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Foetotoxicity of Cadmium in Quackenbush Strain Mice and the Effects of the Metal on Uterine Alkaline Phosphatase during Pseudopregnancy

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Abstract

Experiments were conducted to determine the sensitivity of Quackenbush strain (QS) mice to the foetotoxic effects of single subcutaneous injections of $CdCl_2$ (2, 4 and 8 mg of cadmium per kilogram body weight) on days 1, 2, 4 or 8 of pregnancy. Autopsies performed on day 16 of pregnancy revealed that cadmium was teratogenic and increased the incidence of malformed forelimbs and exencephaly and decreased foetal weight. The metal caused foetal weight changes and exencephaly only when it was administered on day 8 of pregnancy indicating that the post-implantation stage presents the QS mouse with a period of increased sensitivity to the foetotoxic effects of cadmium.

Although injections of cadmium (8 mg per kilogram body weight) during early pseudopregnancy did not prevent the uterus from undergoing a decidual reaction in response to a peanut oil stimulus, they did in some cases prevent alkaline phosphatase from reaching maximal activity levels in decidualized uterine horns. Thus, inhibition of alkaline phosphatase occurred only in response to the metal injected on day 4 of pseudopregnancy and the enzyme was not affected when cadmium was administered on days 1, 2, 3, 5 or 6 of pseudopregnancy.

The results suggested that changes in either progesterone and oestrogen production by the ovary or the alkaline phosphatase activity of the uterus are unlikely to be responsible to any great extent for the teratogenic effects of single subcutaneous injections of cadmium in the QS mouse during early pregnancy.

Introduction

Concern about the potentially harmful effects of increasing levels of cadmium in the environment has stimulated investigations of the biological implications of exposure to the heavy metal (Buell 1975; Weiss 1978). It is evident from these studies that the mammalian reproductive system is a major site where cadmium exerts many of its adverse effects. Thus, in males of several species cadmium induces deleterious effects on the vasculature of the testes and subsequently influences spermatogenesis and androgen production (Gunn and Gould 1970; Saksena *et al.* 1977; Aoki and Hoffer 1978; Lau *et al.* 1978; Niewenhuis and Fende 1978). In female mammals, cadmium may disturb maternal–embryonic relationships and cause placental necrosis, foetal resorption and embryonic developmental malformations (Pařízek 1964; Ferm 1971; Schroeder and Mitchener 1971; Gale and Ferm 1973; Dencker 1975).

It is well known that cadmium inhibits the activity of many enzymes, and particularly those containing either essential thiol groups or a functionally active Zn^{2+} ion (see Dencker 1975; Aoki and Hoffer 1978). In this context, alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from the pregnant mouse uterus is a zinc-containing metalloenzyme and is also inhibited *in vitro* by cadmium

(Murdoch *et al.* 1980). This enzyme is associated with the induction of the decidual cell reaction during early pregnancy (Finn and Hinchliffe 1964; Murdoch *et al.* 1978) and may play an important role in facilitating maternal–embryonic relationships during the peri-implantation period. It is possible, therefore, that *in vivo* inhibition of alkaline phosphatase in uterine decidual cells by cadmium could lead to functional alterations in the decidua which may be detrimental to the developing embryo. This possibility gains credence from the work of Dencker (1975) who showed that, during early gestation in mice, intravenously injected cadmium accumulated not only in the maternal ovary and primitive gut of the developing embryo, but also in the uterine decidua and placenta where alkaline phosphatase activity is normally high (Murdoch *et al.* 1978).

In view of the foregoing considerations, the sensitivity of alkaline phosphatase in the uterus of Quackenbush strain (QS) mice to subcutaneous injections of $CdCl_2$ was investigated in the present study. Before these experiments were conducted, however, the sensitivity of the embryo to maternally administered doses of $CdCl_2$ given at various stages of pregnancy was assessed. This preliminary investigation was necessary because, to our knowledge, there is no literature available that describes acute effects of cadmium in QS mice and different strains of mice differ greatly in their resistance to the metal (Taylor *et al.* 1973; Layton and Layton 1979). However, recent studies by Webster (1978, 1979*a*, 1979*b*) have described chronic effects of the metal on foetal growth retardation in QS mice.

Materials and Methods

Animals and Treatments

Female QS mice, aged 7–9 weeks, were used in all experiments and were housed as previously described (Murdoch *et al.* 1978). Pregnancy and pseudopregnancy were brought about by pairing the females with intact or vasectomized males, respectively. The females were examined for copulation plugs each morning and the day of finding a plug was designated as day 1 or the first day of pregnancy or pseudopregnancy.

