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# IMMUNOTOXICOLOGICAL INVESTIGATION OF SUBACUTE COMBINED EXPOSURE WITH LOW DOSES OF PB, HG AND CD IN RATS

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Detectable interactions between NOEL (No Observed Effect Level) doses of Pb, Hg and Cd in general toxicological, hematological, and immune function parameters were investigated. The metals (Pb-acetate, 20 mg/kg; $HgCl_2$, 0.40 mg/kg; $CdCl_2$, 1.61 mg/kg) were combined. First, the rats received the combination Pb + Hg + Cd for 4 weeks per os. Significant difference vs. control was found only in the weight of lung and popliteal lymph node (PLN). The Pb + Hg and Pb + Cd combinations significantly decreased the PLN to 100 g body weight and PLN to brain weight ratio, and Pb+Hg also decreased the relative adrenal weight. After 12 weeks treatment with the same doses, effects on the thymus, kidney, and adrenal weights in the Pb + Hg, and thymus weight in the Pb + Cd, combination were seen. Pb + Cd also affected the white and red blood cell count and hematocrit. Combined with Hg or Cd, NOEL dose Pb showed toxicity, indicating that exposure limits may be inefficient in combined exposure situations.

*Keywords:* Immunotoxicity – hematology – heavy metals – combination – rat

## INTRODUCTION

Lead, mercury, and cadmium are ubiquitous environmental pollutants causing continuous low dose exposure of the population. The main sources of oral exposure are the consume of certain plants (cereals, cabbage, carrot, radish, tobacco etc.) which accumulate heavy metals when grown in polluted areas, as well as seafood, or products of animals fed on polluted fodders. The inhalation exposure can be significant near coal fired electrical plants, waste incinerators, metal works, or factories emitting heavy metals into the air. The occupational exposure with sometimes much higher doses is rather frequent during mining, metallurgy, metal refining and in different

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other industries (paint, glass, plastic, etc.). For health protection, exposure limits have been worked out for the different metals, below which the risk of adverse health effects caused by them is low. In case of combined exposure, however, the interaction of components can lead to increasing toxicity of the single compounds [4] so exposure limits do not necessarily provide satisfactory protection against health damage in such situations. In our earlier subacute studies, pesticide-heavy metal interactions were investigated paired in LOEL–NOEL dose combinations, and both synergistic and antagonistic effects were registered on the basis of changes in certain toxicological, hematological and immune function parameters [7–11]. The aim of the present study was to investigate whether there are detectable synergistic interactions between inorganic lead, mercury, and cadmium combined in NOEL doses in rats.

## MATERIALS AND METHODS

Sheep red blood cells (SRBC) were produced by Philmaster Ltd. Budapest, Hungary; RPMI-1640 and Freund's Complete Adjuvant (FCA), by SIGMA, USA; Keyhole Limpet Haemocyanin (KLH), by Calbiochem, USA. Lead acetate (Pb), cadmium chloride (Cd), mercury chloride (Hg), and the other materials were supplied by REANAL Factory of Laboratory Chemicals, Budapest, Hungary.

Four-week-old outbred male Wistar rats, obtained from the SPF breed of Charles River Ltd., Hungary, were used. The animals were kept under conventional conditions (up to 5 rats per cage, 12 hour light-dark cycle, 22 ± 2 °C, 70 ± 10% humidity), standard rodent food and water were available at all times. Lead acetate (Pb), $CdCl_2$, and $HgCl_2$ were dissolved in distilled water (that is, the metals were administered in ionic form: $Pb^{2+}$, $Hg^{2+}$, $Cd^{2+}$) and the solution was given per os by glass gavage in a volume of 5 ml/kg.

