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RESEARCH ARTICLE

The effects of henna (hair dye) on the embryonic development of zebrafish (*Danio rerio*)

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Abstract The powder of henna is extensively used as decorative skin paint for nail coloring and as a popular hair dye in Asian countries. Its human health risk is extensive, and it is frequently released as waste into the aquatic environment raising the concerns. Zebrafish (*Danio rerio*) embryos were employed to study the developmental effects of henna. Normal fertilized zebrafish embryos under standard water were selected for the control and test chambers. Three predetermined sublethal concentrations (100, 200, and 275 μM) of henna in 24-well cell culture plates were tested on 1-h postfertilized embryo (pfe) for 96 h. Observation for rates of survival and mortality was recorded; digital camera was used to image morphological anomalies of embryos with a stereomicroscope; and functional abnormalities at 24, 48, 72, and 96 h were performed. The hatching rates of embryos were reduced significantly when treated with 200 and 275 μM or higher concentrations of henna. Slow blood circulation in the whole body was observed with a median effect on hatching exposed to 200 and 275 μM of henna at 48-h pfe. At 72- and 96-h pfe, blood circulation was ceased in the whole body but still had a heartbeat. At 96-h pfe, pericardial sac edema, yolk sac edema, head deformation, spine crooked malformation, and tail malformation (bent tails or hook-like tails) were observed in the surviving larvae at 100 μM. In summary, exposure to henna at 100, 200, and 275 μM causes some altered morphological and physiological abnormalities including increased mortality, hatching delay, slow blood circulation, pericardial sac edema, yolk sac edema, abnormal body axes, twisted notochord, tail deformation, weak heartbeat, and growth retardation and was also detected in some treated embryos and groups having adverse effects on embryonic development of zebrafish provoking potential human developmental risk studies.

Keywords Henna · Zebrafish embryo · Mortality · Hatching · Heart rate · Developmental toxicity

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Introduction

Hair dyes are widely used as cosmetic agents to change the color of hair around the world in recent years. Hair dye poisoning has been emerging as one of the important causes of intentional self-harm in the developing countries like China and India. Henna is used to adorn women's bodies during marriage ceremonies and other social celebrations since the Bronze Age. The use of hair dyes can be traced back to 4,000 B.C., when the hair on Egyptian mummies was dyed with henna (Nohynek et al. 2004). Vegetable hair dyes were used at that time. The first artificial dye was synthesized in the

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laboratory in 1856, and permanent hair colorants have been in commercial use for over 100 years (Wall 1957; Sampathkumar and Yesudas 2009). It is traditionally used in Islamic and Hindu cultures as a hair coloring and as a dye for decorating the nails or making temporary skin tattoos (Al-Suwaidi and Ahmed 2010; Polat et al. 2009). It is more common in women which estimates approximately 35 %, and 10 % of men in Europe, Japan, and the USA have used hair colorants (IARC 1993). The ingredient in most hair dyes is paraphenylenediamine (PPD) in concentration ranging from 2 to 10 %. In Sudan, PPD is mixed with henna, which is a nontoxic herb used to decorate the hands and feet in special social events, such as wedding ceremonies (Sir Hashim et al. 1992), and over 3,159 patients were reported to suffer from PPD poisoning over a 10-year period (1995–2005); among these, 568 (18 %) children were below the age of 14 years (Filali et al. 2006; Hamdouk et al. 2008; Abdelraheem et al. 2009). A study from Morocco described 374 cases of PPD poisoning in adults and children over a 10-year period (Filali et al. 2006). A report from Tunisia showed similar results (Kallel et al. 2005), and a report from Saudi Arabia documented a suicide attempt with PPD of a 14-year-old girl (Ashraf et al. 1994; Dressler and Appelqvist 2006; Abdelraheema et al. 2010). Similarly in India, popular hair dyes contain PPD among other ingredients (Filali et al. 2006; Anuradha et al. 2004). Some product sold as henna also contains PPD, particularly black henna (Hummdi 2012).

