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Teratogenicity of Pb to the central nervous system, heart and somites during early organogenesis using chick embryo model

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Abstract

In the present study, the teratogenic effects of Pb to the CNS, heart and somite development at early organogenesis were examined. In this experiment, chick embryo was the test model and the source of Pb was Pb-acetate. Fresh fertilized eggs are incubated for 24 hours and exposure of embryos to Pb-acetate started after 24 hours of incubation, then the embryos are re-incubated for further 24 hours. The LD_{50} of Pb-acetate is calculated and was found to be 759 ppm. For the experimental work 100 fresh fertilized eggs divided into 5 groups containing 20 eggs in each group. Group I served as master control and was treated with 100 µl of the vehicle (acetic acid), while group II served as the control group and was treated with 100 µl normal saline. The remaining groups (III, IV and V) received treatment with 100 µl Pb-acetate at concentrations of 40 ppm (1/20thLD50), 76 ppm (1/10thLD50) and 150 ppm (1/5thLD50), respectively. The teratogenic effect of Pb on the CNS during early organogenesis is seen in the form of undefined brain lobes, branching of the neural tube and the brain, wavy neural tube and splitting of the brain frontal lobe. The teratogenic effect of Pb on the CNS development was highly significant at the dose of 76 ppm (P<0.001) but at the higher dose (150 ppm) and at the lower dose (40 ppm) the effect was significantly low (P<0.01 and <0.05 respectively). The developmental defects of somites induced by Pb are seen in the form of somite dispersion. The effect was highly significant at the dose of 76 ppm (P<0.001), but it was significantly low at the dose of 150 ppm and 40 ppm (P<0.01 and <0.05 respectively). In addition Pb had no significant effect on the heart development at the dose of 150 ppm and 40 ppm (P>0.05) and had low significant effect at the dose of 76 ppm (P<0.05). From the outcomes of this study, it was found that Pb affects the organogenesis of the CNS and somites. However, its effect on the heart organogenesis seemed to be vanished.

Key words: Lead, Embryo, Nervous system, Heart, Somites, Organogenesis.

Introduction

Lead (Pb) is a toxic contaminant metal and is used in the production of eyeliners, cosmetic, makeups, and as an antiknock compound in gasoline. The global consumption of lead during the period of 1970 to 2000 increased from 4.5 million tons to 6.5 million tons (LDAI, 2001). In Yemen, exposure to Pb has become a major

public health concern. People including pregnant women have been alerted to various Pb hazards due to using of Pb-containing cooking plates in restaurants all over the country, even at homes this kind of cooking plates are used for preparing of hot meals. Some Pb-containing plates local manufacturers used to mix Pb from discharged batteries with aluminum in manufacturing process. The amount of Pb that leaches from a plate depends on how the plate is used and what kind of food is put in it. For example, acidic foods and drinks will leach Pb out of plates much faster than non-acidic foods. The longer the food stays in contact with a plate surface that contains Pb, the more Pb will be leached into the food. Heating up food in a Pb-containing plate can speed up Pb-leaching process. A combination of these factors will make the problem even worse. Exposure to Pb can damage the nervous, hematopoietic and renal systems and is particularly harmful to the developing central nervous systems (CNS) of fetuses and children aged less than 72 months (Lidsky et al., 2003). (Kapoor Neeti and Tiwari Prakash, 2013) Reported that when women encountered to lead during pregnancy that can affects development of fatuous brain, they reported also that toxicity from mercury may harm the developing nervous system of human embryo.

In animal experiments, (Ivan Patrick and Jayzon G. Bitacura, 2016) reported that heavy metals like $ZnSO_4$ induced abnormalities during embryonic development of Tripneustes gratilla . Several studies with chick embryo at late stages of embryonic development reported that Pb exposure causes stunted growth and deformities such as defective beak and legs, hydrocephalus, microphthalmia and anophthalmia (Anwe et al., 1987 & Alhifi et al., 2004). Yara and others reported defects on motor behavior of chicks in the first week after hatching due to single dose of Pb acetate administered into the yolk sac on the fifth incubation day (Yara et al., 2008). Chick embryo model became a preferable model for following up developmental toxicity of environmental contaminants during early organogenesis. Gary and others used chick embryo as a model for the study of Pb encephalopathy (Gary et al., 2004). Roberto and others also used chick embryo for studying the early lesion in the brain induced by Pb (Roberto et al., 2005). But detailed information concerning the early developmental defects of the CNS, heart and somite during the early organogenesis is lacking. So the aim of the current research is to study the teratogenic effect of Pb on embryos' CNS (brain and neural tube), heart and somites during early organogenesis at low concentrations that embryo might be exposed to.

