The effect of iron supplements on pregnancy in rats given a low-zinc diet

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1. Female Wistar rats were given an adequate-zinc (60 μg/g) or low-Zn (7 μg/g) diet for a minimum of 2 weeks and then mated. They were then either continued on the same diets (+Zn−Fe or −Zn−Fe) or given similar diets supplemented with four times the normal level of iron (+Zn+Fe or −Zn+Fe). The day before parturition they were killed and the fetuses removed and analysed.

2. There were no differences in numbers of fetuses or the number of resorption sites. In the absence of Fe supplementation, the mean fetal wet weight was significantly less ($P < 0{\cdot}05$) in the low-Zn group but there was no effect of Zn in the two Fe-supplemented groups. The addition of Fe significantly decreased ($P < 0{\cdot}05$) the mean fetal wet weight in the adequate-Zn groups but had no effect in the low-Zn groups. There were no differences in fetal dry weight, fat, protein or DNA content. Both Fe-supplemented groups produced fetuses of higher Fe concentration ($P < 0{\cdot}01$), and mothers with higher bone Fe-concentration ($P < 0{\cdot}01$) compared with the non-supplemented groups. The low-Zn groups produced fetuses of lower Zn concentration ($P < 0{\cdot}001$) than the adequate-Zn groups but there was no effect on maternal bone Zn concentration.

3. It was concluded that Fe-supplements did not adversely affect fetal growth from mothers given a low-Zn diet, but the addition of Zn to the unsupplemented diet increased fetal wet weight. These findings were not accompanied by any other differences in fetal composition or dry weight, and do not therefore lend support to the suggestion of an Fe–Zn interaction.

The adverse effect of severe zinc deficiency in the rat is well known (Hurley, 1969): the pups are small and severely malformed. Marginal Zn deficiency has also been reported to affect live birth weight (Williams *et al.* 1973) and it has been suggested by Meadows *et al.* (1981) that one of the factors causing mothers to give birth to small-for-gestational-age babies is a diet marginally deficient in Zn. They have also speculated that iron supplements during pregnancy might exacerbate the situation since Fe is believed to reduce intestinal absorption of Zn in non-pregnant adults (Solomons & Jacob, 1981). This study is an investigation into the effect of Fe supplements on pregnancy in rats given an adequate- or low-Zn diet.

MATERIALS AND METHODS

Forty mature female Wistar rats (weighing approximately 200 g) were given a semi-synthetic control diet containing adequate Zn from added zinc carbonate (60 μg Zn/g) and forty were given the control diet with no added Zn (7 μg Zn/g) for 2 weeks. At the end of this period, they were mated overnight with adult male Wistar rats. The occurrence of mating was checked by looking for spermatozoa in vaginal smears the following morning. As soon as this was confirmed, the females were caged separately. Animals that had been on the control diet (+Zn) were either maintained on the same diet (+Zn−Fe) or given a similar diet supplemented with Fe (+Zn+Fe). Those that had been on the control diet low in Zn (−Zn) were either maintained on the same diet (−Zn−Fe) or given a similar diet supplemented with Fe (−Zn+Fe) and food intakes were recorded. The compositions of the diets are described on p. 80.

Table 1. *Composition of control diet (g/kg)*

Starch	335
Sucrose	335
Casein	150
Mineral mix*	40
Vitamin mix†	20
Solka floc	40
Maize oil	80

* Mineral mix (g/kg diet): $CaHPO_4$ 13·0, $CaCO_3$ 8·2, KCl 7·03, Na_2HPO_4 7·4, $MgSO_4 . H_2O$ 4·0, $MnSO_4 . H_2O$ 0·18, $ZnCO_3$ 0·10, $FeSO_4 . 7H_2O$ 0·144, $CuSO_4$ 0·015, KIO_3 0·001.

