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Toxicity of synthetic anti-oxidant butylated hydroxyanisole on growth and development of zebrafish Danio rerio

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***Abstract*---**Butylated hydroxyanisole (BHA), a synthetic antioxidant has been extensively used as a preservative in assorted products including food, cosmetics, pharmaceuticals, animal feeds etc. Despite being an exemplary preservative BHA has its own costs and benefits. High levels of BHA have been reported to cause severe damages at the cellular level in most fishes. On this certitude, the present study was designed to scrutinize the sublethal toxicity impacts of BHA at the molecular level in both the embryos as well as juvenile species of Zebrafish. The acute exposure studies were carried out by exposing the embryos and juveniles to a series of BHA concentration (1, 5, 10, 25, 50 and 100 µM) along with a control. After the acute exposure hatching rate was analyzed along with survival and deformities. The survived individuals were subjected to length analysis to understand the impacts on morphometric characters. The results revealed severe eye, spinal cord and yolk sac deformities in the embryos. 50 and 100 µM BHA revealed very low viability and increased heart rate (≈168bpm at 25µM). The results exhorted severe sublethal anomalies in zebrafish exposed to higher concentration of BHA which may further decrease

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the viability of these fishes at the early stages of life cycle if left unnoticed.

***Keywords*---**toxicity, growth development, zebrafish, deformities, synthetic anti-oxidant.

Introduction

Increasing the shelf life and preventing oxidation in packed products especially food products has been the major concern worldwide. Latterly antioxidants are used comprehensively as preservatives in varied products ranging from packed foods to pharmaceuticals to protect the texture, color, odor and nutritional value of the product (Shahidi & Zhong, 2010; Shahidi & Ambigaipalan, 2015). Apart from the natural plant-based antioxidants such as phenolic compounds, ascorbic acid, flavonoids etc. synthetic antioxidants are also widely used as preservatives owing to their easy production, use and better results. The major synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) and the gallates (Xiu-Qin *et al.,* 2009). Of these 4 major, synthetic antioxidants BHA (E320) which is Generally Recognized as Safe (GRAS) by US Food and Drug Administration (FDA) is widely used in cosmetics, food and pharmaceutical industries. However multifarious researches have revealed that high level of BHA can cause adverse impacts such as delayed sexual maturation, shortened estrous cycle, lower mating rate, slower sperm motility, smaller sperm size etc. (Pop *et al.,* 2013).

BHA which is an isomeric mixture of 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole is an effective antioxidant, nevertheless excess accumulation of BHA is known to cause severe sexual maturation problems in cats, dogs, pigs etc. International Agency for Research on Cancer (IARC) has deemed BHA to be a possible carcinogen to humans (IARC, 1986), however the statement remains controversial as United States Environmental Protection Agency (US EPA) has not included BHA as a carcinogenic agent. Data on the impacts of BHA in fishes is sparse. Various toxicology reports are available on Ethoxyquin and Butylated Hydroxytoluene (Holaas et al., 2008; Victoria et al., 2008; Williams et al., 1990) but not on BHA which makes it highly difficult to understand the fate of BHA in fishes. The scarce reports available have reported severe deformities and delayed sexual maturation in fishes. In order to better understand the impacts of BHA in fishes and their consumers detailed toxicological studies needs to be carried out.

With this conviction, the present study was designed to construct baseline toxicology data of BHA in fishes. Zebra fishes (*Danio rerio*) which share around 70% genetic homology with humans were chosen as animal model for the present study. Zebra fishes are excellent animal models to study the early developmental stages of fishes as they have transparent developmental stages and short developmental period (Harper and Lawrence, 2016). Hence zebra fishes are widely used for developmental toxicology studies besides the 70% genetic similarity helps researchers to prognosticate the impacts of toxicants in humans. Dose dependent toxicology impacts on early developmental stages can be effectively monitored and statistically analyzed in zebrafish embryos (Ingebretson and Masino, 2013; Wullimann, 2009; Tierney, 2011). Hence in the present study zebrafishes were subjected to chronic BHA toxicity analysis on breeding and early developmental stages to understand its impacts on both fishes and humans.

