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IMMUNOMODULATION OF ACIDIFIERS IN CYCLOPHOSHAMIDE TREATED CHICKENS

M. H. H. AWAAD, F. E. SAAD, O. A. ELSHAZLY, Manal A. AFIFY, SAHAR A. ZOUELFKAR and HALA F. EL MENYAWI

Faculty of Veterinary Medicine, Cairo University.

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SUMMARY

In order to challenge the immunopotentiation of the acidifier Nutrilac versus vitamin E, 3 replicate laboratory controlled trials were carried out in cyclophosphamide (CP) immunosuppressed chickens.

The immunosupressing effect of CP on cell mediated and humoral immunity proved to be effective with severe dramatic effect on serum transferrin (Tf) and immunoglobulins (IgG and IgM). Marked alterations in the morphologic features of major lymphoid organs and their weight / body weight indices were evident.

The immunoassay revealed that Nutrilac strongly sustained the production of anti-sheep red blood cells (SRBCs) haemagglutinating (HA) antibodies 7 days post immunization till the end of the experiment with a geometric mean ranging between 2.1-5.5. While vitamin E humbly sustained the HA titer at 14 and 21 days post immunization with a geometric mean of only 0.5 for each. Administration of Nutrilac and vitamin E highly improved the level of serum Tf, IgG and IgM during the entire period of the experiment. They also significantly increased stimulation indices of lymphocyte transformation 7-14 days post immunization with SRBCs till the end of experimental period (21 days) provided that Nutrilac was superior to vitamin E. Bursal / body weight index was significantly increased at 14 and 21 days post treatment with Nutrilac and at 21 days post treatment with vitamin E over CP immunosuppressed non-treated control chickens.

Histomorphologic features of bursa of Fabricus, thymus gland and spleen were markedly improved with Nutrilac and vitamin E by post

treatment day 7 and onwards. The improvement was more obvious in Nutrilac treated chickens. Protection percentage against E.coli challenge in the bioassay study reached 80 % or 68.9% in CP immunosuppressed chicken groups immunopotentiated with Nutriulac or vitamin E respectively as compared with 48.9 % in immunosuppressed non-immunopotentiated group.

INTRODUCTION

Potentiation of normal immune response in poultry occurs by alteration in any step involved in the hosts immunologic reaction either in the classic humoral or in the cell-mediated system (Afify, 1990). The routine use of drugs to modify or modulate an animals immunocompetence as part of the therapeutic management of specific clinical conditions is still at a very preliminary stage in veterinary medicine (Brander et al., 1991).

It is undoubtedly true that factors contributing to immune-suppression would lead to immunodeficiency. The latter is a hazard-anticipating causative agent of serious economic impacts in poultry industry allover the world. Recognition and scientific identification of factors encountered in immune deficiency have lead to increased perusal investigation in the counterattacking modulators to accomplish immune-stimulation. Awaad et al. (1999,b) proved the immunopotentiation of the weak organic acids preparation known as Nutrilac produced by NUTRI-AD (Belgium) for chickens using both immuno and bioassays as criteria. The aim of this study is to challenge the immunopotentiating effect of this acidifier through its administration to immunosuppressed chickens with cyclophosphamid.

MATERIAL AND METHODS

Nutrilac. Nutrilac liquid produced by NUTRI-AD International, Belgium, lot No. NLL 9803 was used.

Vitamin E. 10% vitamin E selenium was used.

Experimental chickens. A total of 324, day-old meat type chickens were divided into 8 groups. The first 7 groups (1-7) were consisting of 45 each and subsequently divided into 3 subgroups as replicates. While the last 8th.group was consisting of 9 birds. All birds were housed in separate, wire-floored pens and fed on a commercial balanced ration ad libitum. The chickens were vaccinated against Newcastle disease using Hitchner and La Sota vaccines at 5 and 18 days of age respectively.

Haemagglutination test. This was carried out after Anon (1971).

Lymphocytic transformation test. This was applied after Charles et al. (1978) and Lucy (1984). Separation of lymphocytes was adopted after Böyum (1968). Determination of viable cell

number was carried out according to Hanks and Wallace (1958). Culturing of lymphocytes was performed as described by Confer et al (1981) using phytohaemagglutinin-P at a concentration of 10 µg / well. Evaluation of lymphocyte blastogenisis response using modified MTT dye uptake assay was adopted after Gärn et al. (1994). The response of lymphocyte was given in terms of stimulation index according to Carpenter et al (1978).

Polyacrylamide gel electrophoresis (PAGE). PAGE was carried out as described by Maurer (1971) using the alternative method with gel system No.1 a (pH 8.9 7 %).

Statistical analysis. This was adopted after Snedecor (1956) and Cochran and Cox (1960).

