UNIVERSITI PUTRA MALAYSIA

NICOTINE-INDUCED PATHOLOGY OF EMBRYONATED CHICKEN EGGS

AMIRULLAH BIN ABDUL RASIF

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FPV 2020 65

NICOTINE-INDUCED PATHOLOGY OF EMBRYONATED CHICKEN EGGS

AMIRULLAH BIN ABDUL RASIF

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

2020/2021

CERTIFICATION

It is hereby certified that we have read this project paper entitled "Nicotine-induced Pathology of Embryonated Chicken Eggs", by Amirullah bin Abdul Rasif and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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PROFESSOR. DR. NOORDIN MOHAMED MUSTAPHA

DVM (UPM), MS (UPM), PhD (Murdoch, Australia)

Professor, Faculty of Veterinary Medicine, Universiti Putra Malaysia

(Supervisor)

_____

DR. MAZLINA MAZLAN

DVM (UPM), MS (UPM), PhD (UPM)

Senior Lecturer, Faculty of Veterinary Medicine, Universiti Putra Malaysia

(Co-Supervisor)

DEDICATION

To my parents, who always picked me up on time, walked by my side all the time, and encouraged me to go on every adventure, especially this one.

ACKNOWLEDGEMENTS

I would like to send my heartiest gratitude to Allah S.W.T for enabling me to accomplish my final year project successfully.

My utmost appreciation and profound thanks go to my project supervisor, Professor. Dr. Noordin Mohamed Mustapha and co supervisor, Dr. Mazlina Mazlina for their time, patience, valuable comments, and guidance throughout this project.

Not forgetting the serology and histopathology laboratory staffs, for assisting me in my laboratory work and sampling.

A big shout out to Ali, Arif, Khidir, Syazwan, Janna and housemates, Izwin and Syuhada for their unconditional support during this project. This project will not be as exciting and fun without you guys. Thank you.

I would also like to acknowledge my loving family for their endless support and encouragement throughout in completing the project.

Million thanks to those who have contributed directly or indirectly to bring this thesis into fruition.

Road to CE 2021

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ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Tahun Akhir.

PATOLOGI ARUHAN NIKOTIN TERHADAP TELUR AYAM BEREMBRIO

Oleh

AMIRULLAH ABDUL RASIF

2020

Penyelia: Professor. Dr. Noordin Mohamed Mustapha

Penyelia Bersama: Dr. Mazlina Mazlan

Nikotin ialah alkaloid terbitan dari ekstrak daun tembakau yang digunakan dalam penghasilan rokok. Kini, nikotin terhadap ibu hamil telah terbukti menyebabkan kesan berbahaya neonat. Bagaimanapun, kesan teratogen nikotin terhadap telur ayam berembrio agak kekurangan. Terdapat kemungkinan bahawa telur ayam berembrio boleh digunakan sebagai model ramalan teratogenesis yang sah. Oleh itu, satu kajian dilakukan untuk menilai kesan matakasar dan mikroskopi pendedahan nikotin terhadap perkembangan embrio ayam. Selain daripada kumpulan kawalan, tiga kepekatan nikotin yang berbeza telah digunakan. Larutan penimbal fosfat dan nikotin telah disuntik secara alantois bermula pada hari kesembilan pengeraman. Dos nikotin

yang digunakan adalah 15, 30, 45 mg/kg (berasaskan berat telur) dan larutan penimbal fosfat disuntik kepada kumpulan kawalan. Telur telah diperiksa setiap hari dan kesemua embrio yang mati sepanjang tempoh ujikaji dan waktu yang ditetapkan telah dituai. Tiga embrio dari setiap kumpulan telah dituai pada hari ke 13, 16 dan 19 pasca-inkubasi. Lanjutan dari penentuan berat embrio dan penilaian lesi, tisu terpilih diawet dalam 10% formalin 10% dan diproses untuk pemeriksaan histologi. Data yang diperolehi telah dianalisis menggunakan ujian ANOVA dua hala dan Chi- square. Kemortalan embrio hanya terjadi pada kumpulan dos pertengahan dan tinggi sahaja. Nikotin secara keertian ($p=0.001$) merencat perkembangan embrio dengan mempamerkan kebengkokan leher dan hiperfleksi digit bersama dengan defisit pada panjang serta berat embrio, jantung dan indeks soma jantung. Interaksi antara dos dan hari pengeraman hanya bererti ($p<0.005$) untuk indeks soma jantung dan panjang embrio. Secara perbandingan, dos yang lebih tinggi terbukti lebih toksik dan menyebabkan kadar merencat perkembangan yang lebih tinggi. Masing-masing, histologi jantung, paru-paru dan hati menunjukkan pelbagai lesi tersandar dos seperti hipertrofi miokardium dan susunan longgar kardiomiosit; degenerasi dan fibrosis sel hati, dan edema berserta penebalan parabronkium. Kesimpulannya, nikotin boleh menrencat pembentukan dan perkembangan embrio ayam pada pelbagai peringkat.

***Kata kunci**: nikotin, tembakau, telur ayam berembrio, teratogenesis, hitologi.*

ABSTRACT

An abstract of the project paper presented to Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999- Final Year Project.

NICOTINE-INDUCED PATHOLOGY OF EMBRYONATED CHICKEN EGGS

By

AMIRULLAH ABDUL RASIF

2020

Supervisor: Professor. Dr. Noordin Mohamed Mustapha

Co-Supervisor: Dr. Mazlina Mazlan

Nicotine is a plant-derived alkaloid extracted from tobacco leaves used in cigarette production. Over the years, the harmful effects of nicotines during pregnancy on neonates via inhalation of tobacco smoke are well established. However, the teratogenic effects of nicotine on embryonated chicken eggs are lacking. It is likely that the embryonated chicken eggs can be used as a valid predictive model of teratogenesis. Thus, a study was conducted to assess gross and microscopic effects of nicotine exposure on embryonic development in chickens. The embryonated chicken eggs used in this experiment apart from a control, were subjected to three concentration of nicotine. The nicotine solution was injected via the allantois commencing on the 9th

day of incubation. The doses of nicotine were 15, 30, 45 mg/kg (based on egg weight) and phosphate buffer solution was used for the control. Eggs were candled daily and any dead embryos along with the those at the designated period were harvested. Three embryos from each group were harvested on the 13th, 16^{th,} and 19th days post-incubation. Following embryo weight determination and gross appraisal of lesions, selected tissues were fixed in 10% buffered formalin and routinely processed for histology. Data obtained was analysed using a two-way ANOVA and Chi-square test. Embryonic mortality was only seen in the middle and high dose groups. It appeared that nicotine significantly ($p=0.001$) affects the general embryonic development manifested by crooked neck and digital hyperflexion, lesser embryonic length and weight, heart weight, and heart somatic index. Likewise, an interaction between dose and incubation days was only significant ($p<0.05$) for the heart somatic index and heart length. Comparatively, the higher dose proved to be much more toxic and caused higher rates of developmental defects. The histology of the heart, lung, and liver revealed varying degrees but dose dependent lesions such as loose and hypertrophied myocardium; degeneration and fibrosis of liver cells; and oedema and thickening of lung parabronchi, respectively. In conclusion, nicotine could impair chicken embryonic development at different stages of development.

Keywords*: nicotine, tobacco, embryonated chicken egg, teratogenesis, histology.*

1.0 INTRODUCTION

The potent parasympathomimetic alkaloid of tobacco, nicotine is responsible for the ill health especially that of the reproductive, cardiovascular, pulmonary, gastrointestinal, and immune systems (Karaconji, 2005; Hossain & Salehuddin, 2013). Apart from population growth, the four-fold increment on tobacco production is attributed to the lifestyle of cigarette-dependent consumers especially in the low and middle-income countries (Mishra *et al.,* 2015; Drope & Schluger, 2018). Thus, it is not suprising that cigarette raked as one of the top leading cause of 4.9 million death annually (Karaconji, 2005; Drope & Schluger, 2018). Despite rigorous and extensive campaign in Malaysia, a death rate contributed by cigarette amount to 23% (Drope & Schluger, 2018).

The environmental tobacco smoke (ETS) or secondhand smoke (SHS) released from tobacco products is considered as a serious health hazard affecting about one-fifth and a third of males and females, respectively (Drope & Schluger, 2018). The main concern of exposure to nicotine during pregnancy has led to premature birth, growth restriction, premature rupture of membranes, spontaneous abortion, and tachycardia (Lambers & Clark 1996). Although uncommon, inevitably animals are also indirectly exposed to SHS with reports serious gastrointestinal, central nervous system, and cardiovascular signs (Novotny *et al.,* 2011).

However, there is still limited research on the health risk of nicotine to animals while *in utero*. Besides, the actual mechanism of nicotine-induced pathology in embryonated chicken eggs is still not well-documented and this study yield detrimental evidence that will sensitise awareness of protecting animals against exposure to

cigarette smoke. In addition, the embryonated chicken eggs is a good model for teratology and embryotoxicity experiment. The earliest stages of birds development correspond to the first half of human gestation period indicating the parallel embryonic development between birds and mammals (Hill & Hoffman, 1984; Bohn *et al.,* 2017).

