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RESEARCH PAPER

Effects of Prenatal Exposure to Urea Fertilizer on the Angiogenesis, Body Growth, and Liver Structure of Duck (*Anas platyrhynchos*) Embryos

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ABSTRACT
The agricultural sector uses fertilizers such as urea to add more nutrients to the soil needed for plant growth. Although it is cost-effective in crop production, indiscriminate use of nitrate-based fertilizer may result in behavioural, morphological, and physiological alterations on non-target organisms. This study determined the angiogenesis activity in the chorioallantoic membrane of urea-exposed duck embryos. It also investigated the weight, morphometries, and liver histopathology to gather more information on urea fertilizer's toxicity. It was observed that urea promoted angiogenesis in the CAM of duck embryos, especially at higher concentrations ($P<0.05$). Embryos treated with urea resulted in an alteration of the head-beak length ($P<0.05$). However, weight, crown-rump length, forelimb length, and hind limb length were not affected. The developing liver of urea-treated embryos showed distortion of the central vein shape and had larger sinusoidal spaces. The presence of Kupffer cells and lipid droplets were observed in the treated section. Congestion of blood cells, haemorrhage, and necrosis of hepatocytes were also observed in the tissue suggesting the extent of damage caused by the fertilizer. The findings of this study showed multiple developmental effects of urea on duck embryos. Further investigations are needed to shed more light on the toxicity of urea fertilizer on vertebrates.

KEYWORDS: Anas platyrhynchos, CAM assay, histology, toxicology, urea

INTRODUCTION

The use of organic chemicals in the agricultural industry has been accelerating to increase food production. Fertilizers such as urea have been widely used because it positively affects plants' growth (Leghari et al., 2016). Urea is a simple fertilizer that provides nitrogen, phosphorus, and potassium to the soil. Soil chemistry is affected by the application of urea in the field; soil microbiota is also altered, enhancing the transformation of the fertilizer (Wagenet et al., 1997). Utilizing urea as an agro-chemical, faster development rates and more crops can be produced in less time (Kim et al., 2001).

Despite the valuable use of urea fertilizer in agriculture, field and laboratory studies have shown that this fertilizer's acute and sub-acute concentrations may cause behavioral, morphological, and physiological changes in multiple vertebrate taxa. Restlessness, erratic swimming, and air gulping were some of the behavioral responses of *Tilapia zilli* fingerlings

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to urea exposure (Ofojekwu et al., 2008). These responses may be attributed to hypoxia since urea may decrease dissolved oxygen and increase free carbon dioxide, dissolved solute, conductivity, and alkalinity (Ofojekwu et al., 2008; do Rosario Gomes et al., 2014). Exposure to urea also increased important hematological parameters in air-breathing fish *Heteropneustes fossilis* due to the stressful condition (Maitra & Nath, 2014). Biphasic organisms such as *Bufo gargarizans* tadpoles were observed to have morphological deformities in their trunk bending shape and eye position upon short-term fertilizer exposure (Zhao et al., 2018). Even in mammals, contamination of urea on drinking water may result in mortality, as observed in suckler cows (Caldow & Wain, 1991).

Indiscriminate use of fertilizer may cause pollution, which is a considerable threat to the environment and human health. Interestingly, the nitrate produced in the transformation of urea fertilizer may be carcinogenic when ingested or introduced to animals (Ahmed et al., 2017). Stomach, testicular, and thyroid cancer were commonly cited adverse effects associated with nitrate contamination (Kristensen et al., 1996; Ward, 2009; Zaldivar & Robinson, 1973). The carcinogenic effects of nitrate occur in three mechanisms: hypoxia, the formation of N-nitroso compounds that can be teratogenic, and impedes iodine uptake, which alters the thyroid gland (Ahmed et al., 2017). Because angiogenesis plays a role in cancer cell growth and tumor metastasis, the effects of acute concentrations of urea fertilizer can be understood using chorioallantoic membrane (CAM) assay.