† Vitamin mix (mg/kg diet): nicotinic acid 60, cyanocobalamin in mannitol 50, calcium-D-pantothenate 40, thiamin hydrochloride 10, riboflavin 10, pyridoxine 10, pteryolmonoglutamic acid 5, D-biotin 1, menadione 1, Rovimix E-25 (Roche) 300, Rovimix A-500 (Roche) 25, Rovimix A-500/D3 (Roche) 15, choline bitartrate 1800.

The morning on which the vaginal smears were positive was designated day 1 of pregnancy. On day 21, i.e. the day before parturition, the animals were weighed and blood taken from the tail for haemoglobin (Hb) and packed cell volume (PCV) measurements. The animals were then killed under carbon dioxide and the fetuses and placentas were dissected out and any abnormalities noted. The litters of each animal were weighed, dried at 90° for 24 h, re-weighed and ground in a pestle and mortar to a fine powder. The number of implantation sites and resorption sites were recorded and checked against the number of corpora lutea. The tibia and fibula from the mothers' left hind-limbs were removed for Zn and Fe analysis. Portions of the fetal homogenate were analysed for fat, and the dried defatted material was then analysed for Fe and Zn by atomic absorption spectrophotometry (AAS) and DNA and protein.

Diets

The composition of the control diet given to group +Zn−Fe is shown in Table 1. Group +Zn+Fe was given the same diet with added Fe in the same proportions as Fe supplements normally given to pregnant women, i.e. four times the recommended daily allowance (US) National Research Council, 1974). This was achieved by adding a further 576 mg ferrous sulphate to the 144 mg already present in each 1 kg diet. Groups −Zn−Fe and −Zn+Fe were given a low-Zn diet, which was achieved by omitting the zinc carbonate usually added to the mineral mix. The diet given to group −Zn+Fe also had Fe added as in that of group +Zn+Fe.

Fats

Accurately-weighed dried samples were defatted in dichloromethane-methanol (9:1, v/v) in Soxhlet thimbles on a Tecator Soxtec 1040 extraction unit. The fat was collected in a cup, the solvent evaporated and the weight of fat deducted from the weight of sample to give the dried defatted weight. The defatted sample was removed from the thimble and dried before further analyses.

DNA

The DNA in defatted fetal homogenate was extracted by the method of Hofert & White (1968) and estimated colorimetrically with indole (Hubbard *et al.* 1970).

Protein

Subsamples of defatted fetal homogenate were analysed for protein by the method of Lowry *et al.* (1951).

Hb and PCV

Hb was determined on freely-flowing blood from the tail vein using an AO haemoglobinometer (American Optical Corporation, Buffalo, New York). PCV was measured using the microhaematocrit technique.

AAS

Samples for Fe and Zn analysis were oven-dried at 90° and ground to a fine powder with a pestle and mortar or stainless-steel coffee grinder. Portions of dry powder were ashed at 480° for 48 h, the ash dissolved in warm concentrated hydrochloric acid, made up to a suitable volume with distilled water, and filtered. The solutions were analysed by flame spectrophotometry on a Varian AA6 AAS with background correction using appropriate standards.

Statistical analysis

As the number of young sharing the blood supply in the uterus in the later stages of gestation has a profound effect on their size at birth (as reviewed by Widdowson, 1968), it was felt that the inclusion of values from animals with very small or very large litters might bias the results and lead to incorrect conclusions. It was therefore decided that outliers would be removed by the procedure of Anscombe (1960). The mean number of fetuses and standard deviations for each group were calculated and any animal that fell more than three standard deviations outside the mean was excluded. Using this technique, four animals were rejected from the experiment, one with a large number of fetuses (group +Zn−Fe) and three with a small number of fetuses (one from group +Zn+Fe and two from group −Zn−Fe). Differences among the four group means were tested using the *F* test modified for heterogeneous variance (Snedecor, 1957*b*). Tests for specific effects of Fe in the adequate-Zn or low-Zn groups, and Zn in the normal-Fe groups were carried out, using a modification of the *t* test (Snedecor, 1957*a*) with the aid of the BMDP computer program (University of California, 1981). The influence of period of time on the Zn-deficient diet on fetal Zn and bone Zn was examined, using the Spearman rank correlation test (Siegel, 1956).