Thus, the hypothesis for this study is that nicotine has the potential to exert an adverse effect on embryonated chicken egg development.

Therefore, this study was aimed at assessing the effect of nicotine on embryonated chicken eggs with the following objectives to evaluate the:

i. viability and deformity of chick embryos
ii. histology of the heart, lung, and liver

2.0 LITERATURE REVIEW

2.1 Tobacco

Tobacco is derived from leaves of the *Nicotiana* genus encompassing 60 species that was discovered as early as 1400 BC (Goodman, 2005). In ancient times, tobacco was smoked for the ritual and religious ceremonies where most common commercially cultivated species are from *Nicotiana rustica* and *Nicotiana tabacum* (Rodgman & Perfetti, 2013). These two species are used in the production of cigarette, pipe, cigar, snuff, and chewing tobaccos (Stedman, 1968; Benowitz, 1996; FAO, 2003; Rodgman & Perfetti, 2013; WHO, 2019).

Dubbed as a chemical factory, tobacco contains 5200 compounds (Hoffman *et al*., 1998; Borgerding *et al*., 2005; Thielen *et al*., 2008; Rodgman & Perfetti, 2013), which are classified mainly into hydrocarbon, oxygen-containing and nitrogen-containing components. Likewise, the chemical compositions of tobacco smoke are estimated to be similar to tobacco's chemical compositions (Rodgman & Perfetti, 2013).

2.2 Cigarette

Existing in various forms, the lethal tobacco smoking is a major cause of morbidity and mortality worldwide affecting 50% of the users. Physically tobacco smoke consists of mainstream (MS) and side stream (SS) smoke which makes up the environmental smoke (ETS) (National Research Council, 1986). Both the MS and SS are equally hazardous with an average 24.1 mg tar, 53 mg carbon monoxide, 4.1 mg nicotine, and 2-3 mg nitrogen oxide (Löfroth, 1989).

2.3 Nicotine

Nicotine, with a molecular formula of $C_5H_4NC_4H_7NCH_3$ or synonymously known as (S)-3-(1-Methyl-2-pyrrolidinyl) pyridine, 1-Methyl-2-(3pyridyl) pyrrolidine or 3-(N-Methyl-2-pyrrolidinyl) pyridine and molecular weight of 162.23 g/mol is categorized as poisonous and hazardous. Nicotine appears physically in a liquid or aerosol form with a density of 1.010 g/ml relative to water density by 1.01:1 (Karaconji, 2005).

Apart from tobacco, nicotine (Karconji, 2005) can be found in leaves of the *Solanaceae* (nightshade) family such as potatoes, tomatoes, green pepper, eggplant (Domino *et al*., 1993; Karaconji, 2005; Hossain & Salehuddin, 2013; Centers for Disease Control and Prevention, 2014). The nicotine is determined to be the most abundant alkaloid in commercial tobacco, comprising around 95% of the total alkaloid (Karaconji, 2005; Benowitz *et al*., 2009). In general, the nicotine concentration of dry weight tobacco leaves represents about 0.3-3% and is about 5% of the tobacco plant weight (Tayoub *et al*., 2015).

Nicotine also serves as one of the chemicals in cigarettes or tobacco-related products (Hossain & Salehuddin, 2013) but has been therapeutically used to aid smoking cessation by relieving the withdrawal symptoms (Casella *et al*., 2010). In 1690 (Tomizawa & Casida, 2005), nicotine was identified as the first natural alternative to chemical pest control substances or a plant-derived insecticide in the form of tobacco extract (Benowitz *et al*., 2009; Rodgman & Perfetti, 2013). Nicotine was proven to have some antihelmintic properties and was historically used against

ectoparasiticide in livestock but later removed from the market when modern anthelmintics became available.

Each cigarette contains 8 to 30 mg of nicotine (Kozlowski *et al*. 1998; Novotny *et al*., 2011; Tayoub *et al*., 2015), but only 0.5 to 2 mg is actually absorbed by the body (Novotny *et al*., 2011). Rickert *et al*. (1984) found that nicotine is the principal alkaloid emitted in cigarette smoke, and about 75-90% contributed to SS.

2.4 Nicotine Effect on Animals Embryo

Nicotine toxicity reports in animals could result in multiple organ failure and fatality, most of the cases caused by cigarette butt consumption (Novotny *et al*., 2011). In the human research field, animal models such as rat, non-primate were extensively used in nicotine studies (Bruin *et al*., 2010). However, there is still a lack of data on prenatal exposure of nicotine in various animal species. The detrimental effects of nicotine on developing foetus were mostly recorded in rodents, rabbits, sheep, and chicken embryos (Maritz, 1988; Chen *et al*., 2001; Hussein *et al*., 2014).

Nicotine receptors can be found in the nervous system, muscle, various organs, and tissues in the body, thus significantly affecting the biological effects throughout the body system (Mishra *et al*., 2015). The microscopically harmful effects of nicotine are remarkable in various organs of the animal embryo (Gilani, 1971; Booyse *et al*., 1981; Maritz, 1988; Bruin *et al*., 2007; Xiao *et al*., 2007; Jensen *et al*., 2012)

2.5 Avian Embryo Development

The avian embryo tissues grow from a continuous division of primitive strikes that consist of ectoderm, endoderm, and mesoderm layers. These three layers of cells

will give rise to organs and tissues of the chick embryo. Table 1 indicates the development of the chicken embryo, where the staging of Hamburger and Hamilton (1951, reprinted 1992) will be used to indicate embryonic age. Relevant stages are correlated with incubation days and major events as a landmark.

Besides, few essential structures are connected with the embryonic tissue but will gradually be diminished during development. They are; yolk sac, allantois, and amnion sac. The yolk sac act as a food source for the embryo, while allantois is responsible for respiratory exchange, storage of the embryo's waste material, and calcium transport from the shell. The amnion is a sac that encloses the embryo and provides protection and preservation (Bellairs & Osmond, 2015).

Table 1: The development of chick embryo by staging and day of incubation

Hamburger and Hamilton (HH)Stages	Time	Major events
Stage 1 - Stage 6	24 hours	The differentiation of the alimentary tract, vertebral column. The development of anterior part (head), eye and formation of nervous system.
Stage 7 - Stage 12	48 hours	The heart has started to develop from primitive tissue.
Stage 13 – Stage 19	72 hours	The primitive lung has started to differentiate from the upper tract. The allantois and amnion sac started to develop. The beginning of legs and wings development.
Stage 20 – Stage 26	5^{th} day	The beginning of tongue formation and eye pigmentation. The spleen has started to differentiate.
Stage 27 – Stage 28	6^{th} day	The reproductive organ and sexual division have started to form. The beginning of voluntary movements. Amnion is prominent.

Stage 29 - Stage 30	7th day	The beginning of beak development. The fusion of the allantois with the chorion to form the chorioallantois begins. The allantois becomes highly vascularised.
Stage 31 – Stage 34	8th day	The first appearance of feathers.
Stage 35	9th day	Toes are formed.
Stage 36	10th day	The beak started to harden. The allantois is prominent.
Stage 37 – Stage 39	11th -13th day	The scales and claws have started to develop. The allantois has started to shrink and develop into the chorioallantois membrane. The inhibition of amniotic fluid function begins.
Stage 40 – Stage 41	14th–15th day	The embryo is repositioned for hatching turns its head toward the large end of the egg.
Stage 42	16th day	Scales, claws, and beak become more prominent.
Stage 43	17th day	Beak turns toward air cell.
Stage 44	19th day	Yolks sac begins to enter or reabsorbed into the abdomen.
Stage 45	20th day	The yolk sac is ultimately drawn into the embryo abdomen lead to closing of the umbilicus. The embryo begins to vocalize and breath.
Stage 46	21st day	Hatching.

Note. Source: "Atlas of chick development", by Bellairs, R., & Osmond, M. (2005), p.418-422

2.6 Heart Development and Histology of Avian

According to Wu *et al*. (2003), the embryonic heart is the first functional organ to develop in vertebrate animals. The formation of the primitive cardiac region is through thickening of mesoderm (Kelly & Buckingham, 2002; Bellairs & Osmond, 2015), leading to the cardiac tube formation. There are four fundamental steps of avian embryonic heart development: heart tube formation, looping, trabeculation, valve

formation, or septation (Lindsey *et al*., 2014). The critical events of cardiogenesis mentioned above happened at stages between 48 to 100 hours (Garcia-Martinez *et al*., 1993; Martinsen, 2005).

The cardiac tube will appear in 24 hours, and then the tube will start to bend or loops inside. The embryo will have two bloodstreams originating from the heart in less than 48 hours (Bellairs & Osmond, 2005). The initiation of heartbeats can be detected in the newly formed heart tube at 29-33 hours, Hamburger–Hamilton (HH) Stage 9 before myocyte contraction begins (Kamino *et al*., 1981). By the end of the stage (HH 10), clear heart rhythm and contractility are visible (Bellairs & Osmond, 2005). The establishment of a strong heart rhythm will eventually lead to effective blood flow during looping.