Intentional self-harm is well reported, hair dye as a means of ingestion (Chrispal et al. 2010), and a growing trend is observed among rural Indian population (Jain et al. 2011). The effects of PPD when ingested are serious which are cervicofacial edema, mucosal injury, respiratory distress, acute renal failure, rhabdomyolysis, and myocardial injury (Ashraf et al. 1994; Ram et al. 2007; Verma et al. 2008; Sampathkumar and Yesudas 2009; Chrispal et al. 2010; Jain et al. 2011). Hair dyes are available in stone, powder, or liquid forms. While the liquid forms are more often ingested with suicidal intentions, mortality is higher with the stone forms (Jain et al. 2011). Henna (*Lawsonia alba*) containing PPD has a high mortality rate (up to 31 %) owing to rhabdomyolysis and renal failure (Rund et al. 2007). Suicide was responsible for about 600,000 deaths in the 1990s (Eddeleston 2000). It is ranked as the third leading cause of death in the age group 15–44 years. Suicide rates have increased by 60 % in the past 50 years (Sampathkumar and Yesudas 2009).

Hair dyes and their ingredients have moderate to low acute toxicity. Contact sensitization to hair dyes has been a safety issue, mainly as a consequence of unprotected professional exposure. Moreover, hair dyes have accounted for most common side effects, such as allergic and irritant contact dermatitis, leukoderma, photosensitivity, purpuric eruptions, angioedema, urticaria and rhinitis, asthma, and syncope (Belton and Chira 1997; Garcia Ortiz et al. 1997; Sahoo et al. 2000; Santucci et al. 1994; Wang et al. 1994). The most important side effect is possibly the toxicity on DNA. This possible toxic effect is more important in women during pregnancy; if such toxicity is effective, it is clear that they are at greater risk of adverse pregnancy outcomes, including complications which may affect the fetus. Besides human health risk, aquatic environmental contamination through wastewater is indispensable, because it poses a great risk to aquatic nontarget organisms including fishes. Moreover, possible interaction of hair dye at early developmental stages may have more complex implications on ecosystem and biota. Hence, the present study was designed and aimed precisely to elucidate the effect of henna (hair dye) on embryonic development of zebrafish.

Materials and methods

Chemical

Henna (hair dye) was purchased from Herbal Henna Export House (New Delhi, India). This compound was dissolved in pure water to make a 20-mM stock solution. The treatment solutions of henna for toxicity tests were obtained by the dilution of the stock solution with embryo medium (Westerfield 1995).

Zebrafish embryos and larvae

The AB line zebrafish used in this study were obtained from Dr. Jingwei Xiong of Harvard Medical School (Boston, MA, USA) and were maintained following the standard procedures (Westerfield 1995). The night before breeding, adult male and female zebrafish were set up in a breeding tank separated by a mesh screen. After the light was turned on the next morning, embryos were generated by natural mating and were then collected within 30 min after spawning. After being rinsed three times, the clean embryos were moved to Petri dishes or a larger container with embryo medium and were cultured at 28.5 °C.

Chemical treatment and phenotype observations

The normal embryos were selected under a stereomicroscope (Olympus SZX16; Tokyo, Japan) and transferred into 24-well microplate with ten embryos per well in a 2-mL pure water. Embryos at 1-h postfertilization (hpf) were exposed to various concentrations of henna, and controls were incubated in embryo medium containing pure water. The developmental phenotypes of the experimental embryos were observed every 24 h for 96 h and were photographed with a charge-coupled device (CCD) camera. The survival rates of embryos were recorded, and death was defined as no visual heartbeat. All tests were repeated three times and were conducted in

accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

Statistical analysis

All data in graphs were presented as mean±standard error (SE). Data were analyzed using analysis of variance (ANOVA). Values of $p<0.05$ were considered as level statistical significance. All statistical analyses were carried out using the SPSS statistical software for Windows, version 12.

