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GESTATIONAL EXPOSURE TO THE AQUACONTAMINANT, NICKEL CHLORIDE IN FEMALE ZEBRA FISH, *Danio rerio* CULMINATES IN BEHAVIORAL AND NEUROCHEMICAL CHANGES IN PROGENY

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ABSTRACT

The prenatal period is essential period for neurodevelopment during which a range of exposures can exert long-term changes in the brain. The water-soluble salts of nickel, a heavy metal toxicant, are crucial contaminants in the environment including drinking water that can cause neurological damage. To assess the impact of gestational exposure to nickel chloride on the nervous system of the adult female fish. In the current study, Nickel chloride (3 µl of 1 M $NiCl_2$) was injected intramuscularly in adult female Zebrafish for 30, 40 and 50 days. The time-dependent changes were assessed in the juvenile fish by performing histopathology, behavioral analysis, oxidant and antioxidant status and also the levels of neurotransmitters were examined. The results revealed that the Chronic stress due to $NiCl_2$ was affected the Purkinje layer, increased anxiety and decreased the memory as evident from the tank diving and maze test in a duration-dependent manner. The oxidative stress as assessed by the level of lipid peroxidation showed increased in comparison to the control ($p<0.001$)). The response of tissue antioxidants as assessed by the activities of SOD,GST, catalase and the content of reduced glutathione were found to be affected at 50thday of induction. The activity of acetylcholine esterase and the level of nor adrenaline were significantly increased ($p<0.001$) on 50th day whereas the levels of serotonin and dopamine showed a significant reduction ($p<0.001$). Collectively the study suggests that the nickel chloride can alter the neuronal morphology, affect the behavior and neurotransmission, owing to possible developmental defects in the brain of the juvenile zebrafish.

Keywords: Behaviour, Gestational exposure, Nickel chloride, Neurotoxin, Zebra fish

1. INTRODUCTION

It is well known that gestational exposure to stressors can impact fetal brain structure and function increasing the long-term susceptibility to neuro developmental and neuropsychiatric complications [1].Such exposures can alter neural outcomes in the progeny through changes in the neurotransmission and the behavioral modulation [2] .Environmental contaminants may affect the physical and biochemical properties of Fish [3] .Human exposure to nickel occurs by breathing air, drinking water, consuming food, stainless steel, water pipes, jewelry, cosmetics but only in negligible doses. However, women employed in nickel refineries if exposed during their early pregnancy are at an elevated risk of pregnancy outcomes as the exposure level can exceed the standard limit of 70-170µgm [4] .Nickel is a potential neurotoxin, and oxidative stress has been found to play a crucial role in nickel-induced neurotoxicity [5]. The primary cause of Ni toxicity is that Ni can bind to the sulfhydryl groups of proteins and alter the oxidant status of the tissue [6].Several studies have indicated that $NiCl_2$ can affect the nervous system by inhibiting the activity of ATP and target the active site of acetylcholine esterase [7]. *Daniorerio* is an excellent model for neurobehavioral study, and its nervous system exhibits 65% homology with that of mammals, and hence they have been used in our current study. The study focused on exploring the effect of maternal stress induction owing to gestational exposure to neurotoxicant, $NiCl_2$ which has not been analyzed for its long-term effect on the juvenile population.

2. MATERIALS AND METHODS

2.1. Zebrafish

Adult female zebrafish were collected from Kolathur,Chennai and housed in light/dark cycle (14/10) and throughtout 27 ±1°C was maintained with biofiltration facility and aeration. The laboratory grade chemicals were purchased from either Sigma Aldrich or LobaChemie or Merck and the required stock solutions

were prepared according to the manufacturers' instruction.The group size was maintained at 50 Numbers.

2.2. Experimental Design

For metal toxicity test, adult female zebrafish were exposed to various concentrations of Nickel chloride for 7 days. After the exposure, the smear test was conducted to determine the effective dosage followed by the survival and mortality analysis. Based on the data(not shown),3 µL (477 µg) of 1M $NiCl_2$ was injected to the female fish intramuscularly using a Hamilton syringe(National Analytical Corporation, Mumbai) once in five days for 30 ,40 and 50 days. After continuous exposure for respective days, the injected female fish were allowed to mate with normal males.