Pregnant mice

Pregnant mice were given a single subcutaneous injection of $CdCl_2$ (Ajax Chemicals, Sydney, N.S.W.) in 0·2 ml of 0·9% (w/v) NaCl at 1000 h on days 1, 2, 4 or 8 using doses of either 0·0, 2·0, 4·0 or 8·0 mg cadmium per kilogram body weight. The mice were killed by cervical dislocation between 0900 and 1000 h on day 16 of pregnancy.

The numbers of foetuses and resorptions were noted and were added together to provide a record of the total number of implantation sites. Foetuses were examined under a dissection microscope for evidence of any external abnormalities. Particular attention was given to the head and limbs and the incidence of apparent malformations at these and other sites was recorded as a percentage of the number of implantation sites. In some cases foetal weights were also noted.

Experiments with pregnant mice were continued until 13 litters with one or more embryos were obtained. This number was arbitrarily set before the experiments were started and was a procedure described by Layton and Layton (1979) to facilitate statistical analysis of the data. Mice in which all embryos were resorbed were not encountered in the present experiments and it was not necessary therefore to adjust the calculations to include observations of this nature (Layton and Layton 1979).

Pseudopregnant mice

Single subcutaneous injections of $CdCl_2$ in 0·2 ml of 0·9% (w/v) NaCl were administered to pseudopregnant mice at 1000 h on days 1–6 using doses of 0·0 or 8·0 mg cadmium per kilogram body weight. Deciduomata were induced in the left uterine horns of these mice on day 4 by the intraluminal injection of 30 μl of peanut oil as previously described (Murdoch *et al.* 1978). Six mice

were used in each treatment group and the animals were killed by cervical dislocation between 0900 and 1000 h on day 7 of pseudopregnancy.

The left (decidualized) and right (non-decidualized) uterine horns were individually excised at the time of autopsy and processed separately. Each uterine horn was weighed, minced throughly with fine scissors, and then homogenized at 4°C in 20 volumes of 0·01 M Tris-HCl buffer, pH 7·4, with a ground-glass Potter–Elvehjem homogenizer.

Alkaline phosphatase activity in the homogenates was assayed by the spectrophotometric measurement of hydrolysis of *p*-nitrophenyl phosphate using a cation-free 50 mM glycine buffer system, pH 10·5, and 6 mM substrate (Murdoch *et al.* 1979). Units of activity are defined as nanomoles of substrate hydrolysed per minute at 37°C.

The protein concentration of samples was determined by the method of Lowry *et al.* (1951) using standards of bovine serum albumin.

Statistical Analyses

The experiments were of factorial design and the significance of the results has been assessed by analysis of variance. In experiments with pregnant mice, the litter rather than the foetus was used as the experimental unit (Haseman and Hogan 1975) and tests were done on arcsine transformations of the original data using orthogonal polynomial coefficients to partition degrees of freedom (Snedecor 1962).

Results

The results of an experiment designed to assess the sensitivity of QS mice to the foetotoxic effects of a single subcutaneous injection of cadmium during early pregnancy are shown in Table 1.

Table 1. Teratogenic effects of cadmium in QS mice

Each dose of cadmium was administered to 13 mice and autopsy was performed on day 16 of pregnancy

Day of cadmium injection	Dose of cadmium (mg/kg body wt)	No. of implantation sites	No. of living foetuses	No. of foetuses (%) with: Exencephaly	Malformed forelimbs	Malformed hindlimbs
1	0	150	137	0·0	0·8	0·0
	2	147	136	0·0	3·9	1·0
	4	145	139	0·0	4·4	0·0
	8	159	142	0·0	2·5	0·0
2	0	143	127	0·0	0·0	0·0
	2	146	132	0·0	5·0	0·7
	4	144	128	0·8	6·6	0·0
	8	154	123	0·0	4·5	1·3
4	0	144	125	0·0	0·7	0·0
	2	145	126	0·0	2·8	0·7
	4	148	130	0·7	2·0	0·0
	8	150	138	0·0	5·3	0·0
8	0	159	140	0·0	0·8	0·0
	2	142	131	4·0	5·8	0·8
	4	161	149	4·3	3·6	0·7
	8	143	128	19·0	5·9	0·3