On the basis of earlier 4-weeks dose-effect studies [7, 10] the applied (NOEL = No Observed Effect Level) doses of the heavy metals were: 20 mg/kg of Pb-acetate, 0.40 mg/kg of $HgCl_2$, and 1.61 mg/kg of $CdCl_2$. In the first experiment (*Exp. 1*) 24 animals were treated with the combination of the three metals (another 24 with distilled water), and 8 rats per group were used for toxicological and hematological studies, 8 for PFC assay, and 8 for DTH reaction. The treatment of DTH groups was continued for another 8 weeks after challenge to determine the same toxicological and hematological parameters as following 4 weeks treatment (see below). In the next experiment (*Exp. 2*) the effects of paired combinations of the metals (Pb + Hg, Pb + Cd, Hg + Cd) were investigated using the same experimental protocol.

### *Toxicological and hematological parameters*

The body weight of the animals was measured once a week (always on the same day). The organ weights of brain, thymus, lung, heart, liver, spleen, kidneys, adrenals, testicles, and popliteal lymph node (PLN) were determined on the 29$^{th}$ day and

on the 85th day from the animals used for the DTH reaction. For hematological studies blood was taken from the abdominal aorta. The absolute white blood cell (WBC) count, red blood cell (RBC) count, hematocrit (Ht), mean cell volume of RBC-s (MCV), and the cell content of the femoral bone marrow were measured by a PS-5 Blood Cell Counter (Medicor, Budapest, Hungary) as described [6]. The hemoglobin content was determined by a Minilab-540 photometer (Medicor, Hungary).

### *IgM-PFC assay*

The animals were immunized with $2 \times 10^9$ SRBC i.p. in 0.4 ml PBS on the 25th day of treatment. On the 29th day, the spleen was removed and the IgM-PFC number, calculated for $10^6$ spleen cells and for the whole spleen, was determined [5].

### *Delayed type hypersensitivity (DTH)*

The animals were immunized s.c. at the base of tail by 1mg KLH in 0.4 ml antigen preparation (KLH was dissolved in sterile PBS and emulgated with equal volume of FCA) on the 14th day of treatment. The reaction was challenged on the 29th day by injecting 17.5 mg KLH in 50 ml PBS into the left hind footpad. Footpad thickness was measured just before, 24, and 48 hours after challenge by a Microstat micrometer (CADAR, U.K.), and specific footpad swelling (D%) was calculated as described [6].

The distribution of data was checked for normality by the Kolmogorov-Smirnov test. Depending on the distribution, the statistical analyses were carried out by ANOVA or by Kruskal-Wallis non-parametric ANOVA setting the probability level to $p < 0.05$. In case of ANOVA, post hoc analysis of group differences was performed by LSD test, while group comparisons following the Kruskal-Wallis ANOVA were performed by Mann-Whitney test. For statistical analysis, Statistica for Windows 4.0 software was used.

## RESULTS

No abnormal behavior of the animals or alterations in the body weight gain was observed during the 4 or 12 weeks treatment. The combination of the three metals had no effect on the immune function parameters following the 4 weeks treatment. When the 3 metals were combined in *Exp. 1*, a significant increase in the lung, and a decrease in the PLN weight ($p < 0.05$ related to both 100 g body weight and brain) was observed. In *Exp. 2,* both Pb + Hg and Pb + Cd combinations decreased the relative weight of the PLN, and the former one also decreased the relative adrenal weight. The Hg + Cd combination resulted in an increase in the relative adrenal weight (Table 1). The hematological parameters examined were not affected.

*Table 1*
Changes of relative organ weights following 4 weeks treatment with the combinations of heavy metals (X ± SEM, n = 8)