Results

Sublethal effects of henna on the development of zebrafish embryos

The cumulative mortality and morphological and physiological abnormalities of zebrafish embryos were evaluated at 24, 48, 72, and 96 hpf (Fig. 1). Development of the embryo were observed at 24, 48, and 72 hpf; hatching began at 48 hpf and was completed at 72 hpf; and normal development of the eye and tail were observed in the control embryos (Fig. 4i). Embryo treated with henna displayed abnormalities in morphology and function; significant decrease in hatching rate was observed at 200 and 275 μM of henna (Fig. 2). The median effective concentration (EC_{50}) of henna for hatching was found to be 140.76 μM with 95 % confidence limits (lower 98.58 μM; upper 178.91 μM) for 96-h postfertilized embryo (pfe).

Prominent morphological defects witnessed are pericardial sac edema, yolk sac edema, head deformation, spine crooked malformation, tail malformation, and weak heartbeat in the

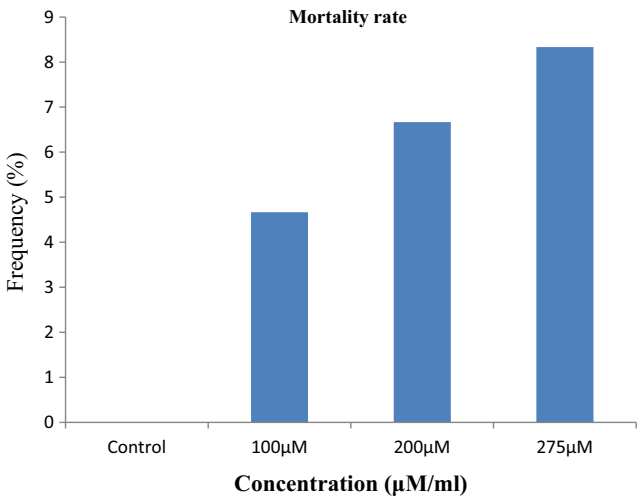

Fig. 1 Effects of henna in terms of mortality at 96 hpf. A significant increase in mortality was observed in 100, 200, and 275 μM of henna-treated embryos. Results are expressed as mean±SE. All experiments were repeated three times

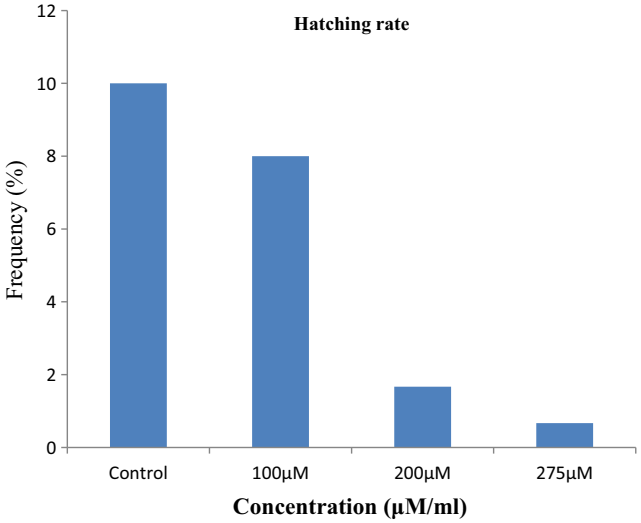

Fig. 2 Effects of henna on hatching rate from the total number of surviving embryos in each concentration. The hatching rate significantly decreased in groups exposed to 100, 200, and 275 μM of henna when compared to the control. Values are expressed as mean±SE of three experiments

treated embryos (Table 1). Embryos exposed to 200 and 275 μM of henna had no blood flow in the whole body at 72 and 96 hpf with an EC_{50} of 115.47 μM (slope was not significantly different from zero; hence, fiducial limits were not computed) but still had a heartbeat. Significant difference in heart rate between the control and treated groups (Fig. 3) was found. Abnormalities were significant in henna-treated embryos at 100, 200, and 275 μM compared to the control (Table 1). Tail malformation was the most marked toxic effect of henna on the development of zebrafish embryo (Fig. 4g, h, k, l, m, n). Embryos (96 hpf) treated with 200 and 275 μM of

