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BIOMARKERS OF HEAVY METAL REPRODUCTIVE EFFECTS AND INTERACTION WITH ESSENTIAL ELEMENTS IN EXPERIMENTAL STUDIES ON FEMALE RATS

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Experimental studies in laboratories in Croatia and U.S.A. were conducted on female rats exposed to lead or cadmium to evaluate effects on the female reproductive integrity. The health condition of the offspring and relationship with essential elements were also evaluated. By using simple biomarkers of reproductive effects it was found that subchronic oral exposure to lead (1500–5500 ppm) or cadmium (50 ppm) during pregnancy and lactation decreased pup body weight, and that lead also decreased pup viability. Acute exposure to cadmium (3 or 5 mg/kg body weight s.c.) *in vivo* suppressed serum concentrations of progesterone and estradiol depending on the reproductive stage. Organ accumulations of lead or cadmium were accompanied by changes in the concentrations of iron and zinc in both mother and pups. Future research should focuss on the effects of metals on endocrine disruption in the ovary and placenta, and on concomitant interaction of toxic and essential metals in mother and offspring.

Key terms:
cadmium, iron, lead, perinatal exposure, reproductive toxicity, steroid hormone concentrations, zinc

The problem of exposure to heavy metals (lead, cadmium, mercury, and arsenic) in developed and less developed countries, and their biological effects have been a source of growing concern in recent years (1-5). Released into the environment by human activities, these metals are ubiquitous. Metal exposure of the general population occurs chiefly through the food chain (e.g. 6), and cigarette smoke

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(cadmium and arsenic). Combined environmental and/or occupational exposure to heavy metals may adversely affect the reproductive health of women and the essential element homeostasis of their descendants. Animal studies have shown the influence of sex and age on metal toxicokinetics and toxicity in the rat (7, 8). It was found that young, pregnant and lactating females had higher absorption of metals from the gastrointestinal tract, and subsequent higher metal retention than other adult animals (8-11). Therefore, females of the reproductive age and the young may present higher at-risk-groups for toxic effects of heavy metals.

A large body of literature (e.g. 3, 12) clearly shows that lead exposure is associated with female reproductive dysfunctions (hypofertility to sterility) and an impaired progression of pregnancy (abortions and miscarriages). Various disorders of the embryo/foetus (retarded growth, malformations, increased neonatal mortality and morbidity, behavioural teratogenicity) have also been reported. Both human and animal data indicate that lead readily crosses the placenta and produces lead concentrations in the neonate comparable to those found in maternal blood (13). In experimental studies conducted elsewhere and in the laboratory in Zagreb it has been shown that perinatal exposure to lead affects essential element homeostasis (14-16).

In contrast to lead, limited and inconclusive evidence exists for an association of cadmium exposure and reproduction in human females (1, 4). Recent data indicate that cadmium accumulates in human ovaries (17) and placentas (18), depending on the age and smoking habits. Animal studies have shown that transfer of essential elements (iron, zinc) and vitamins may be inhibited by placental cadmium accumulation (19-23).

This research is focussed on reproductive effects and maternally-mediated perinatal effects of oral exposure to lead or cadmium in female rats for which human data are limited. We also evaluated the interaction of toxic and essential elements in mother and pups. To study metal action on steroidogenesis, serum steroid hormone concentrations were evaluated in female rats acutely exposed to cadmium.

MATERIAL AND METHODS

Subchronic exposures to lead or cadmium

Experiments were conducted on sexually mature (12-16 weeks old) female laboratory rats (Wistar, Zgim, bred at the Institute's own breeding farm in Zagreb). Animals (at least 50 per group) were exposed to 0 (control), 1500, 3500, or 5500 ppm lead (as acetate), or to 50 ppm cadmium (as chloride; both compounds *pro analysi*, "Kemika", Zagreb) in drinking (deionized) water (Figure 1). Animals had free access to drink and standard pelleted rat feed ("Domžale", Slovenia). Exposure started after mating with unexposed males (over three days and nights), and continued during 22 days of gestation and 21 days of lactation. The reversibility

