

# Nicotine Exposure Before the Onset of Organogenesis Induces Irreversible Embryo Toxicity in Zebrafish

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## Abstract

According to recommendation of World Health Organization (2013), consumption of direct tobacco or through smoking during pregnancy have a greater risk of adverse impact on developing embryo. Exposure to nicotine alone, a constituent of tobacco, compared with exposure to tobacco, in contrast, reported to have lesser negative impact on health and cellular survival. Therefore, there has been a shift in the use of traditional tobacco products to pure nicotine oriented formulations. However, recent reports indicated that pure nicotine itself exert adverse impact on developing embryos. Therefore, in this studied, we have re-evaluated the impact of three sources of nicotine [pure nicotine (P-N), E-liquid (EL) containing 24 mg/ml nicotine (EL-N-2.4%), and tobacco water extract nicotine (TE-N)] on Zebrafish embryo development. Treatment of 3-hour post fertilization (hpf) Zebrafish embryo with varying concentrations of nicotine (0.005 to 1.0 mg/ml) obtained after dilution from above three sources for 24-hour resulted time and concentrations dependent embryo toxicity. TE-N had the most lethal effect followed by EL-N and P-N. 0.02% v/v EL containing 0% nicotine, an equivalent concentration of EL presence in 1.0 mg/ml nicotine got after dilution of EL-N-2.4%, produced no embryo toxicity. But EL concentrations above 0.02% v/v induced embryo toxicity suggesting EL in EL-N could contribute to nicotine induced embryo toxicity. Similarly TE-N non-nicotinic product (NNP-TE) got after photolysis of TE-N containing 1.0 mg/ml nicotine also produced no embryo toxicity. But NNP-TE treatments caused embryo yolks deep brown straining and slows down embryonic plate movement suggesting NNP-TE interfere with the embryo's yolk metabolism. Further, treatment of 3-hpf embryo with nicotine concentration of 1.0 mg/ml for short period (30-minute) induced progressive embryo mortality, but not in 24-hpf treated embryo. This clearly suggest early embryonic insult with nicotine produce irreversible negative impact in the developing embryo.

**Keywords:** Zebrafish; Embryos; Nicotine; E-liquid; Toxicity

## Introduction

World Health Organization, 2013, reported used of tobacco base products or exposure to tobacco smoke either direct or through second hand smoking during pregnancy causes direct adverse impact on the unborn child [1]. These includes increased risk of miscarriage, stillborn or premature infants, infants with low birthweights and Sudden Infant Death Syndrome [2,3]. A large body of works associates tobacco exposure during pregnancy with the long-term neuro-physiological problems on newborn infants that continue into adulthood. For example, exposure to tobacco causes cardiovascular defects [4]; behavioral disorders such as attention deficit/hyperactivity disorder and conduct disorder [5]. However, studies involving, nicotine alone, an important alkaloid constituent of tobacco, have been suggested to produce positive impacts on health and behavior. For example, nicotine

stimulate new blood vessels formation, increases red blood cell count [6,7] and ameliorated behavioral impairments [8]. Based on these observations, there are many pure nicotine delivery system available in the market, which include; nicotine gum, nicotine patches, and electronic cigarettes. Electronic cigarettes commonly known as E-cigarettes, are much more popular compared with the other nicotine delivery system, partly because it mimics cigarettes. E-cigarettes are odorless, and associated with no second-hand smoking impact. Smokeless pure nicotine therapy in pregnant women with tobacco dependence, have shown to improve birth weights and gestational age. Many considered these improvement due to lack of carbon monoxide and other toxicants found in tobacco smoke in the smokeless nicotine delivery system [9]. E-cigarettes vaping release no carbon monoxide and possess no second hand smoking impact. Therefore use of E-cigarettes or even the misuse of E-cigarettes have drastically increased among women [1].