| Combinations | Related to 100 g body weight | | | Related to brain | | |
|---|---|---|---|---|---|---|
| | Lung (g) | PLN (mg) | Adrenals (mg) | Lung (g) | PLN (mg) | Adrenals (mg) |
| Pb + Hg + Cd | 0.60 ± 0.02* | 3.57 ± 0.21* | 21.1 ± 1.48 | 0.90 ± 0.03* | 5.35 ± 0.31* | 31.4 ± 1.44 |
| $Control_{Exp\ 1}$ | 0.51 ± 0.01 | 5.23 ± 0.22 | 19.4 ± 0.94 | 0.77 ± 0.02 | 7.86 ± 0.37 | 29.1 ± 1.43 |
| Pb + Hg | 0.56 ± 0.03 | 3.68 ± 0.53* | 16.3 ± 0.83 | 0.75 ± 0.05 | 5.05 ± 0.80* | 21.9 ± 0.85* |
| Pb + Cd | 0.51 ± 0.03 | 4.57 ± 0.33* | 18.0 ± 1.16 | 0.69 ± 0.03 | 6.30 ± 0.44* | 24.7 ± 1.14 |
| Hg + Cd | 0.58 ± 0.02 | 7.22 ± 0.69 | 20.2 ± 0.74 | 0.78 ± 0.02 | 9.71 ± 1.03 | 27.0 ± 1.27* |
| $Control_{Exp\ 2}$ | 0.57 ± 0.03 | 6.92 ± 0.56 | 18.1 ± 0.93 | 0.73 ± 0.03 | 8.92 ± 0.52 | 23.5 ± 1.04 |

* $p < 0.05$; PLN: popliteal lymph node.

*Table 2*
Effect of the 12 weeks treatment with the paired combinations of the three metals on the relative organ weights of rats (X ± SEM, n = 8)

| Combinations | Related to 100 g body weight | | | Related to brain | | |
|---|---|---|---|---|---|---|
| | Thymus (g) | Kidneys (g) | Adrenals (mg) | Thymus (g) | Kidneys (g) | Adrenals (mg) |
| Pb + Hg | 0.091 ± 0.01* | 0.722 ± 0.02* | 8.87 ± 0.49* | 0.209 ± 0.01* | 1.66 ± 0.05* | 20.3 ± 1.00* |
| Pb + Cd | 0.085 ± 0.01* | 0.700 ± 0.02 | 10.9 ± 1.16 | 0.188 ± 0.01* | 1.55 ± 0.04 | 24.1 ± 2.46 |
| Hg + Cd | 0.094 ± 0.01 | 0.678 ± 0.02 | 10.3 ± 0.76 | 0.221 ± 0.02 | 1.58 ± 0.05 | 24.0 ± 1.72 |
| $Control_{Exp\ 2}$ | 0.115 ± 0.01 | 0.650 ± 0.02 | 11.8 ± 1.05 | 0.265 ± 0.02 | 1.49 ± 0.04 | 27.1 ± 2.49 |

* $p < 0.05$

*Table 3*
Changes in some hematological parameters following 12 weeks treatment with the paired combinations of the heavy metals (X ± SEM, n = 8)

| Combinations | WBC × $10^6$/ml | RBC×$10^9$/ml | Hematocrit (%) |
|---|---|---|---|
| Pb + Hg | 6.81 ± 0.68 | 8.84 ± 0.21 | 51.2 ± 0.82 |
| Pb + Cd | 12.6 ± 1.80* | 8.34 ± 0.78* | 49.2 ± 3.84* |
| Hg + Cd | 7.21 ± 0.50 | 9.20 ± 0.18 | 53.3 ± 1.20 |
| $Control_{Exp\ 2}$ | 6.71 ± 1.21 | 9.60 ± 0.10 | 54.5 ± 0.77 |

* $p < 0.05$

At the end of the 12-week treatment, no changes in the toxicological and hematological parameters were found when the three metals were combined. The Pb + Hg combination significantly decreased the relative thymus and adrenal weight, and increased the relative kidney weight, while the Pb + Cd combination resulted in increased relative thymus weight (Table 2). In Figure 1, the changes of the relative

