Arh hig rada toksikol 1995;46:381-390

ORIGINAL SCIENTIFIC PAPER
UDC 615.916:612.82

TRIMETHYL LEAD NEUROTOXICITY IN THE RAT: CHANGES IN GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)

ZHAOLONG GONG, AR LITTLE JR., HAN EL-FAWAL AND HUGH L. EVANS

Nelson Institute of Environmental Medicine, New York University Medical Center, Tuxedo, NY, U.S.A.

Received November 7, 1995

The literature on the toxicology of lead provides little evidence of the neurotoxicity of organic lead compounds. Toxicant-induced changes in the concentration of glial fibrillary acidic protein (GFAP) in the brain may help clarify at which stage of neurotoxicity astrocytes are affected and whether GFAP may provide an index of toxicity. Male F344 rats (>42 days old) were exposed to 0 (control), 8 or 16 ppm lead as trimethyl lead (TMPb) in drinking water for up to 14 days. Weight gain was significantly reduced in both exposed groups. Control rats had the expected brain regional pattern of GFAP concentration with the highest in the hippocampus and cerebellum and lowest in the cerebral cortex. The hippocampus was the region very sensitive to TMPb, with increased GFAP in rats exposed to 8 and 16 ppm TMPb for 14 days. There was a significant time-response in rats exposed to 8 ppm TMPb with decreases in GFAP on day 7 and increases on day 14. A hypothesis concerning this biphasic change in GFAP concentrations is discussed. The results indicate that GFAP may be used to indicate the role of the astrocyte in the neurotoxicity of TMPb. GFAP concentration, as biomarker of TMPb effect, was as sensitive to TMPb as body weight and thus may provide a marker of neurotoxicity.

Key terms:
astrocytes, body weight, brain, water consumption

Lead is the most ubiquitous toxic metal without any demonstrated biological need (1, 2). Most of the lead in environmental pollution is inorganic, and most experiments with laboratory animals have studied inorganic lead (1). As many as 17 per cent of American children may be at risk because of low level exposure to lead and lead exposure is still a very serious threat to children in many nations (3).

Lead can penetrate the blood brain barrier and accumulate in the brain (4). There is a good correlation between blood lead concentration and brain lead concentration in animals continuously exposed to lead (5-8). Lead in the central

nervous system tends to concentrate in the hippocampus, followed by cerebellum, cerebral cortex, and medulla (9–11). Astroglial processes resemble a sheath which surrounds almost all the vascular surfaces in the mammalian brain, and thus may be an important part of the blood brain barrier (12). Astrocytes can sequester and accumulate lead to a high concentration both *in vivo* (13) and *in vitro* (14). Therefore, astrocytes may serve a protective function for neurons in the brain by acting as a lead sink or filter (15).

Glial fibrillary acidic protein (GFAP) is a cell-specific cytoskeletal intermediate filament protein (16). It provides a convenient marker for changes in the number or size of astrocytes, a major class of glial cells in the brain. GFAP concentration is affected by chemicals (17–20). The cellular mechanism of lead neurotoxicity may be clarified by examining how lead exposure affects GFAP. All published work on GFAP has involved inorganic lead. In one series of *in vivo* experiments, rats were exposed to 10,000 ppm lead acetate in drinking water for 30, 60, and 90 days. After 60 days of exposure to lead, there was an increase of GFAP-staining cells in the hippocampus. The intensity of the GFAP response was enhanced after 90 days of lead exposure (21, 22). Preliminary data in this laboratory indicated that GFAP concentration can decrease under some circumstances in rats exposed to 50, 150 and 450 ppm lead for up to 42 days (23). *In vitro*, lead decreased the expression of GFAP, interpreted as a decrease in the level of differentiation and impairment of glial cell function (24).

Organometals are usually more neurotoxic than inorganic metals (25). The present study investigated the effects of trimethyl lead (TMPb) upon GFAP in the brain. Rats were exposed to 8 and 16 ppm trimethyl lead for up to 14 days. Dose- and time-effect relationships for GFAP levels in the cerebral cortex, hippocampus and cerebellum were examined to test whether continuous exposure to lead produces a different pattern of change in astrocytes at early stages of exposure than at later stages; and whether changes at low doses of lead were different than at higher doses which produce overt toxicity.

