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Review Article

Effects of Nicotine on Chicken Embryo Development: A Review

Ashley Federico[1, 2], Muhammadzohir Hidoyatov[1, 3], Suresh Nimmagadda[1], Poongodi Geetha-Loganathan[1, *]

[1]Department of Biological Sciences, State University New York Oswego, Oswego, USA
[2]Liverpool Animal Health Center, Liverpool, USA
[3]ICON Plc, Whitesboro, USA

Abstract

Background: Studies have shown that 22.3% of the world's population use tobacco and nicotine exposure during pregnancy remains a concern in embryonic development. Cigarette smoke contains several toxic and carcinogenic chemicals and has been known to cause pregnancy complications including premature births, low birthweights, and stillbirths. Purpose: This review aims to study nicotine exposure in chicken embryo development comprehensively. Methods: PubMed, Centers for Disease Control and Prevention (CDC), and Antpedia sites were used to search for studies using chicken embryos as a model. Studies that reported findings on nicotine's effects on various developmental processes were considered for this review. Conclusion: In total, 55 published articles were included in this review to discuss findings of nicotine-induced alterations during chicken embryo development. Findings have shown that nicotine affects angiogenesis, and embryonic and chorioallantoic membrane (CAM) growth by inhibiting cell proliferation. Nicotine affects brain and cerebellar cortex development by suppressing tumor protein p53 reactions. Nicotine also causes abnormal axial rotation and incomplete formation and closure of neural tubes. The compounds like green tree extract, vitamin C, and folic acid can reduce the effects of nicotine to suppress femur growth, decrease the contractility of cardiomyocytes, and reduce survival rates. These compounds are not protective measures to completely overcome the teratogenic effects of nicotine.

Keywords

Nicotine, Prenatal and Postnatal Growth, Embryonic Development, Morphological Defects

1. Introduction

Cigarette smoking is a popular socially practiced habit worldwide. It is estimated that there are 1.1 billion cigarette smokers globally [1] with 10.1% women smokers in the United States alone [2]. More than 22 million women smokers are recorded in the United States alone [3]. From these numbers, approximately 15-25% of women continue smoking during pregnancy [1]. Over 4000 chemicals, toxicants, and carcinogens are present in cigarette smoke [4]. Both active and passive smokers are exposed to these chemicals through the mainstream smoke inhaled by the smoker, and through the

*Corresponding author: p.geethaloganathan@oswego.edu (Poongodi Geetha-Loganathan)

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side stream smoke that burns off the end of cigarettes [5]. Of the thousands of present chemicals, only a few have been extensively studied, including nicotine [5]. This highly addictive, toxic alkaloid compound is dangerous not only to adults, but to a developing fetus, infants, children, and adolescents [3, 5]. Nicotine affects the fertility rate, it alters the morphology of oocytes resulting in mitochondrial alterations, and disrupts fetal development [3, 6]. This highly addictive, toxic alkaloid compound is dangerous not only to adults, but to a developing fetus, infants, children, and adolescents [3, 7]. Nicotine affects the fertility rate, it alters the morphology of oocytes resulting in mitochondrial alterations, and disrupts fetal development [3, 6]. In pregnant women, nicotine readily crosses the placenta, circulates in the fetus' blood, and has been identified in amniotic fluid and umbilical cords [8]. Fetal exposure to nicotine has been a known cause of premature births, spontaneous abortions, low birth weights, sudden infant death syndrome (SIDs), and stillbirths [6]. Nearly 80% of nicotine is metabolized into cotinine by the liver and is present in smoker's blood in higher concentrations due to its longer half-life than nicotine and is therefore commonly used to validate smoking behavior and nicotine intake [9]. Single-cell RNA sequencing of nicotine-treated embryoid bodies derived from human embryonic stem cells showed dysregulated cell-to-cell communication [10, 11] caused due to abnormal Ca^{2+} signaling. Maternal perconceptional smoking results in delayed embryonic morphology explained by lower birth weight and a smaller femur length in the second trimester [12]. Studies have shown that cotinine can be transferred passively to infants via smoking and breastfeeding and advise against smoking even after childbirth [9, 13]. However, few studies have been conducted to observe the direct effect of cotinine exposure during embryonic development.

As nicotine use continues to be a worldwide concern, and with the growing popularity of new e-cigarette technology increasing adolescent exposure and addiction [14], studies have been conducted to better understand the effects of nicotine on developing fetuses. Chicken embryos are common models for these teratogenic studies as they are sensitive to various chemical and physical manipulations that can be conducted at precise developmental stages [15]. Their parallel development of early stages to the beginning half of human pregnancy [16], easy accessibility, and inexpensiveness [15] also make them ideal model organisms to study the potential risks of nicotine in human embryos.

