Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Jul - Sept 2017 / v.19 / n.3 / 371-376
http://dx.doi.org/10.1590/1806-9061-2016-0414

# *Toxic Effects of Ochratoxin A on Calcium Metabolism during Chick Embryo Developmentand in Hatched Chicks*

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■Keywords

Ochratoxicosis, embryotoxicity, blood electrolytes, layer chickens.

Submitted: 14/December/2016
Approved: 03/May/2017

## ABSTRACT

The present study was carried out to study the effect of *in-ovo* ochratoxin A (OTA) injection in Highline layer eggs on calcium metabolism and blood biochemical parameters of embryos and after the hatch. At day 10 of embryonic development, one hundred and sixty-two fertile eggs were individually weighed and divided into two equal treatments. The first treatment (control) consisted of the individual injection of fertile eggs with 50 µL sodium carbonate. In the second treatment (OTA), fertile eggs were individually injected with 12.5 ng OTA dissolved in 50 µL sodium carbonate. On days 12, 14, and 16 of incubation and at the hatch, serum calcium and inorganic phosphorus concentrations were lower ($p$<0.05), while sodium, alkaline phosphatase and triiodothyronine concentrations were higher ($p$<0.05) in the OTA-injected eggs compared with the controls. Serum potassium concentration was not affected ($p$<0.05) by OTA treatment. Lower calcium and phosphorus levels were determined ($p$<0.05) in the allantoic fluid of OTA-injected eggs compared with the controls. On days 12, 14, and 16 of incubation and at the hatch, lower whole body and yolk calcium and phosphorus, but not sodium levels, were measured ($p$<0.05) in the OTA treatment compared with the controls. In conclusion, the injection of eggs with OTA reduced blood calcium and phosphorus levels, which were associated with reduced whole body and yolk content from these electrolytes. Therefore, ochratoxin A had a negative effect on calcium metabolism.

## INTRODUCTION

Mycotoxins are secondary mold metabolites, and are ubiquitous contaminants of food and feed (O'Brien *et al.,* 2001). In crop and animal production, the most important mycotoxins are the aflatoxins (AF) B1, B2, G1, G2, ochratoxin A (OTA), fumonisins (FB1, FB2), zearalenone (ZEA) and trichothecenes (O'Brien *et al.,* 2001).

Ochratoxin A (OTA) is a mycotoxin produced by some toxigenic species of *Aspergillus* and *Penicillium* fungi, and it is an unavoidable contaminant of animal and human food sources (Mukhtar *et al*., 2010). Studies show that this mycotoxin can have nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic effects (El Khoury & Atoui, 2010).

Ochratoxin A inhibits the accumulation of cartilage proteoglycans and general protein synthesis in a dose-related manner (Oliveira *et al.*, 2002). Enhanced calcium uptake by the cells is frequently proposed as the cause of the disruption of cell morphological integrity by specific toxic chemicals. In an experimental study, administration of OTA to the female rabbits resulted in the skeletal defects in the developing embryos (Wangikar *et al*., 2004).

The consumption of OTA-contaminated diets (2 mg/kg PO) by three-week-old male broiler chickens for ten days reduced urinary fractional sodium content and potassium excretion (Glahn *et al*., 1991). On the other hand, Glahn *et al*. (1991) observed lower total plasma calcium levels and higher fractional calcium excretion in AF-fed broilers compared with the controls, and concluded the direct and indirect effects of AF on calcium and phosphorus metabolism may due a reduction in the endogenous synthesis of the parathyroid hormone (PTH) and of the renal sensitivity to PTH (Glahn *et al*., 1991). The supplementation of 2.5 ppm AF (Giroir *et al*., 1991) or 3.5 ppm AF (Smith *et al*., 1992) to broiler chicks for three weeks decreased serum calcium and phosphorus levels.

Therefore, the objective of the present experiment was to study the effects of injection of the mycotoxin ochratoxin A in Highline layer eggs on the calcium metabolism and blood biochemistry of embryos and hatchlings.