MATERIAL AND METHODS

Animals and Lead Exposures

Male F344 rats, body weight about 70 grams, were obtained from Taconic Farms (Germantown, NY). Animals were housed in pairs in stainless mesh cages in a room maintained at temperature of 22±2 °C and humidity between 40 and 80% with a 12-hr light-dark cycle. Lights were on from 04:00 to 16:00. The rats were allowed free access to food (Purina Lab Chow, 5001, Ralston Purina Corp., St. Louis, MO) and distilled drinking water.

After adjustment to the laboratory for one week, rats were divided into three dosing groups of eight rats each of approximately equal average weight. Control

rats continued to receive distilled water as their drinking water. Two groups of rats (n=8) drank 8 or 16 ppm lead (Trimethyl Lead Chloride from Alfa Products, Danvers, MA) in distilled water for seven or 14 days. The system for drinking water was designed to minimize loss of water because of dripping and evaporation and thus to provide a very accurate measure of water consumption (26).

Blood lead concentrations were not determined because of a report that blood concentration is not a good index of exposure or effects of organic lead (27). Historical data from control rats in this laboratory indicated total lead in blood as less than 2 μg/dl.

Body Weight and Water Consumption

Rats and water cans were weighed before and during lead exposure twice a week on a digital balance to an accuracy of 0.1 g. Consumption of food was not measured.

Brain GFAP Assay

Rats were killed by decapitation. For each TMPb exposure level, one group of control rats was killed on day seven of the experiment, and TMPb exposed rats were killed on days seven and 14 of lead exposure. Each dose of TMPb was studied with a separate control group.

The brain was immediately removed and put on a cold plate. Samples were taken from the cerebral cortex, hippocampus and cerebellum. All samples were sonicated with 10 volumes of 1% (w/v) sodium dodecyl sulfate (SDS) which was put on a hot plate with the temperature of approximately 70 °C, then stored at -84 °C for future analysis. Total protein was determined by Pierce Bincinchoninic Acid (BCA) assay (28) and GFAP concentration was analyzed by the enzyme-linked immunosorbent assay (ELISA) method (29). A GFAP standard curve was obtained by serial dilution of a calibrated standard composed of a homogenate of normal rat hippocampus which had been standardized with pure GFAP (IBL Research Products, Corp., Cambridge, MA) by the method of addition.

Statistics

The BMDP computer library was used; the library's name was formerly Biomedical Data Programs (30). Body weight and water consumption data were analyzed by unbalanced repeated measures models with structured analyses of covariance matrices (ANCOVA) (BMDP, Program No. 5V). GFAP data were analyzed by analysis of variance (ANOVA) (BMDP, Program No. 7D), followed by Student-Newman-Keuls multiple range test. $P \leq 0.05$ was considered significant.

RESULTS

Body Weight and Water Consumption

Control rats weighed 90.7±1.5 g (mean±SEM, n=8) before the exposure and 163.5±1.8 g (mean±SEM, n=8) at day 13 of exposure. Rats exposed to TMPb gained less weight than controls (ANCOVA χ^2=16.0, df=2, P<0.001). This reduced weight gain was observed as early as day three in the rats exposed to 8 ppm trimethyl lead. The body weight of rats exposed to 16 ppm TMPb continued to grow more slowly than controls until day 13 (dose X time interaction χ^2=36.2, df=2, P<0.001) (Figure 1). The water consumption of control rats was 24.8±0.3 g/day (mean±SEM, n=8). Water consumption of the rats exposed to TMPb decreased by 21.0% for the 8 ppm group (t=12.82, df=13, P<0.001) and 29.3% (t=8.43, df=14, P<0.001) for the 16 ppm group

