastle vaccination. There were significant differences ($P<0.05$) between the experimental groups at HI titers in response to synthetic vit.A supplementation at the 3rd and 6th week of age (Table, 4).

The immuno-enhancing effect of synthetic vit.A was confirmed by this test, especially the 5.2 mg RA/kg (group 2) followed by 10.4 mg RA/kg (group 3) which recorded the highest values for HI titer compared to the control. Lin *et al.* (2002) found that vitamin A supplementation (12,000 IU retinol/kg diet) had a significant effect on NDV antibody titer in the heat-stressed laying hen. One study showed that 6.66 mg/kg dietary vit.A enhanced antibody titers against NDV (Serman and Mazija, 1985). Sklan *et al.* (1995) reported that dietary vit.A supplementation up to 6.6 mg/kg enhanced immune response parameters, whereas higher levels (13.2 mg) depressed immune responses. For both domestic fowl and turkeys, Friedman *et al.* (1991) and Sklan *et al.* (1995) have observed that increased immune responses (resistance to disease, T lymphocyte proliferation and antibody production) were routinely attained with dosages of 5-10 mg vit.A/kg diet. Vitamin A levels less or higher than this range proved to be suboptimal for immune responses (Friedman and Sklan, 1997). Excess vit.A intake also impaired immune responsiveness in poultry and turkeys (Friedman and Sklan, 1989_b and 1997; Friedman *et al.*, 1991 and Sklan *et al.*, 1995). The impaired immune responses were similar to those observed following low intakes of vitamin A, but the degree of impairment was higher.

The intensity of B-lymphocytic population in spleen and bursa of fabracieous was estimated using alkaline phosphatase reaction. The best activation of B-cell reaction was pronounced in groups 2 & 3 fed on diets supplemented by 5.2 and 10.4 mg RA/kg diet, respectively. The activated B-cell, both nuclear and cytoplasmic took blackish discoloration. In group 2, there was marked increase of the B-cell reaction especially in spleen in which B-cell was observed in the white pulp around the central artery (Fig.3) and in the bursa at the margin of the follicles (Fig.4) compared to the control group (Fig. 1 and 2). The antigenic responses of lymphocytes were increased by dietary vit.A supplementation at low or moderate levels, however, the latter responses decreased when vit.A was supplemented at higher levels (Halevy *et al.*, 1994). The lymphocyte proliferation response was depressed with low dietary vit.A (Friedman and Sklan, $1989_{a,b}$). Lymphocyte proliferation was maximal when the dietary vit.A intake was higher (2-6 μg/g) than NRC recommendation as reported by Friedman and Sklan ($1989_{a,b}$) and Sklan *et al.* (1994, 1995).

The haematological picture of the experimental groups (Table, 5) showed significant ($P<0.05$) increase in the total erythrocytic and leucocytic cells count with marked increase in the percentage of

lymphocyte and monocytes in the chicks fed on diets supplemented with 5.2 or 10.4 mg RA/kg (groups 3 & 4) as well as control group, a matter which indicate that vitamin A affect hematopoiesis both at any level with a decreased number of white blood cells at high level of vitamin A.

This result agreed with that reported by Friedman and Sklan (1993) who reported decreased number of leucocyte at high dietary intake of vitamin A. As a collective end result, this work adds more information on the positive effect of vitamin A on the performance and immune function of broiler chicks reared under heat-stress. The high performance represented by high rate of growth, increased feed intake and improved feed conversion was attained by folding the NRC recommendation ten times. From economical point of view, the high potentiating levels could be only adviced in case of need as in times of heat stress or in vaccination.

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LIST OF FIGURES

Figure 1: Spleen from the control group showing few B-cell reaction, which take blackish discoulouration. Alkaline phosphatase reaction. 10 × 25.

Figure 2: Bursa from the control group showing few B-cell reaction, which take blackish discoulouration. Alkaline phosphatase reaction. 10 × 25.

Figure 3: Spleen from group 2 fed on diet supplemented with 5.2 mg RA/kg showing an increase in the number of activated B-lymphocytes. Alkaline phosphatase reaction. 10 × 10.

Figure 4: Bursa from group 2 fed on diet supplemented with 5.2 mg RA/kg showing an increase in the number of activated B-lymphocytes. Alkaline phosphatase reaction. 10 × 10.

