Pharmacology Biochemistry & Behavior, Vol. 25, pp. 1089–1094, 1986. © Ankho International Inc. Printed in the U.S.A. 0091-3057/86 $3.00 + .00

Lead Retention in Blood and Brain After Preweaning Low-Level Lead Exposure in the Rat[1]

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Received 6 March 1986

LIVESEY, D. J., R. G. DAWSON, P. J. LIVESEY, J. BARRETT AND T. J. SPICKETT. *Lead retention in blood and brain after preweaning low-level lead exposure in the rat.* PHARMACOL BIOCHEM BEHAV **25**(5) 1089–1094, 1986.—Newborn rats of the albino Wistar strain were exposed to lead from birth to 20 days of age through mothers milk, from dams which were fed diets containing 0, 0.25, 0.5 or 1.0% powdered lead. Subsequent determination of tissue lead revealed a direct relationship between the lead levels in both blood and brain of the pups and the lead dosage to which they were indirectly exposed via the dams' milk. Lead retention in both tissues was still evident at 100 days of age, with the relative elevation of lead levels being an order of magnitude higher in brain than in blood. There were no obvious signs of lead intoxication in the pups, apart from mild growth retardation in the group with the highest lead burden. However there was a significant retardation in behavioral development observed on two of four measures which were employed. It was concluded that brief exposure to low lead levels in infancy can have long lasting consequences in the brain and in behavior.

Blood lead level Brain lead level Preweaning lead exposure Nutritional status Eyeopening
Auditory startle Mid-air righting Head-down descent

THERE are now a number of studies that indicate that children exposed to low doses of lead intoxication, i.e. "asymptomatic" or "subclinical" levels, may still suffer behavioral abnormalities [10, 13, 20]. However, in this type of study, inference of a causative relationship between lead loading and behavioral deficit may not be justified [20]. This has resulted in a search for physiological and behavioral concomitants of low-level lead exposure using animal models.

In an earlier study [5] we demonstrated significant behavioral effects following brief exposure to low or "asymptomatic" levels of lead in the rat. Pups ingested lead through their mothers' milk from birth to weaning yielding mean blood-lead levels at weaning ranging from 4.5 μg/100 ml of blood in control animals to 53.5 μg/100 ml in the highest lead group. Behavioral testing occurred at varying times from 3 to 70 days post-natal (PN).

In that study we noted a dose related decline in blood-lead levels between 21 and 74 days PN with evidence of behavioral recovery as lead levels fell. There were, however, indications of behavioral deficit for a considerable time after lead ingestion had ceased. For example, lead treated rats still showed hyperactivity in the open field at PN44 with behavior of the experimental animals becoming increasingly divergent from controls as the rats matured.

Thus a question of considerable significance is the level of persistence of lead in the body following a relatively brief exposure to low-level lead intake, i.e., to lead levels that do not result in obvious signs of neurotoxicity. In the present

[1]This project was supported by Grant No. 80/3548 from the National Health and Medical Research Council of Australia.

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study we traced the course of lead retention in rats subjected to different levels of lead loading from birth to weaning. Lead levels were examined in blood and brain at each of five ages ranging from 12 to 100 days PN.

This study was prompted by three considerations. Firstly Mykkanen, Dickerson and Lancaster [15] noted the need for such studies observing that "there seems to be a surprising lack of data on the concentrations of lead in the tissues of young animals exposed to lead from a very early age" (p. 447). Secondly, as Jason and Kellogg [11] have pointed out, results obtained after prolonged periods of exposure may be caused by lead ingested early in the exposure period. This possibility is given credence by the finding that blood and brain lead levels in young rats declined substantially after 5 weeks of age even though they continued to be exposed to lead [13]. Thirdly there is evidence that abnormalties in brain function [11] and behavior [2,5] persist following the cessation of lead ingestion.

To monitor the toxic effects of the ingested lead, pup weights were recorded. As well, to further assess the neurotoxicity of the lead levels induced in the pups prior to weaning, concomitant behavioral analyses of the development of reflex behavior and coordination were carried out. Tests used were appearance of eye opening, auditory startle, mid-air righting and head-down descent. It was reasoned that retardation in these developmental indices would provide a more sensitive measure of deleterious effects of lead than had been employed previously [1,7].

