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Growth, survival rate, body morphology and color of zebrafish (*Danio rerio*) exposed to Nicotine

Crecimiento, tasa de supervivencia, morfología corporal y color del pez cebra (*Danio rerio*) expuesto a la Nicotina

Koray Umut Yaraş*, *Şehriban Çek-Yalniz*

İskenderun Technical University, Faculty of Marine Science and Technology. İskenderun, Hatay, Turkey.
**Corresponding Author: sehriban.cek@iste.edu.tr*

ABSTRACT

Although Nicotine is one of the most potent toxins in the world, it has been used as replacement therapy and has been given to patients through gums, dermal patches, lozenges and inhalers. Zebrafish are excellent model organisms and are widely used in biomedical studies. The aim of this study was to evaluate the effects of Nicotine on the growth performance and survival rate of zebrafish larvae and juveniles. Four hundred and eighty (480) newly hatched larvae were divided into four experimental groups namely: Control (C, 0 mg·L^{-1}), N1 (N1, 5 mg·L^{-1}), N2 (N2, 10 mg·L^{-1}) and N3 (N3, 20 mg·L^{-1}) per liter of water. The results showed that Nicotine had an adverse effect on the growth and survival rate of zebrafish. In addition, morphological abnormalities were detected. The experimental groups exposed to Nicotine showed lower final weight and length compared to the control and were statistically significant. Of the dosage used in the present study, 20 mg·L^{-1} Nicotine had the most negative effect on growth and survival rate. The survival rate decreased in all exposed groups compared to the control. The maximum growth and the highest survival rate were recorded in the control group. It is proposed that Nicotine when incorporated in water can adversely affect the body morphology, color, growth and survival rate of zebrafish. Cautions should be taken when used as replacement therapy.

Key words: Spinal curvature; Nicotine therapy; model organism

RESUMEN

Aunque la nicotina es una de las toxinas más potentes del mundo, se ha utilizado como terapia de reemplazo y se ha administrado a los pacientes a través de chicles, parches dérmicos, pastillas e inhaladores. El pez cebra es un excelente organismo modelo y se usa ampliamente en estudios biomédicos. El objetivo de este estudio fue evaluar los efectos de la nicotina en el rendimiento del crecimiento y la tasa de supervivencia de las larvas y juveniles de pez cebra. Cuatrocientos ochenta (480) larvas recién nacidas se dividieron en cuatro grupos experimentales, a saber: Control (C, 0 mg·L^{-1}), Nicotina 1 (N1, 5 mg·L^{-1}), Nicotina 2 (N2, 10 mg·L^{-1}) y Nicotina 3 (N3, 20 mg·L^{-1}) por litro de agua. Los resultados mostraron que la nicotina tuvo un efecto adverso en la tasa de crecimiento y supervivencia del pez cebra. Además, se detectaron anomalías morfológicas. Los grupos experimentales expuestos a la nicotina mostraron menor peso final y longitud en comparación con el control y fueron estadísticamente significativos. De la dosis utilizada en el presente estudio, 20 mg·L^{-1} de nicotiana tuvo el efecto más negativo sobre el crecimiento y la tasa de supervivencia. La tasa de supervivencia disminuyó en todos los grupos expuestos en comparación con el control. El crecimiento máximo y la mayor tasa de supervivencia se registraron en el grupo control. Se propone que la nicotina, cuando se incorpora al agua, puede afectar negativamente la morfología corporal, el color, el crecimiento y la tasa de supervivencia del pez cebra. Se deben tomar precauciones cuando se utiliza como terapia de reemplazo.