Experimental design. Chickens of groups 1-6 were immunosuppressed after Jones et al. (1992) by subcutaneous inoculation of 4 mg / bird with cyclophosphamide (CP) (Endoxan, ASTA Medica AG, Germany) in sterile distilled water at one, two and three days of age. Birds of groups1-2 and 3-4 received Nutrilac and vitamin E in a dose of 3 ml and one ml/l drinking water at 1-5 days of age respectively.

For immunoassay; chickens of groups 1, 3, 5 and 7 were immunized intramuscularly at 2 day-old with SRPCs suspension in a dose of 10 mg / bird. Individual blood samples were taken from the immunized groups by heart puncture at 3, 7, 10,14 and 21 days post inoculation. Blood samples were subjected to lymphocyte transformation test. Serum samples were also separated for HA test and equal samples from each group were pooled for PAGE analysis. Feed consumption and final body weight of birds of groups 1, 3, 5 and 7 were carried out at the end of the crop (42 days) for feed conversion ratio (FCR) determination. Three chickens out of each group were sacrificed at 3 days post CP treatment as well as 3, 7, 14 and 21 days post SRBCs immunization. Bursa of Fabricus, spleen and thymus gland were weighted for determination of the relative bursal, spleen and thymus weight indices after Sharma et al. (1989) by the following equation: organ weight in grams X 1000 / total body weight in grams.

For histomorphological examination; specimens kept in 10 % formol saline including bursa of Fabricius, thymus gland and spleen were collected from chickens of the immunized chicken groups as well as from the blank one at 3, 7, 14 and 21 days post immunization.

For bioassay; chickens of groups 2, 4 and 6 were subcutaneously challenged with 10^8 cfu of E.coli serogroup O78 at 12 day-old and were kept under

observation for clinical signs and mortality for 3 weeks.

RESULTS

Obtained results are shown in tables 1-3 and Figs 1-12.

Three days post-treatment with CP, the histomorphologic changes consisted of mild to moderate lymphoid depletion in the spleen and the medulla of the thymic lobules and an almost complete depletion of lymphocytes in the cortex and medulla of the bursal follicles with only reticular cells remaining (Fig. 10A and B). Similar changes were observed in chickens treated with CP and immunized with SRBCs and those immunized and treated either with vitamin E or Nutrilac.

By the day 7, the bursal follicles of CP treated chickens remained atrophied with fibrous thickening of the interfollicular stroma and disappearance of Lymphocytes with only reticular cells were remaining (Fig. 10C). The spleen and thymus of such birds showed lesions similar to those observed 3 days post immunization. In birds treated with either vitamin E or nutrilac, activated lymphoblast cells began to appear in some bursal follicles (Fig.10D) and the medulla and cortex of the thymic lobule (Fig.11A).

The bursal follicles 14 days post CP treatment were markedly atrophied and were widely separated by fibrous stroma (Fig. 11B) and completely depleted from lymphocytes and showed vaculation (Fig.11C). In birds treated either with vitamin E or nutrilac, an increasing in number of lymphoblasts and lymphocytes appeared in some follicles (Fig. 11D). These changes were more obvious in nutrilac treated chickens. The spleen and thymus of such birds were nearly normal, whereas those of CP treated chickens and those post-immunized with SRBCs showed moderate depletion of lymphocytes.

Twenty-one days post CP treatment, lesions in the bursal follicles were more progressive, they were markedly atrophied, fewer in number and were widely separated by thick fibrous interfollicular stroma which appeared edematous in many areas (Fig.12 A). The bursae of birds treated either with vitamin E or Nutrilac showed numerous lymphoid follicles filled with lymphocytes particularly in the latter (Fig. 12B). Lesions observed in the spleen and thymus were similar to those found in day 14-post treatment with CP.

The thymus, spleen and bursa of Fabricius of non-treated, nonimmunized control chickens appeared normal throughout the period of the experiment and contained great numbers of lymphocytes (Fig. 12C and D).

Table 1: Immunomodulatory effect of Nutrilac and vitamin E on lymphocyte transformation, haemagglutinin antibody response and feed conversion ratio (FCR) of cyclophosphamide (CP) treated chickens immunized with sheep red blood cells.