However, many reports question the safety and positive impact of E-cigarettes vaping on health because of the conflicting reports of cellular toxicity induced by E-liquid (EL) containing no nicotine. EL is the liquid used in electronic cigarettes consisting a mixture of propylene glycol, glycerin with or without flavoring ingredients that give vapor body like smoke when heating. Adding different amount of nicotine to EL provide different strength of E-cigarettes (usually 0% to 2.4% or 0 to 24mg nicotine/ml of EL). Some reports showed EL reduced the viability of culture cells while EL aerosol enhanced the production of reactive oxygen species leading to apoptosis and necrosis of culture cells [10,11,12]. In contrast, other reports indicated EL and its aerosol produced no observable cellular toxicity [13]. Survey reports showed a daily similar intake of 37.6mg nicotine among the cigarette chain-smokers (both men and women) but men showed faster nicotine metabolized rate than women [14]. Therefore, daily nicotine intake needed among cigarette chain-smokers, unlikely to be change, even after shifting to E-cigarette from traditional cigarette. Further, another reports on the comparison between chain-smokers using traditional cigarettes and E-cigarette containing 18mg nicotine/ml of EL, showed that E-cigarette vaping took more time each puff (3.5- second) compared with traditional cigarette puff (2.3-second). Blood plasma nicotine concentration also showed robust increased in E-cigarette vaping compared with traditional cigarette smoking [15]. However, consequent of E-cigarette prolong vaping with spike in blood plasma nicotine concentration on neuro-physiological conditions of vapers (both man and women) are not clear yet. Further, studies on the impact of E-cigarette vaping on pregnant women especially on the developing embryo in the womb are lacking. Review and analysis of large bulk of published data inferred more research needed to evaluate both short and long-term impact of E-cigarette use on public health [16]. Recently, use of Zebrafish (*Danio rerio*) as an animal model has been growing rapidly for biomedical research including developmental analysis. Because of the approval of Zebrafish as animal model by Food and Drug Administration (FDA) for drug screening, Zebrafish Embryo Toxicity (ZET) test become more popular choice of technique to evaluate toxicants. Efficiency of ZET analysis is because of external embryo fertilization, transparent embryo and rapid development that enable real time assessment of embryo development [17,18]. Therefore, we have conducted ZET analysis of three sources of nicotine; pure nicotine (P-N), EL containing nicotine (EL-N) and tobacco water extract nicotine (TE-N). Our results showed that nicotine presence in all sources can induce embryo toxicity. TE-N induced the highest embryo toxicity followed by EL-N and P-N. EL alone can induced embryo toxicity in a concentrations dependent manner. In contrast, non-nicotinic photolysis product of TE-N (NNP-TE) severely affected zebrafish embryo developmental rate.

## Materials and Methods

### Materials and solution

Local aquarium store was the source of adults Zebrafish of approximate 3 cm size and 700 mg weight; ocean's sea salt; Aqua-select sterilized bloodworms; API Melfix antibacterial and antifungal; aquarium grade methylene blue. EL containing 2.4% nicotine (EL-N-2.4%) and EL alone were bought from vaporfi website [www.vaporfi.com](http://www.vaporfi.com) while Sigma-Aldrich was the source of pure nicotine in the form of nicotine hydrogen tartrate. Craven A cigarettes full flavor was the source of nicotine from tobacco leaves. Egg media contained 60mg of ocean's sea salt, three drops aquarium grade methylene blue in 1L of distilled water and maintained at 28.5°C until and otherwise stated.

### Zebrafish maintenance and selection

Adult Zebrafish of same size housed separately male and female (10 fish) in each aquarium tank of size (45x60x25cm). The fish maintained under optimum conditions of temperature (28.5°C), water conductivity of ~1,500  $\mu$ S/cm, pH a~ 7 and alternates 14h light and 10h dark cycle as suggested by Westerfield, 2007 [19]. Proper oxygenation of the aquarium was carried out by passing through the air pump continually. The water quality were maintained by replacing tank's water daily with the Millipore filtered water containing appropriate instant ocean salt. Feeding performed twice daily with enough food that can be eaten within 5 minutes.