Materials and Methods

Acclimatization and Breeding

6-8 months old adult male and female fishes were commercially procured and acclimatized to the laboratory conditions. The temperature was maintained at 28^0C and the fishes were maintained in a 14:10 hours light: dark photoperiod (Sulukan et al., 2017 and Westerfield, 2007). Fishes were fed twice a day with *Artemia salina*. For successful breeding 20 males and 10 females were maintained in a tank and allowed to spawn overnight. The pairwise breeding technique of Walker et al., 1995 was employed for successful laboratory breeding.

Evaluation of the Rate of Reproductive Success

Spawning capacity of the selected animals were evaluated using tap water as control. The rate of reproductive success in the selected pairs were examined for 5 days consecutively prior to exposure. During the evaluation of the breeding capacity the females exhibited strong variation in egg production varying between 20-285 eggs. However, the spawning capacity rather than the number of eggs were used as selection criteria. Females that spawned at least once in the 5 days evaluation period and had over 70% fertilization success were selected for the study.

Selection and Treatment of Embryos

The eggs (n=40) were treated using a series of BHA concentrations (control, 1, 5, 10, 25, 50 and 100 μM) prepared in E3 embryonic medium (Westerfield, 2007). Plain E3 medium was used for control embryos. The medium was renewed once in every 24 hours throughout the experimental period. 4 hours post fertilization the resulting embryos were analyzed under dissecting microscope and any unfertilized or dead embryos were immediately removed. The early stages of the embryos were subjected to both acute and chronic toxicity studies in triplicates.

Acute Toxicity Studies

For acute toxicity studies the embryos were exposed to the selected concentration of BHA in 20mL covered Petri dishes with occasional stirring for 4 days. After 4 days of treatment value close to the LC50 value of each concentration will be determined later the treatment will further be narrowed down by choosing 5 different concentration above and below the determined LC50 value with a spacing factor of 2.0 in order to determine the actual LC50 value. The embryos (n=10) in the petri dishes were observed under dissecting microscope to identify and remove unfertilized and dead embryos.

Teratological Analysis

Evaluation of Hatching Rate and Viability

Hatching rate was monitored in different concentrations (0 to 100mM) of BHA successively for 5 days. The rupture of chorion and release of the larvae were monitored using inverted microscope. The rate of mortality was calculated using end points such as absence of heart beat and coagulation after 24, 48 and 72 hpf. The percentage of viable larvae with respect to the total larvae was calculated.

Evaluation of Heart Beat

The heartbeat of the larvae was monitored after 72 hpf visually using inverted microscope. The heart beat was counted using a stop watch by directly viewing the larval cardiac ventricle. The rate of heart beat was measured as number of beats per minute in different concentrations (0 to 25µM) of BHA.

Evaluation of Body Length

72 hpf the body length of the larvae in different concentrations (0 to 100µM) of BHA was measured visually using an inverted microscope connected to a camera system.

Evaluation of Deformities:

Photomicrographs of the larvae were used to analyze the deformities in larvae exposed to BHA concentrations (0 t0 50µM). any deformities in jaw, spinal cord, yolk sac etc were analyzed. The animals were scored based on their morphological deformities (0 = no visible toxic effects; 1 = minor, one to two morphological anomalies; 2 = moderate, three to four effects; 3 = severe, more than four minor toxic effects; and 4 = dead). Mean score for each BHA concentration (0, 10, 25 and 50 µM) was then determined from the individual scores (Fako and Furgeson, 2009).

