Redox and essential metal status in the brain of Wistar rats acutely exposed to a cadmium and lead mixture

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Most Pb and Cd neurotoxicity studies investigate exposure to either of the toxic metals alone, while data on co-exposure are scarce. The aim of our study was to fill that gap by investigating acute combined effects of Pb and Cd on redox and essential metal status in the brain of Wistar rats. Animals were randomised in four groups of six to eight rats, which received 15 or 30 mg/kg of Cd, 150 mg/kg of Pb, or 150 mg/kg of Pb + 15 mg/kg of Cd by gavage. The fifth, control, group received distilled water only. Co-treatment with Pb and Cd induced significant increase in malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS) compared to control and groups receiving either metal alone. This is of special importance, as MDA presence in the brain has been implicated in many neurodegenerative disorders. The groups did not significantly differ in Zn, Cu, Mn, and Fe brain levels. Our findings highlight the importance of metal mixture studies. Neurotoxicity assessments of single chemicals do not provide a real insight into exposure to mixtures in real life. Further research should look into interactions between these metals to reveal complex molecular mechanisms of their neurotoxicity.

KEY WORDS: Cd; Cu; Fe; MDA; Mn; neurotoxicity; oxidative stress; Pb; TBARS; Zn

Lead has been found to produce a wide range of toxic effects in humans and animals, most often targeting the haematopoietic and nervous systems. Its neurotoxicity is often associated with behavioural abnormalities, learning impairment, anxiety, depression, and headache (1–3). Low-level Pb exposure has been shown to impair cognitive function and lead to maladaptive behaviour (4). Children are particularly sensitive as they develop, absorb more Pb through the digestive tract, and are more exposed to it through dust and soil (5). Exposure at early age can later lead to intellectual deficits, hyperactivity, and functional impairment (6–8).

There is also a growing evidence of the toxic effects of Cd on the nervous system and of an association between Cd exposure and neurodegenerative diseases (9, 10). Its neurotoxicity is manifested through symptoms such as vertigo, headache, learning disabilities, Parkinsonism-like symptoms, and impaired vasomotor function (10, 11).

Investigations aiming to explain the mechanisms of their toxicity are numerous. Both metals affect essential metals, such as zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), and magnesium (Mg) status in various tissues. This can be attributed to their similarities and competition for the same biological molecules (12–19). Homeostasis of essential metals is crucial for normal physiological processes in all systems and organs, including the central nervous system. In addition, essential metals are components of antioxidant enzymes that protect the body from free radicals and are therefore closely associated with oxidant/antioxidant processes (20).

One of the suggested mechanisms of their toxicity, oxidative stress, has been recognised as pivotal in the pathogenesis of many nervous system diseases (3, 21). It is known that exposure to Pb and/or Cd could induce oxidative stress in cells increasing production of prooxidants and/or decreasing antioxidant safety mechanisms in organism, which results in lipid peroxidation, DNA damage, and depletion of sulphhydryl (SH) groups (22–25). Studies have shown that Cd-induced production of reactive oxygen species (ROS) functions as an upstream signal to mediate mitophagy in mice brain, contributing to Cd neurotoxicity (26). The results of a study on a mouse model (27) evidenced a correlation between 5-aminolevulinic acid and oxidative stress in mice brain, which implies that oxidative stress might be the main mechanism involved in Pb neurotoxicity.

The fact that humans are exposed to metal combinations rather than a single metal (28) could be of vast importance...
for understanding their toxic effects. The last few decades have seen growing evidence that toxic effects of combined exposure – metal plus metal (28–30) or metal plus chemical (such as persistent organic pollutants) (31–33) – do not correspond to those of a single metal. Similar target organs and mechanisms of toxicity raise concerns about synergistic interactions between Pb and Cd (34, 35). However, animal studies examining the effects of Pb and Cd co-exposure on the central nervous system (CNS) are rather scarce. An acute oral toxicity study (34) showed that co-exposure to Pb and Cd had an additive effect. Other research indicates that Cd could antagonise Pb-induced effects on movement and vertical activity in rats (12), but most of it is still focused on single metals.

The aim of our preliminary study was to shed more light on neurotoxic effects of combined exposure to Pb and Cd by measuring oxidative and essential metal parameters in the brain of Wistar rats. We hoped to get a glimpse of the mechanism(s) of their combined action and interactions at doses that are able to induce toxic effects.

