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Embryonic blood vessel damage and embryotoxicity induced by electronic cigarettes vape juice

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Abstract

This study is an investigation of electronic cigarette's vape juice for its embryonic blood vessel damage and embryotoxicity by the use of Chorioallantoic Membrane Assay. Assessment of vascular areas showed lysis, hemorrhage, coagulation, and hyperemia in the pure grade nicotine treatment, nicotine-containing vape juice experimental treatments, and non-nicotine-containing vape juice treatments. Mean weight, mean length, percentage of dead embryos, and percentage of dysmorphogenic embryos were used as parameters to evaluate embryotoxicity. *Anas platyrhynchos* eggs were used as test organism. Independent Samples Kruskal-Wallis revealed that there is a significant difference in the mean weight and mean lengths of *Anas platyrhynchos* embryos. The percentage of dead embryos conveys possible effect of various concentrations of vape juice treatments in the death of embryos. Dysmorphogenesis in embryos was recorded in treatments containing nicotine. Results of this study can lay foundations in the areas of public awareness, consumption, education, research, and healthcare.

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Introduction

E-cigarettes are electronic nicotine delivery devices that provide an aerosolized combination of flavored liquids and nicotine that the customer inhales. The multiple nicotine concentrations found in e-liquids, various amounts of e-liquids per package, different carrier compounds, ingredients, flavors, and battery voltage all contribute to the wide range of e-cigarettes. E-cigarettes heat and aerosolize an e-liquid or e-juice blend, which usually contains a vehicle humectant like propylene glycol and/or vegetable glycerin, as well as nicotine and flavoring agents (Lerner *et al.,* 2015).

Since the introduction of e-cigarette in 2007, e-cigarettes have gained immense popularity with smokers, nonsmokers, pregnant women, and even teenagers (Qasim *et al.,* 2017). Now, as the utilization of electronic cigarettes continues to spike, emphasizing the use among youth and pregnant women, so does the significance of crucial investigations into the effects of e-cigarette liquid, which is a principal component of e-cigarette device, on the embryonic development and early life.

The chorioallantoic membrane (CAM) is an evolutionary homolog of the mammalian placenta, and the interactions of the CAM and yolk are analogous to those of the placenta and maternal blood. The CAM is a double-layer membrane with a rich, extensive vascular network. Tissue grafts, tumor development and metastasis, drug distribution and toxicologic research and angiogenic and antiangiogenic molecules can all be studied using the CAM (Ribatti, 2016).

Cytotoxic effects on cultured cardiomyoblasts have been documented in studies. Inflammation, inflammatory mediators, and oxidative stress are all increased by e-cigarette smoking. Remarkably, this result was shown to be mostly linked to the byproducts used in flavorings rather than the nicotine itself (Ashour *et al.,* 2020). There is currently no consensus about the precise toxic mechanisms involved in the systemic dysfunction and cellular damage caused by electronic cigarettes. Despite the fact that e-cigarettes produce less nicotine, it is thought that other components in electronic cigarette liquid, especially flavorings, contribute to the cytotoxic impact of electronic cigarette vape (De Vito *et al.,* 2018).

Unfortunately, although the market is filled with these drugs, the precise process of particular flavoring agent induced harm and toxicity is unknown. Despite the fact that e-cigarettes have been touted as a safer alternative than traditional cigarettes, they are mostly used by pregnant women. It is to be noted that aggressive marketing provoked a false perception about the safety of these devices, which further emboldened their use. The aggressive marketing and the fact that e-cigarettes use is growing among all populations, it is paramount to contribute to the pool of growing scientific knowledge regarding the adverse effects of e-cigarette vape juice.

Materials and methods

Test Organism

Three-day old, fertilized duck eggs ranging from 50 grams to 60 grams served as this study's test organism. Ninety-six (96) pieces of *Anas platyrhynchos* eggs were utilized as in ovo models for the treatments. Additional three (3) eggs per treatment were procured as spare egg models, giving 24 spare egg models, for a total of 120 *Anas platyrhynchos* eggs

Preparation of Anas platyrhynchos Egg Model

The duck eggs were procured from an egg dealer located in Binalonan, Pangasinan. The eggs were surface sanitized with 70 percent ethanol to minimize the risk of contamination.