2.3. Fish Breeding

The tanks were divided into two breeding sets of three tanks for each group. Each breeding set was bred every other day for a total of 5 spawnings per set over a 10-day period.For daily breeding set-up, fish were quickly removed using a net and transferred into breeding traps in the same tank, and eggs were collected, allowed to grow without exposure and juvenile obtained at 50^{th} day (10-40 days also assessed but data not shown)were subjected for further investigations from the different groups .Cognitive and biochemical tests were performed in juvenile (untreated F1 progeny) population (50 days post fertilisation) from the exposed mother.

2.4. Group Distribution

Group I-Control Juvenile obtained at 50^{th} day (50 day post fertilisation) from Normal Zebrafish (n=50). Group II –Juvenile obtained from the adult female subjected to $NiCl_2$ for 30 days at 50^{th} day (n=50). Group III- Juvenile obtained from the adultfemale subjected to $NiCl_2$ for 40 days at 50^{th}day (n=50). Group IV- Juvenile obtained from the adult female subjected to $NiCl_2$ for 50 days at 50^{th}day (n=50)

2.5. Histo pathology

Juvenile fishes were euthanazed and dissected as per ethical guidance. Fish were anesthetized with 15°C water and killed by a cut between the brain and the spinal cord and the head was removed. The brain was dissected with a pin and knife. A thin layer of brain tissue was made on a glass slide and stained with Haematoxylin and Eosin respectively for 2 min each followed by Phosphate Buffer Saline (A 10 litre stock of 10x PBS can be prepared by dissolving 800 g NaCl,20 g KCl,144 g $Na_2HPO_4 \cdot 2H_2O$, 24 g KH_2PO_4 make upto 10 L with distilled water) washes. Slides were viewed at 45X magnification using labomed USA, Trinocular Fluorescent and light microscope.

2.6. Anxiety Test

Juvenile Zebrafish were transferred to glass tank of 30cm X 30cm X 15cm (Length X Breadth X Height) and then acclimatized for 30 minutes for dive tank test. Following which, fishes were video captured for a total of 3 min with three consecutive 1 min during a 30-minute period. Time spent at the top or bottom of the tank was calculated using kinovea 8.5 motion analysis software.

2.7. Memory and Learning Test

Juvenile fishes placed in 30 cm tank which was divided into four sections of 7.5 cm each for maze test. They were allowed to acclimatize under the light of 1400 lumens for 15 min., and recordings were made. Measurements made at a period of 12 h juveniles. Fish placed in one end of the tank and a pelleted feed was dropped in the other end of the tank. Time taken for the fish to swim through the maze tank to reach the food pellet was recorded.

2.8. Antioxidant enzymes

2.8.1. Lipid peroxides test

Lipid peroxides in the fish was estimated by thiobarbituric acid reaction method as described by Ohkawa et al. , 1979 [8]. According to the protocol, 0.2 mL fish brain cell extract was taken, and then 0.2 mL of 0.8% SDS, 1.5 mL of 2.5mM acetic acid and 1.5 mL of 100mM Thiobarbituric acid was added. Following which, the mixture was made up to 4.0 mL with distilled water and then heated in a water bath at 95°C for 60 min. After cooling, 1.0 mL water and 5.0 mL n-butanol /pyridine mixture (1:5) were added and mixed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken, and its absorbance read at 532 nm spectrophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA). The activity of lipid peroxides was expressed as nanomoles.

2.8.2. Superoxide dismutase

The SOD activity was measured by the method of Misra and Fridovich., 1976 [9] . The SOD activity of fish brain cells was assayed by their capacity to compete with native or partially succinylated ferricytochrome c for free radicals generated by the xanthine/xanthine oxidase system. The reduction of ferricytochrome c was

monitored with a microplate reader by using the wavelength pair 550 nm minus 557 nm.

2.8.3. Catalase

Catalase activity was measured by the method of Aebi., 1974 [10]. 0. 1 mL of fish brain cell extract was added to a cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 mL of freshly prepared 30mM hydrogen peroxide. The rate of decomposition of H_2O_2 was measured spectrophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) by changes in absorbance at 240 nm. The activity of catalase was expressed as units/mg protein.

