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The cardiovascular function and growth of the developing embryo exposed to nicotine during the early stages of chicken embryo.

Kawakeb.A.O. Saad, Rabeea A. H. Emtawel

Zoology Department, Omar Al-Mukhtar University, Al Bayda', Libya

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A B S T R A C T

Congenital malformations, present at birth, can be caused by genetic factors, environmental influences, or both. Nicotine exposure during pregnancy, whether due to passive smoke exposure or active smoking, is a significant environmental factor contributing to congenital malformations. Nicotine imitates acetylcholine, a natural chemical in the body, and its consumption by expectant mothers leads to growth delays, nervous system and cardiac abnormalities, and other abnormalities.

This study aims to investigate the impact of nicotine on the early stages of embryonic development. The study focused on observing general morphological abnormalities in the development, and alterations in heart rate using domestic chick *Galls galls* embryos. Fertilized eggs were sterilized with 70% ethanol and then injected with three concentrations of nicotine solution (0.1, 0.3, and 0.5 mg/ml) on day zero before being placed in an incubator set at a temperature range of 37.5 - 38 C°. The current research indicated the effects of nicotine on the heart rate (HR) of chicken embryos treated at different stages, HH19 (Day 3), HH27 (Day 5), and HH36 (Day 10), with nicotine solutions in a decrease in HR. This suggests that nicotine has a notable effect on the heart rate of chicken embryos at various developmental stages.

1. Introduction

Maternal exposure to smoking, whether passive or active, in the periconceptional period significantly affects the development of crucial embryonic organs such as the central nervous system (CNS), cardiovascular system (CVS), and skeletal system (SK) (Mone *et al*. 2004) The harmful substances present invigorates are introduced into the body through the act of smoking, referred to as active smoking, or through exposure to a contaminated environment with tobacco-related pollutants, known as passive smoking (Farsalinos *et al*. 2018). Nicotine is one of the 4,700 compounds produced

During cigarette burning, it is colorless compound emitted from cigarette combustion, is a volatile alkaloid with a chemical composition of C10H14N2 and high solubility in water and organic solvents (Vaupel *et al*. 2004). Research has shown a link between smoking and birth defects in infants, with congenital malformations affecting around 3% of births in the US (Honein *et al*. 2001). Smoking also increases the risk of miscarriage and premature birth, leading to microcephaly and abnormally small brain size(Shinawi *et al*. 2010). Nicotine exposure through smoking has been linked to smaller infants and neurobiological impacts, such as stimulation of neurotransmitter release and adrenaline release (Perry and Perry 2004), potentially leading to

***Corresponding author*: E-mail: kawakeb.ali@omu.edu.ly**

cardiac complications. Smoking is a primary neurotoxin, crossing the blood-brain barrier and excreting into breast milk (Somm *et al*. 2009). It also

contributes to cardiovascular diseases, ranking it among the leading risk factors for conditions like coronary heart disease, ischemic stroke, peripheral artery disease, and abdominal aortic aneurysm(Silvey and Brandão 2017).

