(RESEARCH ARTICLE)

An investigation of chronic exposure of lead to the pregnant laboratory animal and its effects on their offspring: Autism investigation to the laboratory animal model in Bangladesh

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Abstract

Lead poisoning is a continuing crisis impacting learning, neurological and behavioral development in children. This study investigated an important outcome from the environmental lead contamination which cause retardation of infant's brain development. A total of 30 Swiss albino mice of both sexes were used in this study and divide into three groups consisting 6 female and 4 male in each group. Two groups of mice were given lead treated drinking (30 ppm and 230 ppm respectively) and rat pellets ad libitum and the control mice were supplied normal drinking water and rat pellets ad libitum. At 20-21th days of pregnancy lead-induced female mouse gave birth to a litter of 5-6 pups; whereas, control mouse gave birth to 10-13 pups at a time. Both lead-induced and controlled offspring were found normal; however, some lead-induced offspring were found comparatively smaller than the control one. Autism spectrum disorder behavioral diagnostic tools were used to explore the level of autism, if any. The anxiety assessment marble burying investigation did not reveal any significant differences among the group's mice. Three chambered social interaction analysis found no significant differences among the mice. Blood serum level of lead for controlled mice were found 0.333µg/dL, whereas, 30 ppm and 230 ppm lead-induced born mice were found 3.833µg/dL and 9.666µg/dL respectively. This study suggested that a genetic predisposition pair with exposure to environmental toxicants play important role in the causes of autism spectrum disorder. Lead is not the pivotal factor of autism development in new born offspring in mice

Keywords: Lead; Autism spectrum disorder; Behavioral activity; Mice

1. Introduction

Lead is one of the most abundant heavy metals in the Earth's crust [1]. Lead exposure has become a grave concern in almost all industrializing and developing countries [1]. Although in developing countries, awareness of lead exposure is growing but only a few numbers of these countries have taken significant steps solving the problem. To date, no research is being investigated in this field in Bangladesh. There is one and only solution to stop this crisis & that is with the diversified researches. Lead has become widely distributed and mobilized in the environment and human exposure and uptake of this element has consequently increased. The high level of exposure damages almost all organs and organ systems, mainly CNS, kidneys and blood, causing death at high levels. Haeme synthesis and other biological processes are also affected due to lower intake of lead, psychological and neuro functions are impaired, and there is a range of other effects [1, 2]. Lead is transported freely through the placenta [3, 4] and also the blood brain barrier. Single doses of lead given to pregnant animals lead to a series of destructive changes in the brains of their offspring during the period when brain formation is complete [5].

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Lead has a long history of public exposure through food and drink. In Roman times lead was used in water pipes and earthenware containers, and in wine storage resulting lead poisoning. Through improved working conditions, lead exposure has largely been controlled in developed countries. However, low level exposure of lead has become concern over the possible adverse effects of environmental lead. In particular, lead poisoning in children experiencing non-occupational exposure has attracted much attention [1, 6]. In Australia, lead poisoning in children was first reported in 1892, although it was not until 12 years later that the source, peeling lead-based paint, a series of ten children was identified with lead colic. In 1943 a study was done over 20 school children in the USA who had experienced acute lead poisoning during infancy or early childhood and found that exposure to environmental lead at levels insufficient to produce clinical encephalopathy was accompanied with long-term deficits in neuropsychological development [7]. Case-control studies on mental retardation [8] reported that acute lead intoxicated children were often left with severe deficits in neurological function. It was subsequently recognized that longer-term squeal were not limited to people affected by excessive exposure but also occurred in children who experienced relatively low-level exposure.

Over the last 30 years many investigations have done, which revealed adverse effects of moderately elevated blood lead levels, on health i.e. below 25 mg/dl. Although lead poisoning related problems have largely decreased in developed countries, chronic exposure to low levels of lead is still a significant public health issue, particularly among some minorities and disadvantaged groups. Several distinct features are observed in autism spectrum disorder patients including the core behavioral hallmarks of stereotyped and repetitive behaviors, incomplete in social interaction and communication.