### Breeding procedure

Three days before breeding, the fish were feds three times each day with bloodworms. The breeding temperature was set at 28.5°C. A breeding net was placed inside the breeding tank, which allowed passing of eggs but not the adult fish. The breeding net adjusted to a height of 20-30 cm, to prevent eating of the eggs by adults. No water filter applied to the breeding tank, to prevent filtration of eggs. Males and females, in a ratio of 2:1 were placed into breeding tank but separated using a glass partition on the evening before spawning and kept in the dark overnight. On the morning before spawning, the partition between males and the female removed, and the laid eggs collected after one hour of spawning.

### Embryo sorting

After removing the adult fish and breeding net, the tanks tilted and the eggs removed by siphoning and transferred to a small tank containing freshly made egg media. Collected eggs were rapidly washed once with egg media containing 3 drops/L of API Melfix antibacterial and antifungal aquarium treatment liquids to ensure no bacterial and fungal contaminant. Then washed the eggs again twice with sterilized egg media (without antibacterial and antifungal treatment liquid). Fertilized eggs sorted out from non-fertilized eggs though scanning with a light Amscope microscope. Treatment of the fertilized eggs begun when they reached age of ~ 3 hours of post-fertilization (hpf).

### Extraction of nicotine from tobacco leaves

Tobacco leaf extract was prepared following the procedure developed by Omara and Attaf [20]. In short, 5 g of finely powder tobacco leaves was treated with 25 ml 1.0M NaOH for 30-minute in a 50 ml Cornell centrifuge tube. The contents of the tube then centrifuged at 6000 rpm for 10 min. After centrifugation, the clear supernatant collected by careful aspiration without touching the pellet. The pellet was then retreated twice by adding 2.5 ml of 1.0M NaOH. The supernatants pulled together and adjust the pH to 7.2 by adding and mixing slowly 2.0 M HCl. The final volume of the collect was then adjusted to 50 ml with distilled water and term as Tobacco water extract (TE-N). 5.0ml of the TE-N was then subjected to repeated ether extractions to minimize the non-nicotinic component of TE-N. This ensured minimizing of non-nicotinic component of TE-N interference

during spectrophotometric determination of nicotine concentration of TE-N [20]. After determination of nicotine concentration, TE-N volume was adjusted to give 2.0mg/ml nicotine with distilled water. The extract store airtight in amber bottle (to prevent photolysis) at room temperature and used within five days of the preparation.

### Preparation of non-nicotinic product of TE-N (NNP-TE)

To prepare NNP-TE, known volume of freshly reconstituted TE-N containing 2.0 mg nicotine/ ml was spread in a glass Petri dish. The content then exposed to solar radiation for 72-hour as suggested by Lian et al, 2017 [21]. Loss of water through evaporation during exposure was then re-adjusted to original volume with distilled water. Nicotine photolysis was identified through observation of increased decoloration of TE-N and lack of TE-N containing 1.0 mg/ml nicotine induced seizure like behavior in adult zebrafish.

### Nicotine Preparation

For Pure nicotine (P-N) stock solutions (5.0mg/ml), 50 mg of nicotine hydrogen tartrate was dissolved in 10 ml of sterile distilled water in a 50 ml amber bottle. Similarly EL-N-2.4% [24 mg nicotine/ml of EL containing 95% propylene glycol and 5% glycerine] and EL without nicotine were also dissolved in sterile distilled water in a 50ml amber bottle.