Selection of Fertilized Eggs through Candling Method

One-hundred twenty (120) *Anas platyrhynchos* eggs were subjected to candling method with the use of a candling apparatus. This method was applied to select the fertilized egg models which were then utilized in the administration of treatments and incubation process. The researchers selected the egg with the presence of a beating embryo with initially visible growing blood vessels in the egg seen upon subjecting

the egg against the light of the candling apparatus. The eggs that were not fertilized were not included as egg models.

Preparation of Different Treatments

The flavored, nicotine-containing vape juice and flavored, non-nicotine-containing vape juice were diluted with Normal Saline Solution to achieve a concentration of 3 (v/v%), 6 (v/v%), and 12 (v/v%). Pure grade nicotine was diluted with NSS to achieve a concentration of 10 (v/v%).

Chorioallantoic Membrane Assay

Chorioallantoic membrane (CAM) assay was utilized to determine the embryonic blood vessel damage and embryotoxicity of electronic cigarette's vape juice on *Anas platyrhynchos* egg animal models. Three-day old *Anas platyrhynchos* eggs were placed in an incubator of 37.5 ^{o}C at 50 percent to 60 percent humidity.

The eggs were surface sanitized to minimize the risk of contamination. At the 5th day of the duck eggs, they were scratched and eventually pierced with 1cc syringe. Then, vape juice treatments were introduced in the egg models and a micropore tape was used to seal the opening. 0.2 mL of different treatments were administered to the air cell space of egg models, in accordance with the in ovo protocols and modified windowing technique applicable to both the control and experimental *Anas platyrhynchos* egg groups of this on day 14, the embryos were harvested for observation and data gathering procedures. The different treatments were compared across the negative and positive control groups. The control and experimental groups' CAM were assessed in terms of lysis, hemorrhage, coagulation, and hyperemia to code for blood vessel damage of the electronic cigarette's vape juice through visual assessment of representative areas.

Analyses of Data

The data on duck embryos' weight and length were expressed as means. For test of differences, the data gathered from the different treatments scores were subjected to preprocessing procedures to establish that the data sets satisfy relevant assumptions related to significant outliers, normality of the distribution, and homogeneity of variance. Through inspection of the exploratory data analysis results, the data obtained for weight of the *Anas platyrhynchos* are not normally distributed according to Shapiro-Wilk's test ($p < .05$).

Additionally, there were significant outliers found in the data set. The same procedure was applied to the data on the length of *Anas platyrhynchos*. The result of the data exploratory analysis indicates that the lengths of *Anas platyrhynchos* are not normally distributed as assessed by Shapiro-Wilk's test ($p < .05$) Therefore, the Independent Samples Kruskal Wallis Test was used to report the findings on the differences in the weight and length of the *Anas platyrhynchos* across the treatments applied.

Percentage of Dead Embryos was calculated through percentage scores. Statistical Package for Social Sciences (SPSS) 64- bit version 26 was used to carry out the aforementioned tests.

Results and discussion

Assessment of Blood Vessel Damage of Electronic Cigarettes Vape Juice

The duck chorioallantoic membrane assay has provided pieces of valuable information on the blood vessel damage of electronic cigarette's vape juice determined by lysis, hemorrhage, coagulation, and hyperemia. Plate 1 presents the indicators of blood vessel damage on the duck's CAM that resulted to lysis, hemorrhage, coagulation, and hyperemia after the introduction of the positive control treatment and experimental treatments.

It can be observed in Plate 1 that the introduction of saline solution into the *Anas platyrhynchos* egg shows no indication of blood vessel damage. For the *Anas platyrhynchos* egg models administered with 10 (v/v%) of pure grade nicotine, lysis, hemorrhage, and coagulation were observed. Moreover, hyperemia was also observed in Plate 1g, suggesting an increased blood flow occurrence. Herein, as the excess blood occurs outside the vascular system, hemorrhage happens due to broken blood vessels.