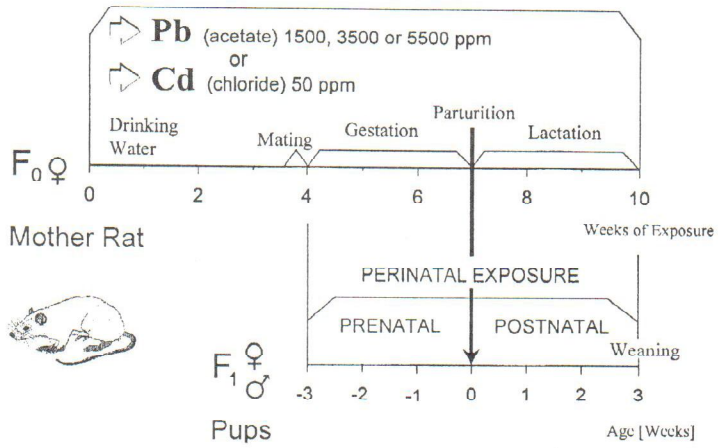

Figure 1 *General study design*

or possibly delayed appearance of metal effects was followed up during eight weeks after cessation of exposure.

At the end of exposure to 5500 ppm lead and 50 ppm cadmium, respectively, mothers and their 21-day-old weanling pups were killed by exsanguination from the abdominal aorta in ether anaesthesia for element analysis. Livers were removed, trimmed, blotted on filter paper, measured for wet weight, and prepared for element analysis by flame or electrothermal atomic absorption spectrometry (Varian AA 375 or SpectrAA 300A, Australia), as described earlier (24, 25).

Biomarkers of reproductive effects were: fertility index (number of pregnant/number of mated x 100), and average body weight and number of live pups per litter on day 0 (in newborn, within 24 h after birth), and on 11th and 21st days after birth (in weanling rats). Biomarkers of exposure were: mother and pup liver concentrations of lead and cadmium. Essential elements analysed in the liver were iron and zinc.

Acute exposure to cadmium

This study was conducted on adult cycling (60-day-old) and timed-pregnant female Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC). Cycling rats were evaluated for stage of oestrus daily by vaginal lavage (26) during four weeks before cadmium exposure, and only regular, four-day cyclers were used in the experiment. Among pregnant animals, the day the lavage was sperm positive was assigned as gestation day 0. Animals were given tap water and pelleted feed (Rat Chow 5001, "Ralston-Purina", St. Louis, MO) *ad libitum*.

Females (at least five per group) were injected with either physiological saline or cadmium chloride (cadmium Cl_2, "Fisher Scientific") in saline at a dose of 3 or 5 mg cadmium/kg body weight (b.w.). A volume of 0.3 ml/100 g b.w. was

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injected subcutaneously (s.c.) between scapulae. In one series of the experiments, 24 hours after cadmium exposure, effects on steroid hormones were evaluated in proestrous rats and in mothers on gestation day 8. In another experiment, four days after the same cadmium dosage, in mothers on gestation day 19, effects on steroid hormones, and concentrations of trace elements (cadmium, iron and zinc) were analysed in the mother's liver and in the whole foetus. In this paper we presented concentrations of progesterone and estradiol in the serum as biomarkers of cadmium effects on steroid hormones.

Animals were anaesthetized with CO_2. From each mother 3–5 ml of blood was collected by cardiac puncture in serum separation tubes. Samples were centrifuged (25,000 rpm, 1000 x g) for 30 min and serum was decanted into siliconized polystyrene vials. Serum progesterone and estradiol concentrations were measured by specific radioimmunoassay after dilution with medium as required using radioimmunoassay kits Coat-A-Count® (TKPG5 and TKE25, Diagnostic Products Corp., LA, CA). The liver and one foetus from each terminal lateral uterine position were removed from each rat for analysis of trace elements. Element analysis was executed as described above.