Gr. No.	Treatment			Stimulation index of lymphocyte transformation measured by MTT (Days post immunization)				Haemagglutinin antibody titer (Days post immunization)					Final Body Wt.	Feed Consumption	FCR
	Imm. Stim.	Sheep RBCs	CP	0	3	7	10	14	21	0	3	7	10	14	21			
1	Vit. E.	+	+	1.31± 0.1	0.79± 1.2	1.00*± 0.6	1.09*± 0.76	1.26*± 0.09	1.19± 0.73	0	0	0	0	0.5	0.5	1479.3± 24.1	3670.40	2.48
3	Nutrilac	+	+	1.31± 0.1	0.80± 1.5	1.08*± 0.7	1.28*± 0.91	1.34*± 0.55	1.14± 1.2	0	0	2.1	4.9	5.5	5.3	1501.5± 11.26	3699.50	2.46
5	Positive control	+	+	1.31± 0.1	0.70± 0.7	0.80± 0.9	0.86± 0.65	0.99± 1.2	1.03± 0.92	0	0	0	0	0	0.5	1332.1± 9.90	3153.99	2.36
7	Negative control	+	-	1.31± 0.1	1.2± 0.04	1.19± 0.32	1.3± 0.014	1.2± 0.247	1.08± 0.024	0	3	5.1	5.6	5.5	4.5	1393± 30.1	2950.9	2.11

* = Significant increase at $P \leq 0.05$

Table 2: Effect of Nutrilac and vitamin E on weight indices of lymphoid organs of experimented chickens.

Gr. No.	Treatment			Time of testing											
				Days post sheep RBCs. immunization											
	Imm. Stim.	Sheep	CP	3 days post CP treatment			3			7			14			21	
				B/ BWt	Sp/ BWt	T/ BWt	B/ BWt	Sp/ BWt	T/ BWt	B/ BWt	Sp/ BWt	T/ BWt	B/ BWt	Sp/ BWt	T/ BWt	B/ BWt	Sp/ BWt	T/ BWt
1	Vit. E.	+	+	1.8	3.4	7.8	2.1	3.1	9.2	3.9	6.1	10.7	3.6	3.4	11.7	6.3*	5.1	18.9
3	Nutrilac	+	+	1.8	3.4	7.8	2.0	3.8	9.1	3.9	6.2	11.3	3.9*	4.9	14.7	7.1*	6.3*	21.4
5	control +ve	+	+	1.8	3.4	7.8	2.2	3.2	9.3	3.1	6.3	10.6	3.1	3.6	12.3	5.5	5.1	17.3
7	control -ve.	+	-	9.57	3.05	13.03	12.77	14.7	12.24	17.67	10.41	34.57	19.84	7.48	32.61	25.07	10.02	29.01

B/B.Wt. = Bursa/body weight index. SP/B.Wt.= Spleen/Body weight index.

Imm. Stim. = Immune stimulant. T.B.Wt.= Thymus/Body weight index.

