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Developmental effects of three textile chemicals on locomotor activity, antioxidant markers and acetylcholine esterase activity in zebrafish

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Textile chemicals discharged into the water bodies cause huge impact on human health and environment. However, the adverse effects of textile chemicals during critical period of brain development are not explored. This study uses zebrafish to assess the developmental toxicity of three textile chemicals. Zebrafish embryos were exposed to different concentrations (1, 5, 10, 20 and 40 PPM) of Naphthalene sulfonic acid (NSA), Metanilic acid (MA) and Acid blue 113 (AB113) from 18 h post-fertilization (HPF) to 96 HPF, respectively. Several endpoints, such as mortality, morphological abnormalities and locomotor activity of embryos and larvae were studied. Biochemical detection of oxidative stress, glutathione and acetylcholine esterase was subsequently tested. The survival rate was decreased (LC (50): 1PPM) by NSA, MSA or AB113 and at > 5PPM a 90% mortality was observed respectively. Exposure to 1 PPM of NSA, MSA or AB113 significantly reduced the locomotor activity in an age dependent manner. However, no neurodegenerative phenotypes were noted. The glutathione and acetylcholine esterase activity (P <0.05) was decreased while malondialdehyde content was accumulated by NSA, MSA or AB113 treatment. The overall findings suggest that the selected textile dyes exposed during critical window development is able to produce oxidative stress and exert noticeable effects on locomotor activity in zebrafish embryos by altering acetylcholine esterase activity.

Keywords: Acetyl cholinesterase (AChE) activity, Acid blue 113, Azo dyes, DOHaD concept, Embryonic development, Metanilic acid, Naphthalene sulfonic acid, Neurodegenerative phenotypes, Oxidative stress Pollution, Textile dyes

In India, textile manufacturing is the second largest employment generator after agriculture[1]. Owing to its huge economic contribution and occupation industrial sector, these industries need proper attention on its environmental concern. Azo dyes were reported to constitute 60-70% of the most commonly used group of dyes in textile industry[2]. In the synthesis of azo dyes, aromatic amines (AAs) are used as intermediates. Dermal, systemic and bacterial biotransformation of azo dyes can release aromatic amines (AAs)[3].

Exposure to certain aromatic amines in textile azo dyes bear risks for various clinical problems in animals as well as human beings including hepatic and renal system damages, mental retardation and degradation of basal ganglia of brain and liver[4,5]. Brüschweilera & CédricMerlot[6] have reported that azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. Also, azo dyes and aromatic amines were detected in women undergarment, which are a potential health hazard for consumers[7,8].

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It has been hypothesized that human behaviour and susceptibility to diseases may arise from prenatal and early postnatal conditions, including exposure to environmental chemicals[9]. This paradigm is known as the developmental origins of health and disease (DOHaD) concept. Altered programming, due to an insult at a critical, sensitive period of early life, can have significant adverse impacts on the process of central nervous system (CNS) development[10]. In addition, some characteristics of neurodevelopmental disorders [e.g., those associated with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD)] can continue into adulthood, implying the adverse effects of a childhood neurodevelopmental alteration can influence health throughout the lifespan[11,12]. Surprisingly in some individuals, the onset of some neurodevelopmental disorders cannot be explained by family history. What triggers the sudden onset and rapid progression of these diseases remains unexplained.

Zebrafish may be a useful model in addressing these uncertainties. Zebrafish are vertebrates, and therefore more closely related to humans than other genetic model organisms[13]. They are relatively small

fishes (3-4 cm long as an adult) and can be easily managed in large numbers in specialized facilities. Zebrafish have a short generation time (3 months) and breed prodigiously (hundreds of offspring per female per week). Embryos develop externally, can be readily manipulated genetically and are transparent[14]. Many factors suggest that the zebrafish is a powerful tool for the study of human diseases: patterning, pathfinding and connectivity in the CNS have all been deciphered and correlate with the human CNS; transparency of embryonic zebrafish facilitates analysis of single neuron activity during the execution of normal and pathological behaviour[15]; touch and behavioural responses, such as movement patterns can be monitored[16]; in zebrafish embryos to those in mammalian systems. Presently, only few studies are available on the possible toxicities of textile chemicals with the help of zebrafish[17]. However, their neurobehaviour impairments have not been conclusively determined.