## MATERIALS AND METHODS

### Location

The present study was carried out at the Biological Applications Department, Nuclear Research Center, Atomic Energy Authority, Inshas City, Sharkia, Egypt.

### Eggs and birds

One hundred and eighty Highline layer fertilized eggs (55-59 g) were used in this study. The eggs were generously donated by Miser Poultry Company (MIPCO). Eggs were individually weighed, and then incubated in a Victoria incubator (Guanzate Co, Italy) at 37.5°C and 60% RH, and turned every 2 h at a 45° angle during the first 19 days of incubation.

### Experimental design

On day 10 of embryonic development (di), eggs were examined by light candling to remove infertile eggs, and to determine the air cell border. Then,162 fertile eggs were individually weighed and divided into two equal groups with three replicates of 27 fertile eggs each. In the first group (control), each egg was injected in the air cell above the mark by 50 µL sodium bicarbonate (2%) using syringe (21 G), while, each egg of the second group (OTA group) was injected in the air cell above the mark with 12.5 ng OTA dissolved in 50 µL sodium bicarbonate (2%).

### Blood samples

Blood samples were collected from the vitelline vein of nine embryos per group (three embryos per replicate) on12, 14 and 16di. At hatch, blood samples were collected from the jugular vein of nine chicks per group (three hatchlings per replicate). Blood samples were stored at room temperature for 30 min to allow blood clotting. The blood was then centrifuged to separate the serum, which was frozen and stored at -20°C until biochemical and hormonal analyses.

### Biochemical analysis

Calcium (Ca), inorganic phosphorus (Pi), and alkaline phosphatase (ALP) serum levels were colorimetrically determined using commercial kits (Stanbio Company, USA) and measured on computerized spectrophotometer (model Spectronic 1201, Milton Roy, USA). Sodium (Na) and potassium (K) serum levels were determined using a flamephotometer (model PFP7/C, Cole-Parmer, UK). Finally, triiodothyronine ($T_3$) concentration was determined using commercial radioimmunoassay (RIA) kit (IZOTOP Co., INSTITUTE OF ISOTOPES Ltd., http://www.izotop.hu) and samples were counted on a Packard Gamma Counter (model 540501 RIA SAR, USA). Chorioallantoic fluid was collected to determine calcium and phosphorus concentrations.

On the same days (12, 14 and 16 di), nine embryos per group (three embryos per replicate) without the embryonic yolk sac were individually weighed (g) then burnt to ash at 550°C and 0.2 g of ash were solubilized in sulfuric acid to determine calcium, phosphorus, sodium, and potassium concentrations.

Nine hatchlings per group (three per replicate) were frozen and burnt to ash as previously described to determine whole body and residual yolk concentrations of calcium, phosphorus, sodium and potassium.

### Statistical analysis

Data were statistically analyzed by one-way analysis of variance using the General Liner Model Procedure of the SAS software (SAS Institute, 2002). Mean values were compared using Duncan's Multiple Range Test (Duncan, 1955) at $p<0.05$. The model applied was:

$$Y_i = \mu + T_i + E_j$$

Where:

$Y_i$ = any value from the overall population.

$\mu$ = overall mean.

$T_i$ = the effect of the $i^{th}$ treatment (i=1, control; 2, OTA).

$E_j$ = the random error associated with the $j^{th}$ individual.

## RESULTS

Table 1 presents the effect of OTA injection infertile eggs on blood biochemical parameters and hormone concentrations. On days 12, 14 and 16 of incubation and at the hatch, the OTA treatment resulted in lower ($p$<0.05) serum calcium and inorganic phosphorus concentrations compared with the control. In addition, higher serum ALP, sodium, and $T_3$ levels were observed ($p$<0.05) in embryos and hatchlings of the OTA group compared with the controls. Serum potassium levels were not affected ($p$<0.05) by OTA treatment in any of the evaluated embryos or hatchlings.

