CONCERTED IMMUNOLOGICAL RESPONSES TO NEWCASTLE DISEASE VIRUS AND SHEEP RED BLOOD CELLS IN CHICKENS SUPPLEMENTED WITH DIFFERENT LEVELS OF VITAMINS C AND E

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ABSTRACT

The effect of dietary supplementation with vitamins C and E on immune responses to Newcastle disease virus and sheep red blood cells was studied in chickens. A commercially supplied basal diet was supplemented with either 120 or 240 mg of vitamin C per kg feed, whereas vitamin E was supplemented at either 140 or 280 mg/kg feed, the two concentrations being taken as low and high supplementation levels of either vitamin, respectively. According to supplementation levels, chickens were grouped as follows: low vitamin C (LC); high vitamin C (HC); low vitamin E (LE); high vitamin E (HE); combined low vitamins C and E (LCE) and combined high vitamins C and E (HCE). A control group received only the basal diet. Antibody responses of the chickens to NDV were determined at 4 and 15 weeks of age. Immunoglobulin response to sheep red blood cells was determined at the age of 8 weeks. The results showed that chickens supplemented with higher vitamin levels (240 and 280 mg of vitamin C and E, respectively, per kg feed) were more immunocompetent and generated higher antibody responses to both, NDV and SRBC, than the chickens supplemented with low vitamin levels (120 and 140 mg of vitamin C and E, respectively, per kg feed). Vitamin supplementation is recommended for higher immunological status and efficient production of chickens.

INTRODUCTION

Improvement of the overall immunological status of poultry as well as resistance to specific pathogens would be beneficial to the growing commercial poultry industry of Tanzania. There are effective methods available for the control of a number of poultry diseases. However, costs of medication and vaccination required for sustainable performance of poultry, particularly layer chickens, can be substantial and unaffordable to small and middle income farmers. Among approaches known to influence the physiological and immunological reactivity of animal organisms, including poultry, balanced nutrition is considered pivotal. However, studies on optimum supplementation levels of immunopotentiating vitamins, i.e. vitamins C and E, for chickens of different production lines have not been exhaustive. Literature search indicates that vitamin C deficiency is linked to compromised immunocompetence and elevated blood levels of this vitamin are linked to increased cellular and humoral immune responses (Edrise *et al.* 1986; Takahashi *et al.* 1991). Vitamin E has been shown to improve immunity to Newcastle disease and increase

performance in commercial broiler chickens with subclinical infectious bursal disease (Franchini *et al.* 1986; McIlroy *et al.* 1992). However, there are other reports denying beneficial effects of supplementation of vitamins C and E, separately or in combination, in different animal species, including poultry (Siegel and Morton, 1984; Franchini *et al.* 1981; Undersson *et al. 1986*, Ndiweni and Finchi, 1991).

The inconsistent reports, notwithstanding, the fact that vitamins, and in particular C and E, influence immune responses is undeniable. In this communication, we report results of our study whose objective was to compare the dose-effect of different levels of vitamins C and E on the immune system of chickens. The antibody responses to Newcastle disease virus (NDV) vaccine and non-pathogenic sheep red blood cell antigens were used as the functional measures of the immune system.

MATERIALS AND METHODS

Experimental chickens and management

Day old layer chickens of a commercial strain (Black nerra) were randomly assigned into 7 groups each comprising 28 chickens. The birds were raised under intensive deep litter system at a density of 5 birds per square metre with free access to water and feed. Artificial light was provided day and night throughout the first 40 days of life.

Feed (basal diet)

Table 1 shows different types of feed used in this study and their proximate composition. Chick starter was given from the 1st to 9th week, growers' mash from 10th to 19th week and layers' mash from the 20th week to the end of the experiment i.e., at the age of 25 weeks.

Supplemental vitamins

The vitamins were obtained by the courtesy of F. Hoffmann La Roche Company, Switzerland with trade marks Rovimix C and Rovimix E-50 SD for vitamins C and E, respectively. Rovimix C is a silicon coated white to yellowish powder which is 96% pure ascorbic acid. Rovimix E-50 SD is a yellowish powder, containing dl-a-tocopheryl acetate (dl-a-TA) finely dispersed in a matrix of fish gelatin and dextrin. It is 50% pure dl-a-TA. The vitamins were thoroughly mixed with the feed to attain levels indicated in Table 2. The final mixtures were fed to chickens within 3 days of preparation.

Experimental design and treatments

The study was set up as a complete randomised design with 7 groups and seven treatments as shown in Table 2. The treatments (supplementations) commenced from day one to the end of the study (25 weeks).

Table 1. Type and composition of the basal diet.