Nicotine rapidly absorbs into the embryo of developing chicks. When nicotine was injected into the air space of a fertilized chick egg, 98% of the dose was actively transferred to the egg proper by day three after injection. By that day, nicotine had begun moving into the embryo however, the most extensive movement of nicotine into the embryo occurred between days 7 and 12 of incubation. Through this movement, embryonic concentrations of nicotine are 3.7 to 7.9 times greater than if nicotine was evenly distributed throughout the egg, with the highest concentration found in the heart and liver. Overall, the percentage of the injected dose transferred to the embryo was small, and the concentration of nicotine found in the embryo was constantly higher than in the other egg components [17].

This review includes the findings of nicotine-induced malformations during chicken embryo development. Various studies exposed embryos to different concentrations of nicotine, mainstream smoke solutions, and side stream smoke solutions to determine their effect on embryonic growth, survival, movements, and the development of the brain, cardiovascular system, neural system, lungs, and chorioallantoic membrane. Compounds including green tea extract, folic acid, and vitamin C were also administered in addition to nicotine to observe their potential protective properties in response to the teratogenic effects induced by nicotine.

2. Effect on Prenatal and Postnatal Growth

2.1. Growth Suppression

Nicotine is one of the thousands of chemicals found in cigarette smoke that are harmful to multiple fetal developmental processes [3, 18]. Free radicals from nicotine and cigarette smoke increase oxidative stress which is associated with many diseases and is responsible for creating hypoxic conditions [19]. Nicotine induces higher vasoconstriction of uterine vessels and decreases oxygen concentration which affects fetal growth by causing embryonic growth and development retardation [18, 19]. Newborns exposed to nicotine during development had lower birthweights and were shorter compared to those who were not exposed to nicotine [3, 18, 20]. Newborns from light and heavy smokers had a 53% and 150% increase in low birth weights, respectively, and between 20-30% of reported low birth weights are a result of smoking [1]. Chick embryos exposed to daily topical nicotine administration had reduced birth weights detected midway through the incubation period, with a higher weight reduction seen in embryos that began nicotine administration earlier [21]. However, if only one dose of nicotine was administered with no continual treatments, embryonic growth returned to normal and produced chicks comparable to control chicks at hatching [22].

2.2. Reduced Bone Development

Decreased embryonic growth induced by nicotine can also be associated with the interference of nicotine with bone development. In adults, smoking affects bone metabolism and can increase the risk of developing osteoporosis, fractures, and reduced bone density [23]. However, nicotine can also cross the placenta, decrease collagen production and alkaline phosphate activity in osteoblasts-like cells, and delay skeletal development in embryos [18]. A study by Shan et al. [18]

observed the effect of nicotine on chick femur development. The femur of a chick begins calcifying on day five of embryonic development, and in this experiment, the chickens were exposed to nicotine both pre- and postnatally: 48 hours after the start of incubation and 48 hours after the chicks hatched. At one month after hatching, the femurs were measured and those exposed to nicotine were significantly shorter in length than those in the control group [18]. These findings correspond to previous studies that found the deleterious effects of nicotine are dose-dependent [23]. and observed delays in bone formation of limb and vertebrae bones in subjects administered toxic doses of nicotine [18, 19].

The retardation of bone development could be due to the inhibition by nicotine of calcium absorption by the embryo [3], therefore interfering with the formation of the bone matrix and the process of bone calcification [18]. Nicotine is also a cholinergically active drug, or it interferes with acetylcholine (ACh) actions and inhibits cholinesterases. In chick embryos, skeletogenesis is regulated by both ACh-dependent and ACh-independent actions as ACh and choline acetyltransferase (ChAT) accelerate bone formation and AChE proteins outline bone formation respectively [23]. Therefore, the chollinergically effect of nicotine can reduce bone mass. Nicotine continues to affect the growth rate of developing embryos after day 12 of incubation, given that the embryo survives [19].