Methodology

Source of fertilized eggs: fresh fertilized eggs (just laid) are obtained from Alzahri Poultry Company, Sana'a, Yemen. The source of lead is lead (Pb)-acetate.

LD_{50} calculation: for calculation the lethal $dose_{50}$ (LD_{50}) of Pb-acetate, 60 Fresh fertilized eggs were kept in the electric incubator provided with shaker and divided into six groups (10 eggs in each group). Group I injected with 100 µl 0.9% NaCl, groups II, III, IV,V and VI injected 100 µl Pb-acetate at concentrations of 10, 20, 50, 100 and 200 ppm, respectively. Solutions were injected into the air sac of each individual egg after 24 hours of incubation and re-incubated for further 24 hours, then the embryos were harvested. Eggs shells were removed and embryos were isolated from their membranes and examined under binuclear. Cessation of heart beat of embryos was considered a sign of death. The died embryos were counted and the LD_{50} values calculated by Trimmed Spearman-Karber Method using software given by EPA, version 1.5. The LD_{50} was found to be 759 ppm (as per the software used, the range was from 605.5 to 952.4 ppm with 95% confidence limit).

The experimental work

Total number of 100 fresh fertilized eggs (just laid) were cleaned with water, marked suitably and divided into 5 groups (containing 20 eggs in each group) and incubated at 38 ± 0.5°C and 60-65 % humidity for 24 hours in an electric incubator provided with shaker. Group I served as master control and was treated with 100 µl of the vehicle "acetic acid", while group II served as the control group and was treated with 100 µl normal saline. The remaining groups (III, IV and V) received treatment with 100 µl Pb-acetate at concentrations of 40 ppm ($1/20^{th}$LD50), 76 ppm ($1/10^{th}$LD50) and 150 ppm ($1/5^{th}$LD50), respectively. Injection have been done by injecting 100 µl of the desired concentration of Pb-acetate, vehicle or sterile normal saline into the air sac after 24 hours of incubation. The injection was performed with the help of 100 µl micro-pipette by making a hole (under aseptic condition) into the air sac. Holes were then sealed with cello-tape after injection and eggs were re-incubated for further 24 hours, then the test and control groups were harvested. Egg shells were removed and the embryos were isolated from their membranes according to (New and D. A. T., 1955) and fixed in Bouin's fluid then stained with Heamatoxylin and Eosin. Permanent slides were made for studying the CNS, somite and heart developmental defects

Table 1: Scheme of score for the damage

Damage		**Score**
No abnormality		0
Somite abnormality		1
CNS abnormality	Neural tube	2
	Brain	4
Heart abnormality		3

The score of the abnormalities represents the degree of severity of developmental defect induced by Pb. The somite abnormality has been given less weightage as such abnormality was observed in control embryos as well of the same age. Similarly, neural tube abnormalities also had been noted in control embryos but the frequency was less (Table 1). Therefore intermittent weightage has been given, finding abnormalities such as brain and heart abnormalities was rather rare and only could be seen when extensive damage to the embryo was caused, so these abnormalities had been given the highest score (Alhifi et al., 2004). Graph pad instate software was used for the statistical analysis, (Wardlaw, 1985).

Results

Trimmed Spearman-Karber analysis gave Pb-acetate LD_{50} values of 759 ppm. In the present study there was no teratogenic effect observed in the embryos treated with vehicle, so the developmental defects in this study were due to Pb. The effect of Pb on the CNS development was highly significant at the dose of 76 ppm (P<0.001) but at the dose of 150 ppm and 40 ppm the effect was less significant (P<0.01 and <0.05, respectively) (Table 2).

Table 2: Teratogenic effect of Pb on CNS development (in terms of score)

Dose (ppm)	**40**	**76**	**150**
Effect	0.43 +/- 0.20	0.75 +/- 0.10	0.63 +/- 0.18
P value	<0.05*	<0.001***	<0.01**

Values are expressed as mean ± SEM. Kreskal-Wallis (Non-parametric ANOVA test) followed by Dunn's test. *** P value considered highly significant, ** P value considered moderately significant and *P value considered low significant. number of embryos = 20

Pb-induced developmental defects in the CNS occurred in different types: 1) branching the neural tube and the brain (Figure-1B), 2) wavy neural tube (Figure-2B), 3) splitting the brain frontal lobe, (Figure-3B) and 4) undefined brain lobes or compacted brain (Figure-4B). On the other hand, Pb teratogenic effect on embryo somites organogenesis occurred in the form of somites dispersion, (Figure-2B). This effect was highly significant at the dose 76 ppm (P<0.001) but at the doses 150 ppm and 40 ppm, the effect was less significant (P<0.01 and <0.05, respectively) (Table 3).