Previous studies

Numerous studies have been conducted on animal models and case studies have been performed on humans. All these studies have consistently shown the significant effects of nicotine exposure during the prenatal period. In the following section, we will present some of the most recent findings. The study on chick embryo found, that early nicotine injections caused growth retardation in chick embryos, with fetal weight decreasing due to the proportional dosage (Rosenbruch, Kniepen *et al*. 1993). Nicotine in the maternal system can cause physiological changes, impacting both the mother and the developing fetus, affecting blood pressure, heart rate, and blood flow (Lambers and Clark 1996). Feng's study indicates that prenatal nicotine exposure can negatively impact fetal cardiovascular systems in sheep and rats, leading to changes in heart rate and arrhythmias, prompting further research for potential interventions (Feng *et al*. 2010). Research conducted on zebrafish revealed that the growth of zebrafish larvae was influenced by exposure to nicotine. The decrease in both notochord length and eye diameter was observed with an increase in nicotine concentration, resulting in adverse effects on startle responses and a decline in survival rate (Parker and Connaughton., 2007). The investigation was carried out during the prenatal period, with nicotine demonstrating an increase in I/R-induced damage to the left ventricle (LV), a reduction in the recovery of LV function post-ischemia, and a decrease in coronary flow rate in offspring of both genders (Ke e*t al*. 2017). The study found that smoking significantly contributes to bone loss in various skeletal locations, with a direct relationship between daily smoking quantity and exposure duration, despite factors like gender, age, body weight, BMI, and lifestyle practices (Kim *et al*. 2012). Research conducted on rodents indicates that newborns exhibit a heightened susceptibility to tobacco smoke, potentially leading to the initiation of cardiac fibrosis. Consequently, initiatives ought to be implemented to dissuade females from smoking while pregnant and in the period following childbirth (Chou and Chen 2014). Recent study indicated ,nicotine exposure during pregnancy has the potential to induce diminished postnatal body weights and reduced blood circulation to all fetal organs, indicating a significant role of nicotine in postnatal health (Aoyagi *et al*. 2020). Chuang and others ,provide evidence of compromised cardiac performance indicative of cardiac fibrosis subsequent to nicotine exposures during pregnancy and early infancy (Chuang *et al*. 2020). A study found that chicks exposed to nicotine experienced delayed developmental progress, stunted growth, and survival issues beyond the 16th day of incubation, possibly due to inadequate oxygen supply as a consequence of cardiac arrest(Chandrakar and Pachlore 2023). Understanding the mechanisms driving developmental nicotine exposure, developing therapeutic strategies, and advocating for policy modifications is crucial for reducing exposure.

2. Materials and methods

2.1 Materials

Fertile eggs of domestic chicken (Gallus gallus) were obtained from a local farm in Shahat city. A total of one hundred and fifty eggs were categorized into five distinct groups. Among these, one group served as the control, while another was administered distilled water. The remaining three groups received varying concentrations of nicotine, specifically 0.1 mg/ml, 0.3 mg/ml, and 0.5 mg/ml. Each group comprised thirty eggs, and samples were collected at three different developmental stages.

Nicotine solution 98% purchased from Sigma-Aldrich 54-11-5 C10H14N2. Medical alcohol ethanol 96% C2H5OH. Formalin stock solution 37%. Phosphate-buffered saline (PBS) tablets. KOH solution 2%. Ammonia solution 2%. Glycerol solution 2%. Alizarin red s (sodium alizarin sulphonate) 100 mg, Acetone puriss 2.5L (Riedel – dehaen 24201). The necessary equipment encompassed an Incubator, water path, and glassware.

2.2 Methods

2.2.1 Chick egg preparation and injection

Chicken's eggs were sterilized using 70% ethyl alcohol, then eggs were candled to determine the embryo positioning through the method of egg candling. A 10-cc syringe equipped with an 18-gauge needle, were used to withdrawal 3 to 4 mL of albumen. The eggs were categorized into groups, including a control group with no treatment, a group injected with 0.5ml of distal water as positive control, and various treated groups receiving injections of nicotine solution at concentrations of 0.1, 0.3, or 0.5 mg/ml. Subsequently,

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the eggs were placed in an incubator at a temperature 37-38 C° and a humidity level of approximately 60-

80%. The developmental stages of all embryos were carefully assessed according to Hamburger and

Hamilton (1951), specifically at stages HH17, HH18, HH19, HH26, HH27, and HH36, upon which the embryos were harvested and fixed for further analysis.

2.2.2 Embryos collection and fixation

Using blunt forceps, the egg shell was scraped directly on top of the embryo, small window (1 cm x 1 cm) was cut into the egg shell, the egg shell was removed first before cutting through the shell membrane, using scissors the blood vessels around the embryos were cut, then embryos lifted with a spoon, and placed in a petri dish and then PBS added, under the microscope, the small membranes surrounding the embryos were removed with micro scissors then embryos were fixed in 10% formalin solution.