The procedure for lead administration was similar to that already described [5]. Ground elemental lead was mixed with dry food fed to the dams. The suckling infants thus received lead via the mother's milk. Elemental lead has been previously utilized in research with sheep [9] and absorption of metallic lead from particles in the gastrointestinal tract has been investigated in the rat [3] using blood-lead levels as an index of lead uptake. In our own research, too [5] we have demonstrated that the use of elemental lead is a viable and consistent means of raising blood-lead levels and avoids the problems of attempting to control for the second radical in lead salts [4]. While a significant source of lead contamination in the environment is from leaded petrol it has been shown that the exhaust product of tetraethyl lead is inorganic [6]. Introduction of lead to the pup via the dam's milk is a realistic approach to lead administration, as the mother is a likely source of lead contamination for the suckling infant.

METHOD

Animals

Suckling dams of the albino Wistar strain were obtained with their litters within 12 hours of birth from the UWA Animal Breeding Unit. Each litter was comprised of 10 male pups made up from the dam's own pups and those from one other litter. After instituting a cross fostering procedure each dam and its new litter was housed in a Perspex cage with a sawdust bedding, for the first 20 days. On day 20 the dams were removed and ad lib food and water were provided for the pups' consumption. On day 30 the pups were housed singly in standard laboratory cages with ad lib food and water for the duration of the experiment. The animal room environment has a temperature of 21°C the humidity was 45–55% and there was a 12 hour light-dark cycle.

Diet

Lead powder (100 mesh to dust BDH chemicals) was combined with ground commercial rat pellets (Milnes' Rat Diet) using a domestic blender to produce four lead diets (1%, 0.5%, 0.25% and 0.1% lead by weight). The control diet was ground pellets alone.

The diets were all stored in plastic bottles fitted with 3 plastic vanes which provided for a thorough mixing of the diet when the bottles were mechanically rotated on their long axes. Diets were thoroughly mixed in this fashion each time the food hoppers were refilled.

Experimental Procedure

This report is the result of six replications of the same experimental procedure carried out over a period of a year. Each experiment involved one batch of 5 litters of rats and their dams. Upon arrival, the dams were weighed and assigned to one of the five dietary conditions (1%, 0.5%, 0.25%, 0.1% lead diet or control diet). The pups were then weighed, marked for identification using a colour code, assigned to one of the five dams systematically so that each dam had 2 pups from each original litter and 10 pups in all. In addition, pup weight was also taken into account in the allocation of pups to dams so that litter of origin and weight were equalized across the five dietary conditions. The dams were fed their lead loaded diets until 18 days post-partum using spill-proof dispensers. On day 19 standard laboratory diets were substituted for the experimental diets as by this stage some pups would begin consuming the contaminated food. Pups and dams were weighed every four days and maternal food consumption was recorded for each successive four-day period until weaning.

Behavioral Testing

A number of developmental parameters were studied in each pup, and the day was noted when each test was positive. Tests were as follows: (1) When both eyes opened fully; (2) When the pup exhibited an auditory startle response. Starting on day 10 each pup was tested once daily by being held by the back of the neck and, when resting quietly, a click was sounded behind its head using a metal toy "clicker" following the technique of Reiter *et al.* [17]. A positive startle consisted of a pronounced leg and body jerk; (3) When a pup could right itself in mid-air. Once each day commencing on day 14, each pup was held upside-down, by the head and the base of the tail, 30 cm above a container of sawdust. Mid-air righting was judged to occur when a pup could land upright on all fours after being dropped from the above position [1]; (4) When the pup could descend a rope head-first. From day 15 on, each pup was tested once each day. It was placed, head upwards, onto a 15 mm diameter rope of length 30 cm which hung vertically from a retort stand above a sawdust container. A fully developed head-down descent was scored when the subject was able to turn around within the top 15 cm of the rope and descend to the sawdust floor [1].