2.3. Lower Survival Rate

Nicotine exposure at early developmental stages affects the embryonic survival rate and is accountable for over five million premature deaths across the world annually [23]. The genotoxic and cytotoxic potential of Areca nut and smokeless tobacco were described [24]. In the study conducted by Shan et al. [19, 25] to observe the survival of nicotine-administered chick embryos observed a 90% survival rate in the control group and in that exposed to green tea extract, a 20% survival rate in the group administered nicotine, and a survival rate of 50% in the chicks exposed to both nicotine and green tea extract. The low survival rate of chicks exposed to nicotine could be a result of the hypoxic conditions created by nicotine-induced oxidative stress [25]. In another study, conducted to determine nicotine's effect on development, only 4% of the nicotine-treated embryos survived [3]. The remaining two embryos from the groups had significant malformations however, due to the small sample size, a valid determination could not be made regarding nicotine-induced developmental malformations [3]. Forsyth et al. [26] also observed mortality in nicotine-treated embryos in a dose-dependent manner with 1%, 5%, and 10% nicotine sulfate solutions producing a mortality rate of 37%, 67%, and 100%, respectively. Exposure of embryos at 48h incubation to 1 mg of nicotine caused a high incidence of embryonic death [27]. Administration of nicotine to chickens following hatching produced no significant differences in growth rate compared to control groups, rather the drug-induced symptoms of loss of appetite, reduced activity, and abstinence from water [28]. Lower survival rates could result from the nicotine-induced decrease in oxygen and nutrient supply to the developing embryo [29].

2.4. Embryonic Movements and Malformations

Nicotine mediates physiological responses by activating neuronal nicotinic acetylcholine receptors and has been associated with infant and toddler motor and sensory defects suggesting that it has a negative effect during early development [1]. To better understand the consequence of prenatal nicotine exposure, Ejaz et al. [1] studied chicken embryonic movements in response to nicotine in mainstream smoke. Swing-like motion and motion of the head, tail, and whole body were observed after the treatment of varying concentrations of nicotine in smoke. Embryonic responses were dose-dependent as lower levels of nicotine resulted in hyperactivity while higher levels led to hypoactivity which matches reports that low doses of nicotine stimulate motor activity while higher doses reduce activity [30]. Increasing concentrations correspond to longer recovery times suggesting low dosages stimulate ganglions while high dosages result in a blockage.

Nicotine is also a known stimulant on cholinoceptors which produces a depolarization blockage in skeletal muscles and ultimately causes paralysis [30]. Forsyth et al. [26] observed embryonic movements for a 5-minute interval, one hour after nicotine administration, and found no movement in the treated embryos. Hamamichi and Nishigori [30] also observed complete stoppage, possibly paralytic, of swing-like movements in embryos treated with 10μg of nicotine and 1 x ACSE (aqueous cigarette smoke extract). Based on normal chicken embryonic movements, unique movements are specific to developmental stages, and altering the movements can interfere with normal embryogenesis processes [1]. Pharmacological activities of nicotine and cotinine caused changes in embryonic movements, however, may not be the only substances affecting behavior [30]. After hatching, administration of nicotine to chickens sometimes resulted in muscular spasms when a non-fatal dose was given, while others experienced no symptoms [28].

3. Morphological Defects Caused by Nicotine Exposure

Deformations were observed in 20% and 100% of chicken embryos treated with 1% and 5% nicotine sulfate solutions, respectively [26]. It is possible that ligamentous contractures or tendinous cause excessive extension and toe deformations in nicotine-treated embryos. More severely affected embryos had vertebra palpable, hypotrophy of leg musculature, and rigid neck, all morphological symptoms of crooked-neck dwarf lethal mutation [26].

3.1. Abnormal Angiogenesis

In chick embryos, nicotine was observed to induce irregular angiogenesis in the chorioallantoic membrane. Melkonian et al. [32] evaluated blood vessel area, diameter, capillary plexus, and blood vessel pattern formation in response to mainstream (MS) and sidestream (SS) cigarette smoke. Each parameter was dose-dependently inhibited, with SS smoke being more inhibitory in all except capillary plexus formation [32]. Blood vessels of smoke-treated CAMs were disorganized as they ran parallel to each other and made random sharp turns rather than branching like control vessels [33]. Collagen, both type I and type II, were approximately two to three times more abundant in CAMs treated with smoke compared to control CAMs. MS-treated CAMs also had a reduction in hyaluronic acid concentrations while it was completely absent from SS-treated CAMs. The overproduction of collagen could result from abnormal hyaluronic acid degradation, which stimulates collagen synthesis, and accounts for the abnormal branching and formation of the CAM blood vessels, as angiogenesis is controlled by collagen metabolism [33]. Exposure to cigarette smoke condensate showed a deviated pattern of blood vessels, hemorrhages, and localized necrosis resulting in embryonic mobility and stunted growth in both chick and mouse embryos [34].

