INTRODUCTION

Lead (Pb) is a metal used by humans for millennia. Its production increased dramatically during the industrial revolution, causing intense release and massive accumulation of this indestructible metal in the environment. Pb poisoning is one of the oldest and most widely studied occupational and environmental hazards. Acute exposure to low dose levels of Pb results in gastrointestinal disturbances (anorexia, nausea, vomiting, and abdominal pain), neurological effects (encephalopathy, malaise, and drowsiness), hepatic and renal damage, and hypertension. Blood Pb levels as low as 10 μg/dl inflict serious damage to the central nervous system of young children, producing developmental delays, lower intelligence quotient (IQ), hyperactivity, learning disabilities, behavioral problems, and school failure (Sen et al., 2002). Moreover, in adult humans, Pb poisoning is a potential factor in brain damage, mental impairment and severe behavioral difficulties, neuromuscular weakness, and coma (Flora et al., 2007a). In experimental Pb poisoning, Flora et al. (2008) reported that Pb causes neurological and behavioral changes in rats chronically ingesting Pb acetate in drinking water.

Several lines of evidence indicate that Pb induces a broad range of physiological, biochemical, and behavioral dysfunctions in experimental Pb poisoning rats as...
well as in humans (Ruff et al., 1996; Wigle et al., 2008). Lanphear et al. (2000) have shown that Pb exposure is associated with significant deficits in IQ of children. However, IQ is a global construct of cognition, and fewer studies have assessed the impact of Pb on specific domains of internalizing behavior such as anxiety (Roy et al., 2009). Recently, we reported that behavioural signs of anxiety and anhedonia as well as changes in neurotransmitters production were found in Pb-exposed rats (Seddik et al., 2010). These changes were reversed by olive leaf extract (OLE) administration. Many authors attribute the neurological symptoms of Pb toxicity to the ability of 5-aminolevulinic acid to inhibit either the K+-stimulated release of γ-aminobutyric acid (GABA) from pre-loaded rat brain synaptosomes or GABA binding to synaptic membranes (Brennan et al., 1995). Furthermore, it has been observed that Pb increases reactive oxygen species (ROS) levels along with intracellular Ca2+ elevation which, in turn, evokes a decline of mitochondrial potential with apoptosis via cytochrome c release (Flora et al., 2007b). Xu et al. (2006) have shown that Pb elicits DNA damage and apoptosis in PC 12 cell line, accompanied by an increase in the Bax/ Bcl2 ratio and caspase-3 activity. Furthermore, Pb is known to possess pro-oxidant catalytic activity with respect to lipid peroxidation (LPO) via ROS generation. Yin and Lin (1995) have demonstrated significant enhancement of the LPO product malondialdehyde when Pb is incubated with linoleic acid, linolenic acid and arachidonic acid. Similarly, a number of researchers have observed an enhanced LPO rate and a decrease of antioxidant defence in the brains and liver of Pb-exposed rats (Bokara et al., 2008). The level of LPO products was directly proportional to Pb concentrations in brain regions (Saxena and Flora, 2006).

The objective of this study, which expands our previous one (Seddik et al., 2010), was to assess the ability of OLE to prevent Pb-induce biochemical changes leading to apoptosis, inflammation, oxidative stress, alteration of cell metabolism as well as to anxiety.

**MATERIALS AND METHODS**

**OLE preparation**

OLE was prepared as described previously (Seddik et al., 2010). Briefly, olive tree leaves were collected from the Oran region of North-West Algeria in summer. They were cleaned, dried, and grounded with a blender. 500 g of leaf powder were submitted to extraction with 1.5 l distilled water in a Soxhlet apparatus for 1 hr at 60°C. After extraction, the solvents were filtered and evaporated. The extract was stored at -20°C until used.

**Animals and experimental design**

4-weeks old male Wistar rats (n = 24) weighing ~70 g were purchased from Charles River (St-Constant, Quebec, Canada) and housed in pairs under standard conditions: 12/12-hr light/dark cycle with light onset at 8:00 AM, 21-25°C temperature, 40-50% relative humidity, and food and water ad libitum.