RESULTS

The Zn and Fe contents (μg/g) of the diets given to the four groups were as follows: group +Zn−Fe: Zn 59·6, Fe 36·3; group +Zn+Fe: Zn 57·6, Fe 161·5; group −Zn−Fe: Zn 6·8, Fe 35·1; group −Zn+Fe: Zn 6·9, Fe 171·5. It was not possible to mate all the animals at the same time and mating occurred sequentially over a period of 9 d. Thus, the period of time on the Zn-deficient diet varied between 14 and 23 d before the rats were made pregnant. There was no correlation, however, between period of time on the Zn-deficient diet and fetal Zn or maternal bone Zn. It was therefore concluded that this difference would not prejudice the results, particularly as the animals were randomly allocated to groups.

Mean daily food intakes, from 7-d periods, are given in Table 2. The unsupplemented low-Zn group ate slightly less in the third trimester ($P < 0{\cdot}05$). Weight gain during pregnancy is shown in Fig. 1. During the third trimester, the mothers in the −Zn−Fe group gained less than the −Zn+Fe group ($P < 0{\cdot}05$). Weight gain in each trimester was positively correlated with food intake ($P < 0{\cdot}001$) in all groups.

The maternal Hb and PCV values are shown in Table 3. There were no significant differences between the groups. There were no differences in maternal tibia and fibula dry weights, and the bone Zn and Fe values are shown in Table 4. The Fe concentration of

Table 2. *Mean daily food intake (g/d) in each trimester of pregnancy in rats given diets ranging in zinc content, with or without iron supplements*

(Mean values with their standard errors)

Group	*n*	1st Trimester Mean	1st Trimester SE	2nd Trimester Mean	2nd Trimester SE	3rd Trimester Mean	3rd Trimester SE
+Zn−Fe	10	20·3	1·4	21·9	1·2	20·2	1·1
+Zn+Fe	11	20·2	0·7	22·1	0·7	20·3	0·8
−Zn−Fe	14	19·2	0·8	21·7	0·9	18·3[a]	0·8
−Zn+Fe	16	20·1	0·8	22·8	0·9	21·3	1·2

+Zn, Zn-adequate; −Zn, low-Zn; +Fe, Fe-supplemented; −Fe, unsupplemented.
[a] −Zn−Fe < −Zn+Fe ($P < 0·05$).