To evaluate neurobehaviour toxicities, there is an urgent need for a molecular biomarker that would be used as an endpoint. To this end, this study aimed to investigate the major physiological alterations occurring during early exposures to azodye (Acid blue 113) and dye intermediates (naphthalene sulfonic acid & metanilic acid). To understand the mechanism underlying the abnormal neurobehaviour and the oxidative stress caused by textile chemicals, we investigate the different endpoints of behaviour analysis and assess the oxidative stress markers and examine the effects of neurotransmitter acetyl cholinesterase (AChE) activity at different developmental stages of zebrafish embryos.

Materials and Methods

Chemicals and Zebrafish

Naphthalene sulfonic acid (NSA), metanilic acid (MA), acid blue 113 (AB113) and other reagents were purchased from Subra scientific Chemicals (Chennai, India). Wild-type adult (<8 months old) zebrafish were reared and maintained at the aquatic research laboratory at Sathyabama Institute of Science and Technology, Chennai. The temperature was maintained at 28°C and the lighting was maintained in a cycle of 14 h of light and 10 h of dark for the fish to breed. The next day morning, the embryos were collected and staged according to the method of [18] and used for analysis. The embryos were maintained in embryo medium (EM) at 28°C in an incubator till 96 HPF. The medium was replaced with fresh medium daily.

Toxicity assays and calculation of LC_{50}

The toxicity assays were performed according to Nellore & Nandita[19] on various developmental stages of zebrafish following exposure to different concentrations of AB113, NSA and MA (1, 5, 10, 20 and 40 PPM) at 18 HPF. The embryos exposed to embryo medium alone were considered as controls. Embryos were collected at every stage (24, 48, 72 and 96 HPF) after exposure at 18HPF for analysis. The teratogenic endpoints were evaluated at various developmental stages viz., 24, 48, 72 and 96 HPF. Based on the concentration survival (%) reported at all the end points the LC_{50} value was determined.

Evaluation of Locomotor activity

Spontaneous tail coiling was evaluated in embryos aged 24HPF following the methodology as described by IWT Selderslaghs *et al.*[20] with minor modifications. The EM treated controls and embryos following various exposures were placed in an alternating manner on the plate and tail movement was observed under a stereozoom microscope (Zeiss) with duration of 5 min. At 48-96 HPF, the locomotor activity of the embryos/larvae was studied in a Petriplate marked with 1 cm grid lines, containing embryo medium[20]. The swimming activity across the grid lines was observed for a period of 1 min, from which the distance covered was calculated. The data sets were subjected to statistical analysis with the Student's t-test to assess the significance of the similarities and differences among control, and treated embryos.

Embryo homogenates preparation

Embryos at various developmental stages viz., 24, 48, 72 and 96 HPF, respectively, were collected for study. The whole embryo homogenates were prepared in 0.1 M phosphate buffer and centrifuged at 3000×*g* for 30 min[19]. The supernatants were used for further analysis.

Measurement of Lipid Peroxidation (LPO)

The assay for membrane lipid peroxidation was carried out by the method of Wright *et al.*[21] with some changes. The mixture of reaction in a total volume of 3.0 mL contained 1.0 mL tissue homogenate, 1.0 mL of TCA (10%), and 1.0mL TBA (0.67%). All the test tubes were placed in a boiling water bath for a period of 45 min. The tubes were shifted to ice bath and then centrifuged at 2500×g for 10 min. The amount of

malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm and expressed as the nmol MDA formed/gram tissue.

Glutathione Estimation (GSH)

The Moron *et al.*[22] methodology was used to estimate GSH content. A mixture made of embryo supernatant and 5% TCA were centrifuged at 5000 rpm for 10 min. The reaction mixture was composed of 2.4 mL of 0.2M phosphate buffer, 0.6 mL of 0.6 mM DTNB [5'5-dithiobis (2-nitrobenzoic acid)] and 0.6 mL of the above supernatant while, the blank contained 2.4 mL of 0.2M phosphate buffer, 0.6 mL of 0.6 mM DTNB and 0.6 mL of 5% TCA. After incubation, the yellow colour developed was read immediately at 412nm on a spectrophotometer and the GSH content was calculated as µmol DTNB conjugate formed/gram tissue.