* = Significant increase at $P \leq 0.05$

CP = Cyclophosphamide

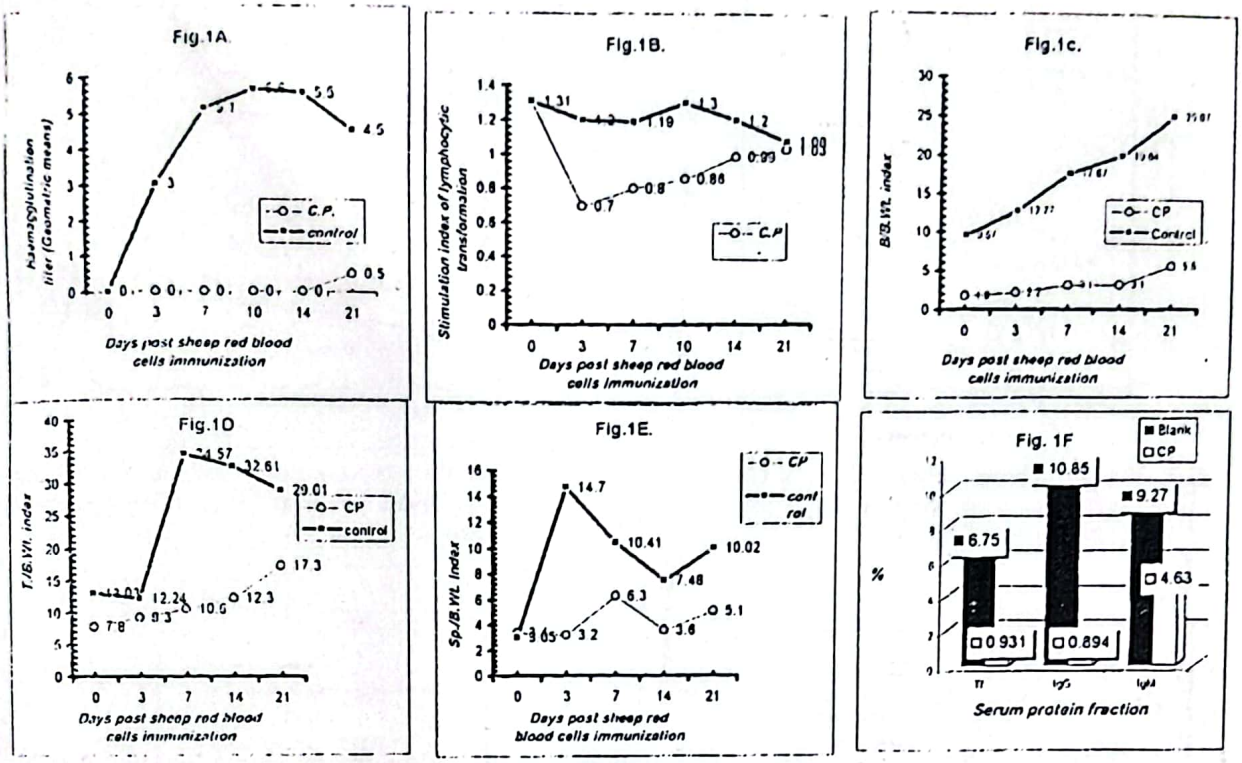

Fig.1: Immunosuppressive effect of cyclophosphamide on chicken immune system.

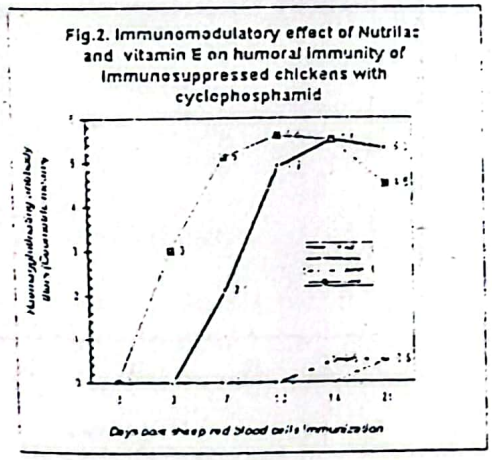

Fig. 2: Immunomodulatory effect of Nutrilac and vitamin E on humoral immunity of immunosuppressed chickens with cyclophosphamid.

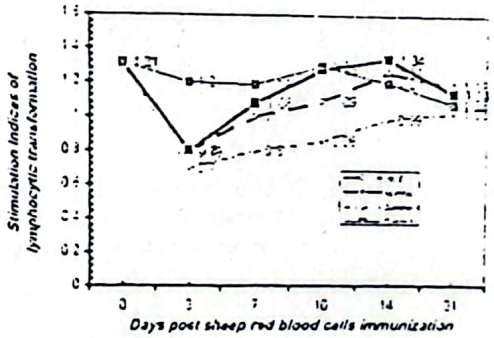

Fig.3: Immunomodulatory effect of Nutrilac and vitamin E on cell mediated immunity of immunosuppressed chickens with cyclophosphamid.

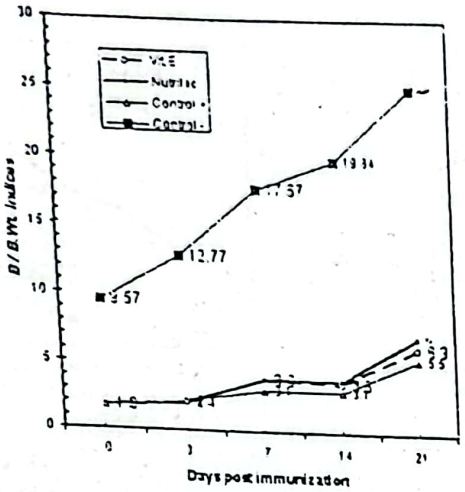

Fig. 4: The bursal/body weight indices of CP immunosuppressed chickens after immunization with SRBCs.

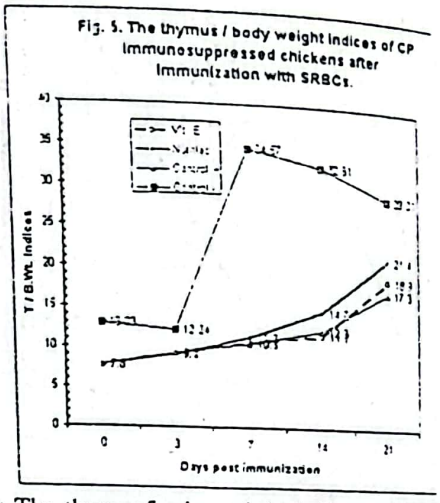

Fig.5: The thymus/body weight indices of CP immunocuppressed chickens after immunization with SRBCs.

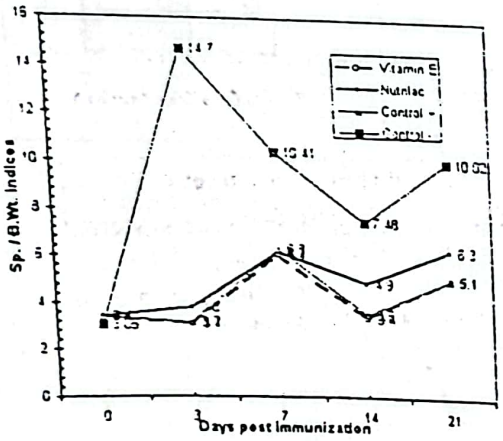

Fig.6: The spleen/body weight indices of CP immunocuppressed chickens after immunization with SRBCs.

