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Research Article

Investigation of Physiological, Developmental and Behavioral Effects of Brilliant Black BN Dye on Zebrafish Embryos and Larvae

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ABSTRACT

This study investigates the developmental, physiological, and behavioral effects of the synthetic azo dye Brilliant Black BN (E151) on zebrafish (*Danio rerio*) embryos. Embryos were exposed to different concentrations (1, 5, 10, and 100 ppm) of Brilliant Black BN until 96 hours post-fertilization (hpf), and their oxidative stress levels, apoptosis, morphological changes, and swimming behaviors were evaluated. The results showed that high-dose exposure to Brilliant Black BN increased oxidative stress, elevated apoptosis levels, reduced body length and eye diameter, and led to an increase in pericardial edema. Additionally, high-dose exposure altered larval swimming activity, inducing anxiety-like behaviors. These findings suggest that Brilliant Black BN may have toxicological effects on zebrafish and highlight the need for further research on the safe use as food additives.

Keywords: Zebrafish, brilliant black BN, azo dye.

1. INTRODUCTION

Food dyes are among the indispensable additives of the modern food industry and are widely used to make foods more attractive and increase consumer interest. Synthetic food dyes are common additives used to improve the colors of foods, preserve the quality of food, and increase its visual appeal[1]. Azo dyes are synthetic dyes that have at least one –N=N– group in their chemical structures and have a wide range of colors. Widely used in the food, textile, pharmaceutical and cosmetic industries, these dyes are preferred for their low cost and stable structure to enhance the colour of food and give it a more attractive appearance[2].

Azo dyes such as brilliant black BN (E151) are commonly found in food products such as ice cream, candy, soft drinks, jelly, desserts, and even some sauces. Interestingly, despite the term "black" in its name, brilliant black BN dye actually appears as a violet purple in aqueous solution and food options. When metabolized in the body, they are converted to aromatic amines, creating toxicity and genotoxic risks. Nitrite compounds can combine with some amino acids to form carcinogenic N-nitroso[3]. The deterioration of foodstuffs, or the excessive breakdown of E151, in food products containing azo dyes, has been observed to be a contributing factor to certain health conditions. These include, but are not limited to, skin irritation, leakage, hyperactive behavioural symptoms (especially in children), asthma-like symptoms and skin rash [4]. There are some studies that can be expanded with attention deficit and hyperactivity disorder (ADHD) in children. For this reason, in EU countries, labels containing undesirable products containing Brilliant Black BN contain warning statements. Studies conducted so far have reported that Brilliant Black BN has no direct carcinogenic effects. However, there are theories that

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some types of cancer may be produced if azo dyes are consumed at high levels [5].

The use of Brilliant Black BN is subject to different regulations in different ways. It is allowed to be used in limited amounts in foods in EU countries. However, special warnings are given for products intended for consumption by children. FDA (American Food and Drug Administration) has banned Brilliant Black BN as a food additive and its use is not approved in the USA [6]. The Turkish Food Codex allows the use of Brilliant Black BN in certain food categories, but specifies certain restrictions. While the recordable acceptable daily intake (ADI) values of food azo dyes such as Brilliant Black BN have been determined by JECFA (Joint Expert Committee on Food Additives) as 1 mg/kg body weight/day, SCF (Scientific Committee on Food) has stated this value as 5 mg/kg body weight/day [7]. However, the data indicate that the daily Brilliant Black BN, particularly in children under 10 years of age, can reach levels of 6.9 mg/kg and thus potentially exceed the established upper limit. Therefore, great importance should be given to dose-dependent toxicological studies in order to better understand the possible harms of Brilliant Black BN [8]. Toxicological studies using model organisms such as zebrafish provide benefits in understanding what these products are. Zebrafish is widely used in the field of toxicology due to its 70% similar genomic structure to humans, embryo transparency and rapid development process [9,10].

This study provides a detailed examination of the toxicity of Brilliant Black BN on zebrafish embryos and larvae. The effects of Brilliant Black BN azo dye on zebrafish embryo development, oxidative stress, apoptosis and behavioral changes were evaluated. Furthermore, the present study aims to furnish significant information on the safe utilisation of food additives by unveiling the dose-dependent effects of Brilliant Black BN.

