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# Effect of Subacute Exposure to Lead on Responses to Estrogen in the Immature Rat Uterus

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Lead is a widely spread environmental pollutant that affects both male and female reproductive systems in humans (Winder, 1993) and in experimental animals (Ronis et al. 1996). Besides occupational lead poisoning, excessive lead exposure originates from leaded gasoline combustion dust, lead-based paint, soil, lead contaminated glassed pottery or water (Needleman, 1990). In women, reported effects include infer-tility, miscarriage, preeclampsia, pregnancy hypertension and premature delivery (Winder 1993). In experimental animals, chronic exposure to lead may cause an inhibition of menstruation, ovulation, and follicular growth in monkeys (Vermande-Van Eck & Meigs 1960), a delay in vaginal opening in pubertal rats (Kimmel et al. 1980) and a decrease in frequency of implanted ova and of pregnancies in mice (Odenbro & Kihlström 1977).

Experimental studies suggest that lead affects female reproductive organs through different mechanisms. It may interact at the enzyme level (Wiebe et al. 1988; Kempinas et al. 1994), may interfere with the action of reproductive hormones at the target organ by modifying the activity of estrogen receptors in the pregnant uterus (Wide & Wide 1980) or inhibit the implantation process that is regulated by estrogens (Wide 1980). Lead may induce imprinting mechanism (Csaba et al. 1986; Tchernitchin & Tchernitchin 1992) causing persistent changes in uterine estrogen receptors (Wiebe & Barr 1988) and ovary LH receptors (Wiebe et al. 1988) following perinatal exposure. Lead may decrease pituitary response to growth hormone releasing factor, affect levels of gonadotropin-releasing hormone, somatostatin, FSH and LH (See Tchernitchin et al. 1998 for a review) and increase blood levels of glucocorticoids (Vyskocil et al. 1991). Additionally, exposure to lead increases stress levels (Vyskocil et al. 1991); which, is followed by a raise in glucocorticoid, catecholamine, growth hormone and prolactin levels. Taking into consideration that increased levels of glucocorticoids (Tchernitchin et al. 1985) and prolactin (Unda et al. 1989) selectively inhibit some responses to estrogen in the uterus and other target organs and that lead interacts with estrogen receptors (Wide & Wide 1980) it becomes clear that the effects of lead exposure on uterine responses to estrogens should be investigated in order to understand the mechanisms involved in lead-induced fertility impairment.

To investigate the effects of exposure to lead on responses to estrogen in the uterus, it is necessary to consider that estrogens induce separate groups of responses through independent mechanisms of hormone action in which different types of estrogen receptors are involved (Tchernitchin et al. 1985). Accordingly, the increase in uterine RNA and protein synthesis are genomic responses induced through hormone interaction with cytosolic-nuclear

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receptors in the various uterine cell-types (Jensen and DeSombre, 1972). Estrogen-induced uterine edema, increase in vascular permeability and release of histamine are non-genomic responses (Tchernitchin et al 1985) induced through hormone interaction with eosinophil leukocyte estrogen receptors (Tchernitchin et al. 1985, 1989) which mediate the migration of these cells from the blood to the uterus (Tchernitchin et al., 1974, 1985), their degranulation (Tchernitchin et al., 1989) and release of agents involved in the development of the eosinophil-mediated responses (Tchernitchin et al. 1985, 1989). Additional mechanisms of estrogen action, involving lower affinity kind II cytosolic and nuclear estrogen receptors, uterine membrane estrogen receptors, cyclic AMP and prostaglandins (see Tchernitchin et al. 1985 for a review) have been proposed as well. The existence of multiple and independent mechanisms of estrogen action for the different responses to hormone stimulation results in a dissociation of estrogenic responses under a number of conditions (see Tchernitchin et al. 1985 for a review). In addition, several estrogenic or antiestrogenic compounds may selectively modify estrogen action in some cell-types only (Tchernitchin et al. 1985; Grunert et al. 1986). Therefore, the study of any agent modifying the action of estrogen in the uterus must consider the different mechanisms of estrogen action and the wide spectrum of responses to hormone stimulation in the different uterine cell-types.

Taking the above into consideration, we earlier reported the effects of a single dose of lead on a subsequent estrogen stimulation of the different uterine cell types in the immature rat, 1 or 24 h after hormone treatment (Tchernitchin et al., 1998). The selective changes in several parameters of hormone action were interpreted as direct effect of lead on receptors, other molecules or processes directly involved in the different mechanisms of hormone action.

The subacute or chronic exposure to lead is the most common situation of environmental lead toxicity. Taking into consideration the possibility that subacute or chronic exposure to lead additionally modifies regulatory mechanisms, such as up- or down-regulation, receptor replenishment, cause time-dependent changes in cells, or induce the synthesis of protective agents against toxicity, the present study describes the effect of a subacute exposure to lead.