Tissue Analyses

At each of five ages (12, 20, 32, 52 and 100 days old), two pups were taken from each litter for blood analysis. Animals were drawn according to their colour code, with two colours being selected randomly for each day. Pups were heavily

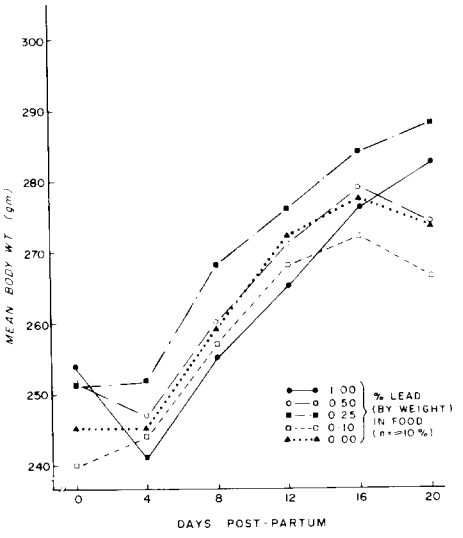

FIG. 1. Mean body weights of dams fed on different lead diets over the suckling period.

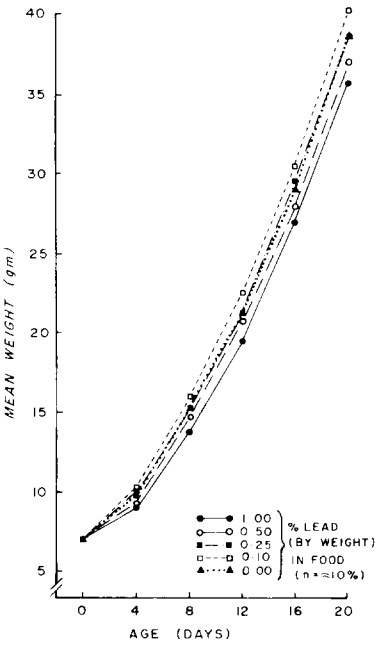

FIG. 2. Mean body weights of pups as a function of lead doses ingested by dams over the first 20 days of age.

anaesthetized with ether and blood samples were taken from the exposed heart using a 2.5 ml heparinized syringe (blood volume obtained ranged from 0.5–2 ml depending upon the age of the animal). The samples were placed in heparinized blood sample tubes, shaken liberally and stored at 5°C until analyzed. Next the brains were removed, placed in glass tubes which had been washed in Triton X-100 and weak acid and then placed in frozen storage until analyzed.

At 20 days post partum the dams were administered a lethal dose of nembutal and, during the anaesthetic stage of the injection, blood samples were taken as described previously for subsequent analysis.

Tissue Lead Analyses

Whole blood (30 μl) was treated with Triton X-100 (600 μl) and analyzed for lead according to the method of Kubasik, Valosin and Murray [12]. Accurately weighed whole brain samples (approximately 1 g of tissue) were digested with mild heating in low lead nitric acid (nitric acid for foodstuffs analysis, BDH Chemicals Ltd., Poole, England). Lead concentration was measured in the prepared blood samples and in the appropriately diluted digested tissue samples using a Varian AA-575 atomic absorption spectrophotometer fitted with a CRA-90 carbon rod atomiser and an ADS-53 automatic sample dispenser.

RESULTS

All results apart from those relating to the developmental indices, were analyzed with a one between (treatment) one within (Days) Analysis of Variance, The Newman-Keuls technique [21] was used for post hoc pairwise comparisons between groups following significant ANOVA results.

Weight of Dams (Fig. 1)

There were no obvious systematic differences in weight gain among groups of nursing dams fed on the different lead diets. An analysis of variance of dam weight over days confirmed that all experimental groups gained weight significantly over the nursing period, $F(5,105)=80.7$, $p<0.001$, at rates not significantly different from controls, $F(4)=0.25$, $p<0.2$.