Measurement of Acetylcholine Esterase (AChE) activity

Detection of AChE activity *in situ* was adapted from Karnovsky & Roots[23]. The AChE activity in supernatants of 24, 48, 72 and 96 HPF zebrafish control and treated embryos were carried out in triplicate, as described by Athitya *et al*[24]. A reaction mixture was made in a cuvette by adding 0.4 mL of homogenate, 2.6 mL of 0.1M phosphate buffer pH 8.0, 100 µL of DTNB reagent and 50 µL of acetylcholine iodide (substrate). The absorbance was measured at 412 nm using the UV probe Spectrophotometer for up to 5 min in every 1 min absorbance. Enzyme rate was calculated using the formula

$$R=5.74(10^{-4})\ \Delta A/C_0$$

Where ΔA= change in absorbance per min
C_0 = original concentration of tissue

Statistical analysis

Quantitative data was expressed as mean ± SEM, depending on the distribution of the data. The Student's t- test was used to test for differences between the textile chemicals exposed and the control zebrafish embryos for locomotor activity and survival data. A multivariate analysis (the developmental stage and treatment as covariates; the lipid peroxidation and GSH levels as dependent variable, respectively) was performed using the SPSS software version 16.0 to evaluate the differences in the mean value of viability, GSH level and lipid peroxidation at various developmental stages exposed to AB113, NA and MA versus control. (**P* <0.05, ***P* <0.001).

Results

Toxicity assay

The primary test was carried out to evaluate the toxicity of AB113, NSA and MA to analyze the rate of mortality followed by studying the neurodegenerative phenotypes. In our study, during the critical window of development viz., 18 HPF zebrafish embryos were exposed to EM alone for control and textile compounds (AB113, NSA and MA) within a concentration range of 1, 5, 10, 20 and 40 PPM for up to various developmental stages of zebrafish viz., 24 HPF (segmentation), 48 HPF (pharyngula), 72 HPF (hatching) and 96 HPF (larval), respectively. As shown in (Fig. 1), the control embryos had a survival percentage of 97.75%. The group that was treated with 1 PPM of textile compounds (AB113, NSA and MA) had 50% survivability, while the other concentrations had extremely low or no viability. Hence, based on the concentration and survival (%) reported at all the endpoints 1 PPM was determined as the LC_{50} value for AB-113, NSA and MA, respectively.

The changes in the morphology of the control and 1PPM AB113, NSA and MA treated embryos were monitored at every developmental stage with the help of the stereo zoom microscope. No significant changes in the appearance of the embryos were

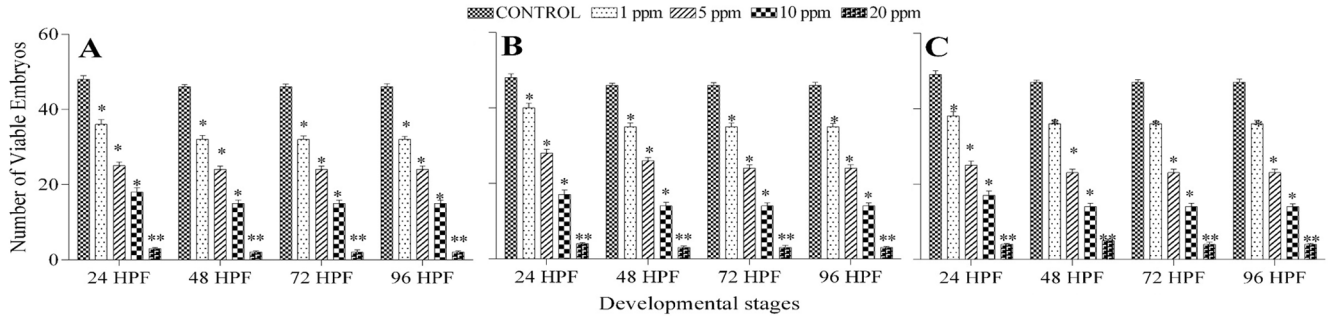

Fig. 1 — Effect of various concentrations of (A) AB113; (B) NSA; and (C) MA on the viability of zebrafish embryos at various developmental stages following exposure at 18HPF. [Results are expressed as mean ± SEM (n = 10), (**P* <0.001, ** *P* <0.05)].

observed in the AB 113, NSA and MA treated groups compared to the control group (Fig. 2). Focusing on the higher concentrations viz., 5 and 10 PPM the malformations were observed more than 90% for AB-113, NSA and MA, respectively (data not shown).