### Treatments

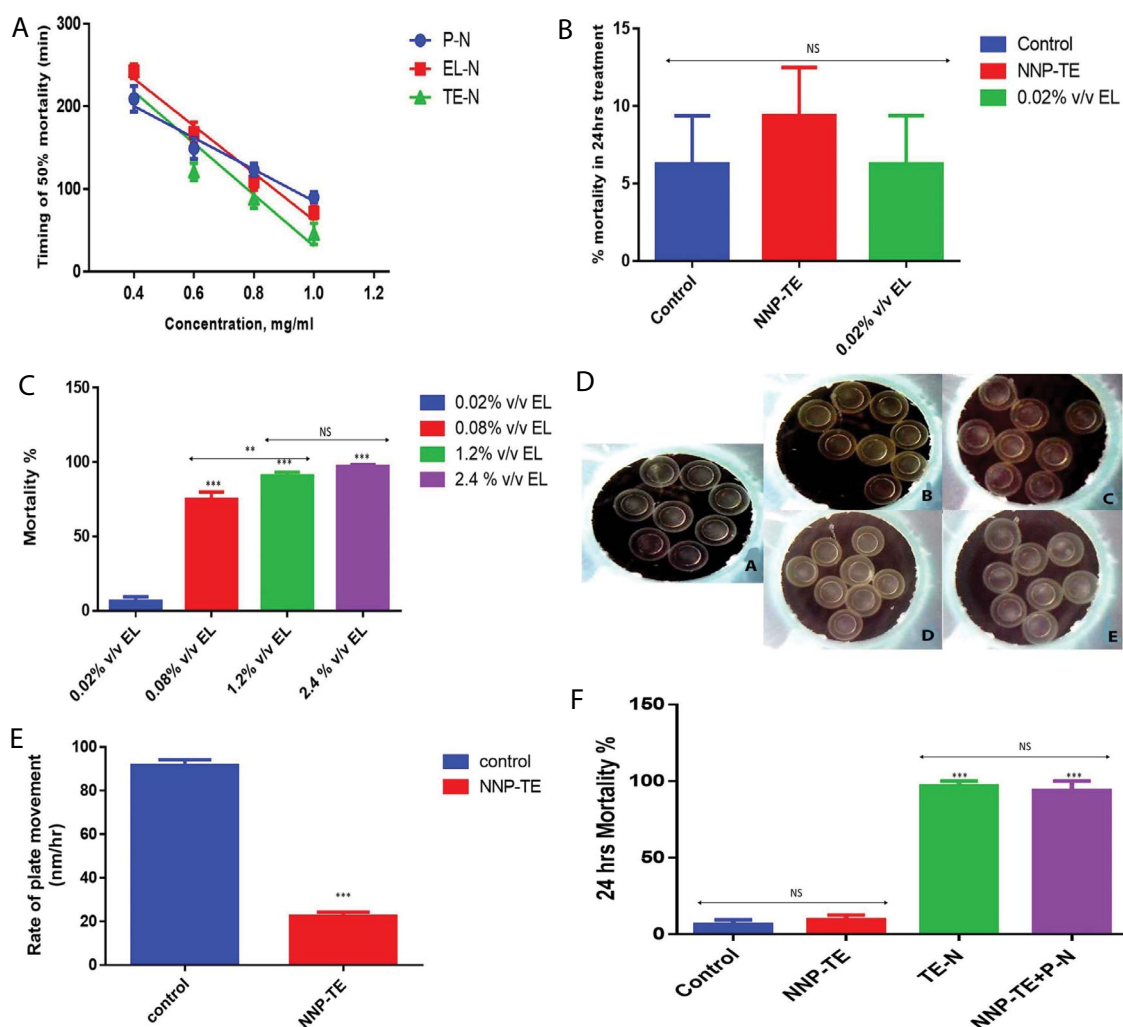
Eggs treatment were carried out by placing the eggs in a series of connected, transparent square containers fitted with an egg holding zone in the center bottom of each containers. Each eggs holding zone can hold maximum of 8 eggs and ensure no eggs displacement during the recording periods. The containers then attached to the sidewall of observation tank. The observation tank was filled with water untill half the height of the eggs container submerged into the water. The water temperature of observation tank maintained at 28.5 °C. The eggs container can hold up to 5.0 ml of egg media. Therefore, working desired nicotine concentrations in 5.0 ml was prepared by subsequent dilutions of nicotine stock solution with egg media and immediately incubated with embryo. An Andonstar microscopic web camera fitted over the eggs container and connected to a computer running iSpy software was used to record the embryo development continually up to 24-hour. Alternatively, embryos were also exposed to nicotine for short period (30-minute), then washed out nicotine and replace with egg media followed by recording embryo development continually up to 24-hour. Recording was carried out under a light projection movie having 4-minute and 50-second of black screen and a 10-second of white screen playing in a continuous loop to minimize the photolysis of nicotine.