CAM is a very simple extraembryonic membrane that serves multiple functions during embryonic development. CAM consists of an epithelium multilayer, ectoderm at the air interface, a mesoderm (or stroma), and endoderm at the allantoic sac interface (Valdes, 2002). CAM assay is the most widely used method to study angiogenesis and tumor invasion due to its thin, transparent, and vascularized structure (Ribatti, 2016; Ribatti, 2017; Lokman et al., 2012). Angiogenic activities caused by agricultural chemicals and their additives were already investigated with the use of this assay.

Increasing evidence of acute urea fertilizer toxicity has been recorded; however, its effects on bird's angiogenesis and liver histopathology are limited. The Mallard *(Anas platyrhynchos)* duck has been used as an experimental animal for toxicological studies. It is also considered a better representative species of wild birds than its chicken avian counterpart. This study aims investigate the effects of pre-natal exposure of urea fertilizer to the angiogenesis, morphometries, and liver tissues of the duck *A. platyrhynchos* embryos. The results of this study can provide important information about urea fertilizer toxicity in birds.

MATERIALS & METHODS

Fertilized duck *A. platyrhynchos* eggs were purchased from a local provider in Butuan City, Philippines. Eggs were thoroughly cleaned by wiping the egg's surface with 95% ethanol to remove dirt and excrements. Eggs of similar weight (around 50 g) were then chosen for the assay. Egg candling was done to check the eggs' viability and locate the air space of the egg. Unfertilized eggs were discarded (Ribatti et al., 2017; Ribatti et al., 2016).

Since urea is soluble in water, dosing solutions of the fertilizer were prepared using distilled water. Before the experiment, toxicity testing using a high concentration of urea fertilizer was performed and induced high mortality of duck embryos. In the end, five concentrations of urea solution were achieved: 0.1, 1.0, 10, 100, and 1000 mg/L assigned as T1, T2, T3, T4, and T5, respectively. Urea concentrations were set to explore the dose-dependent phenotypic effects on duck embryos. Vehicle control (distilled water) and positive control (retinoic acid) were also prepared to compare urea solutions' effects in the in ovo experiment.

Day eight fertilized eggs were used in the study since blood vessel growth is highly active and air sac is more prominent during this age (Hanson, 1954; Miller et al., 2014; Gamallo et al., 2016). Four eggs were used in each treatment group. For the assay proper, the airspace, usually located at the blunt end portion of the egg, was drilled to approximately 5 x 5 mm window. A volume of 40 μL of urea solutions and controls were introduced to each egg and were then sealed using a sterile parafilm tape. Eggs were then incubated for 72 hrs at 37°C. During incubation period, the eggs were tilted to an angle of 5-10 degrees to mimic the natural incubation. After 72 hrs post-treatment, eggs were harvested by reopening the sealed portion, and shells were removed to expose the CAM widely. Each CAM was photo-micrographed three times, which were used for counting the blood vessel vascularity. Branched points were counted and recorded using the TPSdig software of Rohlf (2005). The CAM assay was conducted twice to ensure the validity of the result.

Morphometries of the embryo were measured to test if urea concentrations play a role in developing the duck embryo. Gross morphometries measured include crown-rump length (CRL), head-beak length (HBL), forelimb length (FL), and hind limb length (HL). The weight of embryos was also measured using a digital weighing scale.

The presence of lesions and degree of tissue change in the developing duck liver was analyzed to investigate further the toxicological effects of urea. Three longitudinal sections of the liver were prepared in all replicates of the treatment and control groups. Each histologic slide was counted for lesions under a light microscope. Liver histopathological analysis was done following the identified pathologies by Poleksic and Mitrovic-Tutundzic (1994) and Al-Qudsi and Al-Jahdali (2012).

The vascular density was recorded and arranged in Microsoft Excel Software. Statistical difference of angiogenesis activity and morphometric indices between treatment groups was determined using One-way Analysis of Variance. The statistical test was done using SPSS version 23.0. Differences with $P<0.05$ between experimental groups were considered statistically significant.

