Changes in serum calcium and phosphorus levels and their relationship to egg production in laying hens infected with velogenic Newcastle disease virus

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ABSTRACT

This study evaluated the changes that occurred in the serum levels of calcium and phosphorus in laying hens infected with velogenic Newcastle disease (ND) virus (vNDV), and their relationship to the decrease in egg production usually associated with ND. Two hundred and forty laying hens (32 weeks old) were randomly assigned into four groups of 60 each viz: VAI – vaccinated with ND vaccines and intramuscularly inoculated with vNDV, VAU – vaccinated uninfected, UNI – unvaccinated infected and UNU – unvaccinated uninfected. At weekly intervals blood was collected from six randomly selected hens in each group for serum calcium and phosphorus assays. Groups VAI and UNI showed a significant (p < .05) drop in egg production. Serum phosphorus levels of groups VAI and UNI were significantly (p < .05) lower than those of groups VAU and UNU. There was a highly positive correlation between serum phosphorus levels and egg production which was highly significant (r = .74; p < .01). The changes in serum calcium levels of infected groups were only slight, and the relationship between serum calcium levels and egg production was low, positive and not significant (r = .26; p > .05). Drop in egg production that occurred in the ND-infected laying hens was positively strongly correlated with the drop in serum phosphorus levels.

1. Introduction

Newcastle disease (ND) is a viral disease of poultry caused by a single-stranded, non-segmented, negative-sense RNA virus known as avian paramyxovirus serotype 1, belonging to the genus *Avulavirus*, subfamily Paramyxovirinae within the family Paramyxoviridae, order Mononegavirales (Mayo 2002; Lamb et al. 2005). The disease is worldwide in distribution (Alexander and Senne 2008) and is regarded throughout the world as one of the most important poultry diseases. It causes high flock morbidity and mortality in susceptible birds, leading to serious economic losses (Alexander and Senne 2008). In countries with intensive poultry farming all over the world, outbreaks of ND pose a risk for the sufficient supply to humans with valuable protein. Additionally, ND outbreaks can cause massive economic damage through control efforts and trade losses (Alexander 2001; Alexander and Senne 2008). ND belongs to the notifiable diseases of list A of the World Organisation for Animal Health (OIE 2013).

Infection with vNDV has been associated with severe systemic disease, accompanied by high morbidity and mortality in poultry (Okoye et al. 2000; Igwe et al. 2014). It causes drop in egg production and egg quality and a mortality from 0% to 50% in layers in natural outbreaks (Rao et al. 2002; Alexander 2003; Miller and Koch 2013) and affects the reproductive organs of chicken (Biswal and Morrill 1954; Rao et al. 2002). Drop in egg production and quality/and or poor shell quality are issues that affect producers of high-performing egg layers all around the world. These issues most commonly arise from deficiency, imbalance or malabsorption of calcium, phosphorus or vitamin D3 or due to diseases (Harrison and McDonald 2006; Bohn 2012). Such disorders may primarily affect the anterior pituitary or hypothalamus, thus interfering with normal luteinizing hormone and follicle-stimulating hormone production (Bentley 1998; Dacke 2000).

Calcium and phosphorus are considered the main minerals for laying birds, due to their expressive participation in the metabolism, skeletal structure and metabolism, maintenance of production, and in the quality of the egg shell (Dacke 2000). During the laying process in hens, the calcium levels can become extremely high, reaching levels of 30 mg/dl (Johnson 2000). The involvement of calcium in egg shell formation has been reported to lead to increased intestinal and bone mobilization of calcium which are needed to constantly replenish blood calcium for maximum egg production, quality and hatchability (Johnson 2000). In addition, calcium is required for the production of hard-shelled eggs and the rapid growth rate in young birds (Hurwitz 1989). Serum calcium is essential for bone homeostasis, muscle and nerve conduction, blood coagulation and the control of hormone secretion, particularly vitamin D3 and parathyroid hormone (Stanford 2006). Phosphorus is an essential nutrient for laying hens because of its role in egg shell formation and metabolism (Said et al. 1984). Inorganic phosphorus is derived from the diet. It is a major...
constituent of bone and a vital cellular component, playing important roles in the storage, release, and transfer of energy and in acid–base metabolism. Phosphorus may affect more biological systems than any other element. It is an important element in many body functions including bone formation, acid–base balance, metabolism of fat, carbohydrates, proteins and lipids, and in egg formation (Wideman 1987; Pastore et al. 2012). In poultry, it has been reported that calcium and phosphorus can be affected by poultry vaccination and diseases (Fernandez et al. 1994; Talebi 2006).

