Effects of Commercial Antioxidants Applied \textit{in Ovo} on Chorioallantoic Membrane and Putative Plasma Vitellogenin of Philippine Mallard (\textit{Anas platyrynchos} L.)

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\textbf{Abstract.} Two experiments were conducted to evaluate the effects of different antioxidants and its overdose (4x) \textit{in ovo}. Experiment 1 were grouped as vitamin C, MegaAntioxidant\textsuperscript{®} and Proflavanol C\textsuperscript{®}, while experiment 2 were grouped as 100, 200 and 400 ppm of MegaAntioxidant\textsuperscript{®} having positive and negative control for both. The 200 fertile eggs in each experiment were injected with 100 ul/egg of treatment solution and placebo (positive control), except negative control. Embryonic weight and body measurements were taken weekly (7-21 d). The anti-angiogenesis using CAM at day 4 was accomplished. Determination of putative vitellogenin (VTG) was done at 28\textsuperscript{th} day using SDS-PAGE. Both experiments showed comparable embryonic weight, body length, and head diameter indicating no deleterious effects of different antioxidants and MegaAntioxidants\textsuperscript{®} at high doses. Absence of red blemishes of liver was observed in three different antioxidants. The CAM resulted to lesser blood vessel formation significantly at 200 and 400 ppm MegaAntioxidant\textsuperscript{®}. Further, the 175 kDa putative VTG, was present in vitamin C, Proflavanol C\textsuperscript{®}, positive control and negative control, except MegaAntioxidant\textsuperscript{®}. The results imply that MegaAntioxidant\textsuperscript{®} having 32 different antioxidants could have protected the liver, spared VTG synthesis and had anti-angiogenic property at 200 and 400 ppm doses.

\section{1. Introduction}

Oxidative stress is common in farm animals and man due to pollution, industrialization and global warming. Oxidative stress can cause antioxidant depletion \cite{1} and metal-induced damages to the cells of the liver and kidney \cite{2, 3}. In avian species particularly ducks, it has been found that some commercial feeds and eggs are positive for the presence of heavy metals Pd and Cd beyond the limits imposed by the European Food Safety Authority. The low levels of Cd (0.0025 ppm) in feed have been found to decrease the liver weight and enhancing the first egg lay by 35d at growing \cite{4} and damages to the liver at laying \cite{5}. It has been reported that toxic metals induced oxidative stress in cells \cite{6} and Cd significantly interacts with Ca and Mg to cause oxidative damage in white stork chicks \cite{7}.

Vitellogenin (VTG) is a heavy molecular weight yolk precursor protein released from the liver in the presence of estrogen. The plasma vitellogenin level was associated to egg laying in mallard and pekin duck consistently \cite{8}. Vitellogenin is also considered as metallothionine, a heavy molecular weight peptide having zinc in the cysteine residue but have higher affinity with Cd, Pb, Hg and other...
metals [9]. Heavy metals are known as transition metal in the production of free radical conversion, i.e. from hydrogen peroxide to a very reactive hydroxyl free radical [10-12]. Recently, the puzzling role of vitellogenin in drakes and in immature avian species has been unraveled to serve as endogenous antioxidant and an indicator of cellular damage in avian species [9]. In fishes, VTG was reported to have immune-relevant and antioxidant properties [13].

Heat stress also causes free radical damage affecting metabolic syndrome [14-16], and enhances the aging process [17]. Since oxidative stress is unavoidable it is now imminent to understand the single and combined effects of different antioxidants in animals. In this study, the commercially available antioxidant, that is two different company sources of vitamin C and combined numerous antioxidants in ovo are investigated to determine its observable effects to liver condition, chorioallantoic membrane and putative plasma vitellogenin. In man, some antioxidants are considered anti-angiogenic for metabolic disorder, hence measures of chorioallantoic membrane (CAM) in considered relevant. Furthermore, the effect of different and of overdose antioxidant administered in ovo is also investigated in this study.

2. Materials and Methods

2.1. Experiment 1 – Comparison of Commercial Antioxidants in Ovo

Fertile duck eggs (n=200) were collected from a homogenous flock of female ducks raised under complete confinement fed with Amigo® duck layer pellet in a commercial farm in Victoria, Laguna. The eggs were randomly assigned into five treatments with each having four replications (40 eggs). The egg weight/sizes (small to jumbo) and incubator levels (4 levels) served as blocking factors following a randomized complete block design (RCBD). The eggs were dry cleaned, and all instruments were sanitized using 70% isopropyl alcohol to prevent microbial contamination.

The five treatments used in experiment 1 were T1 = negative control (NC); T2 = positive control (PC); T3 = 100 ppm vitamin C (Vit C); T4 = 100 ppm Mega Antioxidant®; and T5 = 100 ppm Proflavanol C®. The sterilized double distilled water was used as a vehicle for treatments 2 to 5.