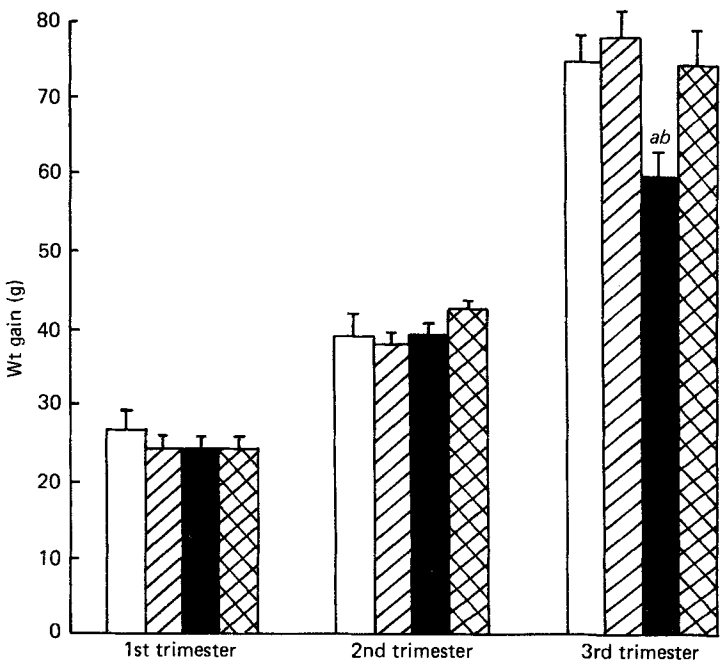

Fig. 1. Weight gain during each trimester of pregnancy in rats given (□), zinc-adequate control diet (+Zn−Fe); (▨), control diet plus iron supplement (+Zn+Fe); (■), marginally Zn-deficient diet (−Zn−Fe); (▩), marginally Zn-deficient diet plus Fe supplement (−Zn+Fe). For details of diets, see p. 80 and Table 1. Values are means, with their standard errors represented by vertical bars. [a] −Zn−Fe < −Zn+Fe ($P < 0·05$). [b] −Zn−Fe < +Zn−Fe ($P < 0·05$).

dried bone was higher in both the adequate-Zn ($P < 0·05$) and low-Zn ($P < 0·01$) Fe-supplemented groups. There were no differences in bone Zn concentrations although there was a trend towards lower levels in the low-Zn groups.

The mean number of resorption sites did not differ between the groups. The number of fetuses for each group, mean wet and dry fetal weights and fat content, are shown in Table 5. There were no differences in numbers of fetuses, dry weight or fat content. In the adequate-Zn groups, the addition of Fe significantly decreased fetal wet weight ($P < 0·05$),

Table 3. *Maternal haemoglobin (Hb) and packed cell volume (PCV) on day 21 of pregnancy in groups of rats given diets ranging in zinc content, with or without iron supplements*

(Mean values with their standard errors)

		Hb (g/l)		PCV (%)	
Group	*n*	Mean	SE	Mean	SE
+Zn−Fe	10	11·8	0·2	33·4	0·8
+Zn+Fe	11	12·4	0·2	35·1	0·9
−Zn−Fe	14	12·4	0·3	35·7	0·5
−Zn+Fe	16	12·9	0·2	36·8	0·6

+Zn, Zn-adequate; −Zn, low-Zn; +Fe, Fe-supplemented; −Fe, unsupplemented.

Table 4. *Maternal bone (tibia and fibula) zinc and iron on day 21 of pregnancy in groups of rats given diets varying in Zn content, with or without Fe supplements*

(Mean values with their standard errors)

		Zn (μg/g dried bone)		Fe (μg/g dried bone)	
Group	*n*	Mean	SE	Mean	SE
+Zn−Fe	10	303·0	33·2	38·4	1·7
+Zn−Fe	11	297·3	26·4	45·6[a]	2·7
−Zn−Fe	14	274·1	28·1	37·7	1·5
−Zn+Fe	16	237·1	24·4	46·9[b]	2·4

+Zn, Zn-adequate; −Zn, low-Zn; +Fe, Fe-supplemented; −Fe, unsupplemented.
[a] +Zn+Fe > +Zn−Fe ($P < 0·05$).
[b] −Zn+Fe > −Zn−Fe ($P < 0·01$).

Table 5. *Number of fetuses per litter, mean wet and dry fetal weights (g) and fat content (g/kg dry weight) on day 21 of pregnancy in groups of rats given diets varying in zinc content, with or without iron supplements*

(Mean values with their standard errors)

		Fetuses		Wet wt		Dry wt		Fat	
Group	*n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE
+Zn−Fe	10	14·1	0·3	4·84[ab]	0·11	0·64	0·05	94·6	4·8
+Zn+Fe	11	15·4	0·5	4·51	0·05	0·61	0·06	91·0	2·8
−Zn−Fe	14	14·5	0·7	4·49	0·08	0·60	0·05	102·4	3·5
−Zn+Fe	16	13·9	0·7	4·67	0·11	0·64	0·08	98·8	3·4

+Zn, Zn-adequate; −Zn, low-Zn; +Fe, Fe-supplemented; −Fe, unsupplemented.
[a] +Zn−Fe > +Zn+Fe ($P < 0·05$).
[b] +Zn−Fe > −Zn−Fe ($P < 0·05$).