Palabras clave: Curvatura espinal; terapia de Nicotina; organismo modelo

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INTRODUCTION

Nicotine is the major component of tobacco (*Nicotiana tabacum*) and is well known to being highly addictive. There are comprehensive studies on the detrimental effects of Nicotine and there is clear evidence that the risk of developing various type of cancer is greater among smokers than non-smokers [1, 2, 3]. Nicotine is believed to contribute to cancer promotion and progression through the activation of Nicotinic ACh receptors (nAChRs). Briefly, when Nicotine is taken by cigarette smoking, it enters the central and peripheral nervous systems and binds to nAChRs that are normally activated by endogenous acetylcholine [4]. Nicotinic ACh receptors are expressed in the cell membrane of all mammalian cells including cancer cells. A study has shown that the mortality rate was 14 times higher in smokers than in non-smokers [5]. In a comprehensive review by Xavier *et al.* [6] has also shown that smoking during pregnancy was linked increased risk of adverse birth outcomes, including low birth weight, prematurity and neonatal mortality.

Despite detrimental effects, some positive effects of Nicotine were observed in the treatment of autoimmune disease [7]; the authors claimed that Nicotine treatment function in different conditions depends on factors such as concentration, how it is employed, treatment duration and other conditons such as body conditions affecting the immune system. Moreover, White and Levin [8], found that Nicotine improved the attention of Alzheimer Diseases patients.

Human studies have shown that Nicotine patches were the best pharmacologic treatments for cessation of smoking without weight gain [9]. The basis of these all claims is Nicotine inhibiting the production of inflammatory cytokines through activating Chlorigenic Anti-inflamatory Pathway (CAP) and therefore can regulate CAP and inhibits the release of pro-inflammatory cytokines and protects the body against damage. There were concerns that starting Nicotine replacement therapy (NRT) in the immediate perioperative period may negatively impact wound healing. The association of NRT with postoperative outcomes among smokers hospitalized for a surgical procedure was investigated [10]. NRT was suggested to be prescribed routinely in the perioperative period.

In humans 75% of Nicotine is converted to Cotinine. In order to determine if zebrafish (*Danio rerio*) metabolize Nicotine similarly to humans Borrego-Soto and Eberhart [11] examined Cotinine levels in Nicotine exposed zebrafish embryos. It was clearly noted that Nicotine was metabolized by zebrafish embryos as in humans. Cotinine levels were first detedted at 36 hours (h) post fertilization (hpf) and continued to increase over time. The same authors also investigated the craniofacial effects produced by embryonic exposure to Nicotine. After Nicotine exposure a dose-dependent reduction in the craniofacial skeleton was detected. In addition they found high mortality in zebrafish adults exposed to Nicotine.

Dean *et al.* [12] investigated the opposing effects of acute and repeated Nicotine exposure on boldness in zebrafish. They suggested that Nicotine could have opposing effects on boldness that vary based on dosage and schedule of exposure. In zebrafish, acute doses of Nicotine have been consistently found to had anxiolytic properties, whereas, chronic exposure elicited anxiogenic effects. In another comprehensive review by Klee *et al.* [13] suggested to use zebrafish for the study of the biological effects of Nicotine. In the review, it was stated that, in zebrafish overactivation of the nAChRs by exposure to Nicotine decreased learning behaviours. A recent study by Victoria *et al.* [14], on zebrafish embryos showed that embryonic exposure to nAChRs Nicotine caused developmental toxicity such as: low hatching success, survival rate, growth and neurobehavioral developmental defects. The authors concluded that chronic exposure to Nicotine impaired hatching success and growth in zebrafish larvae is likely driven by the neurotoxicity and impaired embryonic motor activity [14]. Nicotine significantly altered predator escape response in larvae, specifically the latency and the initial burst movement of the response were impacted. Bhattacherya *et al.* [15] demonstrated that exposure of zebrafish embryos to Nicotine in electronic cigarette liquids during embryonic development results in low hatching success, severely perturbed bone, vascular and cartilage development. Exposure of zebrafish embryos to Nicotine leads to spinal neurons differentiation defects and Nicotine-exposed embryos were shorter than control embryos [16]. Nicotine caused delays in the development of the secondary spinal motoneurons in developing zebrafish embryos. In addition, transient embryonic exposure to Nicotine leads to long-lasting effects on the zebrafish nervous system.