Indicators of blood vessel damage were also observed in the CAM of *Anas platyrhynchos* egg administered with both flavored, nicotine-containing vape juice and flavored, non- nicotine-containing vape juice at 3 (v/v%), 6 (v/v%), and 12 (v/v%) concentrations, respectively (Plate 1c, 1d, 1e, 1f, 1g,1h).

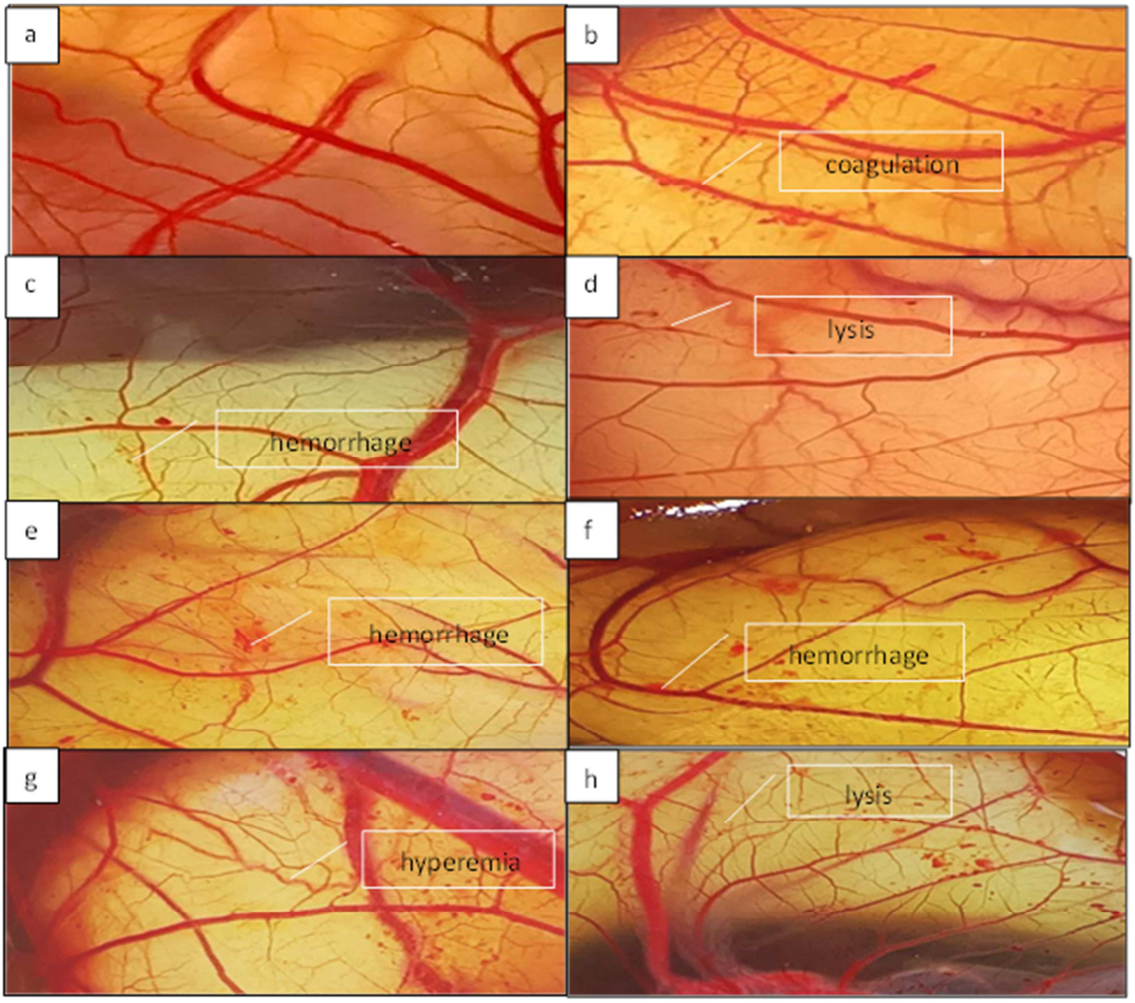

Plate 1. Vascular Area with No Indication of Blood Vessel D amage (a). Vascular areas showing lysis (d, h), Hemorrhage (c, e, f), Coagulation (b) and Hyperemia (g).