2. EXPERIMENTAL

2.1. Materials

2.1.1. Chemical

Brilliant Black BN (E151), an azo compound with a sulfonated structure, is widely used as a food additive (OH, 2021). It has the chemical formula $C_{28}H_{17}N_5O_{14}S_4 \cdot 4Na$ and a molecular weight of 86.7 g/mol. The compound was commercially purchased in solid form and used as a powder.

2.1.2. Zebrafish Feeding and Care

The embryos utilised in the present experiment were obtained from wild-type AB genotype zebrafish (*Danio rerio*), which were obtained from Oregon State University. These fish are kept under constant conditions and growth rates in the Ataturk University Fisheries Faculty Laboratory. Standard zebrafish in the laboratory are kept under a photoperiod that will be recorded as 10 hours of darkness and 14 hours of light care. Fish fed twice daily with Artemia and once with flake food (It is a food containing plenty of natural vitamins, amino acids and mineral salts) are kept at 28±1 ˚C [11]. In order to obtain embryos within the scope of our study, adult female and male fish will be recorded as a barrier according to the spawning containers one day before the experiment, approximately one hour after the last feeding amount. The following morning, upon activation of the zebrafish systems, the barrier within the spawning containers was removed, thereby initiating the spawning process.

2.2. Methods

2.2.1. Exposure to Brilliant Black BN

Eggs were exposed to Brilliant Black BN dye at concentrations of 1, 5, 10, and 100 ppm, while the control group was maintained in E3 medium alone. All groups had their Petri dishes refreshed every 24 hours, and embryos remained exposed to the dye for a total of 96 hours. The experiment was conducted in three independent trials (n = 3) for analysis, with a minimum of 50 embryos per group. The lethal concentration (LC50) and no observed effect concentration (NOEC) were determined based on the chemical toxicity test guidelines of the Organization for Economic Co-operation and Development (OECD). The lower tested concentrations, 1, 5, and 10 ppm, remained unaltered experimentally. Given that the 100 ppm dose is 600–800 times higher than the maximum allowable level for human consumption, excessively high doses beyond this threshold were intentionally avoided. [12]

2.2.2. Morphological and Physiological Measurements

For the measurement of eye size, body length, and pericardial area, 10 larvae were randomly selected from each group, with a total of 30 larvae across three biological replicates. Images of the selected larvae were captured and analyzed using Danio Scope (Noldus) software. To ensure consistency in measurements, specific reference points were established, aligned, and adjusted within a standardized frame while capturing the images. During analysis, eye size was measured based on its maximum diameter, body length was determined along the axis extending from the head to the tail, and the pericardial area was outlined to assess the heart region. Statistical analyses were then performed on the collected data.[13]

2.2.3. Detection of Apoptotic Cells

In each group, 5 randomly selected larvae at 96 hours post-fertilization (hpf) were analyzed, with a total of 15 larvae across three biological replicates. Following a standard protocol, acridine orange, a nucleic acid-selective metachromatic dye that binds to DNA and RNA, was used for analysis. Briefly, the 96 hpf larvae were washed three times with PBS before being incubated with acridine orange at a concentration of 5 mg/mL for 30 minutes at 28.5 °C in darkness. After incubation, the larvae were rinsed three times with PBS and examined under a fluorescence microscope (Zeiss, Discovery V12, Germany).[14]

2.2.4. Determination of Reactive Oxygen Species (ROS)

Five randomly selected 96 hpf (hours post-fertilization) larvae from each group, including the control and those exposed to Brilliant Black BN, were analyzed for oxidative stress using CM-H_2DCFDA. This molecule reacts with various reactive oxygen species (ROS) and serves as a general oxidative stress indicator. CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, which is widely used to detect ROS during development, with fluorescence observed upon oxidation to DCF. The analysis followed a standard protocol. Briefly, larvae were washed three times with ultrapure water before being incubated with CM-H_2DCFDA at a concentration of 1 µg/mL for 2 hours at 28.5 °C in darkness. After incubation, they were rinsed three times with ultrapure water and examined under a fluorescence microscope (Zeiss, Discovery V12, Germany). [15]