### Statistical Analysis

Data were subjected to Single Factor ANOVA using Graphpad prism version 6 statistical software to evaluate the statistical significance between the mean of data sets of different treatments. P values < 0.05 considered to be statistically significant.

### Results

The timing of 50% embryo mortality ( $T-EM_{50}$ ) was identified after watching recorded movies of 3hpf Zebrafish embryo incubated with different concentrations of nicotine ranging from 0.4 – 1.0 mg/ml for 24-hour. Figure 1A showed plots of  $T-EM_{50}$  against the nicotine concentrations used in the treatment. Analysis of plotted data in Figure 1A showed P-N (blue) achieved faster  $T-EM_{50}$  at lower concentrations compared with EL-N (red) ( $F=1, 10=17.5, p=0.001$ ) and TE-N (green) ( $F=1, 10=17.5, p=0.001$ ). However,  $T-EM_{50}$  continuously decreased with increasing nicotine concentrations. Both EL-N and TE-N produced a sharp and significant decrease in the  $T-EM_{50}$  with increasing nicotine concentrations compared with P-N. For example, at 1.0 mg/ml



[Data expressed as Mean±SD of six independent experiments each consisting of eight embryos. \*\*\* statistical significant ( $P < 0.0001$ ).

**Figure 1: (A)** Time dependent variation in  $LC_{50}$  when 3hpf Zebrafish embryos exposed to nicotine of different concentrations ranging from 0.4 - 1.0 mg/ml from three different sources P-N, EL-N and TE-N.

**(B)** Mortality of 3hpf Zebrafish embryos exposed to NNP-TE and 0.02% v/v EL

**(C):** Mortality of 3hpf Zebrafish embryos exposed to increasing concentrations of EL.

**(D):** Deep brown staining of Zebrafish embryos exposed to tobacco extract: (A) Control (egg water), (B) Embryos exposed to TE-N, (C) After thorough washing of embryo exposed to TE-N (D) Embryos exposed to NNP-TE (E) After thorough washing of embryo exposed to NNP-TE.

**(E):** Variation in rate of development of 3 hpf Zebrafish embryos exposed to NNP-TE and the control (embryos exposed to egg water).

**(F):** Mortality of 3hpf Zebrafish embryos exposed to TE-N, NNP-TE and (NNP-TE+P-N) reconstituted NNP-TE with 1.0 mg/ml PN.

nicotine concentration, both TE-N and EL-N achieved faster  $T-EM_{50}$  ( $F=1,10=47.9$ ,  $p<0.0001$  and  $F=1,10=51.6$ ,  $p<0.0001$  respectively) compared to P-N. Further under similar nicotine concentration of 1.0 mg/ml, TE-N showed more pronounced decreases in  $T-EM_{50}$  than EL-N ( $F=1,10=132.3$ ,  $p<0.0001$ ).

Treatment of 3-hpf zebrafish embryo for 24-hour with 0.02% v/v of EL alone, equivalent to concentration of EL presence in 1.0 mg/ml EL-N, showed no sign of embryo mortality ( $F=1,10=0.2$ ,  $p=0.9$ ). (Figure 1 B). However, above 0.02% v/v of EL, the percent embryo mortality observed after 24-hour of treatments increases significantly ( $F=1,10=584.5$ ,  $p<0.0001$ ) in a concentrations dependent manner (Figure 1 C). Nearly 100% embryo mortality were noticed after 24-hour of treatment with 1.2% v/v of EL. Similarly NNP-TE obtained from 1.0 mg/ml TE-N also produced no embryo mortality ( $F=1,10=0.06$ ,  $p=0.82$ ) (Figure 1 B). In contrast, incubation of TE-N containing 1.0 mg/ml nicotine and NNP-TE obtained from 1.0 mg/ml nicotine containing TE-N with 3hpf zebrafish embryo produced a deep brown staining of eggs yolk (Figure 1 D). However, NNP-TE, unlike, TE-N, did not induce embryo mortality

( $F=1,10=0.23$ ,  $p=0.6$ ) but instead slowed down embryo development ( $F=1,24=56.7$ ,  $p<0.0001$ ) (Figure 1E).