Cardiac trabeculation will be in 75 hours of incubation (HH 18), then the primitive atrium and ventricle will be formed, and the chamber's septation will begin (Wittig & Münsterberg, 2016). By five days of incubation, the atrioventricular seption was formed, and all the main chambers can be detected (Garcia-Martinez *et al*., 1993; Martinsen, 2005)

The coronary vasculature formation will start with coronary arteries formed at that stage (HH 32-33) (Manner, 1999). On the other hand, the coronary veins will be visible between days 10 to 14 (HH 38).

The proliferation rate of cardiac myocytes (increase in cell number) declines as the chick embryo develops and stop at hatching but then increases again during post-hatching development. Most of the proliferative phase of the compact layer

(myocardium adjacent to the epicardium) is from day 8 to day 14, resulting in expanding the ventricular wall (Martinsen, 2005).

Unlike chick, mammalian myocytes seem to lose their ability to proliferate shortly after birth ultimately. The process of hypertrophy of chick heart will take place primarily during post-hatching (Li *et al*., 1997). Therefore, the increase in heart mass, especially in the left ventricle during avian post-hatching development, may result from a combination of myocyte proliferation and hypertrophy. The thickness of the avian myocardium on the left side is five times thicker than that of the right ventricle (King & McLelland, 1984; Sedmera *et al*., 2000), allowing efficient pumping.

The histological structure of avian heart muscle is almost similar to mammalian species; only the cardiomyocytes have smaller diameters than those in mammals. This permits faster depolarization and a more rapid heart rate (Abdul-aziz *et al*., 2016).

2.7 Lung Development and Histology of Avian

The larynx, trachea, and lungs are derived from the same endoderm layers and a thicker covering mesoderm. The lung will start to differentiate from the endoderm of pharyngeal in 72 hours. The first prominent respiratory tract structure is laryngeal-tracheal groove, which becomes visible during days 3 to 4 of incubation. (HH, 19). Then, the lung will arise from the groove and growing further into branching. By the end of day 6-7, the lung will appear as distinct as in an adult lung structure. The lung will continue to grow towards the late incubation period (Abdul-aziz *et al*., 2016)

The avian lung histology structures consisted of the bronchial system, atrial/air vesicles, infundibulum, and air capillaries. The bronchial system can be further divided

into the primary bronchus, secondary bronchi, and tertiary bronchi (Del Corral et., al 1992). The primary bronchi give rise to secondary bronchi, and tertiary bronchi arose from secondary bronchi. As described by Abdul-aziz (2016), the primary bronchi are lined by pseudostratified ciliated columnar epithelium with goblet, and mucous glands reside in the connective tissue layer. The wall's deeper layer comprises a muscle layer and is supported by cartilaginous plates (Maina, 2015). The same type of epithelial cell also can be found lining the secondary bronchi, but only contains goblet cells rather than mucous gland (Aughey & Frye, 2001).

The biggest portion of lung parenchyma is covered by the cylindrical-shaped of parabronchi. The lung unit: parabronchi and surrounding tissues are separated in between by interparabronchial septa that contain arterioles, venules, and nerve fibers (Maina, 2015). In general, the wall of parabronchi has three layers from simple squamous (Type 1 pneumocyte) or cuboidal epithelium (Type 2 pneumocytes), a thin layer of loose connective tissue, and a smooth muscle layer (Aughey & Frye, 2001). In contrast to the internal layer of primary and secondary bronchi, there are no cartilages in the parabrochial wall.

The atria bulge from the parabronchus lumen and projected into the parabrochus wall forming structure of small air cavities surrounding the parabronchus lumen (Aughey & Frye, 2001). The entire atrium is lined by the Type II pneumocytes except the area close to the infundibulum opening, where the cuboidal-shaped cells become gradually flat (Bódi *et al*., 2016). The infundibula act like a funnel tube connect the atria with the air capillaries inside the wall of parabronchi. The air capillaries intermingled among blood capillaries, allowing the gas exchange (Aughey

& Frye, 2001; Maina, 2015). The Type 1 pneumocyte is lined by the air capillaries with the absence of Type 2 (Bódi *et al*., 2016).

2.8 Liver Development and Histology of Avian

In chick embryo, liver organogenesis is similar to that of the mammalian embryo (Yokouchi, 2005). The embryonic liver development microstructure is composed of three tissues, namely liver bud from foregut endoderm, septum transversum mesenchyme and endothelial cells of primary sinusoids (Yokouchi, 2005). The liver organogenesis appears in 20-22-somite chicken embryos (50-53 hours of incubation). The liver bud is formed from the endoderm cells called hepatoblast, which possess the capability to differentiate into hepatic parenchymal and biliary system (Spagnoli *et al*., 1998). On the 5th day (HH 26) of development, the hepatic portal has created (Bellairs & Osmond, 2005), the unmature hepatocyte in loosely arrangement and sinusoids has begun to develop (Çöllü & Gürcü, 2017).

The hepatocytes will become more organized, the new branches of liver lobe will be formed, and the proliferation rate of parenchyma cells will become parallel with the growth rate of liver size. After the 8th day (HH, the organogenesis is completed and the liver will enter a rapidly growing phase. The proliferation of parenchymal cell will make the size of the liver increased (Çöllü & Gürcü, 2017). Wong and Cavey (1993) studied liver development in the chick embryo with light and electron microscope. They observed that liver is visible grossly on the 6th to seventh day (HH 30) of development, and its development completed almost by day 14 (HH 40). Subsequently (Stages 36 to 40), cell volume increases, and hepatocytes achieve a relatively uniform size.

Histologically, birds and mammals' embryonic liver appear very different, but some basic features of the avian liver are quite similar to mammals (Aughey *et al*., 2001). The micromorphological liver structures are composed mainly of three distinct cell types: parenchymal cells, epithelial cells of the bile duct, and endothelial cells of the sinusoid (Yokouchi, 2005). The homogenous mass of parenchyma is divided by connective tissue capsule into lobes and lobules; however, the avian liver lacks of distinct lobular structure unlike mammalian liver because of reducing in interlobular septa formation (Hodges, 1972; Abdul-aziz *et al*., 2016).

In mammals, a lobule or a liver unit contains a central vein with hepatic cords radially organized around it. On the other hand, the avian liver is a mass of branching, hollow cords, and bile caniculi are located in the lumina of the cords (Abdul-aziz *et al*., 2016). The hepatic cord of avian are mostly two cells thick and are separated from each other by discontinuous linings of the sinusoids consist of endothelial cells and Kupffer cells (Hodges, 1972; Aughey *et al*., 2001). The portal triads: hepatic artery, hepatic portal vein, and bile duct are placed at between three or more lobules, where the hepatic cords are branching from the central vein (Abdul-aziz *et al*., 2016). Hepatocytes are pyramidal, and they ring the bile canaliculi, which run through the cords' centers.

3.0 MATERIALS AND METHODS

3.1 Chicken Embryonated Egg

Fifty fertile 9-day-old chicken embryonated eggs (*Gallus gallus domesticus*) supplied by a local farm, the hatchery unit of Charoen Pokphand Jaya Farm Sdn Bhd, Rembau Negeri Sembilan were used.

3.2 Inoculum and Dosage

The stock solution was prepared by dissolving 1ml of nicotine (95% pure nicotine, Nacalai Tesque, Inc., Japan) in 39 ml of phosphate buffer saline (PBS). The doses were chosen based on studies conducted by Kozlowsli *et al.* (1998) and Novotney *et al.* (2011) based on a 70kg human where the plasma level of a regular smoker is estimated to be 10 to 50 ng/ml. The equivalent plasma level of nicotine in a chick embryonated egg is in according to mean egg weight calculated to be 40800 ng/ml. For the present experiment, three range of doses were used with the high dose being 45 mg/kg, middle of 30 mg/kg, and a low dose of 15 mg/kg.

The determination of the volume of inoculation was calculated as follow:

$$Inoculation\ volume\ \ ml = \frac{Nicotine\ dose\ mg/kg\ \ \times\ \ Egg\ weight\ mg/kg}{Nicotine\ cocentartion\ mg/ml}$$

3.3 Experimental Design

3.3.1 Grouping

The eggs were divided into four groups that consisted of 12 eggs per group. The first group was kept as a negative control and treated under the same condition but only injected with phosphate buffer solution (PBS). The second group was treated

with a low dose (15 mg/kg) followed by the third group with a middle dose (30 mg/kg) then, the fourth group with a high dose of nicotine solution (45 mg/kg).

3.3.2 Method of Injection

The injections were delivered into the 9-day old embryonated eggs through the allantoisc cavity. First, the viability of the eggs was checked by candling technique before introducing the chemical, then the inoculation site at air sac region of the eggs was marked. The blunt pole of eggs was wiped with 70% alcohol and labelled accordingly. A hole was then made at the marked injection site. Using a sterile BD Ultra-Fine ^{TM} 0.5 ml insulin syringe with 29G ×12.7 mm needle, the calculated inoculation volume was injected vertically into the air space reaching the allantois cavity of 9th day of incubated eggs. The holes were sealed with melted wax, and the eggs were placed in an incubator.

