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Induction of Skeletal Malformations in the Offspring of Rats Fed a Zinc Deficient Diet

Johan Styrud, V. Elisabeth Dahlström and Ulf J. Eriksson

Department of Medical Cell Biology, University of Uppsala, Uppsala, Sweden

ABSTRACT

Pregnant rats were subjected to a trace metal poor diet (1.2 ppm zinc, 5.9 ppm copper, 40 ppm manganese) during the entire gestation. The rat mothers did not gain weight during pregnancy and showed decreased liver weight and lowered serum glucose levels on gestational day 20. The offspring exhibited decreased body and placental weights, delayed ossification of the skeleton, and an increased resorption rate. We also found 4 % skeletal malformations in the offspring (0 % in the controls), which closely resembled a type of malformation previously encountered in rats when the mother was manifest diabetic (i.e. sacral dysgenesis). The zinc levels were decreased and manganese levels increased to the same extent in offspring of trace metal restricted (this study) and manifest diabetic rats (previous studies). Furthermore, when pregnant rats on the trace metal restricted diet were resupplemented with 75 ppm zinc in the drinking water the offspring largely normalized their somatic and placental growth, skeletal maturation, as well as their zinc and manganese levels. In addition, the fetuses of the zinc resupplemented rats did not show any malformations. The possibility of common teratological mechanisms in maternal diabetes and trace metal deficiency may therefore be considered.

INTRODUCTION

The etiology behind disturbed fetal growth and congenital malformations in the offspring of diabetic mothers is at present not clear (10,13,18,20,21). In previous experimental attempts to study this problem we have described a rat model for diabetic pregnancy in which decreased fetal weight, delayed skeletal ossification and an increased number of fetal resorptions and skeletal malformations were found (6,8). Furthermore, the two types of skeletal malformations found in this rat model (micrognathia and sacral dysgenesis, cf. refs. no. 7-10) show a strong genetic dependence to the rat strain used, since we have been unable to induce these malformations in the offspring of manifest diabetic rats from other strains (11,12). In addition, we have shown that the offspring of the diabetic rats in our model are zinc-deficient but have normal levels of copper and increased levels of manganese, thus establishing a link between maternal

diabetes and fetal trace metal disturbances (9). In the present study we have used this malformation-prone rat strain, which is presently kept in a colony in Uppsala (denoted "U rats"), in an attempt to elucidate the teratogenicity of trace metal disturbances in the absence of maternal diabetes.

MATERIALS AND METHODS

Rats from our local colony in Uppsala ("U rats") were used in the present investigation. This colony consists of a Sprague-Dawley derived strain of albino rats in which the offspring consistently exhibit a 15-20% incidence of skeletal malformations when the maternal rat has been made diabetic with streptozotocin before pregnancy (12). The total incidence of all types of fetal malformations is less than 1 % in the control pregnancies of this strain (12). The (U) rats in the present study were all non-diabetic and kept in a light and temperature controlled environment with a 12+12 h light-dark cycle. Two to three female rats were housed per cage and were fed standard rat pellets containing 192 ppm zinc, 33 ppm copper and 132 ppm manganese (R 3, Ewos AB, Södertälje, Sweden) and tap water ad libitum before the onset of pregnancy. They were mated overnight with male rats from the same colony and conception was confirmed by examination of vaginal smears for the presence of sperm in the morning. The day when a positive vaginal smear was obtained was denoted gestational day 0 and from that day each pregnant animal was kept in a stainless steel cage (Stålstandard AS, Norway) with a metal grid floor and without wood cuttings in order to prevent the rats from ingesting trace metals from other sources than the food and drinking water. On gestational day 0 the standard food was changed to a specific trace metal poor diet: 1.2 ppm zinc, 5.9 ppm copper, 40 ppm manganese (Ewos AB, Södertälje, Sweden). About half the pregnant animals received zinc supplemented drinking water with a zinc concentration of 75 ppm (Solvezinc, Tika AB, Lund, Sweden), which was markedly increased in comparison with ordinary tap water (0.1 ppm zinc). The amount of food and water consumed by the pregnant rats was assessed twice a week. On gestational days 0 and 20 the maternal serum glucose levels were estimated (Glucose Analyzer 2, Beckman Inc., Fullerton, CA, USA). The control pregnant rats on standard diet are denoted N, the zinc deficient ZD, and the zinc resupplemented rats are denoted ZDZ in the following.