Therefore, this research work was done to study the following:

- Chronic exposure of Lead to the pregnant animal mice and study the autism spectrum disorder (ASD) in new born mice
- Behavioral study of lead-induced new born to adult life
- Measurement of blood lead
- Gross pathological changes, if any

2. Material and methods

2.1. Experimental animal

2.1.1. Mice

Animal care and treatment were conducted in accordance with the institutional guidelines of Bangladesh Agricultural University, Mymensingh-2202. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Bangladesh Agricultural University. Eight-week-old Swiss albino mice of both sexes were maintained under a constant 12-hour light–dark cycle at an environmental room temperature. They were supplied by Animal Resources Branch, ICDDR, B, Mohakhali, Dhaka. After acclimatization, a total of 30 Swiss albino mice of both sexes were divided into three groups having 6 female and 4 male mice for each group. Out of three groups, two groups of mice were supplied rat pellets as food and lead treated drinking water (30 ppm and 230 ppm respectively) ad libitum until the experimental period. The control mice were supplied rat pellets and normal drinking water ad libitum. Every morning, female mice were checked for their matting status such as swollen and red vulva (proestrus status) or vaginal plug (mating status). At the advanced stage of pregnancy, male mice were separated from the female mice. New born offspring were carefully observed from the day of birth until the eight weeks. At the age of four weeks, male offspring were separated from the female. Morphological, behavioral observation and other autism spectrum disorder were observed to explore the differences, if any, among the group's offspring.

2.1.2. Day old offspring

The female mice got pregnant and gave birth after 19-21 days of pregnancy. In case of control group, one mouse gave birth to a litter, consisting of 10-13 mouse pups; while lead-induced mice gave birth to a litter typically consists of 5-6 pups. From day 1 to 8 weeks old, both control and lead-induced pups were carefully observed; body weight, morphology, distinct characteristics were recorded in every alternate day.

2.2. Autism spectrum disorder investigation tools

Autism spectrum disorder (ASD) represents a heterogeneous group of disorders characterized by difficulties in social interaction, verbal and nonverbal communication, repetitive behaviors [9]. It outlines the combination of impairments in social interactions and repetitive behaviors starting at the early phase in life. The diagnosis of ASD is primarily based

on behavioral evaluation as potential reliable biomarkers are still in an investigative phase [9]. Autism is diagnosed on the basis of early-emerging social and communication impairments and rigid and repetitive patterns of behavior and interests. The demonstration of these varies greatly with age and ability, and the concept of an autism spectrum has been introduced to recognize this diversity [10].

2.3. Marble burying test

Marble burying test is a widely used tool to assess the repetitive behavior relevant to obsessive-compulsive disorder and ASD in mice. It reveals repetitive behavior more than novelty [11]. Mice exhibit various species-typical behaviors such as digging and burrowing. They dig to find food or to create a refuge from predators or cold and to make a safe zone for the young [12]. This protocol is done with some modification stated by Takeuchi et al. [13] and Chang et al. [9]. Marble burying test serves as a measurement of repetitive digging behavior displayed by mice. In the animal laboratory with a quiet environment, three clean standard mouse housing cages were filled with fine shaped wheat husk bedding to 6 cm in depth as densely packed bedding. For consistent results it is important to make sure that the fine wheat husk bedding is evenly distributed. Three furnished cages were placed next to each other to allow recording of all cages at the same time. Twenty four clean marbles were placed on the top of the bedding in each cage with the design that marbles are evenly spaced throughout the cages in a three rows. After each use, the marbles were washed with detergent and rinse thoroughly with tap water, then with 70% ethanol to remove any residual odor and kept it for subsequent use. Photographs were taken before and after placement of mouse on the bedding at 0, 15 and 30 minutes. Video record was taken for 30 minutes to analyze the performance and behavior of each mouse. The experiments were repeatedly investigated for precise understanding of control and lead-induced offspring model mice performance and behavior. A marble is considered buried when more than two-thirds of its volume is covered by fine wheat husk bedding.