Table 1 Compiled data table of morphological and physiological abnormalities among surviving embryos after 72 hpf in each concentration of henna and control

Morphological and physiological abnormalities	Concentration of henna (μM/mL)			
	Control	100 μM	200 μM	275 μM
Head deformation	–		+	+
Tail deformation	–		+	+
Edema	–		+	+
Growth retardation	–	+	+	+
Weak heartbeats	–		+	+
Circulatory disturbance	–		+	+
Spine crooked malformation	–	+	+	+
No hatching	–		+	+

The plus sign (+) indicates that the morphological change parameters are selected as toxicological endpoints at different stages of development

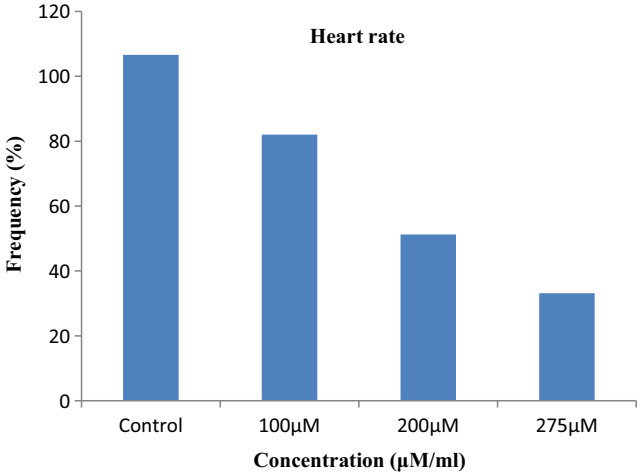

Fig. 3 Effects of henna on the heart rate. Significant difference between the treated groups and controls. Results are expressed as mean±SE. All experiments were repeated three times

henna clearly resulted in bent tails or hook-like tails in greater than 90 % of embryos (Fig. 4m).

Lethal effect of henna on zebrafish embryos

The numbers of dead embryos were determined at 24 h of interval after 24, 48, 72, and 96 hpf in respective pfe. Coagulated or dead embryos appeared white opaque and swollen under the stereomicroscopy (Fig. 4o). At 96-h pfe, mortality rate was 46, 66, and 83 % at 100, 200, and 275 μM of henna, respectively (Fig. 1). After 96-h pfe, all henna-treated embryos died at concentrations 100, 200, and 275 μM (data not shown). However, all embryos incubated in 85 μM or lower concentrations of henna survived for 120 h.

Discussion

The embryo and fingerling toxicity tests are valuable for assessing potential impacts on growth, reproduction, and survival of zebrafish in polluted environment and are important tools for good environmental monitoring (Kristensen 1994; Zagatto 1999). Previous studies suggest that chemicals or drugs can have similar toxic effects in zebrafish embryos and humans (Nagel 2002; Zhang et al. 2003; Milan et al. 2003; Lam et al. 2005). The methods of using zebrafish embryos or larvae as an animal model to assess embryonic and teratogenic effects of chemicals or drugs have been developed (Fraysse et al. 2006; Selderslaghs et al. 2009; Yang et al. 2009). These studies support and accelerate the application of zebrafish embryos or larvae to predict the toxicity of compounds of potential value. The aim of the present study was to use henna as a toxic agent to produce abnormalities in the embryonic development of zebrafish. In this study, zebrafish embryos were used at 1 hpf for 96 h. At the end of the study, anomaly types, %anomalies, %mortality, and EC_{50} values were determined. Developmental deformities such as abnormal embryogenesis, low hatchability, delayed hatching, mortality of newly hatched larvae, and poor survival ratio during the embryo stage due to henna exposure were observed.