The zebrafish is a tropical aquarium fish that has a wide distribution in South Asia, America, Europe and recently scattered almost all over the World. It has several characteristics over the mouse (*Mus musculus*) and rats (*Rattus norvegicus*), such as rapid development with a short life cycle, external fertilization and transparency of embryos, maintains diploid stage, small adult size, ready availability and reasonable cost of maintenance [17, 18]. Most importantly, seventy percent of their genes are similar to those of humans [19]. These are unique characteristics of zebrafish among currently available vertebrate animal models. Zebrafish has been extensively used in a variety of biomedical and contemporary scientific disciplines including angiogenesis, stem cell development, melanoma, cardiology, neurology, ophthalmology, environmental toxicology and organ regeneration.

Previously, the effects of Nicotine on larval growth, morphology and behaviour in zebrafish were investigated by Parker and Connaughton [20]. They concluded that Nicotine exposure significantly reduced notochord length and eye diameter, adversely affected the startle responses and decreased survival. On the basis of their results, they suggested using zebrafish as a model species for invesigating factors affecting vertebrate development.

This study aimed to evaluate the effects of Nicotine on the growth performance and survival rate of larvae and juveniles of zebrafish for a duration of three months.

MATERIALS AND METHODS

Experimental fish, system, water quality parameters and feeding

Facilities of the Marine Science and Technology Faculty, İskenderun Technical University, İskenderun, Turkey, were used to conduct the experiment. A static water system consisting of 12 acrylic tanks of 29 L capacity (23 × 35 × 37 cm) was used. Water quality paramaters was measured and recorded [21]. The weekly water exchange was done manually and the exchange rate was 70%. The water temperature of each tank (27 ± 1°C) was maintained by air conditioning. Water pH ranged from 7.25 to 7.80. Oxygen (YSI PRO 1020, Ohio, USA) level varied from 4.9 to 6 mg·L^{-1} Total alkalinity measured as 225–250 mg·L^{-1} $CaCO_3$. The photoperiod was maintained on a 12 h light: 12 h dark schedule. Zebrafish of known age (3 months old) originating from one pair of spawners, were stocked into each tank at the rates of five females with three males. The mean length and weight of females were measured as 3.34 ± 0.40 cm and 0.38 ± 0.14 g, respectively.

Whereas the mean length and weight of males were measured as 2.97 ± 0.3 cm and 0.25 ± 0.27 g. During the experimental work, fish were fed with two different live bait feeds. The first, one was *Artemia* (Subreme Bay Brand, INC. San Francisco, USA) and the second one was *Daphnia*. Feeding was done twice daily.

Stripping of males (7 males) and females (5 females) was done according to Dede and Cek–Yalnız [18]. Manual stripping was performed. Once the stripping and fertilization had been done, the eggs hatched within approximately 4 days, and the embryos were immediately removed from the aquarium, counted, the average weight and length were recorded and placed in 12 small acrylic aquaria, each containing 29 L of water that was continuously aerated with 4 cm air stone. A total of 480 embryos were separated into four equal treatment groups (40 embryos/aquarium, three aquariums/treatment). The larvae were fed three times a day with *Paramecium aurelia* which were produced in the quarium unit. After two weeks of hatching the larvae were fed three times a day with freshly hatched *Artemia salina* and *Daphnia* spp. throughout the experimental period of three months. Fish experiments were approved by the Iskenderun Technical University in Turkey and were conducted in agreement with the guidelines of The Republic of Turkey University of Iskenderun Technical Laboratory Animal Ethics Committee.