Weight of Pups (Fig. 2)

All groups of animals gained weight rapidly during the preweaning lead ingestion period. An Analysis of Variance revealed a highly significant main effect for Days, $F(2,374)=15089$, $p<0.001$. Less obvious is the observation that the experimental group with the highest lead burden appears to have gained weight less rapidly than any other group. Post hoc independent comparisons using the Newman-Keuls technique following a significant main effect for group weight, $F(4,187)=12.2$, $p<0.001$, and significant Group × Days interaction, $F(8,374)=5.37$, $p<0.01$, confirm this observation by showing that weight gain was, for the most part, independent of lead loading with the exception that the group with the highest lead burden was significantly lighter than all the other groups.

Blood-Level Analyses of Dams and Pups (Fig. 3)

The different groups of dams differed significantly with respect to the levels of lead found in their blood at the end of the nursing period as indicated by the result of a one-way Analysis of Variance, $F(4,21)=40.7$, $p<0.001$, these differences being dose related.

The blood-lead levels in the pups over the 100 days of the experiment show a highly significant, $F(4,128)=77.7$,

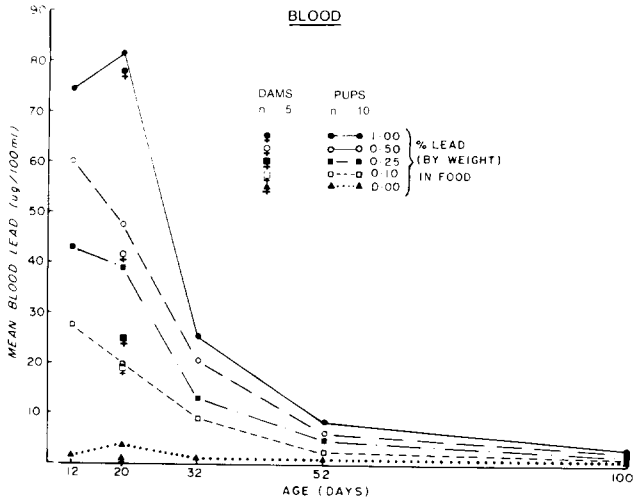

FIG. 3. Mean blood-lead levels of dams at 20 days and of pups from age 12 to 100 days as a function of lead doses ingested by dams.

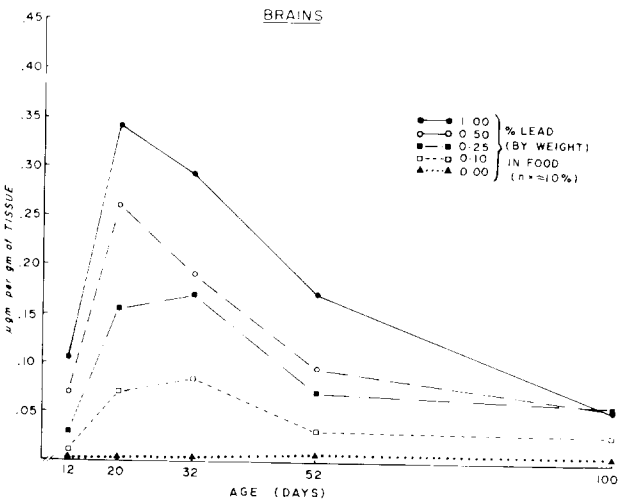

FIG. 4. Mean brain lead levels of pups from age 12 to 100 days as a function of lead doses ingested by dams.

$p<0.001$, dose related elevation of blood-lead followed by an exponential decline. The presence of a significant Dose × Days interaction, $F(16,218)=15.1$, $p<0.001$, confirmed the indications in the figure that the decline was most rapid in the high dose and least in the low dose animals. It is worth noting that the lead levels in the control animals were low compared with ones previously reported in the literature. Mykkanen *et al.* [13] for example recorded mean levels of from 4 to 11 µg/100 ml of blood in groups of their control animals.

Brain-Lead Analyses of Pups (Fig. 4)

This analysis revealed a highly significant dose related elevation of brain-lead levels on experimental animals, $F(4,218)=135.7$, $p<0.001$, and a highly significant Dose × Days interaction, $F(16,218)=12.7$, $p<0.001$. The dose related decline in lead appears to be slower from the brain than from the blood with brain-lead concentrations an order of magnitude higher in the experimental animals than in controls at 100 days of age compared with blood-lead levels.