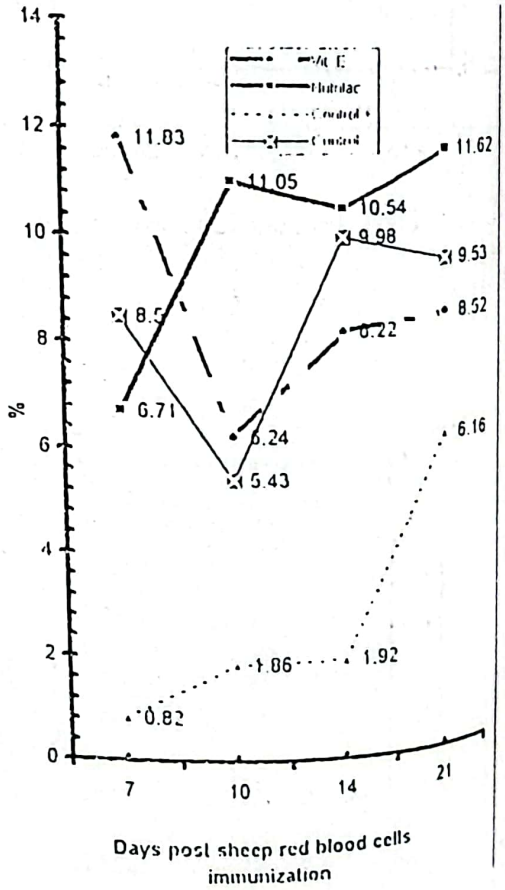

Fig. 7: Immunomodulatory effect of Nutrilac and vitamin E on IgM of immunosuppressed chickens with cyclophosphamid.

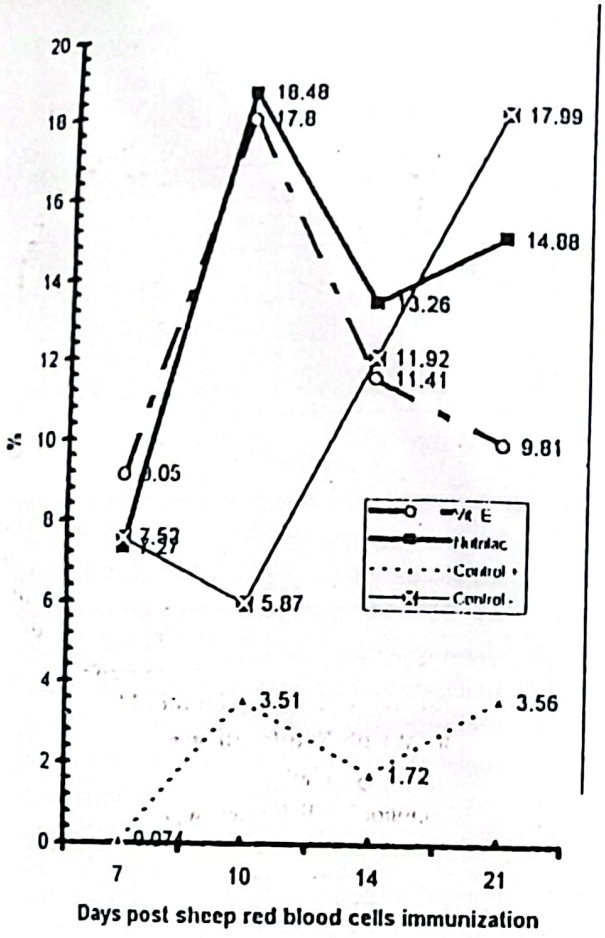

Fig.8: Immunomodulatory effect of Nutrilac and vitamin E on IgG of immunosuppressed chickens with cyclophosphamid.