Results and Discussion

Despite being considered as GRAS BHA is still known to cause severe endocrine disruption hence the advisable daily dosage of BHA in humans is set as 1mg/kg body weight in human adults and children (EFSA, 2011). However, impact of BHA when it reaches above the Advisable Daily Intake (ADI) is not completely deciphered owing to the dissension between scientist regarding the use of BHA and its ADI. In order to obtain comprehensive data regarding BHA *in vivo* studies needs to be carried out on several aspects. With these certitudes, the present study carried out teratological in vivo studies with respect to BHA on Zebra Fish (*Danio rerio*) which are considered as suitable models for developmental toxicity analysis (Simmons et al., 2014 & Antkiewicz et al., 2005)

Viabillity assay

In order to understand the developmental toxicity impacts of BHA on Zebra Fish survival rate of the fishes in different concentrations of BHA was monitored in the present study. The survival rate decreased with increased concentration of BHA in a dose dependent manner after 24, 48 and 72 hours of exposure as depicted in Fig. 1, 2 and 3 respectively. The viability was extremely low in 100µM and 50µM whereas the remaining concentrations the viability percentage varied insignificantly 72 hrs post exposure the viability percentage in control was 88% whereas in 100µM BHA concentration it was merely 23% followed by 62% in 50µM. A delay in hatching was observed after 72 hours of exposure to higher concentrations of BHA (50 and 100µM)

Owing to the sparse literatures on in vivo toxicity studies of BHA on zebra fishes it quite difficult to understand the impacts of BHA on survival rates of zebra fishes. However, the results of the present study are in accordance with Baran et al., 2020 who has reported similar dose dependent BHA impacts on survival rate of Zebra Fishes. Baran et al., confirmed the adverse impacts of BHA on the survival rates of Zebra Fishes at higher concentrations. Baran et al., also reported delayed hatching rate at higher doses after 96 hrs of exposure and stated that no significant changes were observed in hatching rate after 48 and 72 hours of exposures.

