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Perinatal exposure to lead induces morphological, ultrastructural and molecular alterations in the hippocampus

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ABSTRACT

The aim of this paper is to examine if pre- and neonatal exposure to lead (Pb) may intensify or inhibit apoptosis or necroptosis in the developing rat brain. Pregnant experimental females received 0.1% lead acetate (PbAc) in drinking water from the first day of gestation until weaning of the offspring; the control group received distilled water. During the feeding of pups, mothers from the experimental group were still receiving PbAc. Pups were weaned at postnatal day 21 and the young rats of both groups then received only distilled water until postnatal day 28. This treatment protocol resulted in a concentration of Pb in rat offspring whole blood (Pb-B) below the threshold of 10 μg/dL, considered safe for humans.We studied Casp-3 activity and expression, AIF nuclear translocation, DNA fragmentation, as well as Bax, Bcl-2 mRNA and protein expression as well as BDNF concentration in selected structures of the rat brain: forebrain cortex (FC), cerebellum (C) and hippocampus (H). The microscopic examinations showed alterations in hippocampal neurons.Our data shows that pre- and neonatal exposure of rats to Pb, leading to Pb-B below 10 μg/dL, can decrease the number of hippocampus neurons, occurring concomitantly with ultrastructural alterations in this region. We observed no morphological or molecular features of severe apoptosis or necrosis (no active Casp-3 and AIF translocation to nucleus) in young brains, despite the reduced levels of BDNF. The potential protective factor against apoptosis was probably the decreased Bax/Bcl-2 ratio, which requires further investigation. Our findings contribute to further understanding of the mechanisms underlying Pb neurotoxicity and cognition impairment in a Pb-exposed developing brain.

1. Introduction

Lead (Pb) toxicity is still a major health problem, associated with both environmental and occupational exposure (WHO, 2010). In developed countries, growing awareness of the effects of Pb on the environment and on human health has resulted in efforts to restrict the use of Pb in the production of fuels, paints, ceramic products, batteries, solder, and a variety of other consumer products (e.g. artificial turf playing fields made of nylon or nylon/polyethylene blend fibers, plastic toys and jewelry) (WHO, 2010). However, the major current source of early childhood lead exposure is still Pb-contaminated house dust (containing deteriorated Pb-based paint, Pb-contaminated soil) and tap water contaminated by leaching lead pipes. Current sources of Pb in ambient air include smelters, ore mining and processing, lead acid battery manufacturing, and coal combustion activities such as electricity generation (CDC, 2012).

Young children are particularly susceptible to Pb exposure from behavioral factors, such as frequent hand-to-mouth activities, greater gastrointestinal absorption and an immature blood/brain barrier (Lidsky and Schneider, 2003; Goldstein, 1984). The developing central nervous system is a primary target for lead. Acute Pb

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contamination in children (lead concentration in whole blood–Pb-B > 70 μg/dL), which is currently very rare, can have a dramatic effect on the central nervous system, i.e. brain edema, convulsions, coma, and lead encephalopathy (CDC, 2002). However, childhood Pb poisoning on a scale unheard of for decades has been detected in rural northwestern Nigeria. A total of 161 deaths in the two villages have been attributed to the incident from May 2009 to May 2010, with hundreds and potentially thousands more people becoming seriously ill (Moszynski, 2010; Dooyema et al., 2012).

Exposure to lower doses of Pb can lead to subtle non-specific disorders in brain functions—reduced perception; impaired cognition, impaired hearing and sight; even disorders in neurobehavioral functioning, including aggression (Schwartz and Otto, 1987; Bleecker et al., 2005). It has also been shown that Pb-B, even below 10 μg/dL, may be one of the factors that induces lower IQ in schoolchildren (Jakubowski, 2011; Canfield et al., 2003; Lanphear et al., 2005; Bellinger and Dietrich, 1994; Bellinger et al., 1992). Moreover, chelation therapy, which is recommended for children with Pb-B above 45 μg/dL, may reduce the amount of Pb in the organism but will not compensate for cognitive and behavioral problems resulting from Pb exposure earlier in childhood (Rogan et al., 2001).

Despite numerous studies, the precise mechanisms by which Pb exerts neurotoxic effects are not fully understood. It has been shown that this toxic metal may cause apoptosis in cultured rat cerebellar neurons (Oberto et al., 1996), hippocampal neurons (Niu et al., 2002), retinal rod cells (He et al., 2000), and PC12 neuronal cells (Sharifi and Mousavi, 2008). Apoptosis, "programmed" cell death, is an active process characterized by morphological features including chromatin condensation, cell and nuclear shrinkage, and oligonucleosomal DNA fragmentation (Sharifi et al., 2010; Cohen, 1997). It is regulated by a number of anti- and proapoptotic genes expressing homologous proteins and by enzymatic cascades. One of the gene families closely related to these regulatory pathways is the Bcl-2 family, which comprises several Bcl-2 related genes (Hockenbery et al., 1990) that promote (e.g. Bax) (Oltvai et al., 1993) or inhibit apoptosis (e.g. Bcl-2) (He et al., 2003). Apoptotic pathways can be regulated and abolished at several distinct points, especially caspase activation (Chetty et al., 2005). Caspases (being a family of Cys proteases) play an important role in neuronal cell death during development as well as after neuronal impairment. The basic mechanism includes the activation of caspase-3 (Casp-3) precursor protein by upstream signals such as the release of mitochondrial cytochrome C and the cleavage of specific aspartate residues in proteins with various structural and regulatory functions, thus leading to cell apoptosis (Cohen, 1997). It is possible that Pb might play a role in the activation of one of the aforementioned processes, ultimately leading to apoptosis. The consequences of apoptosis in Pb-induced brain damage may be reflected in observed behavioral, motor and cognitive impairments.