Twenty four rats were used in this study and were divided into 3 groups of 8 animals. Group 1 (Control): rats (n = 8) received tap water for 15-weeks ad libitum; Group 2: rats (n = 8) received tap water containing 250 mg/l Pb acetate for 13-weeks ad libitum and tap water for an additional 2-weeks ad libitum; Group 3: rats (n = 8) received tap water containing 250 mg/l Pb acetate for 13-weeks ad libitum and tap water containing 0.1% (w/v) OLE for an additional 2-weeks ad libitum. Water intake and body weight were measured daily and weekly, respectively. The dose of Pb was chosen on the basis of previous studies from our laboratory (Ait Hamadouche et al., 2009) and on literature data (Hamilton and O’Flaherty, 1994). At this concentration, Pb induced wide range of biochemical and physiological dysfunctions in rats. All experimental protocols in the present work were approved by the local animal ethics committee. The open-field (OF) test was conducted after 15 days post-treatment. All rats were sacrificed by decapitation 24 hr after OF test was completed. Serum was prepared from collected blood, and brain regions were identified, separated, frozen in liquid nitrogen and stored at -80°C until biochemical assessment.

**Pb assay**

Pb level was measured in serum and brain (hippocampus and amygdala), but we performed the most of biochemical analysis in the hippocampus. The levels of Pb were 2 fold higher in the hippocampus than the amygdala (42.25 ± 5.1 μg/g tissue and 23.5 ± 1.9 μg/g tissue, respectively). Briefly, 10 mg of powdered brain tissue were digested in 5:1 nitric acid/perchloric acid, as described by Gupta and Gill (Gupta and Gill, 2000). Pb concentration was quantified by atomic absorption spectrophotometry (Model 1100B, Perkin-Elmer, Phoenix, Arizona, USA), and the data were expressed as μg/l serum or μg/g tissue. The limit of detection was 0.1 μg/mg tissue or 0.1 μg/l serum.

**Serum levels of the LPO product**

4-hydroxynonenal (HNE)

Serum levels of HNE-protein adducts were assessed by enzyme-linked immunosorbent assay (ELISA), as described previously (Benderdour et al., 2003), in serum from control and Pb-exposed rats with or without OLE.
treatment. Data were expressed as pg HNE-protein adducts/ml.

**Enzyme assays**

Total activities of hippocampal enzymes, including glutathione-S-transferase (GST) and NADP-isocitrate dehydrogenase (NADP-ICDH), were measured in hippocampal extracts from control and Pb-treated rats with or without OLE. Briefly, 5 mg of powdered tissues were homogenized on ice in 1 ml buffer containing 180 mM KCl, 5 mM MOPS, and 2 mM EDTA (pH 7) and centrifuged for 10 min at 800 g at 4°C, as described previously (Benderdour et al., 2003). Supernatants were used for enzyme assays after 10-min centrifugation at 6,000 g at 4°C. GST activity was measured with commercial kits (Sigma-Aldrich, Oakville, ON, Canada), according to the manufacturer’s instructions. NADP-ICDH activity was assessed as described previously (Benderdour et al., 2003) in the presence of 5 mM *threo*-DS-isocitrate (Sigma-Aldrich), 0.5 mM NADP (Sigma-Aldrich), and 0.1 mM MgCl₂. The enzyme activities were expressed as μmol/min/mg proteins.

**Immunohistochemistry**

Serial 14-μm thick frozen sections from each animal group were cut at the level of the hippocampus and stored at -80°C prior to immunostaining. The sections were air-dried at room temperature, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of 1.5% normal serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse antibody against caspase-3 (EMD Biosciences, Inc., San Diego, CA, USA) at a 1:500 dilution. After washing with tris-buffered saline (TBS), slides were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Sigma-Aldrich) and observed for 30 min at 4°C Leica SP confocal microscope. In parallel, 4,6-diamidino-2-phenylindole (DAPI) staining was used to identify nuclei.