2.2.3. Morphological examination of whole mount embryo and photographing.

Agar's plate was prepared by adding 5g agraous to 100ml distal water and heated until boiling, left to cool down then poured in small size petri dishes, then stored until to use for photographing. Fixed embryos were photographed on the top of the agarous plate by digital camera fixed on optical technology dissected microscope. Photos of whole embryo; Head, limbs and trunk region.

2.2.4 Heart rate calculation.

Nicotine solutions at concentrations of 0.1 mg/ml and 0.5 mg/ml were prepared, and chicken eggs underwent an incubation period of 3, 5, and 10 days. The "window" method described by (Cruz., 2012) was employed to window these eggs. Subsequently, the in vivo heart rate for each embryo assessed over a 15-second duration, repeated three times. Following this, two drops of a separate nicotine concentration were injected to each embryo. The subsequent heart rates were then determined, and the average values were calculated and graphed in an Excel spreadsheet.

3. Results

3.1 Morphological effects of nicotine on devolving embryo.

This study uses early chick embryos as a model to evaluate nicotine's effects on general embryonic development, detecting abnormal phenotypic manifestations at different developmental stages and alteration on heart rate.

3.1.1 Embryos treated with 0.1Mg/ml of nicotine: Eggs were injected with 0.1Mg/ml of nicotine and on day zero, and then collected after incubation in developmental stages HH17, HH26 and HH29. Data for the overall survival, mortality, fertility, and malformation for all experimental stages were illustrated in (Fig 1) presented as percentage. At all stages, fertility rate was between 66.7 to 100%, survival rate was between 66.7 to 100 and malformation rate was 0% in all control groups and distal water groups however, it was 83.3 to 100% in treated groups.

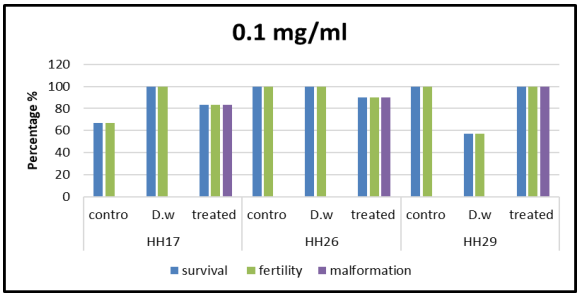

Figure (1) Shows the percentage of fertilization, survival and malformations at HH17, HH26 and HH29.

Figure 2 below shows the effects of nicotine on chicken embryos injected at day zero and collected at different stages, HH17, HH26 and HH29. It showed that control group and a group of distilled water and a group treated with nicotine in stage HH17 were presented in (Fig 2 A) and (Fig 2 A') (Fig 2A") consecutively. Control group embryos show normal growth, lateral body folds, and limb-buds and do not display any abnormalities. They are distinguishable swellings with no alterations in flexures and rotation as shown in (Fig 2A). Treatment with distal water showed no effect on embryos growth as illustrated in (Fig 2 A'). Nicotine-injected group (Fig 2A") the embryos developed with abnormal heart and all embryos in this group showed a general delay in development comparing with control group. The axis of the body was striate line, head was not in the normal shape, and it was not distinct.

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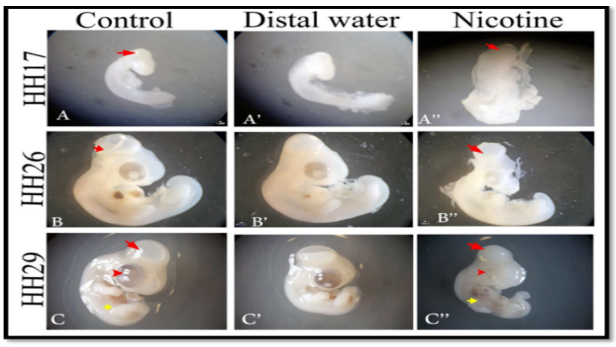

Figure (2) shows the effect of nicotine at 0.1mg/ml on developing chicken embryo at HH17, HH26 and HH29.