Behavioral Observations (Fig. 5)

The results of the four behavioral tests, eye opening, auditory startle, mid-air righting and head-down descent were each analyzed by the use of a χ^2 analysis. Although the testing was conducted from Day 10 to Day 20, data were analyzed only for days on or surrounding the time when the behaviors selected for study were emerging. These analyses showed that the appearance of an auditory startle response, $\chi^2(4)=14.99$, $p<0.01$, and mid-air righting response, $\chi^2(12)=39.85$, $p<0.001$, were delayed significantly in the lead ingestion groups. However, no significant differences were revealed in the appearance of either eye opening or head-down descent.

On day 12 the percentage of animals exhibiting a startle response (Fig. 5A) was far less in the 1.0% and 0.5% lead groups than in the 0.25%, 0.10% and control animals. On Day 13 these differences had disappeared. A similar effect of dose on mid-air righting can be seen in Fig. 5B, where there is an inverse relationship between percent of animals righting

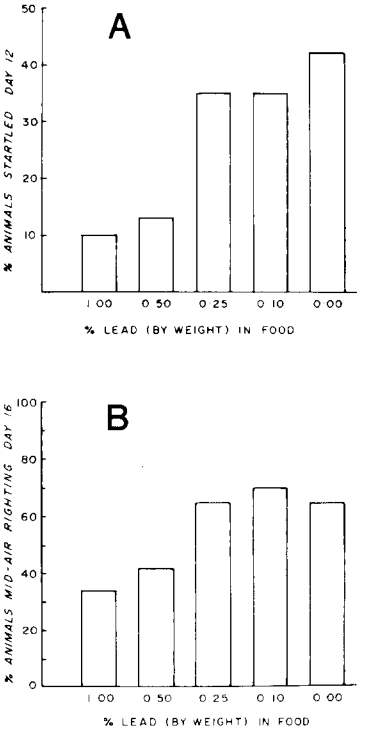

FIG. 5. A. Percentage of pups startled on Day 12 as a function of lead doses ingested by dams. B. Percentage of animals mid-air righting on Day 16 as a function of lead loses ingested by dams.

themselves on Day 16 and lead dose. This retardation was also evidently only a one day delay in development, since by Day 17 group differences were not evident.

DISCUSSION

There are two findings which, taken together, indicate that the nutritional status of the pups reared on milk from dams ingesting different amounts of lead differed only with regard to the lead content of that milk. Firstly there were no obvious systematic differences in weight gain among groups of nursing dams fed different lead diets. From this it seems reasonable to conclude that there are no discernable malnutritive effects that would bias the dams' milk production and thus confound lead effects across the pup groups. Secondly, although it was not possible to analyze milk lead levels directly, dams which received different lead diets had significantly different dose related levels of lead in their own blood at the end of the nursing period and these differences were clearly reflected in the blood lead levels in the pups (Fig. 3). It seems reasonable therefore to conclude that similar systematic differences would be found in the lead content of the milk of the different groups of nursing dams.

The range of doses of lead employed in this study did not produce malnourishment in the dams and only at the highest dose level was the growth of the pups retarded during the ingestion period. This latter finding indicates that effects of lead toxicity and malnutrition can be divorced at low doses. This material also provides evidence that this obvious indication of lead intoxification starts to become evident with blood-lead loadings at weaning of between 50 and 80 μg/100 ml of blood.

Lead loading in blood and brain was dose-specific. The highest levels recorded in blood were of the order of 70–80 μg/100 ml of blood after 12 days of suckling, declining, after dams were taken off lead at 18 days of age, to less than 10 μg/100 ml by day 52. The highest levels recorded in brain were approximately 0.30–0.40 μg/g tissue declining to approximately 0.05 μg/g of tissue by day 100. These comparatively low lead levels in both tissues appear to be partly a result of the use of metallic lead as a source. In a study employing lead acetate and using the same dose by weight, Mykkanen *et al.* [15] revealed substantially higher levels of tissue lead following 21 days exposure (0.5% lead gave a mean level of 47 μg/100 ml while 0.5% lead acetate yielded 335 μg/100 ml of blood).