3.3.3 Embryo Collection

The incubated eggs were tested daily by candling technique to evaluate the viability of the embryos. The eggshell of dead embryos was broken with scalpel and removed from the shell and examined grossly for external deformations at the stipulated period. Three live embryos from each group were randomly selected and harvested on day 13, day 16 and day 19 of incubation. The length (measured from crown to rump) was recorded for every embryo. The heart, liver and lung were dissected out for appraisal of gross lesions. The weight of whole embryos and heart specimen were also measured using a digital analytical balance. All the surviving embryos until day 19 of incubation were also removed for examination.

3.4 Histological Investigation

The heart, lung and liver tissues were fixed in 10% buffered formalin for 24 hours. Then, the fixed heart tissues were trimmed, dehydrated in ascending series of alcohol, embedded in paraffin, sectioned at four-micron thickness and stained with Hematoxylin and Eosin (Feldman and Wolfe, 2014). The histology slides were then examined under a compound microscope equipped with an image analyser software (Motic BA410 TRINOCUL, Speed Fair Co, Hong Kong).

3.5 Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and subjected to a two-way analysis of variance (ANOVA). Meanwhile, differences in external deformation and mortality between treatment groups were evaluated by the Chi-squared test. Only $P<0.05$ was considered to be statistically significant.

4.0 RESULTS

4.1 Viability

Figure 1 shows the number embryonated chicken embryos that remained alive at the end of the experimental period. A total of 88% of all embryonated eggs remained viable during this period.

The lethality and morbidity rates of embryonated chicken embryos is shown in Table 1. Dead embryos were only seen in the 45 and 30mg groups only giving rise to a total lethality rate of 12%. However, there was no significant association between the lethality rate and dose (p= 0.067).

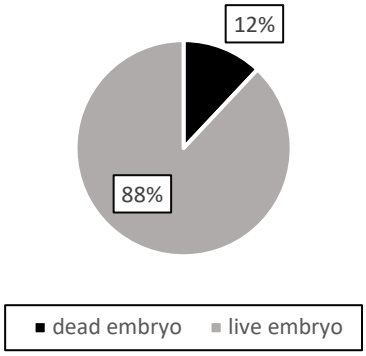


Figure 1. *Pie chart of the frequency of dead versus live embryos (n=50)*

4.2. Gross Findings

4.2.1 Deformation of The Embryo

The rate of deformation of chick embryos is shown in Table 1. The prominent formations observed were crooked or twisted neck and digital hyperflexion in all groups receiving nicotine (Figures 2-4). The incidence of crooked neck and digital hyperflexion was only noticeable in the treatment groups accounting for 62% (31)

Table 2. ***Dose response lethality and deformation rates of embryonated chicken embryos during the experimental period***

Dose	n	% dead chick embryo (n)	% live chick embryo (n)	Deformed chick embryos	
				% crooked neck (n)	% digital hyperflexion (n)
Control	12	0 (0)	100 (12)	0 (0)	0 (0)*
15mg	12	0 (0)	100 (12)	17 (2)	42 (5)
30mg	12	17 (2)	83 (10)	50 (6)	67 (8)
45mg	14	29 (4)	71 (10)	79 (11)*	93 (13)*

* Values between rows are significantly different @ p<0.006 (Bonferroni Post Hoc test)

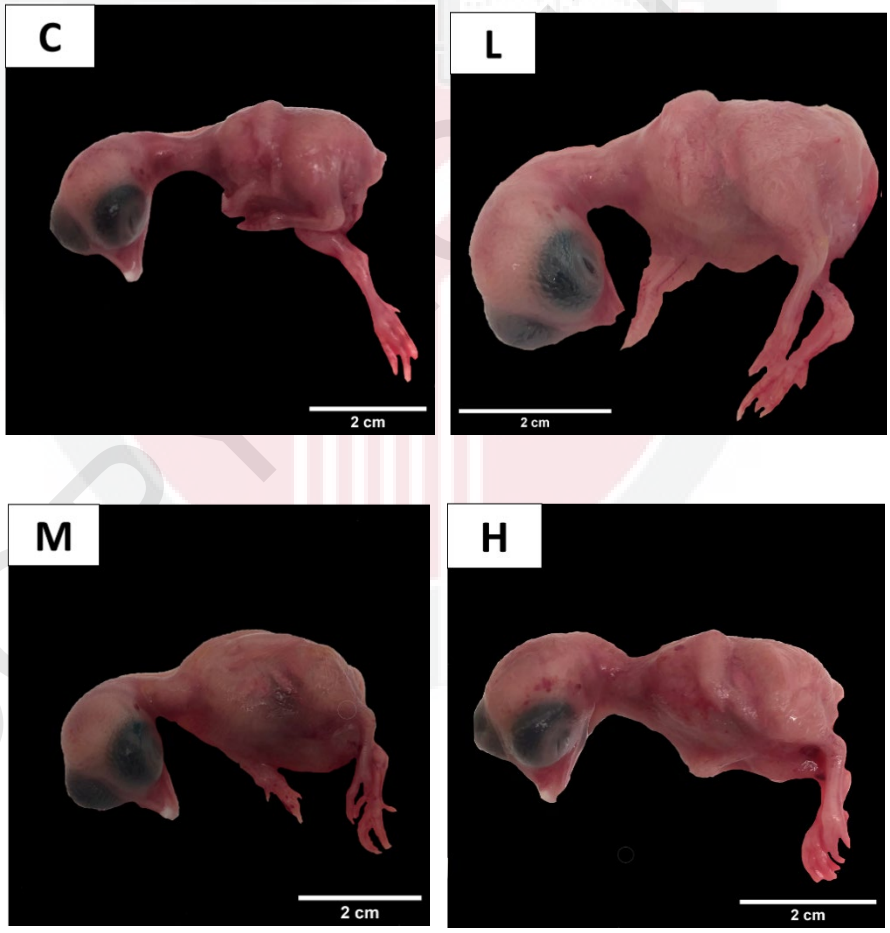


Figure 2. *Photograph of embryo harvested at Day 13 where **C**: control, **L**: 15mg, **M**: 30mg, **H**: 45mg. Varying degrees of crooked or twisted neck and digital hyperflexion were seen only in the treated groups.*

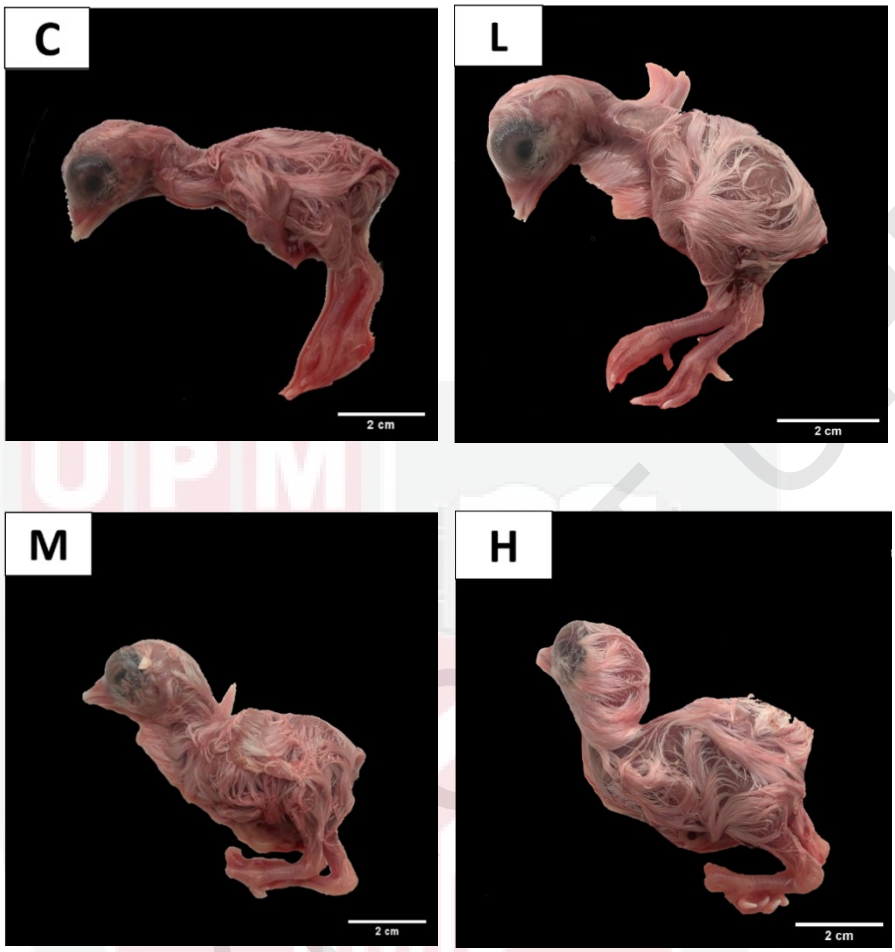


Figure 3. *Photograph of embryo harvested at Day 16 where **C**: control, **L**: 15mg, **M**: 30mg, **H**: 45mg. Varying degrees of crooked or twisted neck and digital hyperflexion were seen only in the treated groups.*

and 52% (26) of total samples (n=50), respectively (Figure 5). The number of deformed embryos was directly proportional to the concentration of inoculated nicotine doses i.e showing a graded response being highest in the 45mg group (Table 1). In addition, based on test of independence, a significant (p=0.001) association between deformed embryos (crooked neck and digital hyperflexion) and doses group was seen between the control and 45mg group ($p<0.006$).