On gestational day 20, the pregnant rats were weighed and killed by a blow to the neck. The maternal liver was dissected out and weighed, the fetuses were exteriorized and examined with respect to weight, viability and occurrence of fetal malformations. From each uterine horn one fetus was randomly chosen and processed for whole body trace metal determination. These fetuses - and the maternal livers - were placed in pre-weighed, acid-washed plastic beakers and cut up thoroughly with a pair of stainless steel scissors and freeze-dried to constant weight for about 24 h (GT 2 Freeze Drier, Leybold-Heraeus AG, West Germany), in order to record the specimens dry weights. They were then transferred to a platinum crucible, ashed overnight in a temperature-programmed furnace (Carbolite, Sheffield, U.K.) and analyzed by atomic absorption

spectrophotometry. Zinc, copper and manganese were determined on a Varian AA-6 instrument in an oxidizing air-acetylene flame at wavelengths of 213.9 nm, 324.7 nm and 279.5 nm, respectively.

One non-malformed (chosen at random) and all the malformed fetuses per individual horn were processed for skeletal staining (24). These fetuses were eviscerated, fixed in 70 % ethanol for 10-30 days, treated with 1 % KOH and stained with Alizarin Red as described previously (7). Subsequent evaluation of skeletal development was made by counting the visualized ossification centers in six different locations in accordance with Aliverti and coworkers (1). The probability (p) of a chance difference between means was estimated by Student's two tailed t-test. Data are given as means ± S.E.M.

Table I. Body weight, body weight gain during pregnancy, liver weight and serum glucose concentration of the normal (N), zinc-deficient (ZD) and zinc-resupplemented (ZDZ) pregnant rats on gestational day 20. Means ± S.E.M.

GROUP	No. of rats	Maternal body weight (g)	Maternal weight gain day 0-day 20 (g)	Maternal liver weight (g)	Maternal serum glucose (mmol/l)
N	3	371 ± 26	137 ± 15	11.7 ± 0.5	5.6 ± 1.3
ZD	11	242 ± 8^{+++}	-1 ± 5^{+++}	7.7 ± 0.4^{+++}	2.3 ± 0.3^{+++}
ZDZ	6	356±11	112±15^{++}	11.0± 0.3	3.2 ± 0.3^{++}

Significances: $^{++}$ $p<0.01$ vs. N rats; $^{+++}$ $p<0.001$ vs. N rats.

RESULTS

During gestational days 0-20 the normal (N), zinc-deficient (ZD) and zinc-deficient and resupplemented rats (ZDZ) consumed the same amount of food and water (data not shown). The body weight and body weight increase during pregnancy differed significantly between the groups, the ZD group did not gain any weight at all, whereas the ZDZ rats showed normal body weights on gestational day 20 (Table I). The ZD maternal rats exhibited decreased liver weights on day 20 in contrast to the ZDZ rats whose maternal liver weight did not differ from the normals (Table I). On gestational day 0 there were no differences in serum glucose levels between the groups (data not shown), but on pregnancy day 20, the serum glucose levels of the ZD and ZDZ rats were both significantly decreased in comparison with the N rats (Table I).

The number of viable offspring was slightly lowered in the ZDZ group (Table II). The number of non-viable fetuses was increased in both the ZD and ZDZ groups, this increase was most pronounced in the ZD group. Fetal body and placental weights were slightly lower than normal in the ZDZ group, whereas they were further decreased in the ZD group (Table II). In four of the eleven ZD litters a total of five malformed fetuses were found.

Table II. Number of viable and non-viable offspring (per uterine horn), fetal body weight and placental weight of the offspring of normal (N), zinc-deficient (ZD) and zinc-resupplemented (ZDZ) rat mothers on gestational day 20. Means ± S.E.M., n denote the number of uterine horns examined.

GROUP	n	No. of viable offspring	No. of non-viable offspring	Fetal body-weight (g)	Placental weight (g)
N	6	6.5 ± 0.6	0.2 ± 0.2	4.6 ± 0.3	0.50 ± 0.01
ZD	22	5.7 ± 0.5	$1.3 \pm 0.3^{+++}$	$2.7 \pm 0.1^{+++}$	$0.39 \pm 0.01^{+++}$
ZDZ	12	$5.3 \pm 0.4^{+}$	$0.6 \pm 0.1^{+++}$	$3.8 \pm 0.2^{+++}$	$0.49 \pm 0.01^{+}$

Significances: $^{+}$ $p<0.05$ vs. N fetuses; $^{++}$ $p<0.01$ vs. N fetuses; $^{+++}$ $p<0.001$ vs. N fetuses.