**MATERIALS AND METHODS**

**Chemicals**

Lead acetate (Pb(CH₃COOH)₂·3H₂O, Centrohem, Staro Pazova, Serbia) and cadmium chloride (CdCl₂·xH₂O, Merck, Darmstadt, Germany) solutions were used for rat treatment. Standard solutions of Cd, Pb, Zn, Cu, Mn, and Fe were used for calibration were purchased from Merck. Nitric acid (65 % HNO₃, Merck) and hydrogen peroxide (30 % H₂O₂, Sigma-Aldrich, Steinheim, Germany) were used for wet digestion of the tissues. All chemicals and reagents for biochemical assays were purchased from Sigma-Aldrich.

**Animals and treatment**

The experiment was performed on thirty-three 8–12-week-old male albino Wistar rats weighing 250–350 g [body weight (bw)]. They were purchased from the Military Medical Academy, Belgrade, Serbia. After acclimatisation to the new surroundings, the animals were randomised into five groups: one control and four experimental. Each group counted between 6 and 8 animals. The control group was administered intraperitoneally 24 h after treatment. The brain was removed, washed with ice-cold saline (0.9 % NaCl), and frozen to -80 °C until metal and oxidative stress analysis. All experimental procedures were approved by the Ethics Committee on Animal Experimentation of the University of Belgrade Faculty of Pharmacy (No. 323-07-11822/2018-05). The study was carried out in accordance with the Animal Welfare Act of the Republic of Serbia (37).

**Metal analysis**

Brain samples for metal analysis were digested with cHNO₃ (7 mL) and H₂O₂ (1 mL) in a microwave digestion system (START D, SK-10T, Milestone Srl, Sorisole, Italy) according to manufacturer’s recommendations. Then we added 25 mL of deionised water to the digested samples. For Pb and Cd measurement we used graphite furnace atomic absorption spectroscopy (GFAAS, with graphite tube atomizer GTA 120 and 200 Series AA; Agilent Technologies, Santa Clara, CA, USA) with 0.5 % NH₄H₂PO₄ as matrix modifier, while for Zn, Cu, Mn, and Fe we used flame atomic absorption spectrometry (FAAS, 240FS AA, Agilent Technologies). The accuracy of AAS analyses was validated with standard reference material (SRM 1577c – bovine liver, National Institute of Standards and Technology, Gaithersburg, MD, USA).

**Redox status analysis**

Brain samples for redox status analysis were homogenised with 0.1 mol/L phosphate buffer (pH 7.4) in the 1:9 weight-to-volume ratio using a T10 basic Ultra-Turrax homogeniser (IKA, Germany). One part of the homogenate was kept at -80 °C to measure thiobarbituric acid-reactive substances (TBARS), and the rest was first centrifuged in a cooling centrifuge at 800×g for 20 min and then at 9500×g for 20 min to obtain a postmitochondrial supernatant. Postmitochondrial supernatant was also stored at -80 °C until biochemical analysis.

Superoxide anion (O₂⁻) was measured as described by Auclair and Voisin (38) by reducing yellow-stained nitroblue tetrazolium (NBT) to blue formazan. The rate of superoxide anion generation was measured as the rate of formation of reduced NBT and expressed as μmol/min/g of protein.

Total oxidative status (TOS) was measured with a spectrophotometric method optimised by Erel (39) and based on oxidant capacity to oxidise ferrous ion–o-dianisidine complex to ferric ion, which forms a coloured complex with xylenol-orange in an acidic medium. Absorbance was measured at 560 nm and reflects the total content of oxidants in the sample, such as H₂O₂ and lipid hydroperoxides. Calibration was performed with hydrogen peroxide, and results were expressed in μmol of H₂O₂ equivalent per g of protein.

The spectrophotometric method we used for measuring total antioxidative status (TAS) has also been described by
Erel (40). The method is based on oxidation of 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonate) (ABTS) to colour ABTS’ radical cation. Discoloration caused by sample antioxidants was measured at 660 nm. The results were expressed in µmol of Trolox equivalent per g protein.