Effects on locomotor activity

To infer the exposure of textile chemicals during critical windows of development to the locomotor activity, 1PPM AB-113, NSA and MA exposed embryos at 24, 48, 72 and 96 HPF, respectively, were selected. When compared to controls, 37.4%, reduction in spontaneous tail coiling was recorded at 24 HPF as shown in Fig. 3A. At 48, 72 and 96 HPF the locomotor activity was significantly reduced by 74.9, 85.2 and 86.2%, respectively in response to 1 PPM AB-113 as shown in Fig. 3B. Similarly, 1PPM NSA exposure significantly reduced the spontaneous tail coiling by 32.5% at 24HPF while 76.1, 80.2 and 85.6% reduction in locomotor activity was recorded at 48, 72 and 96 HPF, respectively compared to age matched controls (Fig. 3A and B.). Surprisingly, similar effects were demonstrated when exposed to 1PPM of MA; at 24 HPF 34.1% reduction in spontaneous tail coiling (Fig. 3A) and 71.8, 79.6 and 85.9% reduction in locomotor activity was noticed at 48, 72 and 96 HPF, respectively (Fig. 3B).

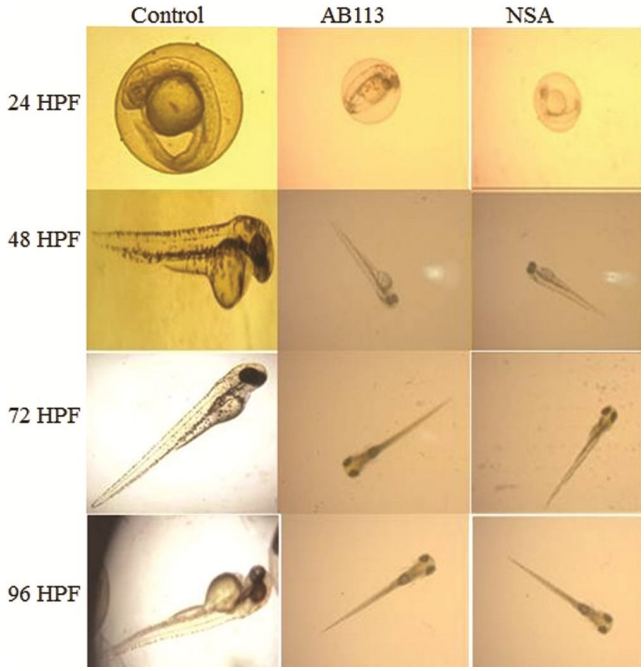

Fig. 2 — Display neurodegenerative phenotypes at various developmental stages of zebrafish exposed to 1PPM AB 113, NSA and MA at 18 HPF when photographed in lateral orientation through stereo zoom microscope at various developmental stage.

Effects on AChE activity

Fig. 4 elucidates the AChE activity at 24, 48, 72 and 96 HPF, respectively. At 24 HPF, 1PPM of AB 113, NSA and MA showed a significant decrease in AChE activity compared to controls. However, 1PPM NSA when compared to AB-113 and MA showed more reduction in AChE activity. At 48 HPF, 1PPM of AB 113, NSA and MA showed a significant

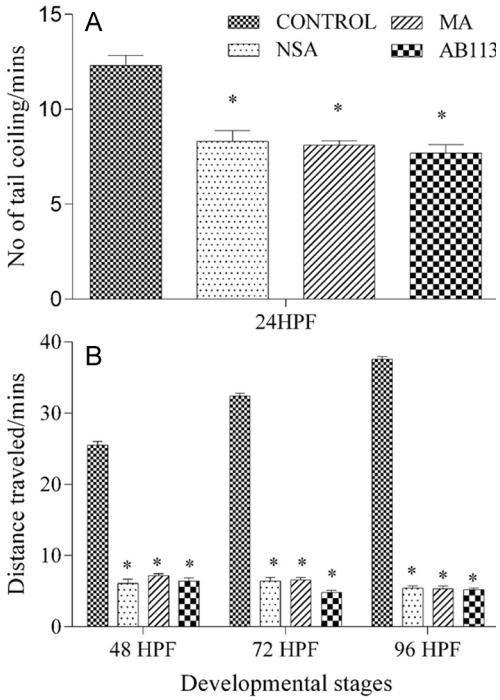

Fig. 3 — Effect of 1 PPM AB 113, NSA and MA on spontaneous tail coiling (A) of zebrafish embryos at 24 HPF and distance moved (cm, B) of zebrafish at 48, 72 and 96 HPF, respectively. [Embryos were exposed during 18HPF. Results were expressed as mean ± SEM (n = 10), (**P* <0.001, ** *P* <0.05)].