RESULTS AND DISCUSSION

The gross morphology of the urea treated-CAMs showed irregular branching with long and thin extensions of the blood vessels (Figure 1). It was observed that the CAM treated with the negative and positive controls have a vascular density, which is equivalent to 115.67±6.89 and 85.33±10.7, respectively (Figure 2). The angiogenesis activity induced by the 0.1 mg/L, 1.0 mg/L, and 10.0 mg/L was almost comparable to the negative control. However, the vascular density of CAM administered with 100.00 mg/L and 1000 mg/L was 2.18-fold and 1.89-fold higher than the negative control, respectively ($P<0.05$). The results suggest that urea solution is proangiogenic at higher concentrations.

Urea is naturally produced as a waste material of some animals. However, urea can be synthetically mass-produced as soil fertilizer that is necessary for agriculture. Although urea biodegrades rapidly and is not known to accumulate (U.S. EPA, 2011), this fertilizer's indiscriminate application may contaminate the environment and may have adverse effects on organisms. Many studies have evaluated urea's adverse effects on animals (do Rosario Gomes et al., 2014; Maitra & Nath, 2014; Ofojekwu et al., 2008; Caldow & Wain, 1991). Some studies have also shown that nitrates from urea can be carcinogenic (Ahmed et al., 2017; Kristensen et al., 1996; Ward, 2009; Zaldivar & Robinson, 1973). However, studies on the angiogenesis activity of urea should be explored further.

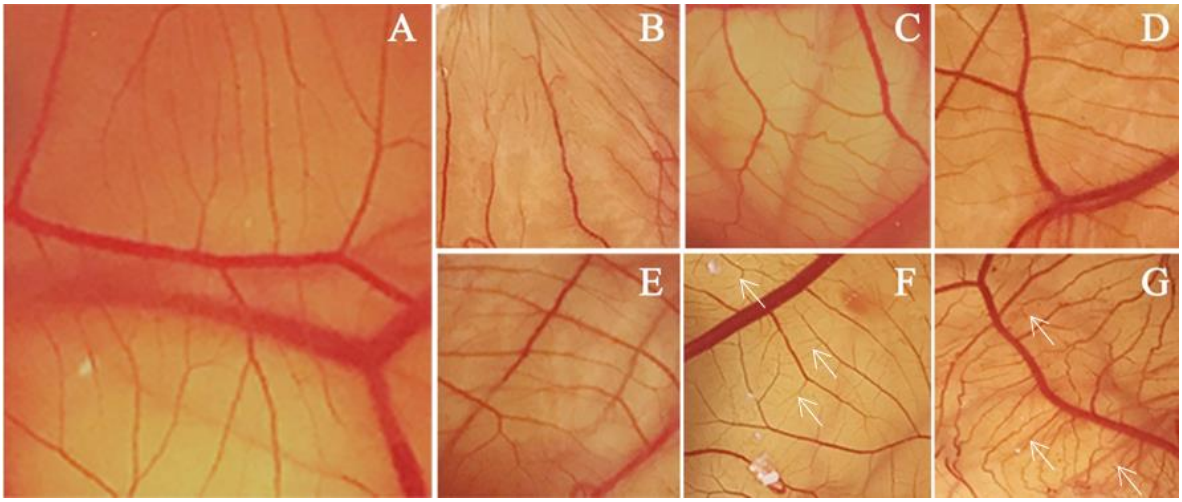

Figure 1. Representative chorioallantoic membranes (CAM) of urea-treated duck embryos. Note the minute vascular growths in the 100 mg/L and 1000 mg/L- treated CAMs (arrows). Negative control (A), positive control (B), 0.1 mg/L (C), 1.0 mg/L (D), 10.0 mg/L (E), 100.0 mg/L (F), and 1000.0 mg/L (G).