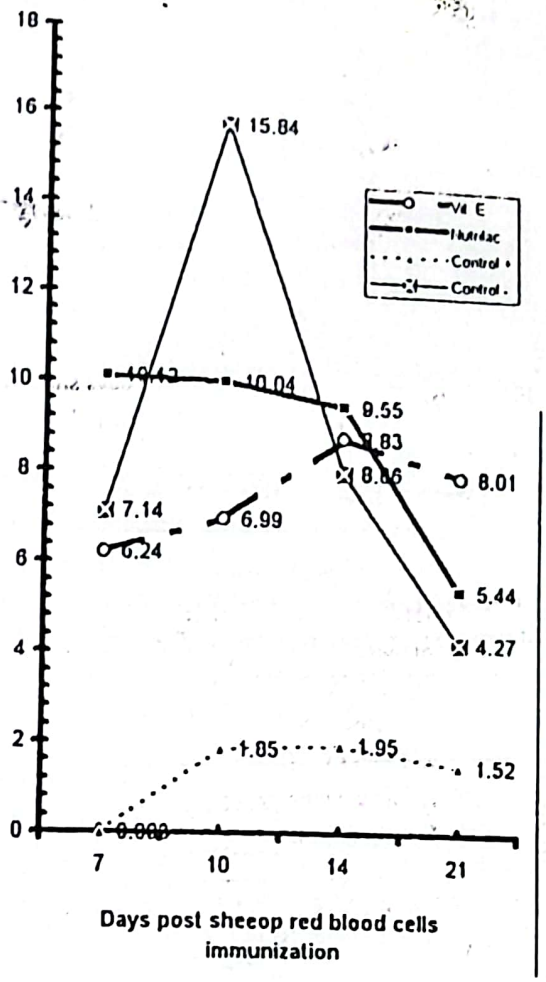

Fig.9: Immunomodulatory effect of Nutrilac and vitamin E on serum transferrin of immunosuppressed chickens.

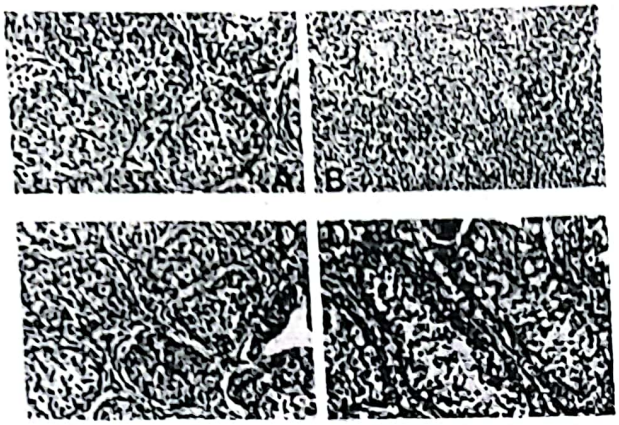

Fig. 10. A) Bursa of Fabricius (B.F). 3 days showing an almost complete depletion of lymphocytes in the lymphoid follicles. Only few lymphocytes could be observed (H & E X 66). B) Thymus gland 3 days showing moderate depletion of lymphocytes in the medulla of the thymic lobule (H & E X 33). C) B.F. 7 days showing atrophy of lymphoid follicles, fibrous thickening of the interfollicular stroma and disappearance of lymphocytes. Only reticular cells are remaining (H & E X 66). D) B.F. 7 days post Nutrilac treatment showing activated lymphoblasts in some lymphoid follicles (H & E X 66).

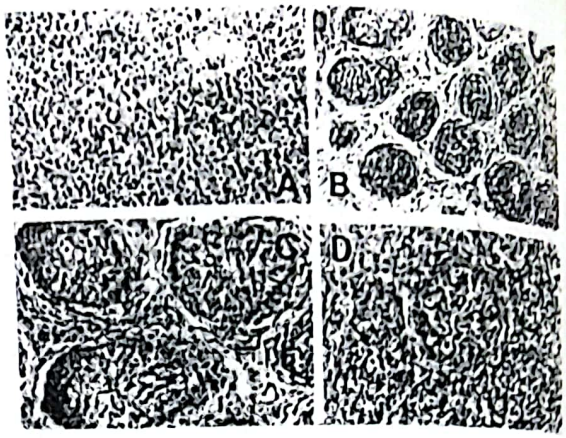

Fig.11. A) Thymus gland 7 days post treatment with Nutr
lac showing activated lymphoblasts in the co
tex and medulla of thymic lobule (H & E X 66
B) B.F. 14 days post immunosuppressio
showing marked atrophy of bursal follicl
which are widely separated by thickened fibro
stroma (H & E X 33). C) B.F. 14 days showi
complete depletion of lymphocytes and vacu
tion (H & E X 66). D) B.F. 14 days post trea
ment with Nutrilac showing an increasing nu
ber of lymphoblasts and lymphocytes in so
lymphoid follicles (arrow) (H & E X 66).