The hatching of embryos was affected by henna in proportion to concentration; as the concentration increased, the hatching rate decreased. One probable reason for the delay or failure of hatching for the above-described developmental abnormalities is that henna can partially or completely limit the ability of developing embryos or larvae to break outer chorion and hatch out. The other reason may be the inhibition of henna to enzymes involved in hatching. Similarly, exposure of zebrafish embryos to other compounds that resulted in disturbance to hatching (Strmac and Braunbeck 1999; David and Pancharatna 2009; Wang et al. 2010) may support this observation besides their differences in the concentration and chemicals. They are of the view that pesticides may affect or inhibit the activity of enzymes involved in hatching, and the same was related to concentration and exposure periods. Our data clearly indicate that low concentration did not cause any detrimental effects on the early life stages and hatching rates of zebrafish. However, higher concentrations (200 and 275 μM) can also affect the hatching rate. The overall hatching success rates differ significantly among the different exposure groups. Furthermore, differences were observed in either mortality or incidence of malformations between the treated and control embryos distinguishing concentrations for the observed cause.

Developmental abnormalities due to henna such as the tail malformation and pericardial sac edema, including no blood flow in the whole body, may result from the cardiotoxicity. Cardiotoxicity of henna may cause mortality, hatching delay, abnormal body axes, twisted notochord, slow blood circulation, pericardial sac edema, yolk sac edema, decreased heart rate, and growth retardation. Tail malformation in zebrafish embryo at 72 and 96 hpf is one of the toxicological endpoints for evaluating the teratogenicity of chemicals; this is consistent with a number of previous studies which indicated that the PPD topically applied reaches the systemic circulation after absorption through the skin (Hummdi 2012; Steiling et al. 2001; Kawakubo et al. 2000). Hummdi (2012) added that the mortality rate in male rats treated with PPD is 21.1 %, 22 % in cases of poisoning from hair dye, 10–35 % in female Wistar rats, and 41.9 % in PPD poisoning cases. In parallel to the present study with henna, bent and hook-like tails were observed in 72-hpf embryos treated with 5 μM of curcumin (Wu et al. 2007) and 1.0 μM of celastrol (Wang et al. 2010).

There were significant differences in the amount of edema between the treated and control groups within 48 hpf. Lethality was the main toxicity of henna, while pericardial sac edema and yolk sac edema were the most severe

