The aim of this study was to investigate the effects of a combination of well-known neurotoxic heavy metals, lead and mercury, with ethanol. For 12 weeks, young adult male Wistar rats were given plain tap water or water containing 5 % (v/v) ethanol to drink, and were treated with two doses of lead acetate or mercuric chloride by gavage. Accordingly, there was a water-drinking and an alcohol-drinking control group. After the treatment period, spontaneous and stimulus-evoked activity from the somatosensory, visual and auditory cortical areas was recorded. The frequency spectrum of the spontaneous activity, as well as latency and duration of the evoked potential were analysed. A shift in frequency was observed in the electrocorticogram, and lengthened latency and duration times in the evoked potentials. Alcohol seemed to influence the effect of the metals. Combined exposure to heavy metals and regular alcohol consumption may result in more severe central and/or peripheral neurotoxic outcomes.

KEY WORDS: combined exposure, electrocorticogram, ethanol, evoked potential, heavy metals, neurotoxicity.

The usual lifestyle of modern societies involves a steady exposure to a mixture of toxic substances for the population. Several of the toxicants consumed with food and drink, or taken up from the environment, are known to affect the nervous system, but our knowledge on possible harmful effects of combined exposures is limited.

Lead in the environment originates from the metal industry and its waste, and from the use of leaded petrol (now banned in many countries). Environmental lead exposes population through the air, water and food originating from contaminated soil. In the mid 1990s, approximately 90 % of all lead emissions into the atmosphere were due to the use of leaded petrol (1). Additional sources of lead include soldered seams in food cans, ceramic glazes and cosmetics (2). Lead, absorbed in any form in humans, is accumulated in the central nervous system (CNS), first of all in the cortex and hippocampus (3). Abnormalities of cortical spontaneous and evoked activity were found in lead-exposed children (4, 5). Changes in central and peripheral evoked activity such as sensory evoked potentials and nerve conduction velocity were described in adults occupationally exposed to lead (6, 7). In animal models, lead induced EEG disorders and learning disability in young rats (8). In earlier studies of our laboratory, similar changes were found in rats after up to 12 weeks of oral exposure by similar doses of Pb^{2+} as used in this investigation (9).

Mercury exposure is an occupational hazard in chloralkali plants and luminescent tube manufacturing. Environmental mercury results from human activity
and is converted to organic form by microbial activity. In long-term occupational exposure to inorganic mercury, alterations of the cortical electrical activity have been reported. Slowed EEG was found in chloralkali workers, as well as amplitude increase of the somatosensory evoked potential and delayed waves in the brainstem auditory evoked potential in several jobs involving mercury exposure. Peripheral axonal neuropathy is another known consequence of mercury exposure. In animal experiments, damage to motor axons by mercuric chloride was described. Mercury affects ion channels in the peripheral and central nervous system, disturbs calcium homeostasis, and influences muscarinic and GABA receptors. In our earlier studies, 

The toxic nature of ethanol itself, including neurotoxicity, is well known. Beyond that, alcohol was reported to enhance carcinogenicity, mutagenicity, and hepatotoxicity of various toxicants. In man, alcoholism is associated with elevated blood lead concentrations, possibly because ethanol enhances the absorption of lead and increases blood alcohol levels. Ethanol potentiated the toxicity of methyl mercury in rats in terms of neurological manifestations (hindleg crossings and abnormal gait) and mortality. The aim of this study was to investigate the neurotoxicity of combined oral administration of the well-known neurotoxic heavy metals, lead and mercury, with another neurotoxic substance, ethanol.

METHODS

Male Wistar rats (12 weeks old, outbred) were obtained at the University’s breeding centre. The animals were housed under controlled standard conditions (up to five rats per cage; temperature, 20 °C to 22 °C; humidity, 60 % to 70 %; 12 hours light-dark cycle, with light on at 6.00 a.m.; standard rodent food and drinking fluid - detailed below - given ad libitum). The rats were treated with 

<table>
<thead>
<tr>
<th>Group</th>
<th>Code</th>
<th>Metal dose / mg kg⁻¹</th>
<th>Volume fraction of ethanol in drinking water / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, water</td>
<td>CW</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Control, alcohol</td>
<td>CA</td>
<td>none</td>
<td>5</td>
</tr>
<tr>
<td>Lead, low dose, water</td>
<td>PbLW</td>
<td>80</td>
<td>none</td>
</tr>
<tr>
<td>Lead, high dose, water</td>
<td>PbHW</td>
<td>320</td>
<td>none</td>
</tr>
<tr>
<td>Lead, low dose, alcohol</td>
<td>PbLA</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>Lead, high dose, alcohol</td>
<td>PbHA</td>
<td>320</td>
<td>5</td>
</tr>
<tr>
<td>Mercury, low dose, water</td>
<td>HgLW</td>
<td>0.4</td>
<td>none</td>
</tr>
<tr>
<td>Mercury, high dose, water</td>
<td>HgHW</td>
<td>1.6</td>
<td>none</td>
</tr>
<tr>
<td>Mercury, low dose, alcohol</td>
<td>HgLA</td>
<td>0.4</td>
<td>5</td>
</tr>
<tr>
<td>Mercury, high dose, alcohol</td>
<td>HgHA</td>
<td>1.6</td>
<td>5</td>
</tr>
<tr>
<td>Mercury, low dose + lead, low dose, water</td>
<td>HgPbLW</td>
<td>0.4+80</td>
<td>none</td>
</tr>
<tr>
<td>Mercury, low dose + lead, low dose, alcohol</td>
<td>HgPbLA</td>
<td>0.4+80</td>
<td>5</td>
</tr>
</tbody>
</table>