Nicotine exposure

The embryos were obtained in the quarium unit. They were raised in dechlorinated tap water and maintained in optimum water conditions as given above. Embryos of the same age, length and weight were randomly selected and divided into 4 groups (12 acrylic aquariums). One control (three replicate) and 3 exposure groups (each three replicate). Each group had 40 embryos. Embryos in the exposure groups were treated with 5, 10, and 20 mg·L^{-1} of Nicotine, respectively. Application doses were identified on the basis of Parker and Connaughton [20]. Nicotine was purchased from Merck company (Merck, 820877; Code Nr, NI035, Germany). The effects of different concentrations [(0 mg·L^{-1}, control), 5, 10, 20 mg·L^{-1} of Nicotine (experimental); once weekly for 90 d] of Nicotine on growth parameters and survival rate were investigated in zebrafish in four trials. Nicotine solutions were freshly prepared the day prior to each water change. This solution was not a stock solution but was prepared on twelve separate occasions (weekly for 90 d). In other words, larvae were exposed 12 times in 90 d. The larvae remained in the solution for an entire week, at which time the water of the aquaria was changed entirely (Every seven days for 90 d). The mean weight (0.24 ± 0.003mg) of the larvae was recorded to the nearest 0.0001 g (Precisa XB 220A, Switzerland). The length (3.47 ± 0.025mm) of larvae was also recorded (Mitutoyo 530–312 Caliper, Japan). These measurements were done each week for a duration of 90 d. The most difficult water quality parameter was pH. Therefore water in each aquarium changed entirely each week. There was no statistical difference in pH between the control and experimental groups. The pH was recorded daily by pH meter (IKA ETS–D6 Germany).

Statistical analysis

The data were presented as the mean ± standard error. Differences between groups in terms of weight and length were determined by the one–way ANOVA test [22]. Differences in mortality between groups were tested with Kruskal–Wallis one–way analysis of variance by ranks (SPSS ver 13 for Windows 10, SPSS, Chicago) followed by Duncan's non–parametric multiple comparison procedure.

RESULTS AND DISCUSSION

Effects of Nicotine on body morphology and color

In all Nicotine exposed groups abnormalities were clearly detected in body shape (spinal curvature, SP) and color (FIG. 1A, B, D). In the control groups, no deformities were observed and all fish developed normally with straight spin and tails with bright intensity of the color (FIG. 1C). In most fish from the Nicotine exposed groups abnormality in body shape (SP) and developmental retardation were recorded. Even when the body shape look normal in some of the fish from Nicotine exposed groups, the body shape was somehow deeper and shorter than that of the control groups. In the control groups body shape was more like stream–line (similar to a fusiform body shape). However, Borrego–Soto and Eberhart [11] exposed zebrafish embryos to Nicotine and did not observe spinal curvature in those embryos. Svoboda *et al.* [16] showed that activation of the nAChR by Nicotine mutated spinal secondary motoneutons and other spinal neurons resulted in reduced growth due to associated changes to body musculature. Parker and Connaughton [20], exposed zebrafish embryos beginning from 0 d post–hatching until 10 d post–hatching and concluded that Nicotine exposure significantly reduced notochord length and eye diameter. The difference between the current and these studies was the duration of the experiment. In the present study, the duration of the experiment was 90 d post–hatching beginning from 0 d post–hatching. The effects of Nicotine was only investigated at embryos and larval stage by these authors. Other study by Bhattacherya *et al.* [15] has investigated effects of exposure to e–cigarette vaping liquids with Nicotine on bone, blood vessel and cartilage development as well as hatching success, in zebrafish embryos. The authors have shown that exposure to vaping liquids wih Nicotine cause skeletal and vascular toxicity. Curved or truncated bodies were also observed by the authors. The present results are in line with these studies.

In the present study, the color of the head, body and tails was faintly blue. In the abnormal fish, the blue color in the caudal abdomen was faint (FIG. 1A, B, D and TABLE I). Other deformities such as distorted lateral lines and retardation in the mandible were clearly recorded in the Nicotine exposed groups (FIG. 1A, B, D). In the present study, positive effects of Nicotine were not observed and the body color of the Nicotine exposed groups of fish was darker than that of the control groups. Similarly, Borrego–Soto and Eberhart [11] observed a reduced number of melanocytes in the high dose Nicotine treated groups. However, the mechanism behind these results needs to be elucidated.