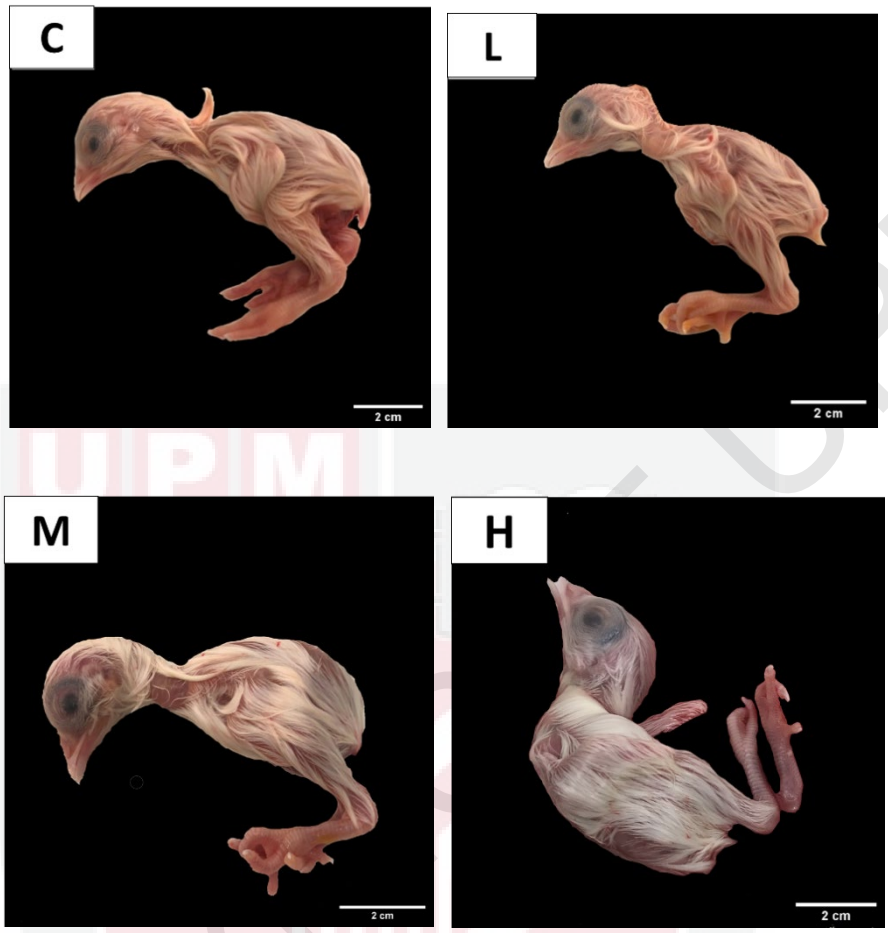


Figure 4. *Photograph of embryo harvested at Day 19 where **C**: control, **L**: 15mg, **M**: 30mg, **H**: 45mg. Varying degrees of crooked or twisted neck and digital hyperflexion were seen only in the treated groups.*

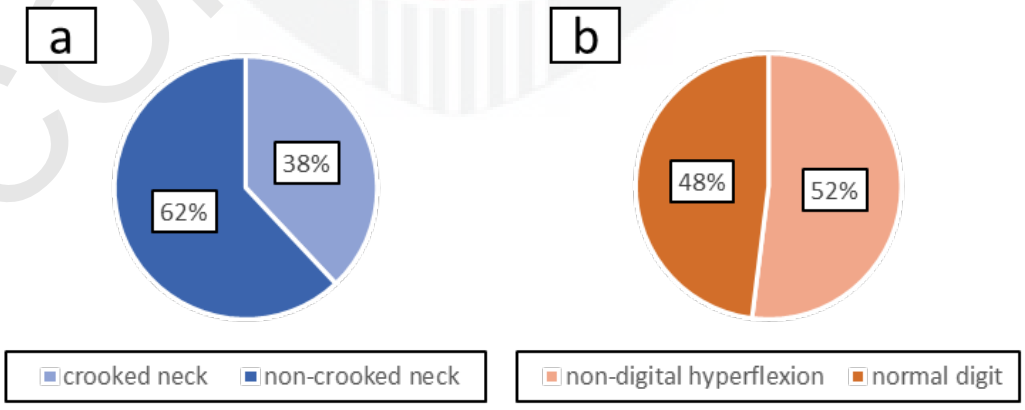


Figure 5. *Pie chart demonstrating the proportion of deformed embryos(n=50)*

A factorial ANOVA was conducted to compare the main effects of dose and incubation days and the interaction effect between dose and incubation days on the embryo body weight, heart weight, body length and heart somatic index. Overall, incubation day effect was statistically ($p<0.05$) significant for all parameters; meanwhile, dose-effect only exhibits significant ($p<0.05$) in heart weight and embryo length parameters. Furthermore, the interaction effects were only found in heart somatic index and embryo length parameters (Appendix).

4.2.2 Embryo Weight

There were no interaction effects between dose and incubation day (P= 0.706). The weight of chick embryo all groups increased significantly (P=0.001) from day 13 until day 19 of incubation, which were expected as embryos were growing over time. In terms of dose, the embryo weight is gradually lower than the control group to high dose nicotine group in all incubation days, except a slightly increased pattern was seen from middle dose to high dose group in day 16 of incubation. The embryo weight high dose groups were significantly lower than control groups.

4.2.3 Heart weight

There was no interaction between dose and incubation day observed (P=0.160). There is an increasing trend of embryo's heart weight as incubation day advances in all groups (P= 0.001), which were expected in this experiment. The heart weight of incubation days 16 and 19 were significantly higher than day 13 but no interaction between day 16 and day 19 of incubation. The heart weight gradually declined from control to high dose group in all incubation days (P= 0.006), where the significant value of control groups was greater than any other group.

4.2.4 Heart Somatic Index

There was a statistically significant interaction between the incubation day and the dose on heart somatic index, F (6,24) = 4.405, p = 0.004. The sample main effect analysis showed that day 13 significantly influenced the heart somatic index than days 16 and 19, whereby the index of nicotine treated groups was always significantly higher than control group. However, significant interactions were not detected between treatment groups.

4.2.5 Embryo Length

The interaction effect for incubation day and dose on embryo's yielded an F ratio of F (6, 24) =2.948, p = 0.027, indicating that the effect was significant. The sample main effect analysis showed that length was significantly affected by nicotine in day 16 than day 13 and 19 of incubation. Within day 16, the length of embryos in the control group was significantly higher than nicotine treated groups, while that of the low dose (15mg/kg) was higher than the high dose (45mg/kg) of nicotine. No interaction was observed between middle dose groups with other nicotine treated groups.

4.2.6 Heart, Lung, and Liver

No significant gross pathological changes were observed on heart, lung, and liver of all the embryonated chicken eggs in control and treatment groups.

Table 3. ***Effect of nicotine on selected parameters of embryonated chicken eggs (means±SD)***

Group	Incubation days	Parameter			
		Embryo weight (g)	Embryo length (cm)	Heart weight (g)	Heart somatic index (%)
Control	13	7.6± 0.15^{c}	5.3±0.06	0.08±0.006^{b,A}	**1.01±0.056^{a}**
	16	16.2±1.10^{b}	**7.2±0.40^{a}**	0.11±0.004^{a,A}	0.65±0.06
	19	24.1±1.8^{a}	8.1±0.23	0.10±0.006^{a,A}	0.41±0.01
15mg	13	6.9±0.45^{c}	5.2±0.06	0.06±0.009^{b}	**0.84±0.10^{b}**
	16	15.2±0.76^{b}	**7.0±0.20^{b}**	0.10±0.011^{a}	0.65±0.06
	19	22.5±2.60^{a}	7.6±0.51	0.10±0.007^{a}	0.44±0.02
30mg	13	6.6±0.47^{c}	5.2±0.12	0.06±0.005^{b,B}	**0.84±0.10^{b}**
	16	13.7±0.06^{b}	**6.7±0.10^{bc}**	0.09±0.009^{a,B}	0.68±0.06
	19	23.2±2.60^{a}	7.6±0.40	0.10±0.009^{a,B}	0.43±0.03
45mg	13	6.6±0.47^{c}	5.2±0.10	0.06±0.003^{b,B}	**0.85±0.03^{b}**
	16	14.4±0.27^{b}	**5.8±0.6^{c}**	0.09±0.015^{a,B}	0.65±0.10
	19	21.5±1.6^{a}	7.1±0.15	0.09±0.008^{a,B}	0.43±0.01

Values in the same column not sharing the same superscript ^{a,b,bc,c} are significantly different at $p < 0.05$.
Values between rows not sharing the same superscript $^{A, B}$ are significantly different at $p<0.05$.
Values in bold indicates interaction effect.