2.2. Experiment 2 – Varying Doses of MegaAntioxidant®

There were five treatments with four replications in the experiment: negative control (T1), positive control (distilled water, T2), 100 ppm (T3), 200 ppm (T4) and 400 ppm (T5) MegaAntioxidant®. The fertile eggs were blocked similar to experiment 1.

2.2.1. In Ovo Treatment Administration

Candling was done to determine the air sac of the eggs, prior to treatment administration and incubation. Eggs assigned to T2 to T5 were punctured at the air sac using a needle and 0.1 mL of the different concentrations of vehicle (T2) and various treatments assigned to T3, T4 and T5, respectively for experiment 1 and 2 were accomplished. After the administration of each treatment, the hole on the top of the egg was sealed with a drop of glue (Elmer’s®) immediately to prevent the entry of microorganisms. Then, the eggs were placed inside the incubator, automatically turned every 5 minutes at required temperature and humidity settings.

2.2.2. Data Collection

2.2.2.1. Embryonic Growth Variables. The head diameter, body length and embryonic weight were sampled in each replicate per treatment at 7th, 14th and 21st day of incubation. The embryonic weight was taken using a digital weighing scale, while the head diameter and body length were measured after each were captured by the EOS® Camera with a 5 cents coin as standard marker. The captured images were uploaded in computer and measured using ImageJ® software (NIH, USA).

2.2.2.2. Angiogenesis and Chorioallantoic Membrane (CAM). Angiogenesis is a normal process in the body characterized by the formation of new blood vessels from existing vasculatures (Maniago, et al, 2014). The CAM was determined by candling the egg first to locate the blood vessels before the shell
was opened carefully and captured using macro lens installed in EOS® (Canon, Japan) camera. The eggs from different treatment replicate were sampled and their CAM was prepared for observation of angiogenic activity. The CAM was determined by candling the egg first to locate the blood vessels before the shell was opened carefully and captured using macro lens installed in EOS® (Canon, Japan) camera.

The shell of each egg was cracked and peeled away from the region over the air space that exists between the shell and inner shell membrane (ISM) at one pole of the egg. The airspace was visualized before the egg was cracked by holding the egg under an intense light (candler). Once upper part of the eggshell was removed, the ISM was peeled away from the CAM with the use of forceps.

In cases where there is extensive blood sinus/capillary bed between the CAM primary stratum layer and the ISM, minor bleeding stopped within 1 to 3 minutes. Hence the CAM of each egg from different treatments was captured using EOS® digital SLR camera with an installed macro lens after 3 minutes. The data were gathered by counting and comparing the number of blood branching in the CAM of the duck eggs.

2.2.2.3. Liver Condition. Duck sampling was done on the 28th day which was the day of hatching wherein the randomly selected samples within replicates from each treatment were sacrificed. In all treatments, sampled ducklings were weighed and put to sleep before they were blood sampled and dissected for liver collection.

The liver of the duck was obtained and weighed after dissection to be able to compute for the hepatosomatic index of the animal. These livers were photographed with the use of a Canon-EOS camera for the determination of macroscopic anatomical irregularities of livers in each treatment. The percent hepatosomatic index (% HIS) was obtained through a ratio between fresh liver weight and live body weight multiplied by 100. This is expressed in percent (%) body weight. HIS indicates hepatic growth and development depending on their age and physiological or physiochemical liver status.

Hepatosomatic Index = (liver weight (g) / body weight (g)) x 100

2.2.2.4. Putative Plasma Vitellogenin (VTG). On the 28th day, the day of hatching (day old chick), blood samples were collected in all treatments with four replications. Di-ethyl ester was placed in a transparent glass chamber to put day old ducklings to sleep then blood was collected through cardiac puncture. The animal is placed right lateral recumbence and palpate the heart at the left lateral thoracic wall. Sterile 25-gauge needle was inserted between the 4th and 5th rib close to the sternum and into the heart. A negative pressure was applied on the syringe to avoid the heart from collapsing. The needle was withdrawn after collection of 0.2 ml of blood per day-old duck. The blood collected was transferred to EDTA-coated vials and then centrifuged in 1500 rpm for 20 minutes to separate the liquid from the solid fraction. The plasma was placed in an Eppendorf® tubes and stored at -40°C until putative VTG assay.

The plasma was assayed for putative plasma vitellogenin using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the INVITROGEN® protocol having 10% running gel. A high molecular weight standard protein ladder Mark 12 (200, 97, 66, 45, and 31 kDa; INVITROGEN®) was placed on the first lane of every gel. Protein separation was done at a constant voltage (200V) for 40 minutes and then the gels were stained with SafeStain® (INVITROGEN®) and washed with distilled water for 60 minutes twice. Images of the gel were taken using EOS® (Canon, Japan) with an installed macro lens.