Statistical analysis

In subchronic studies, differences between an exposed group and a respective control were tested by Student's t-test at the level of significance at $P<0.01$.

For statistical evaluation of the hormonal and element data between more than two groups, analysis of variance in the general linear models procedure with two-tailed test at the level of significance at $P<0.05$ was used (27). All steroid data were logarithmically transformed before analysis to normalize the distributions and eliminate heterogeneity of variance between the groups tested. When significant ($P<0.05$) effects were detected in the analysis, the least-squares means were evaluated for significant differences.

RESULTS AND DISCUSSION

Effects of subchronic lead exposure

No effects on rats' general health (body weight gain, survival) or fertility index were noticed at any lead dose. Figure 2 shows average pup body weights per litter (g), and Figure 3 the number of live newborn, 11-day-old sucklings and 21-day-old weanling pups born to the females exposed to lead in drink during the six-week period of gestation and lactation. Body weight was reduced in the newborn at all lead doses, and in 11- and 21-day-old pups starting at 3500 ppm lead dose (Figure 2). The number of live newborn, and of 11- and 21-day-old pups was significantly reduced starting at 3500 ppm lead dose (Figure 3).

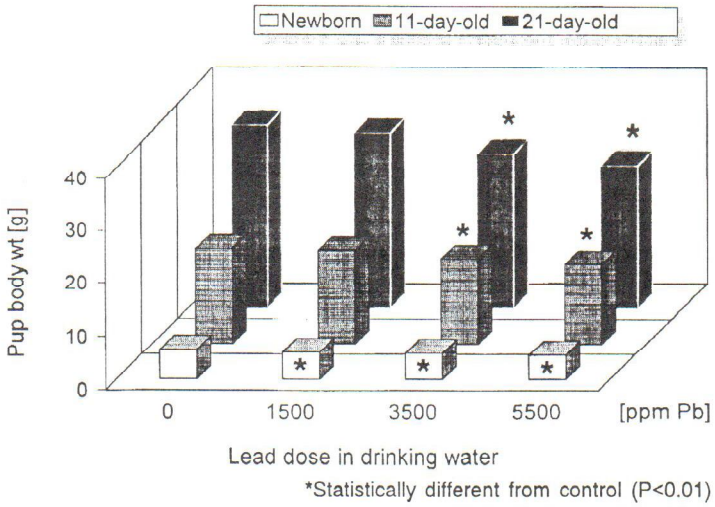

Figure 2 *Mean body weights of pups (g) born to mothers during six-week exposure to lead (1500–5500 ppm in drinking water) during gestation and lactation. N≥20 mothers with litter.*

A follow-up of the offspring of the exposed rats in additional studies showed that the body weights in both male and female F_1 rats reached control values up to the age of 12 weeks. Furthermore, no delayed effects on urine osmolality, kidney structure, blood pressure or reproduction were found in rats perinatally exposed to lead (28–30).

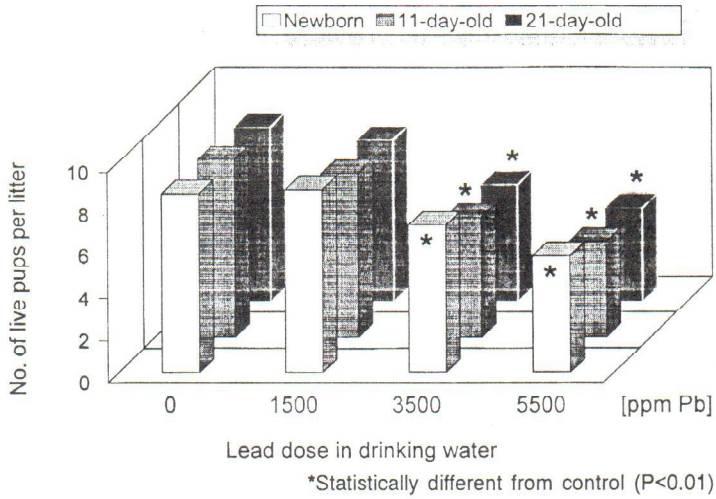

Figure 3 *Mean numbers of live pups per litter born to mothers during six-week exposure to lead (1500–5500 ppm in drinking water) during gestation and lactation. N≥20 mothers with litter.*