TABLE I
Effects of Nicotine on lenght, weight, survival rate, body color in zebrafish (*Danio rerio*).

Exposed groups	Nicotine dosage (mg·L^{-1})	Treatment duration (Day)	Length (cm)	Weight (g)	Survival rate (%)	Fish bodycolor
Control	0	90	2.39 ± 0.2^{a}	0.09 ± 0.029^{a}	91.6 ± 2.9	Vivid and shine blue
N1, n=40	5	90	2.22 ± 0.1^{b}	0.07 ± 0.014^{b}	70.0 ± 13.9	Faint blue
N2, n=40	10	90	2.15 ± 0.1^{c}	0.06 ± 0.016^{b}	58.3 ± 7.8	Faint and pale blue
N3, n=40	20	90	2.10 ± 0.2^{c}	0.063 ± 0.018^{b}	31.7 ± 6.2	Faint and pale blue

Values (mean ± SE of triplicate trials) followed by different letters within the same column are significantly different (P<0.05). In all groups, Nicotine exposure began on the first day of hatching. N1=5 mg·L^{-1}, N2= 10 mg·L^{-1}, N3= 20 mg·L^{-1}

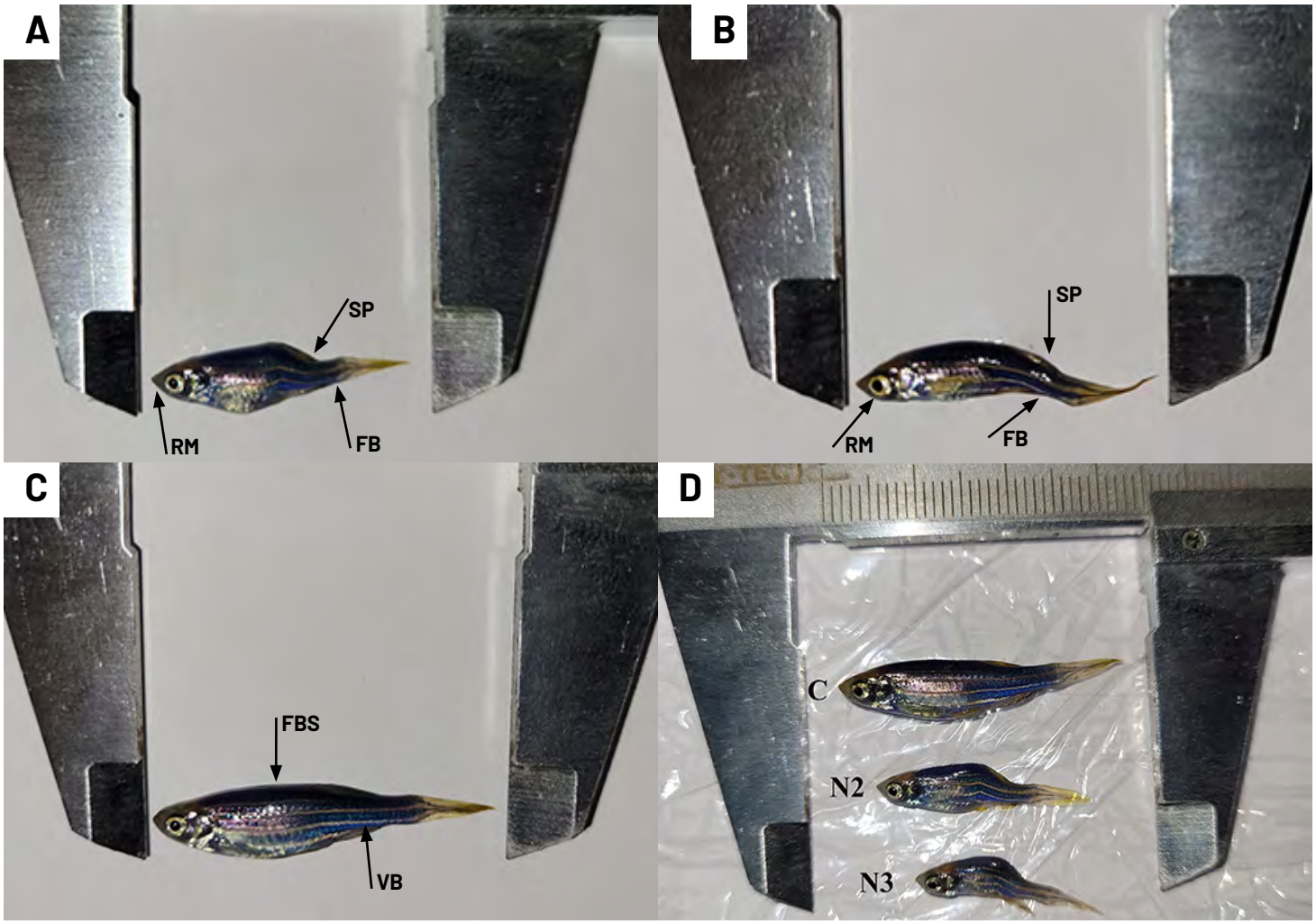

FIGURE 1. Morphological differences between Nicotine–exposed and control groups. A) 20 mg·L^{-1} exposed, spinal curvature, retardation in the mandible and faint body color are shown; B) 10 mg·L^{-1} exposed, spinal curvature, retardation in the mandible and faint body color are shown; C) Control, fusiform body shape is shown; D) Comparison of control and other two exposed groups from different aquariums. SP Spinal curvature, FBS Fusiform body shape, RM Retardation in mandible, FB Faint blue, VB Vivid blue, C control, N2 10 mg·L^{-1}, N3 20 mg·L^{-1}

Effects of Nicotine on growth