2.8.4. Glutathione peroxidase

The glutathione peroxidase was measured according to Wood,J.L.,1970 [11]. About2.0 mL of phosphate buffer (75 $mmolL^{-1}$, pH 7.0), 50µL of (60 $mmolL^{-1}$) glutathione reductase solution, 50µL of (0.12 $molL^{-1}$) NaN_3, 0.1 ml of (0.15 $mmolL^{-1}$) Na_2 EDTA ,100µL of (3.0 $mmolL^{-1}$) NADPH, and 100µL of fish brain cell extract were mixed in a glass tube. Distilled Water was added to make a total volume of 2.9 mL. Then the reaction was started by adding 100µL of (7.5 $mmolL^{-1}$) H_2O_2, and the conversion of NADPH to NADP monitored by a continuous recording of the change of absorbance at 340 nm at 1-min interval for 5 min.

2.8.5. Glutathione reductase

Glutathione reductase was assayed according to Beutler et al.,1963 [12]. 1.0 mL phosphate buffer, 0.5 mL EDTA, 0.5 mL glutathione oxidized and 0.2 mL of NADPH was made up of 3.0 mL with distilled water. After the addition of 0.1 mL of fish brain cell extract, the change in optical density at 340 nm was monitored for 2 min at 30 sec interval.

2.8.6. Acetyl cholinesterase

Acetylcholinesterase (AChE) enzyme activity was estimated by Elman et al.,1961 [13].Fish brain cell extract of a 0.4mL aliquot of homogenate added to a cuvette containing 2.6 mL phosphate buffer (0.1M, pH 8) and 100µL (10 mM) DTNB (5,5-dithio-bis-2-nitrobenzoic acid). The contents of the cuvette were mixed thoroughly by bubbling air and absorbance measured at 412 nm in a spectrophotometer. When absorbance reaches a stable value, 20µl of substrate, *i.e.,* acetylthiocholine was added, and changes in absorbance recorded. Change in the absorbance per min was thus determined.

2.8.7. Serotonin

Serotonin content was estimated according to Schlumpf method 1974 [14]. To 0.2 mL aqueous extract, 0.25 mL of (50mM) O-phthaldialdehyde reagent was added. The fluorophore was developed by heating to 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, readings were taken at 360-470 nm in the spectrofluorimeter. For serotonin tissue blank, 0.25 mL concentrated HCI without OPT was added. Internal standard: (500 μgmL^{-1}) serotonin was prepared in distilled water: HCl-butanol in 1:2 ratio).

2.8.8. Dopamine

The dopamine content was estimated by the method of Schlumpf et al.1974 [14]. To 0.2 mL aqueous phase, 0.05 mL 0.4 M HCl and 0.1 mL EDTA(150 mg) / sodium acetate(12g) buffer (pH6. 9) were added, followed by 0.1 mL iodine solution in 0.1 M in ethanol(10 mg of iodine solution in 100 ml of ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.1 mL 100 mM Na_2SO_3 solution, 0.1 mL acetic acid was added after 1.5 min. The solution was then heated to 100°C for 6 min and then cooled to room temperature; excitation and emission spectra were read from the microplate reader. Tissue blanks for dopamine prepared by adding the reagents of the oxidation step in reversed order (sodium sulfite before iodine). Internal standard: (500 μgmL^{-1} dopamine prepared in distilled water: HCl-butanol in 1:2 ratios. The readings are observed at 330-375 nm for dopamine.

Noradrenaline: Noradrenaline content was estimated by the method of Schlumpf et al., 1974 [14]. To 0.2 mL aqueous phase, 0.05 mL 0.4 M HCl and 0.1 mL EDTA / Sodium acetate buffer (PH6. 9) were added, followed by 0.1 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by addition of 0.1 mL Na_2SO_3 solution. 0.1 mL acetic acid added after 1.5 min. The solution was then heated to 100°C for 6 min and cooled to room temperature. The excitation and emission spectra were immediately read from the microplate reader. Tissue blanks for nor-adrenaline prepared by adding the reagents of the oxidation step in reversed order (sodium sulfite before iodine). Internal standard: (500 μgmL^{-1} Noradrenaline prepared in distilled water: HCl-butanol in 1:2 ratio). The readings are observed at 395-485 nm for nor-adrenaline.