At the end of the treatment period, the rats were anesthetised with urethane and prepared for electrophysiology. The skull over the left hemisphere was opened, and ball-tipped silver electrodes were placed on the primary somatosensory, visual and auditory centres. Electrocorticogram (ECoG) was recorded from these areas for five minutes. Then, cortical sensory evoked potentials (EPs) were recorded by applying somatosensory stimulation (a pair of needles inserted into the whisker pad, delivering weak electric shocks [1 Hz, 3-4 V, 0.05 ms]), visual stimulation (flashes [1 Hz, 60 lux] into the contralateral eye), and acoustic stimulation (clicks [1 Hz, 40 dB]) stimulation. Fifty evoked potentials of each modality were recorded and averaged. Power spectrum was obtained from the ECoG according to the standard EEG bands delta to gamma. Latency and duration were measured for evoked potentials.
The rats were then sacrificed with an overdose of urethane. Weights of the brain, liver, heart, lung, kidneys, thymus and adrenal glands were measured. To obtain relative organ weights, the measured weight of the organs was divided by that of the brain. This calculation is based on our long experience with toxicants potentially affecting the whole body (metabolic effect), being a more reliable method in detecting organ-specific hyper- or hypotrophies than calculating relative weights on the basis of body weight.

All electrophysiological, body weight and organ weight data were tested for normal distribution by the Kolmogorov-Smirnov test, then analysed using the one-way ANOVA and post-hoc LSD test, with p<0.05 set as the criterion of significance.

The study was performed in accordance with the principles of the Ethical Committee for the Protection of Animals in Research of the University of Szeged.

RESULTS

The body weight gain of the animals (between weeks 0 and 12 of treatment) was lower in all treated groups than in the water control group (Figure 1). The difference was significant for HgHW and PbHW groups.

Among relative organ weights (Table 2), liver weight significantly decreased in the metal-treated groups, save for PbHA. The weight of the lungs significantly decreased in groups receiving high-dose metals with water, but not in the alcohol combinations; the weight of the heart showed a similar, although not significant trend. Spleen weight decreased significantly in groups receiving high-dose Hg$^{2+}$. No significant effects were seen for other organ weights.

Control ECoG spectra (Figure 2) were very similar in the three centres (somatosensory, visual and auditory). In the water-drinking groups receiving Pb$^{2+}$ or Hg$^{2+}$, delta-activity decreased compared to water controls; in the alcohol-combined groups, however, delta activity increased vs. alcohol control. Groups receiving both metals combined with alcohol (HgPbLA) showed a decrease in gamma-activity compared to alcohol control, which was not seen in the corresponding water-drinking groups.

Changes in the cortical EPs were more pronounced in the somatosensory (Figure 3) and visual (Figure 4) centre than in the auditory cortex (not shown). In the somatosensory EP, latency was significantly increased by both metals and by their combination, but the effect of Pb$^{2+}$ seemed to be counteracted by alcohol.
Changes in EP duration were less characteristic. Significant changes were seen in the visual centre, both in latency and duration, and the disappearance of lead effect in alcohol-drinking rats was observed here too. The latency of the somatosensory and visual EP was significantly altered by alcohol alone (CA vs. CW groups in Figures 3 and 4).

**DISCUSSION**

In evaluating the effects of alcohol combined with other neurotoxicants, considerations must include interactions in the phase of access to the nervous system and in direct neurotoxicity.

The blood-brain barrier is crucial in protecting the CNS against harmful agents. Ethanol was reported to increase the fluidity of nerve cell membranes (28) and thus to change the permeability of the blood-brain barrier (29). Lead and mercury are both known to damage the blood-brain barrier (30, 31), so that at higher doses, the levels of these metals in the brain can be disproportionately elevated. If alcohol is present, the barrier damage and the concomitant entry of heavy metals in the CNS can be further increased. This, however, did not cause changes in the brain weight in this study. Of the organs measured, the liver seemed to be the most affected both by Pb2+ and Hg2+ and by alcohol, which is a well-known effect (32, 33).