## MATERIALS AND METHODS

Eight groups of female rats (10 to 15 animals per group) from a Sprague-Dawley-derived colony bred at the vivarium of the Faculty of Medicine, University of Chile, were used in the present study. The animals received lead acetate (Merck, Darmstadt, Germany) (172 µg $Pb^{++}$/g body wt s.c.) or saline physiological solution, every second day from the age of 14 days until the age of 20 days (total dose, 688 µg $Pb^{++}$/g body wt) (Table 1). The rats were further treated with estradiol-17 β (Sigma Chemical Co., St. Louis, MO) (300 ng/g body wt i.v.) or its vehicle (absolute ethanol in saline physiological solution 1:9) at the age of 21 days. This age is the most appropriate for the study of the effects of sex steroids on target organs, since estrogen and progesterone levels are extremely low and receptor levels and hormone responsiveness is already fully developed (Tchernitchin et al. 1985). Six or 24 h after treatment the uteri were excised under ether anesthesia. One uterine horn was fixed in 4% neutral formalin and subjected to further histological procedure for eosinophil quantification and morphometry (Tchernitchin & Galand 1983). The other uterine horn was fixed in Bouin alcohol fixative and paraffin sections were subjected to the periodic acid Shiff (PAS) reaction for semiquantiative glycogen determination in the different uterine cell-types. It was previously shown that blood lead levels were 47 µg/100 mL in lead exposed rats, under the same exposure conditions, at the time of uteri excision and 3 µg/100 mL in non-exposed controls (Villagra et al. 1997).

**Table 1.** Experimental conditions

| pretreatment (days 14, 16, 18 and 20) | Treatment (day 21). The uteri were excised 6 h (E6 and V6) or 24 h (E24 and V24) after treatment | |
|---|---|---|
| | Estradiol (E) | Vehicle (V) |
| Lead (L) | L/E6 L/E24 | L/V6 L/V24 |
| Saline physiological solution (S) | S/E6 **S/E24** | **S/V6** S/V24 |

The following parameters of estrogen stimulation were investigated in the uterus: myometrial hypertrophy was measured as increase in the reciprocal value of cell density (RVCD) in circular myometrium, edema in deep and superficial endometrial stroma was evaluated as increases in RVCD in these histological locations (Grunert et al. 1986), and uterine eosinophilia was measured as total number of eosinophils located in both uterine horns (Tchernitchin et al., 1974). Luminal epithelial and glandular epithelial cell hypertrophy were evaluated morphometrically as estrogen-induced increase in cell volume (Tchernitchin et al., 1995), estrogen-induced mitotic response was evaluated as increase in the number of mitotic figures in every cell-type investigated (Grunert et al., 1986) and estrogen-induced apoptotic response was evaluated as increase in the number of morphologically assessed apoptotic figures. Glycogen content in myometrium was visually assessed and expressed semiquantitatively for further non-parametric statistical analysis. All parameters except glygogen were expressed as percent of maximal values obtained under estrogen treatment in animals non-exposed to lead.

Statistics: Uterine eosinophils, mitosis and apoptosis data were subjected to a square root transformation to normalize distribution; the remaining data were not transformed due to their normal distribution (Grunert et al. 1984). Since multiple comparisons were performed between the 4 experimental conditions within the same time of treatment, transformed or non-transformed data were subjected to the least significant difference (LSD) test. The common variance was estimated from a one-way unbalanced analysis of variance (ANOVA) within the same time of treatment, and no significant differences were declared unless ANOVA was significant. The semiquantitative data of myometrial glycogen levels was subjected to the Kohnogorov-Smirnov two-sample test (Siegel 1956).

## RESULTS AND DISCUSSION

The effects of exposure to lead on selected non-genomic parameters of estrogen stimulation are shown in Figure 1. Lead inhibits estrogen-induced uterine eosinophilia at 6 and 24 h after treatment. Lead also inhibits estrogen-induced edema in deep and superficial endometrial stroma at 24 h but not 6 h after treatment, time where a slight potentiation of the response is detected in superficial endometrium. The effects of exposure to lead on selected genomic responses to estrogen are also shown in Figure 1. While estrogen-induced uterine luminal epithelial hypertrophy is not detected at 6 h after treatment in non-exposed animals, the response is fully developed at this time in lead-exposed rats. At 24 h of treatment, exposed and non-exposed rats display similar responses. Estrogen-induced glandular epithelium hypertrophy is not significantly modified under the effect of lead exposure. Myometrial hypertrophy, is inhibited under the effect of exposure at 24 h of treatment. No significant changes in estrogen-induced increase in glandular epithelium apoptosis are detected under the effect of lead exposure.