Component	Type of feed		
	Chick starter	Growers' mash	Layers' mash
Metabolizable energy (kcal/kg)	2800	3000	3000
Crude protein (CP%)	18.1	19.8	16.7
Ether extract (EE%)	4.24	13.73	6.20
Crude fibre (CF%)	9.80	13.73	13.7
Calcium (Ca%)	0.86	0.82	0.71
Phosphorus (P%)	0.66	0.66	0.67
Vitamin C (mg/kg)	40	40	32
Vitamin E (mg/kg)	22	13	7

Table 2: Vitamin C and E content in the final feed mixture.

Vitamin	Group†						
	control	LC	HC	LE	HE	LCE	HCE
Vitamin C (mg/kg)	40*	120	240	40*	40*	120	240
Vitamin E (mg/kg)	22*	22*	22*	140	280	140	280

†control = group receiving neither vitamin C nor E supplementation.
LC, HC = groups receiving low and high levels of vitamin C, respectively.
LE, HE = groups receiving low and high levels of vitamin E, respectively.
LCE, HCE = groups receiving low and high levels of vitamins C and E combined, respectively.

*values indicate the basic concentration of vitamins from commercial suppliers.

Determination of antibody responses

Newcastle disease virus

Before vaccination, the birds were wing-tagged and bled for determination of pre-vaccination NDV antibody levels. Chicks were vaccinated when they reached 4 weeks of age and later boosted at the age of 15 weeks with a lyophylized VP-La Sota strain of the Newcastle disease virus (Ovejero Laboratories, Spain). Before vaccination, the chicks were deprived of water for 4 hours. The vaceine was mixed with distilled water and administered manually *per os* by means of a plastic syringe to ensure total exposure of the entire flock to the vaccine. Antibody response to the vaccine was measured 7 and 14 days post-vaccination using a haemagglutination inhibition (HI) assay.

Sheep red blood cells (SRBC)

The birds were inoculated with SRBC at the age of 60 days. Blood samples were taken prior to inoculations for pre-immunisation measurement of serum immunoglobulin levels. The birds received an injection of 0.5 ml of 50% SRBC suspension in PBS at the sternal muscles. The antibody response was measured 4 and 7 days post-immunization, using a haemagglutination assay (HA) performed in V-bottom microtitration plate wells. Two-fold dilutions of the test sera were prepared in 50 ul volumes of PBS in the range 1:2 to 1:4096. Fifty ul of 1% SRBC suspension were added to each serum dilution and the plates were agitated on a shaker. The plates were covered by plastic sealer and incubated at 37°C for one hour. The titer of total immunoglobulin response was read as the reciprocal of the highest serum dilution which agglutinated the SRBC (seen when holding the plates at 45^0 as the last non-tear forming well). For the determination of immunoglobulins of type G (Ig G), the procedure was modified as follows: the test serum was diluted in PBS - 2-mercaptoethanol (me) solution instead of PBS solution. The serum-PBS-me mixture was left to stand at room temperature for 30 minutes to allow for complete action of me on IgM before subsequent steps as described for measurement of total immunoglobulin.

Statistical analysis

The data was statistically analysed using the SAS® General Linear Models procedure (SAS Institute, 1987). Means were partitioned by Duncan's multiple range test. As an estimate of the persistence of the antibody response to NDV, we have used an index indicating titer rise, which was calculated as follows:

Titer rise = A - B / B,

where A = antibody titer 14 days post secondary vaccination, B = antibody titer before primary vaccination.

RESULTS

Antibody response to NDV

The antibody responses of chickens receiving different vitamin supplementations are shown in Table 3. With the exception of chickens in the LC group, all the other groups had measurable pre-vaccination antibody levels to the NDV antigen. While pre-vaccination antibody titers were randomly distributed within the flock as

suggested by the large P value (P = 0.6058), antibody responses measured on day 7 post-vaccination significantly differed ($P < 0.01$) between groups. Groups supplemented with vitamin E alone (LE and HE) had the highest mean antibody titers relative to the remaining groups. The statistical model used to determine intergroup variance of day 7 post-vaccination titers indicated that 8.5% of the total variation of this parameter was due to vitamin supplementation. This effect (R^2=8.5) was approximately 3-fold greater than the corresponding value (R^2 =2.3) calculated for chickens receiving no supplementation.

The data in Table 3 also demonstrate effect of type and dose of supplemental vitamins on immunocompetence of chickens. Thus, higher supplementation of vitamin C (HC) raised the day 14 primary immune response by 8.6% in comparison to low supplementation (LC). Likewise, in contrast to low dietary content of vitamin E alone (LE) or in combination with vitamin C (LCE), higher supplementation (HE or HCE) increased the chickens' immunocompetence to respond to NDV by 36.6% and 34.9%, respectively. The index calculated to estimate the rise of antibody titers between day 0 (prevaccination) and day 14 post-secondary vaccination showed that the average titer rise was 3-fold in the control group, 14-fold in the LC and HC groups, 5.5-fold in LE and HE groups and 7-fold in LCE and HCE groups.