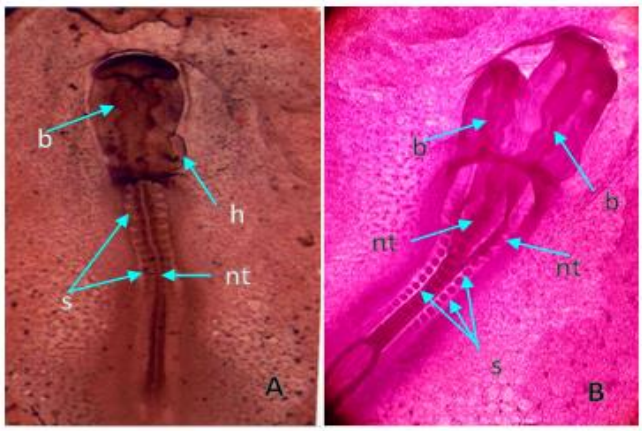

Figure 1: The effect of 40 ppm Pb-acetate in 48 h incubated chick embryo exposed for 24 h (s = somite, nt = neural tube, h = heart and b = brain). (A) control chick embryo, X16, (B) treated chick embryo showing: abnormal; branched into two NT and two abnormal brains, double line and diffused somites, X16

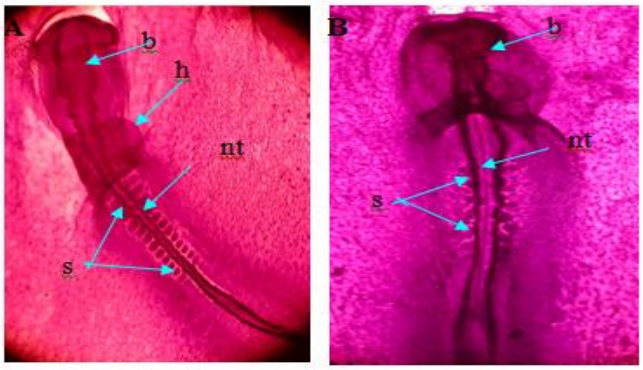

Figure 2: The effect of 76 ppm Pb-acetate in 48 h incubated chick embryo exposed for 24 h (s = somite, nt = neural tube, h = heart and b = brain).(A) control chick embryo, X16, (B) treated chick embryo showing: abnormal undefined brain, wavy NT and less number and diffused somites, X16

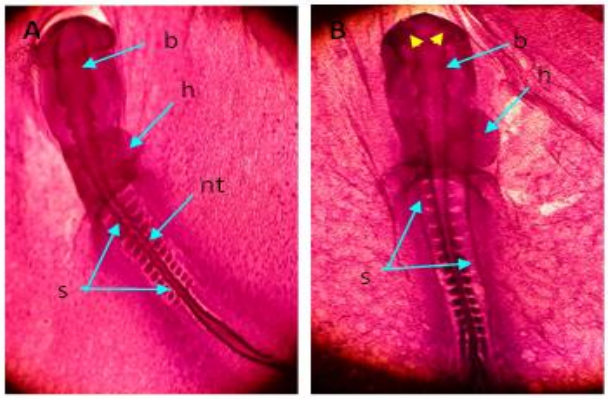

Figure 3: The effect of 76 ppm Pb-acetate in 48 h incubated chick embryo exposed for 24 h (s = somite, nt = neural tube, h = heart and b = brain).(A) control chick embryo, X16, (B) treated chick embryo showing: abnormal splitting in the brain frontal lobe (arrows), abnormal mid and hind lobs and diffused somites, X16