While MS and SS smoke are similar in chemical components, the amounts of the chemicals vary between the two, and the findings indicate that SS smoke has a stronger growth inhibitory effect. The growth and development of CAM vasculature correlates to inhibited cell proliferation which suggests that MS and SS smoke could affect processes dependent on cell division such as wound healing [32]. Previous studies also show that smoke could potentially induce hyperplasia, necrosis, and hemorrhages [32]. The effects of nicotine are dose-dependent, and it was found that while high doses inhibit cell division, low doses could stimulate angiogenesis therefore other dominant chemicals present in cigarette smoke could be responsible for decreased cell division and vessel growth [32]. A recent study has observed that E-cigarette liquid inhibits angiogenesis of the CAM for 5 days due to the upregulation of ATF-3, FOXA2, INHBA, MAPRE-2, and RIPK-1 and downregulation of SERPINA-4 AND VEGF-C genes necessary for embryogenesis as well as angiogenesis [35]. Investigation of pulmonary arteries during chick embryo development in the presence of cigarette smoke extract impaired endothelium-dependent vasodilation proving the effect of cigarette smoke on fetal vascular endothelial vasorelaxant pathways causing the development of pulmonary hypertension in the newborn [36].

3.2. Cardiovascular Defects

Nicotine has been associated with short-term cardiovascular changes in adults including higher heart rate, blood pressure, and arterial stiffness [14]. Studies conducted with mice have also revealed decreased fractional shortening of the left ventricle, an increase in atherosclerotic lesions, autophagy in blood vessels, the development of atherosclerosis, and increased vascular smooth muscle migration capacity in subjects exposed to nicotine from e-cigarettes [14]. Administration of nicotine to chicken embryos produced ventricular and valvular septal defects due to the reduction of acid mucopolysaccharides in the cardiac jelly, from which the cells are derived [31]. Endocardial cushion defects are also possible as reduced acid mucopolysaccharide concentration could lead to congenital malformations as they are essential for normal embryonic cardiac formation [31]. Topical administration of nicotine to chick embryos resulted in hyperemia and hemorrhages, 30 seconds, and 5 minutes after application, respectively, while a second application was fatal in all embryos [21]. These findings support the possibility that nicotine exposure could result in the development of cardiovascular disease.

3.3. Irregular Heart Rate

It was found that high doses of nicotine were fatal to chick embryos, possibly from a reduction of nutrients and oxygen interfering with embryonic development [3]. In the embryos that survived, heart rates immediately increased after exposure to nicotine, and nicotine-treated groups had a higher heart rate by 10-15 beats compared to controls [3]. In this study, a red skin phenotype was observed to have a red skin color as a possible result of nicotine, increasing blood clotting or collateral circulation from increased surface vessels. Nicotine also causes an increase in adrenaline which, when administered to chick embryos, increases heart rate, and produces heart arrhythmias such as asystoly and dysrhythmic phenomena [21]. This increase in heart rate along with smaller, abnormally formed capillaries can increase hemorrhaging as the thinner vessels are unable to support the increased pressure and rate of blood flow. Exposure to nicotine at the early stages of heart development is proven to be toxic causing cardiac anomalies with heart size to be smaller than normal with irregular beats [37]. Nicotine exposure in the chick embryos of 2-4 days of incubation showed aortic stenosis, ventricular and atrial septal defects, malformation of the aortic valve and pulmonic valve, and thin atrial and ventricular wall [37].

3.4. Reduced Cardiac Autonomic Nerve Reactivity

Pappano [38] analyzed a sinoatrial pacemaker to measure nicotine's effect on cardiac autonomic nerve reactivity. The declined impulse frequency and pacemaker inhibition in response to nicotine was maximum at a concentration of 10^{-5} M, with inhibition increasing between days 10 and 12 of incubation. Alternatively, nicotine did not produce a consistent acceleration of the pacemaker during embryonic development, with only an additional elevation in Ca^{++} in the bathing solution being able to accelerate the pacemaker. The significant inhibition of the pacemaker by nicotine administration ap-

peared after the presence of cholinergic neuroeffector transmissions and it was due to a mechanism that is dependent upon cholinergic nerve stimulation, such as the activation of hexamethonium- sensitive receptors and the release of acetylcholine. It was concluded that autonomic nerves influence the pacemaker impulse frequency and the appearance of transmission and morphological innervation of the chick heart through the autonomic nerves corresponds to the quantity of transmitters present for release [38].