Although in most cases "programmed cell death" is achieved via a family of caspases, an important number of regulated apoptosis pathways are caspase-independent (Pradelli et al., 2010). Moreover, more recent data has revealed that there are also active caspase-independent necrotic pathways defined as necroptosis (programmed necrosis) (Delavallée et al., 2011). This Bax-mediated mitochondrial release of apoptosis-inducing factor (AIF) and its translocation to the nucleus promotes chromatinolysis and is a critical factor in programmed necrosis.

The pro-apoptotic effects of Pb in the rat brain have been reported (Han et al., 2007; Chao et al., 2007; Zhang et al., 2004; Sharifi et al., 2010; Liu et al., 2010; Kiran Kumar et al., 2009). However, Pb-induced necroptosis in the rat brain has not been studied yet. Moreover, only a few works have analyzed the neurotoxicity of Pb at blood concentrations considered safe for humans (below 10 μg/dL, CDC, 2007), particularly in the developing brain.

Brain-derived neurotrophic factor (BDNF) plays important roles in the proliferation, differentiation and survival of neurons during development, as well as in the synaptic activity and plasticity of mature neurons, and is critically involved in synaptic transmission in the hippocampus, as well as in learning and memory (Cohen-Cory et al., 2010). It has been shown that BDNF can also protect neurons from apoptosis (Zhang et al., 2011). Neal et al. (2010) have shown that embryonic hippocampal neurons cultured with Pb had decreased expression of BDNF.

Over the past decade attention has been focused on the hippocampus as a target for lead. The hippocampus is functionally related to vital behaviors and intellectual activities such as memory and learning that are affected by Pb, particularly in young children (Sharifi et al., 2010; Marchetti, 2003) through still undefined mechanism(s). Understanding of the mechanisms of Pb neurotoxicity may provide a basis for developing a new therapeutic strategy aimed at preventing vital behavior abnormalities induced by Pb poisoning.

Hence, the aim of this paper is to examine if pre- and neonatal exposure (Pb concentrations below a 'safe level' in rat offspring blood) may intensify or inhibit apoptosis or necroptosis in the developing rat brain. We studied Casp-3 activity and expression, AIF nuclear translocation, DNA fragmentation, as well as Bax, Bcl-2 mRNA and protein expression and BDNF concentration in selected structures of the rat brain: the forebrain cortex (FC), cerebellum (C) and hippocampus (H). Our microscopic examinations showed alterations in hippocampal neurons.

2. Materials and methods

2.1. Animals

Procedures involving animals were carried out in strict accordance with international standards of animal care guidelines and every effort was made to minimize suffering and the number of animals used. Experiments were approved by the Local Ethical Committee on Animal Testing at the Pomeranian Medical University in Szczecin, Poland (approval No 30/2008).

Three-month old female (250 ± 20 g) Wistar rats ($n = 6$) were kept for a week in a cage with sexually mature males (2:1). All animals were allowed free access to food and water and were kept in a room with a controlled temperature under a LD 12/12 regime. After a week, they were separated from the males, and each female was placed in an individual cage. Pregnant females were divided into two groups: control and experimental. Females from the experimental group ($n = 3$) received 0.1% lead acetate (PbAc) in drinking water *ad libitum*, starting from the first day of gestation. The solution of PbAc was prepared daily in disposable plastic bags (hydropac, Anilab, Poland) from solid reagent directly at the desired concentration, the solution was not acidified. Pregnant females from the control group ($n = 3$) received distilled water until weaning of the offspring. The volume of intaken liquids did not differ significantly between the experimental and control rats. Offspring (males and females) stayed with their mothers and were fed by them. During the feeding of pups, mothers from the experimental group were still receiving PbAc in drinking water *ad libitum*. Pups were weaned at postnatal day 21 (PND 21) and placed in separate cages. From that moment, the young rats of the study and control groups received only distilled water *ad libitum* until PND 28.

We chose oral administration of 0.1% lead acetate as it mimics environmental exposure and is used as a common model of lead poisoning in rats (Kang et al., 2009; Xu et al., 2005). In addition, from our preliminary study and previous experiments (Baranowska-Bosiacka et al., 2012), we knew that this treatment protocol results in a concentration of Pb in rat offspring whole blood (Pb-B) below the threshold of 10 μg/dL considered as safe for humans (CDC, 2007). As our aim was to obtain a Pb concentration below 10 μg/dL Pb-B, we stopped Pb administration after the period of breast feeding, because weaning and feeding pups with regular food and water containing PbAc resulted in a rapid increase in whole blood Pb concentration above the desired threshold level.

We randomly selected 12 young animals (6 from the experimental and 6 from the control groups) for each of 6 measurement techniques (light and electron microscopic studies, western blotting, enzyme activity, RT PCR, immunoassay). The total number of pups was 72 (36 experimental rats and 36 controls). There were no significant differences ($p > 0.45$) between female and male pups in measured parameters. Therefore, we used all pups regardless of gender. Proportions of male and female pups in the experimental (18M/18F) and control groups (17M/19F) were not significantly different ($p = 0.5$, Fisher exact test).

The unanaesthetized pups were sacrificed by decapitation using scissors; brains were quickly removed and dissected into three regions: cerebellum (C),