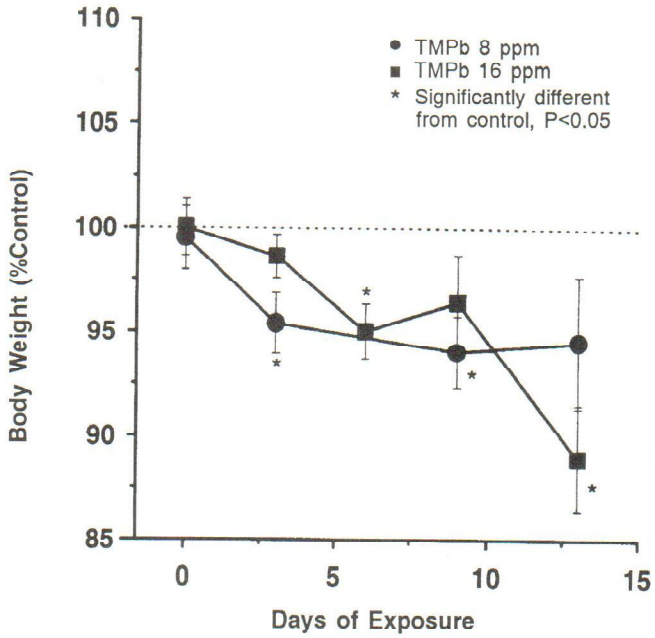

Figure 1 *Body weight of rats during 14-day exposure to TMPb in drinking water. Data are expressed as percentage of the age-matched control group which weighed 103±1.5 g on day 0. Each point shows mean ± SE. Day 0 represents baseline before exposure to lead began.*

GFAP Concentration in Control Rats

GFAP levels of control rats were much higher in the hippocampus and cerebellum than in the cerebral cortex (Table). These results are consistent with differences in GFAP concentration of these brain regions reported in the literature (20, 29). The variability of control samples can be judged by the SE, which were approximately 6% of the control means (Table).

Table. *GFAP Concentration in control rats*

Brain region	n	μg GFAP/mg total protein (mean±SEM)
Cerebral cortex	8	1.99±0.22
Hippocampus	8	5.16±0.24
Cerebellum	8	5.56±0.36

GFAP Dose-Response

GFAP levels were higher in TMPb exposed rats than in controls after 14 days of exposure ($F=4.97$, $df=2, 75$, $P<0.001$). Furthermore, the effects of TMPb differed according to brain region ($F=4.23$, $df=4, 75$, $P<0.01$). The hippocampus was the brain region which was most consistently affected by TMPb (Figures 2 and 3). After 14 days of exposure, GFAP levels in the hippocampus showed a significant dose-related increase (Figure 2). Exposure to either 8 or 16 ppm TMPb caused a significant increase in GFAP. The GFAP concentration in the cerebellum was significantly increased after 14 days exposure to 8 ppm TMPb (Figure 3), but changes with the cerebellum were not significant at other dose and time points.

GFAP Time-Response

There was a significant time-response relationship in GFAP levels in three brain regions of rats exposed to 8 ppm TMPb for seven and 14 days ($F=8.25$, $df=2, 87$, $P<0.001$), with GFAP decreased at seven days (for all three brain regions, 8 ppm TMPb vs control, $F=6.1$, $df=1, 42$, $P=0.02$) and increased at 14 days ($F=21.4$, $df=1, 42$, $P<0.001$) (Figure 3). The multiple range test indicated that GFAP in the hippocampus and cerebellum at day 14 of TMPb exposure was significantly increased above the concentration of the control group. The statistical significance of the decline in GFAP on day seven is shown by the most powerful and appropriate statistical test available for this set of data - an analysis of variance of the entire body of data. Following this, post-hoc tests of individual pairs of control and TMPb data points were evaluated, but the statistical power was reduced because of the smaller N in these post-hoc tests.