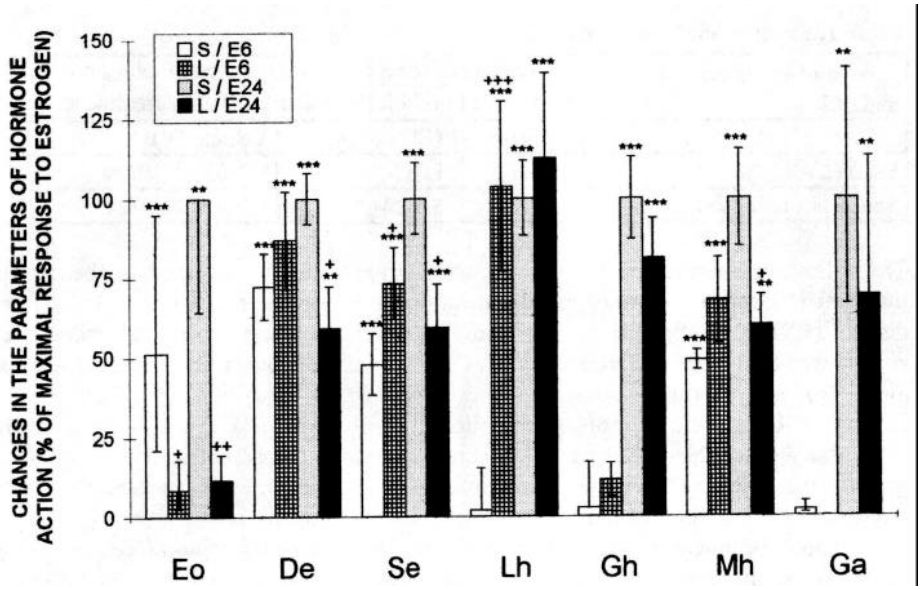


**Figure 1.** Effect of exposure to lead on estrogen-induced uterine eosinophilia (Eo), deep endometrial edema (De), superficial endometrial edema (Se), luminal epithelial hypertrophy (Lh), glandular epithelial hypertrophy (Gh), circular myometrial hypertrophy (Mh) and increase in glandular epithelial apoptosis (Ga), 6 and 24 h after hormone stimulation. Lead exposed rats (L) or saline injected controls (S) were treated with estradiol (E) or vehicle, and killed 6 or 24 h later. Results are means ± SEM of values expressed as % of maximal response to estrogen in animals that were not exposed to lead.. Uterine eosinophilia and glandular cell apoptoses correspond to geometric means ± SEM. Mean values for non-exposed untreated rats are defined as 0. Data for lead-exposed untreated animals is not shown and is closely similar to 0. For analysis of significance, the Least Significant Dfference (LSD) test was used. ** $p<0.01$, *** $p<0.001$, compared to the homologous condition without estrogen; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$, compared to the homologous condition without lead.

The effects of exposure to lead on estrogen-induced mitotic response is shown in Figure 2. At 24 h of hormone stimulation, an important inhibition of the response is detected in luminal epithelium only and a non-significant tendency for inhibition occurs in remaining cell-types. At 6 h after treatment, while a significant response to hormone stimulation is detected in longitudinal myometrium and mesometrium, lead is inhibitory.

Estrogen induced an important increase in glycogen content in myometrial cells at 24 h after treatment ($p<0.01$, Kolmogorov-Smirnov two-sample test) in both non-exposed or lead exposed rats. No change in the response to estrogen was detected under the effect of lead exposure (not shown).

The role of eosinophil leukocytes in estrogen action is well documented (see review in Tchernitchin et al. 1985, 1989). Any condition modifying uterine eosinophilia and/or eosinophil degranulation modify eosinophil-mediated responses, such as endometrial edema. Therefore, the lead inhibition of estrogen-induced endometrial edema at 24 h of treatment is in agreement with the inhibition of uterine eosinophilia. The lack of inhibition