3.1.2 Treatment with nicotine in concentration 0.3Mg\ml at HH18 and HH29:

Nicotine solution was prepared in 0.3Mg/ml and injected on day zero, and then embryos were collected after incubation on different stages of chicken embryos at HH18 and HH29. Data for the overall survival, mortality, fertility, and malformation at all experimental stage were illustrated in (Fig 5) presented as percentage. At all stage, fertility rate was between 60 to 100%, survival rate was between 60 to 100% and malformation rate was 0% in all control groups and distal water groups however, it was 0 to 66.7% in treated groups.

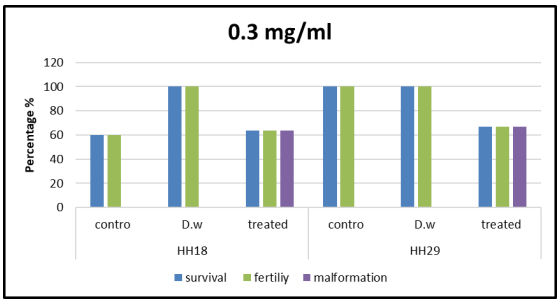

Figure (3) shows the percentage of fertilization, survival and malformation at HH18 and HH29

Figure 4 below showed the effects of nicotine on chicken embryos injected at day zero and collected at different stages, HH18 and HH29. It showed that control group and a group of distilled water and a group treated with nicotine in stage HH18 were presented in (Fig 4 A) and (Fig 4 A') (Fig 4 A") respectfully. The control group embryos show normal growth, consistent flexion and posterior rotation, with torso flexion, tail bud ventrally bending, unsegmented mesoderm, distinct epiphysis, and nose digging. Treatment with distal water showed no effect on embryos growth as illustrated in (Fig 4 A'), however it was observed that nicotine exposure as showed in (Fig 4 A") resulted in developmental delays, reduced heart size, diminished brain size in embryos, craniofacial malformations, blurry vision, and asymmetrically enlarged limb buds. The limb buds, including wing and leg buds, did not protrude from the plastoderin due to the infusion of body folds, appearing as indistinct bulges of varying sizes. There was a lack of skull flexion, posterior rotation, and cervical bending compared to previous stages, with characteristic trunk flexion at the humeral level and absence of ventral tail bud curvature, indicating a lack of nasal digging.

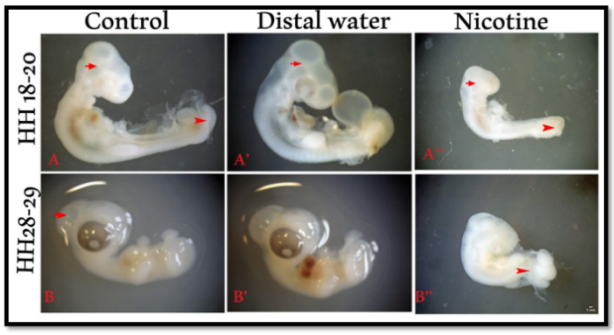

Figure (4) shows Effect of injection 0.3 mg/ml of nicotine on developing chicken embryo development at HH18 and HH29

3.1.3 Treatment with nicotine in concentration 0.5Mg\ ml at HH19, HH28 and HH36:

Nicotine solution was prepared in 0.5Mg/ml and injected on day zero, then embryos were collected after incubation on different stages of chicken embryos at HH19, HH28 and HH36. Data for the overall survival, mortality, fertility, and malformation at all experimental stage were illustrated in (Fig 7) presented as percentage. At all stages, fertility rate was between 50 to 100%, survival rate was between 50 to 100% and malformation rate was 0% in all control groups and distal water groups however, it was 67 to 74% in treated groups.