**Protein detection by Western blotting**

25 μg of total proteins in hippocampal lysates from Wistar rats were loaded for discontinuous 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were then transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada) for protein immunodetection and semi-quantitative measurement. The primary antibodies used were rabbit anti-GSTA4-4 and anti-NADP-ICDH (Abnova, Taipei, Taiwan); anti-caspase-3, anti-Bcl-2 and anti-Bax (EMD Biosciences, Inc.); anti-glucose transporter1 (Glut1) and anti-human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-total and phosphorylated ERK1/2, p38 mitogen activated protein kinase (MAPK), and Akt (Cell Signaling Technology, Inc., Danvers, MA, USA). After serial washes, the primary antibodies were revealed by goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling Technology, Inc.). Immunoreactive proteins were detected with SuperSignal blotting substrate (Pierce, Rockford, IL, USA) and exposed to Kodak X-Omat film (Eastman Kodak Company, Rochester, NY, USA).

**Immunoprecipitation**

Hippocampal tissues were lysed on ice in 1 ml lysis buffer (40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na₃VO₄, 1 mM DTT) supplemented with protease inhibitors cocktail as described previously. A total of 50 μg of total protein was subjected to immunoprecipitation with 1 μg of mouse anti-mouse NADP-ICDH (Abnova) in lysis buffer containing 0.5 M NaCl for overnight at 4°C and then for an additional two hours with protein A (Santa Cruz Biotechnology). The resin was washed with lysis buffer and proteins were removed from the resin by the addition of 50 μl undiluted SDS-loading buffer. The immunoprecipitates were analyzed by Western blotting using rabbit anti-HNE (Cayman Chemical Company, Ann Arbor, MI, USA), as primary antibody.

**Caspase-3 activity**

Enzymatic caspase-3 activity was measured with commercial kits. Hippocampal and frontal cortex tissues (5 mg) were washed with PBS and re-suspended in 200 μl of lysis buffer (Sigma-Aldrich), left on ice for 15 min, and centrifuged. Total proteins (10 μg) were reacted with 200 μM DEVD-pNA substrate in the presence of 100 μl of reaction buffer. Protein concentration of the supernatants was quantified according to the bicinchoninic acid method (Pierce). After 16 hr of incubation at 37°C, p-nitroaniline release was measured at 405 nm for caspase-3.

**DNA fragmentation**

Cytoplasmic histone-associated DNA fragments were quantified with Cell Death Detection ELISA PLUS kits (Roche Applied Science, Laval, QC, Canada) according to the manufacturer’s recommendations. Briefly, hippocampal and frontal cortex tissues (5 mg) were lysed with lysis buffer for 30 min and centrifuged at 200 g for 10 min. The supernatant and a mixture of anti-histone-biotin and anti-DNA-peroxidase were added to streptavidin-
coated microplates and incubated for 2 hr at room temperature. After inclusion of the substrate, absorbance was measured at 405 nm.

**Determination of tumor necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β) and PGE₂**

For quantitative analysis of TNF-α, IL-1β and PGE₂ levels, ELISA kits from R&D Systems (Minneapolis, MN, USA) and enzyme immunoassay (EIA) from Cayman Chemical were used according to the manufacturer’s instructions. TNF-α, IL-1β and PGE₂ levels were measured in rat serum, and the reaction was quantified in a microplate reader (Molecular Devices, Menlo Park, CA, USA).

**The OF test**

Rats were tested for OF locomotor activity in a novel environment as previously described previously (Seddik *et al.*, 2010). Briefly, rats were tested in a fifteen minutes session in an OF box. We reported the locomotor activity in the central area of the OF as an indice of animals’ anxiety level. The time spent in the central area and the numbers of entries into the central area were recorded throughout.

**Statistical analysis**

All values are expressed as means ± S.E.M. unless indicated otherwise. Multiple comparisons were made by 2-way ANOVA, as required, followed by the Bonferroni multiple-comparison post-test. Statistical comparisons were performed with GraphPad Prism software, version 4b (GraphPad Software, San Diego, CA, USA). In all tests, the criterion for statistical significance was p < 0.05.