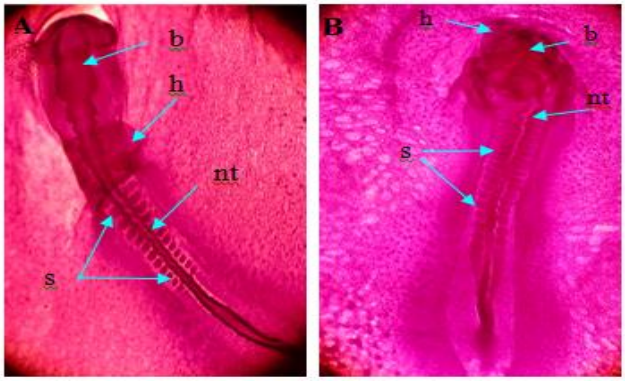

Figure 4: The effect of 150 ppm Pb-acetate in 48 h incubated chick embryo exposed for 24 h (s = somite, nt = neural tube, h = heart and b = brain). (A) control chick embryo, X16, (B) treated chick embryo showing: abnormal compacted brain (arrow), heart misposition and wavy nt, X16

Table 3: Teratogenic effect of Pb on Somite development (in terms of score)

Dose (ppm)	**40**	**76**	**150**
Effect	0.60 +/- 0.25	0.88 +/- 0.13	0.34 +/- 0.18
P value	<0.05*	<0.001***	<0.01**

Values are expressed as mean ± SEM. Kreskal-Wallis (Non-parametric ANOVA test) followed by Dunn's test. *** P value considered highly significant, ** P value considered moderately significant and *P value considered low significant. number of embryos = 20

In addition, the effect of Pb on the heart organogenesis at the doses 150 ppm and 40 ppm was not significant (P>0.05) but it was significantly low at the dose of 76 ppm (P<0.05) (Table 4).

Table 4: Teratogenic effect of Pb on Heart development (in terms of score)

Dose (ppm)	**40**	**76**	**150**
Effect (in terms of score)	0.13 +/- 0.13	0.50 +/- 0.19	0.25 +/- 0.16
P value	> 0.05 ^{ns}	<0.05 *	> 0.05 ^{ns}

Values are expressed as mean ± SEM. Kreskal-Wallis (Non-parametric ANOVA test) followed by Dunn's test. *P value considered low significant, ns = not significant. number of embryos = 20

Discussion

In the current study the data of CNS, somites and heart developmental defects provide evidence of the teratogenic effect of Pb during early stages of embryonic organogenesis. It has been found that the CNS was the most affected embryonic structure by Pb at early stages of embryonic development. In general, heavy metals reported to affect development of the nervous tissues such as branchial nerves, (Ferm et al., 1969 and Van et al., 1996). Pb is reported to interfere with CNS cell micro-molecules during embryonic development (Roberto et al., 2005). Result of the study shown various CNS malformations that may induce by Pb during early embryonic organogenesis. Findings are in consistent with other studies reported that exposure of embryos to Pb has an adverse effect on neurodevelopment which is most pronounced during the

first trimester (Hu et al, 2007). Abnormalities such as microphthalmia and anophthalmia at late stages of chick embryo development are reported by Anwer and others (Anwer et al, 1987). Such abnormalities may be related to the defects in the brain frontal lobes during the early CNS organogenesis as showed in the results of this study. Vertebral, anal, cardiac, tracheoesophageal fistula, renal and limb abnormalities has been reported with prenatal exposure to high lead levels, (Jacobson and Jacobson, 1997). Heavy metals such as mercury show selective inhibition of the neuronal cell, such as the division and migration, so induce CNS developmental defects, (Bose-O'Reilly *et al.,* 2010; Sagiv *et al.,* 2014). In case of human being, the long residence time of Pb in maternal bones may propagate the problem. Then the impairment of the CNS development would be sound due to mobilization of maternal bone Pb, a phenomenon that may constitute a significant public health problem (Gomaa et al., 2002). This propagation in Pb level in the blood is extremely harmful to the rapidly developing central nervous system in the fetus as it crosses the placenta easily (Rastogi et al. 2007). At elevated maternal blood Pb levels, the developing fetus may be at greater risk of Pb exposure from increased maternal plasma Pb than otherwise predicted from whole-blood Pb levels (Lamadrid-Figueroa et al., 2006). Moreover, CNS impairments during early organogenesis may extend to childhood period and affect their behavior. Previous studies reported that in ovo exposure to Pb induces important deficits on motor behavior of chicks during the first postnatal week and such phenomena are related to Pb deposition in the cerebellar tissue during embryonic development (Yara et al., 2008). Pb was also associated with negative outcomes in children, including impaired cognitive, motor, behavioral, and physical abilities. The outcomes of this study may explain the infant attention reported by (Plusquellec et al., 2007), who related infant attention to cord blood-Pb. Behavior changes of children exposed to Pb are more significantly than that of non-exposed children (Mendelsohn et al., 1998). Developmental effect of Pb seems to be complicated; that the low embryo weight was related to high blood Pb levels. Alternatively, low embryo weight may increase Pb absorption and retention in embryo, in such case the effect seems to be sound (Recknor et al, 1998). Glucose uptake also in the brain cortex of the infant is found to be altered by Pb (Nowak et al., 2007), such alteration of glucose uptake certainly will affect the CNS development (Pawlowski et al., 2006). Recent researches have strengthened the evidence that children's physical and mental development can be affected at very low blood Pb levels (Wkly Report, 2007), but still so many factors determine the risk of Pb developmental toxicity. Infants born to women who smoke, drink and maintain poor nutritional status for some nutrients are at a greater risk of Pb toxicity than those born to other women (Lee et al., 2005).