2.4. Three chambered social interaction test

The Three-Chambered test helps to detect general sociability and social novelty in rodent models of CNS disorders. They usually choose to spend more time with another rodent (sociability) and will investigate a novel intruder more than a familiar one (social novelty). Based on these evaluations, the Three Chambered test can assist to identify rodents with deficits in sociability and/or social novelty. The experimental design of this test allows evaluation of two critical but distinguishable aspects of social behavior, such as social affiliation/motivation, as well as social memory and novelty. "Sociability" in this case is defined as tendency to expend time with another mouse, as compared to time spent alone in an identical empty chamber [14]. "Preference for social novelty" is defined as propensity to spend time with a previously unencountered mouse rather than with a familiar mouse. The three-chambered social preference test was assessed with a modified technique stated by Kazdoba et al. [15] and Chang et al. [9] in lead-induced born model mice. It is one of the frequently used techniques preferred to assess the rodent models of autism spectrum disorder (ASD) [16]. This test evaluates the tendency to spend time with another mouse (sociability), and inclinations to social novelty, including the ability to choose between new and familiar mice. Two classes of experimental mouse, a control, naive or "unfamiliarized" animal and other is the test subject needed for the experiment. For the control mouse use a mouse of the same background, age, gender and weight, without any prior contact (not littermates) with the subject mouse.

Each mouse is tested for three trials: Habituation, sociability, social novelty. During the habituation phase, the holding cups in the two side-chambers are empty. Then the mouse, move temporarily to a cage and the investigator sets up for the next trial. For the sociability trial, place an age- and sex-matched novel mouse into one of the holding cups. Then re-introduce the test subject (first mouse) into the middle chamber of the testing apparatus, and allow it to freely explore all three chambers for 10 min. Then the tester mouse was moved temporarily to a clean housing cage while the investigator sets up for social novelty trial. The first novel mouse should be placed randomly and equally distributed between the two holding cups in order to control the chamber preference by the tester mice, which may be a confounding factor.

For the social novelty trial, add a second age and gender-matched novel mouse (N2) into the holding cup that was empty during the previous sociability trial. Then the test mouse should place back into the middle chamber allowing it to freely explore all three chambers for 10 min. Then collection of data and analyze the three-chambered sociability test.

The three-chambered sociability test generally comprises of tracking the location of the test mouse over the duration of the test and determining the time spent in each chambers during all three trials. The presence of a novel mouse is going to elicit stronger exploratory interest, resulting more time spent by the tester mouse near the novel mouse compared to the empty holding cup side. Resulting elicit of more interest in the novel mouse than the familiar mouse. To assess physical interactions between the test mouse and the novel mouse, an interaction zone (defined as 10 cm radius from the base of the metal cup) can be added as a sub-region in the experimental arena design. Additionally, score grooming

and rearing responses to assess parameters that are known to influence social behavior such as anxiety and exploratory drive.

The test mouse is placed in the center chamber. One of the outer chambers comprises an empty restrainer and the other one comprises a restrainer with an unfamiliar mouse. The time, test mouse spends in each chamber and additional parameters of social interaction is recorded. Normal mice shows more interest in the unfamiliar mouse than the empty restrainer, while disturbed one shows no preference. Then another unfamiliar mouse is placed in the empty restrainer. Now, mice with normal sociability should prefer contact with the new unfamiliar mouse

Habituation   Sociability   Novelty

Device set up: ASD diagnostic tool (Three chambered social interaction tool, Pharmacology laboratory, Bangladesh Agricultural University, Mymensingh-2202)

Lead-induced born mice and control mice were investigated in three chambered social interaction test. Habituation, sociability and novelty were recorded for every ten minutes each and repeatedly done for accuracy authentication. A difference score was used to compare the amount of time spent among the group's mice and among the chambers.