**RESULTS**

**Pb accumulation in serum and brain**

We undertook atomic absorption spectrophotometry to measure Pb levels in serum and brain. Rat intoxication with 250 mg/l Pb for 13-weeks was associated with elevated serum Pb levels of 5 ± 0.4 μg/l (p < 0.001) (Table 1). In addition, our results disclosed significant Pb deposits in the hippocampus, reaching 42.25 ± 5.1 μg/g tissue (p < 0.001) with exposure to 250 mg/l Pb. Interestingly, in all intoxicated rats, OLE administration decreased significantly, but not completely, Pb circulation and concentration in serum and brain, respectively. These data suggest that OLE lowers Pb levels in tissues, probably through its Pb-chelating properties.

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**Table 1. Serum and brain Pb concentrations in rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Pb level (μg/ml)</th>
<th>Hippocampal Pb level (μg/g)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
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<tr>
<td>250 mg/l Pb</td>
<td>5.0 ± 0.4 (***p &lt; 0.001)</td>
<td>42.25 ± 5.1 (***p &lt; 0.001)</td>
</tr>
<tr>
<td>250 mg/l Pb+ OLE</td>
<td>1.5 ± 0.2 (**p &lt; 0.01; &amp; p &lt; 0.05)</td>
<td>17.25 ± 3.6 (**p &lt; 0.001; #p &lt; 0.01)</td>
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The data are means ± S.E.M. of n = 4-8 rats and expressed as μg/L serum and μg/g tissue. **p < 0.01, ***p < 0.001 (control rats versus Pb-exposed rats), &p < 0.05, #p < 0.01 (Pb-exposed versus Pb-exposed + OLE rats).

**Pb exposure induces markers of apoptosis in the hippocampus and frontal cortex**

Compared to the controls, Pb-exposed rats presented an increase of caspase-3 activity by 250% (p < 0.01, Fig. 1A). At the protein level, Pb supplementation decreased pro-caspase-3 and elevated active caspase-3 levels probably via the pro-caspase cleavage process (Fig. 1B, p < 0.01). Caspase-3 expression was also assessed by immunohistochemistry in the hippocampus. The staining intensity for caspase-3 was higher in the tissue from Pb-exposed rats than in Pb-exposed rats treated with 0.1% OLE (Fig. 1C). However, there were no positive cells for caspase-3 staining in the control (data not shown). The Bcl-2 family is involved in apoptosis by regulating membrane permeability and induces cytochrome c release from mitochondria into the cytosol (Cory and Adams, 2002). To investigate the effects of Pb on Bcl-2 and Bax expression, membranes were incubated with antibodies against these proteins. The level of the anti-apoptotic protein Bcl-2 was significantly decreased in the hippocampus from Pb-exposed rats (Fig. 1B, p < 0.01). In contrast, the pro-apoptotic protein Bax increased significantly in the same tissues (Fig. 1B, p < 0.001). Interestingly, 0.1% (w/v) OLE administration prevented significantly (p < 0.01) Pb-induced caspase activation and blocked changes in Bcl-2 and Bax protein expression, when compared to Pb-exposed rats.

In addition, an important characteristic feature of apoptosis was the fragmentation of intact DNA into inter-nucleosomal pieces. As shown in Fig. 1D, the levels of cytoplasmic histone-associated DNA fragments were increased by 2.2-fold when DNA was extracted from the hippocampus of Pb-exposed rats (p < 0.01), which was not observed in unexposed (control) animals. Interest-
Fig. 1. Pb exposure induces markers of apoptosis in the hippocampus. (A) Caspase-3 activity and (B) caspase-3, Bcl-2 and Bax protein levels were measured in hippocampal extracts from rat brains with commercial kits and Western blot, respectively. (C) Expression of caspase-3 revealed by immunohistochemistry in the hippocampus section. 4,6-diamidino-2-phenylindole (DAPI) staining was used to identify nuclei. (D) Cytoplasmic histone-associated DNA fragments were assessed by ELISA in the hippocampus section. The data are means ± S.E.M. of n = 4–8 rats. *p < 0.05, **p < 0.01, ***p < 0.001 (control versus Pb-exposed rats), #p < 0.05, ##p < 0.01 (Pb-exposed versus Pb-exposed + OLE rats). CTL: Control, Casp-3: Caspase-3.
ingly, 0.1% OLE supplementation reduced significantly (p < 0.05) but not completely DNA fragmentation. Collectively, the detection of apoptosis by DNA cleavage confirms the occurrence of apoptosis in the hippocampus of rats subjected to Pb. Finally, we investigated the effect of Pb on other brain sections, including the frontal cortex. Our data showed that OLE-abolished Pb-induced caspase-3 activity (Fig. 2A) and DNA fragmentation (Fig. 2B, p < 0.05) in the tissue, indicating that frontal cortex region will be also a target to Pb accumulation.