2.2.5. Detection of lipid droplets

From each group, 5 larvae at 96 hours post-fertilization (hpf) were randomly selected for analysis, with three replicates totaling 15 larvae. The selected larvae, previously exposed, were examined using Nile Red staining, which interacts with intracellular lipid droplets. Following the standard protocol, the larvae were rinsed three times with ultrapure H_2O and then incubated overnight at 28.5 °C in the dark with 10 ng/mL Nile Red. After incubation, they were washed three more times with ultrapure H_2O and imaged using a fluorescence microscope (Zeiss, Discovery V12, Germany). Fluorescence analysis was conducted with ImageJ software. [16]

2.2.6. Larval movement activity analysis

Zebrafish larvae were exposed to black paint. The locomotor activity of the larvae was assessed using a standard protocol. Twenty-four larvae were randomly selected from each group, with three biological replicates, for a total of 72 larvae, for this analysis. Their movement was tracked using an analog camera capable of recording in 25 configurations. To monitor individual larvae, 24-well plates were placed in the DanioVision Observation Room, with one 6 dpf larva per well. A heating/cooling system maintained a constant temperature of 28.5 °C within the observation room.

The lifespan of zebrafish larvae was examined in relation to light conditions, locomotor activity, and dark/light transitions. Initially, 30 individuals underwent a medium adaptation period, followed by an 80-minute video recording session, which included alternating 10-minute dark phases and 10-minute breaks. The adaptation process was analyzed based on the recorded footage. Behavioral assessments of the larvae were carried out using EthoVision software (Noldus Co.), with all analyses conducted in three independent replicates.[17]

2.2.7. Statistical Analysis

Data from all experiments were statistically compared using GraphPad Prism Software version 8.0 for Windows (GraphPad Software, San Diego, CA). Analysis of the results of the control and experimental groups was made with one-way ANOVA. All data are presented as mean ± standard mean error (SEM). P values <0.05 were considered significant. Statistically significant changes are indicated with a symbol (*). Symbol expressions are as follows: *p < 0.05 (significant); **p < 0.01 (very significant); ***p < 0.001 and ****p < 0.0001 (highly significant).

3. RESULTS and DISCUSSION

The biological effects of azo dyes on organisms are an important topic in toxicological studies. This study focuses on the effects of the azo dye Brilliant Black BN (E151) on zebrafish (*Danio rerio*) embryos and larvae. In particular, the effects of the dye on the production of reactive oxygen species (ROS), apoptosis and the amount of lipophilic organic (nile-red) were evaluated. Our results show that Brilliant Black BN azo dye can cause oxidative stress, damage and behavioral changes in zebrafish larvae.

In circumstances where food dyes are present, there is the potential for these dyes to induce oxidative stress in animals, thereby resulting in the generation of reactive oxygen species. Reactive oxygen species (ROS) are oxygen-depleting substances produced as a natural result of metabolism in aerobic organisms. While the antioxidant defense structure is balanced under normal conditions, it can occur by oxidative stress in case of excessive exposure to characteristic factors or chemical agents. Oxidative stress is a negative effect that occurs in structural changes, leading to durability and functional impairments[18]. This spread was investigated by the effect of exposure to the azo dye Brilliant black

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BN (E 151) on ROS levels in larvae. ROS production was evaluated by the average flora density measured in larvae. The data obtained showed that ROS increased in the groups exposed to the dye and this increase was dose-dependent ($p<0.05$) (Figure 1). The results suggest that the azo dye Brilliant black BN may contribute to the formation of oxidative stress and cause potential deterioration. The results of the examination with azo dyes After exposure to the fruit fly of the Brilliant black BN azo dye, the enzymatic antioxidant defense caused deterioration[5]. Allura red dye caused dose-dependent deterioration in antioxidant quality, while carmoisine azo dye increased fluorescence intensity in zebrafish dose-dependently[9,19]. All these results show that azo dyes can be modified and cause varying oxidative stress, are inline with our results.

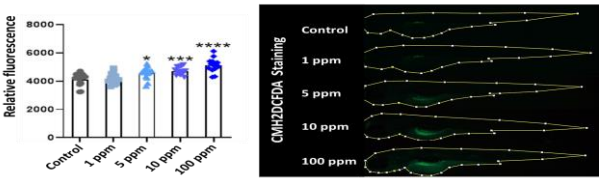

Figure 1. CM-H2DCFDA stain for ROS produced by 96 hpf larvae.