Somite developmental impairment induced by Pb may be explained by its interference with some specific proteins during early organogenesis. On the other hand, somites contribute to the formation of the skull and the backbone of the embryo so any defects in the somites at the early organogenesis will lead to deformities in the skull, notochord and the backbone. This was reported by (Landauer, 1960), who observed the neck malformation after nicotine injection into chick eggs between 0 and 120 hours of incubation. The author concluded that nicotine affects a special protein that has an important function in the developmental steps that precede formation of the spine and the cervical region in particular. The result of this study is in consistent with other studies reported the morphological abnormalities induced by Pb in the African Catfish (Osman et al., 2007), such as irregular head shape, notochordal defect and eye malformation. Moreover, any defects in somite development may lead to improper axonogenesis of the embryo. Studies reported that exposure of embryo to heavy metals such as cadmium induced abnormal somite patterning of the muscle fibers and defects in axonogenesis of the embryo (Chow and Shuk, 2003). The effect of Pb on the heart development at early organogenesis seems to be low as compared to its effect on the CNS and somite organogenesis.

It has been reported by some authors that, Pb developmental toxicity depends on many factors. These intra individual factors may alter Pb developmental toxicity (Bellinger et al., 1992). Such factors could be polymorphic alleles of genes coding for proteins involved in the partitioning of circulating Pb, the most important being δ-aminolevulinic acid dehydratase (ALAD), among unidentified binding sites for Pb (Dietrich et al., 1993 and Wardlaw, 1985). A recent contribution described that polymorphisms in the ALAD genes are strongly associated to plasma/blood Pb ratios (Lanphear et al., 2000). Other findings raise the possibility that fetuses of women with a tendency to have a lower erythrocytic Pb binding capacity, reflected in higher plasma/blood Pb ratios, and consequently greater plasma Pb levels for a given whole blood Pb concentration, would be more exposed to Pb and at a greater risk of developmental toxicity (Needleman et al., 1990). The developmental defects induced by Pb at early stages of embryonic organogenesis found in the present study may impair the survival of embryos to later stages. This may explain the elevated risk of miscarriage associated with increased maternal blood-Pb level (Pawlowski et al., 2006). The plasmatic fraction of Pb represents the toxicologically active fraction that affect the embryo easily, so women with a tendency to have a higher plasma/whole blood Pb ratio could have an elevated risk of miscarriage due to a higher plasma Pb for a given whole blood Pb and would consequently have a history of spontaneous abortion, which could be due to a greater availability of placental barrier-crossing Pb to the embryo (Borja-Aburto et al., 1999 & Lamadrid-Figueroa et al., 2006). The presence of other metals may mitigate Pb intoxication; Pb developmental defects reported to be low in the presence of calcium and zinc (Anwer et al., 1988). Results of the present study showed highly significant effect of Pb at early organogenesis of CNS, somites and heart at the dose of 76 ppm. Low significantly effect was shown at the doses 40 ppm and 150 ppm. This may be related to the high concentration of Pb at the dose of 150 ppm that may decrease or block the permeability of embryonic membranes to lead

molecules at this stage of early organogenesis. On the other hand, Pb concentration at the dose of 40 ppm was low enough to show less effect. This suggests that Pb concentration is important factor and embryonic membranes permeability to lead molecules may get affected and then its intoxication at early organogenesis, so further studies are recommended.

Conclusion

From the outcomes of the current study, organogenesis of the CNS is the most sensitive towards Pb followed by somites organogenesis. The developmental defects, in particular those of the CNS induced by Pb may have relation to the tension, behavioral, and intellectual problems and such developmental complications that may affect children educational achievement. Pb interferes early somites organogenesis and such defect may induce, at early organogenesis, head abnormal shape and neck abnormalities that have been reported to be occurred at late stages of embryonic development exposed to Pb earlier.

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