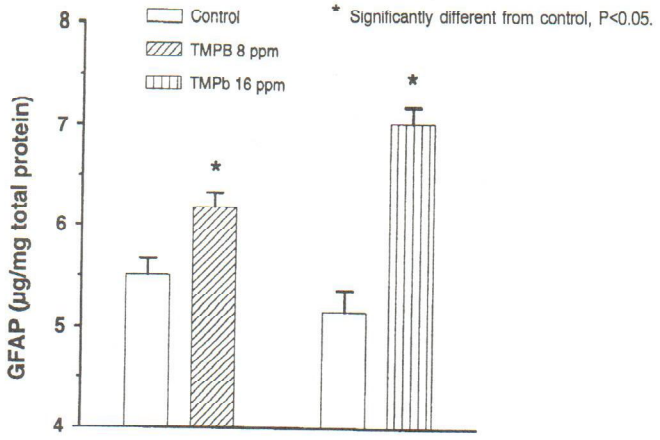

Figure 2 *The concentration of GFAP in the hippocampus in rats exposed to 8 or 16 ppm TMPb for 14 days. Data are shown as mean ± SE, n=8.*

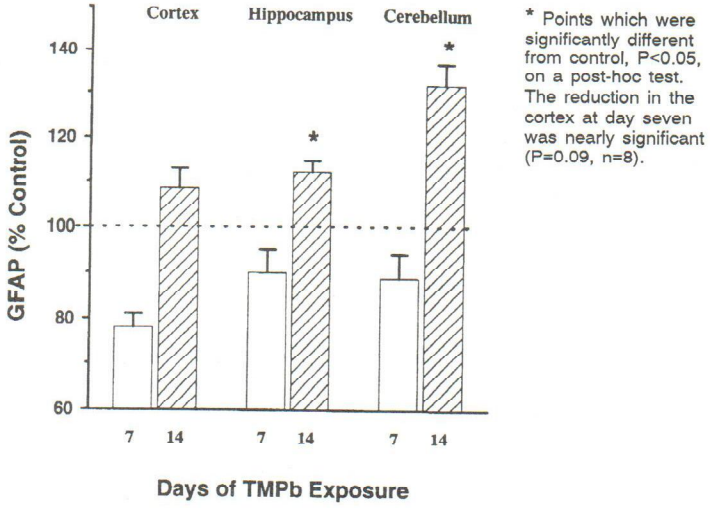

Figure 3 *GFAP concentrations in rats exposed to 8 ppm TMPb differed depending on the duration of exposure*

DISCUSSION

This is the first report of an organo-lead compound affecting brain GFAP. Exposure to TMPb for 14 days produced increases in hippocampal GFAP concentration (Figure 2). Other experiments have shown that reactive gliosis accompanying neuronal damage is the cause of increased GFAP concentration in rats exposed to trimethyl tin (31). The most-frequently reported response of astrocytes to diseases or injuries of the brain is reactive astrogliosis, which is defined primarily as an increase in the number and size of cells expressing GFAP. *In vivo*, the increase of GFAP is primarily due to astrocyte hypertrophy which is most often accompanied by neuronal damage (31, 32).

Of the three brain regions studied here, GFAP concentration of the hippocampus was the most consistently affected by TMPb, followed by the cerebellum, while the GFAP concentration of the cerebral cortex was not affected by TMPb. The brain regions which had the greatest change in GFAP are those having the highest basal levels of GFAP (Table 1) and the highest uptake of lead compounds (9–11).

The effective dose of TMPb (8 ppm Pb in the drinking water, leading to consumption of approximately 1.3 mg Pb/kg/day) is much lower than the dose of inorganic lead necessary to affect brain GFAP (21, 22). This observation fits the pattern in which the organic compounds of many metals have greater neurotoxicity, on a mg/kg basis, than their inorganic counterparts (1, 25).

The small, consistent decreases in GFAP at the earliest time of exposure to the lower concentration of TMPb (Figure 3) deserves additional research. Change in brain GFAP may be the result of direct action of TMPb upon astrocytes, or it may be secondary to damage to neurons. Because the astrocytes absorb lead as an early defense (13), and lead can inhibit GFAP expression *in vitro* (24), lead may inhibit synthesis of GFAP, resulting in a decline of GFAP, since organic lead is known to inhibit the synthesis of other proteins in the nervous system (34–37).