Antibody response to SRBC

The chicken total immunoglobulin (Ig) and immunoglobulin G (IgG) responses to sheep red blood cells influenced by vitamin supplementation are shown in Table 4. The distribution of pre-immunisation total immunoglobulin titers in the different groups was non-random as indicated by a significant difference ($P < 0.01$) between groups. Groups LC, HC, LE and HE had significantly lower pre-immunisation antibody titers than the control, LCE and HCE groups. IgG levels before immunisation were, as expected, low and randomly distributed within the flock. The immunoglobulin responses (Ig and IgG) measured 4 days post-immunization were higher than pre-immunisation levels, although intergroup differences were not significant. The data show that total Ig and IgG responses to SRBC increased steadily from day 0 to day 7 post-immunization, and in particular, the day 7 responses (Ig and Ig G) significantly differed between the groups ($P < 0.001$). LCE and HCE groups had higher total Ig and IgG responses as compared to the remaining groups ($P < 0.05$).

DISCUSSION

The results of the present study describe that immune responses to the Newcastle disease virus and sheep red blood cell antigens are positively influenced by dietary vitamins C and E. The results have shown that, an increase in dosage of either vitamin resulted in higher antibody responses. The significance of these results is that the amplitude of an immune response of an individual is correlated with the individual's overall resistance to disease, which in turn conserves the animal's well-being and production efficiency.

Table 3. Antibody response to ND virus vaccination of chickens receiving different levels of vitamin supplementation (mean ± s.e.).

Group	Prevacci-nation[1]	Post-vaccination			
		Primary Response		Secondary response[2]	
	Day 0	Day 7	Day 14	Day 14	Titer rise[3]
control	365.5 ± 132.4[a]	124.3 ± 144.0[b]	169.6 ± 110.4[b]	1556.6 ± 244.0[b]	3.3
LC	42.2 ± 142.6[a]	347.5±155.1[ab]	368.6±118.9[ab]	1146.7 ± 263.5[b]	26.0
HC	316.9 ± 134.7[a]	419.4±146.6[ab]	403.4±112.4[ab]	872.3 ± 248.4[b]	1.7
LE	104.6 ± 134.7[a]	654.3 ± 146.6[a]	278.3+112.4[ab]	1033.1 ± 243.9[b]	8.8
HE	330.2 ± 137.2[a]	673.2 ± 149.2[a]	439.1±114.4[ab]	1028.9 ± 253.2[b]	2.1
LCE	253.0 ± 132.4[a]	88.7 ± 144.0[b]	187.9±110.4[ab]	1116.7 ± 239.7[b]	3.4
HCE	221.2 ± 130.1[a]	123.9 ± 141.6[b]	537.1 ± 108.5[a]	2648.3 ± 239.7[a]	11.0
R^2 (%)	2.3	8.5	4.5	17.3	
CV(%)	302.6	277.5	174.8	95.0	
Pr > F	0.6058	0.0096	0.1798	0.0001	
Mean	235.5	340.9	340.0	1358.7	

[a,b]Means within each column with the same superscript are not significantly different ($P < 0.05$). [1]Chickens had received vitamin supplementation for 30 days before this treatment. [2]Secondary response was determined following a booster vaccination 3 months after the primary vaccination. [3]Titer rise at day14 post secondary vaccination in relation to day 0 titer before primary vaccination.

Table 4. Antibody response, to SRBC immunisation, of chickens receiving different levels of vitamin C and E, (means ± s.e.).

	Days post-SRBC immunisation					
	0		4		7	
Group	Total Ig	IgG[1]	Total Ig	IgG	Total Ig	IgG
control	8.62 ± 0.94[a]	0.21 ± 0.07[a]	70.90 ± 104.60[a]	0.27 ± 0.19[a]	755.86 ± 143.49[a]	23.86 ± 3.19[b]
LC	4.40 ± 1.01[b]	0.00 ± 0.08[a]	11.76 ± 112.66[a]	0.00 ± 0.20[a]	112.96 ± 154.55[b]	6.64 ± 3.44[c]
HC	4.21 ± 0.96[b]	0.00 ± 0.07[a]	261.79 ± 106.45[a]	0.36 ± 0.19[a]	202.29 ± 146.03[b]	9.50 ± 3.25[c]
LE	2.93 ± 0.96[b]	0.00 ± 0.07[a]	75.26 ± 106.45[a]	0.43 ± 0.19[a]	186.86 ± 146.03[b]	10.00 ± 3.25[c]
HE	4.00 ± 0.98[b]	0.07 ± 0.07[a]	125.55 ± 108.40[a]	0.44 ± 0.20[a]	228.74 ± 148.71[b]	12.11 ± 3.31[c]
LCE	7.65 ± 0.94[a]	0.07 ± 0.07[a]	211.03 ± 104.60[a]	0.07 ± 0.19[a]	1147.59 ± 143.49[a]	33.52 ± 3.19[a]
HCE	8.27 ± 0.92[a]	0.07 ± 0.07[a]	254.67 ± 102.84[a]	0.27 ± 0.19[a]	1173.33 ± 141.08[a]	29.47±3.14[ab]
R^2 (%)	16.4	2.3	2.6	2.3	25.1	26.2
CV(%)	87.3	654.4	381.5	385.3	137.3	93.9
Pr > F	0.0001	0.4526	0.5334	0.6066	0.0001	0.0001
Mean	5.8	0.06	147.6	0.26	562.7	18.30