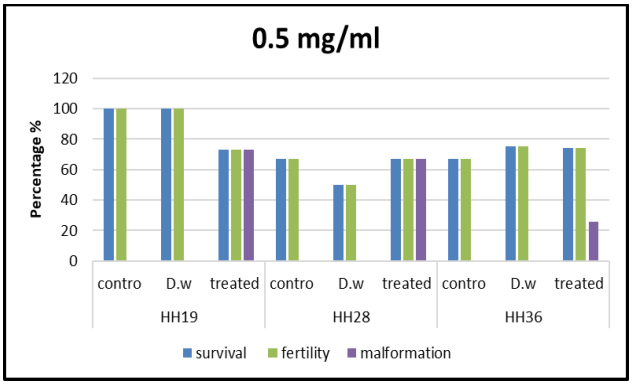

Figure (5) shows the percentage of fertilization, survival and malformations at HH19, HH28 and HH36.

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Figure 6 below shows the effect of nicotine on chicken embryos injected at day zero and collected at HH19 and HH28. It showed that control group and a group of distilled water and a group treated with nicotine in stage HH19 were presented in (Fig 6 A) and (Fig 6 B) (Fig 6 C) respectfully. The control group embryos have normal growth patterns, slightly larger limb-buds than wing-buds, and a closed amnion. They show cervical flexures and rotation, causing an angle between the medulla and posterior trunk. Treatment with distal water exhibited no significant impact on the growth of embryos, as demonstrated in (Fig 6 B). Nonetheless, it was noted that the group treated with nicotine (Fig 6 C) displayed a reduction in growth. The brain, head, and heart exhibited diminished size, while the tail lost its natural curvature. Additionally, deformities in the shape of the trunk were observed, along with a lack of enlargement in the limb buds.

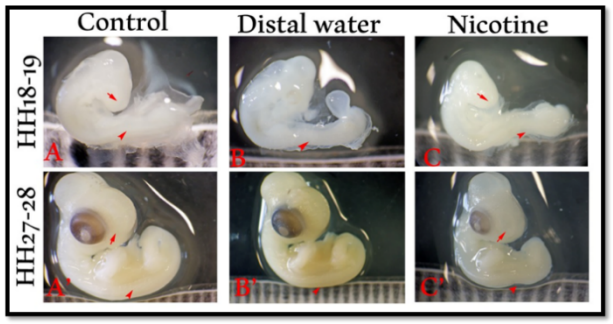

Figure (6) shows effect of injection 0.5 mg/ml of nicotine on developing chicken embryo at HH19 and HH28

In general, nicotine caused a morphological malformations in head craniofacial trunk an libs buds in additional caused embryo death, it was dose dependent, Several previous studies have indicated that nicotine exposure can result in deformities in embryos, as well as distortions in the development of the brain, heart, trunk, limbs, eyes, and skeletal structure (Behnke *et al*. 2013).

This study reveals that early nicotine exposure during development delays cerebral maturation causes neural tube anomalies, deformed limbs, and ocular structures. It also shows reduced cranial and cerebral size, midbrain elongation, and diminished cardiac structure, these findings align with previous research suggesting that nicotine exposure during gestation adversely affects fetal growth, whether through active or passive smoking(Chandrakar and Pachlore 2023). Prior investigations into the impact of nicotine on developmental physiology have established that the physiological responses induced by nicotine are mediated through the activation of neuronal nicotinic acetylcholine receptors (Smith *et al*. 2010). Furthermore, nicotine has been shown to inhibit Ornithine decarboxylase (ODC) (Slotkin and Bartolome., 1986), an enzyme crucial for cellular proliferation. This inhibition may elucidate the observed reduction in embryonic size and developmental retardation. Moreover, numerous studies have identified a correlation between embryonic nicotine exposure and skeletal anomalies; nicotine has also been found to diminish collagen synthesis and alkaline phosphatase activity in osteoblast-like cells, thereby delaying skeletal maturation in embryos (Shan, Qamar *et al*. 2015) The observed retardation in osseous development may be attributable to the inhibitory effects of nicotine on calcium absorption within the embryo(Saad and Husayn 2024).