4. Abnormal Lung Development and Function

Lungs begin development in the first trimester of pregnancy and continue through the third trimester and postnatally as alveolar growth begins before age two. Therefore, fetal, and adolescent lungs are vulnerable to environmental exposures such as nicotine in cigarette smoke. Nicotine may affect fetal lung development by reducing antioxidant enzyme activity and through nicotinic acetylcholine receptors (nAChRs) [14]. Previous studies revealed thickening of lung walls, narrowing of airways, and reduced surface area complexity in embryos exposed to nicotine [14]. Nicotine also induced changes in alveolar development including increased alveolar volume, decreased counts of radial alveoli, and alteration in fibrillar collagen expression, all of which could potentially lead to impaired fetal pulmonary function [6]. Chorioallantois membranes in chick embryos exposed to sidestream cigarette smoke led to an upregulation in type I and II collagens. This supports that nicotine upregulates a7 nicotinic receptors, therefore increasing these collagen depositions and increasing airway wall area and thickness, leading to abnormal lung function [6]. Postnatally, smoke exposure has been linked to asthma, lower respiratory tract illnesses, and reduced lung function in infants and children [14].

5. Brain Growth Inhibited

Brains continue to develop throughout embryonic and fetal stages and are more vulnerable to long-term alterations. Nicotine found in cigarette smoke interferes with normal brain development, especially during the critical period of differentiation and maturation [39]. Ornithine decarboxylase (ODC), regulated by various growth factors, acts as a catalyst in synthesizing polyamines (putrescine, spermine, and spermidine) necessary for cell proliferation [40] and therefore ODC activity is essential in fetal brain growth [41] and Central Nervous System (CNS) differentiation [22] in early development. Initially, nicotine stimulates the brain and then inhibits it, and when it is consumed during pregnancy it compromises the brain and its neural pathway's development [42]. In chicken embryos, nicotine was found to inhibit this ODC activity, and dose-dependently cause paralleled reductions in embryo size and brain weight [23, 41, 42], and a decrease in head size, and trunk diameter [7]. Nicotine-treated embryos had correlated reductions of glucose and Glut 1 55 kilodalton isoform concentrations in the developing brain but produced no changes to the level of Glut 3 transporter proteins [42]. The decreased levels of brain glucose subsequently caused a reduction in both brain Cyclic adenosine monophosphate (AMP) and cyclic AMP binding proteins [22]. Cyclic AMP concentrations along with its dependent protein kinase activity regulate ODC expression in cells, therefore, nicotine exposure disrupts the pathway for normal brain development and growth [22], as ODC plays an essential role in cell division and the synthesis of putrescene, spermidine, and spermine [41]. It was observed that after 144h of development, nicotine-treated embryos had a dose-dependent increase in ODC activity however, it is uncertain as to whether this increase is nicotine-induced given the various growth signals responsible for the elevation and maintenance of embryonic ODC activity [42].

In nicotine-treated embryos, Forsyth et al. [26] observed extensive cranial hemorrhage, with hemorrhage surrounding the rhombencephalon and severe cases with hemorrhage in the fourth ventricle. However, any relationship between the cranial hemorrhage and fatality observed in embryos exposed to nicotine is unclear. In contrast to the previous studies, infusion of nicotine into the chick embryos at later developmental stages, around day 8 incubation for 10 days showed levels of nicotine in the serum and extraembryonic fluid, while the nicotine receptors were not upregulated in the brain showed no change [43]. No difference in thresholds of vestibular response in nicotine-treated embryos compared to the controls might be due to the difference in the exposure time point during development.

5.1. Abnormal Cerebellar Cortex Development

Nicotine exposure also resulted in a reduction in mature Purkinje cells, the largest neuronal cells in the cerebellar cortex [7]. During the fourth day of incubation, the cerebellum in the chicken embryo begins to undergo a series of segmentation. The cerebellar mantle layer divides into an inner mantle layer (IML) and outer mantle layer (OML), in which each layer continues to differentiate. The inner cortical layer and the Purkinje cells derive from the IML. El-Beltagy et al. [7], observed that nicotine induces the development of the cerebellar cortex in chicken embryos. The inner cortical layer could not be identified by day eight of incubation, and Purkinje cell patterns and the cerebellar cortex foliation were irregular by day 16 in nicotine-treated embryos. Cell accumulations in the superficial area of OML ~~(EGL)~~ could also not be recognized suggesting a nicotine-induced suppression of the cell growth and differentiation needed for this process. Nicotine also induced changes in the cellular structure of the Purkinje cells leading to mitochondrial degeneration, rough endoplasmic reticulum (RER) channel destruction, and irregular expansion of Golgi bodies [7].

Normal development of the cerebellar cortex is dependent on P53 protein reactions and caspase-3 [7]. The P53 proteins are responsible for the regulation of cell growth, proliferation, and division [7, 29]. High levels of P53 have been seen in tumors suggesting an inhibition of apoptosis [29]. Nicotine suppressed the reaction of the P53 proteins in the cerebellar cortical layers while control embryos displayed a strong reaction for the P53 protein [7] suggesting that nicotine affected the normal synthesis of the proteins. Activated caspase-3 is essential for apoptosis and plays a role in protein cleavage [44]. Like the P53 protein levels, caspase-3 in the control groups was higher than in the groups treated with nicotine [7] suggesting that nicotine inhibits caspase-3 activity, therefore suppressing apoptosis leading to irregular cerebellar cortex development. As the cerebellum plays a role in balance [7] and learning [45], abnormal development may result in irregular movements and learning disabilities post-hatching.