[a,b]Means within each column with the same superscript are not significantly different ($P < 0.05$).

[1]This response was measured after chickens had been exposed to vitamin supplementation for 60 days.

Our results on dose effect of supplemental vitamins on immune response comply with earlier reports by Davelaar and van Den Bos (1992) who found that the effect of ascorbic acid was dose dependent in reducing tracheal lesions due to infectious bronchitis in broiler chickens. Gross (1988) noted reduced incidence of pericarditis or death following air sac challenge with *E. coli* when vitamin C was supplemented at a dose of 300-330 mg/kg feed. Butcher and Rossi (1993) demonstrated a dose-dependent effect of vitamin E on the immune response to NDV vaccination and severity of tracheal lesions during infectious bronchitis of layer poults. In our work we used 240

and 280 mg of vitamins C and E, respectively, per kg feed as high supplementation levels and 120 and 140 mg of vitamins C and E, respectively, per kg feed as low supplementation levels. The positive effect of higher supplementation was apparent when we compared antibody responses of the chickens to Newcastle disease virus as well as to sheep red blood cell antigens.

Antibodies constitute an important barrier to virus replication and dissemination in the body. Our results show that the chickens used in this study had high levels of prevaccination antibodies to NDV. While these vertically transmitted maternal antibodies play a significant role in the protection of chicks particularly in the first 3-4 weeks of life (Gwakisa, unpublished data), the high levels of prevaccination antibodies reduced the response of the chickens to the vaccine. This negative feedback mechanism of antibodies was particularly vivid 7 days after vaccination in the majority of the chickens. The fall of antibody titer during the first 7 days following vaccination also indicates active involvement of antibodies in the neutralisation of viruses, a trend which became less explicit 14 days after vaccination. It is noteworthy that the prevaccination titers were determined when the chickens had been exposed to supplementation with vitamins for 30 days and accordingly some of the intergroup variation for this parameter may have been influenced by the different supplementation levels. The effect of vitamin supplementation on the immune response was also evident when we utilised sheep red blood cells as the antigen. The underlying assumption for employing sheep red blood cells is that, the immune response to this complex, non-pathogenic T cell-dependent antigen should be a broad indicator of the general immunocompetence (Lamont, 1994). Gross *et al.* (1980) found positive associations between anti-SRBC antibody and resistance to viral and parasitic disease, but negative associations with bacterial diseases. Antibody response to SRBC has been correlated with resistance to several infectious diseases (Gross, 1988; Dunnington *et al.*, 1986) as well as response to NDV vaccination (Gwakisa *et al.*, 1994). In the present study, higher anti-SRBC antibody titers were detected in chicken groups supplemented with higher vitamin levels. This finding agrees with the prior observation with NDV responses. Taken together, the results of antibody responses to NDV and SRBC strongly suggest that dietary supplementation of vitamins C and E at high dose of 240 and 280 mg/kg feed, respectively, exerts a positive effect on the immune status of chickens.

In summary, three points are noteworthy from the present study. First, the results of this study confirm and augment the body of information on the immunopotentiating effect of vitamins C and E on the immune system of chickens. Second, the concordant antibody responses to NDV and SRBC imply a cause-effect relationship between the vitamin treatments and the immunological dynamics exhibited by chickens of different groups. In this aspect, the applicability of the SRBC antigen system for testing of the immune system of chickens has been validated, and this may well be relevant for studies which do not involve vaccinations. Third, the results of this work have demonstrated a contrasting difference between the basal diet and supplemented diet, on their effects on the immune

system, particularly when high levels like those determined in this study are used. Bearing in mind that health and production of chickens is dependent on, among other factors, a properly functioning immune system, the findings of this study have aroused an interest to define the effect of vitamin supplementation on economically important traits of chickens. Our own preliminary findings reported in Max *et al.* (in press) showed that higher levels of supplementation with vitamins C and E reduced morbidity and mortality rates following naturally-occurring infectious epidemics. However, in the cited communication, growth performance and egg weight were not significantly influenced by increased levels of the two vitamins under normal conditions. Work is underway to authenticate these findings using chickens maintained under different systems.

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