3.1.4 Effects of nicotine on heart rate: In this experiment, the eggs were injected with two different concentrations of nicotine solution 0.1 Mg and 0.5Mg on day 0 , and then opened at day 3, day 5 and day 10. Then heart rate was calculated this, as shown in the (Fig 7, 8, 9).

3.1.4.1 Heart rate (bpm) in 3-day chicken embryos.

The study shows that nicotine concentrations of 0.1 and 0.5 mg/ml significantly reduced heart rate in chick embryos it was 90.6 and 81.6 pbm/ minute respectively, compared to the control group and distal water 128 and 127.33 pbm/ minute. The higher the concentration, the lower the pulse.

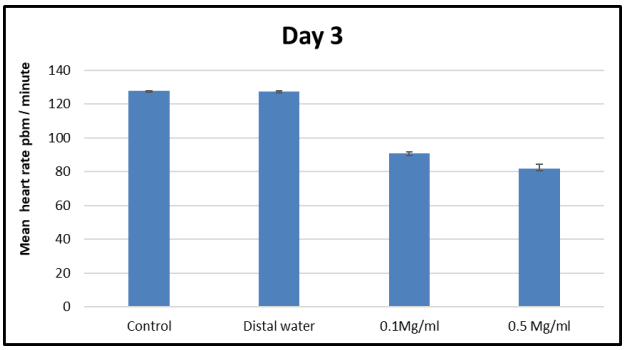

Figure (7) shows the heart rate differences in the control and distal water N. 0.1 Mg and N, 0.5Mg, on the embryo on day3. Not: error bars presented by SD ±

3.1.4.2 Heart rate (bpm) of 5-day chicken embryos:

The chick embryos, which were incubated for 5 days and injected with nicotine at concentrations of 0.1 mg/ml and 0.5 mg/ml, displayed a decrease in heart rate as depicted in the data below 79 and 77.6 pbm / minute respectively. This decrease was noted in comparison to both the control group and the distal water 140 and 136 pbm/ minute respectively. Additionally, embryos at

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higher stages that received nicotine injections exhibited even lower heart rates.

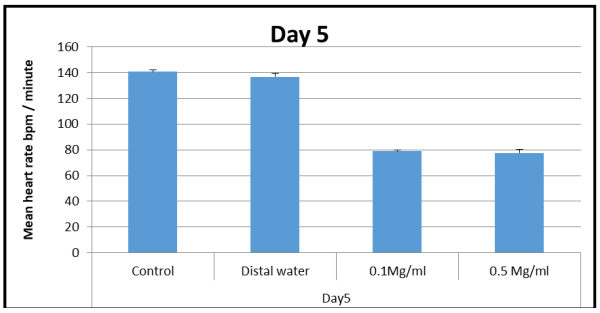

Figure (8) shows the heart rate differences in the control and distal water N 0.1Mg, N 0.5Mg, on the embryo on day 5. Not: error bars presented by SD±

3.1.4.3 Heart rate (bpm) of 10-day chicken embryos: chick embryo 10-day were noticed sharp decline in heart rate in embryos that were injected with concentrations of 0.1 mg/ml and 0.5 mg/ml it was 24 and 22 pbm/ minute respectively, compared to a group control and distal water 69 and 68 pbm/ minute respectively. Whenever the increased embryos stage lowers the rate of heartbeats. The higher the concentration, the lower the pulse.