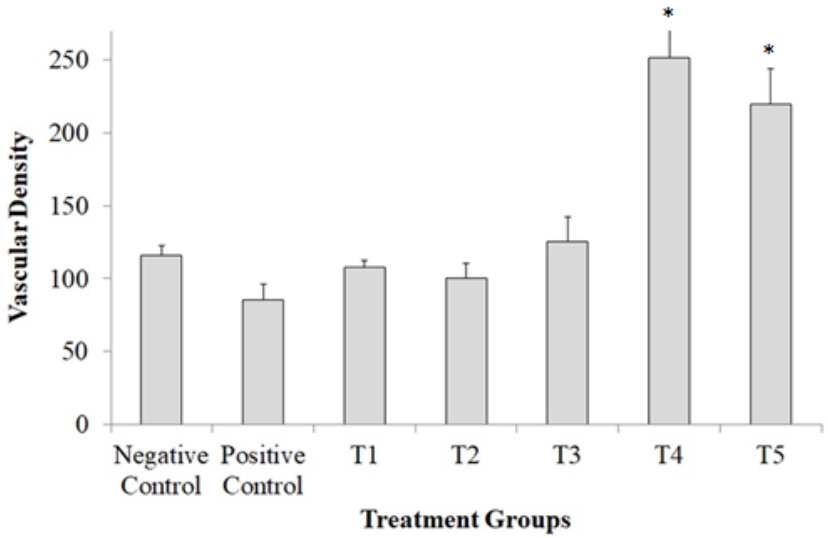

Figure 2. Vascular density of the chorioallantoic membrane of duck embryos topically applied with urea fertilizer ($P<0.05$). 0.1 mg/L (T1), 1.0 mg/L (T2), 10.0 mg/L (T3), 100.0 mg/L (T4), and 1000.0 mg/L (T5). *P<0.05 when compared to the negative control based on Tukey's honestly significant difference test.

The weight, crown-rump length, forelimb length, and the hind limb length of the duck embryos were statistically altered by the urea solution treated on the CAM (Figure 3; Table 1). However, the head-beak length was statistically significant between treatment groups (P<0.05). It was observed that the HBL of the positive control decreased by around 0.88-fold compared to the control (P=0.002). At the same time, the T3 group had the shortest HBL among the treatment groups but was not statistically different from the controls (P=0.108).

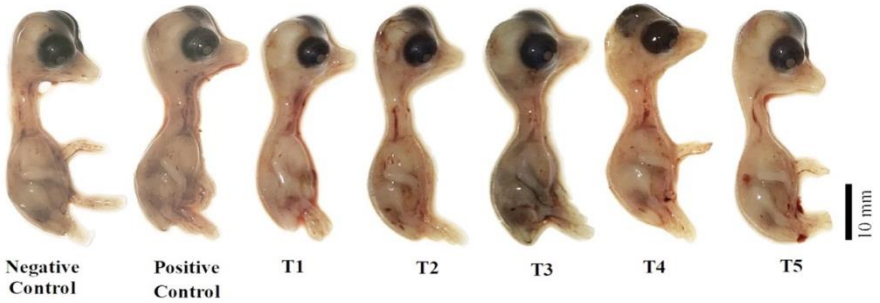

Figure 3. Representative duck embryos exposed to urea fertilizer. 0.1 mg/L (T1), 1.0 mg/L (T2), 10.0 mg/L (T3), 100.0 mg/L (T4), and 1000.0 mg/L (T5).

Table 1. Body growth parameters measured in the embryo of ducks exposed to urea fertilizer.

Parameter	Negative Control	Positive Control	1.0 mg/L	1.0 mg/L	10.0 mg/L	100 mg/L	1000 mg/L	*P-value*
Weight (g)	1.92±0.11	1.84±0.12	1.84±0.04	2.11±0.05	1.92±0.06	1.91±0.07	2.17±0.09	0.072
CRL (mm)	38.97±1.46	38.52±0.34	39.25±0.94	39.03±1.89	36.91±0.46	39.32±0.99	38.36±0.55	0.728
HBL (mm)	17.81±0.36	15.75±0.10	17.51±0.23	17.05±0.27	16.63±0.11	17.61±0.32	17.45±0.41	0.002*
FL (mm)	13.34±1.65	11.05±0.41	12.06±0.26	11.82±0.35	11.28±0.69	13.54±0.08	10.94±0.46	0.126
HL (mm)	13.39±1.10	13.61±0.45	15.57±1.42	14.92±0.87	14.62±0.72	15.60±1.76	15.07±0.29	0.662