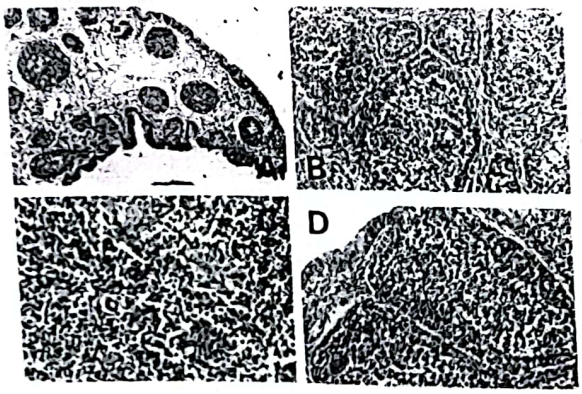

Fig.12. A) B.F. of 21 days post cyclophosphamide immunosuppression showing few severely atrophied lymphoid fo
cles widely separated by thickened oedematous interfollicular stroma (H & E 13.2). B) B.F. 21 days
treatment with Nutrilac showing numerous lymphoid follicles filled with lymphocytes. Other follicles
peared severely atrophied with reticular cells (arrow) (H & E X 33). C) Thymus of blank control chick
showing large numbers of lymphocytes in the cortex and medulla of the thymic lobule (H & E X 66). D)
of blank control chickens showing large lymphoid follicles filled with lymphocytes (H & E X 33).

Table 3: Results of E. coli serogroup 078 infection to Nutrilac and vitamin E immunopotentiated chickens previously immunosuppressed with cyclophosphamide (CP).

Group No.	Treatment	No. of dead/Total examined birds	Mortality %	Protection %
2	Vitamin. E	14/45	31.1%	68.9%
4	Nutrilac	9/45	20%	80%
6	Control	23/45	51.1%	48.9%

DISCUSSION

There are a large number of immunomodulatory agents that are capable of stimulating or suppressing the immune responsiveness of an animal. Only a few of these compounds have been employed to any degree in clinical cases, but many are currently under investigation. A great diversity exists in the chemical nature of the agents that might possess immunomodulatory activity. (Brander et al., 1991). Cyclophosphamide (CP) is one of these immunomodulators. It is an immunosuppressive drug, which are commonly used in immunological experiments (Awaad et al, 1978 Nakamura et al., 1987 and Al-Afaleq and Jones, 1991).

In the present investigation; experimental chickens inoculated over 3 days with CP showed complete suppression of humoral immune response during 14 days post SRBCs immunization with developing of a low haemagglutinating (HA) antibody titer (0.5 geometric mean) only at 21st day post immunization (Table 1 and Fig.1A). These findings demonstrate the effect produced by CP as a medication used to produce chemical bursectomy as described by Warner (1969). Lerman (1970) has already established the effect of CP as a depressor of the humoral response.

CP dramatically affected cell mediated immunity resulted in a lower lymphocytic transformation stimulation indices during the entire period of the experiment (21 days) than non-immunosuppressed chickens (Table 1 and Fig. 1B). Glick (1971) and Hiraga et al. (1976) recorded that CP is primarily a B-cell suppressor, however; it also produces transient T-cell deficiency.

Studying serum electrophoretic profiles of normal and CP treated chickens revealed significant suppression in the level of serum Tf and gammaglobulins (IgG and IgM) using PAGE (Fig.1F). Our results are indicating that CP not only could suppress serum immunoglobulins but also suppress

serum Tf which was considered by Awaad (1975) the first defense line in the immune system of chickens.

CP also induced a marked alteration in the histomorphologic features of major lymphoid organs (bursa of Fabricius, thymus gland and spleen). However the effect on the bursa of Fabricius was much more severe and progressive than its effect on the thymus or spleen, particularly the latter which showed insignificant changes. Bursal changes ranged from almost complete depletion of lymphocytes in bursal follicles (Fig. 10A) to markedly atrophied follicles which were widely separated by fibrous interfollicular stroma with only reticular cells remaining (Fig. 10C) or even vacuolated follicles (Fig.11C). At 21 days post CP treatment bursa of Fabricius showed few severely atrophied lymphoid follicles widely separated by thickened edematous interfollicular stroma (Fig. 12A). These findings are completely accord with that reported by Elmubarak et al. (1981).

In CP immunosuppressed chickens lowered bursa / body weight (B / B.W.), spleen / body weight (Sp. / B.W.) and thymus / body weight (T / B.W.) indices were recorded. Moreover, severe decrease in serum Tf, IgG and IgM during the entire 21 days post CP treatment were observed as compared with the non-immunosuppressed ones (Fig.1C,D, E and F).

For assaying the immunomodulatory effect of studied immunostimulators on the humoral immune response of immunosuppressed chickens; HA test was adopted for detection of antibody titers expressed in geometric means post SRBCs immunization. Detectable HA titers could be determine 3 days post immunization in the non-immunosuppressed control group and maintained till the end of the experiment (21 days). An increase in geometric mean of HA titers appeared only at 14 and 21 days post SRBCs immunization at a low-level (0.5) in vitamin E immunostimulated group. Nutrilac treated group started to overcome the immunosuppression from day 7 and not only sustained the same geometric mean of non-immunosupressed chickens at day 14 but also figured higher mean at 21 days post immunization (Table 1 and Fig. 2).