At the beginning of the experiment mean larvae weight and length were almost the same among experimental groups (P>0.05). However as the experiment progressed, beginning from the fifth week of the experiment, juveniles in control groups were significantly larger than N exposed groups (Groups N1, N2, N3 and TABLE 1). Weight gain in juveniles of the zebrafish was significantly affected by the Nicotine exposure level (TABLE 1, P<0.05). The fish exposed to higher Nicotine doses (Groups $N2_{a, b, c}$ and $N3_{a, b, c}$) attained significantly smaller weights than fish in control groups (TABLE 1, P<0.05, Fig. 2A). The worst weight gain was recorded for the fish exposed 20 mg·L^{-1} of Nicotine. Comparison of the weight gains of fish sampled from all control groups ($C1_a$, $C1_b$ and $C1_c$) indicated that fish grew at similar rates (FIG. 2A). Mean body length of fish was detrimentally affected by the exposure of the Nicotine (TABLE 1, P<0.05, FIG. 2B). The smallest body length were recorded in fish exposed 20 mg·L^{-1} of Nicotine ($N3_{a, b, c}$). Fish in the control groups had the largest body size (C_a, C_b and C_c) (TABLE 1, P<0.05, FIG. 2B). As a whole, the mean body length of fish was significantly higher in control groups compared to fish exposed to Nicotine N1, N2 and N3 (P<0.05; FIG.2B). In all Nicotine exposed groups, mean body weight and length decreased as the dosage of Nicotine increased. Chronic exposure to Nicotine resulted in a significant growth reduction in zebrafish [14, 16]. These results are consistent with the current results.