**Table 1 –** Effect of OTA injection on selected serum biochemical parameters and hormonal concentrations of embryos and hatchlings.

| Parameters | Treat. | Embryonic age (day) | | | At hatch |
|---|---|---|---|---|---|
| | | 12 | 14 | 16 | |
| Calcium (mg/dL) | Cont. | 6.33± 0.02[a]* | 7.44 ± 0.1[a] | 9.04 ± 0.09[a] | 10.84 ± 0.1[a] |
| | OTA | 4.48 ± 0.02[b] | 5.97 ± 0.1[b] | 7.30 ± 0.09[b] | 8.12 ± 0.1[b] |
| Phosphorus (mg/dL) | Cont. | 3.77± 0.06[a] | 4.24± 0.08[a] | 4.98 ± 0.04[a] | 5.69 ± 0.1[a] |
| | OTA | 3.10± 0.06[b] | 3.50±0.08[b] | 3.40 ± 0.04[b] | 4.21 ± 0.1[b] |
| Alkaline phosphatase (IU/L) | Cont. | 43.72± 0.3[b] | 37.58±0.2[b] | 28.76 ± 0.2[b] | 25.24±0. 2[b] |
| | OTA | 48.58± 0.3[a] | 41.54± 0.2[a] | 33.70 ± 0.2[a] | 28.58±0. 2[a] |
| Sodium (MEq/L) | Cont. | 101.82± 0.6[b] | 123.21 ± 0.7[b] | 136.19 ± 0.7[b] | 142.87 ± 0.8[b] |
| | OTA | 123.91 ± 0.6[a] | 139.62 ± 0.7[a] | 149.31 ± 0.7[a] | 153.68 ± 0.8[a] |
| Potassium MEq/L) | Cont. | 5.28 ± 0.02 | 6.55 ± 0.02 | 7.61 ± 0.04 | 8.31 ± 0.03 |
| | OTA | 5.19 ± 0.02 | 6.21 ± 0.02 | 6.96 ± 0.04 | 7.68 ± 0.03 |
| Triiodothy. (ng/mL) | Cont. | 1.35±0.04[b] | 1.74 ±0.04[b] | 2.07±0.06[b] | 2.22±0.03[b] |
| | OTA | 1.77±0.04[a] | 2.34 ±0.04[a] | 2.53±0.06[a] | 2.88±0.03[a] |

*Values are expressed means ± standard error of the mean.

a, b, Means with different superscripts within the same age indicate significant differences between treatments by Duncan's Multiple Range Test ($p$<0.05).

N = 9 per treatment.

Table 2 presents the effect of OTA injection infertile eggs on calcium and phosphorus concentrations in the allantoic fluid. On days 12, 14 and 16 of embryonic development, lower calcium and phosphorus concentrations ($p$<0.05) were determined in OTA group compared with the control group.

**Table 2 –** Effect of OTA injection on the calcium and phosphorus concentrations the allantoic fluid of chicken embryos.

| Parameters | Treat. | Embryonic age (day) | | |
|---|---|---|---|---|
| | | 12 | 14 | 16 |
| Calcium (mg/dL) | Cont. | 1.79 ± 0.06[a] | 2.27 ± 0.04[a] | 2.78 ± 0.02[a] |
| | OTA | 1.25 ± 0.06[b] | 1.79 ± 0.04[b] | 2.15 ± 0.02[b] |
| Phosphorus (mg/dL) | Cont. | 1.03 ± 0.02[a] | 1.20 ± 0.01[a] | 1.43 ± 0.02[a] |
| | OTA | 0.70 ± 0.02[b] | 0.94 ± 0.01[b] | 1.15 ± 0.02[b] |

*Values are expressed as means ± standard error of the mean.

a, b, Means with different superscripts within the same age indicate significant differences between treatments by Duncan's Multiple Range Test ($p$<0.05).