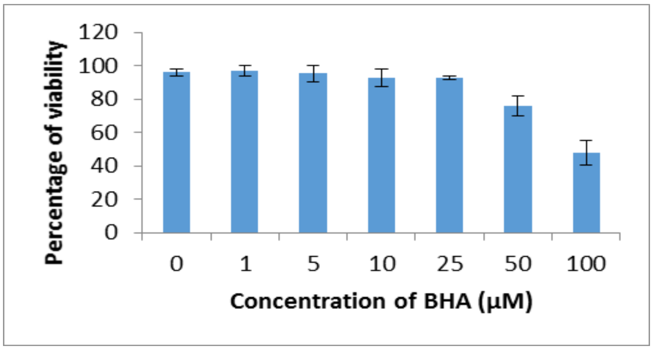


Fig. 1: Percentage of viability of Zebra Fish larvae 24 hours post exposure

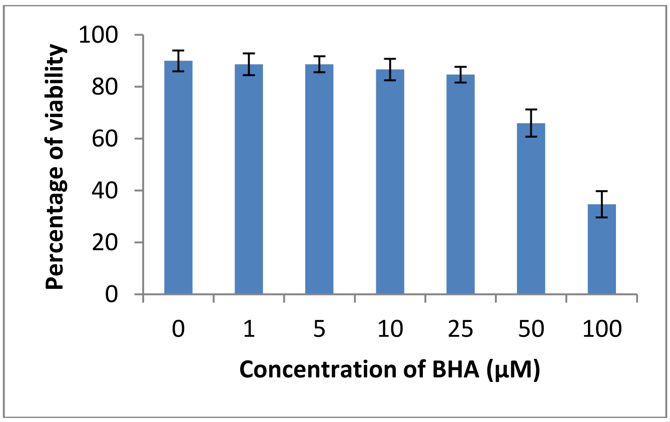


Fig.2: Percentage of viability of Zebra Fish larvae 48 hours post exposure

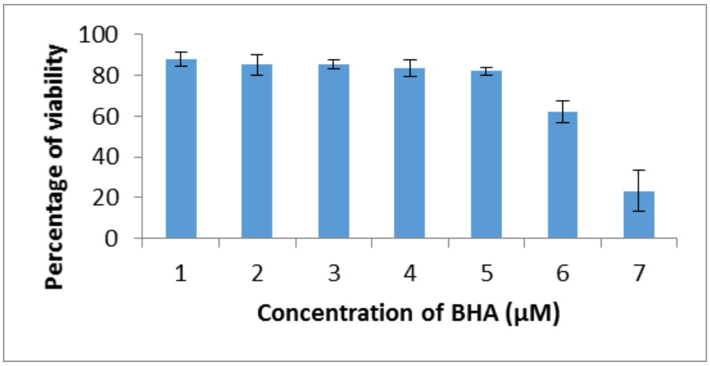


Fig.3: Percentage of viability of Zebra Fish larvae 48 hours post exposure

Evaluation of Heart Beat

Increase in heart beat was observed in zebra fishes exposed to various concentrations of BHA (0, 1, 5, 10, 25µM) in a dose dependent manner as depicted in Fig.4. At the lower concentration of BHA no significant change in heart beat was observed compared to the control. On the other hand, animals exposed to 10 and 25µM showed significant changes. The heart beat increased from 154 bpm in control to 168 bpm in 25µM followed by 167 bpm in 10 µM BHA treated fishes. So far, no data available on impacts of BHA on the cardiac toxicity of Zebra fishes. However, the cardiac development and functionality of Zebra Fishes with respect to various other stressors have already been reported (Chen, 2013; Miura & Yelon, 2011).

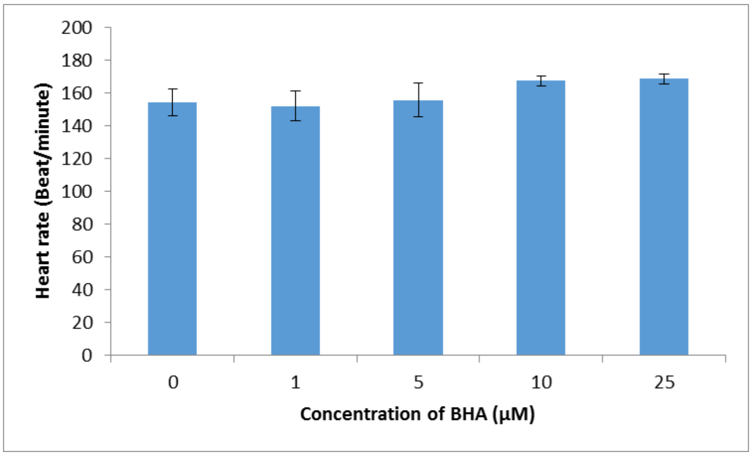


Fig. 4: Heart rate response of Zebra Fishes exposed to several concentrations of BHA

Alafiatayo and his coworkers (2019) reported that they noticed no significant changes in the heart rate of Zebra Fishes exposed to various concentrations (7.80, 15.63, 31.25, and 62.50 µg/mL) of *Curcuma longa* extract but the heart rate was completely absent in fishes exposed to the highest concentration of 125 µg/mL *Curcuma longa* Extract. Martinez et al., 2019 reported insignificant heart rate changes in Zebra Fishes exposed to higher concentrations of multi walled carbon nano tubes but he observed increased heart rate at lower concentration. The results of the present study were discordant with the above-mentioned toxicity studies. Despite being anatomically different from human heart Zebra fishes are still considered as exemplary animal model for studying human heart diseases since all vertebrates show conserved cardiac development (Bakkers, 2011). Hence more in vivo toxicity studies must be carried out to scrutinize the impacts of BHA on cardio toxicity of Zebra fishes which will provide us an insight on the impacts of BHA on cardiovascular development and functionality of not only fishes but humans as well.