2.9. Data analysis

Statistical comparisons were made using GraphPad software. Two-way ANOVA test with a 95% confidence interval was performed at an alpha = 0.05 (95% confidence interval).

3. RESULTS AND DISCUSSION

3.1. Histopathology

Effect of maternal exposure to $NiCl_2$ on the pathology of the brain of the juveniles: Necrosis and purkinghe layer damage was observed in the brain of juveniles(Group I V) (Fig. 1) which was not evident in the case of control juvenile(Group-I).

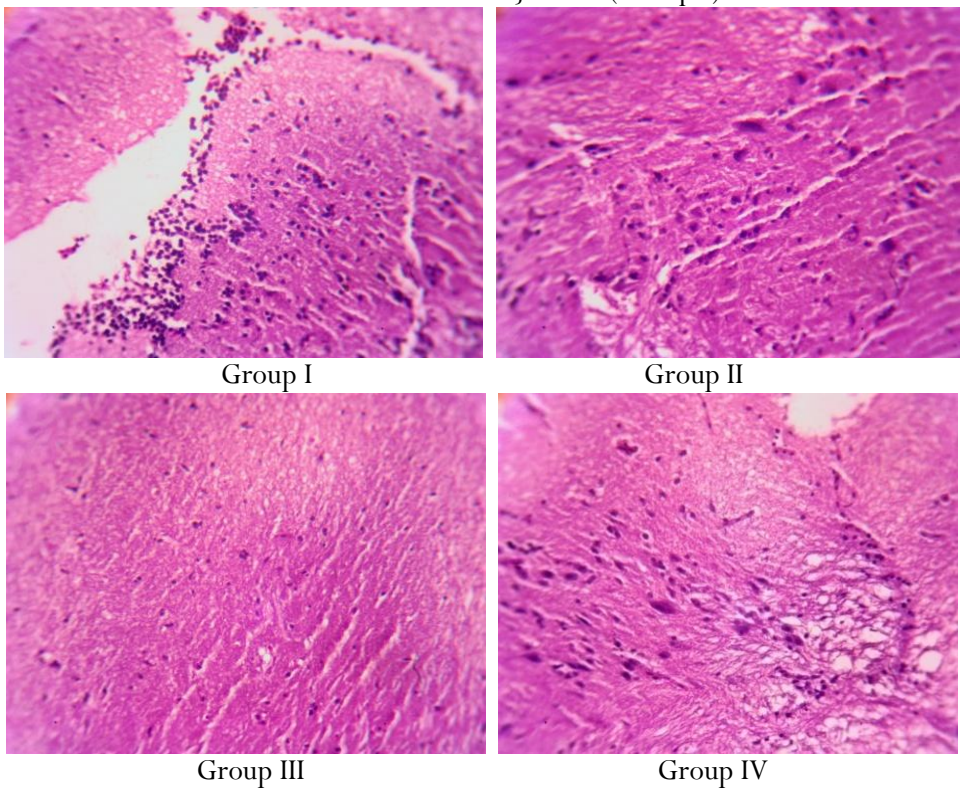

Fig 1: ***Microscopic appearance of cerebellum section of brain of Zebrafish that were subjected to $NiCl_2$ exposure at* 400 X magnification**

Group I, II represents the normal architecture of the brain cells ; Group II represents the mild shrunken cells; Group III represent the enormous number of shrunken cells; Group IV represent the necrotic cells and purkinghe layer is affected.

Smear pathological studies would give a potential toxicological effect of heavy metals in marine organisms [15]. A study has reported that Ni exposure in *Hypophthalmichthys molitrix* can cause necrosis, vacuolation, hypertrophy in the liver, a fusion of the lamellae and hypertrophy in gills and degeneration of tubular cells and hyperplasia of kidney after 10, 20 and 30d exposure to 5.7 mgL^{-1} Ni [16]. Similar to this data, in our study also, we have observed that Ni can cause damage in brain tissues (Fig.1) indicating that Ni induced maternal stress can have a detrimental effect in the F1 offspring as denoted by damaged Purkinje layer in the juvenile brain(Group-I) (Fig.1) .The cerebellum develops much more rapidly in zebrafish than in mammals. The zebrafish cerebellum has three lobes, the corpus cerebelli (CCe), the valvulacerebelli (Va), and the vestibulolateral lobe. CCe and Va have tri-lamellar structures comprising the granule cell, Purkinje cell, and molecular layers. Reports reveal that the most striking differences between autistic and normal brains is the loss of Purkinje cell layer in the cerebellum and activation of microglial cells that are central to the inflammatory response [17]. Our demonstration of loss of the Purkinje cell layer in the cerebellum of juvenile of the stressed female (Fig.1) depicts the undesirable effect of heavy metal exposure during gestation.