The toxicity of TMPb was also indicated by the reduced growth of young rats during exposure to TMPb (Figure 1). Inorganic lead can reduce growth, primarily as a result of its effects upon ingestive mechanisms and appetite (38, 39). The results of Figure 1 indicate that TMPb interferes with the growth of rats in a manner similar to that of inorganic lead. It remains to be determined whether TMPb and inorganic lead compounds share the mechanisms of inorganic lead effects upon growth. Toxicologists seldom evaluate ingestive behavior thoroughly (e.g., 22). However, studies of ingestive behavior can provide both an informative and sensitive index of toxicity (40). In the present experiment, water consumption and body weight proved their value as general indices of toxicity because they were equal to brain GFAP concentration in terms of sensitivity to TMPb.

These results strengthen suggestions that the measurement of GFAP concentration in the brain can provide a quantitative index of neurotoxicity (41, 42).

Acknowledgments This research was supported by grants from the National Institute of Environmental Health Sciences (ES-000260 and ES-04895) and from the American Petroleum Institute. We thank dr. J.P. O'Callaghan for suggestions concerning the GFAP assay and dr. Biserka Kargačin for suggestions concerning the toxicity of metals.

REFERENCES

1. *Bondy SC.* The neurotoxicity of organic and inorganic lead. In: SC Bondy and KN Prasad, eds. Metal Neurotoxicity. Boca Raton: CRC Press, 1988:2-13.
2. *McIntosh MJ, Moore MR, Goldberg A.* Lead toxicology and neurotoxicology. Rev Environ Health 1989; 8:87-118.
3. *U.S. Agency for Toxic Substances and Disease Registry (ATSDR).* The nature and extent of lead poisoning in children in the United States: a report to Congress. Atlanta: U.S. Department of Health and Human Services, 1988.
4. *Bradbury MWB, Deane R.* Permeability of the blood-brain barrier to lead. Neurotoxicology 1993; 14(2-3):131-6.
5. *Savolainen H, Kilpio J.* Brain and blood lead in acute intoxication. Scand J Work Environ Health 1977; 3:104-7.
6. *Rader JI, Peeler JT, Mahaffey KR.* Comparative toxicity and tissue distribution of lead acetate in weanling and adult rats. Environ Health Perspect 1981; 42:187-95.
7. *Bankowska J, Hine C.* Retention of lead in the rat. Arch Environ Contam Toxicol 1985; 14:621-9.
8. *Llobet JM, Domingo JL, Paternain JL, Corbella J.* Treatment of acute lead intoxication. A quantitative comparison of a number of chelating agents. Arch Environ Contam Toxicol 1990; 19:185-9.
9. *Collins MF, Hrdina PD, Whittle E, Singhal RL.* Lead in blood and brain regions of rats chronically exposed to low doses of the metal. Toxicol Appl Pharmacol 1982; 65:314-22.
10. *Lindh U, Conradi NG, Sourander P.* Distribution of lead in the cerebellum of suckling rats following low and high dose lead exposure, A micro-PIXE analysis. Acta Neuropathol 1989; 79:149-53.
11. *Goyer RA.* Toxic effects of metals. In: Amdur MO, Doull J, Klaassen CD eds. Casarett and Doull's Toxicology. The basic science of poisons. 4th ed. New York: Pergamon Press, Inc. 1991:639-46.
12. *Vaquera-Orte J, Cervos-Navarro J, Martin-Giron F, Becerra-Ratia J.* Fine structure of the perivascular-limiting membrane. In: Navarro J., Fitschka E. eds. Cerebral Microcirculation and Metabolism. New York: Raven Press, 1981:129-38.
13. *Holtzman D, DeVries C, Nguyen H.* Maturation of resistance to lead encephalopathy: cellular and subcellular mechanism. Neurotoxicology 1984; 5:97-124.
14. *Tiffany-Castiglioni E.* Cellular targets of lead neurotoxicity: *in vitro* models. Toxicology 1986; 42:303-15.
15. *Tiffany-Castiglioni E, Sierra EM, Wu JN, Rowles TK.* Lead toxicity in neuroglia. Neurotoxicology 1989; 10:417-44.
16. *Eng LF, Smith ME.* Recent studies of the glial fibrillary acidic protein. Ann New York Acad Sci, 1985; 455:525-37.
17. *O'Callaghan JP.* Neurotypic and gliotypic proteins as biochemical markers of neurotoxicity. Neurotoxicol Teratol 1988;10:445-52.