Table 6. *Mean protein (mg/g), DNA (mg/g), zinc (μg/g) and iron (μg/g) content of dried defatted fetuses on day 21 of pregnancy from groups of rats given diets varying in Zn content, with or without Fe supplements*

(Mean values with their standard errors)

		Protein		DNA		Zn		Fe	
Group	*n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE
+Zn−Fe	10	831·6	28·3	22·2	0·7	161·2[a]	4·5	297·8	20·6
+Zn+Fe	11	856·5	13·7	21·3	0·5	149·4	4·0	440·1[b]	18·7
−Zn−Fe	14	847·4	14·7	21·7	0·6	130·6	2·4	292·7	20·7
−Zn+Fe	16	871·2	13·0	21·5	0·7	137·3	4·1	407·4[c]	20·4

+Zn, Zn-adequate, −Zn, low-Zn; +Fe, Fe-supplemented; −Fe, unsupplemented.
[a] +Zn−Fe > −Zn−Fe ($P < 0·001$).
[b] +Zn+Fe > +Zn−Fe ($P < 0·01$).
[c] −Zn+Fe > −Zn−Fe ($P < 0·01$).

and in the groups without Fe supplementation the addition of Zn significantly increased fetal wet weight ($P < 0·05$). It was noticed that the fetuses from the low-Zn mothers showed a higher incidence of morbidity than the others. In both low-Zn groups, 57% of mothers showed evidence of uterine and fetal haemorrhages. Brown patches were observed on the uterine wall in the region of the placenta; in the fetuses severe neck haemorrhages were noted. In the adequate-Zn groups, similar disturbances were observed in only 10% (+Zn−Fe) and 18% (+Zn+Fe) of animals, and these were much less severe.

Results of protein and DNA analysis of the dried defatted fetuses are shown in Table 6, together with the Fe and Zn contents. There were no differences in protein and DNA contents of the fetuses nor in the DNA:protein values. The addition of Fe to the diet increased fetal Fe in both the adequate-Zn ($P < 0·01$) and low-Zn ($P < 0·01$) groups and there was no difference in the effect at the two Zn levels. The adequate-Zn mothers produced fetuses with a higher Zn concentration than those given a low-Zn diet ($P < 0·001$).

DISCUSSION

The suggestion has been made that, in humans, maternal tissue Zn depletion is associated with fetal growth retardation and that Zn deficiency may be exacerbated by the Fe supplements which are prescribed for many pregnant women (Meadows *et al.* 1981). It has been demonstrated that there is an interaction between Fe and Zn in the intestine such that as the Fe:Zn value increases above 1, Zn uptake is increasingly inhibited (Solomons & Jacob, 1981). The Fe:Zn value in an average diet is about 1. However, it is usual practice for pregnant women to be given Fe supplements during pregnancy, such that their daily intake is increased about fourfold. Since their Zn intakes are unlikely to change much, the Fe:Zn value will be greatly increased. In the present study we attempted to model the human situation by giving an Fe-supplemented diet to pregnant rats from day 1 of pregnancy onwards, such that the Fe:Zn value in the adequate-Zn group was increased from 0·6 to 2·8, and in the low-Zn groups from 5·2 to 24·9. Typical commercial breeding diets for rats contain 30–35 mg Fe/kg; our semi-synthetic diet contained 35 mg Fe/kg. However, the maternal tibia and fibula and fetal Fe concentrations were significantly higher in the Fe-supplemented mothers, which suggests that the level of 35 mg Fe/kg may not be optimal for pregnancy. The unsupplemented low-Zn group gained significantly less weight during

the third trimester than both the low-Zn Fe-supplemented and the adequate-Zn groups, and this could be linked to the lower food intake of this group during this time period. There was a significant relationship between food intake and weight gain ($P < 0{\cdot}001$) in each trimester, as might be expected. However, when the weight gain mean was adjusted to a common mean food intake to eliminate the effect of food intake, although the slope of regression was similar, the intercepts were different. Thus, differences in food intake did not account for all the differences in weight gain in the 3rd trimester.