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We found earlier that the maternally-mediated reproductive effects of lead were dependent on the duration of exposure, and thus more pronounced effects on pup weight and viability were observed in the second offspring after longer lead exposure than in the first offspring after shorter exposure of their mothers (31). In addition, it was found that at oral maternal exposure to as much as 7500 ppm lead, adverse effects in pups were reversible. That is, the number and weight of pups per litter in the second offspring born to mothers whose lead exposure ceased after weaning of the first offspring (at the age of 11 days) for four weeks before the second mating, did not differ from the control values (32). Our results on maternally-mediated effects of lead in the pups are in concordance with the data obtained in other studies on rats under similar exposure conditions (e.g. 33, 34). The results also agree with field studies conducted by the Institute in Zagreb in a lead-smeltery area, in which an increase in abortion and a decrease in twin birth rates were found (13, 35).

Concentrations (μg/g wet weight) of iron, zinc and lead in the liver of mother rats and of their 21-day-old weanling pups after mothers' exposure to 5500 ppm lead in drink during the six-week period of gestation and lactation are presented in Figure 4. Element analysis showed an increase in liver lead concentrations in exposed females and 21-day-old weanlings followed by a significant rise in iron concentration in the pups. Liver zinc concentrations did not change either in mother rats or in their pups.

In our earlier studies, in pups perinatally exposed to lead (3500–7500 ppm), we observed liver haemosiderosis with foci of extramedullary erythropoiesis by histopathological examination, and anaemia (reduced red blood cell count, haemo-

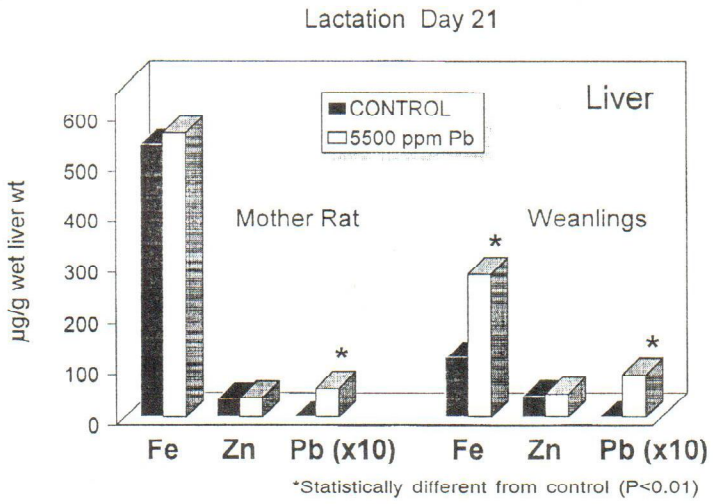

Figure 4 *Element concentrations in the liver (μg/g wet wt) of mothers and their 21-day-old pups after mothers' six-week exposure to lead (5500 ppm in drinking water) during gestation and lactation. N≥10 animals.*

globin, and haematocrit) by haematological analysis of the peripheral blood (15, 36). Both increased liver iron concentration and microscopically visible iron-containing pigment haemosiderin reflected accumulated iron in the liver tissue as a consequence of lead-induced impairment of haemoglobin synthesis. Chronic (14-week) oral exposure of female rats to lead (1500-7500 ppm) showed a different disposition of lead in organs of the adult rats and in those of the young. Liver lead concentrations were higher in sucklings than in female rats, and adult kidney lead concentrations were higher than in the pup kidney (24). Furthermore, kidney iron, zinc, and copper concentrations in female rats decreased, whereas in pups no changes in these concentrations were observed (14). It should be pointed out that the results presented in the paper together with the findings from our other studies clearly show that the lead-induced changes were more pronounced in perinatally exposed young rats than in the directly exposed adult ones, and that organ distribution of both toxic and essential elements was different in the adult than in the young (14, 28, 29).