The heavy metal was teratogenic at all doses used and significantly increased ($P < 0·05$) the incidence of malformed forelimbs at all stages of pregnancy studied. This defect showed a left-sided bias and mainly involved an apparent reduction in post-axial development. The various doses of cadmium were essentially equally

effective in this respect. The incidence of malformed hindlimbs was much lower than that of malformed forelimbs and was not significantly affected by the cadmium injections. In addition, there were no significant effects of the heavy metal on the number of implantation sites, resorption rate, or the number of foetuses still living on day 16 of pregnancy, and no evidence could be found for any malformations of the face, trunk or tail in any treatment group studied. A major effect of cadmium,

Table 2. Foetal weights of QS mice treated with cadmium during early pregnancy

Values are the means ± standard errors for 13 mice. Foetal weights were recorded on day 16 of pregnancy. *Significantly different from corresponding controls, $P < 0{\cdot}05$

Day of cadmium injection	Dose of cadmium (mg/kg body wt)	Mean foetal weight (mg)	Day of cadmium injection	Dose of cadmium (mg/kg body wt)	Mean foetal weight (mg)
1	0	438 ± 12	4	0	457 ± 29
	8	443 ± 17		8	413 ± 15
2	0	455 ± 19	8	0	451 ± 18
	8	438 ± 22		8	403 ± 17*

however, was to produce exencephaly in the foetuses of dams receiving injections during the post-implantation period of pregnancy. The incidence of exencephaly was significantly higher ($P < 0{\cdot}01$) in the high- than in the lower-dose groups and increased only when injections of the heavy metal were administered on day 8 of pregnancy.

Table 3. Effects of cadmium on the alkaline phosphatase activity and weight of uterine horns of QS mice during pseudopregnancy

Values are the means ± standard errors for six mice. An oil injection was given on day 4 in the left uterine horn to induce a decidual reaction; the right uterine horn was not stimulated. Autopsy was performed on day 7 of pseudopregnancy

Day of cadmium injection	Dose of cadmium (mg/kg body wt)	Alkaline phosphatase activity (units/mg protein) in:		Weight of left horn (mg)	Weight of right horn (mg)
		Left horn	Right horn		
1	0	350 ± 47	61 ± 10	339 ± 31	35 ± 2
	8	378 ± 45	59 ± 13	329 ± 16	39 ± 2
2	0	380 ± 16	61 ± 9	298 ± 43	30 ± 5
	8	388 ± 46	51 ± 7	319 ± 49	31 ± 4
3	0	350 ± 33	51 ± 6	345 ± 50	26 ± 2
	8	400 ± 47	59 ± 11	326 ± 42	32 ± 4
4	0	405 ± 44	59 ± 10	314 ± 39	30 ± 2
	8	241 ± 31	53 ± 4	250 ± 34	29 ± 2
5	0	351 ± 49	48 ± 9	311 ± 25	26 ± 3
	8	370 ± 41	58 ± 7	330 ± 40	31 ± 3
6	0	360 ± 49	60 ± 6	320 ± 19	28 ± 2
	8	381 ± 43	47 ± 5	342 ± 37	32 ± 2

The results summarized in Table 2 show that foetal weights were also significantly reduced in mice receiving 8 mg cadmium per kilogram body weight on day 8 of pregnancy. This dose of cadmium was not effective, however, in altering foetal weights when administered during the pre-implantation stages of pregnancy.

When mice were injected with 8 mg of cadmium per kilogram body weight during early pseudopregnancy a decidual cell reaction, in response to a peanut oil stimulus on day 4, occurred in the left uterine horn in all cases and was accompanied by significant increases ($P < 0{\cdot}01$) in uterine horn weight and alkaline phosphatase activity on day 7 (Table 3). Cadmium, however, significantly depressed ($P < 0{\cdot}01$) the alkaline phosphatase activity of decidualized uterine horns when administered on day 4 of pseudopregnancy but failed to have any apparent effect on the enzyme when injected before or after day 4. A slight, but not statistically significant, decrease in the wet weight of decidualized uterine horns also occurred after the injection of cadmium on day 4.