2.3. Statistical Analysis
Analysis of variance was used to analyze the values obtained from the measurement of the growth variables in experiments 1 and 2. PROC ANOVA of SAS [18] was utilized in analyzing the effects of treatments on various variables and the comparison of treatment means used was Scheffe’s Test after ANOVA declared significance at alpha ≤5%.

3. Results
3.1. Experiment 1 - Comparison of Commercial Antioxidants in Ovo
The effect of different commercial antioxidants on embryonic growth and development, i.e. embryonic weights, head diameter and body length at 7, 14, and 21 days of incubation were comparable (P>0.05; Table 1).

**Table 1.** Mean ± SEM of embryonic weekly body weight (g) of fertile duck eggs administered with different antioxidants in ovo.

| Treatments          | Age, days |          |          |          |
|---------------------|-----------|----------|----------|----------|
|                     | 7         | 14       | 21       |          |
| Mega Antiox®, g     | 0.29 ± 0.06| 3.58 ± 0.2| 21.66 ± 2.3|          |
| Proflavanol C®, g   | 0.31 ± 0.03| 3.36 ± 0.1| 19.86 ± 1.3|          |
| Vitamin C, g        | 0.40 ± 0.06| 3.48 ± 0.4| 21.92 ± 1.0|          |
| PC, g               | 0.31 ± 0.01| 3.19 ± 0.2| 20.69 ± 1.0|          |
| NC, g               | 0.36 ± 0.08| 3.39 ± 0.2| 21.66 ± 0.1|          |
| MEAN±SEM, g         | 0.33 ± 0.05| 3.40 ± 0.2| 21.16 ± 1.1|          |

3.1.1. Chorioallantoic Membrane (CAM). Representative CAM per treatment of four replicated sample photos are shown in Figure 1. There were no differences (P>0.05) in the degree of angiogenic branching among the treatments (data not shown). However, it is noticeable and consistent that eggs applied with MegaAntioxidant® in ovo have distinct yellow yolk pigment (Figure 1) compared to the rest.

![Figure 1](image-url)

**Figure 1.** Images of CAM documented at day 3 of incubation from the different treatments. The capital letter symbol indicates the following: (A) MegaAntioxidant®; (B) Proflavanol C®; (C) Vitamin C and; (D) Positive Control; and (E) Negative Control.

3.1.2. Liver Condition. Consistently the liver surface area (Table 2) and different colors of liver were observed with various antioxidant treatments of day-old ducks (Figure 2). The liver of the negative (D) and positive (E) control manifest more red blemish color embedded and the smaller surface area (P<0.05) compared to MegaAntioxidant® (A), Proflavanol C® (B) and Vit C (C) treated eggs.

**Table 2.** Mean ± SD of liver area of day old ducklings administered with different antioxidants in ovo.

| Treatments  | Mean ± SD (mm) |
|-------------|----------------|
| Mega Antiox®| 460.36 ± 4.44³ |
| Proflavanol C®| 449.98 ± 13.03³ |
| Vitamin C   | 458.70 ± 5.44³ |
| PC          | 434.74 ± 9.68³ |
| NC          | 436.42 ± 9.85³ |
| MEAN±SEM    | 448.04±8.49    |

³: Means within the same column having different superscript are significantly different at p<0.05.
3.1.3. Putative Plasma VTG. Surprisingly, plasma sampled from each treatment run through SDS-PAGE showed a distinct noticeable band present circa 175 kDa in negative control, positive control, Vit C and Proflavanol C® but absent in MegaAntioxidant® consistently in four replications/lanes (Figure 3).

3.2. Experiment 2 – Varying Doses of MegaAntioxidant® inOvo

3.1.4. Chorioallantoic Membrane (CAM). The effects of different doses of mega-antioxidant were evaluated for angiogenesis at day 4 of incubation period. Selected photos from four replicated samples are shown in Figure 4. Secondary collaterals of CAM were counted in all doses of MegaAntioxidant® and the positive and negative control groups. Before the data were subjected to statistical analysis, the raw data counts were transformed using the square root transformation. The data is shown in Table 3. Results showed that the doses of 200 and 400 ppm significantly reduced the number of secondary collateral count compared to the control treatments.
Table 3. Mean ± SD secondary collaterals in the CAM at day 4 of incubation period.

| TREATMENT       | CAM 2 Collateral Count |
|-----------------|-------------------------|
| 1 (+ control)   | 2.49±0.55<sup>a</sup>  |
| 2 (- control)   | 2.39±0.55<sup>a</sup>  |
| 3 (100 ppm)     | 2.18±0.50<sup>ab</sup> |
| 4 (200 ppm)     | 2.12±0.29<sup>b</sup>  |
| 5 (400 ppm)     | 2.12±0.33<sup>b</sup>  |
| MEAN±SEM        | 2.26±0.44               |

<sup>a,b</sup> Superscript with similar letter is not significantly different at p<0.05.