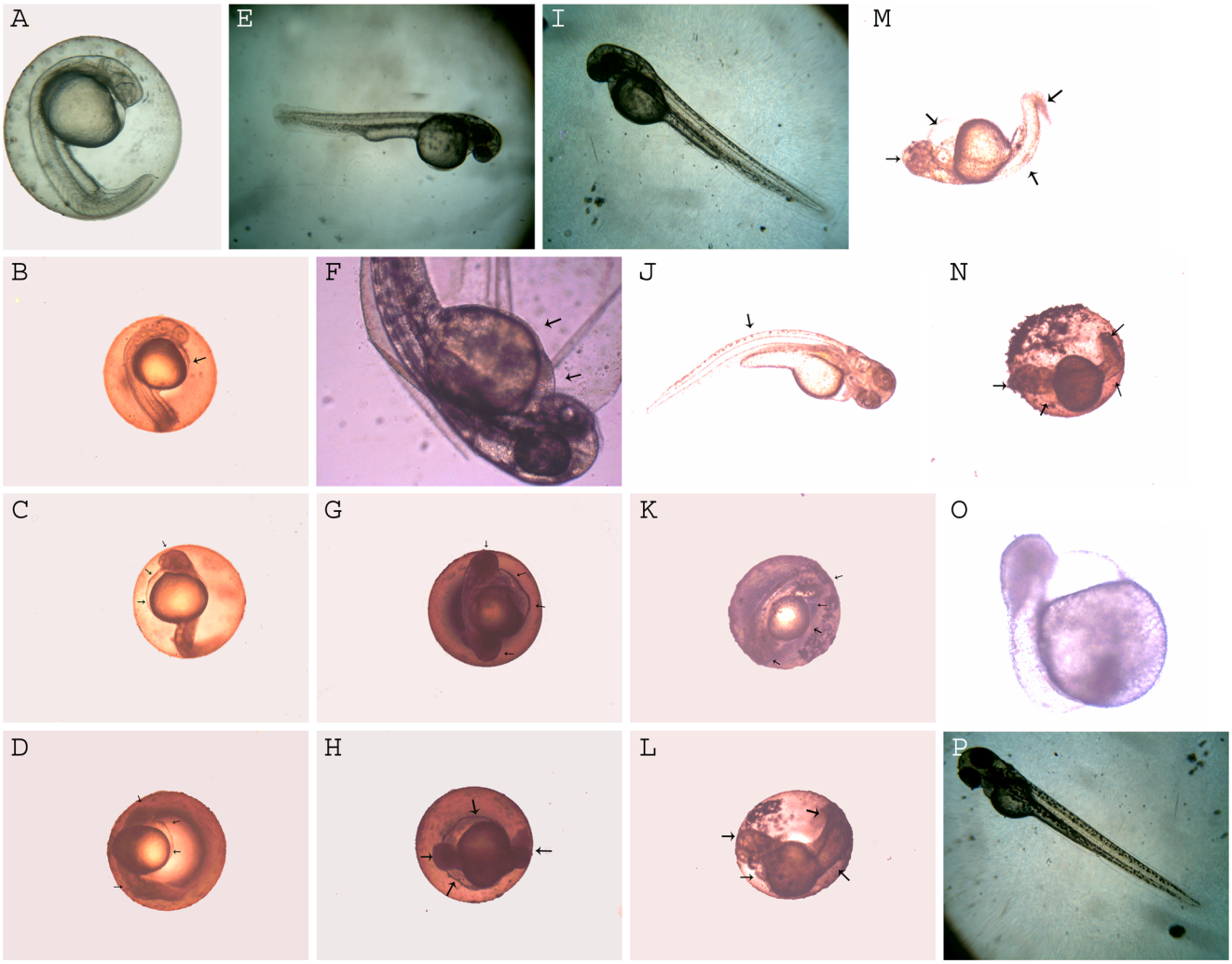

Fig. 4 Toxic effects of henna on the development of zebrafish embryos. **a** A normal embryo at 24 hpf. **b** A 24-hpf embryo with yolk sac edema with 100 μM of henna (*arrow*). **c** A 24-hpf embryo with pericardial sac edema, yolk sac edema, and head deformation with 200 μM of henna (*arrows*). **d** A 24-hpf embryo with pericardial sac edema, yolk sac edema, head deformation, and spine crooked malformation with 275 μM of henna (*arrows*). **e** A normal embryo at 48 hpf. **f** A 48-hpf embryo on hatching with pericardial sac edema and yolk sac edema with 100 μM of henna (*arrows*). **g**, **h** A 48-hpf not hatched embryo with pericardial sac edema, yolk sac edema, head deformation, and tail deformation with 200 and 275 μM of henna (*arrows*). **i** A normal embryo (hatched larva) at 72 hpf. **j** The 72-hpf hatched larva with spine crooked malformation with 100 μM of henna (*arrow*). **k**, **l** A 72-hpf not hatched embryo with pericardial sac edema, yolk sac edema, head deformation, and tail deformation with 200 and 275 μM of henna (*arrows*). **m** Hatched larva with pericardial sac edema, head deformation, spine crooked malformation, and hook-like tail hatched from embryo incubated with 100 μM of henna (*arrows*). **n** A 96-hpf not hatched embryo with pericardial sac edema, head deformation, spine crooked malformation, and tail deformation with 200 μM of henna (*arrows*). **o** A 96-hpf abnormal dead embryo with 275 μM of henna. **p** A normal larva at 96 hpf