**Pb induces alterations in redox status**

Then after, we investigated whether redox status in rats is implicated in part in Pb-induced toxicity. As illustrated in Fig. 3A, chronic Pb exposure resulted in a significant increase of the LPO product HNE compared to the controls. HNE levels (measured as pg of HNE-protein adducts/ml) were found to be significantly higher in animals treated with 250 mg/l Pb than in the control group (Fig. 3A). Maximum levels of HNE-protein adducts reached 90 ± 9.5 pg/ml serum (p < 0.05). However, OLE administration for 2-weeks significantly attenuated the increment of HNE generation in comparison to Pb-treated animals (Fig. 3A). In other experiments, we showed that HNE-detoxifying GST activity and protein...
expression decreased significantly by ~70% (p < 0.05) in Pb-exposed rats compared to the controls (Figs. 3B and 3C). OLE administration significantly prevented Pb-induced inhibition of GST activity and protein expression (p < 0.05). Taken together, these results indicated that Pb evoked oxidative stress through HNE generation and GST inhibition. In addition, OLE may be a helpful antioxidant in counteracting Pb empoisoning via its ability to attenuate LPO and establish normal GST levels.

Energy metabolism was affected by Pb exposure

In the next series of experiments, we investigated whether Pb-exposure impairs the activity of both NADP-ICDH, a crucial enzyme for energy metabolism and redox status (Benderdour et al., 2003), and Glut1, a protein involved in glucose transport and considered as the rate-limiting step in glucose utilization. Our data revealed that Pb inhibited the activity of NADP-ICDH by 40% (p < 0.01, Fig. 4A) without affecting its protein expression (Fig. 4B), suggesting the existence of a posttranslational modification process of this enzyme. Then after, we tested the hypothesis of potential NADP-ICDH inactivation by HNE, a product of free radical-induced LPO. Our choice of HNE was based on our previous studies indicating that this enzyme is a potential target for HNE binding in hypertrophied heart (Benderdour et al., 2003). Our finding revealed an increased of HNE/NADP-ICDH adducts in the hippocampus from Pb-exposed rats by 3.9-fold (Fig. 4C, p < 0.001), suggesting the involvement of HNE in its posttranslational modification. Furthermore, the investigation of Glut1 expression revealed a decrease of its protein level in the intoxicated rats (Fig. 4D). However, all these changes in NADP-ICDH and Glut1 were reversed by OLE administration.

Pb exposure induces the generation of inflammatory mediators

Because brain damages are associated with the generation of inflammatory mediators (Esposito and Cuzzocrea, 2010), we evaluated whether Pb exposure induces TNF-α, IL-1β and PGE₂ production in hippocampus (Figs. 5A-5C). Our finding showed that exposed rats to Pb have an increase level of these inflammatory mediators and reach 79, 17, and 155 pg/ml (p < 0.001), respectively. The increase of these pro-inflammatory mediators was significantly blocked by OLE treatment. Altogether, these data confirm the presence of an inflammatory component in Pb intoxicated rats.

Pb modulates MAPK and Akt phosphorylation

To gain insights into the signalling pathways activated during Pb intoxication, we examined the phosphorylation patterns of MAPKs and Akt after chronic exposure of rats to 250 mg/ml Pb. As shown in Fig. 6, our data indicate that p38 MAPK and Akt phosphorylation levels rose...
in the hippocampus (p < 0.05, p < 0.01)). However, the ERK1/2 phosphorylation levels decrease in the same condition (p < 0.05). Interestingly, OLE administration abolished the modulation of p38 MAPK, ERK and Akt phosphorylation by Pb exposure.