3.1.5. Embryonic Growth Variables. Consistently, it was observed that embryonic weight, head diameter and body length were not significantly different among the varying doses of MegaAntioxidants® (data not shown). Macroscopic data indicates that the increasing doses of MegaAntioxidant® revealed no deleterious effect on body length, head diameter and embryonic weight (P>0.05).

3.1.6. Liver Condition. The varying doses of MegaAntioxidants® showed comparable means for liver weight and hepatosomatic index (data not shown). However, the liver surface area showed that treatments with antioxidants (T3, T4 and T5) revealed significantly greater compared to the positive and negative control. The positive control (Figure 5) appears to have lesion and hematoma while negative control seems to have hematoma. On the other hand, the treatments with 100, 200 and 400 ppm MegaAntioxidant® clearly shows absence of hematoma and lesions.
4. Discussions
Oxidative stress is a common phenomenon to all living organisms, which can be caused by intrinsic and extrinsic factors. Likewise, naturally occurring antioxidants are produced or synthesized by the living animals. However, due to growing population, industrialization and environmental pollution, free radical damage or oxidative stress is enhanced [19]. Therefore, understanding the effects of two commercial sources of vitamin C and the 2x –4x higher recommended doses of combined antioxidants is presented using in ovo procedure.

The different pharmaceutically grade and commercially available antioxidants used were described by the company sources, (1) the MegaAntioxidant® represents a combination of 32 different antioxidants having 4 forms of vitamin C; (2) the Vit C is simply the generic form of ascorbic acid (500mg) while, (3) Proflavanol C® represents the same forms of vitamin C that are found in MegaAntioxidant® but of greater concentration (300mg vs 130mg) with inclusion of grape seed extract (100mg). Tablets are recommended to be taken orally, hence the limitation of the in ovo procedure is that the effect of digestion is not considered.

The comparable weekly (day 7, 14 and 21) embryonic weight, head diameter and body length of various antioxidants may indicate that free radical damage has limited effect on embryonic growth and development (experiment 1 and 2). Our result is supported in principle [20] in cattle that antioxidants administered with FMD vaccine did not attenuate the growth disturbance. This is possible because of the observed long-term damage done by free radicals on tissues of animals [17].

The statistically non-significant branching of CAM in experiment 1 simply means that at 100ppm doses of any of the two forms of vitamin C or MegaAntioxidants do not have anti-angiogenic property. At high doses of vitamin C it has been reported that anti-angiogenic property occur [20], but this was not investigated in this study. High doses, of MegaAntioxidants® significantly reduced the number of branching or blood vessels and revealed seemingly narrow branching of CAM. The anti-angiogenic property of 200 or 400 mg/kg dose of MegaAntioxidants may be relevant in mitigating the metastasis of the carcinoma cells which is not happening in growing animals but can be experienced at old age.

Abnormalities in the liver were reported locally, which includes cirrhosis, hematoma and tumor-like structure in the liver [8]. Greater surface area as an effect of antioxidant is a very good indicator of antioxidants’ action of protecting the hepatic cells from damage. This result is parallel to the effect of Cd in downsizing the liver weight at growing stage of Mallard from 12 to 20 weeks old [4]. Further, the presence of red blemishes in the liver in the positive and negative controls maybe a manifestation of free radical damage, caused by intrinsic factors and may be induced by heavy metals present in fertile eggs which act as transition metal for the formation of hydroxyl radical derived from hydrogen peroxide a reactive oxygen species [21, 22]. Therefore, application of antioxidants offer solution to improve the duck liver condition.

Vitellogenin is a universal indicator of environmental disrupt in avian species. In our study, the absence of the VTG band at around 175 kDa at day old ducklings, implies that MegaAntioxidant®, composed of several antioxidants may have synergistically acted in protecting the liver such that
synthesis of vitellogenin, a natural or endogenous antioxidant [9, 23-24] may no longer be needed. This is the first reported administration of different antioxidants in ovo, showing the blunting of putative plasma VTG by MegaAntioxidant®. Confirmatory evidence should further be conducted at the cellular level and the use of ELISA to quantify the concentration on plasma VTG.

5. Conclusion

In ovo administration of different antioxidants did not have any positive or negative effect on embryonic weight, head diameter and body length even at high doses of MegaAntioxidant®. Commercial antioxidants protected the liver from oxygen free radical damages. Lastly, among the three treatments, the administration of MegaAntioxidant® in ovo implies the sparing of the putative plasma VTG and caused anti-angiogenic effect at higher doses (200 - 400 ppm).

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