5.2. Cognition/Learning Deficiencies

Nicotine exposure has been observed to produce learning defects in post-embryonic development. Lower mental scores and responses to auditory stimuli were observed in children at one and two years of age who were exposed to nicotine during prenatal development [46]. Studies have shown that embryonic exposure to nicotine in chick embryos stunted the learning abilities of chicks after hatching [22, 42]. Detour learning tasks, or a setting in which a selected goal is blocked by a detour that must be completed to reach it [47], have been used to analyze cognition in hatched chicks following nicotine administration during incubation [22]. Nicotine-treated chicks were slower in reaching the warm and light feeding compartments, had lower responses to auditory and visual cues, and had a flatter learning curve [22]. The complexity of detour tasks suggests that nicotine impaired the maturation of neural processes that mediate behavioral plasticity or others, such as cognitive mapping, that are specific to detour problems [22]. The reduced maturation rate correlates to the suppression of CNS growth and functional defects in response to nicotine exposure [22].

5.3. Imprinting Impairment

Prenatal exposure to various factors influences development and produces long-term behavioral defects. Kabadayi et al. [47] exposed chick embryos to nicotine during incubation days 0 and 5 and tested its effect on imprinting behaviors and capabilities post-hatching. The left intermedial part of the hyperstriatum ventral (IMHV) parallels the role of the hippocampus in mammalian cognitive behaviors [48]. The high concentration of muscarinic cholinergic receptors and acetylcholine release in the IMHV along with protein kinase C (PKC) isoforms are responsible for the filial imprinting behavior, or the characteristic of chicks following their first encountered object [48]. The study found that nicotine reduced concentrations of PKCγ~~γ~~ and PKC~~B~~β in the IMHV by 15-25% and 20-30%, respectively, and ultimately decreased chick imprinting. PKC~~a~~α was unaffected by nicotine exposure however, it does not contribute to imprinting behavior [48].

5.4. Neural Tube Defects

Neural tube defects (NTD), which affect the brain and spinal cord, occur in approximately 3000 births in the US each year and correlate to an increase in child mortality within a year after birth [16]. Both passive and active smoking are risk factors associated with NTDs [16] as nicotine decreases growth rate or causes a retraction of neurites, inhibiting central nervous system development [49, 50]. Nicotine-induced abnormal axial rotation, neural tube formation, and synaptic expression.

A chick embryo begins development with its head facing downward and as development progresses, the body follows the rotation of the head until the embryo is positioned on its left side [16]. Bohn et al. [16] found that nicotine-treated embryos compared to the controls had four times as many occurrences of atypical rotation or excessive rotation and uneven cervical regions dorsally. The atypical rotation correlated to incomplete neural tube closure of the cervical region and other spinal cord developmental defects. Treated embryos also had a lack of separation between the embryo's surface and the spinal cord roof [16]. Additionally, Dalgic et al. [4], analyzed changes in stage 12 of development to observe the effects of cotinine, a major metabolite of nicotine, on neural tube closure. Stage 12 embryos have a leftward-turned head, a defined telencephalon, a slight S-shape to the heart, and an anterior closure of the neuropore. However, of the ten embryos treated with high cotinine doses, only six reached stage 12, and four of those six had malformations. The embryos had defective neural tubes in the caudal and cranial sections of the thoracic region and had incomplete neural tube closures as the neural folds were not fused above the neuroepithelium [4].

5.5. Neural Circuit Formation

Nicotine exposure during pregnancy affects neural circuit formation and reduces functional synaptic expression [39]. Nicotine interferes with nicotinic acetylcholine receptors (nAChRs) which control activity in developing the brain and spinal cord and regulate other neural developmental processes such as neural growth, neuronal survival, gene expression, synaptogenesis, cell death, morphological changes of neurites, and plasticity events [51]. Momose-Sato and Sato [39] observed that the spontaneous wave activity mediated by nAChRs in early neural developmental stages was inhibited by exposure to nicotine. The inhibition of this wave activity referred to in this study as the depolarization wave, correlates to disrupted synaptic network formation [39]. When nicotine

was administered at the onset, peak expression of, and maturation of nAChR, each stage experienced a reduction of a8-positive neuron immunoreactivity, proximal dendrites, and neurotic length of cholinoceptive neurons, and MAP-2-positive neurites in the tectum of chick embryos, with the MAP-2-positive neurons being more extensive when visible with staining [51]. Torr ão et al. [51] also observed that nicotine had no impact on neurite density/numbers per cell and, when combined with a-BGT, nicotine produced no change in the neuritic length of tectal cells. These findings suggest that the development of nicotinic receptor-containing neurons in the tectum of chick embryos is influenced by the blockade and activation of nAChRs [51].