Calcium and phosphorus metabolism has been well researched in production in laying hens and pet birds for economic reasons (Harrison and McDonald 2006; Stanford 2006, 2007; Käppeli et al. 2011). There is however a dearth of reports on serum calcium and phosphorus in laying birds in viral diseased states. Knowing that ND affects most body systems, including the nervous, reproductive, renal and digestive systems which are directly involved in both calcium and phosphorus metabolism and egg formation, there is the need to investigate the changes that occur in serum calcium and phosphorus levels of laying hens infected with ND virus (NDV). The objective of this study was therefore to evaluate changes in serum levels of calcium and phosphorus in laying hens infected with vNDV, and correlate these changes with the drop in egg production that occurs in ND. Furthermore, the importance of calcium and phosphorus for egg production in layers has been described in several previous studies. However, just few documents present studies on layers, and under field conditions just like this document.

2. Materials and methods

This study was scrutinized and approved by the University Committee on Medical and Scientific Research Ethics.

2.1. Hens

Two hundred and forty Isa-Brown pullets obtained from Zartech Farms, Nigeria, were used for the study. They were randomly assigned into four groups of 60 each viz: VAI – vaccinated with ND vaccines and inoculated with vNDV, VAU – vaccinated uninoculated, UNI – unvaccinated inoculated and UNU – unvaccinated uninoculated. Pullets in groups VAI and VAU were given Hitchner B1 vaccine intraocularly as day-old chicks, 4 weeks later LaSota was given orally in drinking water, at 9 and 16 weeks of age Komarov was administered intramuscularly (IM), and oil-emulsion-inactivated vaccine IM was given according to National Veterinary Research Institute, Vom, Plateau State, Nigeria and Biovac®, Israel. Brooding of all the pullets was done on deep litter. Each of the groups was brooded separately under the same environmental conditions. Feed and water were provided ad libitum. The hens were kept in isolation in the Poultry Experimental Unit of the Department under strict biosecurity measures. The daily minimum and maximum temperatures were 24.28°C and 32.19°C, with a mean of 28.24°C, and a relative humidity of about 70% during the rainy season that falls to about 20% during the dry season.

General care of the birds was provided in accordance with the Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

2.2. Velogenic NDV inoculum

The virus used was the vNDV strain, duck/Nigeria/903/KUDU–113/1992, which belongs to genotype XV11 (Shittu et al. 2016). The virus was isolated in Kuru, Plateau State of Nigeria from an apparently healthy duck and characterized biologically by Echeonwu et al. (1993). The inoculum had a median embryo infective dose (EID50) of $10^4.46$ per ml. It is a velogenic strain of NDV and this pathotype is enzootic in Nigeria and other African countries, Middle and Far East and America where it causes severe outbreaks of ND in commercial farms and indigenous Nigerian chicken (Echeonwu et al. 1993; Solomon et al. 2012).

2.3. NDV challenge

At the peak of egg production, 32 weeks of age, each hen in groups VAI and UNI was inoculated IM with 0.2 ml of the inoculum on day 0 post infection (PI). Each hen in groups VAU and UNU was inoculated IM with 0.2 ml of phosphate buffered saline (uninfected groups) as placebo.

2.4. Clinical signs

The hens were observed twice daily for clinical signs of ND from days 0 to 49 PI when the last egg samples for the study were collected. The daily and weekly percentage (%) egg production for each group and the egg shell quality parameters were recorded during this period.