**The OF test**

In the center of the arena, analysis of locomotor activity show that rats treated with 250 mg/l of Pb spent 78% (p < 0.001) less time in the center of the arena (Fig. 7A) and displayed 60% (p < 0.01) less amount of activity (Fig. 7B) compared to controls and 250 mg/l Pb+ OLE groups. OLE treatment increased the time spent into the center area (p < 0.01) but not the amount of the activity with respect to the Pb-exposed animals. Finally, between each trial, the OF was thoroughly cleaned with a 10% ethanol solution in order to obviate possible biasing effects due to olfactory influences on subsequently tested animals.
DISCUSSION

Pb remains a significant public health problem and its neurotoxic effect is well known (Verstraeten et al., 2008). There is growing evidence that Pb is an efficient cell signalling molecule and considered as a key mediator of inflammation and oxidative stress-induced neuro-pathological effects. In fact, by modulating the expression of different genes, Pb exhibits a wide array of biological activities, including signal transduction, and gene expression (Schauder et al., 2010). In particular, the relevance of Pb to brain biology and pathology is now becoming clearer. In our recent study, we reported that behavioural signs of anxiety and anhedonia as well as changes in neurotransmitters production were found in Pb-exposed rats (Seddik et al., 2010). These changes were reversed by OLE administration. In the present study, we provide additional evidence that OLE acts as anti-apoptotic, anti-inflammatory, and anti-oxidant mediator in our experimental model of Pb poisoning.

At the starting point, we evaluated the potential role of chronic Pb-exposure in the apoptotic process in the hippocampus and frontal cortex. With the ultimate goal of clarifying this role, we documented the ability of Pb to modulate markers of apoptosis as well as factors known to be involved in this process including oxidative stress, inflammation, energy metabolism, and different signalling pathways. Firstly, in investigating the classical markers of apoptosis, we obtained data showing that Pb induced caspase activation and DNA fragmentation in the hippocampus of Pb-exposed rats. The activation of caspase-3 was also showed in the frontal cortex from these rats, indicating that Pb induced apoptosis process in different areas of the brain. A major question arising with regard to apoptosis in general is whether the apoptotic response of the hippocampus to Pb exposure requires upregulation of pro-apoptotic protein synthesis or whether it relies on pre-existing apoptotic machinery. Our data show that the apoptotic response of the hippocampus to Pb is associated with decreased Bcl-2 expression and increased Bax expression. This suggests that the hippocampus needs to modulate the synthesis of at least several anti- and pro-apoptotic factors to be able to undergo apoptosis when exposed to Pb. It is noteworthy that Pb is capable of inducing apoptosis in several brain areas, including the hippocampus (Sharifi et al., 2010), through pro-apoptotic factors up-regulation (for example, caspases, Bax, and Bcl-2). In cultured cells, it has been shown that Pb at 10 μM can promote apoptotic cell death in PC12 cell line (Sharifi and Mousavi, 2008).

Apoptosis is noticed as a possible mechanism of Pb-induced cell death in central nervous system (Oberto et al., 1996). Apoptosis is regulated by complex gene-regulated pathways and enzymatic cascades. One of the families of genes closely related to these regulatory pathways is the Bel-2 family, which comprises several Bel-2-related genes, some of which promote apoptosis (Bax, Bad) and some exert the opposite effect on the death pathway and inhibit apoptosis (Bcl-2, Bcl-xl, Bcl-w, NR-13, Mcl-1) (Yang and Korsmeyer, 1996). Our results suggested that Pb-induced neurotoxicity in hippocampus might be due to a facilitation of apoptosis by enhancement of Bax protein expression.

Secondly, we reported that Pb significantly induced HNE-protein adduct accumulation in the serum of rats exposed to 250 mg/l of Pb, indicating an increase in oxidative stress. To determine whether the increase in HNE-protein adducts was related to antioxidant enzyme system impairment, we conducted additional experiments to examine GST activity and expression. Our results clearly
showed decreased GST activity and protein expression in serum and hippocampus, respectively. Recently, Hamed et al. (2010) reported that Pb induced toxicity in different brain areas by interfering on the balance between pro-oxidant/antioxidant. The authors demonstrated that Pb induced LPO products accumulation, decreased glutathione pool and inhibited antioxidant enzyme activities, such as GST and superoxide dismutase (SOD). Moreover, Pb may disturb the antioxidant barrier via the inhibition of functional SH groups present also in free radical-scavenging enzymes such as GST (Vallee and Ulmer, 1972).