Superoxide-dismutase (SOD) activity was performed according to the modified method of Misra and Fridovich (41), based on the ability of the enzyme to inhibit auto-oxidation of epinephrine in alkaline medium (pH 10.2). The activity of this enzyme is expressed in relative units, which are obtained by measuring the absorbance of the resulting red oxidation product at 480 nm and 25 °C. SOD activity in the sample is calculated as the percentage of inhibition of epinephrine auto-oxidation (U/g).

Levels of total SH-groups were measured as described by Ellman (42), using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as reagent in alkaline medium (pH 9.0). Absorbance of yellow reaction product (dianion of 5-thio-2-nitrobenzoic acid) was measured at 412 nm.

MDA was determined as TBARS, according to the method described by Girotti (43). It is based on the absorption maximum of MDA and other TBARS with thioharbituric acid at 535 nm. Absorbance was measured after heating samples and reagents at 100 °C for 5 min, then ice-cooling them, and then centrifuging them at 10,000×g and + 4 °C for 10 min. Calibration was performed with MDA solutions.

Protein concentration in brain tissue was determined with the method described by Bradford (44), in which bovine serum albumin is used as the standard. This is a spectrophotometric method based on the binding of Coomassie Brilliant Blue G-250 to a protein molecule in a sample, which causes the absorption maximum to shift from 465 to 595 nm.

Advanced oxidation protein products (AOPP) were determined with a spectrophotometric method described by Witko et al. (45), using glacial acetic acid, 1.16 mol/L potassium-iodide solution, and phosphate buffer (pH 7.4). The absorbance was measured at 340 nm. The reaction was calibrated with chloramine-T, and results expressed as µmol of chloramine-T equivalent per g of protein.

Oxidative stress index (OSI) as an indicator of the degree of oxidative stress was calculated as a ratio between TOS and TAS and presented in arbitrary units (40). All spectrophotometric methods run on ILAB 300 Plus analyser (Instrumentation Laboratory, Milan, Italy), with the exception of TBARS determination, run on a Cary60 UV–VIS spectrophotometer (Agilent Technologies).

Statistical analysis

For all statistical analyses we used the IBM SPSS Statistics version 18 for Windows (IBM, Armonk, NY, USA). After normality and homogeneity of variance check, differences between the groups were analysed with one-way ANOVA or Kruskal-Wallis test, followed by LSD or the Mann-Whitney U test, respectively, with P<0.05 set as the level of significance. All values are expressed as mean ± standard deviation or median and range. Graphs were made using the GraphPad Prism 5 software (GraphPad software, Inc., La Jolla, CA, USA).

RESULTS

Pb and Cd levels in brain

The levels of Pb (Figure 1) and Cd (Figure 2) in rat brains are expressed as ng of metal per g of wet tissue. Lead brain levels were significantly higher in the Pb150 and Cd15+Pb150 groups than controls (P<0.001), but the two groups did not differ significantly. Cd levels were significantly higher in the Cd15 and Cd30 groups compared to control (P<0.01) and in the Cd30 group compared to Cd15. The Cd15+Pb150 group, however, had significantly lower Cd brain levels than the Cd15 group.

Essential metals

There were no significant differences in Zn, Cu, Mn, and Fe brain levels between the groups (Table 1).

Oxidative stress parameters

Table 2 shows the effects of Pb and/or Cd treatment on oxidative stress parameters in rat brain. MDA and TBARS levels were significantly higher in the Cd30 group compared to control (P<0.01) and the Cd15 group (P<0.05). Co-treatment significantly increased MDA and TBARS compared to control (P<0.001) and either metal administered alone (Cd15 P<0.001, Pb150 P<0.05). However, we...
observed no significant changes in AOPP, O$_2^-$, SOD, and SH. Exposure to toxic metals resulted in significant increase in TAS in all treated groups compared to control, but TOS did not change significantly. Consequently, OSI was significantly lower in the Cd15 (P<0.05), Cd30 (P<0.01), and Cd15+Pb150 group (P<0.05) compared to control.

DISCUSSION

Our findings confirm that both metals pass the blood-brain barrier (BBB) and accumulate in the brain. As we expected, Cd levels were almost two times higher in the brains of the Cd30 group than the Cd15 group. However, combined administration with 15 mg/kg bw of Cd led to lower Cd brain levels than administration of Cd alone in the same dose. At the same time, Pb in the combination group was higher, but not significantly, than when administered alone in the same dose. These findings point to toxicokinetic interactions between Pb and Cd – most likely to a competition for metal transporters in the BBB (46, 47).