N = 9 per treatment.

Table 3 shows the effects of OTA injection infertile eggs on yolk calcium, phosphorus, sodium and potassium concentrations. On days 12, 14, and 16 of incubation and at the hatch, OTA injection resulted lower yolk calcium and phosphorus levels ($p$<0.05) and higher sodium levels ($p$<0.05) compared with the control treatment, while, yolk potassium concentration was not affected ($p$>0.05) by the OTA treatment.

The effects of OTA injection infertile eggs on whole body calcium, phosphorus, sodium, and potassium concentrations are shown in Table 4. On days 12, 14, and 16 of incubation and at the hatch, whole body calcium and phosphorus levels were significantly lower ($p$<0.05) and sodium levels were significantly higher ($p$<0.05) in the OTA group than in the control group. Potassium levels were not significantly affected by OTA treatment ($p$>0.05).

**Table 3 –** Effect of OTA injection on calcium, phosphorus, sodium and potassium concentrations in the yolk of embryos and hatchlings.

| Parameters | Treat. | Embryonic age (day) | | | At hatch |
|---|---|---|---|---|---|
| | | 12 | 14 | 16 | |
| Calcium (ppm) | Cont. | 180.82±0.2[a] | 200.57±0.7[a] | 230.40±0.7[a] | 250.92±0.15[a] |
| | OTA | 160.98±0.2[b] | 170.44±0.7[b] | 200.81±0.7[b] | 22o.12±0.15[b] |
| Phosphorus (ppm) | Cont. | 550.12±0.3[a] | 600.34±0.3[a] | 630.24±0.3[a] | 660.99±0.4[a] |
| | OTA | 490.21±0.3[b] | 540.54±0.3[b] | 580.66±0.3[b] | 590.47±0.4[b] |
| Sodium (ppm) | Cont. | 60.12 ±0.8[b] | 60.58±0.9[b] | 70.29 ±0.9[b] | 70.95 ±0.5[b] |
| | OTA | 70.25 ±0.8[a] | 70.84±0.9[a] | 80.18 ±0.9[a] | 80.76±0.5[a] |
| Potassium (ppm) | Cont. | 170.66±0.7 | 190.92±0.8 | 190.85±0.8 | 210.65±0.5 |
| | OA | 160.99±0.7 | 190.67±0.8 | 190.91±0.8 | 210.12±0.5 |

*Values are expressed as means ± standard error of the mean.

a, b, Means with different superscripts within the same age indicate significant differences between treatments by Duncan's Multiple Range Test ($p<0.05$).

N = 9 per treatment. ppm = mg/kg dry weight.

**Table 4 –** Effect of OTA embryo injection on whole body calcium, phosphorus, sodium and potassium concentrations of embryos and hatchlings.

| Parameters | Treat. | Embryonic age (day) | | | At hatch |
|---|---|---|---|---|---|
| | | 12 | 14 | 16 | |
| Calcium (ppm) | Cont. | 170.45±0.6[a] | 340.21±0.3[a] | 770.66±0.9[a] | 1390.1±0.11[a] |
| | OTA | 130.52±0.6[b] | 290.12±0.3[b] | 520.09±0.9[b] | 910.54±0.11[b] |
| Phosphorus (ppm) | Cont. | 80.31±0.3[a] | 120.62±0.3[a] | 220.09±0.7[a] | 310.77±0.7[a] |
| | OTA | 60.92±0.3[b] | 90.23±0.3[b] | 180.35±0.7[b] | 240.82±0.7[b] |
| Sodium (ppm) | Cont. | 740.05±0.6[b] | 740.04±0.9[b] | 880.99±0.4[b] | 960.22±0.5[b] |
| | OTA | 860.09±0.6[a] | 890.99±0.9[a] | 970.19±0.4[a] | 1130.77±0.5[a] |
| Potassium (ppm) | Cont. | 690.44±0.2 | 720.88±0.2 | 840.84±0.1 | 910.67±0. 8 |
| | OTA | 680.85±0.2 | 730.93±0.2 | 850.21±0.1 | 890.72±0. 8 |

*Values are expressed as means ± standard error of the mean.

a, b, Means with different superscripts within the same age indicate significant differences between treatments by Duncan's Multiple Range Test ($p<0.05$).