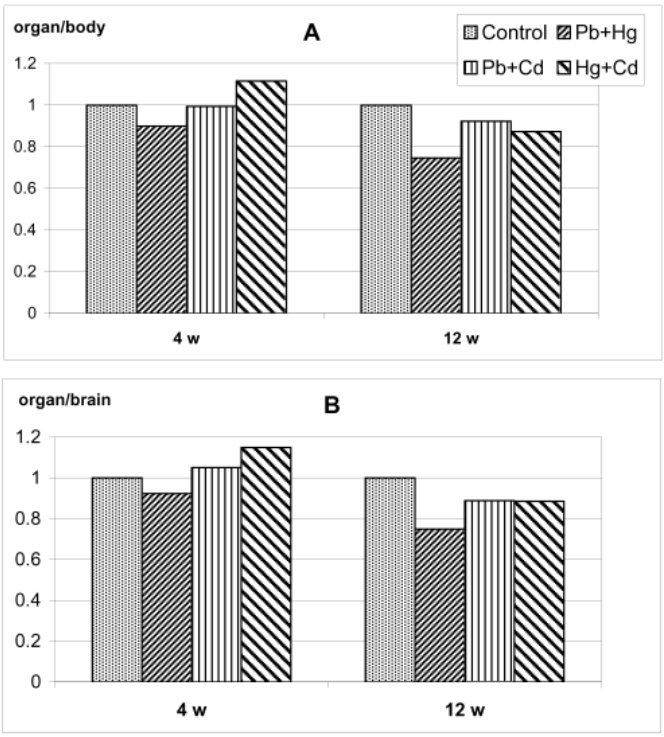


*Fig. 1.* Changes of the relative weight of the adrenals, after 4 and 12 weeks treatment (abscissa) with the double combinations of the three metals (see insert in A). A, organ weight/100 g body weight; B, organ weight/brain weight. The values were normalized to the corresponding controls. For error ranges and significance, see Tables 1 and 2

weight of the adrenals after the 4- and 12-week treatment are compared. As it can be seen, the trend of changes was the same after both treatment periods. Among the hematological parameters measured (Table 3) the Pb + Cd combination significantly increased the white blood cell count ($p < 0.05$), decreased the red blood cell count ($p < 0.05$) and the hematocrit value ($p < 0.05$).

## DISCUSSION

The advantage of the multilevel design in combined toxicology studies is that it minimizes the number of dose groups, as the lower level combinations need not be investigated when negative result is received at the higher combination level [5]. As interactions were not detected by the immune function assays in the triple metals combinations, the paired combinations were not investigated with these assays.

Because of the low dose levels and relatively short duration of treatment only a few parameters showed interactions between the heavy metals. The most sensitive parameters were the relative organ weights as they changed already following a 4 weeks treatment, while changes in some hematological parameters appeared only 8 weeks later, and only in the Pb + Cd combination. Alterations of hematological and related parameters on treatment with heavy metals have been repeatedly described in the literature [4, 12, 13] but the variance in the doses and the fundamental difference of single vs. combined administration precludes a direct comparison.

It should be mentioned that the changes in the adrenal weight were alike both after 4 and 12 weeks of treatment (see Fig. 1) while the alterations of lung (Pb + Hg + Cd) and popliteal lymph node weight (Pb + Hg + Cd, Pb + Hg, Pb + Cd) proved to be temporary. The changes of thymus and kidney weights appeared only after 12 weeks of treatment. The present experiments do not give information about the mechanism of interactions observed. The main targets of Cd toxicity are kidney and liver; that of lead are hemopoiesis, nervous and cardiovascular system; and of Hg, liver, kidney, immune system, and nervous system [1–3]. The low doses investigated failed to produce changes in the weights of the main target organs (except kidney weight in the Pb + Hg combination on the $12^{th}$ week). The hematological alterations appeared in the Pb + Cd combination after 12 weeks treatment, so any interaction between Pb and Cd on the hemopoiesis cannot be excluded even if the cellularity of the femoral bone marrow did not change. In earlier studies, using the same experimental system, 4-week treatment with 80 mg/kg lead-acetate increased the MCV value, while 6.43 mg/kg $CdCl_2$ decreased the hematocrit and MCV without any alteration of the cellularity of the femoral bone marrow [8].

The results cannot be directly extrapolated to man, but the interactions between NOEL doses of the heavy metals (mainly the combinations with lead) point to the possibility of increased toxicity of the single components. So, it is not sure that exposure limits, determined for the metals one by one, can provide a satisfactory protection in such situations.

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