Legend: CRL- crown-rump length, HBL- head-beak length, FL- forelimb length, HL- hind limb length
**statistically significant between groups based on One-way analysis of variance (ANOVA)*

Chemicals topically applied to the CAM may also diffuse in the blood and flow in the systemic circulation in the embryo; thus, it may affect an animal's developmental growth. The developmental effects of the application of urea solutions on the morphology of the duck embryos were investigated. Schuytema & Nebeker (1999) reported significant effects of nitrogen-based fertilizers, including ammonium nitrate, sodium nitrate, and urea on the Pacific treefrogs and African clawed frogs. In anurans, exposure to increasing nitrogenous fertilizer concentration also impaired tadpole growth (Baker et al., 2013; Ortiz-Santaliestra et al., 2012). Fish are also susceptible to the toxicity of N-based fertilizer. Growth retardation has been observed in tilapia (Monsees et al., 2017), zebrafish (Simmons et al., 2012), and catfish (Schram et al., 2014). However, the effects of urea exposure in birds are limited. In this study, the urea did not statistically affect the weight, crown-rump length, forelimb length, and hind limb length of the embryo, but the head-beak length revealed to have been affected by the fertilizer.

Hepatic histopathology was also investigated to determine the extent of toxicity of the urea solution treated on the CAM (Figure 4). Histologic sections showed intact liver covered completely with epithelial cells. The lowest concentrations of urea (0.1 and 1.0 mg/L) showed less damage to the liver since most of the cells were still intact. However, some sinusoids that radially extend from the central vein had a wider lumen than the negative control. Histological liver sections of 10 mg/L- treated embryos had central veins congested with blood cells, while cellular infiltration can be observed in the sinusoids. Liver sections also showed central veins of irregular shape, and the endothelial lining was not clearly observed.

Hepatic tissues of the duck embryos treated with 100 and 1000 mg/L displayed a heavily distorted liver. The central vein can hardly be distinguished, and blood cells can be found at the central vein's junction, while some of these blood cells infiltrated the large lumen of the sinusoids. Congestion and hemorrhage were seen near the central veins. Many hepatocytes in T4 and T5 have an indistinguishable plasma membrane, while the nuclei of other liver cells were absent or can be observed in the extracellular matrix. Phagocytic Kupffer cells were also observed at the junction of the central vein, while minute droplets of lipids were seen in the tissues. Necrotic cells were observed at 100 and 1000 mg/L urea concentrations.

Many studies have demonstrated the histological effects of urea and other N-based fertilizers on vertebrates. The result in this study is consistent with the study of Al-Qudsi and Al-Jahdali (2012), where monosodium glutamate-induced histopathological lesions on chick embryos. In rainbow trout, the sublethal concentration of composite nitrogen-based fertilizer induced deposition of fat droplets and metabolic incision-like lesions in the liver (Capkin et al., 2009). Treatment of ammonium sulfate fertilizer on *Channa punctatus* resulted in hypertrophy on the liver and degranulation, pyknosis, and focal necrosis (Ram and Sathyanesan, 1987). Necrosis and edema were also observed in the hepatic tissue of Clarias gariepinus exposed to the wastewater from a superphosphate fertilizer company (Zebedee et al., 2015). On the other hand, magnesium nitrate resulted in lipid deposition, congestion, damage on hepatocyte membranes, and protein coagulation on the hepatic tissue of *Gallus domesticus* embryos (Teusan et al., 2016). In this study, the most affected liver part was the central vein and sinusoidal spaces. Congestion and cellular infiltration of blood cells were also observed in the study, while there are some regions of the tissue that indicates hemorrhage. In the two highest concentrations (100 and 1000 mg/L), Kupffer cells and minute lipid droplets were observed.