Oxidative stress can basically lead to apoptosis. Apoptosis stimuli released by damaged mitochondria can trigger cell death[20]. In our study, it was observed that apoptosis became more pronounced, especially at 10 and 100 ppm doses (Figure 1). These findings indicate that exposure to Brilliant Black BN can increase cell proliferation and this can lead to developmental defects. Similarly, since azo dyes such as carmoisine and reactive black 5 cause developmental defects that increase apoptosis in zebrafish embryos [9,21]. It is reported that ROS, especially mitochondrial dysfunction, cause apoptosis activation, and tartrazine azo dye is intensely given to apoptosis in the nerve region and head region of zebrafish[22]. These findings support the idea that Brilliant Black BN, which exhibits the toxic effects of azo dyes, may also lead to similar developmental disorders.

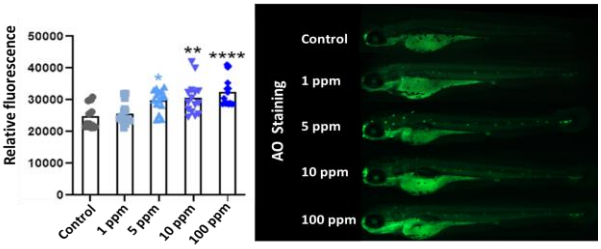

Figure 2. Acridine orange (AO) staining in the apoptotic larvae at 96 hpf.

Nile Red, which is widely used in the detection of lipid droplets in oil, is an important fluorescent marker in pharmaceutical and toxicological studies[23]. It has been shown that organisms exposed to environmental stress can distribute its function through energy metabolism to maintain favorable homeostasis[24]. In the experiment, zebra larvae were exposed to carmoisine, which resulted in a dose-dependent increase in the bloom intensity of Nile Red [9]. In a successful study, it was shown that carmoisine (E122) azo dye together with Bisphenol A (BPA) increases oxidative stress and this effect is not related to increased MDA levels. In the study, it was stated that reactive oxygen species (ROS) oxidize lipids and proteins in the cell at high temperatures; The commercial sector has been highlighted as a biomarker for the release of MDA by the cell as a result of lipid peroxidation and the resulting damage[25]. In our study, it was observed that different areas measured with Nile Red increased in zebrafish larvae exposed to Bright Black BN in a dose-dependent manner (Figure 3). This is an important finding that shows parts of the lipophilic images and possible changes in lipid formation.

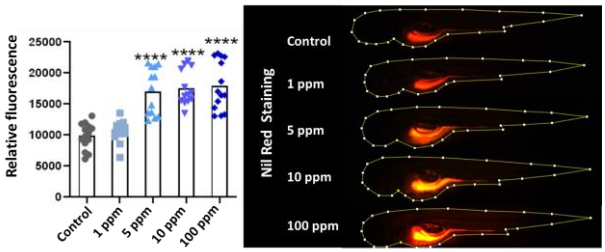

Figure 3. Mean flora density of adipocyte lipid droplets after Nile Red staining and opening.

After exposure of Brilliant Black BN azo dye to zebrafish embryos, a dose-dependent violet-purple dye color is more clearly seen in the chorion at the 48th time (Figure 4). Considering the previous products, the dye used accumulates in the chorion depending on the dose and its feature can pass through the chorion and pass to the embryo. It is predicted that especially at high doses, the dye can accumulate in the embryo and cause color change and this can have possible effects on developmental rates[9]. After 72 and 96 hours, especially at the 100 ppm dose, the decrease in body length and eye gaps and the increase in pericardial edema were determined. These findings show that the dye can cause developmental disorders in zebrafish. Azo dyes, especially with increased apoptosis, are based on the decrease in retinal structure and the resulting small eye phenotype[21]. In our study, microphthalmia (shrinkage of the eyes) was observed in zebrafish larvae exposed to Sunset Yellow[26]. These results suggest that Brilliant Black BN may also have similar negative effects on eye development.