In another set of experiments, 3-hpf zebrafish embryo were incubated with either 0.5 mg/ml of P-N or same concentration of nicotine obtained after dilution of EL-N-2.4% with eggs water for 24-hour. The embryo development was recorded up to 24-hour with recording setup. The percent mortality were calculated through the analysis of recorded movie. Plotting of percent mortality against the time of zebrafish embryo incubation, showed both P-N and EL-N produced sigmoid curve mortality profile. However, EL-N induced sharp increase in embryo mortality after 3-hour of incubation leading to faster 100% embryo mortality within 7-hour of incubation. P-N also induced similar increase in percent zebrafish embryo mortality. But a lower percent embryo mortality were noted at any point of incubation time after 3-hour of incubation when compared with EL-N induced embryo mortality. Further, P-N treated embryo showed 100% mortality only after 15 to 16-hour of incubation (Figure 2).

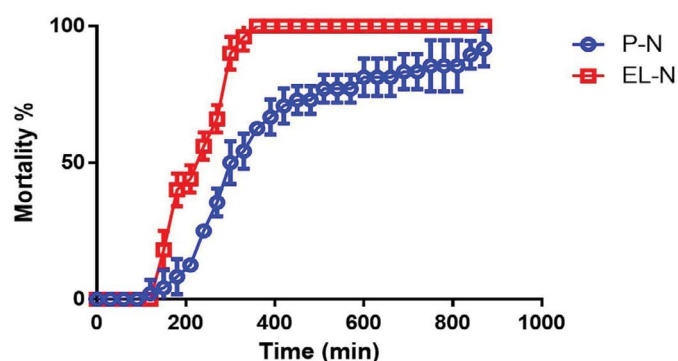
**Table 1:** EL-N dependents percent survival and percent hatching of Zebrafish embryos.

Condition	24hr survival	% hatched
24hrs exposure of 3hpf embryos with 0.005 mg/ml EL-N	100%+/-0%	77%+/-13.25%
24hrs exposure of 3hpf embryos with 0.01 mg/ml EL-N	92%+/-9.4%	0%+/-0%
30 min exposure of 3hpf embryos with 1.0 mg/ml EL-N	2.5% +/-0%	0%+/-0%
30 min exposure of 24hpf embryos with 1mg/ml EL-N	70% +/- 17%	67.5 +/- 17%

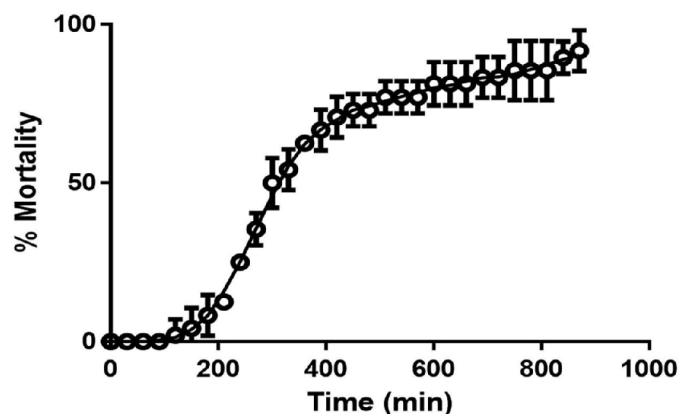
Data expressed as Mean  $\pm$  SD of is a 6 independent experiments each consisting of 8 embryos per group.

In contrast, lowering the nicotine concentration of P-N and EL-N to 0.005 mg/ml in incubates did not showed significant ( $F=1,10=0.25$ ,  $p=0.63$ ) percent embryo mortality during 24-hour of incubation. However, treated embryo showed significant reduction of embryo hatching compared to control ( $F=1,10=44.1$ ,  $p<0.0001$ ; control  $58.2\pm 11.2$  hours and 0.005 mg/ml EL-N  $95\pm 7.5$  hours). And most of the manually hatched larvae did not survive for long (Table 1).