Table 1: Composition (%) of the basal diet

Ingredient	Diet	
	Starter	Grower-finisher
Physical composition:		
White corn, ground	45.62	58.80
Soybean meal, 44%	36.96	27.55
Fish meal	4.00	4.00
Dried fat	9.99	6.78
Dicalcium phosphate	1.42	0.87
Limestone, ground	1.20	1.31
Common salt	0.41	0.37
Mineral mixture[1]	0.10	0.10
Vit.B+K3+Choline[2]	0.10	0.10
Muvco D3[3]	0.10	0.10
DL-methionine	0.10	0.02
Chemical composition:		
Crude protein, %	23.02	20.00
ME, Kcal/kg	3200	3200
C/P ratio	139.1	160.0
Methionine, %	0.50	0.37
Lysine, %	1.33	1.10
Calcium, %	1.01	0.88
Total phosphorus, %	0.69	0.58
Available phosphorus, %	0.47	0.35

[1] Muvco mineral mixture: Each kg contains Mn 60g, Zn 45g, Fe 30g, Cu 5.0g, I 0.5g, Co 0.2g, Se 0.1g and calcium carbonate up to 1000g.

[2] Vitamin B+K3+Choline (Muvco): Each kg contains niacin 20g, vit. B2 4.5g, vit.B6 3.0g, vit.B12 13.0mg, vit.K3 2.0g and choline chloride 100g.

[3] Muvco D3: Each kg contains vit.D3 5,000,000 IU.

Table 2: Body weight development (g) for chicks of the four groups.

Age in weeks	Groups			
	1	2	3	4
Body weight (g)				
0	29.5	30.0	31.0	29.8
1	120±3.50 [a*]	126±5.31 [a]	111±4.50 [a]	113±6.35 [a]
2	332±4.50 [a]	339±5.00 [a]	307±5.10 [ab]	281±4.80 [b]
3	702±8.40 [a]	740±7.58 [a]	611±9.10 [b]	572±7.80 [c]
4	1124±10.15 [ab]	1194±11.5 [a]	1050±9.60 [b]	939±8.20 [c]
5	1356±9.50 [a]	1429±8.12 [a]	1266±10.12 [b]	1141±6.75 [c]
6	1786±11.10 [b]	2100±10.30 [a]	1563±8.90 [c]	1500±12.10 [c]
Times the initial	60.54	70.00	50.42	50.34

* Figures in the same row having the same superscripts are not significantly different (P<0.05)

** Mean±SE

Table 3: Performance of chicks of the four groups.

Age in weeks	Groups			
	1	2	3	4
Body weight gain (g)				
0-1	90.5±3.15 [a*]	96.0±3.50 [a]	80.0±2.10 [b]	83.2±2.80 [b]
1-2	212±3.80 [a]	213±3.10 [a]	196±2.64 [b]	168±2.20 [c]
2-3	370±4.10 [b]	401±3.80 [a]	304±4.15 [c]	291±2.83 [c]
3-4	422±4.15 [b]	454±3.90 [a]	439±4.10 [ab]	367±3.40 [c]
4-5	232±3.16 [a]	235±3.40 [a]	216±2.89 [ab]	202±3.10 [b]
5-6	430±5.12 [b]	671±4.89 [a]	297±3.15 [d]	359±3.01 [c]
Total (0-6)	1756.5 [b]	2070 [a]	1532 [c]	1470.2 [c]
Feed intake (g)				
0-1	131	117	92	104
1-2	333	282	280	288
2-3	568	544	528	513
3-4	786	793	800	807
4-5	993	900	760	997
5-6	999	1257	1152	1015
Total (0-6)	3810	3893	3612	3724
Feed conversion				
0-1	1.45	1.22	1.15	1.25
1-2	1.57	1.32	1.43	1.71
2-3	1.54	1.36	1.74	1.76
3-4	1.86	1.75	1.82	2.20
4-5	4.28	3.83	3.52	4.94
5-6	2.32	1.87	3.88	2.83
Total (0-6)	2.17	1.88	2.36	2.53

* Figures in the same row having the same superscripts are not significantly different ($P<0.05$).

Table 4: Serum vit.A concentration and antibody titers against Newcastle disease virus of the four groups.

Items	Groups			
	1	2	3	4
Vit.A conc. (μg / ml)	0.37±0.001 ^{c*}	0.42±0.001 ^{c}	0.75±0.002 ^{b}	0.90±0.001 ^{a}
Antib.titers(log 2) (age in week)				
3	3.75±0.25 ^{a}	4.25±0.96 ^{a}	4.00±0.50 ^{a}	3.50±0.58 ^{a}
6	4.50±0.33 ^{b}	7.00±0.80 ^{a}	5.00±0.35 ^{b}	4.25±0.38 ^{b}