3.2. Behaviour studies

In juvenile fish, which is obtained from female fish exposed to metal toxicant for 30, 40 and 50 days ,the anxiety was found to be increase as compared to control juvenile (Table 1).

Table 1: Behavioral changes in the juvenile from the female fish following gestational exposure to $NiCl_2$ for different days

Parameters	Group-I	Group-II	Group-III	Group-IV
Time spent at the bottom of the tank (seconds)	30±1	70±3*	107±2*	190±3**
Time to reach the end of the tank in seconds	180±4	173±3*	169±3*	94±2**

*All values were indicated as mean±S.D. Significance was tested statistically by ANOVA test. All asterisks represent statistically significant data *P<0.05; **P<0.01 as compared to control values.*

3.2.1. Assessment of anxiety and memory in juvenile using tank diving test and maze test

During anxiety, zebrafish spent most of the time in the bottom of the tank. The time spent at the bottom of tank was significantly higher in induced group when compared to that of control and was found to be dependent on more prolonged duration of dosage. The cognitive behavior of juvenile as assessed by learning and memory significantly affected by the durations of metal toxicant (Table 1) with drastic changes observed on 50th day treated group. Zebrafish (Daniorerio) is a useful species for studying behaviour patterns and for modelling complex brain disorders. Zebrafish behavioral responses are robust, which are evolutionarily conserved, and resemble those of mammalian species. In this study nickel chloride exposed fish showed variations in the behavior of juveniles with an intense impact in maze test and anxiety test concerning those observed in control. There was a decreased locomotor activity(anxiety test) in our study (Table1) similar to previous reports which had highlighted that a dose of 7.5 mg Ni/L could affect locomotion in Danio rerio [18].

3.3. Effect of maternal exposure on oxidant and antioxidant activities

The status of oxidant/antioxidant in the brain of juvenile Zebrafish subjected to gestational exposure to $NiCl_2$ is presented in Fig.2.

It was evident that while the level of peroxides was significantly elevated ($p<0.001$), the SOD and CAT activity were drastically reduced in 50thday group when compared to that of control. The activities of GR and Gpx also showed a significant decrease in 50 daysof maternal exposures compared to control.

Depending on the nature and duration of the stress, antioxidant enzymes levels will be altered. It also depends on the susceptibility of the living organisms to the heavy metal toxicity.

During fetal development the brain cells will be more susceptible to metal toxicity due to low antioxidant defenses [19]. In a study using daniorerio model, decreased activities of SOD and CAT were reported on exposure to aluminum [20] supporting our current observations made in *D.rerio* on nickel chloride exposure (Fig. 2). Similar to our study, exposure to nickel chloride in *Oncorhynus mykiss* (rainbow trout), was found to cause abnormal biochemical variations [19].

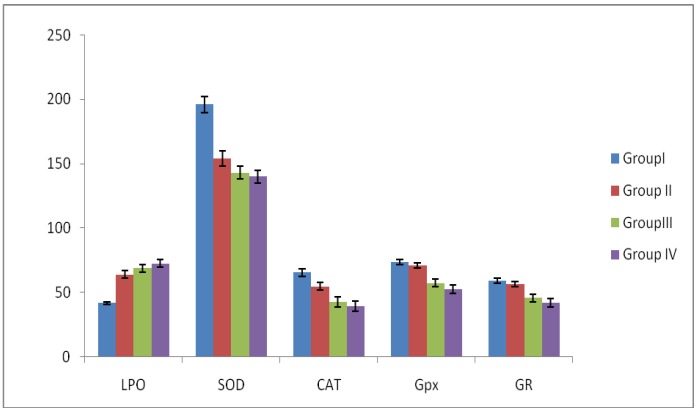

Fig 2: Oxidant and antioxidant status in the brain of the juvenile of female fishes subjected to Nickel chloride

Results were expressed as mean ± standard deviation. SOD, Superoxide dismutase; CAT, Catalase; GR, Glutathione reductase; GPx, Glutathione peroxidase .