All five fetuses had malformation of the sacral dysgenesis type (Figure I, cf. refs. no. 6, 9,11). The rate of malformation was 3.9% (5/128) in the ZD group, whereas it was zero in the N (0/39) and ZDZ (0/63) groups.

The five malformed fetuses in the ZD group weighed the same (2.7 ± 0.1 g) as the non-malformed ones, whereas the placentae of the malformed fetuses were slightly lighter (0.34 ± 0.02 g) than their non-malformed littermates ($p < 0.001$, Table II).

Figure I. Alizarin Red stained day-20 fetuses from normal (N, left), zinc-deficient (ZD, middle), and zinc-resupplemented (ZDZ, right) rats. The ZD fetus shows sacral dysgenesis, (cf. refs. no. 7-10,12) the other two fetuses show normal morphology.

Table III. Maternal liver concentrations of trace metals in normal (N), zinc-deficient (ZD) and zinc-resupplemented (ZDZ) rats on gestational day 20. Means ± S.E.M.

GROUP	No. of rats	CONCENTRATION (mg/kg dry liver weight)		
		ZINC	COPPER	MANGANESE
N	3	104 ± 3	16.0 ± 0.4	8.1 ± 0.3
ZD	11	81 ± 2^{+++}	13.8 ± 0.3^{+++}	7.9 ± 0.4
ZDZ	6	104 ± 2	16.0 ± 0.2	7.2 ± 0.1^{+++}

Significances: $^{+}$ $p<0.05$ vs. N rats; $^{++}$ $p<0.01$ vs. N rats; $^{+++}$ $p<0.001$ vs. N rats.

The maternal livers of the ZD rats accumulated less zinc and copper during gestation (Table III). The ZDZ rat showed normal zinc and copper levels, whereas the accumulation of manganese was lowered (Table III). The ZD fetuses exhibited greatly diminished accumulation of zinc and slightly increased levels of manganese (Table IV).The ZDZ fetuses showed less pronounced alterations in their accumulation of trace metals than the ZD offspring, but nevertheless had lowered zinc and increased manganese levels compared to the controls (Table IV). The copper levels were similar in all groups of fetuses (Table IV). The sum of ossification centers in six different locations (sternum, metacarpus, metatarsus, anterior and posterior proximal phalanges, caudal vertebrae, cf. ref. no. 1) was decreased in the ZD fetuses and normal in the ZDZ offspring (Figure II).

Table IV. Total body concentrations of trace metals in fetuses of normal (N), zinc-deficient (ZD) and zinc-resupplemented (ZDZ) rats on gestational day 20. Means ± S.E.M.

GROUP	No. of fetuses	CONCENTRATION (mg/kg dry body weight)		
		ZINC	COPPER	MANGANESE
N	6	131 ± 1	10.9±0.4	0.9±0.02
ZD	22	105 ± 2^{+++}	10.6 ± 0.4	1.1 ± 0.1^{+++}
ZDZ	12	122 ± 2^{+++}	10.7 ± 0.3	1.0 ± 0.1^{+++}

Significances: $^{+}$ $p<0.05$ vs. N fetuses; $^{++}$ $p<0.01$ vs. N fetuses; $^{+++}$ $p<0.001$ vs. N fetuses.