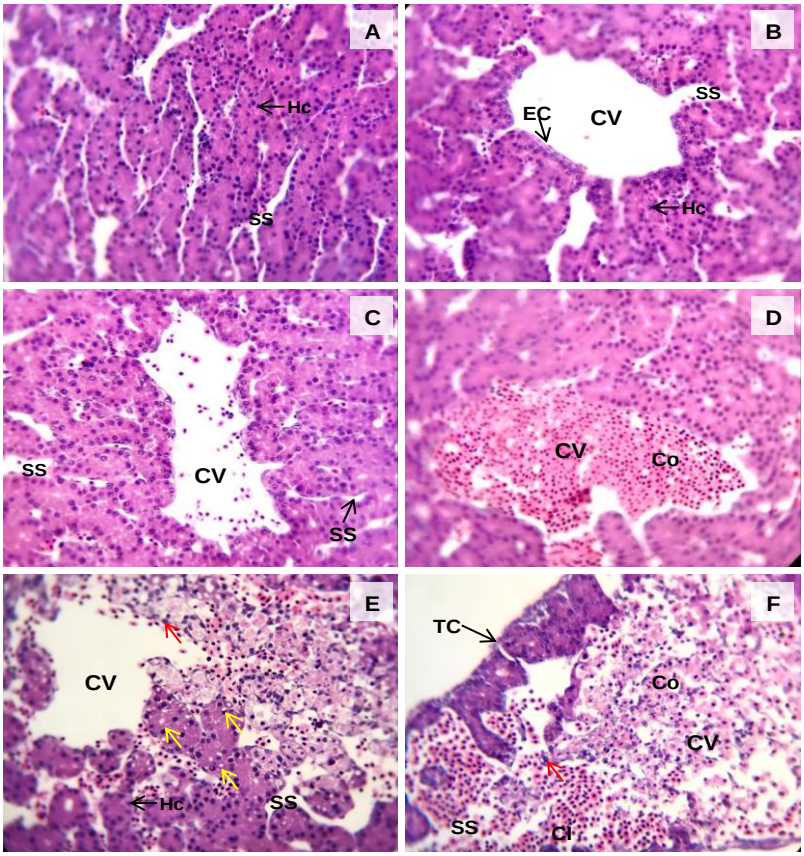

Figure 4. Liver histological sections of day 11 duck embryos. Negative control (A), 0.1 mg/L (B), 1.0 mg/L (C), 10.0 mg/L (D), 100.0 mg/L (E), and 1000.0 mg/L (F). (H&E 400X). (A) Control liver shows narrow sinusoids and intact hepatic nuclei. The central vein is not shown. (B-C) Most parts of the central vein are still intact and endothelial cells can be clearly seen. Some sinusoids are wider than the control. (D) The central vein is distorted and congested with blood cells. (E-F) The liver is surrounded with connective tissue while congestion of blood cells can be observed. Some hepatocytes have an indistinguishable plasma membrane while other hepatocytes underwent karyolitic. Lipid droplets (yellow arrow) and Kupffer cells (red arrows) can be observed in some regions of the section. *Keywords: central vein (CV), endothelial cell (EC), sinusoidal space (SS), tissue capsule (TC), hepatocyte (Hc), congestion (Co),*

CONCLUSION

This study revealed that urea fertilizer has increased angiogenic activity in the chorioallantoic membrane of duck embryos. Body growth parameters such as weight, crown-rump length, forelimb length, and hind limb length were not significantly affected by the fertilizer. However, upon investigation on the liver histology of duck embryos, several lesions were observed, such as congestion, cellular infiltration, hemorrhage, and necrosis, suggesting the adverse effects of urea on the hepatic tissue. This study provides valuable information on the effects of urea fertilizer on multiple developmental aspects of duck embryos. It is recommended that a study on the effects of low urea dosage throughout the stages of duck embryo development must be conducted. Measuring the VEGF expression levels is also an important area to focus on in future studies.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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