On the other hand, for assaying the immunomodulatory effect of the studied immunostimulators on cellular immune response; lymphocytic blastogenesis using dye uptake test after Gänn et al. (1994) was adopted. This test is very useful for assaying the cell survival and proliferation (Slater et al., 1963). Stimulation indices of lymphocytic transformation measured by MTT revealed statistical significant increase in chickens received Nutrilac or vitamin E, on detection at 7, 10 and 14 days over their non-immunostimulated control group provided that Nutrilac treated group gave more lymphocyte transformation stimulation indices than vitamin E treated group (Table 1 and Fig.3).

Electrophoretic analysis in PAGE revealed significant increase in serum Tf and immunoglobulins (IgG and IgM) in CP treated chickens that immunopotentiated with either Nutrilac or vitamin E over their non immunostimmulated control group during the entire period of the experiment. This increase was much higher in Nutrilac treated group.. Morgan (1974) reported that Tf plays a vital and central role in iron metabolism and has a second important function that of participating in the bodys defenses mechanism against infections. Aforementioned results of lymphocytic transformation completely confirm the report of Tormey et al. (1972) who mentioned that lymphocytes may have an iron requirement for transformation, and the function of Tf could be the iron supply which enhances the growth of lymphocytes in response to antigen. The increase in immunoglobulins in the electrophoretic study correlates well with the results of haemagglutination test.

In spite of the increase in the body weight in Nutrilac and vitamin E treated groups (1501.46 g ± 11.26 and 1479.3 g ± 24.1 respectively) as compared with their control (1332.09 g ± 9,9); the FCR in these immunostimulated groups was 10 and 12 points respectively higher than that of the control group. Regarding feed consumption, it was much lower in control group than immunostimulated ones (3153.99 as compared with 3699.50 and 3670.40 in Nutrilac and vitamin E treated groups respectively). This might be attributed to the improvement in the appetite of the birds after compensation of the immunodepression by the used immunostimulants. However, the obtained FCR reflects the bad quality of the used commercial ration.

An alteration in major lymphoid organs / body weight indices were recorded with statistic significant increase in B / B. Wt. index in Nutrilac treated group at 14 and 21 days post immunization and in vitamin E treated group at 21 days post immunization (Figs.4-6).

Histomorphological features of experimented immunopotentiators completely paralleled with results of the immunoassay. By the day 7 immunopotentiated birds showed that the activated lymphoblast cells began to appear in some bursal follicles (Fig.10 D) and the medulla and cortex of the thymic lobule (Fig.11A). Fourteen days post immunization these birds showed significant increase in number of activated lymphoblast cells and many lymphocytes appeared in some follicles (Fig. 11D). These changes were more obvious in Nutrilac treated chickens. The spleen and thymus of such birds were nearly normal, whereas those of CP treated chickens showed moderate depletion of lymphocytes. Twenty-one days post immunization the bursae of vitamin E or nutrilac treated birds revealed an increasing number of lymphocytes in many follicles particularly in Nutrilac treated group (Fig. 12B).

For overall judgement on immunomodulation of

studied acidifiers in immunosuppressed birds; a bioassay was carried out. Challenge with E.coli O 78 was undertaken 7 days post course of administration of immunostimulators. Challenge with E.coli O 78 resulted in 80 % and 68.9 % protection for Nutrilac and vitamin E treated groups respectively as compared with 48.9 % in non-immunostimulated CP immunosuppressed chicken group (Table 3). This means that administration of Nutrilac or vitamin E could overcome the immunosuppression and could increase the protective mechanism of the immunosuppressed hosts against infection.

Regarding our findings and taking in consideration results of Awaad et al (1999,b) it could be concluded that Nutrilac has a stimulatory effect on both cell mediated and humoral immunity. Moreover; it could be concluded that it is not only a potent immunomstimmulator but also a counter-attacking modulator that accomplish immune-stimulation and compensate immunosuppression. Brander et al. (1991) reported that imunostimulants exert their effects when administered prior to antigenic challenge and are useful for protecting immunocompromised animals at risk from opportunistic infections or, alternatively, animals that have been exposed to virulent infectious agents. On the other hand, immunomodulators administered simultaneously with antigens may prove to be effective immunologic adjuvant for the potentiation of a specific immune response, particularly to vaccines. They concluded that immunstimulants, which promote immune function after antigenic exposure, could be used for treating diseases caused by infectious agents for which no satisfactory vaccine or treatment is available. Eventually; Nutrilac as an immunostimulating compound could undoubtedly help in facing antigenic exposure and the hazard-anticipating immunosuppressive agents causing serious economic impacts in poultry industry.

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