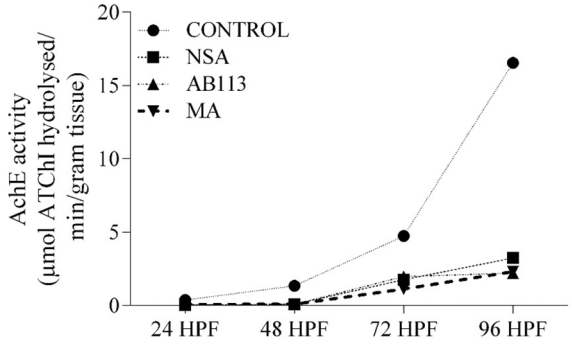

Fig. 4 — Effect of 1 PPMAB 113, NSA and MA on Acetyl choline esterase (AChE) activity at various developmental stages of zebrafish embryos following exposure at 18HPF renewed fresh daily. [Results were expressed as mean ± SEM of 3 replicates (100 larvae/ replicate. ANOVA: Treatments, **P* <0.001; ** *P* <0.05 indicate treatments significantly different from control]

decrease in AChE activity compared at controls. On the other hand, 1 PPM AB 113 showed an extra decrease in AChE activity compared to other textile compounds. At 72 HPF 1PPM of MA showed additional decline in AChE activity when compared to AB113 and NSA. When focusing at 96 HPF AB-113 showed more decline in AChE activity when compared to NSA and MA.

Effects on antioxidant markers

The embryos at different HPF following exposure to the respective azodye and aromatic amines at 18 HPF were collected and the lipid peroxidation was estimated by measuring the amount of malondialdehyde and the results are depicted in Fig. 5. At 24 HPF, 1PPM of AB 113, NSA and MA showed a significant increase in LPO levels compared to controls. However, 1PPM NSA when compared to AB-113 and MA showed more increase in LPO levels. While at 48 HPF, 1PPM of AB 113, NSA and MA showed a significant increase in LPO levels compared to controls. On the other hand, 1 PPM AB 113 showed an extra increase in LPO activity compared to other textile chemicals . At 72 HPF 1PPM of AB 113 showed additional increase in LPO levels when compared to MA and NSA. When focusing at 96 HPF AB-113 showed more increase in LPO levels compared to NSA and MA.

Fig. 6 clearly demonstrates that at 24 HPF 1PPM of AB 113, NSA and MA showed a significant decrease in GSH levels compared to controls. However, 1PPM MA when compared to AB-113 and NSA showed more decrease in GSH levels. While at 48 HPF, 1PPM of AB 113, NSA and MA showed a significant decrease in GSH levels compared to controls. On the other hand, 1 PPM MA showed an extra increase in GSH activity compared to other textile chemicals. At 72 HPF 1PPM of MA showed additional decrease in GSH levels when compared to AB-113 and NSA. When focusing at 96 HPF MA showed more decrease in GSH levels when compared to NSA and AB-113.

Discussion

The current study reported that textile compounds (AB113, NSA and MA) at 1PPM concentration do not have any neurodegenerative phenotypes including flat head, small eyes and pinched midbrain-hindbrain boundary and thin yolk extension. But, AB113, NSA and MA exposure has significantly affected the mortality and locomotor activity. This result is consistent with previous past study showing that exposure to the synthetic dye Basic Red 51 (BR51, used in cosmetic industry) and the natural dye erythrostominone (ERY, a potential commercial dye extracted from fungi) reduced significantly locomotor activity[25]. These observations suggest that during critical period, brain developmental program is highly sensitive to AB113, NSA and MA with neurobehavioural alterations[19].