18. *O'Callaghan JP.* Assessment of neurotoxicity: use of glial fibrillary acidic protein as a biomarker. Biomed Environ Sci 1991;4:197–206.
19. *Cookman GR, Hemmems SE, Keane GJ, King WB, Regan CM.* Chronic low level lead exposure precociously induces rat glial development *in vitro* and *in vivo*. Neuroscience Letters, 1988; 86:33–7.
20. *Evans HL, Little AR, Gong ZL, Duffy JS, Wirgin I, El-Fawal HAN.* Glial fibrillary acidic protein (GFAP) indicates *in vivo* exposure to environmental contaminants: PCBs in the Atlantic Tomcod. Ann New York Acad Sci 1993; 679:402–6.
21. *Selvin-Testa A, Lopez-Costa JJ, Nessi de Avinon AC, Pecci Saavedra J.* Astroglial alterations in rat hippocampus during chronic lead exposure. Glia 1991; 4:384–92.
22. *Selvin-Testa A, Loidl CF, Lopez-Costa JJ, Lopez EM, Pecci Saavedra J.* Chronic lead exposure induces astrogliosis in hippocampus and cerebellum. Neurotoxicology 1994; 15(2):389–402.
23. *Gong ZL, Little AR, El-Fawal HAN, Evans HL.* Lead induces a biphasic glial fibrillary acidic protein response in rat brain. Soc Neuroscience Abstr 1994; 20:1656.
24. *Stark M, Wolff JEA, Korbmacher A.* Modulation of glial cell differentiation by exposure to lead and cadmium. Neurotoxicol Teratol 1992; 14:247–52.
25. *Evans HL, Daniel SA.* Modification of conditioned responding by exposure to organo-metals. In: H.A. Tilson and S.B. Sparber, eds. Neurotoxicants and Neurobiological Function. New York: John Wiley and Sons, 1987: 262–78.
26. *Evans HL, Bushnell PJ, Taylor JD, Monico A, Teal JJ, Pontecorvo, M.J.* A system for assessing toxicity of chemicals by continuous monitoring of homecage behaviors. Fund Appl Toxicol 1986; 6:721–32.
27. *Alessio L, Dell'Orto A, Forni, A.* Alkyl lead compounds. In: Alessio L, Berley A, Boni M, Hol R. eds. Biological indicators for the assessment of human exposure to industrial chemicals. Luxembourg: Commission of the European Committee, 1986:7–15.
28. *Smith PK, Krohn RI, Hermanson GT.* Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150:76–85.
29. *O'Callaghan JP.* Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. Neurotoxicol Teratol 1991; 13:275–81.
30. *Dixon WJ.* BMDP Statistical software manual. Berkeley: University of CA Press, 1990.
31. *Balaban CD, O'Callaghan JP, Billingsley ML.* Trimethyltin-induced neuronal damage in the rat brain: comparative studies using silver degeneration stains, immunocytochemistry and immunoassay for neurotypic and gliotypic proteins. Neuroscience 1988; 26:337–61.
32. *Norton WT, Aquino DA, Hozumi I, Chiu F-C, Brosnan CF.* Quantitative aspects of reactive gliosis: a review. Neurochem Res 1992; 17:877–85.
33. *Eddleston M., Mucke L.* Molecular profile of reactive astrocytes-implications for their role in neurologic disease. Neuroscience 1993; 54:15–36.
34. *Konat G, Offner H, Clausen J.* Triethyllead-restrained myelin deposition and protein synthesis in the developing rat forebrain. Exp Neurol 1976; 52:58–65.
35. *Konat G, Offner H, Clausen J.* Effect of triethyllead on protein synthesis in rat forebrain. Exp Neurol 1978; 59:162–7.
36. *Konat G, Offner H, Clausen J.* The effect of triethyllead on total and myelin protein synthesis in rat forebrain slices. J Neurochem 1979; 32:187–90.
37. *Kennedy JL, Girgis GR, Rakhra GS, Nicholls DM.* Protein synthesis in rat brain following neonatal exposure to lead. J Neurol Sci 1983; 59:57–68.
38. *Minnema DJ, Hammond PB.* Effect of lead exposure on patterns of food intake in weanling rats. Neurotoxicol Teratol 1994; 16(6):623–29.
39. *Hammond PB, Succop PA.* Effects of supplemental nutrition on lead-induced depression of growth and food consumption in weanling rats. Toxicol App Pharmacol 1995;13:80–4.