Increased brain Pb levels were also observed in a chronic interaction study (48), but these were nearly five times higher than control. The main pathway of Pb crossing the BBB is that it mimics and substitutes Ca ions. Another is divalent metal ion transporter (DMT1) (47). One study showed that combined exposure to Pb and Cd synergistically increased the expression of this transporter in the CNS of developing rats treated for 45 days (49). Other in vivo studies have also shown higher Pb levels after co-exposure with other metals. In a recent eight-day study (50) this was attributed to high Pb affinity for specific proteins in the brain, which implies that Pb more efficiently binds to protein target sites than Cd.

Even though exposure to Pb and Cd has been shown to interfere with essential metals in many tissues (15, 30, 51–53), our findings fail to confirm this in the rat brain. No visible interference in the brain was also reported by other similar studies (54, 55). This suggests that changes in brain essential metals are not the major mechanism of Pb and Cd neurotoxicity after acute exposure. However, this hypothesis needs to be investigated further.

The most important finding of our study is that lipid peroxidation in the rat brain observed after acute co-administration of Pb and Cd was significantly higher than the one induced by treatment with either metal alone. This points to additive or even synergistic neurotoxicity of these metals. However, the limitation of our study design is that it cannot inform about the type of toxicodynamic interactions observed in the rat brains.

We were surprised to find that Pb or the lower Cd dose (15 mg/kg bw) did not change MDA and TBARS brain levels when given alone, as many other studies reported this increase with Pb or Cd (9, 23, 54, 56–60). MDA and TBARS significantly increased only at the Cd dose of 30 mg/kg bw, which suggests that the dose can have an important role in redox disturbances in the brain. The

| Essential metals | Control (μg/g) | Cd15 (μg/g) | Cd30 (μg/g) | Pb150 (μg/g) | Cd15+Pb150 (μg/g) |
|------------------|--------------|-------------|-------------|--------------|------------------|
| Zn               | 11.12        | 10.69–15.10 | 12.35       | 11.39–13.71  | 12.12–15.66      |
|                  | 10.69–15.10  | 11.39–13.71 | 12.12–15.66 | 11.16–15.40   |                  |
| Cu               | 1.99±0.41    | 2.24±0.27   | 2.17±0.23   | 2.26±0.26    | 1.88±0.22        |
| Mn               | 1.93±0.44    | 1.38±0.24   | 1.72±0.34   | 1.65±0.40    | 1.90±0.51        |
| Fe               | 22.42        | 18.12       | 19.31       | 19.12–22.86  | 13.44–24.93      |

Values are represented as means ± standard deviations or medians and ranges. Cd15 – the group treated with Cd in the dose of 15 mg/kg bw; Cd30 – the group treated with Cd in the dose of 30 mg/kg bw; Pb150 – the group treated with Pb in the dose of 150 mg/kg bw; Cd15+Pb150 – the group treated with a combination of 150 mg/kg Pb and 15 mg/kg Cd
The question remains, however, to what extent does Cd contribute to MDA and TBARS production when combined with Pb, considering that the Cd brain levels in the Cd15+Pb150 group were lower than in the Cd15 or Cd30 groups.

As for antioxidative defences, acute exposure to Pb and/or Cd did not affect SOD. Significantly higher TAS observed in our study, however, implies that these metals may have induced higher production or redistribution of antioxidants, as reported elsewhere (61), which in turn restored oxidant/antioxidant balance to normal. However, this may not be the case with prolonged exposure to Pb and Cd (as opposed to acute effects we measured), as it seems to deplete antioxidant enzyme defences and tip the balance toward a more oxidative state or stress (23, 24, 54).

We therefore believe that changes in MDA and TBARS levels might be related to direct interaction of Pb and Cd with membrane unsaturated lipids as a mechanism leading to oxidative damage (62, 63).

CONCLUSION

Our findings confirm concerns that single exposure studies can underestimate the toxic effect of Pb and Cd. Further subacute and subchronic experimental studies are warranted to better understand the complex toxicokinetic and toxicodynamic mechanisms of neurotoxic effects and interactions between Pb and Cd in mixtures.

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Conflicts of interest

None to declare.