2.5. Estimation of biochemical blood parameters

Two millilitres of blood was collected from six randomly selected laying hens in each group on day 0 PI and at weekly intervals for the 49 days PI. Serum samples were harvested and used immediately for determination of serum calcium and phosphorus levels, following standard procedures using Quimica Clinica Applica test kits. The determination of serum calcium was based on the ortho-cresolphthalein direct method (Connerty and Briggs 1966), while that of phosphorus was based on the Fiske-SubbaRow method (Fiske and SubbaRow 1925; Godwin 1970).

2.6. Enzyme linked immunosorbent assay

Blood samples were collected from 10 hens in each group on days 0, 10, 15 and 21 PI. The separated serum was stored at $-20°C$ until used for enzyme linked immunosorbent assay (ELISA) test. ELISA was performed on all sera at a 1:500 final working dilution using commercial ND antibody test kits that were purchased from IDEXX Laboratories Inc. and graciously provided by Southeast Poultry Research Laboratory, Athens, Georgia. Duplicate titres were obtained and calculated using XCHEK software (IDEXX Laboratories Inc). An optical density of 650 nm wavelength was used to detect the colour change using an Emax reader (Molecular Devices, Sunnyvale, CA).
2.7. Statistical analyses

Data generated for the study were subjected to one-way analysis of variance. Variant means were separated \textit{post hoc} using the least significant difference method (Okafor 1992). Probabilities less than or each at 0.05 were accepted as significant.

3. Results

3.1. Clinical signs

There was a drastic drop in egg production in both infected groups (VAI and VAU) of hens beginning at week 1 PI, with the egg production of these groups being significantly ($p < .05$) lower than those of groups VAU and UNU (Table 1). The egg production in group UNI was significantly ($p < .05$) lower than those of groups VAU and UNU all through the experiment even when there was improvement from their very low week 2 PI values from week 4 PI onwards. The egg production in group VAI was also significantly ($p < .05$) lower than those of groups VAU and UNU all through except on week 4 PI, and their recovery to almost normal production levels was better than that of group UNI (Table 1). Only in group UNI hens produced white-coloured (bleached) soft-shelled and cracked eggs by day 6 PI. Misshapen and ridged eggs with different shades of colour (bleached and brown) were observed by day 10 PI in hens that survived, and persisted to the end of the experiment (Figure 1). There was no change in egg shell colour and quality in group VAI (Figure 2). Torticollis and paralysis of the wings and limbs were observed in group UNI on day 6–9 PI and persisted to days 18–21 PI. The lesions and the results of the immunohistochemical studies will be presented in another publication.

3.2. Effect on serum calcium levels

The serum calcium levels of group UNI were significantly ($p < .05$) higher than that of group VAU on day 7 PI (Table 2). On day 14 PI, the serum calcium levels of hens in group VAI were significantly ($p < .05$) lower than those in groups VAU and UNU (Table 2). On day 21 PI, the serum calcium level of group VAI was significantly ($p < .05$) lower than those of hens in groups VAU, UNI and UNU, while on day 42 PI, that of group UNI was significantly ($p < .05$) lower than those of groups VAU and UNU (Table 2). The correlation between serum calcium levels and egg production was positive, direct and low, and not significant ($r = .26; p > .05$).

3.3. Effect on serum phosphorus levels

There was a decrease in serum phosphorus levels following infection (Table 3). On day 7 PI, the serum phosphorus levels of the hens in groups VAI and UNI were significantly ($p < .05$) lower than that of group UNU, but on day 14 PI that of group VAI were significantly ($p < .05$) lower than that of hens in groups VAU and UNU (Table 3). On day 21 PI, the serum phosphorus levels of the hens in groups VAI and UNI were significantly ($p < .05$) lower than those in groups VAU and UNU, while on day 28 PI that of group UNI was only significantly ($p < .05$) lower than that of group UNU (Table 3). On day 35 PI, the serum phosphorus levels of group UNI were significantly ($p < .05$) lower than that of group VAU, while on day 42 PI that of group UNI was significantly ($p < .05$) lower than that of group UNU (Table 3). On day 49 PI, the serum phosphorus levels of the hens in groups VAI and UNI were significantly ($p < .05$) lower than those in groups VAU and UNU, while on day 49 PI the serum phosphorus and egg production was high, positive (direct) and significant ($r = .74; p < .01$).