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**Table 2** Relative organ weights (organ weight/brain weight) in the control and treated animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Lungs</th>
<th>Heart</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Adrenal gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW</td>
<td>8.920±1.321</td>
<td>1.126±0.143</td>
<td>0.780±0.073</td>
<td>1.524±0.119</td>
<td>0.342±0.049</td>
<td>0.343±0.058</td>
<td>0.038±0.007</td>
</tr>
<tr>
<td>CA</td>
<td>8.192±1.013</td>
<td>1.043±0.083</td>
<td>0.760±0.168</td>
<td>1.525±0.290</td>
<td>0.334±0.056</td>
<td>0.320±0.074</td>
<td>0.037±0.005</td>
</tr>
<tr>
<td>PbHW</td>
<td>7.810±0.968</td>
<td>0.983±0.071</td>
<td>0.630±0.043</td>
<td>1.500±0.096</td>
<td>0.277±0.035</td>
<td>0.318±0.058</td>
<td>0.033±0.007</td>
</tr>
<tr>
<td>HgHW</td>
<td>6.920±0.777</td>
<td>3.04±0.056</td>
<td>0.726±0.103</td>
<td>1.522±0.158</td>
<td>0.277±0.044</td>
<td>0.299±0.049</td>
<td>0.037±0.007</td>
</tr>
<tr>
<td>PbHA</td>
<td>8.785±1.366</td>
<td>1.093±0.196</td>
<td>0.765±0.206</td>
<td>1.681±0.199</td>
<td>0.339±0.038</td>
<td>0.302±0.067</td>
<td>0.04±0.005</td>
</tr>
<tr>
<td>HgPbHW</td>
<td>7.941±0.369</td>
<td>1.006±0.096</td>
<td>0.638±0.064</td>
<td>1.445±0.178</td>
<td>0.340±0.065</td>
<td>0.302±0.062</td>
<td>0.035±0.006</td>
</tr>
<tr>
<td>HgPbLA</td>
<td>7.601±1.066</td>
<td>0.950±0.130</td>
<td>0.706±0.097</td>
<td>1.500±0.165</td>
<td>0.295±0.065</td>
<td>0.323±0.085</td>
<td>0.038±0.006</td>
</tr>
</tbody>
</table>

Mean ± SD, n=10. * p<0.05 vs. water control. For group codes, see Table 1.

**Figure 3** Latency and duration of the somatosensory cortical evoked potentials, in water-drinking (left) and alcohol-drinking (right) groups. Mean±SD, n=10. Ordinate: latency or duration, ms * p<0.05, treated vs. corresponding (water or alcohol) control; ° p<0.05, alcohol control vs. water control.
One of the likely mechanisms by which Hg\(^{2+}\) acts on the spontaneous cortical activity is to affect the ascending cholinergic activation (34). Hg\(^{2+}\) inhibits choline acetyltransferase (35) and inactivates muscarinic receptors (36), resulting in diminished activation of the cortex. In our study, this was more prominent in the auditory and visual and less in the somatosensory centre. As for Pb\(^{2+}\), literature describes only changes in spontaneous and evoked ACh release (37). This effect did not cause a significant ECoG change in our experiment. Observing the effect on the evoked potentials, Pb\(^{2+}\) and alcohol seemed to be in antagonism, whereby cortical GABAA receptors may have been desensitised due to the 12-week exposure to alcohol as a GABA agonist (38, 39). The resulting disinhibition of the cortical circuits involved in the generation of evoked potentials (40) could partially counteract the depression (lower amplitude, lengthened latency) of the evoked cortical responses caused by Pb\(^{2+}\) (9). This was indicated by comparing identical data of water-drinking and alcohol-drinking animals in Figures 3 and 4. In case of Hg\(^{2+}\), no such antagonism was observed, possibly due to the difference in the cortical effect of the two metals outlined above.

Our results showed that some of the neurotoxic effects of the investigated metals were altered by co-exposure with alcohol. This, together with the effect of alcohol on the access of heavy metals to the brain, suggests that combined exposure to heavy metals and regular alcohol consumption could result in more severe neurotoxic outcomes.

Acknowledgement

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Sažetak

KOMBINIRANI UČINCI SUPKRONIČNE IZLOŽENOSTI OLOVU, ŽIVI I ALKOHOLU NA SPONTANU I PODRAŽAJNO IZAZVANU KORTIKALNU AKTIVNOST KOD ŠTAKORA

Cilj rada bio je istražiti učinke kombinacije poznatih neurotoksičnih teških metala, olova i žive, u kombinaciji s etanolom. Mušjacima štakora soja Wistar (starim 12 tjedana) tijekom 12 tjedana bila je dostupna obična voda ili voda koja je sadržavala 5 % (v/v) etanola te su tretirani oralnom intubacijom s dvije doze olovova acetata ili živina klorida. Kontrolne skupine štakora tretirane su ili samo vodom ili pak vodom s alkoholom. Tijekom tretmana spontana i podražajno izazvana aktivnost iz somatosenzorskih, vizualnih i auditivnih kortikalnih područja bila je snimana. Raspon frekvencija spontane aktivnosti, kao i latentni period te trajanje izazvanih potencijala su analizirani. Na elektrokortikogramu primijećen je pomak frekvencije, a kod izazvanih potencijala produženi latentni period i trajanje. Čini se da alkohol utječe na učinak metala. Kombinacija izloženosti teškim metalima i redovite konzumacije alkohola može rezultirati težim središnjim i/ili perifernim neurotoksičnim učinkom.

KLJUČNE RIJEČI: elektrokortikogram, etanol, evocirani potencijal, kombinirana izloženost, neurotoksičnost, teški metali

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