Evaluation of Body Length

Body length is considered as an important criterion to understand the impacts of stressors on developmental toxicity. In the present study, the length of the zebra fishes showed varying response to different concentrations of BHA. The length of the fishes exposed to lower concentrations of BHA (0 to 25μM) fluctuated insignificantly but the fishes exposed to higher concentration of BHA (50 and 100μM) showed significant decrease in body length compared to control (Fig. 5). The length decreased from 3.6mm in control to 2.06mm in 100μM followed by 2.53mm in 50μM. the results were similar to that of the developmental toxicity studies carried out by Huang et al., 2018. Huang and his coworkers reported a negative correlation between the body length of Zebra fishes and diethyl nitrosamine concentration to which they were exposed. From the results, they concluded that higher concentration of diethyl nitrosamine stressor inhibited body length of Zebrafishes. Various records on developmental toxicity of different stressors are available as published literatures but lack of detailed impacts of BHA on developmental toxicity in zebrafishes calls for more contemporary researches to be carried out in this field to understand the level of growth inhibition imparted by BHA using zebra fish as model so as to predict its fate in humans.

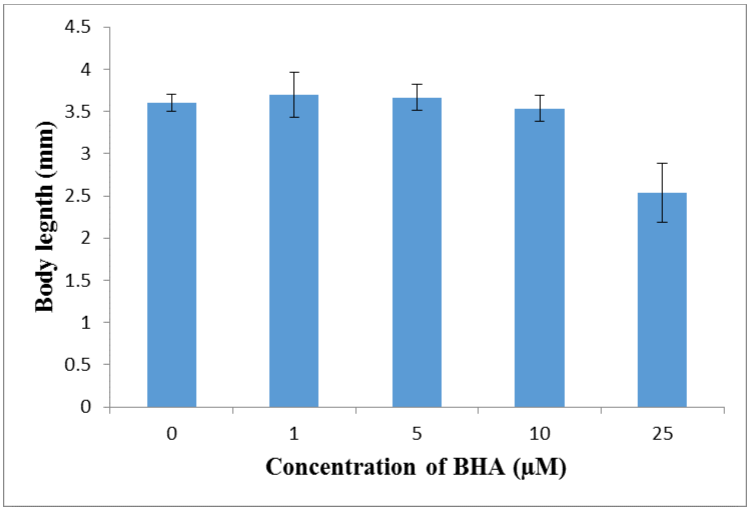


Fig.5: Evaluation of body length after BHA exposure on zebra fish after 72 hrs

Evaluation of Deformities

Baran and his coworkers (2020) reported BHA induced deformities in the early life stages of Zebra Fishes. They reported pericardial edema (PE) which is the most common abnormality, yolk sac edema (YSE), body malformation (BM) and curved body axis (CBA) in a dose dependent manner. Such kind of malformations are attributed to the toxic environments (Antkiewicz et al., 2005; Zhou et al., 2009 & Comakli et al., 2018). Many such deformities were also observed in the present study. Compared to control animals exposed to 10, 25 and 50μM of BHA displayed moderate to severe spinal cord fold deformities (Fig 6a - d). Similarly, moderate to severe deformities were also observed in the yolk sac of embryos exposed to 10, 25 and 50 μM BHA (Fig. 7a - d). Apart from spinal cord and yolk sac malformations mild, moderate and severe deformities were also observed in the eyes of embryos exposed to 10, 25 and 50 μM BHA (Fig. 8a - d).