Parallel to the results of this study that electronic cigarette vape juice showed positive indications of blood vessel damage across 3 (v/v%), 6 (v/v%), and 12 (v/v%) concentrations of flavored, nicotine-containing vape juice and flavored non-nicotine containing vape juice, it was stated that combustible cigarette use has now been related to endothelial-dependent vascular disease indicated by increased dysfunction including nitric oxide (NO)– dependent alterations, oxidative stress, increased thrombosis, cell death, and inflammation. Data from the aforementioned study demonstrated that acute exposure to flavoring chemicals used in tobacco products induced endothelial damage at possibly physiologically relevant amounts (Fetterman *et al.,* 2018).

It was found that aerosols formed from electronic cigarette refill liquids had identical toxicity profiles to non-vaporized refill liquids, making direct exposure of cells to e-liquids reliable for determining relative toxicity (Rowell *et al.,* 2017).

Endothelial integrity was assessed after exposure to six different eliquids with variable nicotine concentrations and serum from e-cigarette users using human-induced pluripotent stem cell–derived endothelial cells (iPSCECs) and a high throughput screening approach. The cytotoxicity of the e-liquids varied significantly, with the cinnamon-flavored product being the most potent, resulting in

significantly reduced cell viability, increased reactive oxygen species (ROS) levels, caspase 3/7 activity, and low-density lipoprotein uptake, activation of an oxidative stress-related pathway, and impaired tube formation and migration, confirming endothelial dysfunction. There was also an increase in inflammatory cytokine expression in e-cigarette users' serum. It was also determined that acute exposure to flavored e-liquids or e-cigarette usage exacerbates endothelial dysfunction, which frequently precedes cardiovascular disease (Lee *et al.,* 2019).

All of the flavoring chemicals examined lowered nitric oxide generation, which could be due to reactive oxygen species scavenging nitric oxide and decreasing eNOS activation. Nitric oxide is a cardioprotective signaling molecule that prevents vascular inflammation and thrombosis and regulates vascular tone. The absence of nitric oxide signaling is known to promote a proinflammatory and prothrombotic endothelium, resulting in vascular dysfunction and the formation of atherosclerotic plaques (Vita, 2011).

Endothelial-derived NO is a key regulator of blood vessel function. Damage to the vascular endothelium, which results in decreased NO and other vasoactive agent release, may lead to hypertension, thrombotic events, and inflammation (Boulanger, 2018).

Comparison on the Embryotoxicity of Electronic Cigarette's Vape Juice

Anas platyrhynchos embryos introduced with Treatment 1 showed the highest mean weight of 5.51 g. It was followed by experimental groups introduced with Treatment 3 having the mean weight of 4.71 g and Treatment 6 having 4.70 g. Experimental groups administered with Treatment 7 having 4.64g mean weight and Treatment 4 having a mean weight of 4.63g follows. Subsequently, Treatment 8 recorded a mean weight of 4.59, and Treatment 5 generated the least mean weight of 4.57g. Ultimately, it can be observed from the graph that there is a drastic decrease in mean weight from Treatment 1 to Treatment 2. Meanwhile, Treatment 3, 6, and 7 have almost similar mean weights. Close mean weights could also be deduced in Treatment 5 and 8. Data on mean weights of *Anas platyrhynchos* embryos served as one of the parameters of embryotoxicity. Independent Samples Kruskal-Wallis Test was used for the statistical analyses performed on the data gathered to determine whether the data on weight of *Anas platyrhynchos* embryos differ significantly with respect to the different concentrations of nicotine-containing vape juice, and non-nicotine containing vape juice, and the negative and positive control. The differences on the means on all the weights of the *Anas platyrhynchos* embryos were measured and were found to be significantly different based on data obtained from the control and experimental groups as indicated in the p- value 0.000. Since p-value is less than 0.05, the null hypothesis was rejected.