4.3 Histology

4.3.1 Incubation Day 13

The histology of the heart, lung and liver tissues of embryos in control group was within normal limits. The lung microstructure appeared to correspond to the late stage of lung development although the parabronchial wall has not fully developed.

Multifocal areas of loose myocardium were seen throughout all nicotine treated group (Figure 6). The walls of parabronchi were thicker in nicotine treated group than control group (Figure 10).

4.3.2 Incubation Day 16

No histological lesions were observed in the heart, lung, and liver tissues of chick embryos in control group albeit the myocardium was much more compact at this instance. Multifocal area of loose myocardium was consistently evident in all nicotine treated groups with evidence of degeneration (vacuolation) and hypertrophy in the 15mg and 45mg groups (Figure 7-8).

Area of fibrosis was also seen in the liver tissue of the 30mg and 45mg groups (Figure 9). In addition, the 45mg group revealed the presence of homogenous pinkish exudates (presumably surfactant) in the parabronchial lumen in (Figure 10).

4.3.3 Incubation Day 19

The microscopy of the heart, lung, and liver tissues of embryos in the control group were within normal limits.

In the 30mg and 45mg groups, similar lesions as seen at Day 16 persisted in addition to diminishing existence of foci of hepatic fibrosis in the 45mg dose group.

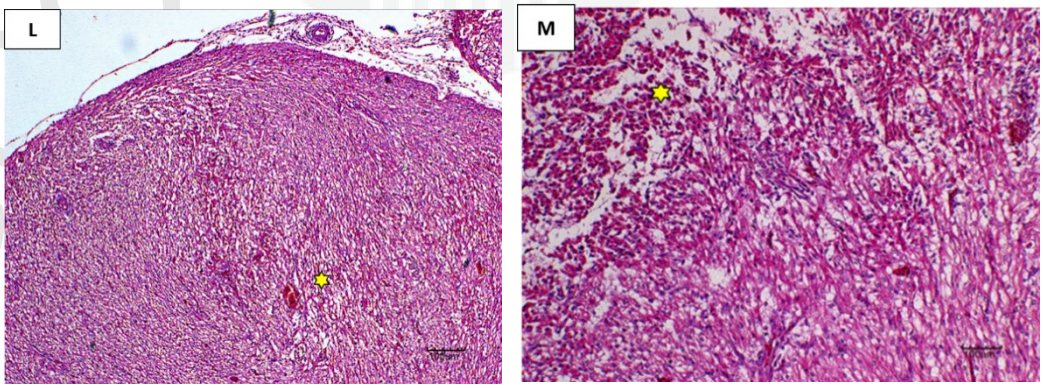


Figure 6. *Photomicrograph of the heart of embryos harvested on Day 13.* ***L****: 15mg group (H&E, 10X).* ***M****: 30mg group. The yellow star indicates loosely connected cardiomyocytes (H&E, 20X).*

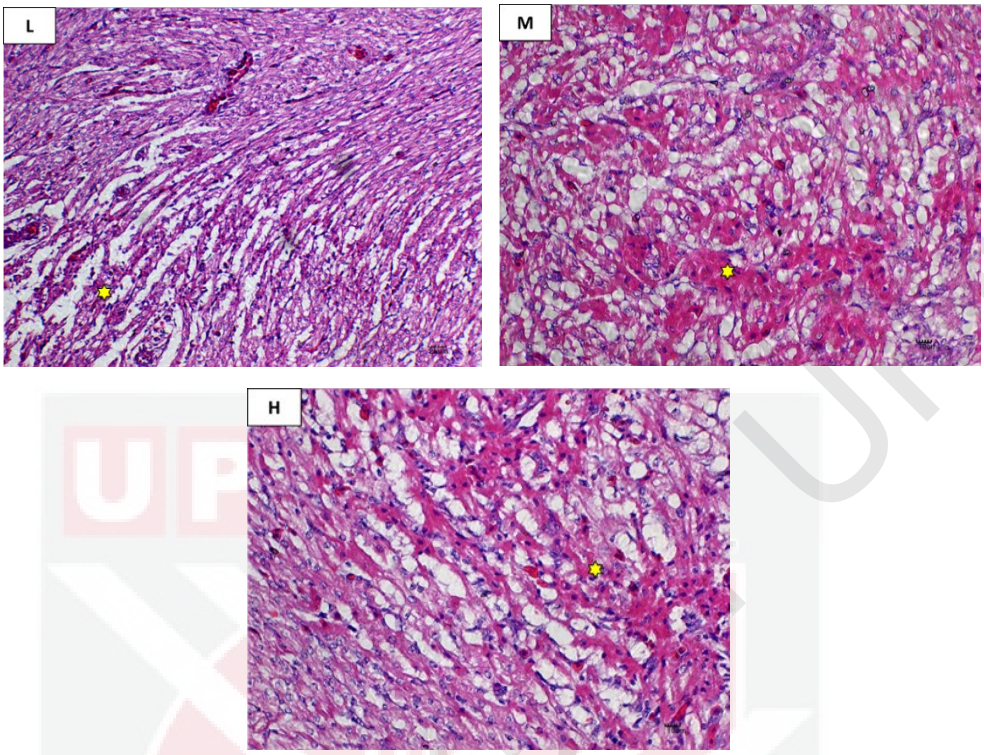


Figure 7. *Photomicrograph of the heart of harvested on Day 16.* ***L****:15mg group with the yellow star indicating loose area of myocardium, (H&E, 20X).* ***M:*** *30mg group with the yellow star indicating hypertrophied myocytes surrounded by loose area with vacuolated cells (H&E, 40X).* ***H****: 45mg group with the yellow star indicating hypertrophied myocytes surrounded by loose area with vacuolated cells (H&E, 40X).*

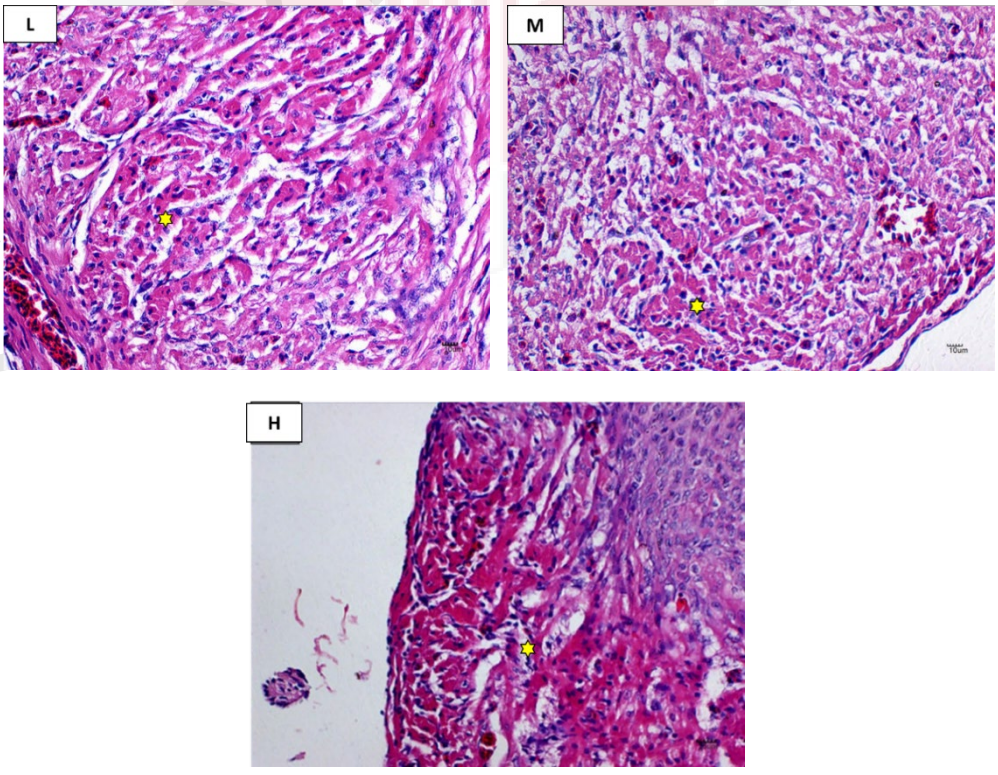


Figure 8. *Photomicrograph of the heart of embryo harvested on Day 19:* ***L****:15mg with the yellow star indicating enlarged myocytes (mild hypertrophy) (H&E, 20X).* ***M****: 30mg group with the yellow star indicating enlarged myocytes (mild hypertrophy), (H&E, 40X).* ***H****: 45mg group with the yellow star indicating a demarcated area of hypertrophied myocardium, (H&E, 40X).*