3.4. Enzyme linked immunosorbent assay

All IDEXX ELISA titres of $\leq 396$ are negative for NDV antibodies. The antibody titres were negative at day 0 PI in the unvaccinated groups while vaccinated laying hens had high average titres of between 1473 and 1484 (Table 4). After challenge, sero conversion occurred and the titres rose in both groups VAI and UNI at days 10, 15 and 21 PI. Throughout the

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{figure1.png}
\caption{Bleached, misshapen and ridged eggs laid between days 10 and 21 PI by group UNI laying hens compared with group UNU.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{figure2.png}
\caption{No appreciable change in egg shell colour and quality of eggs laid by VAI laying hens compared with VAU throughout the experimental period.}
\end{figure}
Fernandez et al. (1994) recorded a decrease in calcium and moderate severe decrease in the level of serum calcium and phosphorus with several diseases in pet birds (Hochleithner 1994). Lloyd and Gibson (2006) observed a decrease in serum calcium in laying hens experimentally infected with vNDV, compared with uninfected groups.

**Table 1.** Means weekly percentage egg production ± standard error mean of laying hens experimentally infected with vNDV, compared with uninfected groups.

| Experimental period (weeks) | Treatment groups |
|-----------------------------|------------------|
|                             | Vaccine infected  | Vaccine uninfected | Unvaccine infected | Unvaccine uninfected |
| 0                           | 86.57 ± 0.84     | 86.29 ± 0.87       | 87.71 ± 0.52       | 87.43 ± 0.69       |
| 1                           | 53.57 ± 5.66     | 88.14 ± 0.63       | 36.86 ± 5.64       | 85.43 ± 1.59       |
| 2                           | 31.14 ± 3.05     | 84.00 ± 1.46       | 17.86 ± 2.69       | 83.00 ± 1.62       |
| 3                           | 35.14 ± 1.30     | 78.00 ± 2.42       | 19.00 ± 3.71       | 71.29 ± 3.64       |
| 4                           | 78.29 ± 3.46     | 83.57 ± 2.82       | 55.57 ± 3.18       | 84.29 ± 3.11       |
| 5                           | 79.29 ± 1.71     | 93.14 ± 1.18       | 77.14 ± 0.74       | 94.71 ± 0.61       |
| 6                           | 82.29 ± 0.61     | 94.14 ± 0.10       | 80.00 ± 0.87       | 94.57 ± 1.02       |
| 7                           | 86.14 ± 0.86     | 93.29 ± 0.89       | 86.29 ± 1.19       | 93.29 ± 0.89       |

Note: a,b,c Different superscripts in a row indicate significant differences between the groups (p < .05).

**Table 2.** Means levels of serum calcium (mg/dl) ± standard error mean of laying hens experimentally infected with vNDV, compared with uninfected groups.

| Experimental period (days) | Treatment groups |
|----------------------------|------------------|
|                            | Vaccine infected  | Vaccine uninfected | Unvaccine infected | Unvaccine uninfected |
| 0                           | 24.35 ± 1.18     | 22.81 ± 1.01       | 24.05 ± 0.82       | 24.80 ± 0.75       |
| 7                           | 24.42 ± 0.84ab   | 21.98 ± 0.49a      | 26.06 ± 0.22b      | 24.67 ± 1.63ab     |
| 14                          | 22.72 ± 0.76     | 26.31 ± 0.87       | 24.00 ± 0.78ab     | 25.68 ± 1.18b      |
| 21                          | 26.50 ± 0.48ab   | 28.86 ± 0.75a      | 25.10 ± 0.92b      | 28.02 ± 1.02a      |
| 28                          | 25.33 ± 1.09     | 26.37 ± 1.38       | 25.21 ± 0.39       | 26.82 ± 1.64       |
| 35                          | 26.78 ± 0.69     | 27.65 ± 0.88       | 25.01 ± 0.81       | 25.18 ± 1.29       |
| 42                          | 22.68 ± 1.56ab   | 30.39 ± 1.04a      | 26.68 ± 1.36b      | 30.17 ± 1.15a      |
| 49                          | 25.26 ± 1.44ab   | 27.84 ± 0.76a      | 24.36 ± 0.69b      | 27.23 ± 0.68a      |

Note: a,b Different superscripts in a row indicate significant differences between the groups (p < .05).