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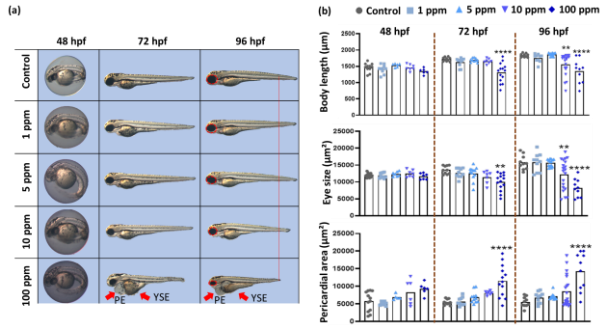

Figure 4. (a) Microscopic ratios of embryos exposed to Brilliant Black BN azo dye at 48, 72 and 96 hours (b) eye size, body length, pericardial area ratio of zebrafish embryo. PE; pericardial area, YSE; yolk sac edema.

The data obtained from these data show that the defects of oxidative stress and modified apoptosis are increased. The behavioral responses of zebrafish larvae were evaluated by observations, especially on thigmotaxis (walking close to the edge) and total distance traveled at night. The findings obtained show that exposure to a range of doses of Brilliant Black BN (E151) led to a significant increase in both behavioral parameters (Figure 5). Thigmotaxis is the behavior of organisms to seek refuge in safe areas by moving to the edge against threats of aggression and is generally associated with anxiety and stress. In this way, exposure to high doses of dye affected the natural course of zebrafish larvae, encouraging them to follow more closely to the edge[27]. This suggests that dye exposure causes anxiety-like behavioral changes in fish and shows an increased defense against the threat of aggression. High doses may cause changes or significant stress on organisms, even those normally considered safe, and may be expressed by causing anxiety. Normally, fish behave more calmly and sheltered during the night, but exposure to the dye caused the fish to cover a wider area. This situation can be evaluated as a result of increased adaptability and activity level, expanding with oxidative stress and flexibility changes in the bodies of zebras [28].

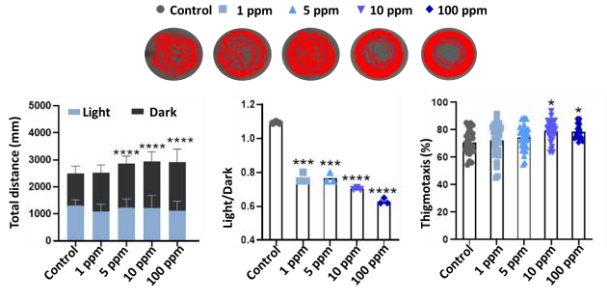

Figure 5. Comparisons of total distance, thigmotaxis and pursuit/darkness ratio data from locomotor activity analyses of zebrafish larvae between groups (n = 16, trial repeated 3 times).

As a result, high doses of Brilliant Black BN exposure, increasing both thigmotaxis behavior and the distance traveled at night in zebrafish larvae, reveal that these dyes can cause behavioral changes such as stress, anxiety and deterioration in organisms. These findings show that high doses can cause behavioral disorders by affecting the natural changes and severity of zebrafish larvae adaptations.

4. CONCLUSIONS and RECOMMENDATIONS

The effects of food dye Brilliant Black BN on zebrafish were examined, and it was observed that toxic effects occurred, particularly at high doses (100 ppm). This exposure caused decreased oxidative stress, cell death and changes in behavior with various appearances. Especially, important tissue parts such as impairments in locomotor activity, increased night movement, increased thigmotaxis level, decreased height and eye distances, and increased pericardial edema were detected. These findings show that Brilliant Black BN carries a risk in terms of developmental and economic durability.

The obtained results show that Brilliant Black BN may have negative effects on the nervous system, growth and general negativities in high dose exposure. However, more detailed and comprehensive studies are required at the molecular and systemic levels to fully elucidate the biological impact and intensity of this dye. Long-term results, particularly at varying doses, reveal disruption in gene expression and provide a basis for further studies to determine the possible healing method.

Ethical approval

For this type of study, formal consent is not required. Zebrafish larvae younger than 5 days old were used in the study. Therefore, the work does not require any license (Directive 86/609/EEC and EU Directive 2010/63/EU). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interests

The authors declare no conflict of interest.

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