malformation caused by henna (Fig. 4g, h). It was reported that many biochemical and molecular mechanisms occur among cell, tissues, and organs during embryogenesis, and a great number of pollutants could specifically influence these mechanisms (Fraysse et al. 2006). Pericardial sac edema was often considered as the result of heart failure or circulatory failure (Fraysse et al. 2006; Merrill 1946), so henna seemed to hurt heart functions significantly. Myocardial damage and myocarditis are reported less frequently in hair dye poisoning but associated with higher mortality (Jain et al. 2011; Singh et al. 2009). Benzene, benzoquinone, and hydroquinone are PPD metabolites. Recently, Lee et al. (2007) suggested that hydroquinone acts as a strong inhibitor of activated macrophages. It suppresses the production of proinflammatory cytokines, secretion and expression of cytotoxic molecules, and activation of CD29. PPD could also induce apoptosis via the involvement of reactive oxygen species (Chen et al. 2010). Further, the effects of henna on lethal response and embryogenesis on the zebrafish were evaluated in different time points; the groups exposed to high concentrations (200 and 275 μM) of henna showed acute severe injury and death. The increased mortality rate of the treated embryos was observed with increase in concentration. Hatching rate decreased significantly in 200 and 275 μM exposed group compared with

the control. The observed responses could be viewed as the sensitivity of embryo for the henna. However, sensitivity of the developing embryo may differ based on the chemicals or compounds tested, for example, firefighting chemicals were more toxic to fathead minnow larvae, and early life stages of *Oryzias latipes* were the most sensitive to toxic effect of triphenyltin (Gaikowski et al. 1996; Fent and Meier 1994). Moreover, new pattern of exposure to PPD has been recognized through henna which increases the risk of developing adverse health effects related to PPD. PPD is shown to cause rhabdomyolysis in rats by promoting leakage of calcium ions from the smooth endoplasmic reticulum resulting in prolonged muscle contraction and irreversible change in muscle structure (Sampathkumar and Yesudas 2009; Chrispal et al. 2010). Similarly, cervicofacial edema, the edema of the face, neck, and laryngeal region causing respiratory distress and hypoxia have been well documented in patients (Ram et al. 2007; Verma et al. 2008; Sampathkumar and Yesudas 2009; Chrispal et al. 2010; Jain et al. 2011; Soni et al. 2009; Filali et al. 2006) affected by PPD.

To the best of our knowledge, this is the first study that demonstrates the effects of henna on mortality, heartbeat, and hatching rates during the developmental stage like zebrafish larval stage and also observes the effects on hatching rate of zebrafish embryos. Therefore, henna should be used with great caution in a sustainable way so that it may not be hazardous to aquatic environment and human beings.

Conclusion

Exposure to henna altered morphological and physiological abnormalities including increased mortality, hatching delay, slow blood circulation, pericardial sac edema, yolk sac edema, abnormal body axes, twisted notochord, tail deformation, weak heartbeat, and growth retardation at 100, 200, and 275 μM. The henna used in this study was in powder form that could easily disperse and flow into aquatic environments, leading to possible adverse effects on aquatic organisms. Therefore, the control and release of untreated henna waste into the environment should be given special attention for clean environment and good quality of life. Henna might play an important role in these serious damages. The pathogenesis after exposure to henna in aquatic organisms remains unclear, suggesting further studies, including the pathological evaluation of affected embryos and other molecular techniques to elucidate the toxic mechanisms.

Conflict of interest The authors are grateful to other members of the Research and Development Platform of Drug Screening of Shandong Academy of Sciences for raising and breeding zebrafish and have no potential conflicts of interest to report.

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