Incubation of 3-hpf eggs with 1.0 mg/ml nicotine containing TE-N for 30-minute followed by washing with eggs water showed no decreased in the embryo mortality. None of the embryo survived up to 24-hour after the treatments (Figure 3). Similarly, incubation of 3-hpf eggs with 1.0 mg/ml for 30-minute followed by washing induced nearly 100% mortality within 24-hour after the treatment. In contrast, nearly 30% embryo mortality were noted when 24-hpf eggs incubated for 24-hour with either 1.0 mg/ml P-N or diluted EL-N containing 1.0 mg/ml nicotine. But the surviving embryo showed significant ( $F=1,10=38.3$ ,  $p<0.0001$ ) delayed in embryo hatching (control  $58.2\pm 11.2$  hours and 1.0 mg/ml EL-N  $89\pm 13.4$  hours).



**Figure 2:** Mortality curve of 1.0 mg/ml nicotine treated Zebrafish embryos. Data expressed as Mean of six independent experiments each consisting of eight embryos.



**Figure 3:** Mortality curve of 3hpf Zebrafish embryos pre-exposed to 1.0 mg/ml P-N for 30-minute during 24-hour after exposure. Data expressed as Mean of six independent experiments each consisting of eight embryos.

## Discussion

The results of incubation with P-N, EL-N and TE-N showed that nicotine alone is enough to induced zebrafish embryo toxicity in a concentrations dependent manner. At low concentrations of nicotine obtained after dilution of EL-N-2.4% and TE-N with eggs water have low toxicity compared to P-N. But at high concentrations, EL-N and TE-N produced significant increase in embryo toxicity compared to P-N.

Non-nicotinic ingredients in EL that is, propylene glycol and glycerine and water soluble non-nicotinic constituents of TE-N appears to enhanced embryo toxicity. However, 24-hour treatment of 3-hpf embryo with 0.02%v/v EL (comparable to % v/v of EL obtained after dilution of EL-N-2.4% to give nicotine concentration of 1.0 mg/ml) showed no embryo toxicity but delay embryo hatching. The results could be due to a difference in accessibility of the 0.02%v/v EL to the developing embryo through the protective chorion and perivitelline viscous fluids [22]. But dechoriation of early stage embryos and exposure to embryo media with or without 0.02% v/v EL produced no change in mortality rate. However, time dependent increase of embryo mortality in EL-N treated embryos compared with P-N treated embryos, point to EL contribution in increasing EL-N induced embryo mortality. Therefore, lack of visible embryo mortality when treated with EL concentrations below 0.02% v/v but delay in embryo hatching suggests that 0.02% v/v EL exert minimum but significant influenced on the nicotine induced embryo mortality. Further analysis showed EL alone at concentrations above 0.02% v/v drastically increased the embryo mortality. In another words, EL alone can induce concentrations and time dependent embryo mortality. This observation, therefore, is more significant because of earlier reports which showed that EL can induce cellular damage [9,10,12,23].

In contrast, in case of TE-N induced zebrafish embryos mortality, the non-nicotinic constituent's presence in the TE-N directly influenced embryo toxicity. For example, both NNP-TE and TE-N showed time-dependent deep brown staining in the embryo's yolk. However, NNP-TE had an insignificant mortality compared with TE-N. But NNP-TE produced slower embryonic plate movements and delayed hatching. Therefore, it is likely that the components in the both TE-N and NNP-TE that produced deep brown staining of embryo yolk could interfere developing Zebrafish embryo yolk metabolism. Further, reconstitution of pure nicotine with the NNP-TE exerted significant embryo toxicity comparable to that of TE-N. Therefore, these results clearly suggest the high sensitivity of developing embryos to nicotine. The results therefore agree with the previous report of Massarsky et al., 2015 on Zebrafish embryo toxicity induced by cigarette smoke total particulate (0.4 and 1.4  $\mu\text{g/ml}$  equi-nicotine units). However disagree with the report of no role of nicotine on the embryo toxicity [23]. This is because another study using zebrafish embryo as well as current observation showed pure nicotine induced concentrations and length of exposure dependent embryo mortality. For example, Palpant et al., 2015 reported pure nicotine concentrations as low as  $34\mu\text{M}$  ( $\sim 5.5\mu\text{g/ml}$ ) significantly increases embryo mortality after 72-hour of treatment compared to 48-hour of treatment [24]. The current studied obtained similar results as incubation of 3hpf Zebrafish embryo with 0.005 to 0.01 mg/ml (or 5 to 10  $\mu\text{g/ml}$ ) nicotine for 24-hour produced negligible embryo mortality