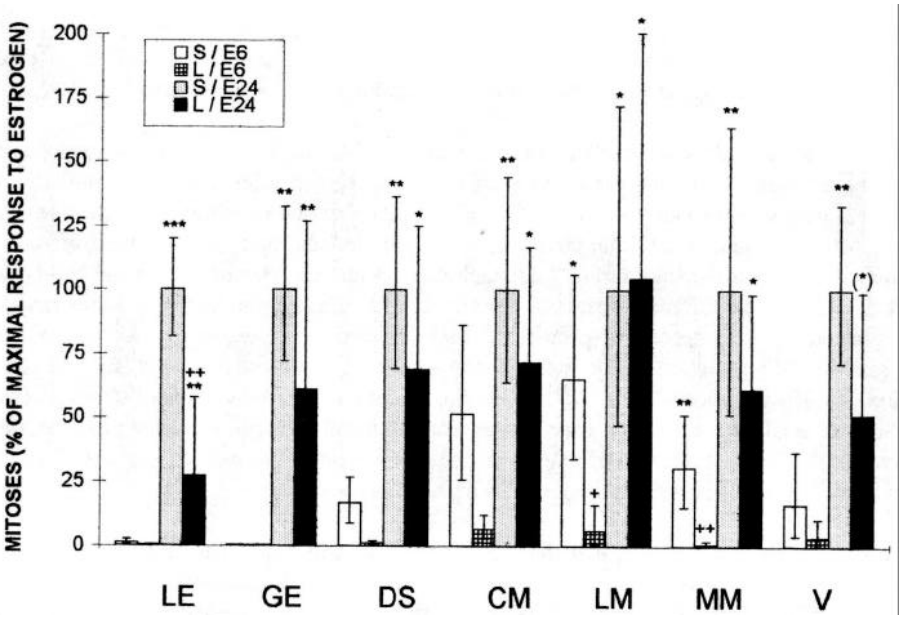


**Figure 2.** Effect of exposure to lead on estrogen-induced increase in mitoses in uterine luminal epithelial (LE), glandular epithelial (GE), deep endometrial stromal (DS), circular myometrial (CM), longitudinal myometrial (LM), mesometrial (MM) and vascular (V) cells, 6 and 24 h after hormone stimulation. Lead exposed rats (L) or saline injected controls (S) were treated with estradiol (E) or vehicle, and killed 6 or 24 h later. Results correspond to geometric means ± SEM of square root transformed values, expressed as % of maximal response to estrogen in animals that were not exposed to lead. Mean values for non-exposed untreated rats are defined as 0. Data for lead-exposed untreated animals is not shown and is closely similar to 0. For analysis of significance, the Least Significant Difference (LSD) test was used. *$p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to the homologous condition without estrogen; + $p<0.05$, ++ $p<0.01$, compared to the homologous condition without lead.

of edema at 6 h of treatment in deep endometrium and its slight potentiation in superficial endometrium can be explained by the increase in eosinophil degranulation in the blood under the effect of lead (Villagra et al, 1997), which potentiates non-genomic responses to estrogen at early times (6 h) and further inhibit them. The inhibition can be explained by the loss of eosinophil granule content through a process of degranulation, which renders these cells unable to mediate a full non-genomic response (Tchernitchin et al. 1989). The inhibition by lead exposure of estrogen induced uterine eosinophilia can be explained either by the increase in glucocorticoid and prolactin under the effect of lead; which, in turn, inhibit uterine eosinophilia (*vide supra*), or by a direct effect of the eosinophils themselves.

The finding of a potentiation of estrogen-induced luminal epithelial hypertrophy under effect of lead exposure, and that the response develops earlier than in non-exposed rats, while no significant change was detected in glandular epithelium and an inhibition of response occurs in circular myometrium, is another example of the dissociation between genomic responses in the rat uterus. Although this finding, together with the inhibition of mitoses that affect mostly luminal epithelial cells, points to differences in the regulation

mechanisms in the different uterine cell-types, the differences can be also explained by the high concentration of lead that occurs in uterine luminal epithelial cells (Nilsson et al. 1991), the first step before its secretion into uterine lumen (Jin & Nilsson, 1993).

The previous study on the effect of acute exposure to lead in the mitotic response to estrogen revealed a strong potentiation of the response 6 h after hormone stimulation in several uterine cell-types, that was followed by normalization or inhibition of the response at 24 h of stimulation (Tchernitchin et al. 1998). In contraposition to these effects, a subacute exposure to lead during 7 days inhibits the mitotic response in several cell-types, at 6 or 24 h. The difference between the effects of acute and subacute exposures may be explained by time-dependent processes, such as change in regulatory mechanisms or regulation of hormone receptor levels, or the synthesis of protective agents, such as stress proteins. It was reported that lead induces the synthesis of various kinds of stress proteins (Shelton et al., 1986), which may protect some but not all cell-types; for instance, they may inhibit lead potentiation of mitotic response at 6 h after estrogen stimulation, observed in acutely exposed animals but not following subacute exposure.

This study clearly demonstrated that prepubertal rat exposure to lead affected estrogen action in different uterine cell-types, and that the interaction by the pollutant with hormone action differed from that caused following acute exposure. Further work is in progress to investigate the mechanisms explaining the difference.

Taking into account that similar blood lead levels in humans are considered in many countries below the legal safety limits in occupational exposure, present findings suggest a need for further studies to investigate if these changes occur in humans under similar blood lead levels and whether these alterations have a meaning to female lead infertility in both humans and experimental animals.

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