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Table 2 Effects of acute Pb and/or Cd treatment on oxidative stress parameters in the brain of Wistar rats (N=6–8 per group)

| Parameter     | Control | Cd15 | Cd30 | Pb150 | Cd15+Pb150 |
|---------------|---------|------|------|-------|------------|
| TBARS (μmol/mg protein) | 161.36±47.74 | 175.25±43.23 | **309.64±106.78**: | 230.95±47.82 | **390.57±95.15**: |
| AOPP (μmol/min/g protein) | 27.57±6.28 | 24.76±9.40 | 27.68±6.43 | 25.4±6.18 | 24.83±8.83 |
| O₂⁻ (μmol/min/g protein) | 91.74±6.65 | 99.78±17.30 | 91.38±18.94 | 95.13±11.82 | 90.24±7.92 |
| SOD (U/g) | 57.35±10.63 | 47.52±5.03 | 57.73±18.82 | 58.29±5.87 | 49.32±10.92 |
| SH (mmol/g protein) | 0.14±0.03 | 0.15±0.02 | 0.13±0.02 | 0.15±0.02 | 0.12±0.02 |
| TAS (μmol Trolox Equiv. /g protein) | 130.00 | 268.66 | **300.07**: | 222.61 | **264.91**: |
| TOS (μmol H₂O₂ Equiv. /g protein) | 2.36±0.68 | 2.46±0.53 | 2.05±0.54 | 2.77±0.65 | 2.89±1.89 |

OSI

0.0162±0.0078 | 0.0106±0.0049*: | 0.0068±0.0016**: | 0.0149±0.0087‡: | 0.0099±0.0037*: |

Values are represented as means ± standard deviations or medians and ranges. * P<0.05, ** P<0.01, *** P<0.001 compared to control; † P<0.05, †† P<0.01 compared to Cd15; ‡ P<0.05, ‡‡ P<0.01 compared to Cd30; § P<0.05 compared to Pb150 (one-way ANOVA or Kruskal-Wallis test followed by LSD or the Mann-Whitney U test). Cd15 – the group treated with Cd in the dose of 15 mg/kg bw; Cd30 – the group treated with Cd in the dose of 30 mg/kg bw; Pb150 – the group treated with Pb in the dose of 150 mg/kg bw; Cd15+Pb150 – the group treated with a combination of 150 mg/kg Pb and 15 mg/kg Cd; TBARS – thiobarbituric acid-reactive substances; O₂⁻ – superoxide anion; AOPP – advanced oxidation protein products level; SH – total thiol groups; SOD – superoxide dismutase; TAS – total antioxidant status; TOS – total oxidative status; OSI – oxidative stress index (TOS/TAS)
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Dosadašnji podatci o neurotoksičnosti olova (Pb) i kadmiya (Cd) uglavnom su opisani nakon izloženosti pojedinačnim metalima, a podatci o neurotoksičnom djelovanju smjese tih dvaju metala prilično su oskudni. Cilj ovoga rada bio je ispitati učinak akutnoga izlaganja smjesi olova i kadmiya na parametre oksidacijskoga stresa i status bioelemenata u mozgu štakora. Životinje su bile podijeljene u pet skupina: Cd15 (15 mg/kg), Cd30 (30 mg/kg), Pb150 (150 mg/kg), Pb150+Cd15 i kontrolna, netretirana skupina. Smjesa olova i kadmiya dovela je do značajno viših vrijednosti malondialdehida (MDA) i drugih produkata u mozgu štakora u usporedbi s kontrolnom skupinom i obama pojedinačnim metalima. To je iznimno važno, imajući u vidu da je prisutnost MDA-a u mozgu povezana s mnogim neurodegenerativnim poremećajima. Nije dobivena statistički značajna razlika između ispitivanih skupina u razinama cinka (Zn), bakra (Cu), mangana (Mn) i željeza (Fe). Rezultati ovoga istraživanja važan su doprinos budućim istraživanjima smjesa i upućuju na to da istraživanja toksičnosti u kojima se procjenjuje neurotoksičnost pojedinačnih kemikalija ne pružaju stvarni uvid u neurotoksičnost njihovih smjesa kojima smo izloženi u stvarnom životu. Dobiveni rezultati otvaraju daljnja pitanja o interakciji i mehanizmima toksičnosti smjese tih metala.

KLJUČNE RIJEČI: Cd; Cu; Fe; MDA; Mn; neurotoksičnost; oksidacijski stres; Pb; TBARS; Zn