like untreated control. But all the nicotine treated embryos showed hatching delay and manually hatched larvae could not survive long. This clearly suggest 24-hour treatment even with low concentration of nicotine is enough to produced long lasting impact on the developing embryo. Further, nicotine-induced embryo toxicity were not only limited to zebrafish but also observed in other animal species as well [25-27]. For example, treatment of mouse embryo with pure nicotine at a concentration between 1.0 to 3.0 mg/kg/day were reported to decrease number of hatched mice blastocysts but at higher concentration (5.0mg/kg/day), embryo development were shown to stop at morula stage [25]. Similarly, nicotine concentration of 1.0 mM (~160 µg/ml) were reported to induced severe morphological abnormality and apoptotic cell death of mouse whole embryo culture [26,27]. Current investigation also showed Zebrafish embryo developmental stage at which embryo was exposed to nicotine and nicotine concentrations and exposure length influenced the embryo survival. For example, incubating 3-hpf embryos with 1.0 mg/ml for 30-minute followed by rinsing to remove the nicotine, resulted in embryo death within 24-hour after treatment. However treatment of 24-hpf embryos with 1.0 mg/ml for 30-minute, resulted nearly 30% embryo mortality and a delay in hatching of surviving embryo. But hatched larvae had a similar survival rate like control. This results showed that incubation of nicotine with early stages embryo produced higher mortality compared with exposures at later developmental stages. Similar studied with bovine embryo showed exposure to nicotine (0.6mg/ml) before 16-cell stage of development reduced embryo cleavage rates. Not only this, nicotine also reported to induced formation of polynuclei with various abnormal nuclear structures [28]. Therefore, formation of such polynuclei abnormal nuclear structures, if occurs, before the onset of organogenesis, then this likely to hinder organogenesis. This could be lethal to the developing embryo but might not be in the same extent in fully developed individual. In another words, high-dose nicotine insult, for short periods, to the developing embryo before the onset of organogenesis can lead to embryo mortality. The results, therefore, clearly showed direct Zebrafish embryo development monitoring in the presence of nicotine could be useful tool to evaluate the impact of nicotine during various stages of development. This can benefited in understanding the birth defect associated the newborns from pregnancy mother with nicotine dependency.

## Conclusion

Exposure of Zebrafish embryo to nicotine containing different sources such as E-cigarette and tobacco reduced embryos survival. Nicotine played primary role in inducing embryo toxicity. However, non-nicotinic ingredients presence in E-cigarette as well as non-nicotinic constituents of tobacco increases impact of nicotine on embryo toxicity. Non-nicotinic constituents of tobacco slow-down embryonic plate movement but non-nicotinic ingredients of E-cigarette alone at higher concentrations induced significant embryo mortality. The extent of nicotine induced embryo mortality depend on the stage (ages) at which the embryo was exposed to nicotine and length of exposure. Acute high-dose of nicotine insult before the onset of organogenesis was enough for induction long-term negative impact on the developing Zebrafish embryo. Therefore, prospective women planning for pregnancy with nicotine dependency should start avoiding second hand smoking or used of any sources nicotine before the onset of pregnancy and during pregnancy periods. This precaution could only prevent tobacco/nicotine related birth defect and long-term adverse impact on the health of new born infant.

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## Author's Contribution

**Mohammad Kutub Ali:** Research supervisor and coordinator, Protocols designer, experimenter, data analysis and manuscript drafting.

**Derron Ricardo Taite:** Conducted these experiments as part of a master's thesis, at the Department of Basic Medical Science, The University of the West Indies, Mona campus, Kingston, Jamaica.

## Ethics

This research was conducted after the approval of ethic committee Department of Basic Medical Science, The University of the West Indies Mona campus, Kingston, Jamaica.

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