Electronic cigarette usage during pregnancy was associated with an increased prevalence of Low Birth Weight (LBW) compared to non-use in a study conducted by [16]. E-cigarette usage was associated with a greater prevalence of LBW and preterm birth among respondents who did not simultaneously smoke combustible cigarettes during pregnancy. Only for daily e-cigarette users were associations seen when stratified further by frequency of e-cigarette usage. Thus, e-cigarette use during pregnancy, especially when used on a daily basis by those who do not concurrently smoke combustible cigarettes, is linked to adverse birth outcomes.

Treatment 1 recorded the highest mean length of 57.83mm, followed by Treatment 6 having 53.66mm, and Treatment 3 with 53.63mm. Then, it was followed by Treatment 7 which has 53.14mm, Treatment 4 with 53.00mm mean weight, Treatment 8 with 52.50mm, and Treatment 5 having 51.14mm. Among the treatments, Treatment 2 shows the lowest in mean length, with 50.50mm. A drastic drop in mean length can be observed when Treatment 1 is compared among the other treatments. Similar to mean weights, data on mean lengths of *Anas platyrhynchos* embryos served as one of the parameters of embryotoxicity. Independent Samples Kruskal-Wallis Test was used to determine whether the data on length of *Anas platyrhynchos* embryos differ significantly with respect to the different

concentrations of nicotine-containing vape juice and non-nicotine containing vape juice, and the negative and the positive control. The differences on the means on all the lengths of the *Anas platyrhynchos* embryos were measured and were found to be significantly different based on data obtained from the control and experimental groups as indicated by the p-value 0.000. Since p-value is less than 0.05, the null hypothesis is less than 0.05, the null hypothesis is rejected.

Two recent human epidemiological studies have found that e-cigarette use during pregnancy is associated with an increased risk of fetal growth and low birth weight (Cardenas *et al.,* 2019) and (Munro *et al.,* 2016). The use of e- cigarettes eliminates exposure to the majority of tobacco smoke constituents (Regan *et al.,* 2021). However, nicotine, which has been linked to decreased fetal growth in animal studies, is commonly found in e-cigarettes (Schweitzer *et al.,* 2015). Furthermore, sole vapers and dual users in pregnancy had a higher risk of Small for Gestational-Age (SGA) than non-users. This observation was consistent with previous research.

According to a population-based cohort study conducted in Sweden, maternal use of snus during pregnancy is associated with an increased risk of SGA (Baba *et al.,* 2013).

In utero nicotine exposure causes a number of pregnancy and birth complications. Smoking increases the risk of both early pregnancy loss and intrauterine fetal death in pregnant women (Baba *et al.,* 2014). The compared autopsy specimens from unexplained intrauterine fetal deaths or unexplained postnatal deaths to those from perinatal deaths caused by other known causes through a histological analysis. They discovered abnormal maturation and/or migration of cerebellar Purkinje cells, which they believe was caused by maternal cigarette smoke exposure. They also discovered abnormal cellular architecture of the choroid plexus within the 4th ventricle, as well as a link between these abnormal choroid plexus findings and maternal smoking during pregnancy. Herein, there is a possible link between nicotine and Sudden Infant Death Syndrome (SIDS) (Lavezzi *et al.,* 2013). It was noted that nicotine exposure during pregnancy, whether from cigarettes, nicotine patches, or e-cigarettes, increases the risk of sudden infant death syndrome.

This study reveals that using nicotine, such as nicotine patches or electronic cigarettes, is not a safe alternative to smoking during pregnancy because nicotine exposure, in any form, can impair an infant's cardiorespiratory function and raise the chance of SIDS. From the results of the experiment and the claims of other published studies, it could be concluded that use of e-cigarette could potentially cause mortality to the embryo (Li *et al.,* 2018).