However it is dangerous to compare dose effects directly across these two studies since control animals in the Mykkanen *et al.* study had levels of lead which fell well within the range of our experimental groups (e.g., a control level of 0.15 μg/g of brain tissue in that study compares with an experimental group in the present study fed from a dam receiving 0.25% of lead by weight and assayed at 20 days of age). it should be noted too that the higher lead levels induced in the Mykkanen *et al.* animals produced a significant weight loss in all lead loaded groups of pups.

While these lower lead burdens produced no obvious signs of gross behavioral abnormality in the pups there was evidence that some aspects of development were briefly but significantly retarded in their appearance. It should be noted however that malnutrition may have contributed to these delays in development since the analysis included one group (1.0% lead ingestion) which was malnourished.

This evidence of some developmental delay with mean blood-lead levels as low as 45 μg/100 ml is in accord with findings from the earlier study [5]. In that study behavioral testing covering more complex cognitive tasks revealed significant behavioral defects that persisted over a considerable period of time following discontinuation of lead administration.

While the earlier study sampled a small number of animals, employed a more limited range of lead ingestion levels and sampled less frequently, a comparison of those results with the ones from the present study has confirmed the consistency of the lead administration procedure in terms of blood-lead levels evident in the pups. This study indicates, as well, that relatively high levels of lead are retained in the brain for long periods following the cessation of lead administration, with decline in the lead loading of that organ being closely reflected by changing levels of blood-lead. The behavioral findings reported in the earlier study also relate closely to the declining brain-lead levels demonstrated in the present work.

Taken in conjunction with the earlier work this study confirms that relatively small amounts of lead consumed during the suckling period show remarkable persistance in the brain of rats and this persistance is reflected in blood lead. This lead loading appears to be expressed in terms of deleterous behavioral effects in the maturing animal, particularly in relation to the animal's ability to adapt quickly to a novel situation [5].

Causal relationships between lead ingestion and behavioral deficit are still being actively explored (e.g., [18,20]). Evidence that the brain absorbs and retains relatively high levels of lead clearly provides the basis for this relationship and numerous studies have examined the effects of lead on brain morphology and function. Winder [20] has presented a comprehensive review of these studies. One significant lead effect appears to be the modification of neurotransmitter release and uptake.

While most studies have again focussed on effects of high levels of lead ingestion (i.e., leading to blood-lead levels substantially above 100 μg/100 ml of blood) a number of studies have examined the effects of low levels of lead toxicity on neurotransmitter availability. Much of this work has been directed to the amine and choline systems. Wide discrepencies in findings between different laboratories have been observed in these studies [18,20]. However Shih and Hanin [18] argue that overall the evidence suggests a trend towards elevated activity in catecholamine function. In studies of cholinergic system function the least contradictory data relate to turnover rates with relatively strong evidence for a depression in the rates of production and utilization of this transmitter [20]. Louis-Ferdinand *et al.* [13] for example, reported significant reduction in ACHE activity (40%) in the hippocampus of 10 day old lead-treated rats. Silbergeld and Goldberg [19] have hypothesised that these dual changes in the two neurotransmitter systems, with increase in aminergic and decrease in cholinergic activity, are interrelated and result in lead induced hyperactivity as observed in their mice.

Evidence of more basic underlying disturbances in neural metabolism that could account for the observed disruption of amine and choline system function have also been reported. Winder [20], for example, reported studies revealing disturbance in oxidative or energy metabolism with blood lead levels down to 10 μg/100 ml, such disturbance being evident in both adult and neonate rats. He also noted that biopterin metabolism is also very sensitive to lead, this enzyme system

playing a significant role in the cycle of aminergic synthesis. Disturbance of this enzyme system would thus have serious effects on catecholamine transmitter systems.

Calcium levels too have been shown to influence lead induced neurochemical effects. It is argued that the likely cause of these changes is competitive replacement of calcium at presynaptic sites with a consequent effect on the release of acetylcholine and uptake of choline this also affecting acetylcholine turn over [18,20].

Thus the links between brain intoxification and behavioral abnormalities are being forged in the area of the "pharmocodynamics" of lead action.

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