Effects of subchronic cadmium exposure

Cadmium-related effects in pups born to females exposed to 50 ppm cadmium in drink during gestation and lactation (six weeks) were expressed as significantly reduced body weights of newborn, and of 11- and 21-day-old sucklings (Figure 5). No cadmium-induced effects were observed on either the number of live pups per litter or on pup survival in the postnatal period (data not shown). It was shown elsewhere that the decrease in body weight in perinatally exposed offspring

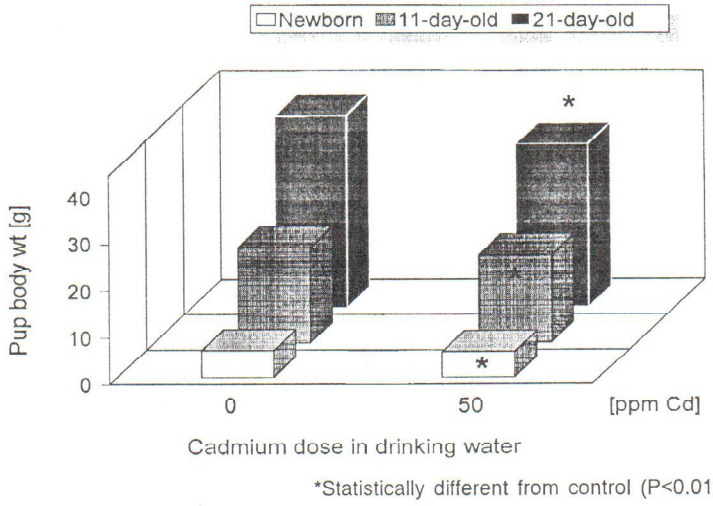

Figure 5 *Mean body weights of pups (g) born to mothers during six-week exposure to cadmium (50 ppm in drinking water) during gestation and lactation. N≥13 mothers with litter.*

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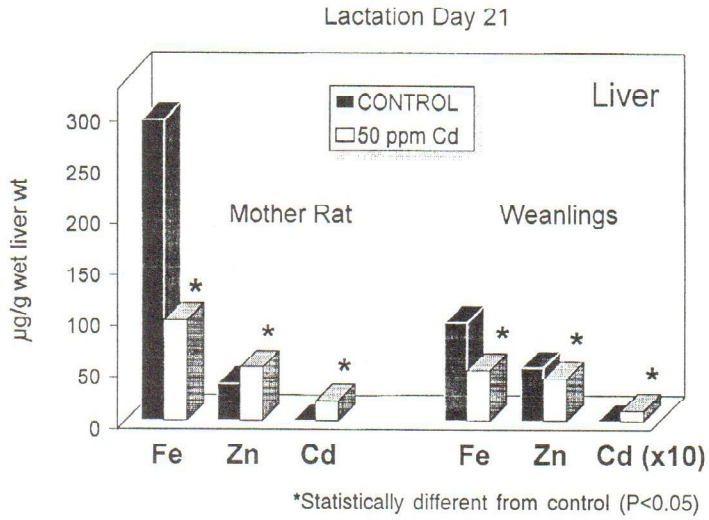

Figure 6 *Element concentrations in the liver (µg/g wet wt) of mothers and their 21-day-old pups after mothers' six-week exposure to cadmium (50 ppm in drinking water) during gestation and lactation. N=10–12 animals.*

persisted for four weeks after cessation of exposure (at the age of three weeks), that is, up to the age of seven weeks (37).

Results of analysis of iron, zinc and cadmium in the liver of rats and of their 21-day-old weanling pups after mothers' exposure to 50 ppm cadmium during gestation and lactation (six weeks) are shown in Figure 6. A significant decrease in liver iron concentrations was detected both in mothers and pups. It was reported elsewhere that in pups at weaning lower iron concentrations were accompanied by anaemia with reduced red blood cell count, lowered haemoglobin and haematocrit, and increased percentage of reticulocytes, while no changes in haematological parameters were found in their mothers (38).