The mechanism of Zn depletion of mothers in the UK suggested by Meadows *et al.* (1981) is likely to be dietary. Zn, like Fe, is only partially available for absorption by the body and its availability depends on many factors; for example, phytate (present in cereals and pulses) makes Zn less available from vegetables than from meat (O'Dell *et al.* 1972). There is, therefore, some concern about the adequacy of vegan or vegetarian diets for groups of the population with high requirements for Zn, i.e. infants, growing children and pregnant women. There are difficulties in assessing Zn status, and measurement of Zn intake is of limited use without some idea of availability. Therefore, it is difficult to judge whether a dietary regimen is adequate for any particular group of people. However, one method of assessing Zn status that has been commonly used in animal studies is bone Zn (Williams & Mills, 1970). We found no significant reduction in maternal bone Zn in mothers given a low-Zn diet, which agrees with findings by Hurley (1969) and suggests that bone acts as a long-term sink which is relatively unavailable in periods of acute stress, e.g. pregnancy. This makes the adequacy of dietary Zn during pregnancy an even more important factor, since there appears to be no substantial reserve of Zn for times when requirements exceed availability. Apgar (1968) also suggests that Zn stores are not available in amounts adequate for the stress of pregnancy and parturition.

Cunnane (1982) has demonstrated that Zn-deficiency does not affect the duration of gestation and parturition. It was, therefore, quite valid to compare fetal weights on day 21 of pregnancy since fetuses from Zn-deficient and control animals were of similar gestational age. Although there were no differences in numbers of fetuses, mean dry weight, protein, DNA or fat content, the mean wet fetal weight appeared to be affected by the Zn and Fe levels of the diet. In the adequate-Zn groups, the addition of Fe to the diet significantly reduced the fetal wet weight but not in the low-Zn groups. A separate effect of Zn was noted in the unsupplemented groups in that the wet weight was increased in mothers given an adequate-Zn diet compared with a low-Zn diet. The reduction in fetal wet weight with a low-Zn diet is not surprising, but the adverse effect on fetal wet weight of adding Fe in the adequate-Zn diet is not supported by any other variables, e.g. dry weight, Zn concentration, etc. It is clear from the results that the differences in wet weight, although significant at the 5% level, are small, and because there were no other differences in fetal composition, it would be premature to place much emphasis or indeed draw any firm conclusions from these observations.

It is not possible to comment on the effect of Zn deficiency on fetal survival at parturition because Caesarian section deliveries were carried out on day 21 of pregnancy. The severe fetal haemorrhaging observed *in utero*, however, suggests that parturition losses would have been greater in the low-Zn groups. This is supported by the evidence of other workers (Williams *et al.* 1973; Cunnane, 1982) who found an adverse effect of Zn deficiency on fetal survival at delivery. Indeed, both Apgar (1968) and Cunnane (1982) found that maternal Zn deficiency *per se* did not affect birth weight of the young but it significantly reduced survival rate.

There was a substantial fall in fetal Zn in both the low-Zn groups, but the Fe-supplemented group was no worse than the unsupplemented group. The addition of Fe to the adequate-Zn diet did not produce a significant reduction in fetal Zn concentration and it is therefore

clear that the Fe supplements did not adversely affect Zn availability from the adequate- or low-Zn diets. There was a much higher concentration of Fe in the fetuses of both Fe-supplemented groups, as was found in the bone measurements. The observation that there were insufficient mobilizable maternal Zn stores to maintain normal fetal Zn levels in animals given an adequate-Zn diet until conception but maintained on a low-Zn diet throughout pregnancy, has important implications. It emphasizes the necessity for adequate dietary Zn throughout pregnancy and points to the fact that the fetus depends mainly on dietary Zn for its supply rather than drawing on maternal stores. The addition of Fe to an adequate- or low-Zn diet at levels similar to those in human pregnancy does not have any major influence on the composition or size of the fetuses the day before term.

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