Significance was tested statistically by two way ANOVA with a 95% confidence interval was performed at an alpha = 0.05 (95% confidence interval) with in the same exposure concentration between different durations of gestational exposure of $NiCl_2$. All asterisks statistically significant

**P<0.05; **P<0.01 as compared to control values.*

Comparisons were made between Group I vs III and IV

**P<0.05-Group-II vs Group-I; *P<0.05-Group III vs Group I*

***P<0.01-Group IV vs Group I*

3.4. Effect of maternal exposure on the activities of neurotransmitters

The activity of acetylcholinesterase and levels of serotonin, dopamine and norepinephrine in juvenile zebrafish following the gestational exposure by metal toxicant is presented in Table 2.

The activity of acetylcholine esterase and noradrenaline showed a significant increase after 50 days of $NiCl_2$ maternal induction compared to control. Table 2 also showed that the levels of serotonin and dopamine were

significantly decreased by maternal exposure to $NiCl_2$when compared to those of control.

The acetyl choline esterase in fish is essential for healthy behavior and muscular function [21]. Alterations observed in the activity of acetyl choline esterase (Table 2) reflect the effect of increased free radical production which affects the behavior of the fishes including diving due to nickel exposure. This altered activity of cholinergic enzyme [22] due to altered behaviour pattern can cause improper impulse transmission resulting in the loss of coordination.

Table 2: The activities of Acetyl choline Esterase, Serotonin, Dopamine, Norepinephrine in the juvenile of Danio rerio subjected to gestational exposure to Nickel chloride for different days

Parameters	Group-I	Group-II	Group-III	Group-IV
Acetyl choline esterase(µ moles per mg)	16.26±1	17.57±1*	19.74±1*	21.62±1**
Seratonin(nano moles per mg)	130.2±4	101.5±4*	91.7±3*	58.8±3**
Dopamine(nano moles per mg)	186±3	145±2*	131±2*	84±1**
Nor Adrenaline(nano moles per mg)	24.39±1	26.36±1*	29.61±1*	32.43±1**

Results are expressed as mean ± standard deviation. Significance was tested statistically by two way ANOVA with a 95% confidence interval was performed at an alpha = 0.05 (95% confidence interval) within the same exposure concentration between different durations of gestational exposure of $NiCl_2$.All asterisks statistically significant

**P<0.05; **P<0.01; as compared to control values.*

Comparisons were made between Group I vs Group-II, III and IV.

Daniorerio possesses a complex serotonergic system featuring all major genes for 5-HT synthesis, metabolism and signaling [23] similar to those observed in humans and rodents. The serotonergic system is linked to many behavioral traits. Studies have shown that heavy metals such as mercury, cadmium, lead, aluminum, nickel, and tin affect chemical synaptic transmission and neurotransmitters in the brain [24]. Coinciding with such reports, we have found depressed levels of serotonin. Another study has revealed the effect of heavy metals on serotonin which shows the altered behaviour pattern and permeability [25]. Such influence was prominent in the 50th day group in which neurotransmitter and behavior was drastically affected than that displayed in the control group ($p< 0.001$) since behaviour was correlated with the changes in the serotonin level.

The axonal projection of dopaminergic neurons can be well studied in Zebrafish. It has been proposed that the zebrafish dopaminogeric system is homologous to the A11 system in mammals, an area involved in motor control and anatomically associated with the nigrostriatal Dopaminergic system [26]. In our study, there is altered dopamine level compared to that of the control.

The altered dopamine levels in this 50th day group (Table 2) were anticipated to affect the motor behavior of fish as evident from the diving ability of juvenile (Table 1).

4. CONCLUSION

The study suggested that gestational exposure to $NiCl_2$ showed significant impact on behavior and morphological changes in the brain tissue of F1 progeny as evident from the damaged Purkinje layer in the cerebellum layer. Nickel chloride affects the antioxidant system and alters the neurotransmitter levels. This study is alarming for neuro disorders caused by chronic exposure to nickel chloride, even though their toxicity is not widely prevalent. Collectively, the study shows the neurotoxic effects of nickel chloride in F1 progeny of Zebrafish.

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