N = 9 per treatment. ppm = mg/kg dry weight.

## DISCUSSION

The injection of chicken embryos with OTA reduced serum calcium and phosphorus levels compared with the control treatment, but did not influence serum potassium levels. These results are in agreement with the results of Kececi *et al.* (1998), Gokhan *et al.* (2005) in broilers and Qubih (2012) in layers, who found that mycotoxicosis (aflatoxicosis and ochratoxicosis) lowered serum calcium and phosphorus levels. Gokhan *et al.* (2005) did not observe any significant changes in the blood potassium level of broilers fed aflatoxin either.

Serum sodium levels significantly increased with OTA injection compared with the saline solution, in agreement with Glahn *et al*. (1991), who found that after 10 d of aflatoxin treatment (AF; 2 mg/kg *per os*), serum sodium levels increased, but no statistically significant changes in serum potassium levels between AF-treated and control birds.

Although the role of alkaline phosphatases (ALP) in bone formation has not been fully elucidated yet, the activity of ALP is used an indicator of the "normality" of bone development (Mcwhinne & Robert, 1973). Firschein & Urist (1971) indicated that the presence or absence of the pituitary gland influences the growth of long bones, total bone alkaline phosphatase content, and the specific activity of this enzyme in the bone. In normal chicken embryos, femur growth is roughly linear in the latter half of incubation (on days 13-15). Together with the increase in bone mass, total alkaline phosphatase activity increases. Our results were agreement with Denli *et al.* (2008) who found that a diet contaminated with OTA significantly ($p<0.05$) increased serum ALP activity in layers compared with a non-contaminated diet.

OTA treatment increased serum triiodothyronine levels. Our data are in agreement with Hatab (2003) and Elaroussi *et al.* (2006), who reported that the presence of OTA in broiler diets caused a significant increase in serum triiodothyronine concentration when compared with the control birds. Those authors concluded that the effects of OTA on thyroid hormones were dose – and time – dependent. El-Barkouky & Abu-Taleb

(2008) also observed that broiler chicks subjected to dietary intoxication with OTA at 50 and 100 ppb from one day to five weeks of age developed a significant increase in serum $T_3$ concentration at both examined OTA levels compared with the control chicks.

Yolks contain more than 90% of the calcium, iron, phosphorus, zinc, thiamine, B6, folate, and B12, and pantothenic acid present in the chicken egg. In addition, the yolk includes all of the fat-soluble vitamins A, D, E, and K of the egg, as well as all of essential fatty acids (EFAs), as determined in the chart of nutrient amounts per 100 g of egg yolk (Kruszelnicki, 2007). Ochratoxin A causes poor shell strength (Hermann, 2002), as previously demonstrated by the rubbery eggshells, which break more readily than normal field, observed during field outbreaks of ochratoxicosis (Jewers, 1990).

Dietary ochratoxin, but not aflatoxin, increased the bone elasticity of young chickens (William *et al.*, 1980), who reported that bone material properties can be altered during mycotoxicoses. Therefore, the bones of young chickens suffering ochratoxicosis may be more brittle than normal bones (William *et al.,* 1980). In the present study, the injection of embryos with OTA resulted in significantly lower calcium and phosphorus concentrations in yolk and in the whole body of embryos and hatchlings.

## CONCLUSION

In conclusion, the injection of OTA chicken eggs with reduced yolk calcium and phosphorus levels, resulting in a reduction of the concentrations of these electrolytes in the serum and whole body of embryos and hatchlings. Therefore, ochratoxin A had a negative effect on calcium metabolism.

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