2.5. Self-grooming

Mice are sensitive to stress and several genetic and manipulations, which may change its activity. [17] Grooming pattern permits assessment of stress levels allowing distinguishing of grooming phenotypes in numerous mouse strains. [17]

Self-grooming is one of the forms of grooming behavior that is usual in mice. It is estimated that a mouse grooms for about 40% of its walking time. Mice have face validity to repetitive behaviors in autism spectrum disorder, normal patterns but unusually long bouts of self-grooming are considered a behavior of autism model mice. Self-grooming test was assessed as stated by Chadman et al. [18]. The subject mouse was placed in a clean standard mouse cage (beaker) for a 5 minute acclimation period. Following acclimation, each mouse was placed individually into a standard mouse cage and scored with a stopwatch for 10 min for cumulative time spent grooming all body regions [19].

2.6. Blood Serum Lead level and gross morphology observation

After completing all behavioral investigations, mice were anesthetized, chest was opened and heart blood was collected via syringe puncture at the heart apex as much as possible. Blood serum was used for blood lead concentration detection. The whole body was opened and different vital organs including brain observed carefully for differences, if any. Blood serum lead was determined by the instruction of lead test kit (Lead test kit, Maxim product Ltd.)

2.7. Data analysis

Standard statistical procedures were applied in the analysis of data obtained from experiments and analysis.

3. Results

3.1. Generations of lead-induced autism spectrum disorder offspring mice

Lead-induced ASD offspring mice were generated in Swiss albino mice. Eight-week-old of both sexes 30 mice were divided into three groups and adapted in the laboratory maintaining a constant 12-hour light–dark cycle at an environmental room temperature. Two groups of mice were supplied rat pellets and lead treated drinking water (30 ppm and 230 ppm respectively) ad libitum and the controlled mice were supplied rat pellets and normal drinking ad libitum. When the pregnancy was established, male mice were separated from the female mice. New born pups were carefully observed until eight weeks and male offspring were separated from female at four weeks age. The offspring were observed for morphological, behavioral and other autism spectrum disorder to explore the differences. Like this way, three generations of lead-induced pups were born and investigated. A significant finding is observed that one

control mouse gave birth to a litter consisting of 10-13 pups; while lead-induced mice gave birth to a litter consisting of 5-6 pups (Figure 1). No major gross abnormalities were observed, however, few offspring from lead induced parents were found comparatively smaller at the time of birth than the control one (Figure 2). Lead levels of 30 ppm and 230 ppm were administered and found no observable effects on mortality or morbidity of the exposed mice and offspring and had no observable adverse physical effects, such as abnormal motor function or ataxia.