Furthermore, this study demonstrates that developmental exposure to AB113, NSA and MA significantly decreases AChE levels in an age dependent manner. During brain development, acetylcholine and cholinergic projection play crucial roles in proliferation, migration and synaptogenesis and in the development of neural cytoarchitecture[26]. So,

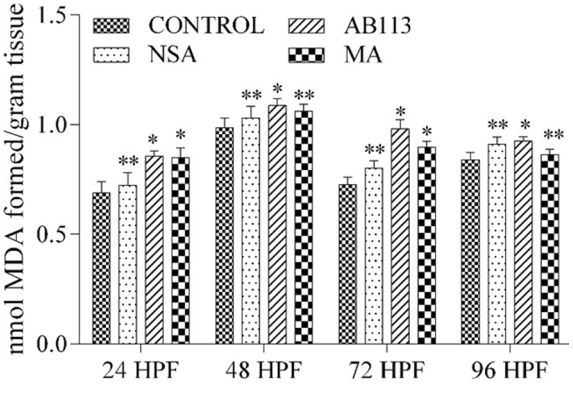

Fig. 5 — Effect of AB 113, NSA and MA on lipid peroxidation at various developmental stages of zebrafish embryos following exposure at 18HPF renewed fresh daily. [Results were expressed as mean ± SEM of 3 replicates (100 larvae/ replicate. ANOVA: Treatments, * $P < 0.001$; ** $P < 0.05$ indicate treatments significantly different from control].

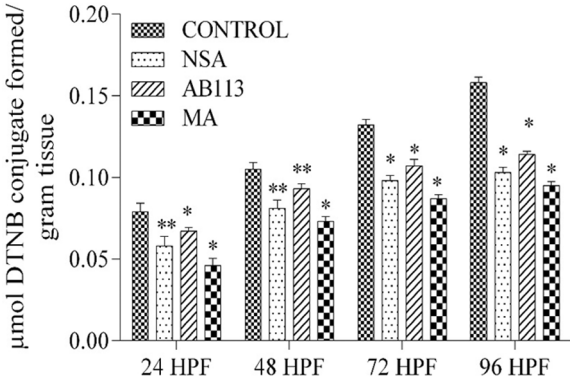

Fig. 6 — Effect of AB 113, NSA and MA on on Glutathione activity at various developmental stages of zebrafish embryos following exposure at 18HPF renewed fresh daily. [Results were expressed as mean ± SEM of 3 replicates (100 larvae/ replicate. ANOVA: Treatments, * $P < 0.001$; ** $P < 0.05$ indicate treatments significantly different from control].

interrupted cholinergic transmission consequently to AChE inhibition, might be a factor of developmental neurotoxicity, including physiologic aberrations ranging from behavioural impairment to death[27]. Our study revealed neurobehavioral deficits are correlated to AChE activity inhibition. This differential decrease in AChE activity at different developmental stages may be due the differential experimental modulation of the cholinergic system, AChE activity or ache gene expression by the AB113, NSA and MA exposure during development and tissue differentiation[28]. Our results are in concurrence with the previous reports where embryos bathed with organophosphate (chlorpyrifos, monocrotophos), AChE was totally inhibited, and somitogenesis was altered[29-32].

Previous evidences suggest that in oxidative stress by an imbalance between the generation of radical oxygen species (ROS) and the antioxidant defences to be important factor in the pathogenesis of many abnormalities and the cholinergic dysregulation. This biomarker has also been considered in pollution monitoring studies using invertebrates and fish[33].Our observation in the present study demonstrated differential increase in LPO levels, while GSH activity decreased as compared to controls for AB-113, NSA and MA, respectively. This differential decrease in GSH levels is proportional to the differential increase in LPO levels and reduction in AChE activity following exposure to AB-113, NSA and MA, respectively. These results demonstrate that oxidative stress is relevant mode of action for AB-113, NSA and MA. Therefore, the significant correlation found between AChE activity and locomotor activity, along with the presence of lipid peroxidation, strongly indicates that inhibition of AChE and GSH activity are the primary key event in the pathophysiology of AB-113, NSA and MA developmental toxicity at various developmental stages, respectively[34-36]. Overall, the present study supports the idea that textile dyes may represent a threat to organisms during embryogenesis, since they impair physiological processes essential to a normal development and, consequently, survival.

Conclusion

In this study, the toxicity of textile chemicals (AB113, NSA and MA) was investigated by analysing the behavioural changes during critical windows of development. These effects suggest that it is developmental stage dependent and correlated with changes in oxidative stress, antioxidant activity and imbalance of acetylcholine esterase activity. Immature brain is vulnerable to AB113, NSA and MA, and exposure to environmental toxicants early in the life may trigger the onset neurodevelopmental disorders. The toxic effects of textile chemicals on behaviour as revealed by the results may be useful for effective countermeasures development about motor deficits under extreme environments by present possible biochemical markers.

Conflict of Interest

Authors declare no conflict of interests.

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