Discussion

The present study of the sensitivity of the QS mouse to the foetotoxic effects of cadmium during early pregnancy describes two types of malformations in the day 16 foetus which have previously been found to occur in other strains of mice (Keino and Yamamura 1974; Layton and Layton 1979) and other species (Ferm and Carpenter 1968; Barr 1973) as a result of exposure to the heavy metal. Firstly, forelimb defects were a feature of cadmium treatment. The incidence of these malformations, however, was relatively low, was similar when cadmium was administered either before or after the expected time of implantation, and was not dose dependent. The second major malformation was exencephaly. This defect, unlike that of the forelimbs, only occurred in response to cadmium administered after the time of implantation and with an incidence that was dose dependent. The effects of the metal on exencephaly, together with those on foetal weight (Table 2), indicate that the post-implantation stage of pregnancy presents the QS mouse with a period of increased sensitivity to the adverse actions of cadmium. These findings are consistent with those of earlier studies which showed that susceptibility to the teratogenic effects of cadmium and other extraneous factors varies with the developmental stage at the time of exposure (Chiquoine 1965; Ferm 1971; Barr 1973; Gale and Ferm 1973; Wolkowski 1974; Hall 1977). The rate of uptake and accumulation of cadmium in embryonic, placental, and ovarian tissues also depends on the day of pregnancy on which the metal is administered (Dencker 1975).

Although increased resorption rates and several other types of malformations resulting from exposure to cadmium have been reported in various strains of mice (see Layton and Layton 1979), the QS mice used in the present study were resistant to such effects when the metal was administered as a single subcutaneous injection. However, not all malformations may have been detected because a detailed internal examination of the foetuses was not conducted.

The results presented in Table 3 show that injections of cadmium at a dose sufficient to cause exencephaly and foetal weight changes did not prevent the uterus from undergoing a decidual cell reaction in response to an artificial stimulus (peanut oil) on day 4 of pseudopregnancy. Thus, single injections of the metal at 8 mg per kilogram body weight cannot be considered to seriously disturb the delicate balance that must be established between progesterone and oestrogen if the decidual reaction is to occur (see Finn and Porter 1975). The failure of cadmium to significantly influence either the resorption rate or the number of implantation sites in pregnant animals provides further evidence in support of this proposal. It is unlikely, therefore,

that changes in progesterone and oestrogen production by the ovary following treatment with cadmium are responsible to any great extent for the foetal malformations observed in the present study. However, cadmium does accumulate in the ovary (Dencker 1975) and can decrease the activity (Unger and Clausen 1973) of cytochrome P-450 which is involved in steroid biosynthesis.

The results in Table 3 also show that both the weight and alkaline phosphatase activity of uterine horns increased on day 7 of pseudopregnancy following deciduoma induction with peanut oil on day 4. These are characteristic features of the decidual reaction and have been described previously (Finn and Hinchliffe 1964; Murdoch *et al.* 1978). Under normal circumstances alkaline phosphatase activity reaches maximal levels in decidualized uterine horns of mice on day 7 of pregnancy and pseudopregnancy and rapidly decreases thereafter (Murdoch *et al.* 1978). In the present study, however, the uterine enzyme failed to reach maximal activity levels when cadmium was administered on the same day (day 4) as the mice received a deciduoma-inducing stimulus. This effect may be facilitated by increases in the vascular permeability of the uterus that normally follow a decidualizing stimulus (Psychoyos 1973) and which allow the stromal cells to be exposed to increased amounts of cadmium during this time. Further experimental work is required to determine whether this effect is exerted either directly on the enzyme or indirectly by causing a slight retardation in the rate of development of the decidual cell reaction. The latter possibility may not be of major importance, however, since cadmium injected on day 4 of pregnancy did not significantly influence foetal weight (Table 2).

Although these results make it tempting to suggest that an inhibitory action on decidual alkaline phosphatase is, in part, responsible for the teratogenic effects of cadmium, there are three reasons why such a proposal cannot be considered as valid. Firstly, there was no increase in the incidence of foetal malformations when cadmium was administered on day 4 of pregnancy. Secondly, alkaline phosphatase activity in the decidualized tissue was not affected by cadmium when injections were given either before or after the time of deciduoma induction. Thirdly, the major teratogenic effects of the metal were observed when injections were administered at a time (day 8) that was coincident with a period of rapidly decreasing levels of uterine alkaline phosphatase activity (Murdoch *et al.* 1978). These results, however, do not eliminate the possibility that cadmium exerts its adverse effects on the foetus by interfering with enzyme systems other than alkaline phosphatase in the decidua (see Dencker 1975; Aoki and Hoffer 1978). Thus, the question of whether the primary site of action of cadmium is on the embryo or decidua (placenta) still remains to be answered.

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