A research regarding the effects of different pharmacologic smoking cessation treatments on body weight changes in patients with Nicotine dependence was investigated [9]. The authors made a conclusion that Nicotine patches plus Fluoxetine were associated with the least weight gain of all smoking cessation treatments and should be used as a pharmacologic smoking cessation treatment [9]. The side effects of Nicotine patches, vitamin D absorption and osteoclasis in participants were not investigated by the authors.

Previous study on humans [9] and current study on zebrafish, these two studies can be compare and discuss because, zebrafish and humans possess ortholog genes [19]. In addition, approximately 82% of human disease–related genes (enumerated in Online Mendelian Inheritance in Man (OMIM)) have a zebrafish ortholog and thousands of zebrafish

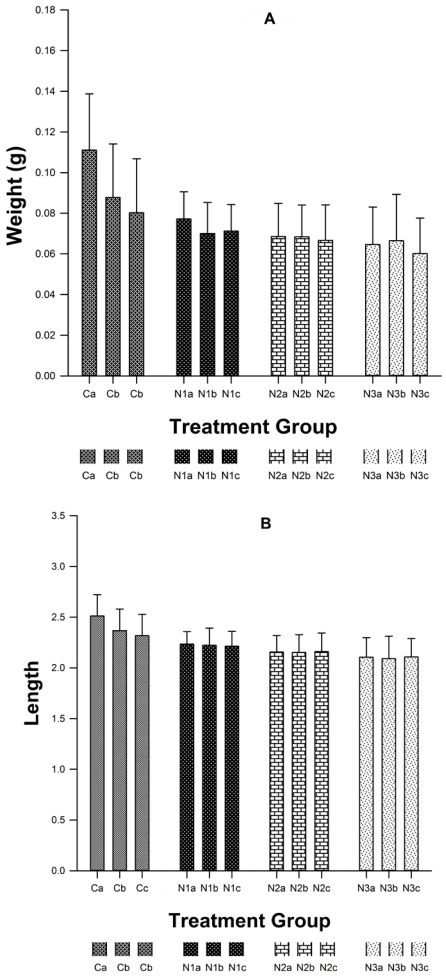

FIGURE 2. Effects of Nicotine on total body weight (A), and total length (B) of zebrafish. Values: Mean ± SE of triplicate trials, (n=40; *P*<0.05)

gene mutations have already been annotated and archived. The same signaling pathways may be highly conserved between humans and zebrafish and the genes involved in human bone differentiation and perhaps weight gain may have orthologs in zebrafish. In addition, Borrego-Soto and Eberhart [11] proved that Nicotine was metabolized by zebrafish embryos as in humans. Zebrafish can be used as a model species in order to investigate the detrimental effects of Nicotine on weight loss, morphology, skin color and spinal curvature in humans.

In the present study it was crystal-clear that Nicotine exposure groups weighed less than that of the control groups. This finding is similar to that of Hsieh *et al.* [9] where, Nicotine patches were associated with less weight gain than control groups, This study was performed in humans.

In humans, Nicotine appears to have various effects on the pituitary-hypothalamic axis that release growth hormones and that can lead to reduced growth such as altering DNA content in cells and initiating apoptosis [7]. It is suggested that higher concentrations of Nicotine are required to induce apoptosis and cell death in different cell lines. These effects are selective for cells expressing nicotinic ACh receptors which are found predominantly throughout the central and peripheral nervous system. In particular, when applied during larval development, Nicotine up-regulates and prematurely activates ACh receptors, altering the timing of developmental events, and leading to detrimental effects on physiological processes and morphological processes.

Effects of Nicotine on survival rate

FIG. 3 shows the survival rates of both control and Nicotine exposed groups. Total survival rates in all the control groups (C_a, C_b and C_c) were uniformly high ranging from 90 to 95% (*P*>0.05). All groups of Nicotine exposed fish exhibited a low survival rate compared to the control groups (*P*<0.05, FIG.3). As a whole, the survival rate decreased as the dosage of Nicotine increased. But the lowest survival rate was recorded in the fish exposed to 20 mg·L^{-1} (*P*<0.05, FIG.3). In this series of the experiment ($N3_{a, b, c}$), the survival rate (31.7%) were significantly decreased compared to the controls (91.6%). This clearly indicates that Nicotine has detrimental effects on the survival rate of the zebrafish.