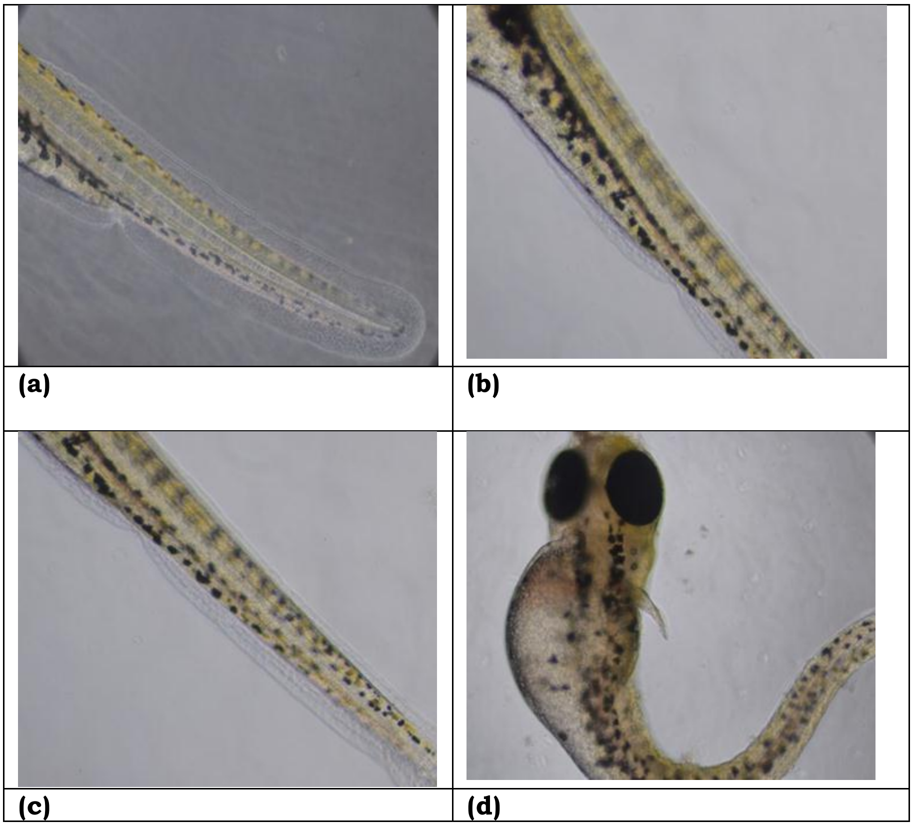
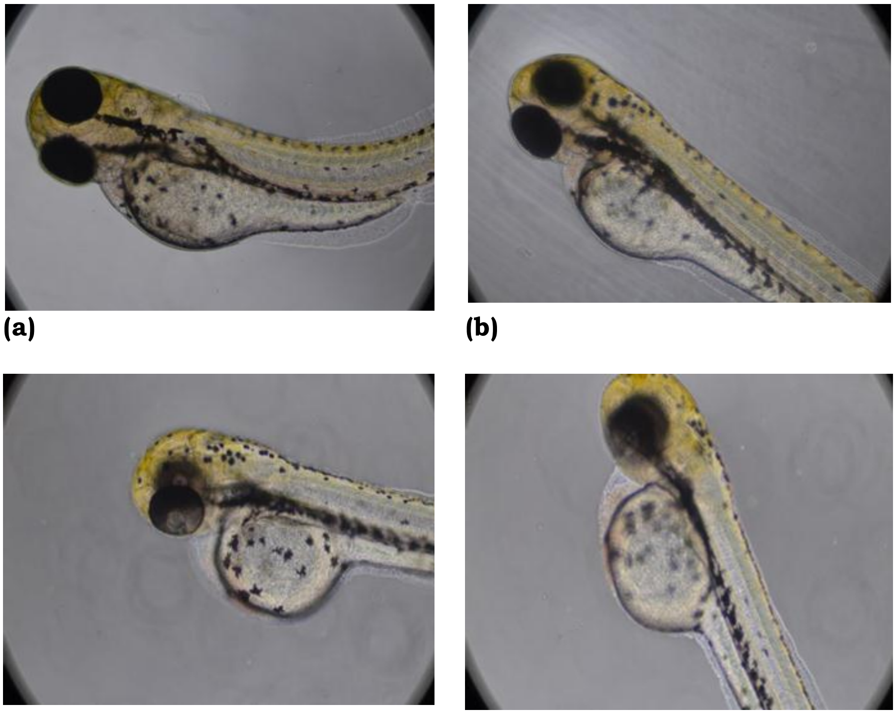