Thirdly, we have focused on analyzing the energy status and tissue metabolism in the Pb-exposed hippocampus. Significant inhibition of Glut-1 protein expression and NADP-ICDH activity was observed. Pb is known to be a potent inhibitor of many enzymes working in the brain, thus possibly inducing functional problems under pathophysiological conditions. These enzymes include those involved in glucose metabolism and energy production: hexokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, pyruvate dehydrogenase complex, and succinate dehydrogenase (Verma et al., 2005, Yun and Hoyer, 2000). This suggests that exposure to Pb may increase the risk of cerebral hypometabolism by direct inhibition of specific glucose utilizing enzymes and citric acid cycle enzymes. In this context, Pb might be regarded as a risk factor in the abnormal metabolism of glucose seen in some kinds of neurodegenerative disorders (Yun and Hoyer, 2000). Furthermore, we showed an increase of HNE/NADP-ICDH adducts in the Pb-exposed hippocampus. These data may explain the inhibition of NADP-ICDH activity, since its protein expression was not changed. The modification of this enzyme by HNE results in its inactivation, as reported by our previous report (Benderdour et al., 2003).

The next set of experiments was focused on analyzing inflammation status in exposed rats to Pb. Significant increase of proinflammatory mediators, such as TNF-α, IL-1β and PGE₂ was observed in the serum. Our data are in concordance with that of Suresh et al. (2006) indicating that Pb induced PGE₂ in the hippocampus from Pb-exposed rats. In culture system, Pb is able to modulate the expression of TNF-α in glial cells (Cheng et al., 2002). TNF-α not only increases apoptosis in neuronal cells but also disturbs the integrity of blood-brain barrier (BBB) (Tsao et al., 2001). An excess production of TNF-α in the brain, which is mainly released by microglia and astroglia, is related to neuronal damage in the CNS after a long-term exposure to a low-level of Pb or bacterial infection, as beautifully depicted in a dedicated review (Viviani et al., 2004). Inflammatory factors, such as TNF-α and IL-1β-released by glia or other cell types, are thought to interfere with different types of brain cells and lead to neuron degeneration in vivo (Zindler and Zipp, 2010). In addition, elevated expression of TNF-α has been found in brain trauma (Li et al., 2011). Since TNF-α is able to trigger neuronal cell death by promoting apoptotic pathways (Knoblach et al., 1999) and suppressing survival signals (Venters et al., 1999), TNF-α may play an important role in Pb-induced brain damage.

Investigation of the molecular mechanisms of Pb-induced apoptosis in Pb-exposed rats showed that Pb induced serine/threonine kinase Akt and p38 MAPK activity but in contrast reduced ERK1/2. It has been established that Pb-elicited phospho-Akt (Ser473) participated in the induction of cyclin D1 required in the death of neurons (Li et al., 2008). Akt activation results in the phosphorylation of numerous other proteins involved in the regulation of glucose metabolism, cell proliferation, apoptosis, cell migration, and gene expression (Brazil and Hemmings, 2001). Depending upon the cell context, the MAPK family, which includes JNK/SAPK, p38 and ERKs, is involved in the survival, proliferation and differentiation of nervous cells. Some of the MAPKs promote the differentiation towards the neuron lineage, others towards the glial one. The MAPKs are also involved in apoptosis and may, therefore, play a role in neurodegeneration (Yen et al., 2011). The p38 MAPK and JNKs pathways are implicated in inhibiting bcl-2, which is an anti-apoptotic factor, and phosphorylation of an essential transcription factor, c-Jun (Matsuzawa and Ichijo, 2001). However, Satoh et al. (2011) reported that abrogation of ERK1/2 in the central nervous system caused impaired neurogenesis and apoptotic degeneration, leading to neonatal death.