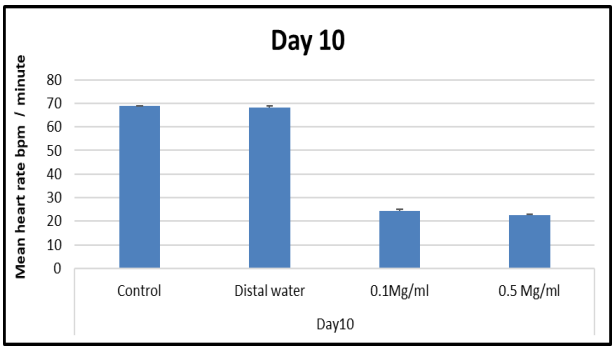

Figure (9) shows the heart rate difference in the control and distal water N 0.1Mg, N O.5Mg, on the embryo on day 10. Note: error bars presented by SD±.

4. Discussion

The results derived from the current investigation suggest that nicotine administration precipitates a decrease in heart rate, a phenomenon that is consistent with a plethora of studies that have yielded similar findings. For example, research conducted by Piano has elucidated that smoking exerts deleterious effects on developmental trajectories, thereby engendering a spectrum of pregnancy-associated complications, which include adverse impacts on cardiac growth and performance (Piano, *et al*. 2010) and impedes the angiogenesis process (Melkonian, *et al*. 2000). Furthermore, the compounds nicotine and carbon monoxide frequently emerge as subjects of inquiry due to their ramifications on hemodynamics and the contractile capacity of blood vessels. These constituents found within cigarette smoke have been linked to vascular disorders, as they potentially attenuate angiogenesis, exacerbate tissue degradation, and promote thrombosis (JA., 2004). Additionally, numerous systematic reviews have established a relationship between maternal smoking and the incidence of congenital heart anomalies (Karatza, *et al*. 2011). Moreover, nicotine has been documented to elevate blood pressure (BP) and is associated with a range of cardiovascular and obstetric complications (Li, *et al*. 2015). A recent study suggested that exposure to nicotine during the formative phases of cardiac development may precipitate congenital heart defects, including aortic stenosis, septal defects, valvular malformations, and thinning of atrial and ventricular walls in chick embryos, ultimately culminating in diminutive cardiac structures and arrhythmias (Greco, *et al*. 2022). Additionally, investigations conducted on murine models have indicated a reduction in left ventricular fractional shortening, an escalation of atherosclerotic lesions, and the activation of autophagy within vascular tissues (McGrath-Morrow, *et al*. 2020). It is hypothesized that fetuses of women who engage in smoking are at an increased risk for congenital heart defects, as the noxious agents in cigarette smoke may induce fluctuations in blood pressure and fetal hypoxia, which could further contribute to the manifestation of these abnormalities (Zhang, *et al*. 2017). In summary, the aggregated findings imply that nicotine exerts a significant influence on hematopoiesis and cardiac development, potentially resulting in modifications to heart rate

Research conducted utilizing animal models indicates that exposure to nicotine may lead to inappropriate activation or deactivation of nicotinic acetylcholine receptors (nAChRs) during critical phases of cerebral development (Yuan, *et al*. 2015). The phenomenon of nicotine dependence is associated with an upregulation in the expression of nAChRs (Govind, *et al*. 2009).

A cohort study involving live births emphasized the correlation between nicotine exposure and the prevalence of birth defects, revealing that maternal smoking during the initial trimester significantly heightened the risk of various anomalies, such as limb reduction defects, gastroschisis, and oral clefts (Perry, *et al*. 2019). Smoking during the preconception period has been shown to increase the risk of gastroschisis by 40%. Furthermore the found , the persistence of smoking into the first trimester is correlated with augmented risks of defects, including gastroschisis and

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limb reduction, even when controlling for additional confounding variables (Perry, *et al*. 2019).

5. Conclusions

This investigation employed chicken embryos to assess the influence of nicotine on morphological development during the early stages of HH17 to HH29, as well as its impact on heart rate at stages HH19, HH27, and HH36. The study's results indicate that early exposure to nicotine can lead to general growth retardation, morphological defects in the brain, heart, trunk, and limbs, as well as a reduction in heart rate.

Conflict of interest: The authors declare that there are no conflicts of interest.

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