DISCUSSION

The major finding in this study was the demonstration of fetal malformations in the trace metal-restricted pregnancies, of a type similar to those appearing in the offspring of manifest diabetic rats (7,8). In addition, the pregnant (U) rats on the trace metal poor diet produced fetuses with several characteristics in common with offspring of manifest diabetic (U) rats (on standard diet): decreased fetal weight (6,7), increased resorption rate (6,7), delayed skeletal ossification (7,8), as well as decreased zinc and increased manganese accumulation in the fetuses (9). This suggests, that the teratogenic processes in the two different types of (U) rat pregnancy - i.e. maternal trace metal deficiency and maternal diabetes - may share some etiological mechanisms. The importance of zinc in this context is illustrated by the effects of its administration to the trace metal restricted rat mothers. In the ZDZ litters there were no malformations, the skeletal ossification was normal and the fetal resorption rate, body and placental weights were also largely normalized. Other authors have suggested that changes in maternal levels of trace metals may be involved in the induction of congenital malformations both in humans (2,3,14,17,23) and animals (15,16). Diabetic individuals are furthermore known to lose trace metals, via increased urinary output in relation to their degree of metabolic control (19). Altered levels of trace metals, in particular zinc, may therefore be of significance for the induction of malformations in diabetic pregnancy. The possible teratogenic actions of an embryo-fetal zinc de-

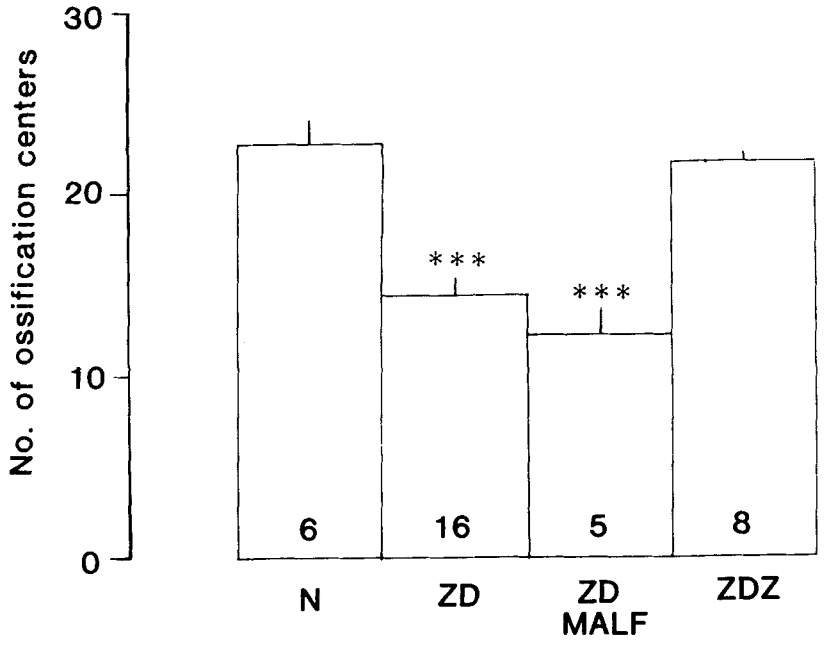

Figure II. Total number of ossification centers in six anatomical locations (cf. ref. no. 1) in the non-malformed and malformed (MALF) fetuses of normal (N), zinc-deficient (ZD) and zinc-resupplemented (ZDZ) rats on gestational day 20. Means ± S.E.M. Significance: $^{+++}$ $p<0.001$ vs. N fetuses.

ficiency are several. This trace metal serves as a co-factor of a number of enzymes, e.g. thymidine kinase, DNA-polymerase and superoxide dismutase, and a deficiency may therefore inhibit the biosynthesis of DNA or affect the defense against free oxygen radicals in the conceptus (4,5,22). The significance of the concomitantly increased fetal manganese levels in this study as well as in fetuses of manifest diabetic rats (9) remains to be elucidated.

A trace metal disturbance of the type produced in the present study may not, however, explain all the malformations seen in the diabetic rat pregnancy, since the frequency of skeletal malformations was markedly lower in this investigation than in the offspring of manifest diabetic (U) rats (4% versus 15-20%, cf. refs. no. 7-8). Furthermore, all the malformations were of the sacral dysgenesis type in the offspring of trace metal restricted rats, whereas in diabetic rat pregnancy both micrognathia and sacral dysgenesis are encountered (7-10). In addition, manifest diabetic rats of this malformation-prone strain appear to give birth to a greater proportion of micrognathias, when the mother is zinc-supplemented (Eriksson, unpublished). Altered fetal zinc levels may therefore play a role in the induction of congenital malformations, and may be of specific significance for the etiology of the sacral dysgenesis syndrome. This idea awaits further investigation.

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Adress reprint requests to:

Dr. Ulf J. Eriksson
Department of Medical Cell Biology
University of Uppsala
Biomedicum
P.O. Box 571
S-751 23 Uppsala
Sweden