Figure 1 First generation lead-induced offspring. A, control offspring; B, 30 ppm lead-induced offspring; C, 230 ppm lead-induced offspring.
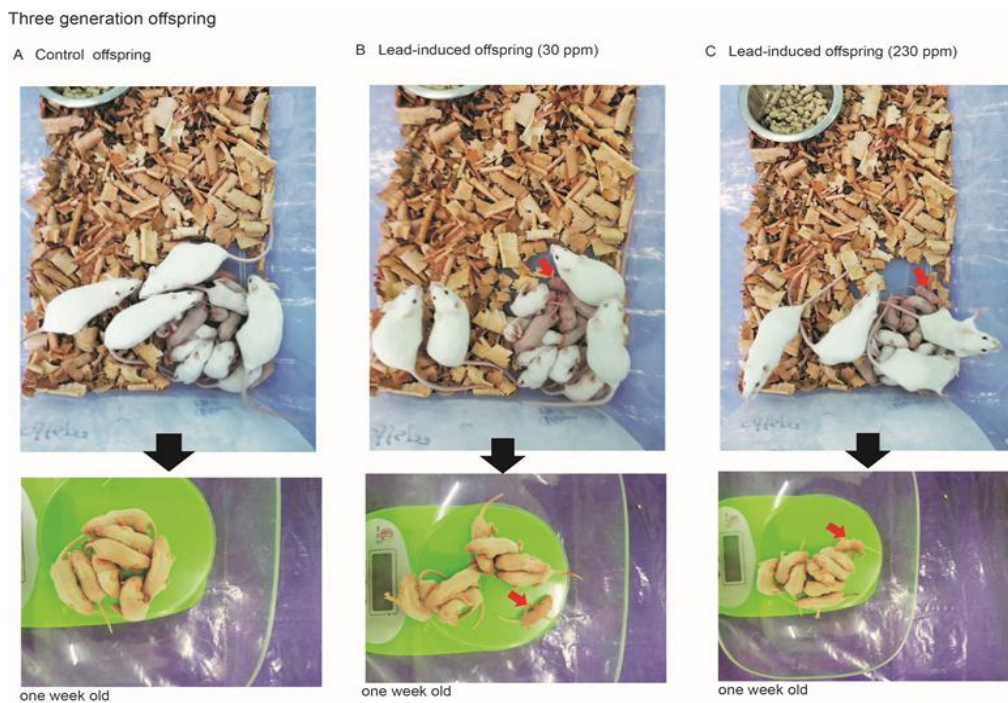

Figure 2 Three generations lead-induced offspring. A, control offspring; B, 30 ppm lead-induced offspring, arrow indicates abnormal small size offspring; C, 230 ppm lead-induced offspring, arrow indicates abnormal small size offspring.

3.2. Marble burying test:

The marble burying test was performed for 30 minutes to assess the anxiety level and repetitive behavior. The marble that completely buried was counted. More marbles were buried in lead-induced mice; however these were not statistically significant (Figure 3) compared with the control mice. These findings indicated that there are no major influences of lead to induce autism in the lead-induced born offspring.
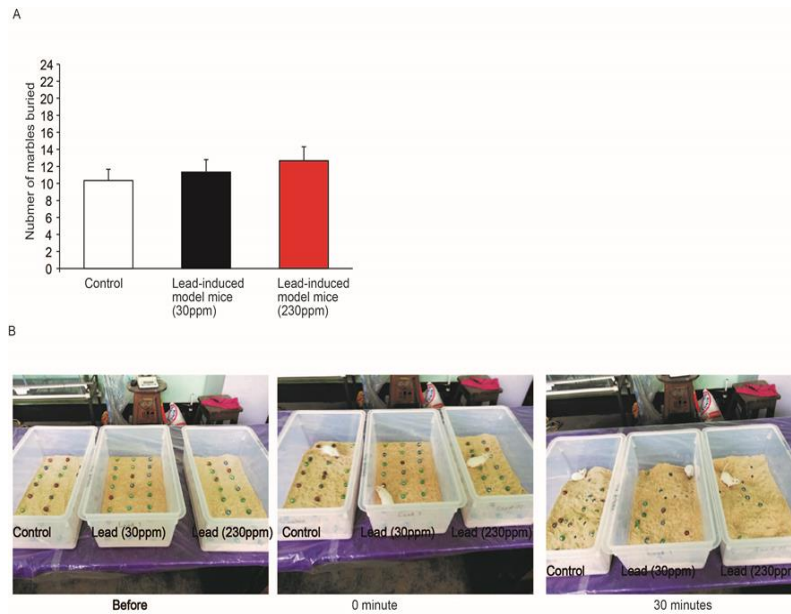
Figure 3 Marble burying test. A, graphical representation of marble burying of control and lead-induced mice. B, marble burying screen shots taken before, 0 minute and 30th minute trial time. n=5