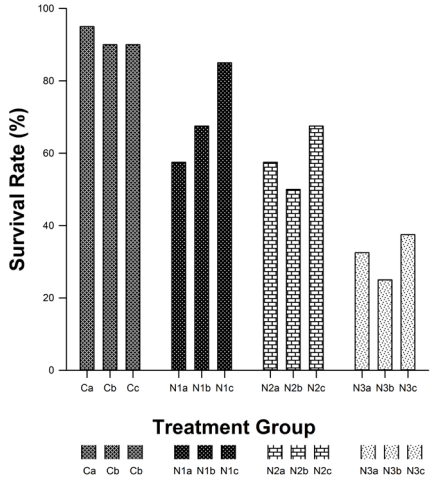

FIGURE 3. Effects of Nicotine on the survival rate of zebrafish. Values: Mean ± SE of triplicate trials, (n=40; *P*<0.05)

Smoking during pregnancy is associated with the detrimental progression and adverse outcomes of low birth weigth, prematurity and survival [6]. Cotinine is the main Nicotine metabolite, and has been widely used as a cigarette smoke exposure biomarker in humans. Borrego-Soto and Eberhart [11] showed that the zebrafish embryo is able to oxidize Nicotine to Cotinine. Same authors recorded high mortality rate, low weight gain in Nicotine exposed zebrafish embryos. These findings are similar to the current results. Low survival rate recorded in Nicotine exposure groups. In the current study, a dose-dependent reduction in the survival rate recorded in zebrafish. Among the fish exposed to the higher dosage (20 mg·L^{-1}) fifteen of the treated individuals were found dead in the third week of the experiment. Although the present research provides clear evidence that Nicotine exposure results in low survival rates, whether this mortality is caused by increases and/or decreases in apoptosis cannot be deduced from the present results as the current study did not investigate cancer and/or apoptosis during the experiment. Moreover, the current study did not measure plasma and urine Nicotine levels during the experiment. Neverthless, the present

results are consistent with the reported results from another study on zebrafish [20]. There is a lack of information in the literature on clearance and exact pathways of Nicotine in zebrafish and other fish species. However peripheral nicotinic receptors have been found in muscle, neuroendocrine cells, peripheral-blood, leukocytes and ganglia in zebrafish [23]. More recently, zebrafish has been used as a model organism, in order to examine ventilation frequency [24]. Exposure to Nicotine increased ventilation frequency. Ventilation frequency in zebrafish was stimulated by both ACh and Nicotine. The present study assume that after Nicotine exposure the plasma and urine Nicotine concentrations of zebrafish are elevated through inhalation and/or through semi permeable body skin. Therefore low survival rate recorded. Nevertheless, direct evidence is awaited inclearance and exact pathways of Nicotine in the body of zebrafish.

CONCLUSIONS

Zebrafish can be used successfully to find out the detrimental and/or beneficial effects of Nicotine. Although the exact mechanism of the adverse effects of Nicotine on zebrafish is not known, In the current study, the detrimental effects of Nicotine on body morphology, body color, weight and survival rate were clearly detected. A dose dependent reduction in the survival and growth rate recorded and the most harmful effects noted on the N3, 20 $mg{\cdot}L^{-1}$ per liter of water groups. Finally, it is emphasized that Nicotine is certainly a harmful factor for zebrafish body color, spinal curvature, weight, length and survival rate. Although Nicotine may have beneficial anti-inflammatory effects and has the potential for cessation of smoking, and perhaps in preventing obesity, further research is needed by using zebrafish as a model organism and caution must be taken when used in replacement therapy.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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