6. Oculomotor Complex (OMN) and Retina

Through afferent and target-derived trophic factors, the central nervous system regulates the chick oculomotor complex (OMN). Comprised of one ventromedial+lateral (vOMN), one dorsolateral, and one dorsomedial nuclear pair, the OMN makes up the cranial nerve III motoneurons which respond to afferent impulses through nAChRs [52]. Wielgus et al. [52] studied the effect of nicotine on the vOMN. They found that nicotine-induced an increase in vOMN cell density by 37%, hypertrophy of the developing vOMN neurons, and a 78% vOMN apoptotic suppression at E11 when maximal apoptosis usually occurs in normal development. The relationship between nicotine dose and inhibition of apoptosis was linear, however, significant differences to the control were only present at doses of 0.20 ug and higher, therefore approximating the threshold for significant vOMN apoptosis inhibition lies between 0.10 and 0.20 um yolk nicotine concentration. However, nicotine's neuroprotective effect could not be concluded through the parameters of this study [52].

The chicken eye contains the same anatomical structures as the human eye except for being oblate. During development, neurons in the chicken retina are produced excessively and two cell death waves occur between embryonic days 4-7 and 10-14 respectively, during which more than half of the cells are eliminated. In between the waves, the regulation level of apoptosis is generally low [20]. The study conducted to investigate the effect of an e-cigarette cinnamon flavoring agent on the neural retina development of chick embryos showed induced apoptosis in all layers of the retina and the sclera between the early and late cell death waves [53]. The down-regulation of CASP-3 expression in the neural retina development indicates that cell death is independent of CASP-3 [53].

7. Chorioallantoic Membrane

Nicotine was observed to inhibit chick chorioallantoic membrane development including blood vessel pattern formation, major blood vessel area and diameter, and capillary plexus formation [32]. MS and SS smoke also interfered with CAM growth in a dose-dependent manner [5] as embryos treated with smoke solutions had smaller CAM areas compared to the controls [32]. Nicotine additionally altered the composition of the CAM as the mesodermal layer was thicker, had more extracellular matrix, and had more fibroblasts than in control CAMs [33]. Nicotine also inhibited cell proliferation which impaired CAM growth [32]. A study by Ji et al. [5] was conducted to determine which chemical components of the smoke primarily affected tissue growth. Each twelve pyridine derivatives identified in MS smoke dose-dependently inhibited CAM growth in chick embryos, with all except two (di- and trimethyl substituted pyridines) having over 70% maximum inhibition [5]. 2- and 3-ethylpyridine were the most potent inhibitors with a maximum of about 87% inhibition [5]. Pyridine itself was not a potent inhibitor, however, substitutions altered its inhibition capacity. Single linear ethyl and methyl substitutions increased the inhibitory activity by 10 million and 1000-fold respectively, while most other substitutions had minor effects [5]. The inhibition of growth by 2- and 3-ethylpyridine could contribute to the low fetus birthweights of active and passive smokers [5].

It is suggested that SS smoke is more toxic than MS smoke to CAMs [33] as methyl and ethyl substitutions are up to 13 times more abundant in SS smoke [5]. SS smoke-treated CAMs also contained seven times the amount of fibronectin compared to MS-treated and control CAMs, which closely resembled each other [33]. However, both active and passive smokers are exposed to these chemicals in cigarette smoke as the tested pyridine derivate is present in both MS and SS smoke [5] but active smokers are at higher risk as they are exposed to MS smoke along with SS smoke [33]. The CAM model is commonly used to measure tumor growth and cancer progression *in vivo*. Squamous cell carcinoma of the lungs. Nicotine shown to promote lung cancer proliferation via the 7-nicotinic acetylcholine receptor (7-nAChR) subtype in H520 cells implanted on chicken CAM [54].