3.3. Three chambered social analysis

The evaluation of three-chamber sociability test was performed for duration of 10 minutes (Figure 4A). It was found that regardless of sex and age both genotype groups spent the most time exploring the novel mouse chamber, it indicates their preference for social vicinity (Figure 4B). Deficits in sociability and ability to recognize social novelty have not been markedly observed in lead-induced mice (Figure 4A, 4B).
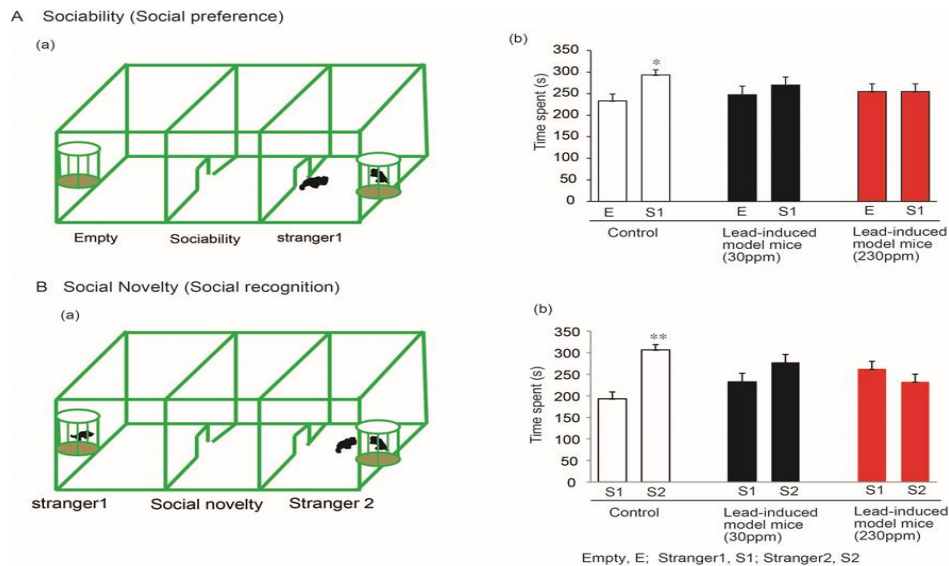

Figure 4: Social behaviour: three-chamber sociability test. Sociability behaviour was determined using the three-chamber test. 4(A)-a, representative photograph showing social ability. 4A-b, control mice spent more time with the stranger1 mouse than the lead-induced mice. 4B-a, representative photograph showing social novelty. 4(B)-b, control mice spent more time with the stranger2 mouse than the lead-induced mice. Animals from at least three different litters were tested for each experimental group. Data are presented as mean ± SEM, *P < 0.05, **P<0.01, n=5

3.4. Self-grooming test

The self-grooming level wasn't that much high among the group's mice. Both 30 ppm and 230 ppm lead-lead induced offspring were found higher level of self-grooming pattern, however, it was not statistically significant (Figure 5).
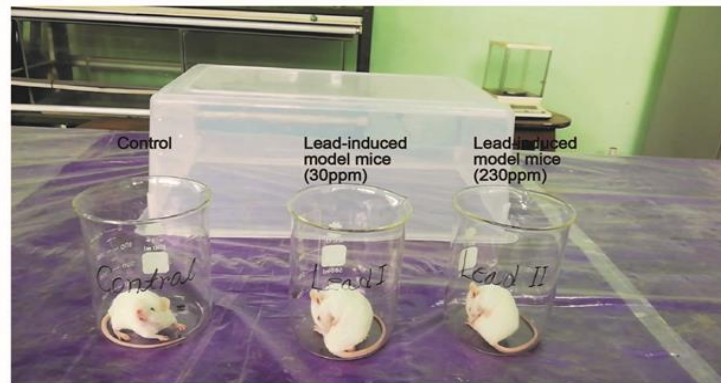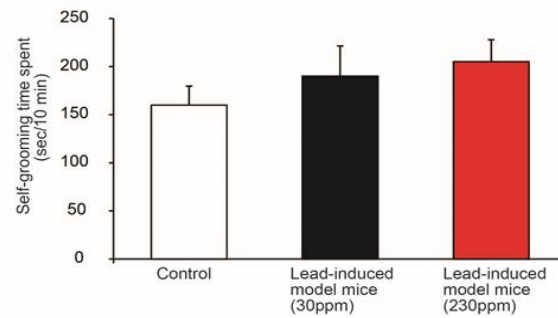
Figure 5 Self-grooming in mice. A, representative demonstration of self-grooming pattern of different groups of mice. B, self-grooming pattern of control, 30 ppm and 230 ppm lead-induced mice. There was no statistical significance. n=5