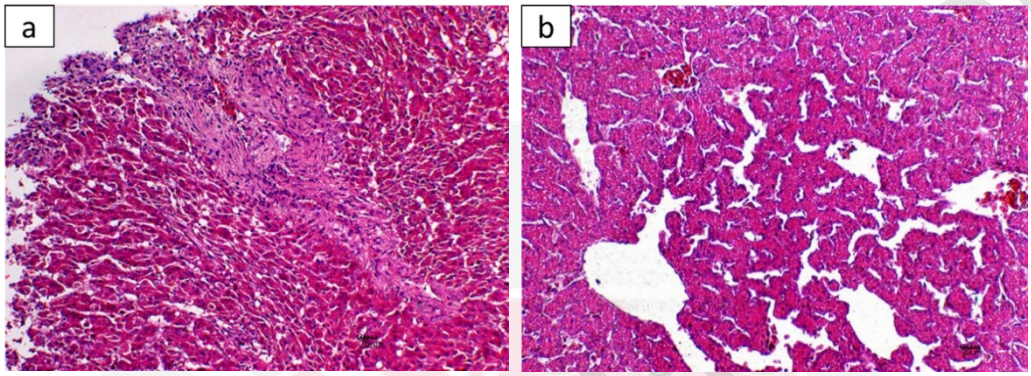
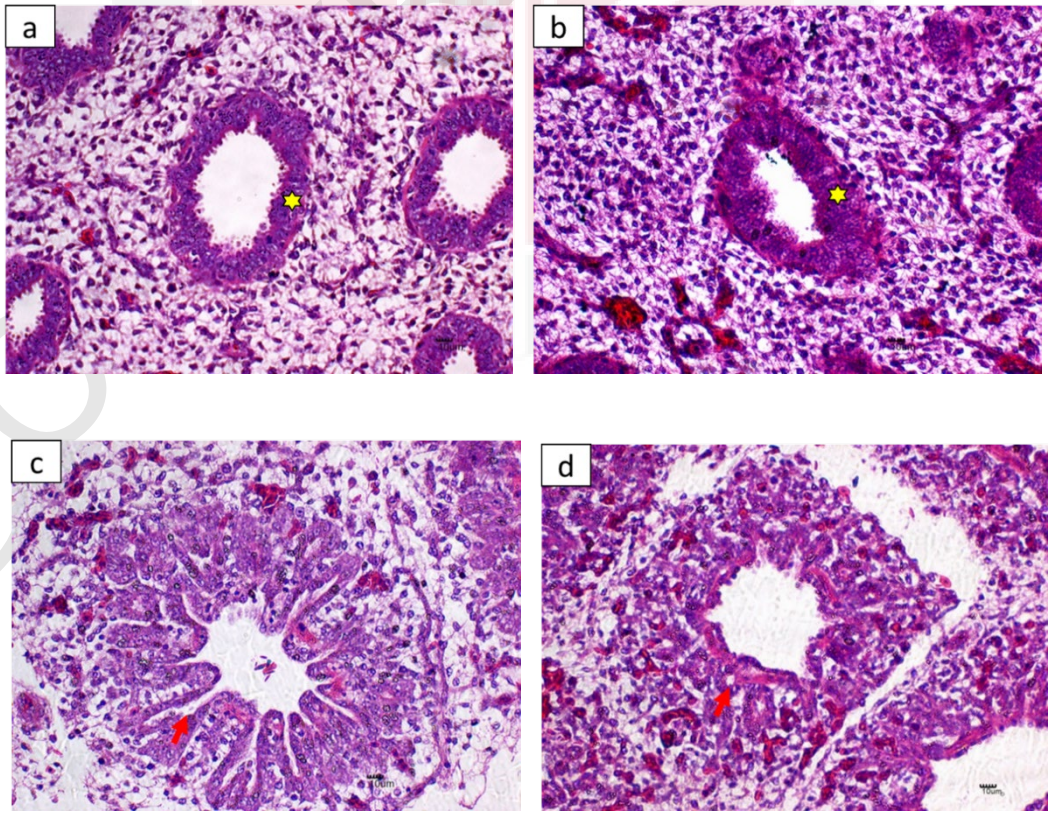


Figure 9. *Photomicrograph of the liver of chick embryos.* ***a****: fibrosis of hepatocytes (H&E, 20X).* ***b****: degeneration of hepatocyte and loss of hepatic cord (H&E, 40X).*



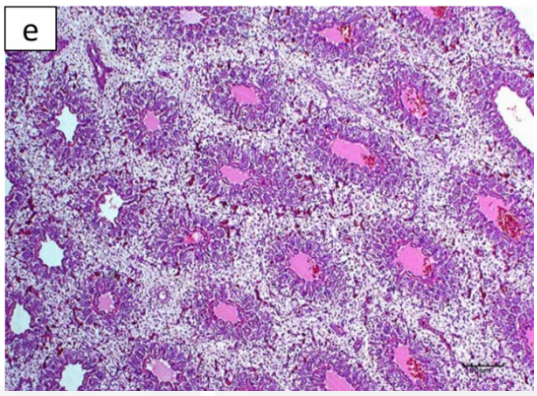


Figure 10. *Photomicrograph of the lung of embryos harvested at Day 13.**a**: Control and **b**: 30mg group. The yellow star shows that the parabronchi wall of the 30mg group is thicker than that of the control. (H&E, 40X). Embryo harvested at D16 **c**: Control and **d**: 45 mg group. The red arrows indicate the atria, the parabronchial wall in control group is well-developed than that of the nicotine treated group (H&E, 40X). **e**: Lung of the embryo from the 45mg group showing the presence of pinkish exudates possibly surfactant within the parabronchi lumen, (H&E, 10X).*

4.3.4 Ventricular Wall Ratio

The ratio between the left to right ventricle thickness was measured to asses significance if the histology (Figure 11) as shown in Table 3. An insignificant increasing ratio pattern was seen in all groups although it was much lower in the 45mg group.

Further examination of liver was done; the number of blood vessels in the liver histology section were counted and presented as Portal triad to Central vein ratio. The data was obtained from five areas viewed under at 20X magnification in a battle manner (Table 3). No significant differences were seen between all groups at all instances.

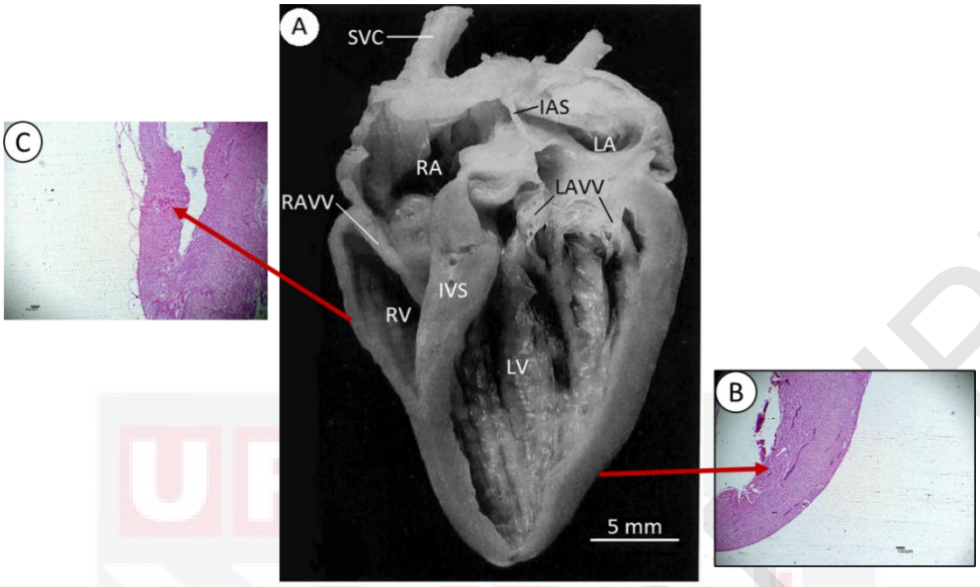


Figure 11. *Illustration of Left and Right heart ventricle thickness measurement.* ***C****: RV (Right ventricle), (H&E,10X),* ***B****: LV (Left ventricle), (H&E,10X). Diagram A was retrieved and modified from Lu, Y., James, T. N., Bootsma, M., & Terasaki, F. (1993). Histological organization of the right and left atrioventricular valves of the chicken heart and their relationship to the atrioventricular Purkinje ring and the middle bundle branch. The Anatomical Record, 235(1), 74-86.*

Table 4. ***The thickness of chick embryo's heart ventricles and number of blood vessels in liver on histological section.***

Incubation day	Group	Ventricle L: R (μm)	Hepatic vessels Pt: Cv (μm)
13	Control	1.17± 0.094	1.86± 0.065
	15mg	1.32± 0.224	2.18± 0.035
	30mg	1.11± 0.067	1.08± 0.687
	45mg	1.15± 1.210	1.56± 0.883
16	Control	1.21± 0.096	1.44± 0.371
	15mg	1.34± 0.057	1.82± 0.401
	30mg	1.54± 0.389	2.52± 1.208
	45mg	1.31± 0.129	1.24± 0.250
19	Control	1.31± 0.135	3.29± 0.766
	15mg	1.44± 0.445	4.20± 1.980
	30mg	1.52± 0.308	3.06± 0.079
	45mg	1.30± 0.166	4.56± 2.033

Values are expressed as means± SD of 8-12 embryos per group.
L: R = Left to Right, Pt:Cv=Portal triad to Central vein.