A substantial increase in cadmium accumulation has been shown to occur during lactation in both the mother and pups (39). As seen in Figure 6, considerably higher values of organ cadmium concentrations were found in mothers than in weanlings, and zinc concentrations were elevated in mothers and reduced in pups. This effect of zinc in adult rats appears to be a consequence of the cadmium-zinc interaction (e.g. 40, 41). That is, cadmium exposure of adult female rats with elevated organ cadmium accumulation induces synthesis of increased amounts of the protein metallothionein, a physiologically binding protein for zinc and copper in the liver, kidney and some other organs, with a consequent increase in liver zinc (and copper) concentrations. In the young, on the other hand, it has been reported that, due to cadmium accumulation in the placenta with a consequently lower transplacental transfer of essential elements from the mother, zinc, iron and other element concentrations in the foetus are likely to be reduced (21–23).

The above findings in subchronically cadmium-exposed female rats agree with the work of the collaborating US EPA laboratory. It was reported that cadmium accumulated in the liver and kidney more rapidly during pregnancy and lactation (42 days) than with a similar exposure duration of non-pregnant (Sprague-Dawley) rats (11, 42). With exposure to 5 ppm cadmium in drinking water effects on reproductive outcome included an increase in preimplantation deaths and a decrease in foetal kidney weight, whereas serum progesterone concentration in mother rats was depressed roughly by 25 per cent at the term.

Effects of acute exposure to cadmium on ovarian steroidogenesis

Concentrations of progesterone and estradiol in serum (ng/ml) of non-pregnant rats in proestrous stage and of pregnant rats on gestation day 8, twenty-four hours after treatment with 3 or 5 mg cadmium/kg b.w. are shown in Figure 7. Reductions in concentrations of serum estradiol were found in females both in proestrus and in early pregnancy (gestation day 8), while serum progesterone concentrations did not change. Progesterone concentrations were several times higher in pregnant than in non-pregnant, proestrous rats. At this stage of rat pregnancy (first trimester), the placenta has not yet developed and thus serum progesterone concentrations reflect only ovarian progesterone production. It had been shown earlier that, by the same treatment, at a later stage of gestation (gestation day 16, third trimester) serum concentrations of steroid hormones were relatively unaffected when evaluated 24 h following cadmium exposure (43).

In near-to-term female rats, on gestation day 19, four days (96 h) after s.c. cadmium injection, a significant depression in serum progesterone concentration

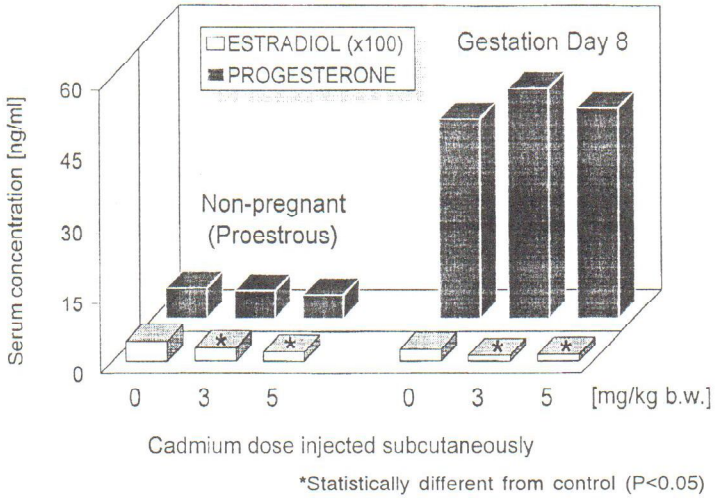

Figure 7 *Concentrations of progesterone and estradiol in serum (ng/ml) in non-pregnant and in early pregnant rats, 24 h after a single cadmium administration (3 and 5 mg/kg s.c.). N≥5 animals.*