40. *Evans HL.* Neurotoxicity expressed in naturally occurring behavior. In: B. Weiss and J. O' Donoghue, eds. Neurobehavioral Toxicity: Analysis and interpretation. New York: Raven Press, 1994:111-35.
41. *U.S. Environmental Protection Agency.* Proposed guidelines for neurotoxicity risk assessment and request for comments. Federal Register 1995; 60 (192):52031-56.
42. *O'Callaghan JP, Jensen KF.* Enhanced expression of glial fibrillary acidic protein and the cupric silver degeneration reaction can be used as sensitive and early indicators of neurotoxicity. Neurotoxicol 1992; 13:113-22.

Sažetak

NEUROTOKSIČNOST TRIMETILNOG OLOVA U ŠTAKORA: PROMJENE U GLIJALNOM FIBRILARNOM KISELOM PROTEINU (GFKP)

Među literaturnim podacima o toksičnosti olova ima malo podataka o neurotoksičnosti organskih spojeva olova. Otrovom izazvane promjene u koncentraciji glijalnog fibrilarnog kiselog proteina (GFKP) u mozgu mogu pokazati u kojoj fazi neurotoksičnosti dolazi do oštećivanja astrocita te da li GFKP može služiti kao praktični pokazatelj otrovnog djelovanja. U ovom radu procjenjivano je izaziva li izloženost štakora organskom olovu promjene u koncentraciji GFKP te kako promjene ovise o dozi i o trajanju izloženosti. Mužjaci F344 štakora u dobi iznad 42 dana izlagani su dozi od 0 (kontrola), 8 ili 16 ppm olova u obliku trimetilnog olova (TMPb) u pitkoj vodi do ukupno 14 dana. U svakoj skupini bilo je osam životinja. Prirast tjelesne težine bio je značajno smanjen u obje izložene skupine. U kontrolnih štakora izmjerene su očekivane koncentracije GFKP u ispitivanim područjima mozga, s najvišim vrijednostima u hipokampusu i u malom mozgu i s najnižim vrijednostima u moždanoj kori. Područje hipokampusa bilo je veoma osjetljivo na TMPb, s porastom GFKP u štakora izloženih 8 i 16 ppm TMPb tijekom 14 dana. U štakora izloženih 8 ppm TMPb opažena je značajna ovisnost učinka o vremenu, tako da su koncentracije TMPb nakon sedam dana bile snižene, a nakon 14 dana povišene. U radu se raspravlja o hipotezi o ovim bifazičnim promjenama u koncentracijama GFKP. Rezultati pokazuju da GFKP može poslužiti kao pokazatelj mogućih staničnih mehanizama i uloge astrocita u neurotoksičnom djelovanju TMPb. Pokazano je također da je GFKP, kao biološki pokazatelj učinaka TMPb, jednako osjetljiv kao i tjelesna težina, pa stoga može poslužiti kao praktični pokazatelj neurotoksičnosti.

Ključne riječi:
astrociti, mozak, potrošnja vode, tjelesna težina

Requests for reprints:

Dr. Hugh L. Evans
Nelson Institute
New York University Medical Center
Tuxedo, N.Y. 10987
U.S.A.