5.0 DISCUSSION

The study demonstrated an *in vivo* process of chicken embryo maturation, following doses of nicotine from the middle stages of its growth. Although the mechanisms of observed effects remain to be elucidated, most previous studies suggested that nicotine exposure at early embryonic development may lead to various abnormality (Khan et al., 1981; Forsyth et al., 1984; Zhao et al., 2005; Bohn et al., 2017). In short, much of the studies tended to focus on early development rather than late stages of development.

In the present study, the embryos were introduced to nicotine on the 9th day of incubation with a follow through analysis from the commencement stage until the hatchability stage. Considerable attention was taken into selecting the suitable doses to simulate SHS and ETS maternofoetal exposure in chick embryo. Adaptations were made based on embryo staging (exposure time), route of induction, and in consideration of previous report on nicotine poisoning in avian species (Novotny et al.,2011). Thus, since the nicotine dose in chicks is 50 times higher than humans (ElBeltagy-Ael et al., 2015), a much higher dose was used in this study.

The limitations to extrapolate of results of this study to humans lies on nicotine pharmacokinetics between embryonated chick egg and human embryo. In pregnant humans, inhaled nicotine crossed the placenta leading to high rates of nicotine metabolism (Dempsey et al., 2002). On the contrary, in embryonated chicken egg, the non-fully functional liver and kidneys will lead to the injected nicotine to remain unmetabolized for a long period (Bolin and Burggren, 2013). Furthermore, very little

is known about the the nicotinic metabolic pathway in chickens (Hukkanen et al., 2005).

Nevertheless, the lethality rate found in this study provide evidence of a partial dose-dependent nicotine toxicity despite the likelihood of not being fully metabolized (Landauer, 1975). External and internal factors such as egg sources, humidity, temperature, and physical characteristics could contribute to the survivability of the eggs (King'Ori, 2011).

The percentage of live embryos on day 19 of incubation was 88% indicating an acceptable hatchability rate of embryonated chicken eggs by (Khalil et al., 2016). Thus, the assumption mortality was solely induced by nicotine in this experiment is valid and adequately justified.

In line with human studies, this embryonic exposure significantly resulted in developmental defects in high dose groups exhibited as crooked neck and digital hyperflexion. These malformations were also previously stated in several studies on chick embryos in dose dependant manner (Landauer, 1975; Forsyth et al., 1994). In the study reported here, the proportion of the lesion in the cervical region (crooked neck) to distal limb seems higher at all stages. This could be influenced by the development stages of vertebral column which is more primary than that of the limb. Alternatively, the effect could have arisen from proven effect of nicotine on the developing cartilaginous vertebrae (Khan, 1975). In addition, tendinous and/or ligamentous contractures following nicotine exposure could also lead to limb deformation (Forsyth et al., 1994). Thus, further studies should be conducted to elucidate nicotine exerts direct effect on either the differentiating chondroblasts

(defective chondrogenesis) or indirectly on associated myoblasts or tendon and ligament.

Based on the embryo's weight, length, heart weight, and somatic index, in general, it appeared that the impact of nicotine was not significant embryonic growth. Only the relation between dose and incubation day was only obvious on heart somatic index and embryo length. The embryo length gradually reduced at increasing dose as reported earlier (Rosenburh et al., 1993; Zhao & Reece, 2005; Mukytiyaz et al., 2014). Khan et al. (1975) described the teratogenic effects of high doses of nicotine interfering with skeletogenesis of chick embryos leading to growth retardation.

The explanation for the heart to body ratio being significantly reduced in the 45mg group only at Day 13 is probably due to the chick cardiogenesis event. It is most proliferatively intense at day 8 to day 14 (Martinsen, 2005).

Suprisingly, at the celullar level, allantoic inoculated nicotine generated pathology of heart, liver and lung of embryonated chicken eggs in a dose-dependent manner. It is conceivable that since the nicotinic acetylcholine receptor (nAChR) was found in almost all tissues, their presence had exerted lesions in the developing embryo in this study. Nicotine binds to the nAChR on plasma membrane causing a conformational change (Benowitz & Burbank, 2016) additional to the release of norepinephrine (sympathetic vasoconstriction) manifested as lesions. Lung showing a rather spectacular lesions due to rapid absorption in this tissue owing to its extensive surface area and small airways. The accompanying vasoconstriction (norepinephrine release) would lower the blood vessel volume and restrict the blood flow to the organs

and tissue. Thus, less essential nutrients in arriving to the embryonic tissues which impairs the cellular development (Karaconji, 2005).

Undoubtedly, the rise in vascular resistance following vasoconstriction will result in hypertension. The increase in blood pressure will lead to build-up of fluid in the tissues as seen in the lungs in this study. The thickening of the lung parenchyma and parabronchi wall of embryo might be associated with an increase in the Type II cell. Apart from being oedema fluid accumulation in the lung, the pinkish homogenous exudate could be that of surfactant secreted by the proliferating Type II cell. Similar features of oedema following nicotine exposure has been reported in in adult and neonates of laboratory animal (Maritz & Thomas, 1995).

Another event can than arise succeeding vasoconstriction is hypertension via the renin-angiotensin system through ANG II action (Mueller et al., 2014). This can influence cardiovascular development leading to cardiac hypertrophy (Mueller et al., 2014) and nicotine modulation of fibroblast favouring fibrosis (Jensen et al., 2012) as seen in this study. All in all, the evidences found in the studied organs were not paradoxical and bears similarities to effect of a plant toxin in chicken (Abdul-aziz et al., 2016).

6.0 CONCLUSION

Although further studies should be conducted to elucidate the mechanism of nicotine-induced pathology on embryogenesis, it can be concluded that the intra-allantoic administration of nicotine at the selected doses produced teratogenic effects on embryonated chicken eggs to that was similar to SHS and ETS.

7.0 RECOMMENDATIONS

Prolong and in-depth studies should be carried out as there is still inadequate information and lack of research done on the effects of nicotine on animals.

In our study, the precaution and quality control measures were established when handling the eggs to minimize egg contamination and experimental errors. Although, it would be best to use specific pathogen-free (SPF) eggs to ensure the pathological effects have not resulted from any specific pathogens vertically or externally transmitted. These would be likely to mask the nicotine effect on embryonated chicken eggs.

We recommend using different methods and routes of nicotine exposure on embryonated chicken eggs such as yolk sac, chorioallantois, and intravascular, which might need skills and much experience in handling the eggs.

Further study should be conducted on the other animal species, especially in mammal. The anatomical characteristics of egg are classified as a closed biological system that constrains the maternofoetal interaction seen in mammals. Exposure of nicotine to animals via inhalation is the appropriate way to mimic secondhand smoke (SHS) mechanism entering the body.

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APPENDIX

Appendix 1

Table 1: Two-way ANOVA results using embryo length as the criterion

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39.056[a]	11	3.551	39.820	.000
Intercept	1530.114	1	1530.114	17160.153	.000
Group	3.339	3	1.113	12.481	.000
Incubation	34.141	2	17.070	191.442	**.000**
Group * Incubation	1.577	6	.263	2.948	**.027**
Error	2.140	24	.089		
Total	1571.310	36			
Corrected Total	41.196	35			

a. $R^2 = 0.948$ (Adjusted $R^2 = 0.924$)

Appendix 2

Table 2: Two-way ANOVA results using heart somatic index as the criterion

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.487[a]	11	.135	35.880	.000
Intercept	15.822	1	15.822	4198.911	.000
Group	.032	3	.011	2.820	.060
Incubation	1.356	2	.678	179.896	**.000**
Group * Incubation	.100	6	.017	4.405	**.004**
Error	.090	24	.004		
Total	17.400	36			
Corrected Total	1.578	35			

a. $R^2 = 0.943$ (Adjusted $R^2 = 0.916$)

Appendix 3

Table 3: Two-way ANOVA results using heart weight as the criterion

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.011[a]	11	.001	15.337	.000
Intercept	.266	1	.266	3951.139	.000
Group	.001	3	.000	5.240	.006
Incubation	.010	2	.005	71.341	**.000**
Group * Incubation	.001	6	.000	1.719	.160
Error	.002	24	6.739		
Total	.279	36			
Corrected Total	.013	35			

a. $R^2 = 0.875$ (Adjusted $R^2 = 0.818$)

Appendix 4

Table 4: Two-way ANOVA results using embryo weight as the criterion

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1543.291[a]	11	140.299	76.208	.000
Intercept	7972.629	1	7972.629	4330.603	.000
Group	16.435	3	5.478	2.976	.052
Incubation	1519.914	2	759.957	412.796	**.000**
Group * Incubation	6.942	6	1.157	.628	.706
Error	44.184	24	1.841		
Total	9560.103	36			
Corrected Total	1587.475	35			

a. $R^2 = 0.972$ (Adjusted $R^2 = 0.959$)