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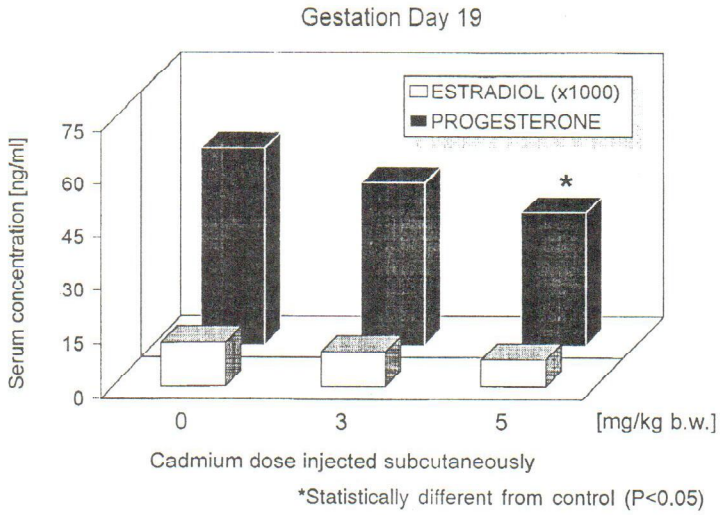

Figure 8 *Concentrations of progesterone and estradiol in serum (ng/ml) in near-to-term rats, 96 h after a single cadmium administration (3 and 5 mg/kg s.c.). N≥6 animals.*

at 5 mg cadmium/kg b.w. dose was found, while no effect on serum estradiol concentration was observed (Figure 8). At this stage of gestation, the placenta was fully developed (present after gestation day 12). Thus, serum progesterone concentrations might reflect both ovarian and placental progesterone production.

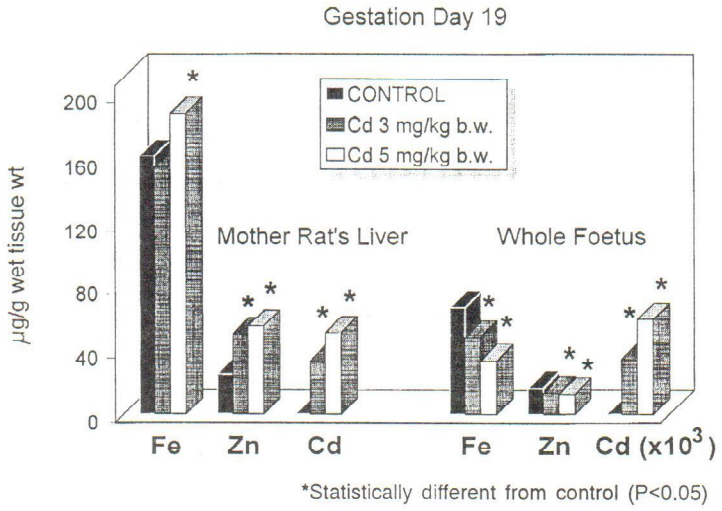

Figure 9 *Element concentrations in the liver (µg/g wet wt) of mothers and in the whole foetuses 96 h after a single administration of cadmium (3 and 5 mg/kg s.c). N≥7 animals.*

However, it should be mentioned that at this cadmium dose (5 mg/kg b.w.) the placentas were moderately to severely necrotic and most of the foetuses had died and/or were resorbed. Under these exposure conditions, the mean number of live foetuses was significantly lower than in control rats; 14.7±2.34 (n=6) in control, 6.11±2.11 (n=8; $P<0.01$) at 3 mg cadmium/kg b.w. and 0.222±0.147 (n=9; $P<0.01$) at 5 mg cadmium/kg b.w.