Table 2 and Plate 2 present limb deficiencies on the *Anas platyrhynchos* as observed in this study, particularly on the treatment group administered with pure grade nicotine (Treatment 2) and treatment group administered with flavored, nicotine-containing vape juice (Treatment 5). Limb deficiencies (LDs) are characterized by the failure in formation or disruption of a portion of the entire upper or lower limb or digits during fetal development; the prevalence of LDs is estimated to be 5-8 per 10 000 live births.

Table 1. Percentage Dead Embryos after the Introduction of Different Treatments.

Treatments	Percentage (%)
1	0.00
2	50.00
3	33.33
4	41.67
5	41.67
6	25.00
7	41.67
8	33.33

Table 2. Percentage of Dysmorphogenic Embryos after the Introduction of Different Treatments.

Treatments	Percentage (%)
1	0.00
2	50.00
3	33.33
4	41.67
5	41.67
6	25.00
7	41.67
8	33.33

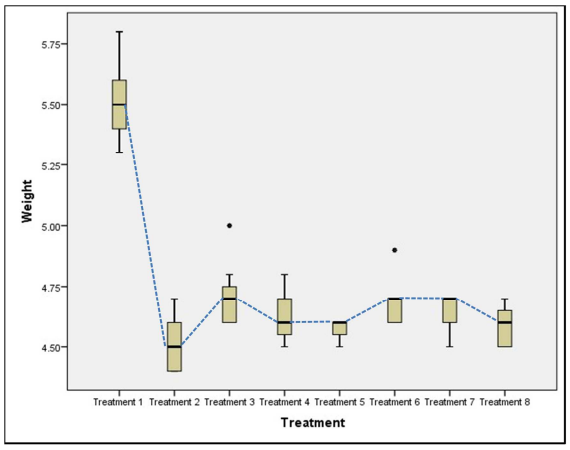

Fig. 1. Box Plot and Whiskers of Length of *Anas platyrhynchos* Embryo.

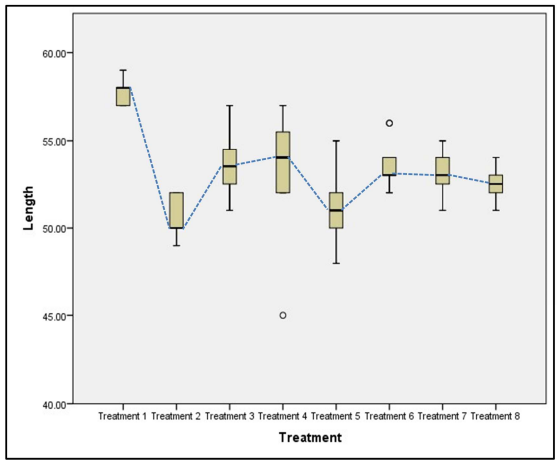

Fig. 2. Box Plot and Whiskers of Length of *A. platyrhynchos* Embryo.

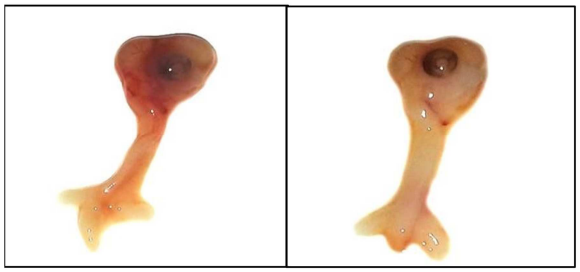

Plate 2. *Anas platyrhynchos* Embryos Introduced with Treatment 2 and Treatment 5 Showing Limb Deficiency.

Maternal active smoking and exposure to passive cigarette smoke emerged as a potential teratogen that affects limb and digit formation (Caspers *et al.,* 2013). Based on the findings of the study maternal cigarette exposure during the periconceptional phase is a possible teratogen that alters limb and digit formation. The study demonstrates that passive cigarette smoke exposure may have an effect on limb development regardless of maternal active smoking status (Caspers *et al.,* 2020). It can therefore be concluded that nicotine and nicotine-containing vape juice potentially causes dysmorphogenesis in embryos.

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