As a consequence of Pb poisoning in the behaviour, we performed the OF test because the association of blood Pb level with anxiety is important. Rats treated with 250 mg/l of Pb spent significantly less time and less activity in the center of the arena suggesting that they were more anxious than controls. As discussed above, we showed an increase in apoptotic markers in hippocampus and frontal cortex: both areas known to be important in anxiety (Adhikari et al., 2010). In accordance with this study, several other studies with both animals (Antonio and Leret, 2000) and humans (Fraser et al., 2006) have reported that Pb exposure causes neurotoxicity, which is characterized by histological, ultrastructural and neurochemical changes in the central nervous system, as well as behavioral deficits. A study by Bellinger et al. (1994) reported increased internalizing behavior and noted higher anxiety among children. Evidence from animal research indi-
cates that early Pb exposure causes permanent changes in the hypothalamic–pituitary axis, glucocorticoid dysregulation, and altered dopaminergic and GABA-ergic (γ-aminobutyric acid–containing) systems, which are associated with increased anxiety and decreased socializing behavior (Nieto-Fernandez et al., 2006). Moreover studies have shown that rats exposed to low levels of Pb during the first postnatal month have deficits in memory and long-term potentiation that persist in the adult age (Murphy and Regan, 1999). Finally, several studies indicate that Pb affects the activity of the monoaminergic system during development of the CNS (Devi et al., 2005, Sansar et al., 2011). In one hand, some evidence indicates that Pb affects monoamine levels in the brain including serotonin (Leret et al., 2002), which is widely distributed throughout the brain and, in particular, in regions associated with anxiety (Linthorst, 2005). In another hand, the serotonin neurotransmission modulates several physiological and behavioural functions that include affective states, as well as stress-related responses and anxiety (Takase et al., 2004).

Finally, we tested the ability of OLE to prevent Pb-induced abnormalities in exposed rats to Pb. Our finding revealed that the administration of OLE abolished apoptosis process, reduced markers of inflammation and oxidative stress, normalized cell metabolism, and internalize behaviour such as anxiety. Indeed, OLE treatment increased the time spent into the center area but not the amount of the activity with respect to the Pb-exposed animals. These data suggest that OLE reverse the anxiety but not the locomotory activity. We do not currently have an adequate explanation for these data. Future studies need to be directed to elucidate this issue. It has been well-documented that chronic human intoxication with a wide range of metals can be treated with considerable efficiency by the administration of natural chelating agents (Flora et al., 2008). Particularly, extensive research is now focusing on herbal products, to establish a better strategy in treating Pb poisoning. Results obtained in animal studies support the idea that OLE has potentially beneficial effects on certain health conditions, including hypertension, cardiovascular diseases, dia­etes, and hyperlipidemia (El and Karakaya, 2009). These favourable outcomes may be due to its ability to chelate metal ions, which catalyze free radical generation (Yu et al., 2006), and to inhibit inflammatory mediators (Visioli et al., 2002). The analysis of OLE composition showed the presence of large amount of bioactive molecules such as oleuropein and flavonoids (Goulas et al., 2010). These compounds have powerful antioxidant activity both in vivo and in vitro. The mode of antioxidant action has been described as being through scavenging of peroxy radicals, hydroxyl radicals and superoxide anions and the chelation of transition metal ions that catalyze oxidation processes (Paiva-Martins and Gordon, 2005). Flavonoids are known to possess highly reactive hydroxyl groups that become oxidized by electron donation and thus stabilize the radical to a less reactive molecule (Schmitt-Schillig et al., 2005). Furthermore, flavonoids are known to chelate divalent metal ions, such Pb, and thus might be one of the factors responsible for preventing free radical formation (Gautam and Flora, 2009).

In conclusion, our findings showed for the first time that OLE administration reduced Pb accumulation in the brain. They suggest that OLE offers significant protection against Pb-induced brain damage through inhibition of 1) apoptosis, 2) oxidative stress, 3) inflammation and 4) cell metabolism impairments. Moreover, OLE administration was shown to play a role in preventing Pb-induced abnormal behavior in Pb-exposed rats. Although results indicate that the OLE treatment could be effective and could be recommended for treatment for preventing Pb-induced neurological disorders, more extensive studies are required in this important area before a final conclusion can be drawn.

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REFERENCES


Neuroprotective effect of olive leaf against lead exposure