3.5. Blood Serum lead level:

After sacrificing, blood serum level was determined and presented in figure 6. Lead level of control, 30 ppm lead-induced and 230 ppm lead-induced mice were 0.333µg/dL, 3.833µg/dL and 9.666µg/dL respectively. An interesting finding was that despite of presence of high level of lead in blood no visible demonstration of autism spectrum disorder in any generations of lead-induced offspring and mice.
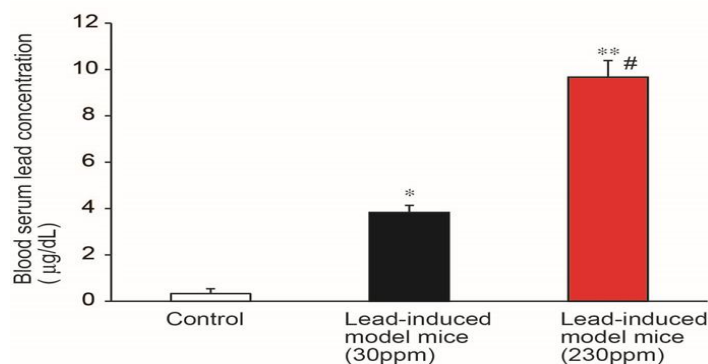

Figure 6 Blood serum lead level. Both lead-induced mice were found significantly higher level of blood serum lead concentration. Data are presented as mean ± SEM, *P < 0.05, **P<0.01; compared with control and #, compared between 30 ppm lead-induced and 230 lead-induced mice. n=5

4. Discussion

Autism spectrum disorder (ASD) is one of the most common neurodevelopmental disorders [20] that affects communication and behavior and is thought to be associated with some environmental contaminations. ASD is

measurable disorder that can be determined by gene analysis, social behavior, reactions and so on. Although autism can be diagnosed at any age, however, symptoms usually appear in the early stage of life. People with autism express difficulty with social communication, interaction, specific interests and repetitive behaviors. Although genetic aberration is the main factors for ASD, however, environmental factors are associated with ASD has been considered. Research has suggested that chronic low-level lead exposure diminishes neurocognitive function in children [21]. Mouse is a useful model for study effects of early chronic low level lead exposure on ASD. This is first animal model study in Bangladesh about ASD. The children's exposure to higher levels of environmental lead has been reduced using substantial steps; however, early chronic low-level lead exposure remains an unresolved child public health problem and child health disparity [21, 22]. Over the past 30 years more than 50 longitudinal and cross-sectional studies have shown that blood lead levels as low as 2µg/dL are associated with lower measured intelligence, reduced neurocognitive function and/or impaired motor functions [21].

Eight-week-old of both sexes Swiss albino mice were used for breeding purpose. They were maintained under a constant 12-hour light–dark cycle at an environmental room temperature. Mice were accustomed and mice of both sexes were divided into four groups having 6 female and 4 male mice for each group. Out of three groups, two groups of mice were supplied rat pellets as food and lead treated drinking water (30 ppm and 230 ppm respectively) ad libitum until the experimental period. The control mice were supplied rat pellets and normal drinking ad libitum. Every morning, female mice were checked for their matting status such as swollen and red vulva (proestrus status) or vaginal plug (mating status). At the advanced stage of pregnancy, male mice were separated from the female mice. New born pups were carefully observed from the day one of birth until the eight weeks. At the age of four weeks, male offspring were separated from the female. Morphological, behavioral observation and other autism spectrum disorder were observed to explore the differences, if any, among the group's offspring. Like this way, three generations of lead-induced pups were born and investigated. One interested finding is observed that one control mouse gave birth to a litter; each litter consists of 10-13 mouse pups; while lead-induced mice gave birth to a litter typically consists of 5-6 pups. No major gross abnormalities were observed, however, few offspring from lead induced parents were found comparatively smaller at the time of birth than the control one. Lead levels of 30ppm and 230 ppm were administered found no observable effects on mortality or morbidity of the exposed mice and offspring and had no observable adverse physical effects, such as abnormal motor function or ataxia.