**Table 3.** Means levels of serum phosphorus levels (mg/dl) ± standard error mean of laying hens experimentally infected with vNDV, compared with uninfected groups.

| Experimental period (days) | Treatment groups |
|----------------------------|------------------|
|                            | Vaccine infected  | Vaccine uninfected | Unvaccine infected | Unvaccine uninfected |
| 0                           | 4.88 ± 0.36      | 7.55 ± 0.24        | 4.67 ± 0.16        | 7.07 ± 0.63b       |
| 7                           | 5.07 ± 0.28a     | 5.78 ± 0.86ab      | 5.09 ± 0.29a       | 6.84 ± 0.66b       |
| 14                          | 3.91 ± 0.80a     | 6.82 ± 0.34ab      | 5.39 ± 0.40a       | 7.04 ± 0.64b       |
| 21                          | 3.51 ± 0.29a     | 5.85 ± 0.49ab      | 3.52 ± 0.40a       | 6.95 ± 0.39ab      |
| 28                          | 5.45 ± 0.67ab    | 6.65 ± 0.78ac      | 4.26 ± 0.27ab      | 7.15 ± 0.38ab      |
| 35                          | 7.00 ± 0.35ab    | 8.59 ± 0.34b       | 6.06 ± 0.64ab      | 7.05 ± 0.63ab      |
| 42                          | 5.96 ± 0.54ab    | 6.23 ± 0.41ab      | 5.35 ± 0.71a       | 7.13 ± 0.49b       |
| 49                          | 4.88 ± 0.36      | 7.55 ± 0.24        | 4.67 ± 0.16        | 7.07 ± 0.63b       |

Note: a,b Different superscripts in a row indicate significant differences between the groups (p < .05).

4. Discussion

The findings in this present study of significantly lower serum phosphorus levels in the infected groups and its high, positive (direct) and significant correlation with drastic drop in egg production showed a significant adverse effect of ND on serum phosphorus concentrations. This corresponds to the findings in psittacine birds (Stanford 2006) and with several diseases in pet birds (Hochleithner 1994) in which dietary phosphate deficiency in egg laying birds reduces both egg numbers and fertility rates in psittacine birds (Stanford 2006) and in which changes in inorganic phosphorus concentration can occur with several diseases in pet birds (Hochleithner 1994). In laying hens and young birds inorganic phosphate could be affected by poultry vaccinations and diseases (Fernandez et al. 1994; Talebi 2006). Lloyd and Gibson (2006) observed a decrease in the level of serum calcium and phosphorus with moderate and severe Spironucleus-infected pheasants. Similarly, Fernandez et al. (1994) recorded a decrease in calcium and phosphorus concentrations in laying hens fed aflatoxin-containing feed. Inorganic phosphorus is derived from the diet and is important in egg shell formation, acid–base metabolism, muscle and nerve conduction, a major constituent of bone, and a vital cellular component, playing important roles in the storage, release and transfer of energy. Drop in egg production, egg abnormalities and significantly lower serum phosphorus levels were found to be strongly related in this study. Decreased serum inorganic phosphate levels may occur from hypovitaminosis D due to reduced production of metabolite 1,25(OH)2D3 in the degenerated kidneys observed in this study. ND has been reported to cause degeneration and necrosis of kidneys (Brown et al. 1999; Okoye et al. 2000; Igwe et al. 2013, 2014). Significantly decreased serum phosphorus levels in the infected groups (VAI and UNI) of laying hens were also found to be correlated to the period of drop in egg production and quality and has several practical implications, apart from drop in egg production, because serum phosphorus is important in egg shell formation, acid–base metabolism and muscle and nerve conduction. Neurological signs were observed in unvaccinated infected group during the infection, while ND in chicken caused severe gastrointestinal, kidney and reproductive lesions which could have affected calcium and phosphorus.
metabolism and homeostasis and the active form of vitamin D₃ involved in the biosyntheses of Ca-binding protein. In birds, this protein is involved in active transport of Ca across the intestinal and uterine wall for bone and egg shell formation (Keshavarz 2003; Bohn 2012; Campbell 2012). This might have led to reduction in egg production in infected groups, and egg quality in unvaccinated infected group observed in the present study.