8. Neutralizing Compounds

Nicotine has been observed to decrease the survival rate of chick embryos, suppress femur development, decrease contractions of cardiomyocytes, and retard growth however, the use of camellia sinesis, vitamin C, and folic acid could help neutralize these effects. Studies found that green tea extract, and antioxidants from the *Camellia sinesis* plant, neutralized the effects of nicotine on survival rates by protecting against free radicals and oxidative stress caused by tobacco use [19] and inhibited the effects of nicotine to suppress bone matrix formation [18]. Embryos treated with green tea extract in addition to nicotine had survival rates and femur development higher than nicotine-treated groups and were comparable to the controls [18, 19] While green tea extract could help

counteract the effects of nicotine, it is not capable of fully overcoming or recovering these effects [18, 19].

The addition of folic acid and vitamin C to nicotine-treated chicken embryos helped reverse nicotine-induced teratogenic effects however, they were not able to reverse those induced by cadmium chloride, a heavy metal present in cigarette smoke [55]. Embryos treated with nicotine had less cardiomyocyte contractile activity and were growth retarded however, those treated with nicotine in addition to folic acid and vitamin C had results comparable to the controls and were not growth retarded [55]. Folic acid and vitamin C could protect against decreased contractions of cardiomyocytes induced by cadmium chloride, as both the groups treated only with cadmium chloride and those with the addition of folic acid and vitamin C had low contractile activity [55].

9. Implications

Even though tobacco smoking is well associated with health risks, several people including pregnant women continue smoking. Few studies have elucidated the teratogenic effects of nicotine exposure, but the biological mechanisms are not well understood. This review demonstrates that the chicken model of nicotine exposure in different forms provides background information to investigate the biological effects of alternative nicotine exposure including electronic cigarette use, and signaling mechanisms underlying the transition to compulsive nicotine intake. This review highlights the findings of using chicken embryos to study the effects of developmental nicotine exposure and comprehensive analysis will be helpful for future studies to better understand the etiological complexity of tobacco and other nicotine consumption.

10. Conclusions

Due to the easy accessibility, inexpensiveness, and similarity to human embryonic development, chick embryos have been used as a model organism to study nicotine-induced alterations in embryonic development as concerns increase regarding worldwide tobacco use. Findings suggest that nicotine inhibited angiogenesis, embryonic, and CAM growth by inhibiting cell proliferation. Nicotine exposure also led to low survival rates and growth retardation by inducing maternal and placental vasoconstriction and inhibiting calcium absorption for regular skeletal development. The development of the brain and cerebellar cortex were also affected as nicotine altered Purkinje development and suppressed P53 reactions. The upregulation ~~ing~~ of a7 nicotinic receptors and altered alveolar development in response to nicotine exposure impaired fetal pulmonary functions and nicotine's interference with nAChRs inhibited neural wave activity and synaptogenesis and altered embryonic movements. Other nicotine-induced neural defects were abnormal axial rotation and incomplete formation and closure of the neural tube in chick embryos. Additionally, green tea extract from *Camellia sinesis*, folic acid, and vitamin C were observed to protect against nicotine-induced effects including suppressed femur and embryonic growth, decreased contractility of cardiomyocytes, and low survival rates. These compounds, however, are not preventative measures and cannot fully overcome the teratogenic effects of nicotine. Therefore, more studies are warranted to investigate the process of treating nicotine-induced effects on embryonic development.

Abbreviations

CDC: Centers for Disease Control and Prevention
CAM: Chorioallantoic Membrane
SIDs: Sudden Infant Death Syndrome
Ach: Acetylcholine
ChAT: Choline Acetyltransferase
ACSE: Aqueous Cigarette Smoke Extract
MS smoke: Mainstream Smoke
SS smoke: Side Stream Smoke
ATF-3: Activating Transcription Factor 3
FOXA2: Forkhead Protein A2
INHBA: Inhibin Subunit BetaA
MAPRE-2: Microtubule Associated Protein RP/EB Family Member 2
RIPK-1: Receptor-Interacting Protein
SERPINA-4: Serpin Family Member 4
VEGF-C: Vascular Endothelial Growth Factor C
nAChRs: Nicotinic Acetylcholine Receptors
ODC: Ornithine Decarboxylase
CNS: Central Nervous System
AMP: Adenosine Monophosphate
IML: Inner Mantle Layer
OML: Outer Mantle Layer
RER: Rough Endoplasmic Reticulum
IMHV: Intermedial Part of the Hyperstriatum Ventral
PKC: Protein Kinase C
NTD: Neural Tube Defects
OMN: Oculomotor Complex
vOMN: Ventromedial+lateral

Author Contributions

Ashley Federico: Conceptualization, Writing - original draft

Muhammadzohir Hidoyatov: Conceptualization, Writing - original draft

Suresh Nimmagadda: Writing - review & editing

Poongodi Geetha-Loganathan: Funding acquisition, Project administration, Writing - review & editing

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Conflicts of Interest

The authors declare no conflicts of interest.

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