Fig. 6: Spinal cord fold abnormality of Zebrafish after treatment of BHA at differentconcentration a: Control (0 μM); b: 10 μM; c: 25 μM; d: 50 μM



(a) (b) (c) (d)

Fig.7: Yolk sac deformities of Zebrafish after treatment of BHA at different concentration a: Control (0 μM); b: 10 μM; c: 25 μM; d: 50 μM

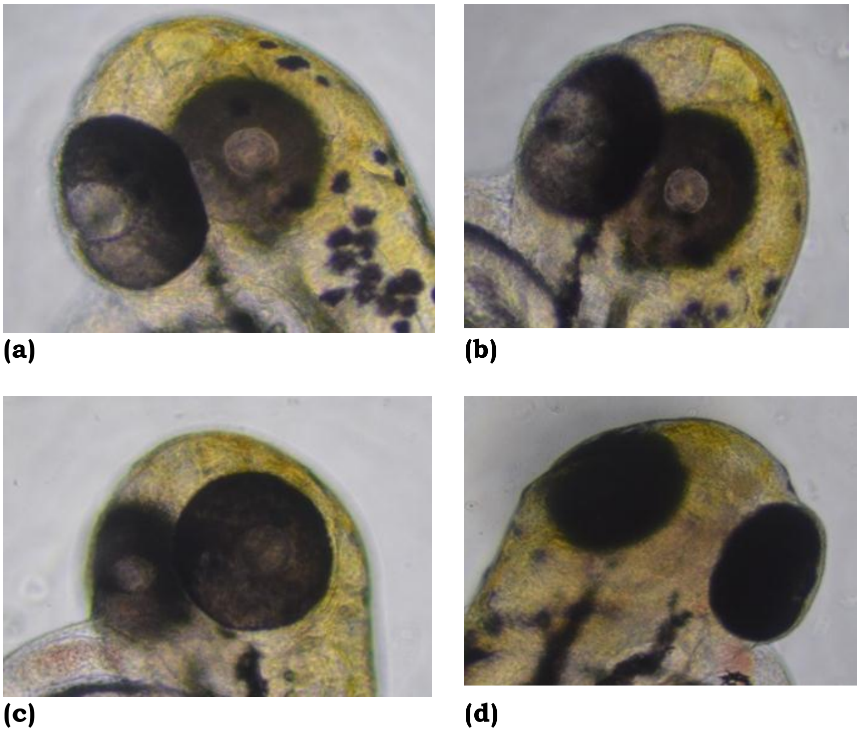


Fig. 7: Eye deformities of Zebrafish after treatment of BHA at different concentration

a: Control (0 µM); b: 10 µM; c: 25 µM; d: 50 µM

Conclusion

BHA being a major antioxidant has been widely used as preservative (E320) in various products including food, cosmetics, medicines etc. BHA is generally considered as safe but on the other hand BHA is capable of transforming itself into various metabolites such as tertiary butyl hydroquinone (TBHQ) whose impacts may be far more precarious than BHA itself. There are very few published literatures on BHA toxicity studies which makes it difficult to understand the fate of BHA and estimate its ADI. In the present study, toxicological impacts of BHA has been examined and clarified at the molecular level using various teratological assays. From the results of the assays, it is concluded that BHA despite being GRAS is capable of inducing deformities in embryos of Zebra Fishes at the early stages of life cycle and is likely to affect the fertility of the fishes at higher concentration. It is also known to disrupt growth and cardiovascular function beyond certain level. Hence the authors suggest that more comprehensive researches need to be carried out to understand the in vivo toxicity of BHA and there by determine a uniform ADI for BHA for more prudent industrial applications.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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