There was a gradual continual increase in serum calcium in the two uninfected groups (groups VAU and UNI) from week 01 and throughout the period of the experiment. This is in agreement with reports of increase in plasma Ca levels at the beginning of laying period of hens and subsequent gradual increase reported by Pavlík et al. (2009) and Käppeli et al. (2011).

The slightly higher serum calcium levels of unvaccinated infected hens recorded in the present study could be a result of dehydration which was one of the clinical signs of ND which could have been caused by reduced feed and water consumption and diarrhoea observed in this group within this period. This corresponds to the findings where significant increase in serum calcium levels has been reported with dehydration and disease states affecting the digestive tract in pet birds/avian species (Hochleithner 1994; Harr 2009). The slightly higher serum calcium levels in unvaccinated infected hens at day 7 PI may also be closely related to severe inflammatory changes, severe morphological damage caused by vNDV in reproductive tract of laying hens notably the shell gland affecting its functional role of calcium absorption, with resultant effect of calcium accumulation in the blood, and not depletion of calcium stores due to ND. The slight increase in the serum calcium level at day 7 PI was followed by a varying pattern of decreased serum calcium levels in the infected groups (VAI and UNI) throughout the experimental period. Campbell (2012) reported that calcium for egg formation is derived from intestinal absorption and bone mobilization. Decreased serum calcium levels have been found to be associated with malabsorption or maldigestion disease process in animals (Bohn 2012) and this malabsorption could interfere with the intestinal and uterine absorption of calcium across the intestinal and uterine wall for bone and eggshell formation (Keshavarz 2003; Bohn 2012; Campbell 2012).

Vaccination is used as a control measure and to improve productivity; however, the present study showed that vaccination may not fully protect hens against drop in egg production due to vNDV infection. This may suggest that vaccination was however not effective in preventing damage of the organs and tissues that are directly or indirectly related to egg laying which could account for slight decrease in calcium and significant decrease in phosphorus levels in the serum.

5. Conclusion

Based on the results of this study, it was concluded that NDV infection in laying hens is associated with significant decrease in serum phosphorus levels which were strongly positively correlated with the drop in egg production.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 4. Means levels of serum ELISA antibody titres (log₁₀) ± standard error mean of laying hens experimentally infected with vNDV, compared with uninfected groups.

| Groups            | Days post challenge |
|-------------------|---------------------|
|                   | 0                   | 10                  | 15                  | 21                  |
| Vaccinated infected | 1473 ± 187.05 a (n = 10) | 1158.4 ± 127.22 a (n = 10) | 1010.5 ± 124.64 a (n = 10) | 1335.1 ± 100.68 b (n = 10) |
| Vaccinated uninfected | 1484.2 ± 211.98 a (n = 10) | 821.1 ± 105.34 a (n = 10) | 1070.2 ± 233.25 a (n = 10) | 707.5 ± 136.18 b (n = 10) |
| Unvaccinated infected | 0 ± 0.00 b (n = 10) | 2335.3 ± 183.71 a (n = 10) | 1856.4 ± 168.78 a (n = 10) | 2132.8 ± 104.84 b (n = 8) |
| Unvaccinated uninfected | 0 ± 0.00 b (n = 10) | 0 ± 0.00 b (n = 10) | 0 ± 0.00 b (n = 10) | 0 ± 0.00 b (n = 10) |

Note: Different superscripts in a column indicate significant differences between the groups, p < .05. Titres >396 are considered positive.
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