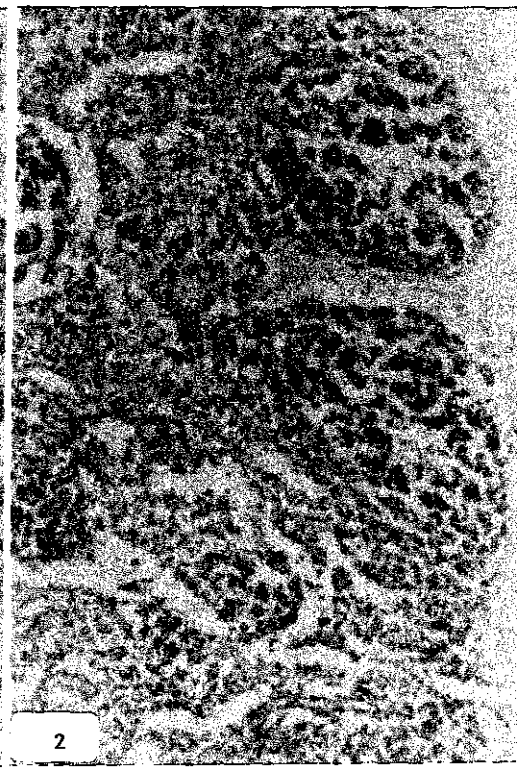
* Figures in the same row having the same superscripts are not significantly different (P<0.05).

Table 5: Hematological picture of chicks of the four groups.

Blood cells	Groups			
	1	2	3	4
Total erythrocytes ($\times 10^6$ / μl)	3.81±0.09 ^{a*}	3.95±0.07 ^{a}	4.13±0.05 ^{a}	3.8±0.17 ^{a}
Total leucocytes ($\times 10^3$ / μl)	11.5±0.11 ^{a}	11.7±0.01 ^{a}	12.1±0.03 ^{a}	8.7±0.05 ^{b}
Heterophils (%)	30±0.13 ^{a}	28±0.16 ^{b}	25±0.17 ^{b}	33±0.23 ^{a}
Lymphoctes (%)	54±0.30 ^{a}	56±0.18 ^{a}	59±0.25 ^{a}	48±0.29 ^{b}
Monocytes (%)	12±0.11 ^{ab}	13±0.13 ^{a}	13±0.16 ^{a}	9±0.02 ^{b}
Eosinophils (%)	3±0.01 ^{b}	3±0.01 ^{b}	2±0.03 ^{b}	8±0.01 ^{a}
Basophils (%)	1±0.009	0±0.00	1±0.009	1±0.002

* Figures in the same row having the same superscripts are not significantly different (P<0.05).

1



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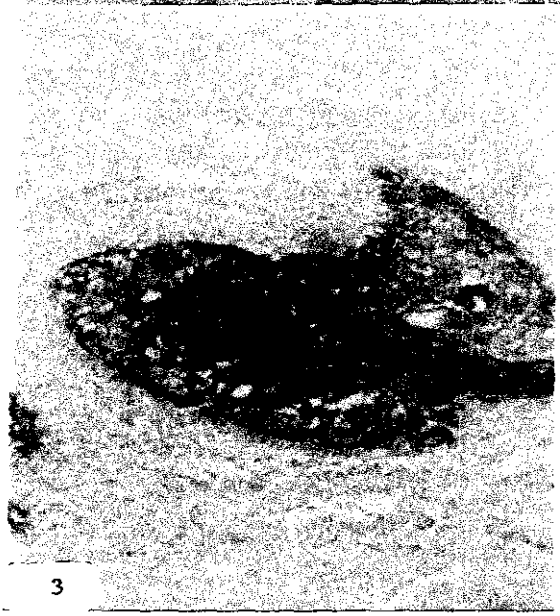

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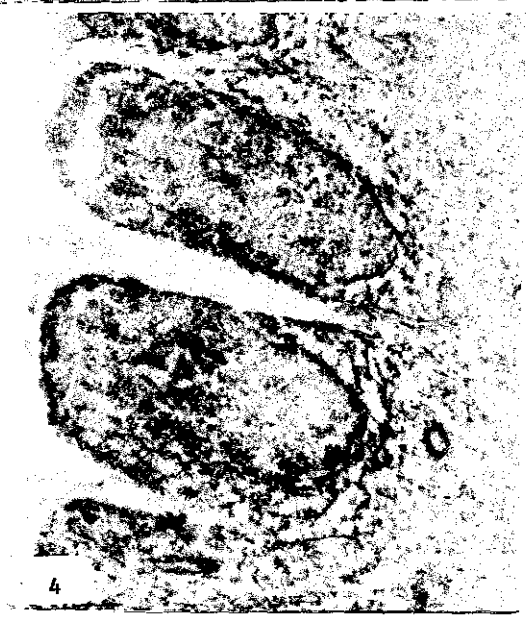

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