The marble burying test was investigated to assess anxiety-linked and repetitive behavior for 30 minutes. The marble that completely buried was counted. An increased number of marbles buried was seen in lead-induced mice; however these were not statistically significant (Figure 3) compared with the control mice. Marble burying test is investigated to measure the anxiety level of the object. These findings indicated that there are no major influences of lead to induce autism in the lead-induced born offspring.

Three chambered social interaction analysis is an important investigation to analyze the ASD, if any and any levels [19]. The main principle of this test is based on the free choice by a subject mouse to spend time in any of three box's compartments during two experimental sessions: the sociability session and the social novelty session. The subject mouse was adapted into the three chamber arena and then placed into the middle chamber. In the left chamber, a stimulus mouse is placed under a wire cage and in the right chamber, a similar empty wire cage is located. The time it spends in each chamber and the time spent at each wire cage are measured to quantify the sociability of the experimental mouse. Normal mice will expend more time with the stimulus mouse, while mice with disruptions in social interactions will show no specific behavior. It may spend similar time in both chamber and middle chamber. In the Social Novelty phase, a novel mouse is placed in the empty right chamber under the wire cage, while the already familiar mouse stays in the left chamber. This time normal mice will expend more time with the new unfamiliar mouse due to an inclination for novelty, but socially disrupted mice might not recognize the familiar conspecific and thus may expend similar time in each chamber or the middle chamber. Mice with normal sociability would spend more time in a chamber or in physical contact with a novel mouse than with an empty cage and mice with normal ability to distinguish social novelty would spend more time with an unfamiliar novel mouse than with a familiar mouse. Social behavior was evaluated with the three-chamber sociability test for duration of 10 minutes. Regardless of sex or age, I found that both genotype groups spent the most time exploring the novel mouse chamber, indicative of their preference for social proximity. Deficits in sociability and ability to recognize social novelty have not been markedly observed in lead-induced mice.

Repetitive self-grooming in mice is an important tool to measure the level of autism spectrum disorder developed in mice. High level of self-grooming has not been observed among the group's mice such control, 30 ppm and 230 ppm lead-induced offspring mice. Both groups of lead-lead induced offspring were found higher level of self-grooming pattern, however, it was not statistically significant.

At the end of the treatment period, the mice were sacrificed and blood serum was collected. Blood serum was determined and presented in figure 6. Lead level of control, 30 ppm lead-induced and 230 ppm lead-induced mice were 0.333μg/dL, 3.833μg/dL and 9.666μg/dL respectively. It is important to mention that despite of presence of high level of lead in blood did not demonstrate any visible and remarkable autism spectrum disorder in any generations of lead-induced offspring and mice.

5. Conclusion

Lead is not the pivotal factor of autism development in new born offspring in mice. This study suggested that a genetic predisposition pair with exposure to environmental toxicants play important role in the causes of autism spectrum disorder.

Compliance with ethical standards

Acknowledgments

Disclosure of conflict of interest

The authors state no conflict of interests.

Statement of ethical approval

The laboratory experimental mice were generated and at the end of the experiment sacrificed humanely following the ethical and welfare guidelines set by the Animal Welfare and Experimental Ethics Committee of Bangladesh Agricultural University [approval number: AWEEC/ BAU/2021(08)].

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