Figure 9 shows concentrations (μg/g wet weight) of iron, zinc and cadmium in the liver of mothers and in whole foetuses four days (96 h) after s.c. cadmium injection. In rats on gestation day 19, liver zinc concentrations were decreased while iron concentrations were not changed. The latest data on iron concentration were in concordance with our earlier finding that following the acute cadmium exposure (24 h after 3 or 5 mg cadmium/kg dose), liver iron concentrations decreased in proestrous rats and females in early pregnancy (gestation day 8), but were not changed in late pregnancy (gestation day 17) (43). In the foetuses of cadmium-exposed female rats, again, both iron and zinc concentrations decreased (Figure 9). Due to placental cadmium accumulation and its relative impermeability for cadmium, concentrations of cadmium in the whole foetus were markedly lower than in the mother's liver.

CONCLUDING COMMENT

Animal studies show that cadmium alters microcirculation in reproductive organs (ovary/testis, uterus, placenta) and that both lead and cadmium may interfere with the normal functioning of the hypothalamus-pituitary-ovarian axis (44-46). There is yet little information on this possibility in humans. Furthermore, even for lead, a well known reproductive toxicant, reproductive effects of chronic low exposure are less known. Human data on lead or cadmium maternally-mediated perinatal effects are limited (47). Data on lead or cadmium effects on steroidogenesis are lacking. More human and animal data are needed to elucidate association(s) between toxic metal concentrations and potential endocrine disruption(s) in the steroid producing organs, i.e. ovary, testis and placenta.

In conclusion, by using biomarkers of reproductive effects, our study showed effects of both lead and cadmium on female reproduction under certain exposure conditions. Future research of metal effects on female reproduction should focus on the ovarian and placental endocrine disruption in both man and animals. The results from animal studies should be used to compare the effects in the two species, and to provide a foundation for risk assessment of reproductive and perinatal effects of toxic metals in humans.

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Sažetak

BIOPOKAZATELJI UČINAKA TEŠKIH METALA NA RASPLOĐIVANJE I MEĐUDJELOVANJA OTROVNIH I ESENCIJALNIH METALA U POKUSIMA NA ŠTAKORICAMA

Žene u dobi za rađanje i malena djeca skupine su s velikom sklonošću pomanjkanju esencijalnih elemenata i stoga podložne ozbiljnim učincima teških metala. U pokusima na životinjama pokazano je da skotne i laktirajuće ženke i mladunčad imaju povećanu apsorpciju iz probavnog trakta i povećane retencije metala. Otrovni metali djeluju na homeostazu esencijalnih elemenata. Podaci o maternalno posredovanim perinatalnim učincima metala su nedostatni, a učinci kadmija na žensku reprodukciju su manjkavi. U radu su prikazani rezultati eksperimentalnih istraživanja reprodukcijskih i perinatalnih učinaka olova i kadmija koja su provedena u laboratorijima u Zagrebu i u Sjevernoj Karolini, SAD. Tijekom istraživanja u izloženih ženki i u njihovim potomcima također su procjenjivana djelovanja metala na koncentracije esencijalnih elemenata. Uporabom jednostavnih biopokazatelja učinaka na rasplođivanje nađeno je da supkronična peroralna izloženost olovu odnosno kadmiju tijekom skotnosti i laktacije značajno snizuje tjelesne težine mladunčadi, a olovo također smanjuje preživljavanje potomaka. Kao biopokazatelji učinaka kadmija na steroidogenezu procjenjivane su koncentracije steroidnih hormona u serumu. Opaženo je da akutna *in vivo* izloženost kadmiju snizuje koncentracije progesterona i estradiola u ovisnosti o stadiju graviditeta. Tijekom izloženosti nakupljanje olova odnosno kadmija u organima popraćeno je promjenama koncentracija željeza i cinka u organima u

štakorica i u njihove mladunčadi. Zaključeno je da bi u budućim procjenjivanjima rizika učinaka teških metala trebalo istražiti endokrine poremećaje u jajnicima i u posteljici te istodobna djelovanja otrovnih i esencijalnih elemenata i u izloženih majki i u njihovih potomaka.

Ključne riječi:
cink, kadmij, koncentracija steroidnih hormona, olovo, perinatalna izloženost, reprodukcijska toksičnost, željezo

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